Vitamin D signaling, infectious diseases and regulation of innate immunity.

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Vitamin D.

Vitamin D was first identified as a cure for nutritional rickets, a disease of bone growth caused by an inadequate uptake of dietary calcium. It is now known that vitamin D can be obtained through two independent pathways; limited dietary sources, and from the photochemical action of solar ultraviolet light in skin. Cod liver oil was discovered as an excellent source of anti-rachitic activity in 1827, although it wasn’t until several decades later that the active ingredient was identified as vitamin D₃. Even earlier, in 1822, a Polish physician studying children reached the remarkable conclusion that sunlight cured rickets after noting that rickets was relatively rare in the clearer air of rural areas. Almost 100 years later, in 1919, it was shown that artificial ultraviolet light cured rickets (49,92). Indeed, secosteroidal vitamin D₃ is produced in skin via photochemical and thermal conversion of 7-dehydrocholesterol in the presence of UVB (~295-320nm) light. While it would seem that vitamin D is readily accessible via dietary or solar routes, vitamin D insufficiency or deficiency is, in fact, quite widespread. Solar UVB irradiation is absorbed by atmospheric ozone; consequently, surface UVB varies markedly in intensity with latitude and time of year. Moreover, as vitamin D intake is generally inadequate in most diets (38-40), rates of vitamin D insufficiency or deficiency rise with increasing latitude.

The term vitamin D refers collectively to vitamin D₃ and to vitamin D₂, which is derived from irradiation of the steroid ergosterol in yeast. Biologically active vitamin D is generated via largely hepatic 25-hydroxylation catalyzed by CYP2R1, CYP27A1 and possibly other enzymes to produce 25-hydroxvitamin D (25D; refs. 21,40,43,67,81), which has a long half-life and is the major circulating vitamin D metabolite. 25D is modified by 1α-hydroxylation catalyzed by CYP27B1 to produce hormonal 1,25-dihydroxyvitamin D (1,25D; refs. 40,43,67). Vitamin D
compounds are catabolized via 24-hydroxylation by CYP24, whose expression is strongly inducible by 1,25D, constituting a negative feedback loop (40,43,67).

While the kidneys represent a major site of 1α-hydroxylation of 25D, it has recently become clear that generation of hormonal 1,25D in peripheral tissues is critical to the full scope of its physiological actions. Renal 1α-hydroxylation is tightly controlled by calcium homeostatic signals, in particular circulating parathyroid hormone (PTH). Although initially characterized as a calcium homeostatic agent, vitamin D is now known to have pleiotropic actions, including a key role in immune system regulation (49). Importantly in this regard, recent research detailed in this review has uncovered critical, cell-specific differences in both the regulation of 1α-hydroxylation of 25D, and 24-hydroxylation that are relevant to the role of 1,25D as an immune system regulator.

**Vitamin D insufficiency/deficiency and disease.**

While there is no strict definition, vitamin D deficiency is widely defined as circulating 25D levels of less than 20ng/ml (50nM; refs. 11,39,40,53,88), whereas one is generally considered to be vitamin D sufficient with circulating 25D concentrations of greater than 30-32ng/ml (75-80nM; refs. 19,37,88). 25D levels are inversely associated with circulating PTH until 25D rises above 30-40ng/ml, at which point PTH levels bottom-out. While vitamin D intoxication can occur, it is not observed until 25D levels reach 150ng/ml (375nM) or more (40), and is associated with hypercalcemia, which if chronic can result in urinary calculi (renal or bladder stones) and renal failure.
While cases of vitamin D toxicity do occur, vitamin D insufficiency/deficiency is far more common. In temperate regions, surface solar UVB irradiation is insufficient to induce cutaneous vitamin D$_3$ synthesis for periods around the winter solstice of up to 6 months or more at higher latitudes (38), a period that is known as vitamin D winter. For obvious reasons, cutaneous vitamin D synthesis is also strongly influenced by skin colour (55). Lack of cutaneous vitamin D synthesis, coupled with vitamin D-poor diets, has contributed to high levels of vitamin D insufficiency or deficiency in European and North American populations (38-40,60). For example, a survey of healthy females across northern Europe found widespread vitamin D deficiency (6), and a recent study found that 42% of African American woman in the U.S. were seriously 25D deficient (<15 ng/ml; ref. 19).

