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Vitamin D and Haematopoiesis

Geoffrey Brown¹ · Andrzej Kutner² · Ewa Marcinkowska³

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Abstract

Purpose of Review This review examines the influences of active vitamin D on ‘developmental’ haematopoiesis and the immune cells produced. Haematopoiesis gives rise to the platelets, erythrocytes and a wide range of immune cell types each of which performs a specific role to protect the organism from a myriad of infectious agents. The newly produced immune cells, for example, monocytes, dendritic cells and T and B lymphocytes, are activated in response to the presence of an infectious agent and differentiate further to perform their roles.

Recent Findings Binding of $1\alpha,25$ -dihydroxyvitamin D₃, the most active metabolite of vitamin D₃, to its receptor, the vitamin D receptor, regulates the expression of very many different genes and therefore a mode(s) of action of vitamin D relates to the regulation of expression of cell-specific genes. The haematopoietic cytokines are essential regulators of haematopoiesis and the further maturation and functionality of the immune cell types. We now know that some cytokines also instruct the development of a particular type of blood cell.

Summary Vitamin D influences the ‘early’ development of monocytes and invariant natural killer T cells and the further maturation of some immune cell types. Findings regarding the regulation of gene expression have revealed that there are links between the actions of vitamin D and cytokines. Whilst we do not have as yet an entirely clear picture on this matter, there are benefits to ‘health’ of the immune system from vitamin D supplementation.

Keywords Vitamin D · Haematopoiesis · Immune cells · Haematopoietic cytokines · Vitamin D and health

Introduction

A clear picture of the architecture of the haematopoietic cell system is vital to developing an understanding of the role of vitamin D in haematopoiesis. For many years, investigators have generally used tree-like diagrams to depict the ancestry of the different types of the haematopoietic cells: this type of model set a paradigm for the behaviour of tissue-specific stem

cells, such as those of the small intestinal mucosa. An underlying principle is that developing cells are restricted to making a choice between just two fates at any particular time as argued by Holzer in 1979 [1]. With regard to this type of model, haematopoietic progenitor cells (HPCs), the progeny of the pluripotent haematopoietic stem cells (HSCs), follow prescribed routes to each end cell type. They undergo binary choices in a stepwise manner to restrict their fate to just one option [2]. Commitment to maturation towards a functional cell type is the final stage in this process.

However, our understanding of the nature of HSCs and their development has changed radically to accommodate many recent findings leading to revision of the aforementioned ‘classic’ model of haematopoiesis. In particular, affiliation/commitment to just one cell lineage can occur at the level of the HSC and therefore much earlier than investigators envisaged in stepwise models. Instead of HSCs being a homogeneous population of pluripotent cells, they are therefore a mixed population of cells with lineage affiliations/biases. Human adult bone marrow CD34⁺ cells are many cells with uni-potent myeloid or erythroid potential and some pluripotent cells [3]. Our view of the process of decision-making

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by HSCs is that the end cell options are all available to the HSC as a continuum whereby the HSC selects one and differentiates towards that cell type. We have described the model and the evidence that supports this viewpoint elsewhere [4•, 5, 6•] (Fig. 1). The model depicts close relationships between particular cell types, as for tree-like models. In constructing the model, we placed the cell lineages adjacent to one another from the sets of options that are available to bi-potent and oligo-potent progenitors, as revealed by cell culture experiments. Whilst HSCs can ‘choose’ a cell lineage, haematopoiesis is a dynamic non-linear ecosystem because HSCs and HPCs that have selected an option can still ‘change their mind’ to differentiate towards a different fate.

