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Vitamin D and Its Analogues in Immune System Regulation

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Running Title: Vitamin D analogues in immune regulation

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Abstract

Vitamin D was discovered as the cure for nutritional rickets, a disease of bone growth arising from inadequate intestinal calcium absorption, and for much of the 20th century was studied for its critical role in calcium homeostasis. However, we now recognize that the vitamin D receptor (VDR) and vitamin D metabolic enzymes are expressed in numerous tissues unrelated to calcium homeostasis. Notably, vitamin D signaling can induce cellular differentiation and cell cycle arrest. Moreover, the VDR and the enzyme CYP27B1, which produces the hormonal form of vitamin D, 1,25-dihydroxyvitamin D (1,25D), are expressed throughout the immune system. In addition, *CYP27B1* expression in immune cells is regulated by physiological inputs independent of those controlling its expression in calcium homeostatic tissues. These observations have driven the development of 1,25D-like secosteroidal analogues and non-secosteroidal analogues in an effort to separate the effects of vitamin D on cell differentiation and function from its calcemic activities. Notably, some of these analogues have had considerable success in the clinic in the treatment of inflammatory and immune-related disorders. In this review, we describe in detail the mechanisms of vitamin D signaling, and the physiological signals controlling 1,25D synthesis and catabolism, with a focus on the immune system. We also survey the effects of 1,25D and its analogues on regulation of immune system function and their implications for human immune-related disorders. Finally, we describe the potential of vitamin D analogues as anti-cancer therapeutics, in particular, their use as adjuncts to cancer immunotherapy.

Significance Statement

Vitamin D signaling is active in both the innate and adaptive arms of the immune system. Numerous vitamin D analogues, developed primarily to minimize the dose-limiting hypercalcemia of the active form of vitamin D, have been used widely in preclinical and clinical studies of immune system regulation. This review presents a description of the mechanisms of action of vitamin D signaling, an overview of analogue development, and an in-depth discussion of the immunoregulatory roles of vitamin D analogues.

List of Abbreviations

1,25D	1,25-dihydroxyvitamin D
1αD	1α-hydroxyvitamin D (alfacalcidol)
22-Oxacalcitol	OCT, Maxacalcitol
25D	25-hydroxyvitamin D
AIRE	Autoimmune regulator
AMP	Antimicrobial peptide
APC	Antigen presenting cell
APECED	Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
ARI	Acute respiratory tract infection
ASL	Airway surface liquid
BCAA	Branched chain amino acid
Calcipotriol	MC309
CAMP/LL37	Cathelicidin antimicrobial peptide
CD	Crohn's disease
CKD	Chronic kidney disease
ChIP	Chromatin immunoprecipitation
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
DBP	Vitamin D binding protein
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
EB1089	Seocalcitol
E.coli	Escherichia coli
GC	Group-specific component of serum
HBD2	human b-defensin 2
HDACi	Histone deacetylase inhibitor
IBD	inflammatory bowel disease
ICI	Immune checkpoint inhibition
IFNγ	Interferon gamma

87	IIH	Infantile idiopathic hypercalcemia
88	iNKT	Invariant natural killer T cell
89	irAE	Immune-related adverse effect
90	LPS	Lipopolysaccharide
91	LXR	Liver X receptor
92	MHC	Major histocompatibility complex
93	MS	Multiple sclerosis
94	mTEC	Medullary thymic epithelial cell
95	mTOR	Mammalian target of rapamycin
96	NK	Natural killer cell
97	NOD	Non-obese diabetic
98	NPFFR2	Neuropeptide FF receptor 2
99	OCT	22-Oxacalcitriol
100	OVA	Chicken egg ovalbumin
101	PBMC	Peripheral blood mononuclear cell
102	PD-1	Programmed death 1
103	PPAR	Peroxisomal proliferator activated receptor
104	P. aeruginosa	Pseudomonas aeruginosa
105	PTH	Parathyroid hormone
106	PRR	Pattern recognition receptor
107	RAG	Recombinase activating gene
108	RAR	Retinoic acid receptor
109	RCT	Randomized, double blind, placebo-controlled trial
110	RXR	Retinoid X receptor
111	SARS COV2	Severe acute respiratory syndrome coronavirus 2
112	SLE	Systemic lupus erythematosus
113	T1D	Type 1 diabetes
114	TB	Tuberculosis
115	TCR	T cell receptor

TEC	Thymic epithelial cell
TGFβ	Transforming growth factor beta
Tfh	Follicular helper T cell
TLR	Toll-like receptor
TR	Thyroid hormone receptor
Treg	Regulatory T cell
UTI	Urinary tract infections
UVB	Ultraviolet B
VDR	Vitamin D receptor
VDRE	Vitamin D response element

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1. A Brief History of Vitamin D

Vitamin D was discovered as the cure for nutritional rickets, a disease of bone growth arising from inadequate dietary calcium uptake, and vitamin D status has long been linked to diet or sun exposure. Rickets was first described as a clinical phenomenon in the early 17th century, and became widespread in the rapidly industrializing, polluted cities of 18th and 19th century Europe (Discovery; Guy, 1923; Jones, 2022). The 2020's represent significant anniversaries of advances in our understanding of vitamin D biology in several regards. In 1822, a Polish physician, who noticed that the condition was rare outside of polluted cities, concluded that sun exposure cured rickets. The anti-rachitic properties of cod liver oil were first noted by a French researcher in 1827 but did not initially gain widespread attention because of a lack of understanding at the time about micronutrients. Approximately 100 years later, Elmer McCollum (1922) showed that cod liver oil heated in the presence of oxygen to inactivate vitamin A retained its anti-rachitic activity, confirming the presence of a distinct active substance. In the same decade, multiple groups showed that UV irradiation of excised skin or food substances was effective at protecting against rickets (Discovery; Guy, 1923; Jones, 2022). The structure of vitamin D3 was determined in 1936.

While vitamin D was initially identified and characterized for its anti-rachitic properties, there are also longstanding links between vitamin D status or sun exposure and immunity. In ancient Greece, heliotherapy (sun therapy) was used as a treatment to alleviate the symptoms of phthisis (tuberculosis, TB), which is caused by an uncontrolled infection of the intracellular pathogen *Mycobacterium tuberculosis* (*M.tb.*) (Clancy et al., 2013). Use of cod liver oil as a treatment for chronic rheumatism dates from the 18th century. Vitamin D was subsequently used as a therapy for scrofula, a form of tuberculosis infecting the lymph nodes (Guy, 1923), and the idea of using sun exposure to treat TB was reborn in the 1800's with the advent of the sanatorium movement in Europe, when disease incidence was at its peak (Grad, 2004; Martineau et al., 2007). In 1903, Niels Finsen received

the Nobel Prize for Medicine for his work demonstrating that cutaneous UV exposure was an efficacious treatment for cutaneous TB (lupus vulgaris) (Grad, 2004; Martineau et al., 2007). Finally, in the 1980's, the active form of vitamin D was shown to control *M.tb.* proliferation in macrophages infected in tissue culture (Rook et al., 1986).

2. Introduction to Vitamin D Metabolism and Signaling.

2.1 Sources of Vitamin D.

Vitamin D (calciferol) is a secosteroid that is found in two principal forms, vitamin D_3 and vitamin D_2. Vitamin D_3 can be produced in skin in the presence of adequate ultraviolet B (UVB) irradiation by photochemical cleavage and thermal rearrangement of the last intermediate in cholesterol biosynthesis, 7-dehydrocholesterol (**Fig. 1**). Because of its relationship to cholesterol biosynthesis, vitamin D_3 is also known as cholecalciferol. It is distinguished from vitamin D_2, or ergocalciferol, which is derived from the steroid ergosterol, and differs from vitamin D_3 by a double bond and a methyl group in its sidechain (**Fig. 1**). Cholecalciferol is available from animal sources, whereas ergocalciferol is found in fungi and a limited number of plant species, notably alfalfa and lichen (Horst et al., 1984; Mau et al., 1998; Wang et al., 2001). Lichen also produces vitamin D_3 and thus serves as a source of supplements for vegan diets (Mangels, 2014). While both D_3 and D_2 are active, vitamin D_3 produces a more sustained rise in circulating vitamin D metabolite levels and greater than 2-fold more subcutaneous storage than vitamin D_2 at identical doses (Armas et al., 2004; Heaney et al., 2011). While cod liver oil and fatty fish are relatively rich sources of vitamin D_3, and sun-dried shiitake mushrooms contain high levels of vitamin D_2, most western diets are vitamin D-poor. There are only limited amounts of vitamin D in meat, eggs and dairy products (Baeke et al., 2010), and many countries resort to dietary supplementation, notably of dairy products, which are also important sources of dietary calcium. Even with supplementation, sun exposure in many populations is the major source of vitamin D. However, solar UVB irradiation is absorbed by the ozone layer, and at sea level

its intensity is insufficient for cutaneous vitamin D synthesis when the sun is below 45º. This period, known as vitamin D winter, can last several months at temperate latitudes, notably in populous regions of northern Europe (Tavera-Mendoza, 2007). Thus, in the absence of appropriate supplementation, circulating vitamin D metabolite levels vary seasonally (Hyppönen and Power, 2007). Moreover, the amount of vitamin D_3 produced in skin exposed to a given UVB dose is dependent on both skin color and age (Webb, 2006).

2.2 Serum 25D levels in populations worldwide.

Serum 25D levels are used as biomarkers of vitamin D status. In past, there was not universal agreement on 25D levels that constituted vitamin D sufficiency, although those of the U.S. Endocrine Society were widely used (Holick et al., 2011). Vitamin D sufficiency was defined as circulating 25D levels above 75 nM (30ng/mL), based in part on the inverse relationship between circulating 25D and levels of parathyroid hormone (PTH), which is released upon a drop in tightly controlled circulating calcium concentrations (Holick et al., 2011) (NB: Metabolite levels are measured in ng/mL in the US and nM elsewhere, with a conversion factor of 2.5–fold). Recently, however, a new Endocrine Society panel was convened to assess clinical trial evidence supporting establishment of 25D threshold levels associated with outcome-specific benefits (Demay et al., 2024). Notably, the panel did not endorse a 25D level of 30 ng/mL (75 nM) as a threshold for sufficiency, nor did it endorse specific 25D levels to define sufficiency, insufficiency, or deficiency. Therefore, we will describe specific vitamin D metabolite levels whenever possible to avoid the use of the term deficiency. These findings are significant for clinicians as vitamin D-poor diets and vitamin D winter combined with sun avoidance, and in some areas, conservative dress, can lead to low levels of circulating 25D in several populations worldwide (Arabi et al., 2010). A survey of >1,000 adolescents across Europe found that 80% had circulating vitamin D metabolite levels below 75 nmol/L, and that 15% were below 27.5 nmol/L (Gonzalez-Gross et al., 2012), findings consistent with estimates of poor dietary vitamin D intake in adolescents

(Diethelm et al., 2014). They are also in agreement with the broader European population; an analysis of 55,844 Europeans of all age groups found that 40% had circulating 25D levels below 50 nmol/L (Cashman et al., 2016). Such observations are not limited to Europe, as low vitamin D is also common in the Middle East and India (Kamboj et al., 2018; Lips et al., 2019). A systematic review and meta-analysis, which included 21,676 participants from 23 African countries, found that 17.3% had serum 25D levels <30 nM and 34.2% had levels <50 nM. As expected, mean serum 25D levels were lower in northern countries and South Africa than in sub-Saharan Africa (Mogire et al., 2020). Other parameters in addition to latitude and diet can also control vitamin D status. The European study cited above found that poor vitamin D status (< 30 nmol/L) was more frequent in dark-skinned people, consistent with reduced cutaneous synthesis (Cashman et al., 2016). Finally, as vitamin D is fat-soluble, deficiency in circulating metabolite levels can be exacerbated by high body mass index (Rabufetti et al., 2019).

2.3. Vitamin D Metabolic Enzymes.

Vitamin D derived from dietary sources, supplementation or cutaneous UVB exposure is not a biologically active molecule. It must undergo sequential hydroxylations, first at C25 on the end of the cholesterol sidechain and then at the 1α position (**Fig. 1**) to produce the active form, 1,25-dihydroxyvitamin D (1,25D). 25-hydroxylation occurs largely but not exclusively upon passage through the liver. The enzyme CYP2R1 is the major but not only vitamin D 25-hydroxylase (Shinkyo et al., 2004). Ablation of the mouse *Cyp2r1 gene* led to a greater than 50% reduction in circulating 25-hydroxyvitamin D (25D) levels (Zhu et al., 2013). Similarly, humans with *CYP2R1* gene mutations are characterized by reduced serum 25D levels and symptoms of vitamin D deficiency (Al Mutair et al., 2012; Cheng et al., 2004). Residual 25-hydroxylase activity may be accounted for by several other enzymes, including CYP27A1 and CYP3A4 (Zhu and DeLuca, 2012). 25D metabolites are the major circulating forms of vitamin D, with the half-life of 25-hydroxyvitamin D_3 being 2–3 weeks, whereas

that of the D_2 form is somewhat shorter (Jones et al., 2014). 25D is a substrate for the unique 25-hydroxyvitamin D 1α-hydroxylase, a mitochondrial enzyme encoded by the nuclear *CYP27B1* gene. *Cyp27b1* was first cloned from the rat by St-Arnaud *et al* in 1997 (St-Arnaud et al., 1997), followed by genes from other species, including human (Prosser and Jones, 2004). Loss of CYP27B1 expression in humans and mice leads to vitamin D-dependent rickets type 1A (Fu et al., 1997; Glorieux and St-Arnaud, 2024). Circulating hydroxylated forms of vitamin D were first identified over 50 years ago, and initial studies on 1α-hydroxylase activity suggested that CYP27B1 expression was largely restricted to kidney (Lawson et al., 1971). Subsequently, the central role of renal CYP27B1 in calcium homeostasis became clear; transcription of the *CYP27B1* gene is under control of Ca^{++} and phosphate regulatory signals (Prosser and Jones, 2004). Notably, a drop in circulating Ca^{++} attenuates the break on PTH release from the parathyroids controlled by the calcium-sensing receptor, and the subsequent increase in PTH binding to its cognate receptor on proximal renal tubular epithelial cells induces expression of *CYP27B1.* 1,25D thus produced is released into the circulation, which, in addition to its pleiotropic roles, inhibits PTH production in a negative feedback loop. Moreover, renal CYP27B1 activity is inhibited by fibroblast growth factor 23 (FGF23), whose production from bone cells is stimulated by high phosphate levels but acts independently of PTH (Bikle, 2000; Saito et al., 2003). Collectively, the above leads to the classical model of vitamin D activation, featuring renal expression of CYP27B1 under control of calcium regulatory inputs and 1,25D acting largely as an endocrine hormone.

