Vitamin D in Defense of the Human Immune Response

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ABSTRACT: Defensin is a generic name reserved for an endogenously synthesized antimicrobial agent. The purpose of this review is to describe a series of discoveries that led to the proposal that 25-hydroxylated metabolites of vitamin D are key, intracellular regulators of the synthesis and action of naturally occurring defensin molecules against bacterial antigens. The discussion will (1) highlight the basic elements of human immune response that is responsive to vitamin D, (2) recount work relevant to the extrarenal expression of the vitamin D-1-hydroxlase (CYP27b1) in the macrophage as an initiator of the innate immune response, and (3) describe recent work on the relevance of the vitamin D intracrine–autocrine–paracrine system in a model of a common and devastating human disease, tuberculosis.

KEYWORDS: vitamin D; innate immunity; adaptive immunity; macrophage; dendritic cell; tuberculosis

IMMUNOACTIONS OF VITAMIN D

The concept of active vitamin D metabolites acting as immunomodulatory cytokines sprang from two seminal observations: when activated with mitogen or specific antigen, human lymphocytes, and monocyte/macrophages expressed the gene product for the vitamin D receptor (VDR);[1,2] and when primed with LPS or interferon-gamma (IFN-γ) human monocyte/macrophages possessed the ability to synthesize and release into the pericellular space 1-hydroxylated vitamin D metabolites, the preferred activating ligands for the

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Ann. N.Y. Acad. Sci. 1117: 94–105 (2007).
doi: 10.1196/annals.1402.036

VDR.[3,4] The most extensively studied facet of vitamin D and the immune system has been the ability of $1,25(OH)_2D$ to modulate T cell responses. Inhibition of T cell proliferation and suppression of B cell immunoglobulin (Ig) production *in vitro* were two of the initial observations indicating that VDR-saturating concentrations of $1,25(OH)_2D_3$ had an effect on lymphocyte function when added to the extracellular medium of those cells.[5–7] In fact, it is now clear that $1,25(OH)_2D_2$, an active vitamin D metabolite of plant origin, as well as hormone $1,25(OH)_2D_3$ not only suppresses T cell proliferation but is a potent modulator of T cell functional phenotype. Initial reports focused on the ability of $1,25(OH)_2D$ to inhibit lymphokines, such as interleukin-2 (IL-2), GM-CSF, and IFN.[8] However, the regulation of Ig production has been shown to be due to indirect effects on T cells rather than direct action of the hormone on B cells.[6] Subsequent work expanded these observations to show that $1,25(OH)_2D$ suppresses the type-1 T-helper cell (Th1) cytokine profile, thereby favoring suppressor T cells or Th2 cells and supporting a role for $1,25(OH)_2D$ as an immunosuppressive hormone with a potential to limit immunologically directed disease.[9] These events have since been refined by several groups who recognized that these Th2 cells were, in fact, the regulatory T cells (Tregs) that are now synonymous with immune tolerance.[10–12] The overall conclusion to be drawn from these studies is that $1,25(OH)_2D$ and its 1-hydroxylated synthetic analogs are potent suppressors of adaptive immunity with putative applications as therapy for autoimmune disease and induction of tolerance in host-graft rejection.[13]

In fact, recent studies performed *in vitro*[14] and *in vivo*[15] indicate that the mechanism by which tolerance occurs is likely to be more complex than a simple balance between Th1 cell-directed humoral and Th2-directed cellular immunity. For example, it has been postulated that vitamin D insufficiency can deregulate the balance between type-1 and type-2 immunity, with Th1-inducing cytokines being disproportionately overexpressed owing to the general lack of macrophage-produced $1,25(OH)_2D$ from extracellular substrate 25OHD. As a result, vitamin D insufficiency may incite the failure of tolerance mechanisms leading to autoreactive cytotoxic T cell activation.[16,17] Further, it has been reported that vitamin D insufficiency results in acceleration of allograft rejection[18] and induction of autoimmune diseases.[19] For instance, the VDR-null/IL-10-null mouse develops severe intestinal inflammation, resembling Crohn's disease, associated with local expression of IL-2, IFN, IL-1I (IL-1β), TNF-alpha (TNF-α), and IL-12.[20] Recently, Chen *et al*.[21] demonstrated that $1,25(OH)_2D_3$ significantly inhibits phospholigand-induced gammadelta T cell expansion, IFN production, and CD25 expression.

