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Review

THE IMMUNOLOGICAL FUNCTIONS OF THE VITAMIN D ENDOCRINE SYSTEM

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Abstract - The discoveries that activated macrophages produce 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$), and that immune system cells express the vitamin D receptor (VDR), suggested that the vitamin D endocrine system influences immune system function. In this review, we compare and contrast how 1α,25-$(OH)_2D_3$ synthesis and degradation is regulated in kidney cells and activated macrophages, summarize data on hormone receptor function and expression in lymphocytes and myeloid lineage cells, and discuss how locally-produced 1α,25-$(OH)_2D_3$ may activate a negative feed-back loop at sites of inflammation. Studies of immunity in humans and animals lacking VDR function, or lacking vitamin D, are reviewed to gain insight into the immunological functions of the vitamin D endocrine system. The strong associations between poor vitamin D nutrition, particular *VDR* alleles, and susceptibility to chronic mycobacterial infections, together with evidence that 1α,25-$(OH)_2D_3$ served as a vaccine adjuvant enhancing antibody-mediated immunity, suggest a model wherein high levels of 1α,25-$(OH)_2D_3$-liganded VDR transcriptional activity may promote the $CD4^+$ T helper 2 (Th2) cell-mediated and mucosal antibody responses to cutaneous antigens *in vivo*. We also review a diverse and rapidly growing body of epidemiological, climatological, genetic, nutritional and biological evidence indicating that the vitamin D endocrine system functions in the establishment and/or maintenance of immunological self tolerance. Studies done in animal models of multiple sclerosis (MS), insulin-dependent diabetes mellitus (IDDM), inflammatory bowel disease (IBD), and transplantation support a model wherein the 1α,25-$(OH)_2D_3$ may augment the function of suppressor T cells that maintain self tolerance to organ-specific self antigens. The recent progress in infectious disease, autoimmunity and transplantation has stimulated a gratifying renaissance of interest in the vitamin D endocrine system and its role in immunological health.

Key words: ??

Abbreviations: **1α-OHase**: 25-hydroxyvitamin D_3-1α-hydroxylase; **1α,25-$(OH)_2D_3$**: 1a,25-dihydroxyvitamin D_3; **24-OHase**: 1a,25-dihydroxyvitamin D_3-24-hydroxylase; **25-OHase**: vitamin D_3-25-hydroxylase; **25-$(OH)D_3$**: 25-hydroxyvitamin D_3; **APC**: antigen-presenting cell; **CNS**: central nervous system; **DBP**: vitamin D binding protein; **DC**: dendritic cell; **EAE**: experimental autoimmune encephalomyelitis; **Gc**: group-specific component of plasma α2-globulin; **GM-CSF**: granulocyte-macrophage colony stimulating factor; **HVDRR**: hereditary vitamin D-resistant rickets; **IBD**: inflammatory bowel disease; **IDDM**: insulin-dependent diabetes mellitus; **IFN-γ**: interferon-gamma; **IL**: interleukin; **MBP**: myelin basic protein; **MHC**: major histocompatibility complex; **MS**: multiple sclerosis; **NOD**: non-obese diabetic; **RXR**: retinoid X receptor; **STAT**: signal transducer and activator of transcription; **TCR**: T cell receptor; **TGF-β**: transforming growth factor beta; **Th1**: CD4+ T helper type 1 cells; **Th2**: CD4+ T helper type 2 cells; **TNF-α**: tumor necrosis factor alpha; **UC**: ulcerative colitis; **UVB**: ultraviolet B; **VDDR-1**: vitamin D-dependent rickets; **VDR**: vitamin D receptor; **VDRE**: vitamin D responsive element

INTRODUCTION

The vitamin D endocrine system is one of the most sensitive and complex biological systems that terrestrial vertebrates use to sense sunlight. Until two decades ago, the human vitamin D endocrine system was recognized only for it's regulation of Ca^{2+} and phosphorous homeostasis and skeletal formation and maintenance (100). New evidence showing that activated macrophages produce the hormone 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) (2,3,4), and that the nuclear vitamin D receptor (VDR) is present in immune system cells (27,199,208), suggested new immunological functions for the light-sensing vitamin D endocrine system.

This article will provide background information on the synthesis and degradation of the vitamin D hormone, 1α,25-$(OH)_2D_3$ and the structure and function of the VDR.

It will then review recent advances in our understanding of how the vitamin D endocrine system regulates infectious disease, autoimmune disease, and transplantation tolerance, emphasizing insights into the molecular basis for these immunological functions of 1α,25-$(OH)_2D_3$. Many reviews on the subject of vitamin D and the immune system have been published (9,32,38,44,101,102, 103,104,137,138,139,150,151,153,155,157,171,221,258, 264). Therefore, this article will not attempt to provide a comprehensive review of the literature on vitamin D and the immune system, particularly as regards *in vitro* investigations of the hormone's immunological functions. Rather, it will focus on the insights gained through the study of vitamin D and immune system function *in vivo*, and attempt to integrate the evidence into cohesive models describing how 1α,25-$(OH)_2D_3$ regulates immune system function. The article will close with a discussion of unresolved issues that warrant continued active investigation.

THE VITAMIN D HORMONE AND ITS NUCLEAR VITAMIN D RECEPTOR

Biosynthesis and degradation of 1α,25-$(OH)_2D_3$

Terrestrial vertebrates acquire vitamin D_3 mainly through exposure of skin to sunlight (108). There is a common misconception that fortified foods and vitamins supply adequate vitamin D (274). However, some of these sources provide the plant secosteroid, vitamin D_2, which has less vitamin D activity than vitamin D_3 (263). In addition, the vitamin D dose supplied by these sources is insufficient to prevent bone loss (109). The amount of vitamin D required to fulfill it's immunological functions is not known and may be significantly higher than the amount required for mineral ion homeostasis and bone health (272). Most importantly though, only vitamin D_3 derived from sunlight exposure can contribute to vitamin D_3 stores (mainly in muscle and adipose tissue) and thus supply vitamin D for future use. Dietary vitamin D is transported to the liver via the chylomicron remnant, where it is rapidly and completely metabolized. Thus, dietary sources provide an inconstant and usually meager supply of vitamin D that cannot be stored for the future. In addition, if highly localized vitamin D metabolism is required for the immunological functions of vitamin D (see below), then stored vitamin D, acquired through sunlight exposure, may be very important for immunological health.

Exposure of skin to sunlight catalyzes the first step in vitamin D_3 biosynthesis. Ultraviolet B (UVB) photons (290-320 nm) rupture the 9-10 bond of 7-dehydrocholesterol, generating pre-vitamin D_3, which spontaneously isomerizes to vitamin D_3 (Fig. 1). The solar radiation intensity, which varies with latitude and season, determines the cutaneous vitamin D synthesis rate, and hence vitamin D nutrition (108). In Boston (42ºN), vitamin D synthesis rates in skin exposed to mid-day sun are negligible from November through February, because the UVB photons do not have enough energy to mediate the photolysis reaction. The higher the latitude, the greater is the period of negligible vitamin D synthesis, and hence, the higher is the probability that vitamin D insufficiency will occur. The cutaneous vitamin D synthesis rate also decreases with increasing skin pigmentation, advancing age, clothing and sunscreen use (108).

Two enzymatic activation steps are required to produce 1α,25-$(OH)_2D_3$, the biologically active vitamin D hormone (35, 110, 183). The three hydroxylase enzymes that carry out the metabolic activation and degradation of vitamin D_3 are all mitochondrial enzymes belonging to the cytochrome P450 superfamily of hydroxylase enzymes (192). The vitamin D binding protein (DBP), encoded by the *Gc* locus (group-specific component of plasma a2-globulin), transports vitamin D_3 to the liver (121). The liver constitutively expresses the *CYP2D25* gene encoding the vitamin D_3-25-hydroxylase (25-OHase) that catalyzes the

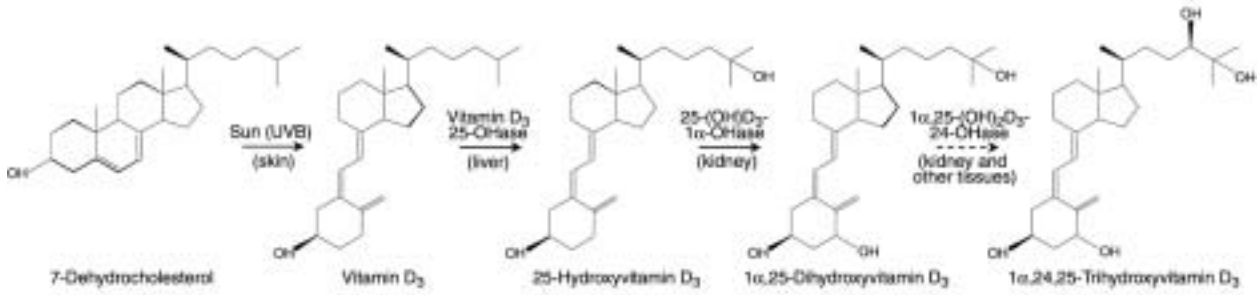


Fig. 1 Vitamin D_3 metabolism (81,121). Biologically inert vitamin D_3, derived mainly from the UVB light catalyzed photolysis of 7-dehydrocholesterol in the skin, is C-25 hydroxylated in the liver and C-1 hydroxylated in the kidney and other tissues to generate the biologically active hormone 1α,25-$(OH)_2D_3$. The enzymes vitamin D_3-25-hydroxylase (25-OHase) and 25-hydroxyvitamin D_3-1-a-hydroxylase (1α-OHase) catalyze the metabolic activation of vitamin D_3. The enzyme 1α,25-dihydroxyvitamin D_3-24-hydroxylase (24-OHase) catalyzes the first step in 1α,25-$(OH)_2D_3$ inactivation.

C-25 hydroxylation of vitamin D_3 (111). Therefore, the vitamin D_3 delivered to the liver is rapidly converted into 25-hydroxyvitamin D_3 (25-$(OH)D_3$).