Intrinsic events that govern fate options within HSCs/HPCs, such as the accessibility of genes and the binding of appropriate transcription factors, clearly shape the production of the various types of blood and immune cells. However, a major change to our understanding of lineage decision-making by HSCs/HPCs is that nurture by environment factors, and presumably proximal interactions with niche cells, is equally important. Since the discovery of the various haematopoietic cytokines, we viewed them as permissive to cell behaviour in that they provide the signals that are essential to the survival and proliferation of HSCs, their immediate progeny and the mature end cell types [7]. In this manner and for example, erythropoietin (Epo) supports erythropoiesis [8], granulocyte colony-stimulating factor (G-CSF) supports the development of granulocytes [9, 10], macrophage colony-stimulating factor (M-CSF) the development of macrophages and dendritic cells [11] and granulocyte/macrophage colony-

stimulating factor (GM-CSF) developing granulocyte and macrophage cells [10, 12]. A major change to this viewpoint is that recent studies have shown that these cytokines also instruct lineage choice by HSCs/HPCs. Epo directs multipotent HPCs towards erythropoiesis [13•], M-CSF instructs myeloid lineage fate in HSCs [14•] and macrophage fate in granulocyte/macrophage progenitors [15] and G-CSF and GM-CSF drive granulocyte/macrophage progenitors to differentiate towards neutrophils [15, 16]. Moreover, HSCs selectively express the receptors for Epo [17], M-CSF [14, 18, 19] and G-CSF [20] at their cell surface providing further evidence to support the existence of lineage biases/affiliations within these cells. Below, we examine the influence of vitamin D taking into consideration the above substantial changes to our understanding of haematopoiesis.

Vitamin D

Humans synthesise the most active metabolite of vitamin D₃, namely 1 α ,25-dihydroxyvitamin D₃ (1,25D₃, calcitriol), from dietary precursors. In the skin, ultraviolet irradiation of 7-dehydrocholesterol generates pre-vitamin D₃ which, via non-enzymatic and thermal means, is isomerised into cholecalciferol (vitamin D₃, D₃). Bio-activation to 1,25D₃ occurs first in the liver, by vitamin D 25-hydroxylase (25-OHase or CYP27A1) to calcidiol (25-hydroxyvitamin D, 25D₃) and subsequently in the kidney, by 25(OH)D-1 α -hydroxylase (1 α -OHase or CYP27B1) [21]. In addition, hydroxylation at C-1 occurs outside of the kidneys, for example, in immune cells [22]. Investigators first identified 1,25D₃ as playing a role in the absorption and transport of calcium, phosphorous and magnesium, and therefore essential to proper bone development [23]. However, 1,25D₃ has pleiotropic functions as to regulating the transcription of around 3% of genes of the human genome through its nuclear receptor the vitamin D receptor (VDR) [24]. We now view 1,25D₃ as having a wide ‘hormonal’ role, including the maintenance of the blood cells, cardiovascular, musculoskeletal, renal, nervous and adipose systems.

The Influence of 1,25D₃ on the Niches that Stem and Progenitor Cells Reside in

The relative contribution of nature, all of the genes and their actions, versus nurture, the environmental variables, and the importance of their interplay to the status and behaviour of cells is an age-old debate. Inherent events within HSCs/HPCs and external influences that include cytokines and proximal stromal cells both play a role in the proper conduct of haematopoiesis. It is therefore important to bear in mind that 1,25D₃ may influence one or both of these controls on blood cell development.