$1,25(OH)_2D$ and its 1-hydroxylated-analogs have consistently shown potential as inducers of myeloid cell differentiation along the macrophage lineage *in vitro.*[22] Because both macrophages and dendritic cells express the CYP27b1-hydroxylase,[23] the enzyme that converts 25OHD substrate to

$1,25(OH)_2D$ product, it has been proposed by a number of laboratories that the production of $1,25(OH)_2D$ by these cells plays a key intracrine, autocrine, and paracrine role in the regulation of the human immune response at local sites of inflammation. In this regard, Penna and colleagues[24] recently showed that, in addition to its direct effects on T cells, $1,25(OH)_2D$ can also influence adaptive immunity indirectly by modulating antigen presentation. Specifically, they showed that, alongside other antigen-presenting cells, such as macrophages, dendritic cells express the VDR and are targets for $1,25(OH)_2D$. While the hormone promotes the differentiation of myeloid precursors toward the macrophage/dendritic cell phenotype, $1,25(OH)_2D$ generally acts to stall the more distal maturation of these cells;[25,26] as such, it has been proposed that the maintenance of dendritic cells in a relatively immature state not only attenuates T cell proliferation but is also a key factor in the generation of Tregs.[27]

Using peripheral blood mononuclear cells as a model, we have shown that expression and activity of CYP27b1 is induced as monocytes differentiate toward immature dendritic cells, with dendritic cell maturation stimuli inducing the enzyme still further.[28] By contrast, VDR expression is relatively high in monocytes-macrophages and low in mature dendritic cells.[29] Further analyses showed that both $25OHD_3$ and $1,25(OH)_2D_3$ are able to suppress dendritic cell maturation as defined by cell-surface antigen expression and antigen presentation in mixed lymphocyte reactions.[28] In view of the differential patterns of CYP27b1 and VDR expression in dendritic cells as they mature, we have proposed a paracrine mode of action *in vivo* in which mature dendritic cells actively synthesize $1,25(OH)_2D$, enabling a paracrine effect of the hormone on VDR-rich immature dendritic cells.[30] This mechanism would allow normal adaptive immune responses to pathogens but would prevent that response from becoming overexuberant and detrimental to the host. Based on this model we would predict that the "brake" on adaptive immunity provided by local synthesis of $1,25(OH)_2D$ is highly dependent on the availability of substrate 25OHD, and this mechanism would be compromised under conditions of vitamin D deficiency.

MACROPHAGE CYP27B1-HYDROXYLASE AND HUMAN DISEASE ASSOCIATED WITH THE ENDOGENOUS OVERPRODUCTION OF THE ACTIVE VITAMIN D METABOLITE

It is now almost 30 years since the first publication of data demonstrating a clear link between vitamin D metabolism and human immunity (see TABLES 1 and 2), including the fact that patients afflicted with macrophage-centric, granuloma-forming diseases of noninfectious (i.e., sarcoidosis), infectious (i.e., tuberculosis), and neoplastic origin (TABLE 3) can suffer from

TABLE 1. Representative, referenced inhibitory effects of 1,25-dihydroxyvitamin D (1,25-D) on the adaptive immune response