Catabolism of both 25D and 1,25D is initiated by the enzyme encoded by the *CYP24A1* gene. Initially characterized for its capacity to hydroxylate 25D or 1,25D at the 24 position, we now know that CYP24A1 catalyzes a multi-step catabolic pathway that leads to elimination of much of the sidechain, culminating in the production of calcitroic acid (**Fig. 1**) (Jones et al., 2012). *CYP24A1* expression is strongly induced in the presence of 1,25D, constituting a negative feedback loop (Prosser and Jones, 2004). The phenotype of *Cyp24a1*-null mice (St-Arnaud, 1999) underlines the importance of the

enzyme in vitamin D metabolite catabolism and calcium homeostasis. Null mice are hypercalcemic, can exhibit renal calcification, and half of them die before weaning. Similarly, in humans, *CYP24A1* mutations cause infantile idiopathic hypercalcemia (IIH) (Jones et al., 2012; Schlingmann et al., 2011). Increased incidence of IIH in the United Kingdom corresponded with increased vitamin D supplementation of infant formula and fortification of milk. Like the mouse knockout model, the initial cohort of IIH patients presented with nephrocalcinosis in addition to profound hypercalcemia and suppressed PTH levels. Even after elimination of vitamin D supplementation and implementation of a low-calcium diet, circulating Ca^{++} concentrations remain elevated and PTH suppressed in IIH patients (Schlingmann et al., 2011).

2.4 Vitamin D Binding Protein.

Group-specific component of serum (GC globulin) or vitamin D binding protein (DBP) is present in the circulation at micromolar concentrations. The transport of circulating lipophilic vitamin D metabolites by a binding globulin parallels that of steroid hormones, which are also bound by specific binding globulins. GC globulin was first identified in 1959 by electrophoresis of serum proteins (Hirschfeld, 1959). Several groups subsequently showed that it acts as a binding protein for serum metabolites of vitamin D. Other work revealed that DBP is also a scavenger of globular actin and binds fatty acids (Bouillon et al., 2024). It is genetically and structurally related to albumin and □-fetoprotein, and all family members share conserved 3-domain alpha-helical topologies, although the orientations of the individual domains vary widely between members. Vitamin D binding residues lie within the first domain of DBP (Swamy et al., 2002; Verboven et al., 2002), and differences in local folding likely explain the specificity of DBP for vitamin D metabolites (Bouillon et al., 2024). DBP is produced almost exclusively by the liver. Its serum concentrations do not vary with vitamin D status, and there is no evidence to date that 1,25D regulates the expression of its gene. It is noteworthy that the estimated turnover rate of DBP is several-fold faster than that of its principal ligand, 25D (Haddad et al., 1981),

indicating that 25D must be recycled. It is also noteworthy that the affinity of the 25-hydroxy metabolite of vitamin D_2 for human DBP is somewhat lower than that of its D_3 counterpart (HADDAD et al., 1976), which may explain the longer circulating half-life of the D_3 form (Jones et al., 2014).

The high affinity of vitamin D metabolites and other steroid hormones for their binding globulins has led to the free hormone hypothesis, wherein most circulating ligand is globulin-bound, with only a small fraction free to enter cells (Mendel, 1989). Under these conditions, bound fractions represent circulating reservoirs, and bound hormone can be released continuously, replacing free hormone taken up by cells. However, the kidney, parathyroid glands, and placenta express megalin and cubilin, which form an endocytic complex that allows for the uptake of DBP-bound vitamin D metabolites into cells. Loss of megalin/cubilin results in osteomalacia and poor survival, demonstrating the importance of vitamin D transport into key cells via uptake of DBP-bound metabolites for regulation of vitamin D and calcium homeostasis (Bikle, 2000; Nykjaer et al., 1999; Nykjaer et al., 2001). The central role of DBP in maintaining circulating vitamin D metabolite levels, and support for the free hormone hypothesis, is manifested in DBP-null mice (Safadi et al., 1999). Under vitamin D-replete conditions, null animals are normocalcemic in spite of having extremely low levels of circulating 25D and 1,25D. Remarkably, while 1,25D levels are below the limit of detection of many assays, tissue-resident concentrations appear normal (Zella et al., 2008). However, null mice develop vitamin D deficiency more rapidly than their wild-type counterparts when on a vitamin D deficient diet, but, intriguingly, are resistant to vitamin D toxicity (hypercalcemia) (Safadi et al., 1999).

To date, there have been two patients identified with undetectable circulating DBP. In one case (Henderson et al., 2019), a 58-year-old woman presented with largely normal bone metabolic markers, in spite of the extremely low concentration of serum 25D of 0.25nM. Her major clinical feature was debilitating ankylosing spondylitis, an inflammatory condition affecting the spine and joints, although the connection of the condition to DBP loss was not established. The patient carried two chromosome deletions: one of 139kb eliminating the entire *GC* gene, and another of 144kb deletion, which deleted

part of the *NPFFR2* (neuropeptide FF receptor 2) gene. The clinical significance of the latter deletion is not clear. In a second case of a 60-year-old male, bone markers and serum calcium and phosphate were normal, and, unlike the first patient, there was no sign of rheumatological disease. Aggressive vitamin D_3 supplementation of the patient failed to restore normal 25D levels, and eventually a homozygous G>A substitution was identified, which led to exon 7 skipping and production of a truncated protein subject to nonsense-mediated decay (Banerjee et al., 2021). Taken together, phenotypes of these patients show that the effects of loss of DBP in humans largely phenocopy those seen in null mice.

3. The Vitamin D Receptor.

1,25D as a free hormone can enter cells, where it binds to the vitamin D receptor (VDR), a member of the nuclear receptor family of ligand-regulated transcription factors. There are 48 genes encoding nuclear receptors in the human genome (Robinson-Rechavi et al., 2003). The first cDNAs encoding nuclear receptors were cloned in the mid-late 1980's and their domain structures analyzed. That encoding the human VDR was cloned in 1988 (Baker et al., 1988). Typical of other classes of transcription factors, nuclear receptors are composed of a minimum of two structural and functional domains. There is a highly conserved site-specific DNA binding domain composed of two zinc finger motifs. Notably, the first *VDR* genes cloned from patients with hypocalcemic vitamin D-resistant rickets contained point mutations in sequences encoding the DNA binding domain (Hughes et al., 1988). C-terminal to the DNA binding domain lies a less conserved-terminal □-helical ligand-binding domain, which in most receptors serves as a ligand-regulated transcriptional regulatory domain (Mangelsdorf et al., 1995; Robinson-Rechavi et al., 2003; Weikum et al., 2018). Receptors also contain N-terminal domains that can contribute to transcriptional regulation. However, these are highly variable in sequence and length, and that of the VDR is essentially non-existent.

As cDNAs for steroid receptors predominated among the earliest cloned nuclear receptors, subsequent studies on their modes of action became paradigms for receptor action. Steroid receptors function as homodimers and bind in a hormone-dependent manner to cognate DNA sequences in the form of palindromes of hexanucleotide repeats. The universality of this paradigm was shaken by the observation that several nuclear receptors, in addition to recognizing response elements in the form of direct repeats with variable spacing, functioned as heterodimers with common retinoid X receptor (RXR) heterodimeric partners (Leid et al., 1992; Victor et al., 1991). The VDR is a member of this class of receptors, and VDR/RXRs recognize vitamin D response elements (VDREs) in the form of direct repeats of hexameric PuG/TTCA motifs separated by 3bp (so-called DR3 motifs) (Umesono et al., 1991) (**Fig. 2**). Dimerization of most nuclear receptors is essential to create a "footprint" large enough for stable DNA binding. However, even this characteristic is not universal; a subset of receptors possess extended DNA binding domains and can bind stably to DNA as monomers.

Human nuclear receptors have been divided into a series of 6 subgroups. The VDR (NR1I1) is in sub-group 1, which also includes receptors for all-trans retinoic acid (RARs), thyroid hormone (TRs), as well as receptors controlling lipid metabolism, liver X receptors (LXRs) and peroxisomal proliferator activated receptors (PPARs), among others. Unfortunately, reviews that provide high altitude views of nuclear receptor action tend to lump these receptors to together functionally. However, in some respects, function of VDR/RXRs is distinct from other members of group 1. For example, RXRs heterodimerized with RARs, TRs or LXRs bind DNA constitutively and, in the absence of cognate ligands, recruit transcriptional corepressor complexes, thus inhibiting transcription of adjacent genes. Subsequent hormone binding leads to eviction of corepressor complexes and recruitment of coactivators (Hu et al., 2003; Nagy et al., 1999; Perissi et al., 2004). Evidence for such a mechanism of regulation by VDR/RXRs is limited, largely because, unlike the receptors cited above, the association of VDR/RXRs with chromatin is strongly 1,25D-dependent (Jones and Pike, 2020; Meyer et al., 2012) (**Fig. 2**). Like other receptors, VDR agonist binding (and to some degree DNA binding)

controls the movement of C-terminal helix 12 of the ligand binding domain. This generates a conformation optimal for recruitment of coactivator complexes required for transactivation (Zhang et al., 2011; Zheng et al., 2017). Competitive VDR antagonists prevent the movement of helix 12, thus blocking coactivator recruitment (Belorusova et al., 2020). Finally, unlike VDR/RXRs, LXR/RXRs and PPAR/RXRs function as so-called permissive heterodimers, where transcriptional activation can occur through specific ligand binding to either of the heterodimeric partners or to both (Pérez et al., 2012). In contrast, in VDR/RXR heterodimers, the VDR is the unique signaling partner.

Analyses of gene expression profiling studies (either by microarrays or RNAseq experiments) have shown that effects of 1,25D on transcription lead to transactivation or transrepression in roughly equal measure, and that expression profiles are highly cell specific (Dimitrov et al., 2021). It is often assumed that VDR-regulated gene transcription occurs essentially through its interaction with VDREs. However, the reality is considerably more complex. ChIP-seq (chromatin immunoprecipitation followed by high-throughput DNA sequencing) studies have provided valuable information about the genome-wide distribution of the VDR (and RXRs) (Heikkinen et al., 2011; John et al., 2014; Meyer et al., 2014; Meyer et al., 2012; Ramagopalan et al., 2010), notably confirming that 1,25D-induced binding occurs predominantly at DR3-type VDREs. However, some peaks are associated with DNA motifs bound by other types of transcription factors. Importantly, combining ChIP-seq studies with gene expression profiles revealed that VDREs are not enriched in peaks adjacent to downregulated genes. These and other data reveal that transrepression occurs through heterogeneous and cell-specific mechanisms (White et al., 2024). A good example is inhibition by 1,25D of genes whose transcription is driven by cMYC. In the presence of 1,25D, expression of the *MYC* gene is inhibited by ~50% via downregulation of □-catenin, a coactivator of the TCF/LEF transcription factor family that drives *MYC* gene expression. In addition, the 1,25D-bound VDR also induces turnover of MYC protein by recruitment of proteasomal subunits to DNA-bound cMYC (Salehi-Tabar et al., 2019; Salehi-Tabar et al., 2012). Combined, these mechanisms can lead to repression of cMYC-driven transcription and essentially complete loss of

411 cMYC protein. Neither requires direct, sequence-specific DNA binding by the VDR. Other mechanisms
412 of transcriptional regulation by the VDR in immune cells will be presented below.
413
414 **4. Development of Vitamin D Analogues.**

415 **4.1 Secosteroidal Analogues.**

416 From the beginning, the vitamin D field has been the subject of intense analogue development,
417 with likely over 1,000 secosteroidal and non-secosteroidal compounds generated to date (Jones and
418 Pike, 2020; Maestro, 2024). The hydroxylated forms of vitamin D_3, 25D and 1,25D, were discovered
419 in the 1970's and their chemical syntheses followed soon after. Perhaps the most straightforward of
420 the vitamin D analogues is alfacalcidol, or 1α-hydroxyvitamin D (1αD_3) **(Fig. 3),** which has been in use
421 since 1981 (Kubodera, 2009). It is a pro-drug and substrate for 25-hydroxylases, which is converted
422 to 1,25D upon passage through the liver (Kubodera, 2009). Weekly injections of 1αD are well tolerated,
423 and in one clinical trial, were more efficacious than calcium plus vitamin D in correcting bone mineral
424 density in osteoporotic patients (Nuti et al., 2006). Similarly, the 1α-analogue of vitamin D_2,
425 doxercalciferol, is used to treat secondary hyperparathyroidism (Brown, 2001). As described below,
426 alfacalcidol has also demonstrated efficacy in the treatment of immune-related disorders.

427 In addition to its critical role in calcium homeostasis, it has been recognized since the early
428 1980's that 1,25D can induce cellular differentiation and cell cycle arrest (Miyaura et al., 1981; Tsoukas
429 et al., 1984). As a result, analogue development has been driven largely by a desire to separate the
430 calcemic actions of 1,25D from its capacity to induce cell differentiation and growth arrest. Academic
431 laboratories and the pharmaceutical industry have been at least partially successful in this regard,
432 producing several analogues that present substantially reduced, if not absent, calcemic activity while
433 retaining efficacy in inducing cell differentiation and antiproliferative activities. Hundreds of analogues
434 have been produced and an in-depth treatment of their development is beyond the scope of this
435 review. For more information on analogue structures and pharmacological properties, readers are

referred to the comprehensive survey by Glenville Jones and J. Wesley Pike (Jones and Pike, 2020), and the review by (Maestro, 2024). However, a few relevant examples of representative analogues are presented here.

While a number of prodrug variants of vitamin D have been developed (Jones and Pike, 2020), the majority are analogues of the hormonal form 1,25D. Several of this latter group contain (multiple) sidechain modifications, which can substantially modify their pharmacological properties. Three excellent examples are Calcipotriol (MC903) (Calverley, 1987), 22-Oxacalcitriol (OCT, Maxacalcitol) (Murayama et al., 1986), and EB1089 (Seocalcitol) (Binderup et al., 1991a) (**Fig. 3**). Calcipotriol features a C22-23 double bond, a 24-OH group and a terminal cyclopropane ring, whereas in OCT an oxygen replaces the C22 carbon. The extended sidechain of EB1089 also contains conjugated double bonds, notably at the 24 position. Many such sidechain modifications do not substantially compromise the affinity for the VDR, and several compounds act as potent VDR agonists. Importantly, however, they generally substantially diminish affinity of compounds for DBP and alter analogue metabolic breakdown. CYP24A1 catalyzed catabolism of 1,25D is initiated by hydroxylation of the C24 carbon of the cholesterol sidechain, and EB1089 features a double bond at the 24 position. We performed comparative gene expression profiling studies of EB1089 and 1,25D in a human head and neck squamous carcinoma model and found, as expected, that there was extensive overlap between the two profiles. However, induction of expression by 1,25D of several target genes was more transient than that observed in the presence of EB1089, a difference that disappeared in the presence of a CYP450 inhibitor (Lin et al., 2002). This suggests that EB1089 is metabolically more stable in this model. Indeed, studies from Glenville Jones' group have provided evidence that EB1089 is hydroxylated on the C26 carbon in a reaction that does not appear to be catalyzed by CYP24A1 (Shankar et al., 1997). In contrast, the same group showed that CYP24A1 is implicated in the catabolism of calcipotriol and OCT (Masuda et al., 1996; Masuda et al., 1994). Notably, calcipotriol

460 breakdown led, among other products, to the formation of calcitroic acid, the end product of 1,25D
461 catabolism (Masuda et al., 1994).