The blood 25-$(OH)D_3$ level is widely used as an indicator of vitamin D nutrition (273). The 25-$(OH)D_3$ is the most abundant vitamin D metabolite, reflecting the quantity of vitamin D impinging on the liver from the diet, the skin and storage sites. The blood 25-$(OH)D_3$ also has a relatively long half-life, approximately 15-30 days. Since sunlight exposure is the main vitamin D source, and sunlight availability and intensity vary seasonally, the 25-$(OH)D_3$ also varies seasonally, reaching a peak in early fall and a nadir in early spring (108). The combination of abundance, variability, and accessibility make the blood 25-$(OH)D_3$ measurements particularly useful for assessment of nutritional status.

The liver exports 25-$(OH)D_3$ bound to DBP. The secosteroid-carrier complex enters the renal proximal tubules via cubilin binding and megalin-mediated endocytosis (106,283). A tightly-regulated kidney enzyme, 25-hydroxyvitamin D_3-1α-hydroxylase (1α-OHase), catalyzes the rate-limiting C-1 hydroxylation step in 1α,25-$(OH)_2D_3$ synthesis (192). The *CYP27B1* gene encoding the 1α-OHase has been cloned (255). Loss-of-function mutations in this gene cause the inherited disorder vitamin D-dependent rickets type 1 (VDDR-1), which can be corrected by administering 1α,25-$(OH)_2D_3$ (125,129,246, 279). The 1,25-$(OH)_2D_3$ circulates in blood bound to the DBP at a concentration typically between 0.01-0.1 nmol/l (36).

The metabolic inactivation of 1α,25-$(OH)_2D_3$ also occurs in the kidney, as well as in other target tissues such as intestine and bone (121). It begins with a C-24 hydroxylation to 1α,24,25-trihydroxyvitamin D_3 catalyzed by 1α,25-dihydroxyvitamin D_3-24-hydroxylase (24-OHase) (192). Catabolism continues with further oxidation, hydroxylation, side-chain cleavage to the C-23 alcohol, and finally oxidation to the excreted, water soluble C-23 carboxylic acid, calcitroic acid. The *CYP24A1* gene encoding the 24-OHase was cloned from rat kidney cells (189). A *CYP24A1*-null mouse has been generated; it had high 1α,25-$(OH)_2D_3$ levels confirming the role of the 24-OHase in hormone catabolism (245).

Regulation of 1,25$(OH)_2D_3$ biosynthesis and degradation

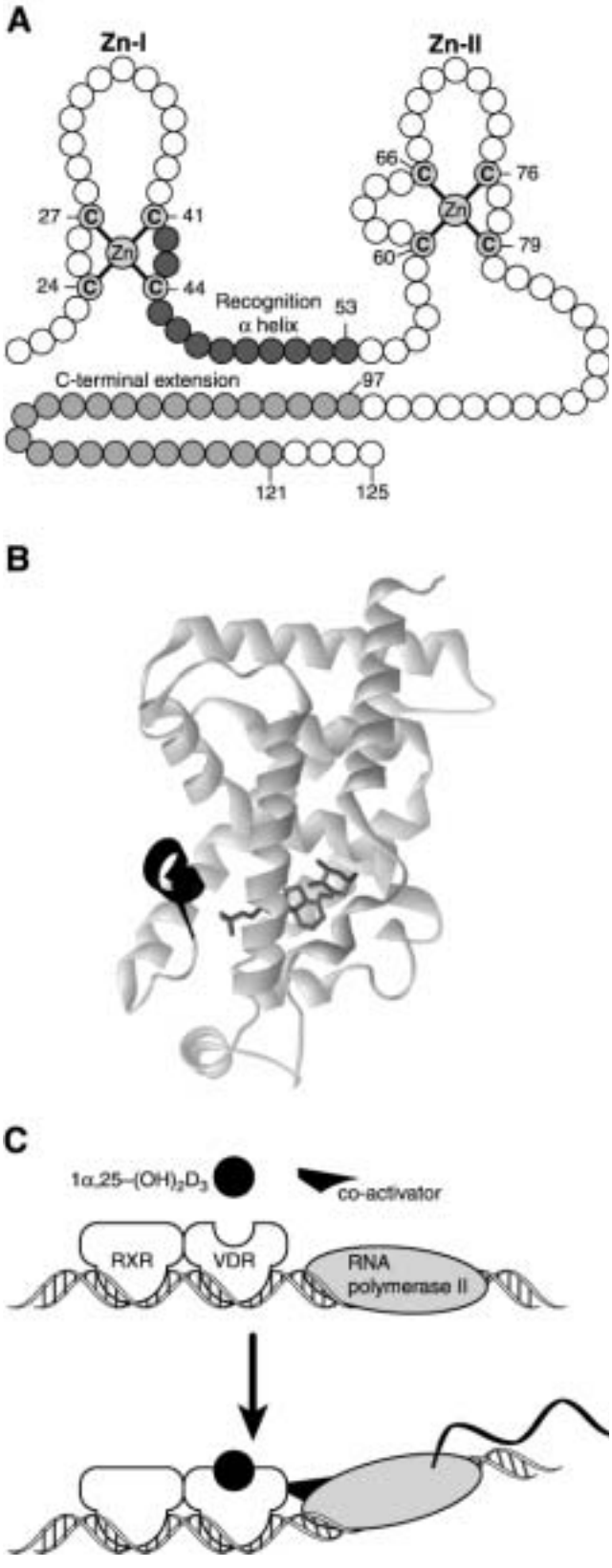
Feedback regulation mechanisms govern systemic hormone synthesis and degradation, such that the blood 1α,25-$(OH)_2D_3$ levels are nearly invariant (105). In the intact animal, the parathyroid glands sense low serum calcium levels and release parathyroid hormone. The parathyroid hormone binds to a receptor on kidney cells, initiating a cAMP signaling cascade that stimulates *CYP27B1* gene transcription by means of four cAMP-responsive elements in the promoter (132). The enhanced *CYP27B1* gene transcription leads to increased 1α,25-$(OH)_2D_3$ synthesis and rising serum calcium levels. The rising serum 1α,25-$(OH)_2D_3$ levels activate VDR-dependent feed-back loops that repress *CYP27B1* gene transcription (132,255), and stimulate *CYP24A1* gene transcription, slowing hormone synthesis and accelerating hormone degradation. It is noteworthy that the *CYP27B1* promoter has sequences corresponding to canonical AP-1, AP-2, Sp1 and NF-κB elements, suggesting that its regulation is very complex and possibly distinct in different cell types (132).

Vitamin D receptor (NR1I1) structure and function

The VDR is an ancient member of the superfamily of nuclear receptors for steroid hormones. The VDR forms a heterodimer complex with the retinoid X receptor (RXR) and functions as a ligand-activated transcription regulator (31,61,73,100). Like the other steroid hormone receptor family members, the VDR exhibits a modular structure. It has an N-terminal DNA-binding domain linked by a flexible hinge region to a C-terminal ligand-binding domain that includes the RXR dimerization interface. The RXR-VDR complex regulates gene expression through vitamin D responsive elements (VDRE) in the promoters of 1α,25-$(OH)_2D_3$-responsive genes (31,61,73,100). The VDRE is composed of two hexameric half-sites, arranged as direct repeats, separated by three random base pairs. For example, the VDRE in the osteopontin promoter, one of the highest affinity elements known, had the sequence GGTTCACGAGGTTCA (181,260). The *CYP24A1* gene actually has two VDRE in the promoter (126).

High resolution crystal structures have been reported for the DNA-binding domain bound to a VDRE (236), and for the holo VDR ligand-binding domain (lacking residues 165-215) (226). The protein core of the VDR DNA-binding domain is organized into two zinc-nucleated modules (Fig. 2A). The half-site recognition helix formed by residues on the C-terminal side of the first zinc finger inserts directly into the major groove of the VDRE half-site. The adjacent C-terminal extension imparts additional specificity (113). A nuclear localization signal is located between the two zinc fingers (112). The VDR ligand-binding domain has an activation helix that undergoes a major conformational change upon ligand binding (Fig. 2B). The re-positioning of the activation helix allows the VDR-RXR complex to recruit VDR interacting proteins termed co-activators that promote chromatin remodeling, recruitment of RNA polymerase II holoenzyme, and gene transcription (47,148,213) (Fig. 2C).

The VDR subdomains important for DNA binding, hormone binding, dimerization, and transactivation are mostly conserved in all vertebrate species studied, including fish, frog, chicken, mouse, rat and human (146,252). The expression of a highly conserved VDR in



species that span a considerable evolutionary distance suggests that this receptor has pleiotrophic functions coordinating the availability of light, interpreted biologically as 1α,25-$(OH)_2D_3$ abundance, with a variety of biological processes. The classical functions of the VDR are regulation of blood calcium and phosphate concentrations and bone metabolism through control of gene expression in the intestine, bone, kidney, and parathyroid gland (31,61,73,100).

Allelic variants of the chromosome 12 *VDR* gene occur naturally in the human population (267,280,294). The natural variants are distinguishable by the sensitivity of the DNA to the restriction endonucleases *Fok*I (VDR^f), *Apa*I (VDR^a), *Bsm*I (VDR^b) and *Taq*I (VDR^t), with the lower case letter denoting the presence of the endonuclease site. The *Fok*I restriction enzyme detects a start codon polymorphism (231). The VDR^f allele uses the first start codon and encodes a VDR that is three amino acids longer than the VDR^F allele, which uses the second start codon (124). The VDR^f allele was transcriptionally less active (124,280). The *Bsm*I and *Apa*I enzymes detect intronic polymorphisms, whereas the *Taq*I enzyme detects a silent base change in codon 352 (168,267,280). These three polymorphisms are clustered near the 3' end of the *VDR* gene. They are in strong linkage disequilibrium with a singlet (A) repeat in exon 9 that results in long (*L*) and short (*S*) alleles (168,267,280). The VDR^L allele was transcriptionally more active than the VDR^S allele (280). In European and Asian populations, the three common haplotypes involving the 3' end polymorphisms were *baTL*, *BAtS*, and *bATL*, with the *BAtS* haplotype being transcriptionally less active (267,280).