VDR expressed in activated human lymphocytes	Provvedini. *Science* 221:1181, 1983 Bhalla. *JCEM* 57:1308, 1983
1,25-D inhibits T cell differentiation	Abe. *Proc. Natl. Acad. Sci.* 78:4990, 1981
1,25-D inhibits T cell proliferation	Lemire. *J. Clin. Invest.* 74:657, 1984
1,25-D inhibits Th1 cell activity	Lemire. *J. Immunol.* 134:3032, 1985
1,25-D inhibits IL-2-driven B cell Ig production	Lemire. *J. Clin. Invest*. 74: 657, 1984 Jordan. *Transplant Proc.* 28:901, 1986

TABLE 2. Representative, referenced potentiating effects of 1,25-dihydroxyvitamin D (1,25-D) on the innate immune response

VDR expressed in mitogen- and antigen (TB)-activated macrophages	Abe. *Proc. Natl. Acad. Sci.* 78:4990, 1981 Barnes. *J. Clin. Invest*. 83: 1989
1,25-D promotes antigen processing	Rigby. *Blood* 64:1110, 1984
1,25-D increases phagocytosis	Cohen. *J. Immunol.* 136:1049, 1985
1,25-D increases superoxide synthesis	Roux. *Cell Immunol.* 97:286, 1986
1,25-D increases bacterial killing	Provvedini. *Bone* 7:23, 1985
1,25-D increases IL-1β and TNF-α production	Fagan. *Mol. Endocrinol.* 5:179, 1991 Prehn. *Blood* 80:2811, 1992

$1,25(OH)_2D$-driven hypercalciuria or hypercalcemia as a consequence of overproduction of the hormone by macrophages. This provided the first conclusive evidence that synthesis of the active vitamin D metabolite can occur outside of the confines of the renal tubular epithelial cell, the acknowledged endocrine source of the hormone, and highlighted a potential role for locally produced $1,25(OH)_2D$ as a tissue-specific modulator of the human immune response in those diseases. As well as seeking to discern the role of CYP27b1 in defining adaptive dendritic cell–T cell interactions described above, we have also revisited original studies from our group and others, which described vitamin D metabolism and function in macrophages. Recent experiments have shed new light on the function of macrophage CYP27b1 in normal human immunity.[31] Specifically, we have shown that, whereas the enzyme in dendritic cells functions to attenuate adaptive immunity, in macrophages it acts to enhance innate immunity (TABLE 2).

TOLL-LIKE RECEPTORS (TLRS): A LINK AMONG GRANULOMA-FORMING DISEASES, MACROPHAGE VITAMIN D METABOLISM AND ACTION, AND HUMAN INNATE IMMUNITY

Elucidation of the mechanism by which macrophage-centric human granuloma-forming diseases engage the vitamin D system was initiated by the discovery by Janeway of the means by which the mammalian innate im-

TABLE 3. Human granuloma-forming diseases reported to be complicated by 1,25-dihydroxyvitamin D-driven hypercalcemia or hypercalciuria

Noninfectious	Infectious	Neoplastic
Sarcoidosis	Tuberculosis	B cell lymphoma
Crohn's disease	Leprosy	Hodgkin's disease
Silicone granulomata	Candidiasis	Lymphomatoid granulomata
Paraffin granulomata	Histoplasmosis	Dysgerminoma
Berylliosis	Coccidiomycosis	Seminoma
Wegener's infantile fat necrosis slack skin disease	Cat scratch fever	Mesothelioma

mune system recognizes microbial pathogens.[32] Janeway proposed that recognition must involve evolutionarily primitive receptors that bind conserved microbial constituents. These receptors were termed pattern recognition receptors of which the mammalian TLRs are prototypical. TLRs have been shown to have specificity for recognition of microbial ligands and mediate functions of the innate immune system. For example, TLR4 was shown to signal the innate immune activation of the adaptive immune response and mediate responses to LPS.[33] Experiments performed in the[34,35] Modlin labs led to the finding that microbial lipoproteins also trigger host responses via TLR2, subsequently demonstrating recognition via TLR2/1 heterodimers. The identification of TLR ligands made possible experiments to investigate the functional role of TLRs in the innate immune response. TLRs can regulate phagocytosis either through enhancing endosomal fusion with the lysosomal compartment[36] or through induction of a phagocytic gene program including multiple scavenger receptors.[37] TLRs also fulfill another function of innate immunity, the induction of direct antibacterial activity.[38]