Epidemiological studies link vitamin D deficiency to increased rates of cancer, as well as autoimmune and infectious diseases (80). U.S. rates of bladder, breast, colon, ovary and rectal cancer increase 2-fold from south to north (34). North-south gradients of autoimmune conditions such as multiple sclerosis, Crohn’s disease, and type 1 diabetes and have also been documented (2,15,47,59). Connections between vitamin D insufficiency and infectious diseases go back over 100 years, with the recognition in the 19th century that solar radiation was beneficial for patients suffering from tuberculosis (TB). Associations between vitamin D deficiency and TB susceptibility were made over 20 years ago (22,33). A more recent study of a genetically homogeneous immigrant population of Gujarati Asians in the London area with high rates of TB found an association between active disease and 25D deficiency and an even stronger association of disease with undetectable serum levels of 25D (102). In addition, we have known for over 20 years that 1,25D inhibits the growth of M. tuberculosis in cultured human macrophages (73). Interest in the connection between vitamin D supplementation and treatment of TB has been
rekindled lately by several studies (e.g. 50-52,54,75), not least the recent observations of Martineau and colleagues in a double-blind randomized controlled trial that a single dose of 100,000U of vitamin D$_3$ (2.5mg) enhanced anti-mycobacterial immunity in healthy tuberculin skin test-positive donors (54). In addition, critical links described below have recently been made between molecular events controlling vitamin D signaling pathways and innate immune responses against mycobacterial infection.

While the potential protective effects against TB infection have attracted the most attention, data is accumulating for several sources that vitamin D may also be beneficial in combating a range of other infectious agents of bacterial or viral origin. One small but intriguing study worthy of follow-up found that elderly women undergoing long-term treatment with vitamin D as an anti-osteoporosis agent had a significantly lower rate of *Helicobacter pylori* infections than an untreated control group (44). There are also a number of studies examining the potential role of vitamin D in protection against upper and lower respiratory tract infections, which can be caused by a variety of etiological agents, many of them viral in origin (14,57,103). Subclinical vitamin D deficiency was associated with severe lower respiratory tract infection in an Indian study (98), and clinical vitamin D deficiency was associated with 13-fold increased risk of pneumonia in Ethiopian children (58). A Finnish study found an association between serum 25D concentrations of less than 40nM (16ng/ml) and a range of acute respiratory infections (sinusitis, tonsillitis, otitis, bronchitis, pneumonia, pharyngitis, and laryngitis) in young army recruits (46). In addition, Cannell and several colleagues have persuasively argued based on a range of epidemiological data that cutaneous vitamin D production provides the ‘seasonal stimulus’ associated with solar radiation that underlies the seasonality of epidemic influenza (16,17). Finally, clinical and genetic evidence is accumulating that vitamin D may play a role in
modulating human immunodeficiency virus (HIV) infection, although more work needs to be
done to clarify the relationship between vitamin D physiology and HIV infection. A positive
correlation was established between vitamin D supplementation and CD4-positive T cell counts
in seropositive individuals (95). A correlation between mortality from HIV infection and vitamin
D deficiency has not been clearly established. However, interpretation of the vitamin D status of
HIV-positive individuals is complicated by the potential confounding effects of antiretroviral
therapy on vitamin D metabolism (95). This is an area that merits further clarification, because,
as detailed below, a potential role for 1,25D signaling in modulating HIV infection is supported
by genetic studies on vitamin D receptor (VDR) gene polymorphisms.