VITAMIN D METABOLISM AND VITAMIN D RECEPTOR EXPRESSION IN IMMUNE SYSTEM CELLS

Vitamin D metabolism in immune system cells

In 1982-83, two seminal discoveries introduced a new era in vitamin D research, the study of the vitamin D

Fig. 2 Structure and function of the VDR. Panel **A**) the VDR core DNA-binding domain has a darkly-shaded DNA half-site recognition helix on the C-terminal side of the first of two zinc finger motifs (236). The lightly-shaded residues form a C-terminal extension that is involved in DNA response element discrimination. Panel **B**) the VDR ligand-binding domain, represented as a ribbon diagram, complexed with 1α,25-$(OH)_2D_3$ (226). The position of the shaded helix (H14 in the Protein Data Base entry 1DB1; H12 in ref. 226) is ligand dependent and critical for co-activator binding and transactivation function. The image was created with Accelrys WebLabViewer Lite (version 3.2 for Macintosh OS 9). Panel **C**) the RXR-VDR-ligand complex recruits co-activator proteins and the RNA polymerase II holoenzyme to activate transcription (47, 61).

endocrine system's immunological functions. Two research groups found evidence of VDR expression in hematopoietic cells (27, 199, 208). Moreover, a third research group reported that pulmonary alveolar macrophages from sarcoidosis patients synthesized 1α,25-$(OH)_2D_3$, which was the first report of extra-renal 1α,25-$(OH)_2D_3$ synthesis (2,3,4). Together, these observations suggested that beyond the established functions of the hormone in mineral ion homeostasis and bone biology, locally produced 1α,25-$(OH)_2D_3$ might perform regulatory functions in immune system cells.

The enzymes that catalyze 1α,25-$(OH)_2D_3$ synthesis and degradation in kidney cells and sarcoid macrophages are identical, but these cells differ significantly in how the enzyme levels are regulated, and therefore in hormone production. The renal and sarcoid macrophage 1α-OHases had similar affinity and specificity for 25-hydroxylated substrates (2,4) and identical cDNA sequences (193). However, the 1α,25-$(OH)_2D_3$ suppressed its own synthesis in kidney cells, but not in sarcoid macrophages (105). In addition, in macrophages but not in kidney cells, interferon-gamma (IFN-γ) treatment stimulated a 6-fold increase in the *CYP27B1* transcripts encoding the 1α-OHase (193). This IFN-γ-mediated increase in *CYP27B1* transcripts was also observed in macrophages from other granulomatous diseases (21) and from normal tissue (65,105,293).

A second significant difference between kidney cells and activated macrophages relates to 1α,25-$(OH)_2D_3$ degradation. In kidney cells, the 1α,25-$(OH)_2D_3$ induced the *CYP24A1* transcripts encoding the 24-OHase, thereby increasing the hormone's degradation (192). In contrast, the 1α,25-$(OH)_2D_3$ did not induce *CYP24A1* transcripts in the IFN-γ-activated macrophages (2,4). Instead, the IFN-γ triggered activation of the signal transducer and activator of transcription 1 (STAT1), the STAT1 sequestered the VDR, and without the VDR, transactivation of the *CYP24A1* promoter via the tandem VDRE did not occur (67, 271). Thus in IFN-γ-activated macrophages, the *CYP24A1* gene was resistant to 1α,25-$(OH)_2D_3$-mediated induction.

The IFN-γ-activated macrophages can produce 1α,25-$(OH)_2D_3$ at a very high rate, because they have a high ratio of the 1α-OHase to the 24-OHase. This high 1α,25-$(OH)_2D_3$ synthetic capability led to the hypothesis that the 1α,25-$(OH)_2D_3$ produced locally by activated macrophages might perform immunoregulatory functions at sites of inflammation (218) (Fig. 3A). High level 1α,25-$(OH)_2D_3$ synthesis may only occur in activated macrophages that have some minimum level of 25-$(OH)D_3$ substrate for the 1α-OHase. Individuals with low sunlight exposure may have a 25-$(OH)D_3$ level that is too low to support high level 1α,25-$(OH)_2D_3$ synthesis by activated macrophages, which may compromise the postulated hormone-dependent immunoregulatory feed back loop.

Immunological phenotype of 1α-OHase-mutant humans and animals

The immune system functions of 1α,25-$(OH)_2D_3$ *in vivo* are not well understood. One approach to understanding these functions is to examine immune dysfunction in humans and animals lacking the 1α-OHase. Humans with loss-of-function mutations in *CYP27B1* have the disease VDDR-1, due to insufficient 1α,25-$(OH)_2D_3$ synthesis (125,129,246,279). In mice, a targeted disruption of the *CYP27B1* gene generated an animal model of VDDR-1 (56,195). The humans and mice with VDDR-1 are normal at birth, but develop hypocalcemia, hypophosphatemia, secondary hyperparathyroidism, skeletal deformities, and reproductive problems as a consequence of very low serum 1α,25-$(OH)_2D_3$. The *CYP27B1*-null mice developed enlarged lymph nodes and had decreased numbers of peripheral $CD4^+$ and $CD8^+$ T cells (195). These immunological abnormalities were not observed in humans or rodents lacking VDR function (see below), so they may not relate directly to lack of 1α,25-$(OH)_2D_3$-VDR function. The *CYP27B1*-null mice did not spontaneously develop autoimmune disease, so a loss-of-function mutation in *CYP27B1* was not sufficient to precipitate autoimmune disease. The relative susceptibility of these mice to infection or to induced autoimmunity has not been investigated. A *CYP24A1*-null mouse has been generated (245), but the immunological phenotype of the mutant has not been reported.

VDR expression in immunologically relevant cells

Reports that the VDR was expressed in hematopoietic cells (27, 199, 208) contributed to the hypothesis that locally-produced 1α,25-$(OH)_2D_3$ may perform regulatory functions in immune system cells at the site of inflammation (218) (Fig. 3A). The VDR was expressed constitutively in myeloid lineage cells (27,199,208), in particular monocytes, dendritic cells (DC), microglia, and astrocytes (Table 1 and references therein). Mature, mitotically active, medullary thymocytes also constitutively expressed the VDR (205,207), consistent with the possibility that the 1α,25-$(OH)_2D_3$ may perform some functions in these cells.

Mature, peripheral T lymphocytes significantly increased their VDR expression after activation. For example, VDR expression increased from 1800 to 8700 molecules/cell when resting murine $CD4^+$ T cells underwent activation (134). High level VDR gene expression was demonstrated in activated murine $CD4^+$ T helper (Th) type-1 and type-2 cells (177) and $CD8^+$ T cells (270). Similarly, activated human $CD4^+$ and $CD8^+$ T cells expressed the VDR (206), as did activated human B lymphocytes (166,167,208,209). In rheumatoid arthritis patients, the lymphocytes expressed the VDR without further *in vitro* activation, suggesting they had undergone activation *in vivo* (154,257).

Table 1 VDR expression in immunologically relevant cells

Cell Type	Species	Analytical Method	References
Myeloid lineage cells			
Monocytes	human	Ligand binding and sedimentation	(27,208)
Dendritic cells	human	Ligand binding and sedimentation	(33)
Lymphocytes			
B cells, activated	human	Ligand binding and sedimentation	(208,209,278)
Thymocytes, mature medullary	rat	Ligand binding and sedimentation	(205,206,207)
T cells, activated	human	Ligand binding and sedimentation; immunochemistry; PCR of cDNA	(27,207,208,291)
$CD4^+$ T cells, activated	human	Ligand binding and sedimentation	(206)
$CD4^+$ T cells, activated	mouse	Ligand binding and sedimentation; immunochemistry	(270)
$CD4^+$ Th1 cells, activated	mouse	Ligand binding and sedimentation; PCR of cDNA	(29,177)
$CD4^+$ Th2 cells, activated	mouse	Ligand binding and sedimentation; PCR of cDNA	(134,177)
$CD8^+$ T cells, activated	human	Ligand binding and sedimentation	(206)
$CD8^+$ T cells, activated	mouse	Ligand binding and sedimentation; immunochemistry	(270)
T cells, rheumatoid arthritis	human	Ligand binding and sedimentation	(209)
CNS			
Astrocytes	rat	Immunochemistry	(136)
Glial cells	hamster	Autoradiography	(251)
Neurons	hamster	Autoradiography	(251)
Neurons	rat	Immunochemistry	(34,136,211,277)
Oligodendrocytes	rat	*In situ* hybridization; immunochemistry	(17)

The VDR expression data suggest that myeloid cells would be constitutively 1α,25-$(OH)_2D_3$ responsive, but lymphocytes would be responsive only after activation. It is important to note that as monocytes differentiated into macrophages, they increased their 1α,25-$(OH)_2D_3$ production, but decreased their VDR expression (133). This observation suggests that at the site of an inflammation, the activated macrophages may produce hormone that acts via a paracrine rather than an autocrine pathway to regulate nearby lymphocytes. In B lymphocytes activated through the B cell antigen receptor and CD40 in the presence of interleukin (IL)-4, the 1α,25-$(OH)_2D_3$ induced expression of the *CYP24A1* gene encoding the 24-OHase that degrades the hormone (166, 167)**.** Thus, activated B cells might inactivate the locally-produced 1α,25-$(OH)_2D_3$. Therefore, the activated T lymphocytes expressing high levels of the VDR and lacking the 24-OHase might be important targets of locally-produced 1α,25-$(OH)_2D_3$ (Fig. 3A).