THE INNATE IMMUNE RESPONSE IN TUBERCULOSIS: THE ROLE OF VITAMIN D

A role for vitamin D in the antimicrobial activity of human monocytes and macrophages against *Mycobacterium tuberculosis* (M.tb.) was first suggested by experiments in the labs of Rook in 1986[39] and Crowle et al. in 1987.[40] These experiments were performed by adding $1,25(OH)_2D_3$ to the culture medium of human monocytes and macrophages *in vitro*. Yet Crowle et al. write "Concentrations of $1,25(OH)_2D_3$ near 4 μg/mL were needed for good protection. These seem unphysiologically high compared with the 26 to 70 pg/mL in the normal circulating range." Nevertheless, these studies prompted investigation of the mechanism by which supraphysiological concentrations of $1,25(OH)_2D_3$ led to antimycobacterial activity. However, recent advances in our understanding of what constitutes normal vitamin D status (25OHD>30 ng/mL)[41] have underpinned a diverse array of epidemiological studies showing association

between vitamin D insufficiency (i.e., 25OHD<30 ng/mL) and susceptibility to human disease, notably immune-related disorders. A fundamental feature of these reports is that in each case vitamin D deficiency was defined by circulating levels of 25OHD, not $1,25(OH)_2D$. The most logical conclusion from this is that immunologically active $1,25(OH)_2D$ is generated locally in a manner similar to that originally described for sarcoidosis patients, with resulting effects being either autocrine or paracrine, or both. We have postulated that this is a crucial cog in the mechanism linking vitamin D and human immunity, and data from our groups (see below) have shown how this may apply to both innate and adaptive immunity.

ANTIMICROBIAL ACTIONS OF VITAMIN D

The initiating step in the mammalian innate immune response is a breech in the epithelial barrier between the microbe-laden outside environment and the relatively sterile internal environment of the host. Once the bacterial products, so-called pathogen-associated molecular patterns or PAMPs, gain access to the host interior, they are recognized by TLRs. The liganded TLRs recruit MyD88 adaptor proteins, which, in turn, trip a number of intracellular signaling pathways, many of which terminate in the nuclear localization and transaction of NF-κB. The end result is antigen destruction and initiation of the T- and B-cell adaptive immune response. Recent work by us[31] now demonstrates that interaction of PAMPs shed from the cell wall of M.tb. with the TLR2/1 dimer pair on the macrophage triggers upregulation of expression of both the CYP27b1 and vitamin D receptor (VDR). This permits the macrophage to internalize serum vitamin D binding protein (DBP)-bound 25OHD from the extracellular fluid by facilitated endocytosis and use that 25OHD as substrate for the upregulated CYP27b1. $1,25(OH)_2D$ synthesized intracellularly via the CYP27b1 is then free to interact in an intracrine mode with the VDR, engage the retinoid X receptor, transactivate the endogenous defensin gene, cathelicidin, and promote cathelicidin gene product (LL-37)-directed killing of ingested M.tb. These data predict that a decrement in the extracellular content of 25OHD may be a limiting factor for effective M.tb. killing; in fact, data showed that extracellular $25OHD_3$ was as or more effective than equimolar concentrations of the hormone $1,25(OH)_2D_3$ in inducing cathelicidin expression in macrophages despite the fact that $1,25(OH)_2D_3$ is bound by the VDR with a 1,000-fold greater affinity that is $25OHD_3$. To test this hypothesis human monocyte-macrophage cathelicidin expression and M.tb. killing was examined in cells incubated with 25OHD-deficient serum from sunlight-deprived African Americans and from a group of vitamin D-sufficient Caucasians. The 25OHD-deficient serum was significantly less capable of supporting cathelicidin gene expression and bacterial killing than was vitamin D-sufficient serum; importantly both events could be rescued by restoring the extracellular 25OHD levels to normal.