462 Several sidechain-modified compounds have been studied in an array of pre-clinical disease
463 models and some have entered clinical trials. These compounds are generally less calcemic than the
464 parent hormone 1,25D. For example, in our animal studies, 1,25D at a daily dose in mice of 0.25□g/kg
465 inhibited tumor growth but induced hypercalcemia in a head and neck squamous carcinoma model. In
466 contrast, EB1089 at the same dose was a more efficacious anti-tumor agent and did not raise serum
467 Ca^{++} concentrations (Prudencio et al., 2001). On the surface, it seems paradoxical that a compound
468 that is resistant to CYP24A1-catalyzed catabolism is less calcemic than the parent hormone, given
469 the devastating hypercalcemia described above in IIC patients deficient in CYP24A1 (Jones et al.,
470 2012; Schlingmann et al., 2011). However, sidechain modifications usually substantially reduce the
471 affinity of compounds for DBP, and EB1089, OCT and calcipotriol are no exception in this regard
472 (Hansen and Mäenpää, 1997; Jones and Pike, 2020). *In vitro*, DBP in serum acts as a strong
473 antagonist of 1,25D-induced gene expression in tissue culture experiments. Consistent with their
474 reduced affinity for DBP, sidechain-modified analogues, some with substantially lower affinity for the
475 VDR, behaved as highly potent VDR agonists in tissue culture experiments (Ferrara et al., 1994). *In*
476 *vivo*, the reduced affinity of analogues for DBP has profound implications for their circulating half-lives
477 as well as tissue distribution (Jones and Pike, 2020), and is consistent with the accelerated clearance
478 of vitamin D metabolites observed in DBP null mice, which are resistant to hypercalcemia (Safadi et
479 al., 1999).

480

481 **4.2. Non-secosteroidal analogues.**

482 While secosteroidal 1,25D analogues have had considerable success, including, as discussed
483 below, in the clinic, their syntheses are relatively laborious. In an effort to generate synthetically more
484 accessible compounds, non-secosteroidal 1,25D analogues have been identified from chemical

485 libraries using high-throughput screens for VDR agonism based on reporter gene assays. Two of
486 these, LG190178 and LY2108491 (Boehm et al., 1999; Ma et al., 2006) are shown in **Fig. 3**. There
487 are remarkable parallels in the core structures of the two compounds, and the 25-hydroxy surrogates,
488 which terminate with tert-butyl groups, are identical. While there is some sacrifice in affinity for the
489 VDR in these compounds relative to 1,25D or other secosteroid analogues, they have the virtue of
490 being relatively straightforward to synthesize (Demizu et al., 2011). Of the two, LY2108491 has been
491 tested more thoroughly *in vivo.* Notably, it displayed a dose-responsive efficacy in a surrogate model
492 of psoriasis in the absence of hypercalcemia, unlike 1,25D whose efficacy was paralleled by increasing
493 serum calcium levels (Ma et al., 2006). As described below, secosteroidal and non-secosteroidal
494 1,25D analogues have been used extensively in pre-clinical and clinical studies of immunity and
495 immune-related disorders.
496
497 **5. Vitamin D Signaling in the immune system.**

498 **5.1 Overview of immune system function**

499 The immune system consists of a complex network of distinct immune cell types working
500 together with the aid of physical barriers and non-immune cells to protect the host from pathogens
501 such as bacteria, viruses, parasites, and fungi, while providing surveillance of and protection from
502 internal threats like malignancies (Chaplin, 2010). The cells of the immune system develop in the bone
503 marrow from common progenitors and can be broadly classified into innate and adaptive arms (Rieger
504 and Schroeder, 2012). As their names suggest, innate immune cells are first responders and provide
505 a basal level of defense, whereas adaptive immune cells tailor their response to the specific type of
506 pathogen encountered and generate long last memory. Physical barriers including the skin, mucous
507 membranes, and endothelium represent the first line of defense, which have evolved their own innate
508 immune mechanisms to contain the infection and stimulate the adaptive immune system (Chaplin,

2010). Physiologic barriers including pH, temperature, and chemical mediators work in tandem with physical barriers to destroy invading pathogens (Chaplin, 2010).

Pathogen recognition by innate immune cells and initiation of inflammatory responses relies on detection of microbial antigens by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) (Mogensen, 2009). For example, TLR4 and its co-receptor CD14 recognize lipopolysaccharide from gram-negative bacteria. Downstream signaling results in transcriptional changes and the production of pro-inflammatory cytokines. This stimulates effector responses to combat infection and antimicrobial peptide (AMP) production, which limits pathogen replication or directly kills them via membrane lysis (Mogensen, 2009). Broadly speaking, these effector functions include phagocytosis, degranulation, release of cytokines, growth factors, or enzymes, cytotoxic killing of infected cells, and antigen presentation to adaptive immune cells (Marshall et al., 2018). Phagocytes include monocytes, macrophages, dendritic cells (DCs), neutrophils, and mast cells. Phagocytosis of pathogens and damaged cells is aided by the complement system, a network of plasma proteins, which coat target cells or bacteria and trigger a proteolytic cascade that enhances their detection or induces directed lysis. Cell types such as neutrophils, eosinophils, natural killer cells, and cytotoxic T cells utilize degranulation to directly lyse target cells.

Specialized innate immune cells like DCs, termed professional antigen presenting cells (APCs), bridge the gap between the innate and adaptive immune system through their capacity to efficiently acquire and present pathogen-derived antigen to adaptive immune cells called lymphocytes, while providing additional signals that polarize lymphocyte differentiation and proliferation (Marshall et al., 2018). Antibody secreting lymphocytes (B lymphocytes) differentiate to generate high affinity antibodies. A subset of T lymphocytes ($CD8^+$ cells) acquires cytotoxic effector functions, while the other major subset ($CD4^+$ cells) acquires helper functions. These helper functions include the capacity to stimulate activation, proliferation, effector function, and recruitment of innate and adaptive immune cells, antibody production, and cytotoxic T lymphocyte activity (Marshall et al., 2018). Helper T cell

subsets represent distinct differentiation states with limited plasticity between subsets and notably include highly pro-inflammatory T helper 1 (T_H1) and T_H17 cells, as well as T_H2 cells, follicular helper T cells (Tfh), and regulatory T (Treg) cells (Zhu et al., 2010). Cell fate decisions during differentiation are guided by signals provided by APCs during activation. In this way, the innate and adaptive arms of the immune system coordinate to effectively eliminate pathogens.

Classically, an important distinction between the two arms is the acquisition by B and T cells of the adaptive immune system to form immunologic memory after the infection is cleared, allowing for rapid clearance upon reinfection with the same or similar pathogens (Marshall et al., 2018). However, recent work has described a form of memory in innate immune cells, called trained immunity. This results from epigenetic changes in innate immune cells and may explain how vaccines like the BCG vaccine, which was designed to provide protection from *Mtb*, provides broader immunity to a variety of pathogens (Covian et al., 2019). The expression of the VDR and key metabolic enzymes, and the important effects of their downstream signaling, have been characterized in numerous immune cell types and will be described in the following sections.

5.2 The VDR and vitamin D metabolic enzymes in the immune system.

Studies over the last 40 years or so have provided evidence that CYP27B1 is expressed in several tissues with functions unrelated to calcium homeostasis (Bikle et al., 2018). The largest group of these are epithelial cells (Bikle et al., 2018). Notably, epidermal keratinocytes express higher levels of CYP27B1 than kidney cells and could in theory contribute to circulating levels of 1,25D (Bikle et al., 2018). However, much of 1,25D produced in the epidermis acts locally, suggesting that epidermal CYP27B1 expression is necessary to supply the local demand for 1,25D (Bikle and Christakos, 2020). There are several other extra-renal sites of CYP27B1 expression, including the liver, endocrine glands, thymus, brain, placenta, endothelia, bone. Importantly, CYP27B1 is expressed in several cell types of the innate and adaptive arms of the immune system including DCs, macrophages, monocytes, T cells

559 and B cells (Bikle et al., 2018). Initial studies revealed VDR expression in peripheral blood
560 mononuclear cells (PBMCs) isolated from healthy human donors (Bhalla et al., 1983; Provvedini et al.,
561 1983). While VDR expression was high in monocyte-enriched fractions, no expression was observed
562 in lymphocyte-enriched ones until activated with one of several mitogens. Subsequent studies have
563 confirmed that the VDR is expressed in monocytes (Kreutz et al., 1993), macrophages (Kreutz et al.,
564 1993), and DCs (Brennan et al., 1987). More recently, Vdr expression was detected in rarer cell types
565 in the mouse, including natural killer cells and invariant natural killer T (iNKT) cells (Arora et al., 2022;
566 Yu and Cantorna, 2008).

567 A sensitive approach to track Vdr expression utilizes mice carrying a floxed tdTomato reporter
568 gene under the control of Cre recombinase linked to Vdr expression (Arora et al., 2022). This results
569 in irreversible reporter gene expression in cells that express the Vdr. While traditional tools revealed
570 little to no VDR expression in naïve lymphocyte populations, this approach found that 78% and 60%
571 of splenic B and T cells, respectively, were tdTomato$^+$, including the majority of naïve CD4$^+$ and CD8$^+$
572 T cells. Correspondingly, the majority of bone marrow precursors and thymic T cell progenitors were
573 tdTomato$^+$. As these cells are antigen-inexperienced, the results demonstrate (Arora et al., 2022) that
574 the Vdr is expressed during important developmental stages in both T cell and hematopoietic
575 progenitors without the need for antigenic stimulation, as previous data has suggested (Arora et al.,
576 2022). Furthermore, distinct subsets of innate lymphoid cells (ILC1 and ILC3, but not ILC2) were
577 tdTomato$^+$. Indeed, we recently showed that both the Vdr and Cyp27b1 are expressed at low levels in
578 developing murine thymocytes, as well as in thymic DCs, B cells, and epithelial cells at higher levels
579 (Artusa et al., 2023).

580 **5.3 Signaling pathways controlling *CYP27B1* expression in immune cells.**

581 It is important to note that CYP27B1 expression in immune cells is independent of calcium
582 homeostatic inputs (Ismailova and White, 2022) **(Table 1)**. (N.B. data shown in Table 1 is derived only
583 from studies showing primary or direct cell-specific effects on VDR or CYP27B1 expression or direct

584 effects of 1,25D signaling on immune cell phenotype). In innate immune cells, CYP27B1 is induced
585 downstream of cytokine and PRR signaling, for example, in response to stimuli such as IFNγ and LPS
586 (Overbergh et al., 2000), and thus represents a primary response to pathogen detection **(Fig. 4)**.
587 Incubation of human macrophages with TLR2 ligands resulted in increased VDR and CYP27B1
588 expression (Liu et al., 2006). Further work demonstrated that NF-κB signaling following TLR2/1
589 stimulation induced the expression of IL-15 and IL-1β, which respectively act to stimulate CYP27B1
590 expression and induce T_H1 cell differentiation (Krutzik et al., 2008; Liu et al., 2009). T_H1-derived IFNγ
591 then feeds back on macrophages, potently stimulating the vitamin D regulatory network through NF-
592 κB. Mast cells and neutrophils are granulocytes that act as potent mediators of initial inflammatory
593 cascades. While both express the VDR (Yip et al., 2014), neutrophils do not appear to express
594 substantial levels of CYP27B1 (Szymczak and Pawliczak, 2016). DCs and T cells upregulate
595 CYP27B1 when activated by bacterial cell components or signaling through the T cell receptor (TCR),
596 respectively, and 1,25D signaling in these cell types induces a more tolerogenic T cell phenotype (Wei
597 and Christakos, 2015). Intracrine 1,25D signaling in DCs controls their maturation and capacity to
598 present antigen to T cells. Interestingly, DC differentiation is characterized by increased CYP27B1
599 expression but decreased expression of the VDR, suggesting that 1,25D production by mature DCs is
600 utilized in a paracrine fashion (Hewison et al., 2003). Like T cells, B cells have low expression of the
601 VDR and CYP27B1 in resting conditions but upregulate both in response to stimulation with mitogens
602 (Adams et al., 2014; Provvedini et al., 1983). Evidence suggests that the role of autocrine or paracrine
603 1,25D signaling is not redundant with renal-derived 1,25D in the circulation (Lindner et al., 2017).
604 Specifically, T cell but not B cell specific Cyp27b1-deficient mice mirrored the elevated IgE response
605 of total Cyp27b1 KO mice in a model of ovalbumin sensitization, suggesting that loss of 1,25D
606 production in T cells only is sufficient to drive the hyper-IgE response observed. The numerous
607 signaling pathways controlling CYP27B1 expression in various immune cell types underline the
608 importance of 1,25D signaling in immune system regulation.

5.4 Vitamin D signaling and immune system regulation.

The roles of vitamin D signaling in immunity have been studied extensively, ranging from stimulation of antibacterial and antiviral responses to suppression of autoimmunity (Bouillon et al., 2019). One of the important breakthroughs in this regard was the discovery that vitamin D signaling induces the expression of genes encoding antimicrobial peptides (AMPs). Cathelicidins and defensins represent two major classes of AMPs. VDRE's adjacent to the transcription start sites of human *CAMP* and *HBD2/DEFB4* (β-defensin 2) genes have been characterized (Gombart et al., 2005; Wang et al., 2004), revealing that the genes encoding these AMPs are direct targets of the VDR. *HBD2/DEFB4* induction is epithelial cell-specific, whereas CAMP expression was strongly induced in a wide array of cell types (Wang et al., 2004). This regulation appears to be human/primate-specific; e.g. 1,25D stimulated production of antimicrobial activity in cultured human, but not mouse, epithelial cells against *Escherichia coli* (E. coli), and the lung pathogen *Pseudomonas aeruginosa* (P. aeruginosa) (Dimitrov and White, 2016). In the clinic, circulating levels of CAMP were significantly increased in vitamin D-supplemented Crohn's Disease (CD) patients versus those receiving placebo in a small placebo-controlled trial with 27 participants (Raftery et al., 2015).