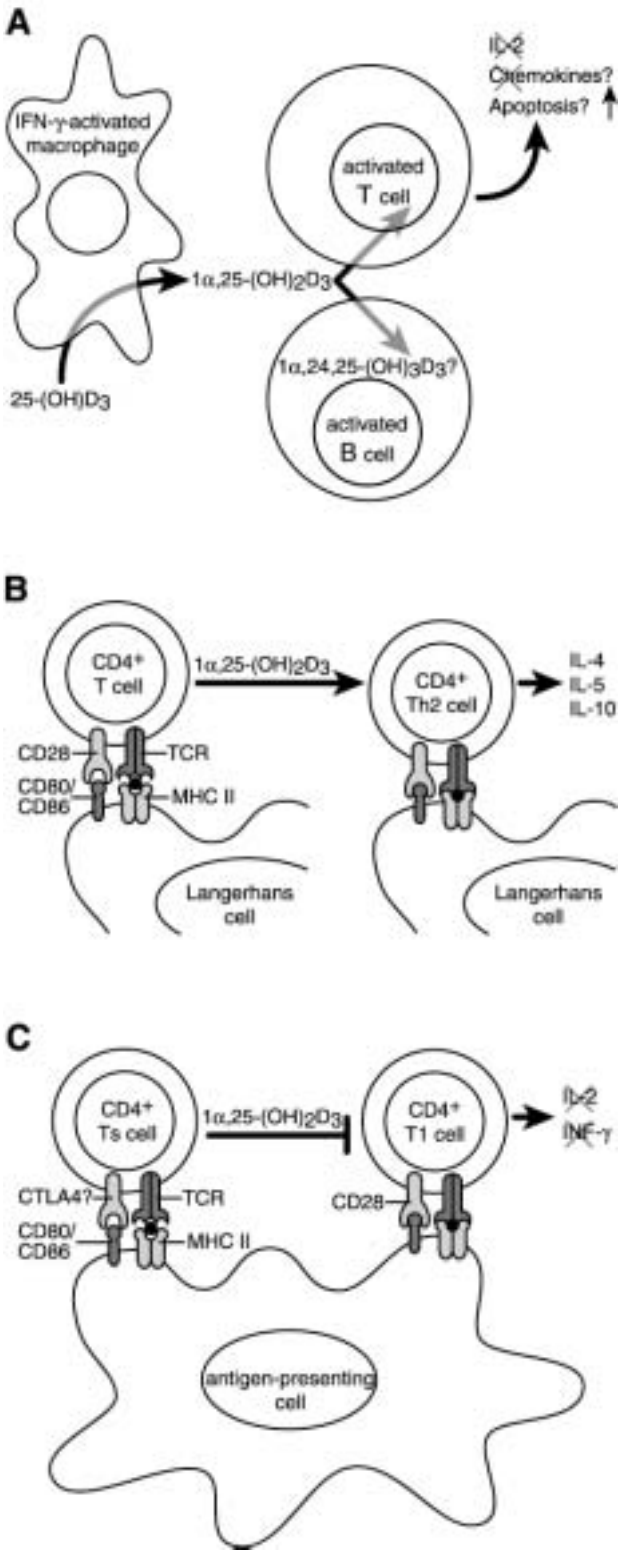
Immunological phenotype of VDR-mutant humans and animals

There is a widely-held and often articulated belief that the major immunological functions of 1α,25-$(OH)_2D_3$ are to inhibit cytokine synthesis by myeloid lineage cells and Th1 cells. The *in vitro* experiments that support this belief are summarized in Table 2 (myeloid cell studies) and Table 3 (T lymphocyte studies). In particular, it is commonly stated that 1α,25-$(OH)_2D_3$ inhibits IL-12 mRNA synthesis by antigen-presenting cells and also IFN-γ synthesis by Th1 cells, and in combination, these activities diminish the Th1 cell-mediated responses (139). However, the *in vitro* data are often conflicting, for example IL-1 and tumor necrosis factor-alpha (TNF-α) (Table 2), or cannot be reproduced *in vivo*, for example IL-12 (Table 2) and IFN-γ (Table 3).

Consequently, a clear understanding of the immunological functions of the vitamin D endocrine system must be derived from studies done *in vivo*.

Studies of immune function in humans and animals lacking VDR function have provided important insights into the immunological functions of 1α,25-$(OH)_2D_3$. Mutations in the *VDR* gene cause hereditary vitamin D-resistant rickets (HVDRR). The HVDRR patients had normal myeloid and lymphoid cell development, as determined by analysis of the cells populating the bone marrow, blood, and peripheral lymphoid organs (128,176). However, the HVDRR patients had frequent and severe episodes of infection (128). This result suggests that abnormalities do exist with respect to the innate and/or adaptive immune responses to infection when VDR function is impaired.

Like the HVDRR patients, mice lacking VDR function due to a targeted disruption of exon 2 had normal myeloid and lymphoid cell development (289). A flow cytometric analysis of neutrophils, macrophages, T cells, B cells, and natural killer cells in the bone marrow, thymus, spleen, and mesenteric lymph node showed no differences between the *VDR*-null and wild-type mice (289). A second study reported that the *VDR*-null and wild-type mice did not differ



as regards myelopoiesis, macrophage IL-12 synthesis, the Th1 or Th2 cell fate of $CD4^+$ T cells stimulated with antibodies to the CD3 component of the T cell receptor (TCR) complex plus antibodies to the CD28 co-stimulatory molecule, or the amount of Th1 cell IFN-γ synthesis (188). However, the *VDR*-null mice had impaired production of the Th1-promoting factor IL-18, a decreased Th1 cell proliferative response to CD3 and CD28 stimulation in the presence of exogenous IL-12, and decreased expression of STAT4, a Th1 cell transcription factor. Together, these results suggested that a functional VDR was essential for Th1 cell development (188). A third study reported no abnormalities of myelopoiesis or lymphopoiesis in the *VDR*-null mice, but noted a moderately lower proliferative response to CD3 stimulation in the *VDR*-null T cells (159). In addition, the *VDR*-null macrophages had normal phagocytosis and killing responses, but decreased chemotactic responses. Importantly, correcting the hypocalcemia of the *VDR*-null mice fully restored the macrophage chemotactic response, so this particular defect was a direct consequence of hypocalcemia, not the *VDR* mutation (159). Taken together, these definitive *in vivo* experiments in *VDR*-null mice contradicted a large number of *in vitro* studies reporting that $1\alpha,25\text{-}(OH)_2D_3$ inhibited IFN-γ and IL-12 mRNA synthesis and inhibited Th1 development.

VITAMIN D AND INFECTION

Vitamin D deficiency, VDR polymorphism, and frequency of infection

Vitamin D deficiency has long been correlated with a high incidence of infection, suggesting that this deficiency may enhance susceptibility to infection. The strongest

Fig. 3 Immunological functions of $1\alpha,25\text{-}(OH)_2D_3$. Panel **A**) locally-produced $1\alpha,25\text{-}(OH)_2D_3$ may limit inflammation (218). IFN-γ-activated macrophages synthesize $1\alpha,25\text{-}(OH)_2D_3$ at a high rate if there is sufficient $25\text{-}(OH)D_3$ substrate to saturate the 1α-OHase active sites (2,3,4, 21,65,67,131,193,216,217). The activated, VDR-expressing T lymphocytes adjacent to the $1\alpha,25\text{-}(OH)_2D_3$-producing macrophages may respond to the elevated hormone through altered gene expression and function. Possible activated T cell responses to the elevated $1\alpha,25\text{-}(OH)_2D_3$ might be increased apoptosis or decreased IL-2 and chemokine synthesis. The activated B lymphocytes may inactivate the $1\alpha,25(OH)_2D_3$, since they express the 24-OHase (166,167). Panel **B**) the $1\alpha,25\text{-}(OH)_2D_3$ enhanced the Th2 cell response to cutaneous antigens, as evidence by increased IL-4, IL-5, and IL-10 synthesis (60). Panel **C**) the $1\alpha,25\text{-}(OH)_2D_3$ $1\alpha,25\text{-}(OH)_2D_3$ $1\alpha,25\text{-}(OH)_2D_3$ may inhibit autoimmunity and transplanted tissue rejection by enhancing suppressor T (Ts) cell function. Whether the hormone acts directly on the VDR-expressing T cells or the antigen-presenting cell (APC), or both, is unknown.

evidence of this relationship involves mycobacterial diseases (24,57). Low serum 25-$(OH)D_3$ levels have been linked to increased susceptibility to *M. tuberculosis* and *M. leprae* infection (45,58,87,281). Conversely, vitamin D and sunlight were used successfully to treat mycobacterial diseases before anti-mycobacterial drugs became available (22). Additional studies showed that children with vitamin D-deficiency rickets suffered frequent infections and had a decrease in T lymphocytes (287). These results are consistent with the report that human HVDRR patients suffered frequent infections (128). These correlations suggest that vitamin D supports the immune defenses against mycobacterial diseases.

If there is enough vitamin D to support an immune response against mycobacterial diseases, it appears that subtle differences in VDR function may determine the quality of that response. The evidence suggesting this possibility is comprised of correlations between *VDR* locus polymorphisms and the type of anti-mycobacterial immune response that is made. A Th1 cell-mediated response is protective against mycobacterial (and viral) diseases. The less active VDR^t allele was associated with Th1 cell-mediated responses to tuberculin antigens in an Indian population (235). This allele was also associated with a low risk of chronic *M. tuberculosis* infection and chronic hepatitis B virus infection in Gambians, implying association with a Th1 cell-mediated response to these agents (22,23). Similarly, the less active VDR^f allele was associated with a low risk of chronic *M. malmoense* infection in UK patients, again suggesting a Th1 cell response (83). Together, these results imply that the less active VDR^t and VDR^f alleles in some way facilitated a strong, protective Th1 cell-mediated immune response.