**VDR gene polymorphisms and infectious diseases.**

The association between vitamin D physiology and infectious disease is also supported by
genetic studies implicating polymorphisms in the gene encoding the VDR in disease susceptibility (91). There are numerous VDR polymorphisms, including a common Fok1
restriction fragment length polymorphism (RFLP) that shifts translational initiation to an ATG 3
codons downstream, and Taq1 and Bsm1 RFLPs in the 3’ untranslated region. Genetic studies
have linked VDR polymorphisms with a number of infectious diseases, including susceptibility
to *Mycobacterium tuberculosis* infection and treatment outcome. A Peruvian study found and an
association between specific genotypes of both the Taq1 and Fok1 RFLPs and time to
microbiologic resolution of pulmonary TB (75). In a case-control study of 2,015 African
subjects, homozygotes for Taq1 polymorphism (genotype tt) were significantly underrepresented
in tuberculosis patients (8). In the above mentioned study on Gujarati Asians, the ff genotype of
the Fok1 RFLP was associated with the extent of pulmonary TB in 25D-deficient patients (102).
VDR polymorphisms have also been linked to leprosy, which is caused by a distinct mycobacterial agent *Mycobacterium leprae* (29). Tuberculoid leprosy presents with few bacilli in macrophages and a strong cell-mediated response, whereas the more severe lepromatous leprosy is characterized by numerous bacilli and a weak cellular response. The *tt* VDR polymorphism was associated with tuberculoid leprosy whereas the *TT* genotype was associated with lepromatous leprosy in Bengali patients (77). Although the *tt* genotype was associated with susceptibility to leprosy in a case-control study of patients in the Karonga district of Malawi (30), the expected frequency of *tt* homozygotes was low (5%) and apparent differences between patient and control populations could have been due to chance.

A recent analysis in young children found that the *ff* genotype was associated with an adjusted relative odds of acute lower respiratory tract infection (predominantly viral bronchiolitis) that was 7 times that of the *FF* genotype (76). VDR polymorphisms have been linked to HIV infection, although clear conclusions regarding the role of vitamin D signaling in controlling HIV infection have been difficult to draw. No associations were found between *BsmI* polymorphisms and HIV infection, whereas an association was established between the *BB* genotype and disease progression based on several criteria (7). However, it is difficult to ascribe variations in the *BsmI* genotype to changes in VDR function. Another recent study found no association between a specific polymorphism and protection against HIV infection in a population of injection drug users, but did find a correlation between specific VDR haplotypes (blocks of polymorphisms; ref. 23). The authors concluded that protective VDR polymorphisms were associated with reduced VDR function, consistent with vitamin D signaling promoting HIV infection, and noted an *in vitro* study that the 1,25D-bound VDR could activate the HIV1 long terminal repeat (61).
Molecular mechanisms of action of vitamin D.

Much of the action of 1,25D can be explained by its binding to and activation of the vitamin D receptor. The VDR is a nuclear receptor and ligand-activated transcription factor (20,49) composed of a highly conserved DNA binding domain, and an \( \alpha \)-helical ligand binding domain (72). The ligand-bound VDR activates transcription by heterodimerization with retinoid X receptors (RXRs), which is essential for high affinity DNA binding to cognate vitamin D response elements (VDREs) located in the regulatory regions of 1,25D target genes. VDREs are composed of direct repeats of PuG(G/T)TCA motifs separated by 3bp (DR3) or everted repeats with 6bp spacing (ER6; refs. 20,26,49,89,90). [Note that everted repeats are palindromic, but with the opposite symmetry (toes pointing out) to so-called inverted repeats (toes pointing in) originally identified as response elements for steroid receptors]. ER8 motifs can also function as response elements for the VDR and related retinoic acid receptors (86), thus partially integrating 1,25D and retinoid signaling. DNA-bound VDR/RXR heterodimers act to recruit numerous so-called coregulatory proteins, which control histone modifications, chromatin remodeling and RNA polymerase II binding and transcriptional initiation (24,31,56,70,74). The ligand-bound VDR can also repress transcription. For example, in the presence of 1,25D, VDR/RXR heterodimers can displace DNA-bound nuclear factor of activated T cells (NF-AT), thus repressing cytokine gene expression (5,85). While numerous VDREs have been identified in relatively promoter proximal locations, recent work has provided evidence that the DNA-bound VDR can function at distances as great as 75kb to regulate adjacent target gene transcription (45).
Vitamin D signaling and metabolism in the immune system.