Regulation of antimicrobial responses by 1,25D is multi-layered; in addition to inducing AMP production, 1,25D stimulates PRR expression and autophagy (Ismailova and White, 2022). Autophagy is a key process of immunity that ties the innate and adaptive immune systems together by enhancing antigen presentation and regulating cytokine production, in addition to its role in eliminating intracellular pathogens (Deretic, 2016). Vitamin D can activate autophagy in numerous cell types including keratinocytes, hepatocytes, and endothelial cells in response to cellular damage and oxidative stress (Bhutia, 2022). 1,25D induces autophagy in macrophages by enhancing branched chain amino acid (BCAA) catabolism (Dimitrov et al., 2021). BCAAs are essential amino acids that act as indicators of metabolic status in macrophages and activate signaling by the key metabolic kinase mammalian target of rapamycin (mTOR) (Dimitrov et al., 2021), a key inhibitor of autophagy. PRR

634 expression is also regulated by 1,25D. Monocytic cells and keratinocytes treated with 1,25D strongly
635 upregulated *CD14* gene expression, the co-receptor of TLR4 (Oberg et al., 1993). 1,25D also induces
636 the expression of genes encoding the PRR, NOD2/CARD15, whose gene is mutated in a subset of
637 patients with CD, further supporting a role for vitamin D signaling in barrier immunity (Wang et al.,
638 2010). Cytokines and chemokines, a type of cytokine which stimulates cell migration and recruitment
639 to tissues, are critical messenger signals in the immune system. In macrophages, 1,25D stimulates
640 the production of IL-1β and chemokines including CCL3, CCL4, CCL8, IL-8/CXCL8 (Verway et al.,
641 2013). Co-culture of *Mtb*-infected macrophages with primary human airway epithelial cells revealed
642 that 1,25D-dependent induction of IL-1β enhanced infected macrophage survival (Verway et al., 2013).
643 Collectively, these data show that vitamin D signaling positively regulates the anti-microbial activity of
644 first responders to infection, which include macrophages, neutrophils, and stromal cells.

645 Although 1,25D signaling stimulates antimicrobial immunity, it can also paradoxically function
646 to dampen the immune response, particularly in the adaptive arm of the immune system. For example,
647 1,25D suppressed the production of pro-inflammatory cytokines IL-6, TNFα, and IFNγ in *Mtb*-infected
648 human peripheral blood mononuclear cells (PBMCs), in a dose-dependent manner (Khoo et al., 2011).
649 Stimulation with lipopolysaccharide (LPS), a component of the outer cell wall of gram-negative bacteria
650 and the ligand for TLR4, induced IL-6 and TNFα production in human and murine
651 monocytes/macrophages. Notably, this induction was suppressed by 1,25D treatment (Zhang et al.,
652 2012). In NK cells, 1,25D in combination with the synthetic glucocorticoid dexamethasone enhanced
653 the expression the anti-inflammatory cytokine IL-10 and induced a regulatory phenotype (Deniz et al.,
654 2008). Similarly, intracrine production of 1,25D by DCs induces a tolerogenic phenotype, characterized
655 by IL-10 production, decreased IL-12 and IL-23 production, and decreased expression of MHC-II and
656 co-stimulatory molecules including CD40, CD83, and CD86 (Adorini and Penna, 2009). This, in turn,
657 has significant downstream effects on the phenotype of lymphocytes including reducing B cell

658 proliferation and antibody production, as well as reduced differentiation of pro-inflammatory Th1 and
659 Th17 T cell populations.

660 In addition to the cell extrinsic effects of 1,25D on T cell activation and differentiation, there
661 are also intrinsic effects as well. As mentioned above, TCR triggering with co-stimulation results in
662 upregulation of the VDR (Bishop et al., 2021). In human T cells, 1,25D signaling through the VDR
663 results in enhanced expression of the VDRE containing gene *PLCG1*, which encodes PLC-γ1, a
664 central component of the TCR signaling cascade (von Essen et al., 2010). Therefore, 1,25D signaling
665 in human cells contributes to T cell priming in a cell intrinsic manner. This may assist with the rapid
666 activation of T cells when a threat is detected, however, 1,25D signaling through the VDR in T cells
667 also attenuates *IL2* transcription, which is fundamental to the differentiation and proliferation of both
668 $CD4^+$ and $CD8^+$ T cells (Chen et al., 2014; Rigby et al., 1984). This may be essential to limit excessive
669 proliferation of T cells after antigen exposure. Consistent with this, Vdr or Cyp27b1-deficient murine T
670 cells have a hyperproliferative phenotype.

671 Regulation of T helper cell differentiation by vitamin D is well documented. $CD4^+$ T cells derived
672 from *Vdr* KO versus wildtype mice cultured in the presence of T_H17 inducing cytokines (transforming
673 growth factor beta (TGF-β1) and IL-6) with TCR stimulation resulted in double the amount of T_H17
674 cells in KO cultures (Bruce et al., 2011). Conversely, treatment of wildtype T cells with 100nM 1,25D
675 reduced the frequency of T_H17 cells by half. 1,25D treatment also inhibits T_H1 differentiation and IFNγ
676 production (Bruce et al., 2011). In addition, complement binding to CD46 on differentiated human T_H1
677 cells induced VDR and CYP27B1 production and subsequent changes in gene expression leading to
678 attenuation of their pro-inflammatory phenotype (Chauss et al., 2022). While the frequency of naturally
679 occurring anti-inflammatory regulatory T cells (Tregs) in the circulation or in secondary lymphoid
680 organs is unaltered in *Vdr* or *Cyp27b1* KO versus wild-type mice (Artusa et al., 2024), *in vitro*
681 polarization of Vdr KO Tregs using TGF-β1 and TCR stimulation is significantly impaired relative to

controls. Collectively, these results show that vitamin D signaling influences T cell differentiation at several levels, attenuating inflammatory T cell responses.

Recently, a randomized controlled trial of 25,871 participants, vitamin D and marine omega 3 fatty acid supplementation and incident autoimmune disease (VITAL), examined the effect of daily intake of 2,000 IU of vitamin D, 1,000 mg of omega 3 fatty acids, or matched placebos, respectively, on autoimmune disease incidence within a 5 year period (Hahn et al., 2022). Participants included diverse but mostly non-Hispanic white (70%) ethnicities, made up of roughly 50% men and women over the age of 50 in the U.S. Vitamin D supplementation, with or without omega 3 fatty acid intake, reduced the incidence of all self-reported autoimmune diseases by 22% (hazard ratio 0.78, confidence interval 0.61–0.99, P=0.05). Intriguingly, the protective effects of vitamin D supplementation dissipated 2 years after the trial ended (Costenbader et al., 2024). Consistent with the results of the trial, low vitamin D status exacerbates disease severity and mortality in a variety of murine models of human autoimmune diseases. In a chemical injury model of gut colitis, which is dominated by pathogenic T_H1 and T_H17 responses (Froicu and Cantorna, 2007), Vdr-deficient mice exhibited increased T_H17 differentiation and disease pathology. In the same study, it was shown that vitamin D supplementation of wildtype mice reduced disease severity. 1,25D treatment also slows the progression of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS) (Lemire and Archer, 1991). The protective effects of 1,25D in EAE pathogenesis was abrogated by selective deletion of the Vdr in T cells (Mayne et al., 2011). Dietary intake of 1,25D in mice also prevented or abrogated symptoms associated with two murine models of arthritis (Cantorna et al., 1998). Similarly, dietary intake of 1,25D in combination with intraperitoneal injections attenuated the symptoms of a mouse model mimicking human systemic lupus erythematosus (SLE) (Lemire et al., 1992). Low vitamin D status is also a risk factor for conditions such as asthma, allergic rhinitis, and wheezing (Bener et al., 2014), where T_H2 cells play a pathogenic role. Mice fed a vitamin D deficient diet had increased T cell dependent antibody titers associated with enhanced T_H2 responses in a mouse model of allergic

707 airway hypersensitivity to chicken egg ovalbumin (OVA) (Heine et al., 2014). Therefore, vitamin D
708 signaling controls autoinflammatory disease progression in models with distinct pathogenic
709 mechanisms.

710 Numerous pre-clinical and clinical studies have linked decreased vitamin D levels to increased
711 incidence of type 1 diabetes (T1D) (Daskalopoulou et al., 2022; Soltesz et al., 2007), which is
712 characterized by autoimmune destruction of insulin-producing beta cells of the pancreas. Non obese
713 diabetic (NOD) mice, which have mutations in a variety of loci encoding proteins that regulate T cell
714 activation, are the most common murine model of T1D (Chen et al., 2018). Results of studies
715 examining the role of vitamin D signaling in incidence of autoimmune diabetes in NOD mice are
716 conflicting. Early evidence suggested that vitamin D supplementation in female NOD mice reduced
717 the incidence of disease from 56% to 8% (Mathieu et al., 1994). Another study found that both male
718 and female mice fed a vitamin D-depleted diet in the first 100 days of life led to increased T1D
719 incidence during adulthood (Giulietti et al., 2004). Unexpectedly, Vdr-deficient NOD mice developed
720 diabetes at the same rate as control NOD mice (Gysemans et al., 2008). Thus, more investigation of
721 vitamin D signaling in disease incidence in the NOD mouse model is merited. It is important to note
722 that vitamin D signaling in pancreatic beta cells is important for their proliferation and survival (Wang
723 et al., 2016). Collectively, poor vitamin D status is linked to the pathogenesis of multiple models of
724 autoinflammatory disease, mirroring clinical findings made in humans. However, the potential
725 therapeutic benefits of 1,25D supplementation in the prevention and treatment of human autoimmune
726 diseases is still under investigation.

727 Given the current literature, it is highly probable that 1,25D signaling in both arms of the
728 immune system is involved in autoinflammatory disease initiation and progression. In addition, other
729 evidence exists that vitamin D signaling in non-immune cells may shape the immune response.
730 Adoptive transfer of T cells into recombinase activating gene (RAG) deficient mice results in T cell
731 driven intestinal inflammation and mortality. Interestingly, in reciprocal bone marrow chimera

732 experiments, wildtype T cells that developed in *Vdr* deficient hosts were far more pathogenic than Vdr
733 KO T cells that developed in wildtype mice when adoptively transferred into RAG KO mice (Giulietti et
734 al., 2004). Furthermore, *Vdr* KO T cells that developed in wildtype hosts did not have significantly
735 differences in weight loss when compared to wildtype T cells that developed in wildtype mice. This
736 data strongly argues for the involvement of non-hematopoietic cell types in long lasting phenotypic
737 changes observed in T cells.

738 While vitamin D signaling in keratinocytes and other non-immune stromal cell types has been
739 carefully examined, vitamin D action in stromal cells of the thymus, which shapes T cell development,
740 has been largely overlooked until recently. The thymus regulates the maturation of bone marrow
741 derived T cell precursors into mature naïve T cells. Critical developmental checkpoints including MHC
742 restriction, lineage commitment, and the negative selection of highly autoreactive T cells, all of which
743 occur in waves through interactions between the TCR and peptide-MHC expressed by antigen
744 presenting thymic epithelial cells (TECs) (Shichkin and Antica, 2022). Importantly, defects in negative
745 selection result in compromised central tolerance and the escape of autoreactive T cells into the
746 circulation, which leads to autoimmunity. The activity of the transcription factor autoimmune regulator
747 (AIRE) is critical to the process of negative selection, as it induces the ectopic expression of tissue
748 restricted antigen (TRA) in medullary TECs (mTECs) (Marx et al., 2021). Loss of AIRE function in
749 humans results in autoimmune polyendocrinopathy candidiasis ecto-dermal dystrophy (APECED), a
750 multifaceted autoimmune disorder. Thus far, it has been shown that thymocyte development in the
751 absence of 1,25D signaling is largely normal, with little to no differences in the proportion of major
752 thymocyte subsets (Artusa et al., 2024; Yu and Cantorna, 2011). However, the intra-thymic
753 development of regulatory innate-like immune cells including iNKT and CD8αα-intraepithelial
754 lymphocytes is defective in Vdr deficient mice (Artusa et al., 2024; Yu and Cantorna, 2008; 2011).

755 We found that the Vdr and Cyp27b1 are expressed in multiple non-lymphoid cell types in the
756 thymus, notably Aire^{+} mTECs, and that 1,25D signaling was intact in TECs (Artusa et al., 2023).

Notably, 1,25D treatment induced Aire and TRA gene expression, suggesting that vitamin D signaling may regulate key aspects of central tolerance. Consistent with this, the proportion of Aire^{+} mTECs and TRA gene expression was reduced in the thymus of Vdr and Cyp27b1 deficient mice (Artusa et al., 2024). Moreover, markers of T cell negative selection were diminished in Cyp27b1 deficient mice. Evidence of spontaneous autoimmunity could be observed in the pancreas and stomach of Cyp27b1 deficient mice. Additionally, we found that thymi were significantly smaller in Cyp27b1-deficient mice by 8 weeks of age, and that loss of vitamin D signaling led to a premature thymic aging phenotype. These results are noteworthy as aging increases susceptibility to infection and cancer due to accumulated defects in immune function (Liang et al., 2022). This is in part attributable to loss of thymic function, as thymic activity peaks in our infancy and childhood. This suggests that vitamin D signaling contributes to thymic function by promoting tissue longevity and the development of self-tolerant T cell repertoire.

AIRE regulates the expression of thousands of genes, but it is poorly understood how it is recruited to chromatin due to the absence of a sequence-specific DNA-binding domain. To date it's been shown that distinct domains of AIRE recognize H3K4me0 marks on chromatin, bind to methylated CpG islands, or are anchored to Z-DNA (Fang et al., 2024; Org et al., 2008; Waterfield et al., 2014). While these interactions contribute to Aire activity, disruption of their interactions do not alter the breadth of AIRE targets, suggesting that other recruitment mechanisms are active. The presence of 4 conserved LXXLL motifs, nuclear receptor binding sites, in AIRE strongly suggests that it can bind to nuclear receptors *in vivo* (Nagamine et al., 1997). We found that AIRE was a coactivator of the Vdr in a model *in vitro* system and that the two proteins are recruited to chromatin in a 1,25D-dependent manner in primary murine mTECs. This supports the notion that AIRE may interact with and be recruited to DNA by its interaction partners, which include the Vdr and potentially other nuclear receptors (Artusa et al., 2023). Since nuclear receptors target genes also number in the thousands, such interactions may function to enhance the breadth of Aire targets.