A particularly interesting study involved *M. leprae* infection in Calcutta, India (229). The infections were classified as tuberculoid or lepromatous leprosy. Tuberculoid leprosy patients made a strong, protective Th1 lymphocyte-driven immune response to the bacterial pathogen, so they developed mild skin lesions with tuberculoid granulomata containing very few bacilli (285). Lepromatous leprosy patients made a strong but poorly protective Th2 lymphocyte-driven humoral immune response to the pathogen, so they developed severe skin lesions with bacilli-laden macrophages. The less active

Table 2 $1,25\text{-}(OH)_2D_3$-mediated control of myeloid cell cytokines

Cytokine	Cells	Species	Regulation	References
IL-1	Peripheral blood MC	human	enhancement *in vitro*	(28)
IL-1	Peripheral blood MC	human	inhibition *in vitro* and *in vivo*	(172,265)
IL-6	Peripheral blood MC	human	inhibition *in vivo*	(172)
TNF-α	HL-60 cells, U937 cells	human	enhancement *in vitro*	(204,248)
TNF-α	Peripheral blood monocytes, peritoneal macrophages	human	inhibition *in vitro*	(50,173)
TNF-α	Peripheral blood MC	human	inhibition *in vivo*	(172)
IL-12	RAW264.7 cells	mouse	inhibition *in vitro*	(54,143)
IL-12	Activated macrophages	mouse	no effect *in vivo*	(188)

MC: mononuclear cells

Table 3 $1,25\text{-}(OH)_2D_3$-mediated control of T lymphocyte cytokines

Gene	Cells	Species	Regulation	References
IL-2	Antigen-stimulated T lymphocyte hybridomas	mouse	inhibition *in vitro*	(29,139)
IL-2	Mitogen-stimulated peripheral blood MC	human	inhibition *in vitro*	(209,222,225,264)
IL-2	Peripheral blood mononuclear cells	human	no effect *in vivo*	(172)
IFN-γ	Mitogen-stimulated peripheral blood MC	human	inhibition *in vitro*	(173,175,219,225)
IFN-γ	Jurkat T cells	human	inhibition *in vitro*	(54)
IFN-γ	Activated Th1 cells	mouse	no effect *in vivo*	(39,43,177,188)
IFN-γ	Peripheral blood mononuclear cells	human	no effect *in vivo*	(172)
GM-CSF	Mitogen-stimulated peripheral blood MC	human	inhibition *in vitro*	(219,259)
GM-CSF	Jurkat T cells	human	inhibition *in vitro*	(261,262)
Osteopontin	Osteoblast	mouse	stimulation	(181)
FasL	T hybridoma cells	mouse	inhibition *in vitro*	(48)

MC: mononuclear cells

VDR^t allele was associated with tuberculoid leprosy (presumably a protective Th1 cell response), whereas the more active *VDR*^{T} allele was associated with lepromatous leprosy (presumably a non-protective Th2 cell response). Thus, the *VDR* genotype may have influenced the Th1 or Th2 cell lineage choice of newly-activated $CD4^+$ T cells specific for cutaneous *M. leprae* antigens, with the more transcriptionally active alleles possibly favoring the Th2 cell lineage (Fig. 3B). It is important to note that the association between the *VDR* genotype and tuberculosis, hepatitis B, and *M. leprae* infection was stronger in subjects with limited serum 25-$(OH)D_3$ levels than in subjects with substantial serum 25-$(OH)D_3$ levels (24,235,281,294). In this manner the genetic risk factor, *VDR* genotype, appeared to combine with an environmental risk factor, insufficient sunlight, to generate the phenotype of susceptibility to infection.

1α,25-$(OH)_2D_3$ as a vaccine adjuvant

Further evidence that vitamin D may enhance immunity to infection derives from studies of 1α,25-$(OH)_2D_3$ as a possible vaccine adjuvant (59). When mice were immunized with hepatitis B surface protein, and 1α,25-$(OH)_2D_3$ was applied to the skin at the immunization site, or included directly in the vaccine innoculum, the hormone increased the mucosal IgG1 and IgA responses to hepatitis antigen about 3-fold (60). Similar findings were reported for *Haemophilus influenzae* type b oligosaccharide-protein conjugate immunization (71). Consistent with the increased IgG1 and IgA responses, the 1α,25-$(OH)_2D_3$-treated animals developed a higher frequency of IL-4, IL-5 and IL-10-producing Th2 cells in the lymph nodes draining the subcutaneous immunization site than the controls. These *in vivo* results refute the idea (derived from *in vitro* studies) that the 1α,25-$(OH)_2D_3$ abrogated Th2 function and reduced IgG responses (122,140). Moreover, the results are consistent with the mycobacterial disease studies described above, and reinforce a model wherein a high level of 1α,25-$(OH)_2D_3$-liganded VDR transcriptional activity may promote newly activated $CD4^+$ T cells to adopt the Th2 cell fate in response to cutaneous antigens (Fig. 3B). The common mucosal immune system is integral to the host defense mechanisms that protect mucosal surfaces from colonization with infectious agents.

Experiments done *in vitro* have confirmed the ability of 1α,25-$(OH)_2D_3$ to promote $CD4^+$ Th2 cell development under some circumstances (30). $CD4^+$ T cells were increasingly driven to the Th2 cell fate, rather than the Th1 cell fate, when they were stimulated with antibodies to CD3 and to CD28 in the presence of 1α,25-$(OH)_2D_3$. The Th2 cell fate was characterized by *GATA-3* and *c-maf* gene expression, and IL-4-, IL-5- and IL-10-production. Details of the mechanism underlying this hormone action are not yet known.

1α,25-$(OH)_2D_3$-induced anti-mycobacterial activity in macrophages

The resistance of human peripheral blood monocyte-derived macrophages to *M. tuberculosis* infection has been studied *in vitro* (242). The 1α,25-$(OH)_2D_3$ treatment increased the membrane assembly of a functional NADPH-dependent phagocyte oxidase, which increased superoxide anion production and decreased *M. tuberculosis* growth. This vitamin D activity appeared to occur independently of VDR-mediated *de novo* transcription from a classical VDRE. Instead, it involved a rapid activation of the class I phosphatidylinositol 3-kinase. Other investigators have implicated protein-protein interactions between the VDR and this phosphatidyl-inositol 3-kinase in control of monocyte differentiation *in vitro* (107). These *in vitro* studies suggest that a novel non-genomic signaling pathway may mediate some effects of 1α,25-$(OH)_2D_3$ on monocyte differentiation and macrophage function. However, detailed studies documenting the importance of the proposed non-genomic signaling pathway *in vivo* will be needed to confirm this pathway's physiological relevance.

In summary, there is considerable evidence that 1α,25-$(OH)_2D_3$ and the VDR have important biological functions as regards the immune response to infectious disease. The associations between vitamin D nutrition, particular *VDR* alleles, and susceptibility or resistance to mycobacterial and viral infections indicates a likely causal relationship between VDR function as a ligand-activated transcription regulator and innate and adaptive immunity to infections. The intriguing studies on *M. leprae* disease phenotypes (229) and 1α,25-$(OH)_2D_3$ as a vaccine adjuvant (60) suggest that high levels of 1α,25$(OH)_2D_3$-liganded VDR transcriptional activity may promote the $CD4^+$ Th2 cell-mediated and mucosal antibody responses to cutaneous antigens *in vivo* (Fig. 3B).

VITAMIN D AND AUTOIMMUNE DISEASE

A diverse and rapidly growing body of evidence indicates that the vitamin D endocrine system has an important but poorly understood role in the establishment and/or maintenance of immunological self tolerance. Seminal studies demonstrated that administering 1α,25-$(OH)_2D_3$ inhibited disease induction in animal models of thyroiditis (74), experimental autoimmune encephalo-myelitis (EAE), a model of multiple sclerosis (MS) (39,142), systemic lupus erythematosis (144), psoriasis (69), insulin-dependent diabetes mellitus (IDDM) (160), and both collagen-induced arthritis and Lyme arthritis (40). The immune responses in these animal models of autoimmune diseases vary with respect to immune response type, target tissue, and autoantigens, indicating that the vitamin D endocrine system may be regulating an

immunological process that is common to all of these models. For example, locally-produced 1α,25-$(OH)_2D_3$ from activated macrophages may be acting on nearby VDR^+ T lymphocytes in a negative feed-back loop that resolves an inflammatory response before self tolerance mechanisms fail and autoimmunity results (**Fig. 3**).

Multiple sclerosis

The striking geographic distribution of MS suggested to others (84) and to us the possibility of a link between sunlight, vitamin D and MS risk (101,102). MS disease prevalence increased with increasing latitude in both hemispheres from a low of 1-2 cases per 10^5 population near the equator, to a high of >200 cases per 10^5 population at latitudes >50º (1). Among latitude-associated variables, average December solar radiation correlated most strongly (r = -0.8) with MS prevalence, implying that sunlight might be protective in MS (1). Three recent reports have reinforced this possibility. In the United States, individuals with the highest residential and occupational sunlight exposure had the lowest risk of mortality from MS (odds ratio 0.24) and highest risk of mortality from melanoma (odds ratio 1.38) (76). The lower MS risk among individuals with high sunlight exposure was independent of country of origin, age, sex, race and socioeconomic status (47). Importantly, immigration from a low to a high solar radiation region reduced MS risk in populations that carried MS-susceptibility genes (68). For example, Irish immigrants to Hobart, Australia (42.8ºS) had a 5-fold higher MS prevalence than Irish immigrants to Queensland, Australia (25.1ºS), regardless of age at migration (97). In Australia, there was a higher negative correlation between MS prevalence and UVB radiation (r = -0.91) than the positive correlation between UVB radiation and malignant melanoma (r = 0.75 for males; r = 0.8 for females) (269).

Additional evidence for a link between sunlight, vitamin D, and decreased MS severity comes from studies on seasonal variations in MS disease. Disease onset and exacerbations frequently occurred in the spring (18,86,120,284), when vitamin D supplies were lowest. When the seasonal variation in MS lesion frequency (16) was compared to serum 25-$(OH)D_3$ levels for individuals living in the same German town, it was clear that lesion frequency peaked about two months after the nadir of serum 25-$(OH)D_3$, and serum 25-$(OH)D_3$ peaked about two months before the nadir of lesion frequency (70). This important temporal correlation points to a possible cause and effect relationship between lack of vitamin D and increased MS severity (70).

If the seasonal variations in MS disease onset and severity (16,18,86,120,284) are related to vitamin D_3 supplies derived from sunlight, as we suggested (101, 102), then the seasonal variations in MS disease provide an important insight into vitamin D metabolism and immune system function. The serum 1α,25-$(OH)_2D_3$ level does not vary seasonally (105), so the hormone supplied by the serum may not be the most as regards immune system function. However, the stored vitamin D_3 and the serum 25-$(OH)D_3$ levels do vary seasonally. Thus, it may be that highly localized 1α,25-$(OH)_2D_3$ synthesis, supported by the stored vitamin D_3 (acquired through sunlight exposure) and/or serum 25-$(OH)D_3$, is essential for immunological health.