Evidence for a role of vitamin D signaling in the immune system in general and in innate immune responses in particular has been accumulating from a variety of sources. The VDR is present in most cells of the immune system, including T lymphocytes, neutrophils and antigen presenting cells such as macrophages and dendritic cells (3,10,12,62,69). 1,25D is an inhibitor of maturation of dendritic cells, the most potent of the antigen presenting cells, and acts directly on T lymphocytes to inhibit T cell proliferation (93). 1,25D signaling represses the transcription of genes encoding key T helper 1 (Th1) cytokines, such as interferon γ and interleukin-2 (5,93). 1,25D is thus a suppressor of antigen presentation to, and activation and recruitment of Th1 cells. The net effect of 1,25D action is to polarize T helper responses towards a more regulatory T helper 2 phenotype, which is considered to be a key component its capacity to suppress Th1-driven autoimmune responses (93).

In the last few years, researchers in the vitamin D field, and particularly those interested in its immunomodulatory functions, have come to appreciate the important contributions of extra-renal 1α-hydroxylase (CYP27B1) to vitamin D physiology. Activated macrophages and dendritic cells express CYP27B1 (1,64,65,84), which, unlike the renal enzyme, is not regulated by Ca++ homeostatic signals, but primarily by immune inputs, mainly interferon-γ and agonists of TLR (Toll-like receptor) pattern recognition receptors. Critically, this renders the immune system responsive to circulating levels of 25D. Modlin and colleagues found in microarray studies that signaling through human macrophage TLR1/2 toll-like receptor heterodimers stimulated with bacterial lipopeptides induced expression of both CYP27B1 and the VDR (50; see Fig. 1). Most importantly, they showed that, in TLR2/1-stimulated human macrophages cultured in the presence of human serum, downstream VDR-driven responses were strongly dependent on
serum 25D concentrations. VDR-driven responses were strongly blunted or absent in serum from vitamin D-deficient individuals; a defect that could be overcome by 25D supplementation. Moreover, consistent with previous findings (60,82), 25D levels serum from African Americans used in the study were markedly lower than those of Caucasian Americans (50). This study thus provided a clear demonstration of the dependence immune responses on circulating 25D levels. Similarly, stimulation of the TLR4/CD14 receptor complexes by lipopolysaccharide (LPS) induces CYP27B1 expression (84, our unpublished results), consistent with correlations others have seen between TLR4 and CYP27B1 expression (27,28).

Remarkably, while expression of CYP24, the mitochondrial enzyme that initiates 1,25D catabolism, is exquisitely sensitive to the presence of 1,25D, the negative feedback loop appears to be defective in macrophages (Fig. 1). Adams, Hewison and colleagues have recently shown that, while expression of CYP24 transcripts is induced by 1,25D in macrophages as in other cells, the corresponding enzymatic activity is virtually undetectable (71). 1,25D induces the expression in macrophages of a splice variant form (CYP24-SV) that encodes a truncated enzyme lacking the critical amino-terminal mitochondrial targeting sequence (71). Although the substrate binding pocket of CYP24-SV is apparently functional, the enzyme, trapped in the cytosol, appears to be catalytically inactive. This would suggest that, in macrophages, robust 1,25D signaling is maintained over extended period of time, which would be advantageous for combating intracellular pathogens such as M. tuberculosis. It also provides at least part of the molecular basis for the excessive production of 1,25D by macrophages in granulomatous diseases such as sarcoidosis (41).