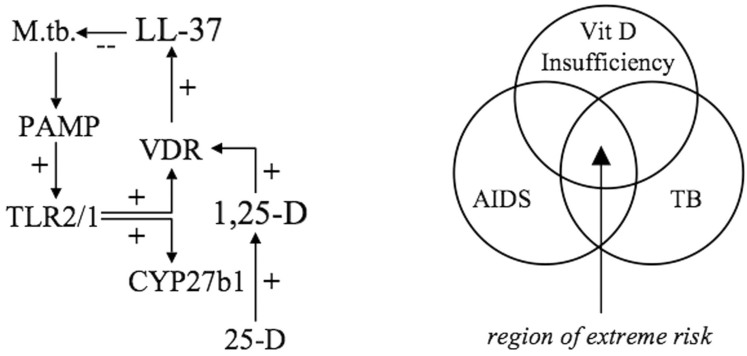

FIGURE 1. Vitamin D and Tuberculosis. The left panel schematic shows the stimulation of the human macrophage TLR2/1 by pathogen-associated molecular pattern (PAMP) ligand from mycobacterium tuberculosis (M.tb.) leading to induction of expression of the CYP27b1-hydroxylase and vitamin D receptor (VDR). In the presence of substrate 25-hydroxyvitamin D (25-D), internalized of the serum vitamin D-binding protein (not shown), the mitochondrial CYP27b1-hydroxylase is able to generate sufficient quantities of 1,25-dihydroxyvitamin D (1,25-D) to ligand the VDR in an intracrine mode, permit dimerization with the retinoid X receptor (not shown) and greatly enhance cathelicidin gene expression leading to the generation of endogenous defensin-like LL-37 molecule and subsequent mycobacterial killing. The right panel diagram describes the modern-day confluence of AIDS, TB, and vitamin D insufficiency pandemics affecting many parts of the world. The arrow indicates the "region of extreme risk" to the host created by the concurrence of these disorders.

Collectively, these observations have underlined the pivotal role of macrophage CYP27b1 in mediating the effects of vitamin D on innate immunity and illustrate the potential impact of vitamin D status of the host on nonskeletal aspects of human physiology (FIG. 1).

We have chosen to study tuberculosis (TB) as a model, because it is a disease of global proportions that poses a major infectious disease risk and it provides a model for studying the human immune response to an intracellular pathogen. According to the World Health Organization[42] infection with M.tb. kills a human being every 15 sec. In 2005 alone, there were estimated to be nearly 2 million deaths worldwide attributed to TB. At any one time, fully a third of the world's population is infected with M.tb., and 8.8 million of these will progress to an active form of the disease during the course of the next 12 months. TB is one of the leading causes of death worldwide in women of reproductive age[43] and in individuals infected with HIV.[44] To bring this pandemic closer to home, estimates are that 10–15 million people residing in the United States are infected with M.tb. And, like the situation worldwide, mycobacterial infection is a leading cause of death among patients with AIDS in this country. The concern for the AIDS patient is acute given the recent emergence of extensively drug-resistant (XDR) TB and its high mortality rate.