A few nutritional studies also point to a link between vitamin D and MS. Fish is a good vitamin D source, and MS prevalence was lower along the Norwegian coast than it was inland (253), which has been attributed to a high fish diet along the coast (84,253). Furthermore, two small, uncontrolled, non-blinded trials have suggested that fish oil consumption may lower MS severity and exacerbations (85,182). The nutritional status of the MS patients in these trials as regards vitamin D or other nutrients was not determined before or after fish oil supplementation. In the context of vitamin D nutrition, it is noteworthy that vitamin D insufficiency was common in MS patients. The serum 25-$(OH)D_3$ level was insufficient (<50 nmol/l) in 69% of MS patients (179), and these patients had significantly reduced bone mass, and increased bone loss and fracture rates compared to age- and sex-matched controls (51). These findings indicate that significant vitamin D insufficiency of long duration may exist in most MS patients.

Genetic studies have correlated variant alleles of genes involved in vitamin D metabolism with MS disease. Such correlations may imply a causal relationship between the genotype and the MS-susceptible phenotype. No associations between MS and *VDR*, *CYP27B1*, or *Gc* allelic variations were found in Canadians (247), but the *Gc-1f* allele was associated with MS in Icelanders (10), and the VDR^b allele was associated with MS in the Japanese (77). The VDR^b allele and the major histocompatibility complex (*MHC*) *DPB1*0501* allele commonly occurred together in MS patients (180). Similar results were reported for a large North American pedigree of Pennsylvania Dutch extraction in which MS segregated as an autosomal dominant trait (275). In this important study, all seven MS patients and none of the eleven unaffected family members had the *MHC DR15,DQ6* genotype together with a candidate susceptibility locus on Chromosome 12p12. The markers *D12S1715* and *GATA63D01* delineated the 18 centimorgan region encompassing the proposed Chromosome 12p12 susceptibility locus (275). Although the *VDR* gene was not considered in this study, it should be pointed out that the *VDR* gene is in the delineated region. Thus, the Pennsylvania Dutch study may be a second example of particular *MHC* and *VDR* genotypes combining to influence MS susceptibility. How the genes

may interact is not known. However, based on the known function of MHC class II molecules in antigen presentation and the known high level VDR expression in activated $CD4^+$ T cells, it is tempting to speculate that the interacting genes may influence which peptides are presented to the $CD4^+$ T cells and what fate the T cells follow after encountering the peptides during thymic development (e.g. during central tolerance induction) or during peripheral immune responses.

Very strong support for the concept that the vitamin D endocrine system has an important role in the establishment and/or maintenance of immunological self tolerance derives from studies on EAE. Immunizing rodents with spinal cord homogenate or myelin basic protein (MBP) induces a progressively paralytic autoimmune disease with strong similarities to MS (190). The biologically active hormone, 1α,25-$(OH)_2D_3$, partially inhibited EAE morbidity and mortality in MBP-primed SJL/J mice (142). Moreover, 1α,25-$(OH)_2D_3$ pretreatment completely blocked EAE induction in MBP-primed B10.PL mice (39). Further, 1α,25-$(OH)_2D_3$ treatment rapidly reversed the paralytic symptoms of mice with severe acute EAE (178). Together these experiments indicate that 1α,25-$(OH)_2D_3$ is a profoundly important EAE inhibitor.

The mechanisms by which the vitamin D endocrine system may influence MS or EAE are not known. The 1α,25-$(OH)_2D_3$ prevented EAE in *CD8*-null mice (164) but not *VDR*-null mice (165), indicating that the VDR is necessary, but $CD8^+$ T cells are not necessary for the inhibition mechanism. The lower the dietary calcium level, the higher was the 1α,25-$(OH)_2D_3$ dose needed to completely prevent EAE symptoms, suggesting a role for calcium in the disease inhibition mechanism (41). MS is thought to develop when $CD4^+$ Th1 lymphocytes initiate an abnormal autoimmune response to a neural protein, causing mononuclear cell infiltration, demyelination, oligodendrocyte loss and axonal degeneration (7). Similarly, neural protein-specific $CD4^+$ Th1 lymphocytes producing IFN-γ are pathogenic in EAE (292). For these reasons, current research in the EAE model has focused on the possible involvement of VDR^+CD4^+ T cells as targets of 1α,25-$(OH)_2D_3$ action.

Many previous *in vitro* studies conducted on peripheral blood mononuclear cells reported that 1α,25-$(OH)_2D_3$ addition inhibited antigen- and mitogen-induced T cell proliferation through IL-2 downregulation (8,28,29,122, 123,134,140,141,152,161,209,222,223,224,225,230,237,254,264) and cell cycle arrest (223,225). This inhibitory effect appeared to be a direct action on T cells, because the 1α,25-$(OH)_2D_3$ also inhibited the proliferation of highly purified T cells that were stimulated with antibodies to CD3 and to CD28 (170,173,174). In addition, the hormone inhibited mitogen-induced IFN-γ synthesis *in vitro* (54,170,173,219,225). The molecular basis for 1α,25-$(OH)_2D_3$-mediated repression of IL-2 secretion has been studied in a transient transfection system. When an IL-2 promoter-reporter construct and a VDR construct were transiently transfected into Jurkat T cells, and the cells were stimulated in the presence of 1α,25-$(OH)_2D_3$, the liganded VDR-RXR heterodimers blocked the formation of the NFATp and Fos-Jun protein dimers that are involved in activating the IL-2 promoter (8). Together, these *in vitro* studies fostered the idea that 1α,25-$(OH)_2D_3$ inhibited $CD4^+$ Th1 cell proliferation and IFN-γ synthesis, and this mechanism accounted for the hormoneís ability to inhibit EAE (139).

Our laboratory has been interested in the mechanisms by which the vitamin D endocrine system controls EAE. We reported that activated $CD4^+$ Th1 and Th2 cells expressed the VDR, so both could be hormone targets (177). When we tested the $CD4^+$ Th1 cell inhibition hypothesis *in vivo*, the results did not support it (177). The B10.PL mice pretreated with 1α,25-$(OH)_2D_3$ did not develop EAE when they were primed with MBP, but contrary to the Th1 inhibition hypothesis, the lymph nodes of these mice had a high frequency of $CD4^+$ Th1 cells that proliferated and produced IFN-γ in response to MBP. In addition, when MBP-specific, IFN-γ-producing $CD4^+$ Th1 cells were subjected to 1α,25-$(OH)_2D_3$ treatment *in vitro*, the IFN-γ synthesis did not decline. Finally, when MBP-specific, IFN-γ-producing $CD4^+$ Th1 cells were transferred into unprimed recipient mice, the 1α,25-$(OH)_2D_3$ treatment did not inhibit their ability to cause EAE. Thus, our *in vivo* results in the EAE model ruled out a simple mechanism of 1α,25-$(OH)_2D_3$-mediated inhibition of $CD4^+$ Th1 cell proliferation and IFN-γ synthesis.

Studies done with human cells have reinforced the conclusion that the 1α,25-$(OH)_2D_3$ has no direct effect on IFN-γ synthesis in T cells. Firstly, the 1α,25-$(OH)_2D_3$ did not inhibit IFN-γ secretion from highly purified human T cell lines that were stimulated with antibodies to CD3 and to CD28 (174). Secondly, when healthy human volunteers were dosed with 1α,25-$(OH)_2D_3$, it had no effect on the IL-2 or IFN-γ produced by their peripheral blood mononuclear cells (172). Thus, *in vivo* studies in mice and humans indicate that 1α,25-$(OH)_2D_3$ does not inhibit T cell IFN-γ synthesis.

A second hypothesis was that the 1α,25-$(OH)_2D_3$ might influence $CD4^+$ T cells to follow a Th2 cell fate, which was observed *in vitro* (30, 82) and under some circumstances *in vivo* (40,59,60). We used the adoptive transfer of TCR transgenic cells specific for MBP to trace the fate of the MBP-specific T cells in B10.PL mice. When the recipients of the TCR-transgenic cells were treated with 1α,25-$(OH)_2D_3$ and immunized with MBP, they did not develop EAE. No increase in Th2 cell IL-4 transcripts, either in the lymph nodes or in the CNS, accompanied 1α,25-$(OH)_2D_3$-

mediated prevention of EAE in these mice (177). Similarly, Th2 cell generation did not accompany 1,25-$(OH)_2D_3$-mediated prevention of EAE in myelin oligodendrocyte glycoprotein-primed Biozzi AB/H mice (162). Others showed that the 1α,25-$(OH)_2D_3$ prevented EAE in mice fed a low calcium diet and immunized with MBP, but no increase in Th2 cell IL-4 transcripts occurred in these mice (41). Furthermore, the 1α,25-$(OH)_2D_3$ was only slightly less protective in *IL-4*-null mice than in wild-type controls (42). Thus, studies from three laboratories have ruled out 1,25-$(OH)_2D_3$-mediated enhancement of Th2 development as an obligatory step in the EAE inhibition mechanism.