1,25D is a direct inducer of antimicrobial innate immunity.
We have had molecular evidence that 1,25D is a regulator of innate immune responses for several years. It has been known since the early 90’s that expression of the coreceptor of TLR4, CD14, is strongly induced by 1,25D in human cells (63). This regulation is conserved in the mouse; for example, recent work showed that induction of CD14 expression by 25D was abrogated in mice lacking CYP27B1 (79). The study also showed that vitamin D signaling enhanced the expression of TLR2 approximately 2-fold in human keratinocytes. Given that signaling through either TLR2 or TLR4 enhances vitamin D signaling by upregulating expression of the VDR and CYP27B1, the effects of 1,25D on TLR2 and CD14 expression in keratinocytes constitutes a positive feedback loop (see Fig. 1). Notably, however, recent findings suggest that such a loop does not function in monocytes. Treatment of human monocytes with 1,25D suppressed expression of both TLR2 and TLR4 mRNA and protein in a time- and dose-dependent manner (78). Signaling through TLR2 was suppressed in 1,25D-treated monocytes, as was signaling through TLR4 in the presence of LPS, even though CD14 expression was induced by 1,25D. The authors speculated that downregulation of pattern recognition receptors by 1,25D in antigen presenting cells may contribute to its capacity to attenuate excessive Th1-driven inflammatory responses and potential downstream autoimmunity (78).

Given that the VDR is a transcription factor and acts as a ligand-regulated gene switch, its signaling is ideally suited to analysis using genomic approaches. We have used a combination of microarrays and in silico screens for VDREs to identify several hundred 1,25D target genes (4,48,97,101). Microarray analysis showed that CD14 was induced 27-fold by 1,25D in well-differentiated human squamous carcinoma cells, for example (48), and in silico analysis identified an upstream VDRE in the human CD14 gene (97). In the course of in silico screening for VDREs, we noted that two genes encoding antimicrobial peptides (AMPs) CAMP
(cathelicidin antimicrobial peptide, hCAP18, LL37) and DEFB2 (DEFB4, β-defensin 2) contained promoter-proximal consensus DR3-type response elements (96). Further analysis of the CAMP and DEFB2 VDREs showed that both elements bound VDR/RXR heterodimers in a 1,25D-dependent manner in vitro and in cells in culture, and functioned in reporter gene assays (96). CAMP expression was strongly stimulated by 1,25D in all cell types examined (epithelial cells, macrophages/monocytes, and neutrophils), whereas DEFB2 expression was modestly induced in cells of epithelial origin. The strong induction of CAMP by 1,25D was subsequently observed by others in a range of cell types (32,50), including in 1,25D-treated or ultraviolet B-irradiated human skin biopsies (99), clearly indicating that 1,25D is a primary inducer of the gene. On the other hand, 1,25D is likely to be a secondary regulator of DEFB2 expression. For example, while 1,25D augmented basal DEFB2 expression ~2-fold, it enhanced the strong stimulation induced by IL-1β to the same degree (96).

AMPs are vanguards of innate immune responses against bacterial, fungal and viral attack, and many act directly by disrupting the integrity of pathogen membranes (35,42,66). In addition, CAMP and some β-defensins can function as chemoattractants for neutrophils, monocytes and other cellular components of immune responses (51). The induction of AMP expression by 1,25D in humans provides a potential molecular basis for the accumulating evidence, documented above, that a vitamin D replete state provides broad protection against a range of bacterial and viral pathogens. For example, defensin expression is induced in response to H. pylori infection in the gastric mucosa (100), and rhinovirus infection in airway epithelia (68), suggesting that 1,25D-induced DEFB2 expression may provide a measure of protection against these agents. In addition, while there are conflicting results concerning the role of vitamin D signaling in controlling HIV infection, it should be noted that human cathelicidin inhibited the
replication of a number of HIV isolates (9), and that the human and porcine homologues reduced the infectivity of lentiviral vectors (83), suggesting that vitamin D signaling may indeed induce antiretroviral activity.