6. Vitamin D and its analogues in immune-related disorders.

Several publications have alluded to connections between seasonality of infectious diseases and fluctuating vitamin D metabolite levels. However, the seasonality of vector-borne infections also correlates with climatic conditions favoring their replication (Coussens, 2017). Nonetheless, as presented above, the expression of the VDR and CYP27B1 in immune cells is widespread, and there is growing preclinical evidence for roles of vitamin D signaling in controlling innate immune and inflammatory responses **(Fig. 4)**. As a result, there have been numerous clinical trials to test the potential benefits of vitamin D supplementation to prevent or attenuate the incidence or severity of immune-related disorders. The ultimate test of the efficacy of vitamin D supplementation is the randomized, double blind, placebo-controlled trial (RCT). However, RCTs with vitamin D supplementation are fraught with complications because vitamin D is a nutrient and not a drug, and assessment of vitamin D intake is complicated by the cutaneous synthesis that occurs in the presence of adequate solar UVB. Finally, as it is unethical to leave a patient vitamin D deficient, placebo wings frequently include low-dose vitamin D supplementation (often 400IU/day). As a result, many trials are conducted on vitamin D-sufficient populations. Moreover, as described below, dosing schedules appear to be key for efficacy.

6.1 Vitamin D supplementation in bacterial infections.

In vitro studies described above showed that vitamin D signaling in humans induces expression of the genes encoding antimicrobial peptides CAMP and HBD2, as well as the secretion of antimicrobial activity (Wang et al., 2004). In an RCT of patients treated with placebo or 1,000 IU/day of vitamin for 90 days, supplementation enhanced antimicrobial activity of lung airway surface liquid (ASL) (Vargas Buonfiglio et al., 2017). Moreover, supplementation eliminated seasonal variations in ASL antimicrobial activity consistent with fluctuating vitamin D metabolite levels. Moreover, ASL

807 antimicrobial activity was inhibited by a blocking antibody recognizing the active form of CAMP, LL37
808 (Vargas Buonfiglio et al., 2017). These studies thus link vitamin D status to mucosal antibacterial
809 activity in humans.

810 Consistent with a role of vitamin status in controlling antibacterial activity, several cross-
811 sectional and observational studies as well as RCTs link low vitamin D status to dental caries
812 (Ismailova and White, 2024; Li et al., 2023; Olczak-Kowalczyk et al., 2021; Suárez-Calleja et al., 2021).
813 These were supported by umbrella analyses of systematic reviews and meta-analyses (Hujoel, 2013;
814 Theodoratou et al., 2014). One, encompassing a broad array health outcomes, singled out an inverse
815 association of dental caries with vitamin D status (Theodoratou et al., 2014). While the quality and
816 reliability of trials varied considerably, the study noted that “restricting the analysis to studies with
817 nonbiased treatment assignment increased the percent reduction in caries rate from 47% to 54%”
818 (Theodoratou et al., 2014). Robust innate immune responses, including AMP production, are important
819 for oral health (Dale et al., 2006; Ozturk et al., 2010). Notably, CAMP/LL-37 is efficacious against
820 bacterial species, such as *Streptococcus mutans*, present in plaque (Yoshida et al., 2019), and its
821 concentrations in saliva correlate positively with vitamin D status (Gyll et al., 2018). Another study
822 found an inverse correlation with salivary 25D levels and severity of caries (Nireeksha et al., 2024).
823 Vitamin D status has also been linked with AMP levels in other diseases of oral health such as gingivitis
824 and chronic periodontitis (Bayirli et al., 2020).

825 Bacterial infections of the urinary tract (urinary tract infections, UTI) are also linked to low
826 serum vitamin D levels. Vitamin D signaling induces CAMP expression in the urinary bladder (Hertting
827 et al., 2010), and urinary LL-37 levels are correlated with vitamin D metabolite levels in young children
828 (Georgieva et al., 2019). Clinically, recurrence of UTI in infants, children, and premenopausal women
829 are linked to low vitamin D status (Gan et al., 2023; Georgieva et al., 2019; Hacihamdioglu et al., 2016;
830 Nseir et al., 2013; Tekin et al., 2015). These findings are supported by a meta-analysis of 9 studies
831 and 1921 participants, which confirmed the correlation between rates of UTI and low vitamin D status

(Deng et al., 2019). Numerous publications have also linked poor vitamin D status to severity of bacterial sepsis, and correlations have been made between seasonal variations in vitamin D status and seasonality of sepsis, whose incidence is highest in the winter and lowest in the fall (Danai et al., 2007; Kempker et al., 2012). Moreover, in the US, seasonal variations increase with latitude (Danai et al., 2007). A systematic review of observational cohort studies encompassing 9,715 critically ill patients concluded that low serum vitamin D levels increased the risk of sepsis and mortality in critically ill patients (de Haan et al., 2014). Subsequent studies reached similar conclusions, including one in neonates (Seok et al., 2023; Upala et al., 2015; Workneh Bitew et al., 2021). In a small RCT (20 patients only) in new-onset sepsis patients, high-dose vitamin D supplementation trial raised 25D levels by 5–15 ng/mL (200,000 and 400,000 IU, respectively) and increased circulating levels of LL-37, but did not analyze sepsis-related outcomes (Quraishi et al., 2015). Unfortunately, this trial is typical of other intervention trials, which were underpowered to assess effects of supplementation on clinical outcomes in sepsis patients. Clearly, larger RCTs, to assess the efficacy of supplementation with vitamin D or one of its analogues, are warranted.

6.2 Vitamin D supplementation in viral infections.

There has been widespread interest in the potential protective effects of vitamin D in respiratory infections, many of which are viral in origin. In an RCT conducted on Japanese school children (Urashima et al., 2010), vitamin D supplementation reduced the rates of seasonal influenza A, with the most striking effects in children who had not previously received supplements. While these results are compelling, globally, the results of intervention trials are mixed. Adrian Martineau and coworkers have published two meta-analyses of studies examining the potential benefits of vitamin D supplementation on incidence and severity of acute respiratory tract infections (ARIs) (Jolliffe et al., 2021; Martineau et al., 2017). The findings are important for several reasons. Both the 2017 study and its 2021 update highlighted the fact that daily dosing of vitamin D, as opposed to bolus dosing, was

efficacious in reducing the incidence of ARIs. The 2021 update noted “No significant effect of vitamin D supplementation on the risk of having one or more ARIs was observed for any of the subgroups defined by baseline 25(OH)D concentration.” (Jolliffe et al., 2021). As pointed out in a recent viewpoint article (Giustina et al., 2024), of mega supplementation trials in New Zealand, Australia and the U.S., two employed monthly bolus dosing schedules, which other studies have found are not efficacious. There is thus a need for population-based RCTs of daily vitamin D supplementation that are sufficiently powered to detect any effects of vitamin D on a variety of indications, immune-related or otherwise. There is also a need for further analysis of individual participant data from existing trials aimed at sub-group analysis to assess the potential benefits of supplementation of individual with low vitamin D levels (25D levels <25nM) (Giustina et al., 2024).

The growing links between vitamin D signaling and antiviral innate immunity (White, 2022) spurred an interest in a potential role of vitamin D status and incidence and outcome of COVID-19, which is caused by severe acute respiratory syndrome coronavirus 2 (SARS CoV2) infection. Immune evasion strategies by SARS CoV2 can be accompanied by elevated proinflammatory cytokine release, pneumonia (Wang et al., 2020), sepsis and potentially fatal acute respiratory distress syndrome (Zhou et al., 2020), symptoms whose incidence is also associated with poor vitamin D status (Quesada-Gomez et al., 2020; Tsujino et al., 2019; Zhou et al., 2019). It is noteworthy that 1,25D was identified as a repurposed drug for treatment of H5N1 influenza A virus-induced lung injury, whose clinical features are similar to those of COVID-19 (Huang et al., 2021). During the pandemic, numerous clinical studies were conducted to determine the potential relationship between vitamin D status and COVID-19 incidence and outcome. A recent systematic review and meta-analysis of 16 studies assessed the potential protective role of vitamin D supplementation in COVID-19 incidence, mortality, and patient intensive care unit admission (Sartini et al., 2024). Significantly, the analysis, which included a mix of RCTs, as well as prospective, retrospective and cohort studies with different dosing regimens, concluded that supplementation has a protective effect against COVID-19 incidence in RCTs and

against both incidence and intensive care unit admission in other study types. While these results are encouraging, further large-scale RCTs will be necessary to firmly establish the protective effects of vitamin D in COVID-19.

6.3 Inflammatory Bowel Diseases.

Crohn's disease (CD) is a relapsing-recurring form of IBD arising from defective intestinal innate immune homeostasis (White, 2018). The incidence of CD is rising worldwide (Ng et al., 2017), and, notably, its frequency increases with increasing latitude. In addition, there is an association of CD risk with low sun exposure early in life (Holmes et al., 2019; Jantchou et al., 2014). Given the roles of vitamin D signaling in enhancing innate immunity and suppression of inflammatory immune responses in general, and the relevance of its target gene regulation to CD specifically, there has been considerable interest in vitamin D (analogue) supplementation in CD therapy (Vernia et al., 2022). Sidechain analogues of 1,25D, BXL-62 [1α,25$(OH)_2$-16-ene-20-cyclopropyl-vitamin D_3], TX 527 [19-nor-14,20-bisepi-23-yne-1,25$(OH)_2D_3$] and ZK-191784 (with a cyclopropane- and oxazole-substituted sidechain) have demonstrated efficacy in reducing inflammatory markers and maintaining barrier integrity in preclinical or *ex vivo* models of IBD (Laverny et al.; Martinesi et al., 2014; Stio et al., 2007). A number of generally small intervention trials have, collectively, supported a role for supplementation in reduction of disease activity (White, 2018). Meta-analyses of observational studies and RCTs linked poor vitamin D status to increased disease activity and confirmed the benefits of supplementation in reducing rates of clinical relapse (Gubatan et al., 2019; Li et al., 2018; Valvano et al., 2024). It is important to note, however, that, in some CD patients, supplementation is complicated by intestinal malabsorption after bowel resections or ostomy procedures associated with CD management. For example, in one study, patients who did not respond to oral supplementation benefitted from high-dose sublingual vitamin D_2 treatment (McCullough and Heaney, 2017). Moderate UVB exposure is also an alternative in these cases (Koutkia et al., 2001).

It is likely that therapeutic benefit of vitamin D supplementation of CD patients also arises from the effects of vitamin D sufficiency on composition of the gut microbiome (Cantorna and Arora, 2024). A healthy microbiome contributes to normal immune system development, extraction of energy and nutrients from dietary components, and competitive displacement of pathogens. It also appears that the nature of microbial colonization that occurs early in life is important for immune system development and may affect disease risk later in life (Rinninella et al., 2019). CD is characterized by dysbiosis of the gut microbiome, with an enrichment of species associated with inflammation (Kaakoush Nadeem et al., 2020; Marie et al., 2011; Seksik et al., 2003). In an RCT conducted on healthy adults, serum 25D levels were associated with enrichment of beneficial bacteria at the expense of pathogenic species. Notably, supplementation led to a dose-dependent increase in species associated with decreased IBD activity (Charoenngam et al., 2020). Given all of the above, it would be important to undertake larger scale RCTs to determine optimum dosing schedules for supplementation and to assess the potential interactions of supplementation with other CD therapeutics (Cantorna and Arora, 2024).

6.4 Vitamin D and allergies.

Allergies arise from an overreaction of the immune system to allergens due to genetic, environmental, and nutritional factors. The pathogenesis of allergic reactions can be attributed to excessive type 2 responses with participation from T_H2 cells, mast cells, basophils, eosinophils, and IgE secreting B cells (Zhang et al., 2024). In addition to less common excessive responses to food allergens, allergic diseases including asthma, allergic rhinitis, and atopic dermatitis can place a heavy burden on the affected persons and the healthcare system. While allergies are not strictly geographically restricted, there are observations of increased frequencies of allergies in populations at higher latitudes (Camargo et al., 2007; Mullins et al., 2009). Moreover, there is data suggesting that sunlight exposure may be critical in the first two years of life to reduce the risk of developing food

allergies, asthma, allergic rhinitis, and atopic dermatitis (Hwang et al., 2016). This suggests that vitamin D status early in life may be an important factor in preventing childhood onset of allergic diseases.

Studies examining asthma risk in healthy and asthmatic individuals have found that vitamin D status is negatively correlated with asthma risk and severity in both children (Bener et al., 2012; Kolokotroni et al., 2015), and adults (Chang et al., 2023; Niruban et al., 2015; Zhu et al., 2022). Various studies have reported an inverse relationship between vitamin D status and rates of atopic dermatitis in children and adults (Baek et al., 2014; Cicek and Kole, 2023; Ng and Yew, 2022). Similarly, the majority of studies are in agreement that vitamin D levels are lower in atopic rhinitis patients than in healthy controls (Coban et al., 2021; Restimulia et al., 2018; Saad et al., 2020). However, a number of studies have found no links between vitamin D status and the risk of developing atopic dermatitis or allergic rhinitis, in both adults and children (Baiz et al., 2014; Berents et al., 2016; Cheng et al., 2014; Wu et al., 2017). Thus, more work is required firmly establish a relationship between low vitamin D status and disease risk. We note that differences in risk for each of these diseases have also been attributed to gender, race, ethnicity, genetics, and lifestyle choices.

6.5 Vitamin D and autoimmunity

Poor vitamin D status is implicated in all major autoimmune diseases (Bouillon et al., 2019). Intriguingly, the incidence of T1D and MS are associated with latitude (Bach, 2018). A study of white army recruits in the U.S. found that 25D levels of less than 50 nmol/L at the time of recruitment were linked to an almost twofold increased risk of MS later in life (Munger et al., 2006). Maternal 25D levels of less than 30 nmol/L were associated with a 1.9-fold increased risk of development of MS in the offspring of Finnish women (Munger et al., 2016). Increased MS risk in multiple studies was also correlated with genetic variants predisposing to lower circulating 25D levels (Mathieu et al., 2005). A

low frequency variant of *CYP2R1* which conferred a large effect on circulating 25D levels was associated with a 1.4-fold increased risk of developing MS (Manousaki et al., 2017). A meta-analysis of cohort studies conducted in the U.S. and Sweden analyzing the effects of single nucleotide polymorphisms associated with decreased 25D levels also concluded that poor vitamin D status was a risk factor for MS (Rhead et al., 2016).