A third hypothesis was that the 1α,25-$(OH)_2D_3$ might inhibit DC maturation, resulting in decreased $CD4^+$ Th1 cell priming. This hypothesis derived from *in vitro* studies showing that 1α,25-$(OH)_2D_3$ inhibited DC maturation in bone marrow or peripheral blood cell cultures supplemented with granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4 (26,37,93,200,201). The criteria of DC immaturity were retention of high mannose receptor levels and endocytic activity, and failure to up-regulate CD40, CD80, CD83, CD86 and class II MHC molecules, and to activate T cells in mixed lymphocyte culture. The DC derived from the 1α,25-$(OH)_2D_3$ supplemented cultures retained the capacity to produce IL-10 upon activation (37,200). Further, 1α,25-$(OH)_2D_3$ treatment *in vitro* decreased costimulatory molecule expression (49), inhibited IL-12 production (37,54,137,200), and promoted apoptosis (200). Compared with wild-type animals, *VDR*-null mice had an increase in mature DC in lymph nodes but not in spleen (92). We reasoned that if the 1α,25-$(OH)_2D_3$ directly prevented DC maturation and subsequent priming of Th1 cells, then the hormone should prevent EAE in mice that expressed a transgenic TCR specific for MBP, whether or not these mice had other T and B lymphocytes. However, we found that the 1α,25-$(OH)_2D_3$ did not inhibit EAE in TCR-transgenic B10.PL mice that had a non-functional *Rag-1* gene, although it inhibited MBP-induced EAE in TCR-transgenic B10.PL mice that had a functional *Rag-1* gene (177). These data do not rule out an indirect effect of 1α,25-$(OH)_2D_3$ on DC, but they are not consistent with a simple mechanism whereby the 1α,25-$(OH)_2D_3$ acts directly on immature DC to prevent their maturation. Our results suggest that *Rag-1*-dependent T or B lymphocytes are necessary for 1α,25-$(OH)_2D_3$-mediated inhibition of EAE. Thus, it is possible that the hormone acts on a *Rag-1*-dependent cell, and this cell subsequently influences DC function.

Additional studies from our laboratory examined the fate of unprimed, MBP-specific, TCR-transgenic T cells that were transferred into 1α,25-$(OH)_2D_3$- or placebo-treated B10.PL mice prior to MBP priming (177). In the placebo-treated mice that had severe acute EAE, activated, IFN-γ-producing, TCR-transgenic T cells were detected in the lymph nodes and in the central nervous system (CNS). In the 1α,25-$(OH)_2D_3$-treated mice without EAE signs, activated, IFN-γ-producing, TCR-transgenic T cells were detected in the lymph nodes but not in the CNS. These results suggest that in this EAE model, CNS resident or recruited cells participated in the mechanism whereby the 1α,25-$(OH)_2D_3$ inhibited EAE induction. These CNS resident or recruited cells might be the *Rag-1*-dependent, $CD4^+TCRab^+$ regulatory T cells that suppressed the activation of neural peptide-specific T cells in the CNS (191,268). Thus, our working model postulates that the 1α,25-$(OH)_2D_3$ treatment may augment the function of these CNS resident or recruited suppressor T cells that maintain self tolerance to neural proteins in the CNS by suppressing neural antigen-specific $CD4^+$ Th1 cell activation, possibly by influencing the antigen-presenting cell (177) (Fig. 3C).

Our laboratory has also studied the process by which 1α,25-$(OH)_2D_3$ reversed EAE (178). Mice with severe acute EAE (complete hind limb paralysis) were randomized to receive 1α,25-$(OH)_2D_3$ or placebo treatment. The hormone-treated animals began walking with a wobbly gate at 3 days post treatment, whereas placebo-treated mice remained paralyzed. A histopathological examination at 3 days post treatment showed the hormone-treated mice had a 50% decrease in white matter and meningeal inflammation. A flow cytometric analysis at 1-2 days post treatment showed that the hormone-treated mice had 70% fewer $CD11b^+$ cells per spinal cord sample than the placebo-treated mice (178). Gene expression studies at 1 day post treatment have shown that the decline in $CD11b^+$ cells was attributable to a 1α,25-$(OH)_2D_3$-mediated decrease in the chemokines that attract these cells (L. Pedersen, F. Nashold and C. Hayes, submitted to ?). Together, these data clearly showed that the 1α,25-$(OH)_2D_3$ contributed to the resolution of inflammation in mice with established EAE by reducing the burden of $CD11b^+$ inflammatory cells. Others confirmed that 1α,25-$(OH)_2D_3$ treatment rapidly improved clinical EAE disease in the Lewis rat model (80). These investigators reported hormone-mediated inhibition of CD4, MHC class II and type II nitric oxide synthase expression in the posterior areas of the CNS. They hypothesized that the 1α,25-$(OH)_2D_3$ may directly inhibit the type II nitric oxide synthase promoter in microglia and astrocytes.

Transforming growth factor-β1 (TGF-β1) is widely recognized as an anti-inflammatory cytokine that may play an important role in immunological self tolerance (210). The possibility that this cytokine participates in 1α,25-$(OH)_2D_3$-mediated inhibition of EAE has been considered. We reported that 1α,25-$(OH)_2D_3$ treatment prior to EAE induction enhanced TGF-β1 transcripts in the lymph nodes, but we were unable to detect an enhancement of TGF-β1 proteins (43). Similarly, we reported that 1α,25-$(OH)_2D_3$

treatment after EAE induction enhanced TGF-β1 transcripts in the CNS (43). However, we were unable to detect an enhancement of TGF-β1, TGF-β1, TGF-β2 or TGF-b3 proteins, or their receptors, in spinal cord samples from 1α,25-$(OH)_2D_3$ compared to placebo-treated mice with EAE (C. Hayes, K. Flanders, F. Nashold, M. Rude and K. Spach, unpublished). Other investigators found no effect of short-term 1α,25-$(OH)_2D_3$ treatment on TGF-β1 transcripts in the CNS (80). Thus, the possibility that TGF-β1 participates in 1α,25-$(OH)_2D_3$-mediated inhibition of EAE remains an unsettled question.

Diabetes

Like MS, there is compelling evidence from epidemiological, genetic, nutritional, and immunological studies for a link between sunlight, vitamin D and IDDM risk. Firstly, IDDM incidence increased with increasing latitude in Europe (78,88,94,212,228,266,282), Scandanavia (6,55,186), China (145) and Canada (290). Furthermore, IDDM incidence varied inversely with solar radiation exposure (203), establishing a link between sunlight and IDDM risk. Vitamin D insufficiency may exist in most IDDM patients, as evidenced by their lower mean 1α,25-$(OH)_2D_3$ concentrations and higher molar ratios of 24,25-$(OH)_2D_3$ to 25-$(OH)D_3$ compared to healthy controls (11,20,75,185,227). Low bone density has also been reported in IDDM patients, but the interpretation of this observation is controversial (202). Most significantly, large population-based studies have shown that high dietary vitamin D supplementation in infancy correlated with a significantly reduced risk of IDDM in later life (95,99,116,184,249). Thus, there is a solid correlation between inadequate vitamin D nutrition and elevated IDDM risk.

A possible causal relationship between inadequate vitamin D endocrine system function and increased IDDM susceptibility is further strengthened by genetic studies correlating variant *VDR* alleles with IDDM. The VDR^b allele was implicated in IDDM susceptibility in Indian Asians (163), Germans (72,196,197) and Taiwanese (46). In the Dalmatian population of south Croatia, the VDR^t allele was a risk factor for IDDM (241). In Japanese families, the VDR^F genotype was associated with IDDM (19,288). In French families, the VDR^t allele was associated with a high risk for severe diabetic retinopathy (256). To date, no *Gc* (130) or *CYP27B1* (198) polymorphisms have been associated with IDDM. Thus, in Indian, German, Taiwanese, Japanese and French families, associations between *VDR* alleles and IDDM susceptibility have been reported, and in one report, a gender-specific association was observed (96).

The basis for a protective role of 1α,25-$(OH)_2D_3$ in IDDM has been studied in the non-obese diabetic (NOD) mouse, which develops IDDM spontaneously and is widely used as an IDDM model (15). A seminal study reported that treatment of NOD mice with 1α,25-$(OH)_2D_3$ prevented pancreatic insulitis (158). These investigators subsequently reported that 1α,25-$(OH)_2D_3$ treatment also reduced the incidence of IDDM in NOD mice (160). It is significant that the NOD macrophages had a defect in 1α,25-$(OH)_2D_3$ synthesis (194), which may be related to the IDDM disease prone phenotype of NOD mice. This result strongly suggests that a negative feed-back loop initiated by activated macrophage 1α,25-$(OH)_2D_3$ synthesis has some role in protection from IDDM (Fig. 3A). A second postulated role for 1α,25-$(OH)_2D_3$ in IDDM is reducing the vulnerability of pancreatic islet cells to a cytotoxic T cell-mediated attack (220). Yet another mechanism was suggested by data showing that the 1α,25-$(OH)_2D_3$-mediated prevention of IDDM in NOD mice was accompanied by an increase in Th2 cell IL-4 production and a decrease in Th1 cell IFN-γ production in response to pancreatic autoantigens, both in the pancreas and in the peripheral lymph nodes (194). The dominance of the IL-4 response suggests that the hormone may have stimulated the pancreatic autoantigen-specific T cells to follow the Th2 cell fate (Fig. 3B). It is noteworthy that the 1α,25-$(OH)_2D_3$ treatment did not stimulate ovalbumin-specific T cells to follow the Th2 cell fate in NOD mice, indicating that the mechanism for the immune deviation effect was complex and autoantigen specific. A final mechanism considered was induction of suppressor cells. One group found that the protection against IDDM afforded by 1α,25-$(OH)_2D_3$ treatment of NOD mice appeared to be independent of suppressor cells (44). However, another group showed that treatment of NOD mice with 1α,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor vitamin D_3, an analog of 1α,25-$(OH)_2D_3$, inhibited IDDM (91). In this study, no marked development of Th2 cells was noted. Rather, the analog enhanced the function of $CD4^+CD25^+CD38^+$ suppressor T cells. These suppressor T cells inhibited activation of $CD4^+$ T cells specific for pancreatic proteins in the pancreatic lymph node but not in the spleen. This result is similar to our finding that 1α,25-$(OH)_2D_3$ enhanced the function of *Rag*-1-dependent cells that inhibited activation of $CD4^+$ T cells specific for neural proteins in the CNS but not in the spleen in mice immunized to induce EAE (177). Together, these results from two disparate systems point to a role for suppressor T cells in the mechanism whereby the vitamin D endocrine system supports immunological self tolerance (Fig. 3C). These suppressor T cells may function within the tissues that express their cognate self epitopes.