**Species-specific mechanisms of AMP expression.**

Although classes of AMPs are conserved, there is considerable inter-species variation in both gene sequence and number, and in tissue distribution and regulation of expression. α, β and θ defensins contain six disulfide bond-forming cysteines (66), with subclasses distinguished by different spacings of Cys residues. While β-defensins are widespread in vertebrates, α-defensins are mammalian, and θ-defensins are primate-specific. The 5 human α-defensins are expressed in myeloid or enteric tissues, whereas the 19 murine genes (cryptdins) are enteric only. Cathelicidins are cationic and defined for their N-terminal cathelin domain, which is cleaved during maturation. Mice and humans have single cathelicidin genes, whereas there are multiple cathelicidin peptides in bovine species (104).

Apart from variations in gene number and tissue distribution of expression, there are also differences in gene regulation between species. Notably, neither of the VDREs in the *CAMP* and *DEFB2* genes is conserved in mice, and Gombart et al (32) noted that the *CAMP* VDRE is imbedded in an *Alu* repeat, which is a human or primate-specific transposable element. This lack of conservation is noteworthy in light of differences that have emerged in regulation of AMP expression in humans and mice. It was established by 2001 that stimulation of TLR2 on either human or mouse macrophages led to induction of antimicrobial activity against TB infection (13,87). Induction of antimicrobial activity in murine macrophages is dependent on inducible
nitric oxide synthase (iNOS) activity. Remarkably, however, whereas iNOS inhibitors blocked induction of AMP activity in mouse macrophages, they had no such effect in human cells.

The mechanism of induction is human macrophages was unclear until the discovery of the TLR2/1-stimulated expression of both CYP27B1 and the VDR in human cells, leading to the induction of CAMP under conditions of 25D sufficiency (50; Fig. 1). Moreover, in 25D-treated human cells, CAMP protein was shown to colocalize with mycobacteria in phagolysosomal structures. Subsequently, knockdown of CAMP expression in TB-infected human THP-1 macrophage-like cells confirmed that its induction is essential for 1,25D-stimulated antimycobacterial activity (52). Whether vitamin D signaling is induced in murine macrophages remains unclear. However, even if induction of CYP27B1 and the VDR does occur, it would be unlikely to lead to CAMP expression because of the lack of a VDRE in the murine CAMP gene.

CAMP expression is also strongly induced human keratinocytes under conditions of epithelial wound healing (18,25,36). In CYP27B -/- mice, however, under conditions where CYP27B1 ablation completely eliminated the strong injury-induced expression of CD14, induction of CAMP expression was mildly attenuated, but the effect did not achieve statistical significance (79). It has been argued that regulation of AMP expression in mice and humans has diverged because mice use nitric oxide as an intermediate in innate immune signaling and are nocturnal, whereas humans acquire vitamin D from exposed skin during the daytime (50).

Taken together, the interplay between 1,25D and TLR signaling and the direct induction by 1,25D of AMP gene expression provide a strong molecular basis for epidemiological evidence for the protective effects of a vitamin D-replete state against infectious diseases. They also underline a growing consensus among researchers (e.g. 11,38,40,94) that the widespread vitamin D insufficiency/deficiency observed in North American and European populations strongly
supports revising upwards the recommendations for adequate daily intake of vitamin D (currently at 200IU for children, 400-600IU for adults in these populations), and possibly extending vitamin D supplementation beyond dairy products, as is now practiced in the U.S.
References.


**Figure Legend.**

FIG. 1. Schematic representation of the interplay between toll-like receptor and vitamin D signaling detailed in the text. Arrows in blue refer to pathways that are active in keratinocytes but not in monocyte/macrophages. Nonstandard abbreviations used: 27B1, CYP27B1; lipopep., triacyl lipopeptides.