In a study identifying 310 T1D cases (along with 613 controls) found that 25D levels > 100nmol/L were associated with a 44% lower risk of T1D in non-Hispanic US military personnel. In contrast, those in the bottom 20% of 25D status had the highest risk of developing T1D (Munger et al., 2013). Several retrospective studies found that vitamin D supplementation in the first year of life lowered the risk of later development of T1D. A large cohort study of 10,366 children born in northern Finland in 1966 and published in 2001 found that vitamin D supplementation was associated with dramatically decreased incidence of T1D (Hyppönen et al., 2001). Specifically, the relative risk of regular versus no supplementation was 0.12. In addition, children who took the recommended 2,000 IU per day dose had a relative risk of 0.22 compared to those who received lower doses. Finally, children displaying symptoms of rickets had a 3.0 increased relative risk of developing T1D (Hyppönen et al., 2001). These findings are significant as interpretation of many studies is complicated by the effects of sun exposure on vitamin D status. However, sun exposure is not a significant source of vitamin D in northern Finland due to the year-round vitamin D winter.

6.6 Vitamin D analogues in inflammatory disorders

In addition to their therapeutic applications for indications associated with disrupted calcium homeostasis such as osteoporosis and chronic kidney disease (CKD)(Maestro et al., 2019), vitamin D analogues have demonstrated efficacy for the treatment of various inflammatory disorders. Numerous classes of analogues have shown superior efficacy relative to 1,25D in the suppression of inflammation and modification of immune population phenotypes. Evidence is derived from pre-clinical studies using

human PBMCs and mouse models, in addition to clinical data examining their effects on psoriatic lesions. One of the earliest analogues whose immune regulatory function was described was alfacalcidol. In agreement with its potential anti-inflammatory properties, alfacalcidol treatment attenuated disease severity in a rat model of type II collagen-induced arthritis (Tsuji et al., 1994). In the clinic, one case report claimed that alfacalcidol treatment cured psoriatic lesions (Berth-Jones and Hutchinson, 1992). Two studies in humans undergoing hemodialysis for chronic kidney disease oral administration of alfacalcidol for four weeks enhanced IL-2 production and lymphoproliferative responses of isolated PBMCs, respectively (Tabata et al., 1988; Tabata et al., 1986). These findings are intriguing as they are in contrast to our current understanding of the function of vitamin D signaling in suppression of *IL2* transcription and T cell proliferation. However, it is unclear how the diseased state in CKD patients affected these outcomes.

Newer vitamin D analogues have yielded results consistent with our current understanding of vitamin D biology. Topical application of calcipotriol **(Fig. 3)** on psoriatic lesions reduced epidermal differentiation, the production of pro-inflammatory cytokines such as IL-6 and attenuated the recruitment of T cells and neutrophils. Treatment of human PBMCs with calcipotriol reduced LPS-dependent IL-1 production, and in separate work, attenuated IgM, IgG, and IgA antibody production in mitogen stimulated cells (Berth-Jones and Hutchinson, 1992). In the clinic, topical calcipotriol is an efficacious treatment of psoriasis vulgaris (Kragballe et al., 1991) and is currently one of the most commonly prescribed medications alongside betamethasone, a corticosteroid (Patel et al., 2008). Importantly, calcipotriol is efficacious without causing hypercalcemia - a major limitation for usage of naturally occurring 1,25D at therapeutic dosages. Mechanistically, this appears to be due to reduced affinity for DBP and more rapid clearance (Berth-Jones and Hutchinson, 1992) (see also section 4 on vitamin D analogues). While highly efficacious, up to 20% of patients treated with topical calcipotriol experience cutaneous irritation when applied to the face or intertriginous areas. Notably, a comparative clinical study assessing the safety and efficacy of calcipotriol versus calcitriol found that patients using

calcitriol had a significantly greater improvement due to effects on flexural region without any clinically significant hypercalcemia observed.

As such, newer analogues such as tacalcitol and maxacalcitol were developed. Tacalcitol (1α,24-dihydroxyvitamin D_3) is hydroxylated at the 24 position but lacks other sidechain modifications. Its topical application was effective at reducing PMN, T cell, and monocyte numbers in psoriatic lesions while inhibiting epidermal cell proliferation. While less cutaneous irritation was reported in humans, there is evidence that tacalcitol may also be less efficacious as a therapy. Maxacalcitol **(Fig. 3)** suppressed keratinocyte proliferation *in vitro* significantly better than both calcipotriol and tacalcitol (Barker et al., 1999). Furthermore, once-daily topical application of maxacalcitol markedly improved or cleared psoriasis in 55% of participants, compared to 46% for those receiving calcipotriol (Barker et al., 1999). Maxacalcitol also has also demonstrated efficacy in treating palmoplantar pustulosis, a chronic inflammatory skin condition characterized by pustules on the palms and soles (Yamamoto, 2019). Pre-clinical studies using murine models have demonstrated that topical maxacalcitol treatment on imiquimod-induced psoriatic skin inflammation reduced MHC-II$^+$ cell infiltration, expression of pro-inflammatory cytokines IL-17, IL-22, IL-12p40, TNFα, and IL-6 mRNA in the skin, and significantly increased FoxP3$^+$ regulatory T cell infiltration and IL-10 expression when compared to betamethasone ointments or vehicle controls (Hau et al., 2018). Interestingly, maxacalcitol uniquely suppressed the expression of IL-23p19, which is elevated in psoriatic lesions and contributes to the stabilization of pathogenic Th17 signature secretion. This is noteworthy as pro-inflammatory Th17 cells are pathogenic in a variety of autoimmune and autoinflammatory conditions (Yasuda et al., 2019), providing a clear rationale for investigating the use of vitamin D and its analogues in the treatment of such diseases. Notably, one study found that oral maxacalcitol treatment significantly reduced markers of arthritis and systemic lupus erythematosus in autoimmune MRL/lpr mice without elevating serum calcium (Abe et al., 1990), and separately, it was shown that oral maxacalcitol had a smaller effect on serum calcium in mice receiving doses even 30X higher than that of 1,25D. Therefore, oral

maxacalcitol therapy could be a promising drug candidate for use in humans with autoimmune disease.

Maxacalcitol, tacalcitol, calcipotriol, alfacalcidol, and doxercalciferol are currently approved for topical use to treat psoriasis in various countries including Canada, the United States, Europe, and Japan (Leyssens et al., 2014). However, efficacy in diverse pre-clinical disease models has been shown for several other vitamin D analogues yet to be used clinically. Systemic administration of MC-1288, a C20 epimer of 1,25D, suppressed the development of type I collagen induced arthritis in mice when given before the onset of disease and reduced the severity of joint inflammation when given at the onset of disease, without causing hypercalcemia (Larsson et al., 1998). KH-1060, another C20 epimer of 1,25D with an extended sidechain bearing an ether, was shown to prevent the onset of T1D in NOD mice at non-hypercalcemic doses (Mathieu et al., 1995). Another study showed that KH-1060 bound to the VDR with a similar affinity as 1,25D but more potently suppressed IL-2 production by mitogen stimulated human PBMCs. Furthermore, KH-1060 was 633,000 times more potent than 1,25D at suppressing murine thymocyte proliferation, albeit with a 1.3-fold increased calcemic effect (Binderup et al., 1991b).

Several analogues have been tested for efficacy in treatment of gut inflammation and have shown promising results. ZK191784, a secosteroidal sidechain analogue, suppressed Th1 mediated colitis (Daniel et al., 2006; Strauch et al., 2007) in mice and modulated NF-κB signaling in murine preadipocytes and macrophages while suppressing their pro-inflammatory phenotype (Zhu and Wilding, 2020). In human PBMCs, ZK191784 demonstrated improved suppression of IFNγ, TNFα, IL-1β, while enhancing induction of IL-4 and IL-10 (Daniel et al., 2005), consistent with its anti-inflammatory effects in murine disease models. Similarly, 1α,25-dihydroxy-16-ene-20-cyclopropyl-24-oxo-vitamin D_3 exhibited significantly enhanced suppression of IL-6, IL-12, IFNγ, TNFα production in human PBMCs compared to 1,25D, while super-inducing *CYP24A1* and *CAMP* (Laverny et al., 2009).

Furthermore, it was markedly less calcemic than 1,25D when administered *in vivo* to mice (Lemire et al., 1994). Importantly, data from primary lamina propria mononuclear cells derived from IBD patients revealed that this analogue inhibited pro-inflammatory cytokine production more efficaciously than 1,25D (Laverny et al., 2010).

While the analogues described above consist of primarily of 1,25D-like secosteroids with various side-chain modifications, non-secosteroid analogues have also been generated and investigated for clinical efficacy. Compound A, a non-secosteroid with a diarylpentane core identical to those of LG190178 and LY2108491, appeared to act in a cell-specific manner; it promoted an anti-inflammatory phenotype in activated human PBMCs, characterized by enhanced Th2 cytokine secretion and diminished Th1 and Th17 cytokine secretion without strongly inducing vitamin D-responsive genes in human intestinal cell lines (Na et al., 2011). Importantly, mouse studies demonstrated that Compound A is not hypercalcemic *in vivo* at high doses and is more efficacious than 1,25D at diminishing EAE severity (Na et al., 2011). Collectively, the increased efficacy and decreased calcemic effects of vitamin D analogues relative to natural vitamin D metabolites support the utility in the clinic. Currently approved analogues are utilized for topical treatment of psoriasis, however, promising results with these and the other analogues suggest they may represent efficacious treatments for other autoinflammatory human diseases.

7. Vitamin D analogues and their potential as adjuncts to cancer immunotherapy.

The potential association between vitamin D status and increased cancer risk was first proposed in a seminal study that found increased colon cancer mortality in populations residing in the northeast of the United States, which has less UV exposure year-round, than those living in the south (Garland and Garland, 1980). Other data demonstrating an association between low serum vitamin D levels and increased risk of prostate, colorectal and breast cancers further supported this hypothesis

(Ahonen et al., 2000; Bertone-Johnson et al., 2005; Garland et al., 1989). A series of studies published in 2010 analyzing serum 25D concentrations and risk of developing rarer cancers, including endometrial, esophageal, gastric, kidney, non-Hodgkin lymphoma, ovarian, and pancreatic cancer, did find a reduced risk of developing cancer in those with serum 25D >30ng/mL. However, whether low vitamin D levels are causative or merely associative is still under debate.

Numerous studies have noted the antiproliferative and pro-differentiation effects of 1,25D on cancer cells (Deeb et al., 2007; Fleet et al., 2012), leading to an interest in vitamin D and its analogues as potential cancer therapeutics. As discussed above, vitamin D also has many immune-modulatory functions, which suggests that its regulation of immune cell function may affect cancer surveillance or control. Several RCTs of vitamin D supplementation have been conducted to test the potential anti-cancer efficacy of vitamin D. A post-hoc analysis of the AMATERASU trial, which included colorectal cancer patients receiving placebo or 2,000 IU/day of vitamin D_3, found a significant benefit for relapse-free survival in the treatment group (Urashima et al., 2019). The SUNSHINE trial, which compared the effect of high-dose vitamin D_3 treatment (8,000 IU/day for 2 weeks, then 4,000 IU/day) to 400 IU/day in advanced colorectal cancer patients receiving chemotherapy did not find a significant difference in progression-free survival but a decreased risk of death (Ng et al., 2019). Similarly, a meta-analysis of 10 RCTs did not find any benefit of vitamin D supplementation on total cancer incidence. However, the same study found an association between supplementation and reduced total cancer mortality, which was largely attributable to supplementation by daily dosing and not infrequent bolus dosing (Keum et al., 2019).

Clinical trials utilizing calcitriol as a single agent or in combination with other drugs have shown varying levels of success. The anti-tumor efficacy of calcitriol is best observed when it is used at high concentrations. While this raises concerns for dose-limiting hypercalcemia, available data indicates that it is safe when administered intermittently (Woloszynska-Read et al., 2011), since mild to moderate hypercalcemia is rapidly reversible. Moreover, oral daily administration in prostate cancer

patients could be safely given for up to 15 months (Gross et al., 1998; Woloszynska-Read et al., 2011). While calcitriol may be administered safely in humans, its efficacy as an anti-cancer therapeutic agent is limited at non-toxic doses. As discussed previously, many vitamin D analogues exhibit increased efficacy relative to calcitriol while largely avoiding hypercalcemic side effects. Inecalcitol (TX-522, 19-nor-14-epi-23-yne-1,25-$(OH)_2D_3$), a side-chain analogue of calcitriol, more potently decreased tumor growth in various cancer models including breast (Verlinden et al., 2000), squamous cell (Ma et al., 2013), and prostate (Okamoto et al., 2012) compared to calcitriol. Furthermore, in these mouse experiments inecalcitol induced tumor regression without significantly affecting serum calcium levels (Ma et al., 2013; Okamoto et al., 2012). Phase I trials in a cohort of 54 advanced prostate cancer patients using inecalcitol in combination with the chemotherapy docetaxel resulted in hypercalcemia in two of four patients receiving 8,000 µg of inecalcitol, which normalized after a few days of drug removal (Medioni et al., 2014). The maximum tolerated dose was determined to be 4,000 µg, 100-times that of calcitriol when in combination with docetaxel (Beer and Myrthue, 2004). Seocalcitol (EB1089; Fig. 3) also had potent antiproliferative effects *in vitro* and significantly decreased tumor growth in vivo in animal models of head and neck squamous cell carcinoma (Prudencio et al., 2001). Seocalcitol and paricalcitol have been examined in phase I and phase II trials. However, neither demonstrated significant anti-tumor efficacy as monotherapies in pancreatic (Evans et al., 2002), hepatocellular carcinoma (Dalhoff et al., 2003) or prostate cancer patients (Schwartz et al., 2008). This may be, in part, because of acquired tumor resistance to vitamin D and its analogues *in vivo*. Intriguingly, resistant cells retain active vitamin D signaling, providing a rationale for potential use of vitamin D analogues in combination therapy, which has become the norm in cancer treatment.

There has been a recent surge in interest in links between vitamin D status and response to immunotherapy, and the potential use of vitamin D and its analogues as adjuncts for cancer immunotherapy **(Fig 5)**. Immune checkpoint inhibition (ICI), a kind of immunotherapy, functions by blocking interactions between cancer cells and inhibitory receptors expressed on T cells, eliciting

anticancer T cell activity. This is achieved through the administration of monoclonal antibodies targeting inhibitory receptors expressed on T cells such as programmed-death 1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4). One study analyzed the relationship between PD-1 ICI efficacy and serum 25D levels in 77 advanced lung cancer patients and found that the baseline 25D levels of partial response patients was significantly higher than that of non-responders. Moreover, overall survival was significantly improved in patients with 25D ≥ 20 ng/mL versus 10–20 ng/mL or < 10 ng/mL (You et al., 2023). In a survey of 703 primary melanoma biopsies, elevated VDR expression was correlated with a reduced risk of melanoma-related death (Muralidhar et al., 2019), along with upregulation of pathways mediating anti-tumor immunity. It was also associated with increased levels of tumor-infiltrating lymphocytes. These results suggest that VDR expression may be useful as a biomarker for response to immunotherapy. In a clinical study with 200 advanced melanoma patients receiving anti-PD-1 immunotherapy, vitamin D_3 supplementation significantly increased the response rate to immune checkpoint inhibition (36.2% in those with 25D ≤ 30 ng/mL and not-supplemented versus 56.0% in supplemented and with 25D > 30 ng/mL; p =0.01)(Galus et al., 2023). Importantly, progression free survival (5.75 versus 11.25 months; p=0.03) and overall survival (27 versus 31.5 months; p = 0.39) was higher in the supplemented group. These data strongly suggest that vitamin D status is associated with, and supplementation increases, ICI efficacy and prognosis in patients with advanced cancers.