A second reason to study TB is that it provides an important model for investigation of the human immune response to an intracellular pathogen. By investigating human TB, we have shown that: the human innate immune system recognizes microbial lipoproteins via TLR2;[34] that activation via TLR2 of human monocyte-macrophages leads to instruction of the adaptive immune response via (i) release of IL-12[34] and (ii) dendritic cell differentiation and maturation;[45,46] and activation of monocyte-macrophages via TLR2 triggers (i) macrophage differentiation,[45] (ii) a nitric oxide-dependent antimicrobial pathway in mice,[47] and (iii) a vitamin D-dependent antimicrobial pathway in humans.[31] Furthermore, TLR2 has been shown to be important for resistance to TB in mouse models[37] and polymorphisms in TLR2 are associated with susceptibility to TB in humans.[48–50]

There have been many genetic and epidemiological studies linking various factors to susceptibility to TB, many of which have focused on various aspects of the vitamin D pathway. Several studies have linked serum 25OHD levels to both TB disease progression and susceptibility. In 1985, Grange *et al*.[51] reported that of 40 Indonesian patients with active TB and treated with anti-TB chemotherapy, the 10 patients with the highest 25OHD levels at the outset of therapy had "less active pulmonary disease." In 2000, Wilkinson *et al*.[52] investigated the relationship between vitamin D deficiency and VDR polymorphisms with tuberculosis in the Gujarati Asians living in West London. They found that severity of vitamin D deficiency correlated with increasing risk of TB, and that certain VDR genotypes correlated with disease only when 25OHD levels were deficient. Our study discussed above demonstrated the novel finding that TLR2/1-activated human monocyte-macrophages required sufficient levels of $25OHD_3$ in culture to initiate host defense mechanisms. Taken together, these studies indicate that host defense against M.tb. is dependent upon the serum 25OHD level of the host and not that of the active $1,25(OH)_2D$ hormone.

There is a long history of using orally administered vitamin D to treat mycobacterial infections with apparent success. In 1946, Dowling[53] reported the treatment of patients with lupus vulgaris (a form of cutaneous TB) with oral vitamin D2. Eighteen of 32 patients appeared to be cured, nine improved. Morcos *et al*. treated 24 newly diagnosed cases of TB in children with standard chemotherapy with and without vitamin D.[54] They noted clinical and radiological improvement in the group treated with vitamin D. Nursyam *et al*. administered vitamin D or placebo to 67 TB patients during a 6-week course of standard TB treatment;[55] 100% of the vitamin D-supplemented group had radiological improvement as well as a significantly higher sputum conversion rate from AFB-positive to AFB-negative (100%) than placebo group (76.7%; $P = 0.002$). The most recent supporting evidence comes from the just published work of Martineau[56] who performed a randomized, double-blind, controlled trial of placebo or a one-time dose of 1 M IU of vitamin D in a group 192 healthy tuberculosis contacts in London City. They found that the whole blood

from vitamin D-treated contacts was significantly more capable of suppressing the proliferation of the M.tb. surrogate BCG *in vitro* compared to that of placebo-treated subjects ($P < 0.03$) and the 25OHD content of both extracts of the serum and the conditioned medium of autologously harvested and cultured monocytes-macrophages from vitamin D-treated subjects was increased significantly ($P < 0.001$) compared to placebo controls. These results in vitamin D-treated vitamin D-insufficient subjects (Hewison, unpublished) suggest that the TB-challenged human monocyte-macrophage can effectively generate enough of its own $1,25(OH)_2D$ from substrate 25OHD to provide a positive therapeutic outcome in humans infected with M.tb.

Progress in curtailing the human death rate from TB has been hampered by access to, cost, and effectiveness of current antibiotic regimens. As such, developing safe, simple, and cost-effective means of boosting the endogenous innate immune response to M.tb. in susceptible populations will continue to be a major focus for work in the field. A clinical trial is under way (Adams and Hewison, unpublished) to rescue the antimicrobial action of M.tb.-challenged human macrophages using paired sera from human subjects pre- and post-repair of vitamin D insufficiency *in vivo*. Considering the current coordinate pandemics in vitamin D insufficiency, tuberculosis, and AIDS across sub-Saharan Africa and South Asia, simple repair of vitamin D insufficiency in those populations may prove to be effective adjuvant therapy for the immunocompromised host with tuberculosis.

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