Other autoimmune diseases

There is some evidence for a link between sunlight, vitamin D, and reduced risk of the inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis (UC), although the evidence is much less compelling than

the evidence for such a link in MS or IDDM. IBD is a chronic inflammatory disease of the gastrointestinal tract with an uncertain etiology. The key pathological mechanism in IBD appears to involve a dysregulated immune response to gastrointestinal tract antigens (119). There are some reports that IBD risk varies with latitude. The death rates from Crohn's disease and UC were high in England, Germany and the Scandinavian countries, and low in Mediterranean countries (214,244). In Europe, the Crohn's disease rate was 80% higher in the northern than in the southern countries (238). Furthermore, both Crohn's disease and UC appeared to be more frequent in the northern than in the southern United States (244). The IBD risk reportedly also varies by occupation, with indoor work increasing the risk (52,243), and by season, with symptom onset mainly in the winter (169). These correlations may signal a relationship between low sun exposure and IBD risk. Hypovitaminosis D and low bone mineral density have been documented in IBD patients (63,98,117,118, 127,135,233,234,239,276). The interpretation of the relationship between IBD, hypovitaminosis D, and bone mineral density is complex, because IBD disturbs nutrient absorption, and some of the drugs use to treat IBD have effects on bone mineral density. Small dietary studies have shown that fish oil supplements lessened the clinical IBD symptoms in UC patients (14, 250). These studies are also difficult to interpret, because there are several anti-inflammatory components of fish oil, and no further information is available on which of them may be beneficial in IBD. Finally, an IBD susceptibility locus was mapped to Chromosome 12 (53,64,232). Genetic fine mapping of the Chromosome 12 IBD susceptiblity locus showed that the less active *VDR*^{t} allele was associated with Crohn's disease in German families (156) and in a larger sample of Europeans (240).

The combined geographic, ecological, nutritional, and genetic evidence led us to hypothesize that high sunlight exposure or supplemental vitamin D_3 might reduce IBD risk by increasing the immunoregulatory functions of 1α,25-$(OH)_2D_3$. We explored this possibility experimentally using the dextran sodium sulfate-induced colitis model in C3H/HeJ mice (149). We found that 1,25-$(OH)_2D_3$ pre-treatment reduced colon histopathology by 61% in the acute colitis phase of IBD (C. Hayes and F. Nashold, unpublished). Moreover, when 1α,25-$(OH)_2D_3$ was administered to mice with chronic dextran sodium sulfate-induced colitis, the hormone treatment reduced colon histopathology by 40% (Hayes and Nashold, unpublished). Others reported that 1α,25-$(OH)_2D_3$ treatment reduced spontaneous colitis in IL-10-knockout mice (42), but had no effect on spontaneous colitis in IL-2-knockout mice (25). Thus, experiments in animal models of IBD are beginning to document a protective effect of 1α,25-$(OH)_2D_3$ in IBD.

Other autoimmune diseases may also be vitamin D-responsive. In murine Lyme arthritis and collagen-induced arthritis, we found that dietary 1α,25-$(OH)_2D_3$ supplementation minimized or prevented arthritis symptoms (40). In addition, when given to mice with early arthritis symptoms, dietary 1α,25-$(OH)_2D_3$ supplementation prevented symptom progression. Others reported a weak association between the *VDR*^{b} allele and early onset rheumatoid arthritis in Spanish women (79). Patients with arthritis-associated *MHC* alleles and *VDR* alleles had the earliest disease onset. Similarly, for the autoimmune disease spontaneous lupus erythematosis, the*VDR*^{b} allele was associated with lupus in Chinese patients (114). Also, adding 1α,25-$(OH)_2D_3$ to peripheral blood cells from lupus patients inhibited the spontaneous immunoglobulin synthesis by these cells (147). Finally, the 1α,25-$(OH)_2D_3$ inhibited lupus in MRL/1 mice (144). No further information on these suggestive links between vitamin D and arthritis or lupus is yet available.

VITAMIN D AND TRANSPLANTATION

Research into the immunoregulatory activities of 1α,25-$(OH)_2D_3$ suggested to us and to others that 1α,25-$(OH)_2D_3$ (or its analogs) might inhibit the rejection of transplanted tissue. The effects of 1α,25-$(OH)_2D_3$ in tissue transplantation are reviewed here. The effects of its analogs in tissue transplantation have been reviewed previously (157).

Heart transplantation

We tested the hypothesis that 1α,25-$(OH)_2D_3$ might delay the rejection of transplanted tissue in a cardiac allograft model system (115). Neonatal murine heart tissue was transplanted into MHC-incompatible recipient mice. Administering 1α,25-$(OH)_2D_3$ to the recipient mice prolonged the heart allograft survival from 13 to 51 days, compared to the placebo-treated mice. The 1α,25-$(OH)_2D_3$ was more efficacious than cyclosporine in prolonging graft survival. Similar results were obtained in a rat heart allograft model (115). Prolonged graft survival was achieved without an increase in susceptibility to fungal or viral infection and without hypercalcemia (40). These results indicated that the 1α,25-$(OH)_2D_3$ might be a clinically useful immunomodulatory agent in human organ transplantation.

Kidney transplantation

Because the kidney is the major site of 1α,25-$(OH)_2D_3$ synthesis, kidney transplant patients commonly receive supplementary 1α,25-$(OH)_2D_3$ to maintain mineral ion homeostasis and skeletal integrity. This clinical practice afforded the opportunity to investigate the effect of the supplementary hormone on renal allograft survival in humans. A case-control study showed that the 1α,25-

$(OH)_2D_3$ treatment significantly prolonged the function of the transplanted kidney (12,187). One possible mechanism for prolonging renal graft function might be a hormone-mediated decrease in intra-graft fibrosis (13). In rodent renal transplant models, the 1α,25-$(OH)_2D_3$ treatment reduced the amount of bioactive TGF-β1 protein in the renal lysates, which would be expected to reduce fibrosis. The treatment also increased the formation of a complex between Smad3, a downstream mediator of TGF-β1 signaling (62), and the VDR. The finding of decreased TGF-β1 protein and increased Smad3-VDR complex formation is somewhat puzzling, because one might have expected the decrease in TGF-β1 protein to yield a decrease in active Smad3. Others reported that formation of a Smad3-VDR complex increased the ligand-induced VDR transactivation function (286). Clearly, further investigation will be required to understanding crosstalk between the TGF-b and the VDR pathways and how it may influence renal allograft survival.

Pancreatic islet transplantation

Interesting information has also come from studies exploring a combination of 1α,25-$(OH)_2D_3$ and the immunosuppressive drug mycophenolate mofetil for prolonging pancreatic islet allograft survival (5, 89, 90). The 1α,25-$(OH)_2D_3$ treatment alone delayed islet allograft rejection in 50% of the recipients. However, the combined 1α,25-$(OH)_2D_3$ plus mycophenolate mofetil treatment induced long-term tolerance to the allografts. The investigators implicated an increased frequency of transferable $CD4^+CD25^+$ suppressor T cells and changes in $CD11c^+$ DC function as part of the tolerogenic mechanism. The DC recruited to the allograft in the tolerant mice displayed lower levels of the co-stimulatory molecules CD40, CD80 and CD86, secreted less IL-12p75, and elicited a lower T cell-mediated IFN-γ response than the DC recruited to the allograft in the acutely rejecting mice. It remains to be elucidated whether the target of the 1α,25-$(OH)_2D_3$ action in this system was the suppressor T cell or the DC cell or both. However, the conclusion that the mechanism of 1α,25-$(OH)_2D_3$ action in this system involves $CD4^+CD25^+$ suppressor T cells is reminiscent of results obtained in autoimmune disease models as illustrated in Fig. 3C.

Liver transplantation

The ability of 1α,25-$(OH)_2D_3$ to prolong liver allograft survival has also been studied (215). Rats were treated with 1α,25-$(OH)_2D_3$ prior to transplantation, and graft survival and cytokine indicators of an immune response were measured. The 1α,25-$(OH)_2D_3$ prolonged the liver allograft survival as evidenced by a decrease in the release of liver enzymes into the serum. The hormone treatment also reduced the intra-graft IL-2 and IL-12 concentrations, while increasing the IL-4 and IL-10 concentrations. These data suggest a possible shift to a Th2-mediated immune response as illustrated in Fig. 3B.

SUMMARY

A renaissance of interest in the immunological functions of the vitamin D endocrine system has been stimulated by recent progress in the areas of infectious disease, autoimmune disease, and transplantation. It is clear that considerable additional experimentation in these emerging research areas will be required to develop detailed mechanistic understandings of how 1α,25-$(OH)_2D_3$ influences immunity. Good evidence indicates that the IFN-γ-activated macrophage functions as a source of 1α,25-$(OH)_2D_3$ at sites of inflammation, provided there is sufficient 25-OH-D_3 to supply substrate to the 1α-OHase. However, we do not yet know exactly which immune system cells are the targets of this highly localized hormone synthesis, or how the 1α,25-$(OH)_2D_3$ alters the functions of those cells. The decreasing VDR expression in the activated macrophages, together with the increasing VDR expression in activated T and B lymphocytes, suggests that the locally-produced 1α,25-$(OH)_2D_3$ probably functions in a paracrine rather than autocrine regulatory loop. Studies on vitamin D deficiency and *VDR*-mutant humans and rodents indicate that the vitamin D endocrine system is essential for effective immune responses to infectious agents, but not for lymphopoiesis or myelopoiesis. There are indications that a high level of the 1α,25-$(OH)_2D_3$ and transcriptionally active *VDR* alleles may enhance the development of strong Th2 cell-mediated responses, but mechanistic details of how this may occur are lacking. A wide variety of epidemiological, genetic, nutritional and biological studies done in humans and rodents are pointing to an important role for the vitamin D endocrine system in maintaining immunological self tolerance. The most encouraging studies in this regard showed that supplementary vitamin D in childhood correlated with a much reduced IDDM incidence in adulthood. Once again, the mechanisms underlying the 1α,25-$(OH)_2D_3$-mediated enhancement of self tolerance, and tolerance to allografts, are not yet clear. The mechanisms may relate to a paracrine feed-back loop resolving inflammation, or influence over the differentiation fate of activated CD4 T cells, or to enhancement of suppressor T cell functions, or all of these. It will be exciting to see the progress made in these rapidly developing areas when the subject of vitamin D and the immune system is next reviewed.

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