ICI has revolutionized the cancer treatment field, but like many therapies, treatment comes with unintended off-target effects. In this context, ICI is associated with immune-related adverse effects (irAEs), which cause organ-specific toxicities due to disrupted T cell tolerance and reactivation of autoreactive T cells, thus mimicking autoimmune diseases. Importantly, vitamin D status was shown to correlate negatively with irAE occurrence in anti-PD-1 treated lung cancer patients (You et al., 2023), and significantly reduced the chances of developing ICI-induced colitis in melanoma patients receiving PD-1 or CTLA-4 inhibitors (Grover et al., 2020). Moreover, in a case-report of a man with

dermatological irAEs resistant to topical steroid treatment, phototherapy with UV-B light resolved his symptoms (Donaldson et al., 2018), suggesting that the synthesized vitamin D suppressed ongoing inflammation. Collectively, vitamin D in combination with ICI therapy has shown promising results in both bolstering ICI efficacy while managing immune-related side effects.

These results suggest that vitamin D analogues may be useful adjuncts to ICI therapy because they are efficacious at concentrations where they do not induce hypercalcemia. We have exploited the combinatorial effects of vitamin D analogues and histone deacetylase inhibitors (HDACi) in 1,25D-resistant cancer models (Banwell et al., 2004; Rashid et al., 2001) to develop analogues that integrate HDACi into the backbone of a non-secosteroidal VDR agonist (Barbier et al., 2022; Sarmadi et al., 2024). Histone deacetylases (HDACs) regulate the acetylation of histones, transcription factors, and cofactors in the nucleus in addition to some cytoplasmic proteins. The nuclear actions of HDACs have significant effects on regulation of gene expression, and HDACi such as SAHA (suberoylanilide hydroxamic acid; vorinostat) are used clinically as therapies for cutaneous and peripheral T cell lymphoma and multiple myeloma (Sun et al., 2018). VDR agonist/HDACi hybrids are robustly bifunctional *in vitro* in a series of cancer models and are bioavailable (Aslakson and Miller, 1992). The most recently developed analogue, ZG-126 (Fig. 3), reduced tumor size more than 1,25D or SAHA in B16-F10 melanoma tumors while displaying comparable efficacy to gemcitabine, a chemotherapeutic agent (Sarmadi et al., 2024). Importantly, high dose ZG-126 treatment in the 4T1 mouse model of triple negative breast cancer reduced tumor burden and metastases more than the combination of 1,25D with SAHA, supporting the benefit of bifunctional compounds relative to traditional combination therapy (Sarmadi et al., 2024). In addition, published (Barbier et al., 2022) and unpublished gene expression profiling studies have provided evidence that hybrid compounds such as ZG-126 should render cancer cells more susceptible to immune checkpoint inhibitor therapy. For example, ZG-126 treatment reduced the accumulation and gene signature of anti-inflammatory macrophages in 4T1

mouse tumors (Sarmadi et al., 2024). Collectively, the above studies suggest that it would be important to investigate the potential of vitamin D analogues as adjuncts of immune checkpoint inhibitor therapy.

8. Concluding statement.

Since 1,25D was discovered as the biologically active mediator of vitamin D signaling in 1971 (Holick et al., 1971a; Holick et al., 1971b), its therapeutic application in human health has been subject of vigorous investigation **(Fig. 6)**. Initially used solely as a drug for diseases derived from perturbations in calcium homeostasis, the anti-proliferative, pro-differentiation, and immunoregulatory activities of 1,25D are now well-recognized. This has resulted in clinical success in the treatment of various inflammatory disorders by 1,25D and its analogues, particularly for the treatment psoriasis vulgaris. Notably, utilization of vitamin D analogues have expanded the potential for vitamin D-targeted therapies in the clinic due to their increased efficacy and diminished calcemic effects. Thus, future applications of vitamin D analogues may endeavor to elicit systemic activity through oral administration to target and treat complex internal inflammatory disorders including autoimmune diseases or cancer as either single agents or combination therapies.

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Data Availability Statement

This review article contains no datasets generated or analyzed during the current study.

Author Contributions

P. Artusa and J.H. White contributed equally to the writing and editing of the manuscript.

Footnotes

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Figure Captions

Figure 1. Overview of Vitamin D biosynthesis. Significant levels of vitamin D_2 or D_3 can be obtain through dietary consumption of fatty fish, fortified foods such as milk, fungi, and limited plant sources, or supplementation. Vitamin D3 can also be generated in cutaneous keratinocytes from 7-dehydrocholesterol given sufficient UV-B radiation and heat. Vitamin D is hydroxylated to its major circulating form, 25-hydroxyvitamin D (25D), largely in the liver by CYP2R1. Other hepatic enzymes also have some 25-hydroxylase activity, such as CYP27A1 and CYP3A4. Under normal conditions, the majority of the circulating biologically active form of vitamin D, 1α, 25-dihydroxyvitamin D (1,25D), is generated in the kidney through the activity of the 1α-hydroxylase (CYP27B1). In addition, CYP27B1 is expressed and active in many extra-renal cell tissues and cell types. Finally, catabolism is initiated by CYP24A1, whose expression is induced by 1,25D in a physiological negative feedback loop.

Figure 2. Mechanism of action of the VDR. Extracellular or intracrine-derived 1,25D binds to the vitamin D receptor (VDR), inducing a conformational change and heterodimerization with related retinoid X receptors (RXRs) and translocation to the nucleus. In the nucleus, ligand-bound VDR/RXR complexes recognize and bind to vitamin D response elements (VDREs). VDREs consist of hexameric PuG/TTCA motifs separated by 3 bp spacers, called DR3 elements. DNA-bound RXRs recruit coactivators to stimulate chromatin remodeling necessary for induction of gene transcription.

Figure 3. Chemical structures of 1,25D_3 and some notable vitamin D analogues. A. 1,25D_3 (left) and three of its analogues containing cholesterol side chain modifications. **B.** Chemical structures of non-secosteroidal analogues, including ZG-126, a bifunctional analogue that incorporates an HDAC inhibitory hydroxamic acid moiety (circled) into the backbone of a VDR agonist.

Figure 4. Vitamin D signaling in the immune system. A. Physical barriers such as the skin represent the first line of defense against pathogens. Similar defense mechanisms are also active in other epithelial barrier tissue (lung, bladder, gut). 1,25D production by local immune cells and epithelial cells enhances the production of antimicrobial peptides (ex: CAMP/LL-37), the expression of PRRs, cytotoxic killing of infected cells by natural killer cells, and the recruitment of neutrophils. In addition to being a source of antimicrobial activity, monocytes also differentiate into macrophages or dendritic cells. 1,25D retards dendritic cell differentiation and induces a tolerogenic phenotype, inhibiting antigen presentation. **B.** In relevant draining lymph nodes, dendritic cells activate naive T cells, which then proliferate and differentiate into different TH subtypes or cytotoxic T cells, depending on their lineage. Cytokine production by TH1 cells promotes cytotoxic T cell proliferation and enhances VDR and CYP27B1 expression in dendritic cells. This results in increased 1,25D production which negatively feeds back to inhibit TH1 (and TH17) cell function, while promoting regulatory phenotypes such as TH2 and Treg cells. 1,25D also inhibits antigen presentation by DCs, restricting T cell activation and proliferation. In addition, 1,25D inhibits the proliferation and production of immunoglobulins by B cells. Notably, T and B cells only express significant amounts of the VDR and CYP27B1 when activated.

Figure 5. The anti-cancer effects of vitamin D signaling in combination with immune checkpoint inhibitor therapy. A. Anti-cancer effects of vitamin D signaling either through direct induction of apoptosis or inhibition of proliferation, or through modulation of immune cells and the gut microbiome. **B.** Example of the anti-tumor effects of immune checkpoint inhibitor (ICI) therapy, which releases the inhibition of T cells via blockade of key immune regulatory molecules such as PD-1. This may also result in immune related adverse events (irAE's) including skin irritation, colitis, or more severe autoinflammatory phenotypes. **C.** Example of how combining ICI therapy with vitamin D

supplementation can result in inhibition of irAE's due to the anti-inflammatory roles of vitamin D and greater reduction in tumor burden due to the combined anti-cancer effects of both therapies.

Figure 6. Immune-related health effects of vitamin D. Enhanced antimicrobial responses and gut microbial homeostasis in vitamin D-sufficient individuals, in addition to tolerance to self-antigens by the adaptive immune system (left). Increased risk and severity, in pre-clinical models, of autoimmunity, cancer, inflammatory bowel disease, and allergies in vitamin D deficient individuals (right).

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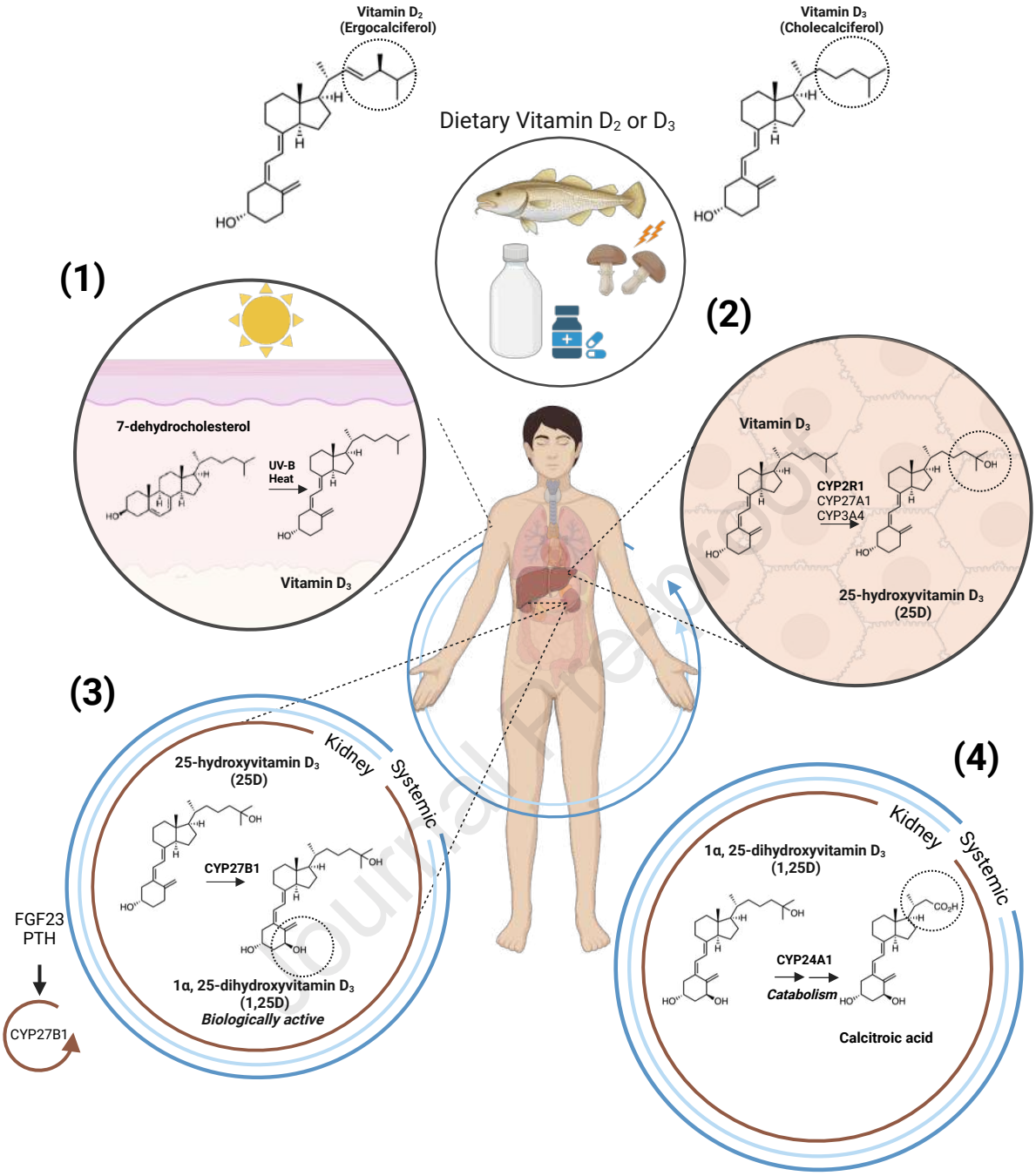
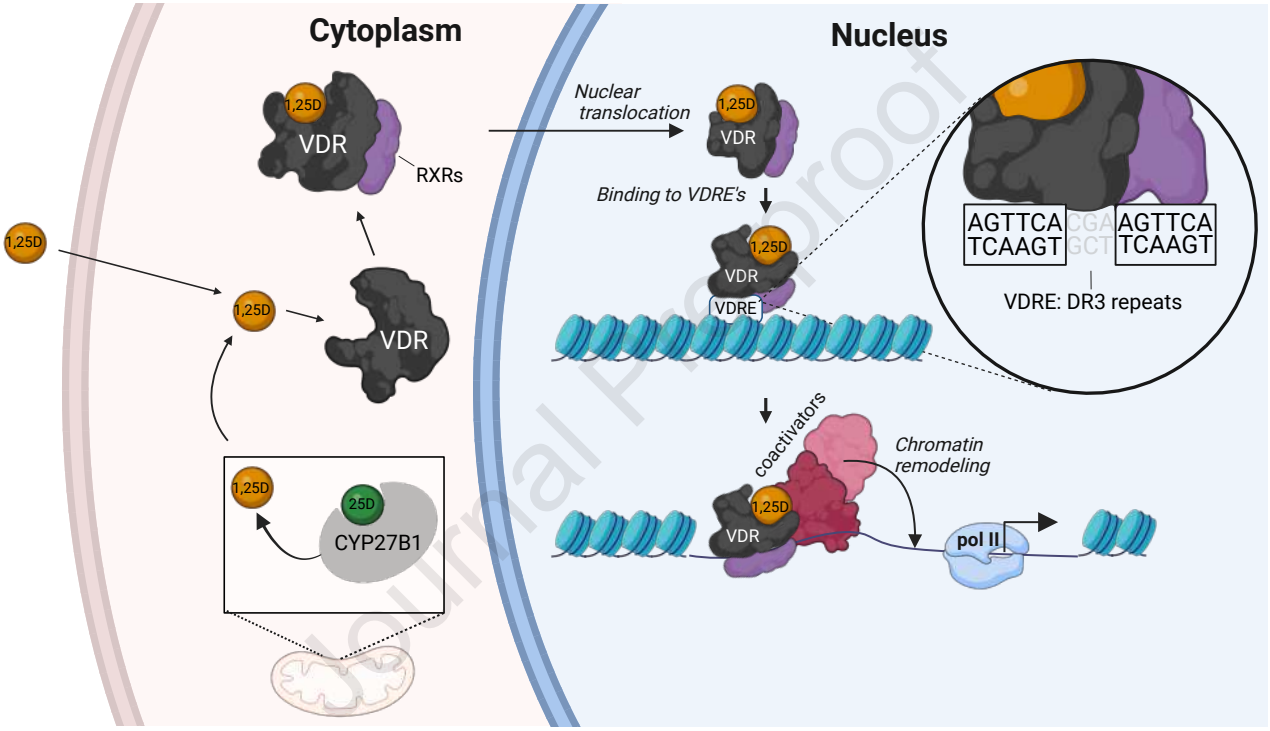
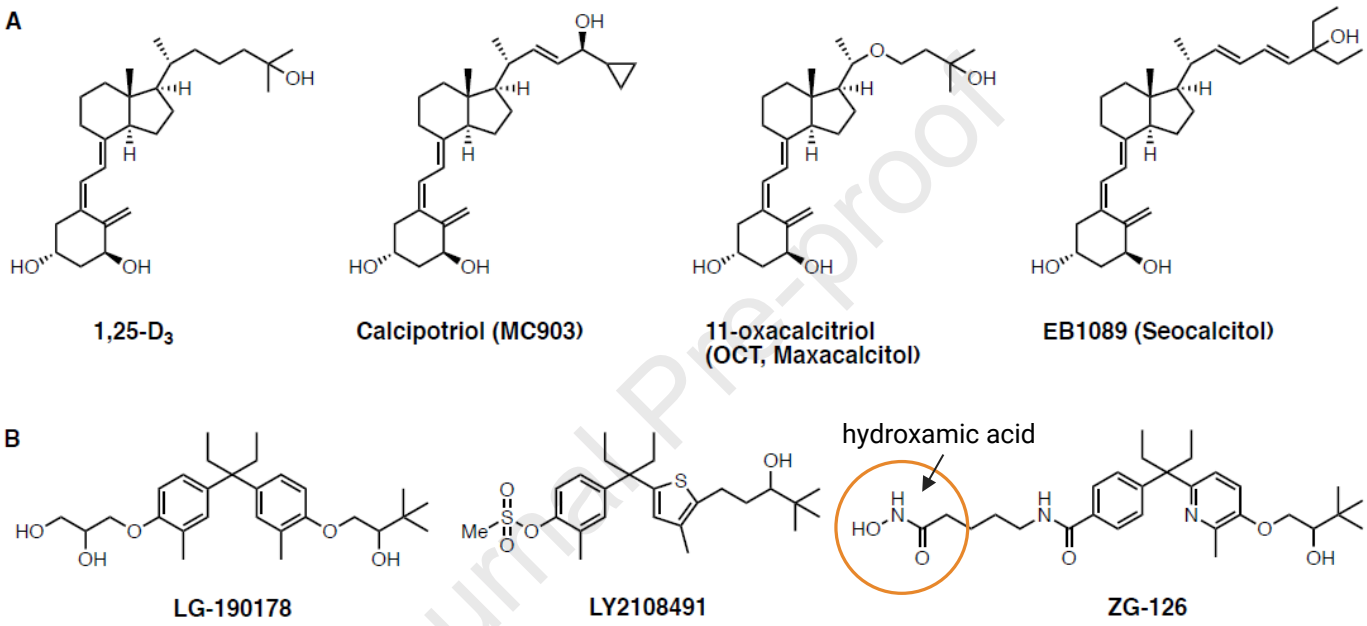
Table 1. Overview of regulation of vitamin D metabolite expression in immune cells and effects of vitamin D signaling on immune cell function.

Cell type	Regulation of VDR expression	Regulation of CYP27B1 expression	Regulation of function by vitamin D signaling	Immunological effect/ consequences	References
Monocytes/ Macrophages	▲ *M.tb.*[a] lipopeptide, IL-15	▲ *M.tb.*[a] lipopeptide, IFNγ, LPS[d], IL-15	▲ AMP[b] production, PRR[c] expression, IL-1β, IL-8, CCL3, CCL4, CCL8, IL-8, PPARγ, autophagy. ▼ IL-6, TNFα.	Enhanced microbial killing, antigen presentation, inflammation, chemotaxis, altered macrophage phenotypes.	Oberg et al. 1993, Overbergh et al. 2000, Liu et al. 2006, 2009, Krutzik et al. 2008, Wang et al. 2004, Khoo et al. 2011, Verway et al. 2013, Dimitrov and White, 2016, Ismailova and White 2022.
Dendritic cells	▼ Differentiation (CD40 signaling)	▲ LPS[d], differentiation (CD40 signaling)	▲ IL-10, dendritic cell maturation, CCL3, CCL4, PD-L1. ▼ IL-12, TNFα, IFNγ, CCL5, MHC-II, CD40, CD80, CD86.	Inhibition of antigen presentation, reduced pro-inflammatory helper T cell differentiation.	Hewison et al. 2003, Adorini and Penna 2009, Ismailova and White 2022.
Neutrophils	*Not known*	*Not expressed*	▲ AMP[b] production, PRR[c] expression, formation of NET-like structures. ▼ Apoptosis (autocrine IL-4 signaling).	Enhanced microbial killing, potentially prolonged antimicrobial activity	Agerberth et al. 2000, Takahashi et al. 2002, Agraz-Cibrian et al. 2019, Ismailova and White 2022.
αβ-T cells	▲ TCR[e] signaling/activation	▲ TCR[e] signaling/activation	▲ FoxP3 transcription. ▼ IL-2, IFNγ, IL-17A.	Decreased pro-inflammatory helper T cell differentiation/function, increased Treg differentiation, decreased CTL cytotoxicity.	Chen et al. 2014, Rigby et al. 1984, Adams et al. 2014, Provvedini et al. 1983, Bruce et al. 2011, Bishop et al. 2021.
B cells	▲ BCR[f] signaling, CD40/IL-21	▲ BCR[f] signaling, CD40/IL-21	▲ Apoptosis, altered class switching, IL-10. ▼ Plasma cell maturation, proliferation, immunoglobulin production.	Diminished antibody production and T-cell stimulation	Adams et al. 2014, Provvedini et al. 1983, Chen et al. 2007.
γδ-T cells	▲ Nonpeptidic monoalkyl phosphate stimulation	*Not known*	▼ Phospholigand-induced expansion, IFNγ, cytotoxicity.	*Not known*	Chen et al. 2005, Bernicke et al. 2022.
Natural killer cells	*Not known*	*Not known*	▲ Granzyme A (LAK[g] cells), IL-10 ▼ Differentiation from cord blood, cytotoxicity, IFNγ.	*Not known*	Deniz et al. 2008, Lee et al. 2018.
Invariant natural killer cells	*Not known*	*Not known*	▲ Thymic development, functional maturity.	*Not known*	Yu and Cantorna 2008.
Innate lymphoid cells (3)	▲ IL-23+IL-1β	*Not known*	▲ IL-1β pathway, proliferation. ▼ IL-22, IL-17F, GMCSF.	*Not known*	Konya et al. 2018, He et al. 2019.
Eosinophils	▲ *Calcitriol (Eol-1[h] cells)*	*Not known*	▼ Activation.	*Not known*	Lu et al. 2017.
Mast cells	▲ *Calcitriol*	*Not known*	▼ Histamine, TNFα, , IL-6, activation.	*Not known*	Yip et al. 2015, Liu et al. 2017.

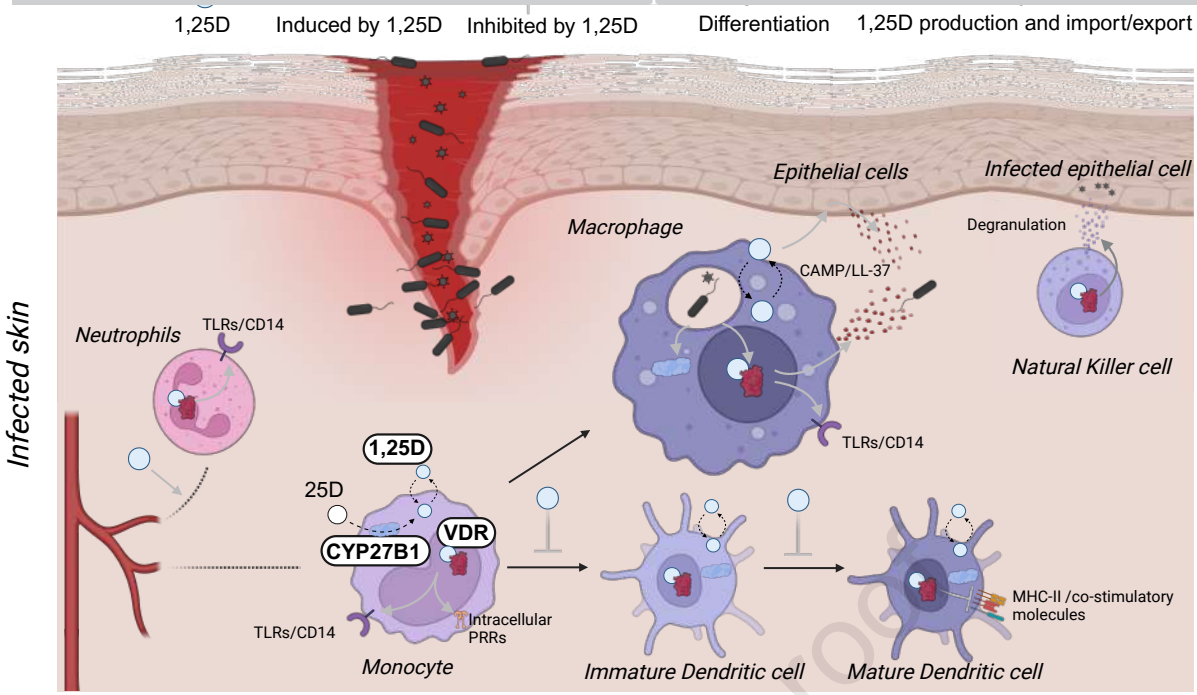
[a] *M.tb., Mycobacterium tuberculosis.* [b] *AMP, antimicrobial peptides.* [c] *PRR, pattern recognition receptor.* [d] *LPS, lipopolysaccharide.*
[e] *TCR, T cell receptor.* [f] *BCR, B cell receptor.* [g] *LAK cells, lymphokine activated killer cells (mostly NK, but also NKT and T cells).* [h] *Eol-1 cells, eosinophilic leukemic cells.*


Vitamin D2
(Ergocalciferol)
Vitamin D3
(Cholecalciferol)
Dietary Vitamin D2 or D3
(1)
7-dehydrocholesterol
UV-B
Heat
Vitamin D3
(2)
Vitamin D3
CYP2R1
CYP27A1
CYP3A4
25-hydroxyvitamin D3
(25D)
(3)
Kidney
Systemic
25-hydroxyvitamin D3
(25D)
CYP27B1
1α, 25-dihydroxyvitamin D3
(1,25D)
Biologically active
FGF23
PTH
CYP27B1
(4)
Kidney
Systemic
1α, 25-dihydroxyvitamin D3
(1,25D)
CYP24A1
Catabolism
Calcitroic acid

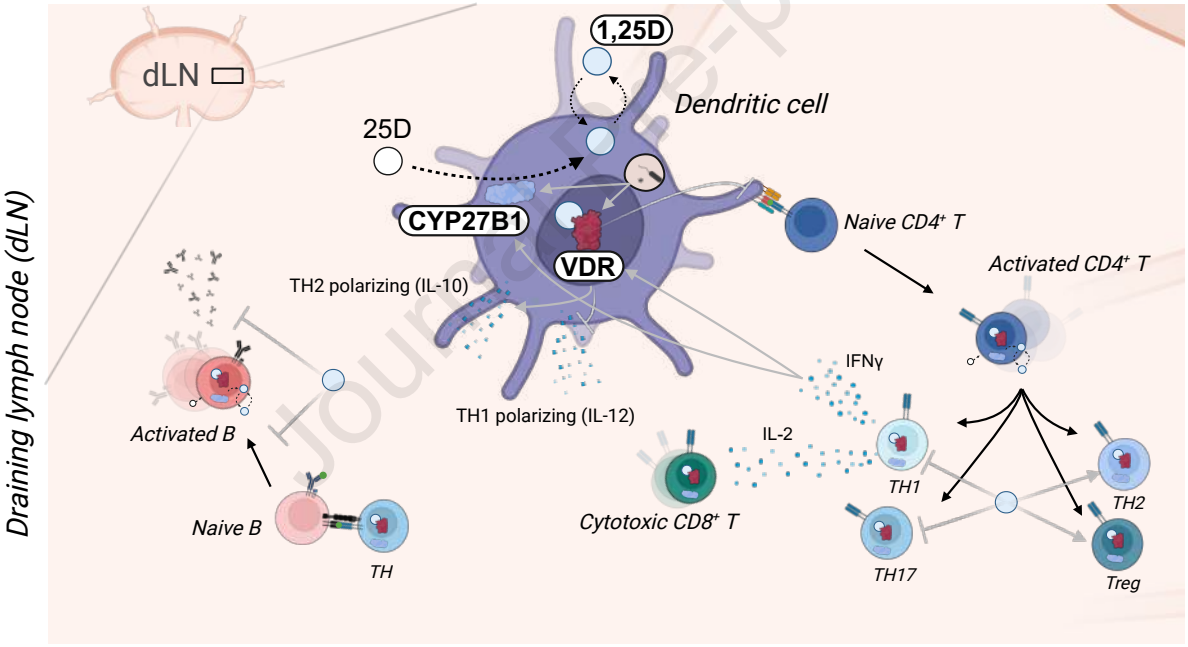




A



B



VDR - Vitamin D receptor 1,25D - VDR ligand TLRs - Toll-like receptors PRR - pattern recognition receptor

TH - T helper IL - Interleukin IFNγ - Interferon gamma