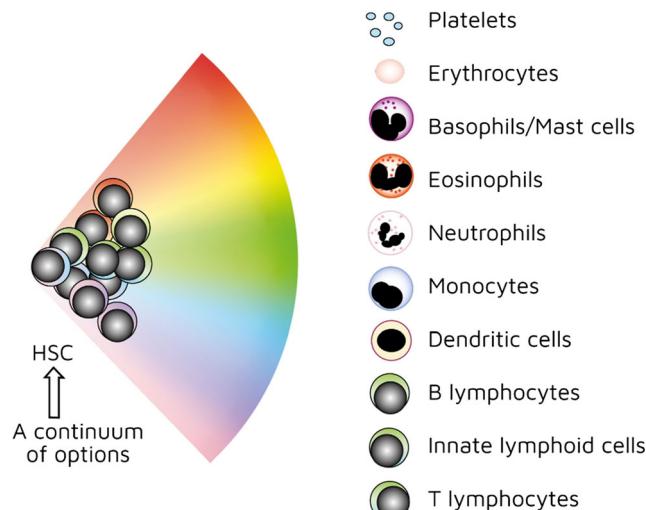


Fig. 1 A continuum model of haematopoiesis. The end cell options are all available to the haematopoietic stem cell (HSC) as a continuum whereby the HSC selects one and differentiates towards that cell type. HSC and haematopoietic progenitor cells that have selected an option can still ‘change their mind’ to differentiate towards a different fate. There are particular close relationships between the cell lineages, inferred from the sets of options available to bi-potent and oligo-potent progenitors, as revealed by cell culture experiments

In the embryo, the development of blood cells occurs in regions that include the aorta-gonad-mesonephros and foetal liver. Haematopoietic stem and progenitor cells (HSPCs) arise from the haemogenic endothelium in the dorsal aorta and hedgehog and notch signalling, leading to Runx1 expression, specifying the haematopoietic niche. Studies of the influence of the 1,25D3 precursor D3 on zebrafish embryos, by means of treatment or inhibiting the enzyme (Cyp2r1) that is required for D3 hydroxylation (to 25D3), revealed that an accumulation of D3 antagonises hedgehog signalling and impairs the formation of the haemogenic vascular niche/its identity, leading to reduced HSPCs. This effect was specific to D3 and not the active 1,25D3 [25]. Zebrafish studies have also shown that the availability of 1,25D3 during embryonic development has an impact on the development of HSPCs. Loss of 1,25D3 biosynthesis, mediated by Cyp27b1, or loss of VDR reduced the number of embryonic HSPCs and 1,25D3 acts in vitro and ex vivo to stimulate proliferation of these cells. This effect was independent of the calcium regulation of cell proliferation and gene expression analysis indicated that an increase in CXCL8 (interleukin-8) in HSPCs plays an autocrine or paracrine role. Similarly, the effect 1,25D3 on CD34+ve human umbilical cord HSPCs ex vivo is to increase survival, proliferation and the formation of multi-lineage colonies [26]. As mentioned above, the foetal liver supports HSPC self-renewal and differentiation, as does the adult organ in certain disease states when there is reactivation of haematopoiesis. Though microenvironment influences on definitive haematopoiesis in foetal liver remain poorly understood, there is now an integrated map for the human foetal liver haematopoietic cell lineages [27].

For haematopoiesis in adult mice, investigators observed an effect of 1,25D3 on the environment that HSCs/HPCs reside in. Deletion of VDR in haematopoietic cells in VDR-deficient mice ($VDR^{-/-}$) did not affect the function of HSCs/HPCs per se. In adult mice, haematopoiesis preferentially takes place in the bone marrow and the $VDR^{-/-}$ mice showed a significant increase in the number of HSCs/HPCs in the spleen and increased primitive splenic haematopoiesis. Investigators placed the mice on a rescue diet (containing lactose, calcium and vitamin D) that corrects many of the abnormalities in non-haematopoietic cells and this enabled them to test whether the increased residence of HSCs/HPCs in the spleen was due to loss of VDR in the haematopoietic environment. The protocol reversed the changes seen in the $VDR^{-/-}$ mice leading investigators to the conclusion that the maintenance of bone calcium homeostasis is important to adult haematopoiesis localising within the bone marrow [28].

HSCs/HPCs reside in precise anatomic niches in the bone marrow and, for example, proximal mesenchymal cell populations regulate the self-renewal, proliferation and differentiation of HSCs. The cytokine G-CSF releases HSCs/HPCs from their niches in the marrow into the circulation by means of osteoblast suppression, and the possible breakdown of

osteoblastic niches. Studies of $VDR^{-/-}$ mice have revealed that VDR plays a role in G-CSF-induced osteoblast suppression and the subsequent mobilisation of HSCs/HPCs. The mobilisation of these cells is severely impaired in $VDR^{-/-}$ mice. $\beta 2$ -Adrenergic receptor agonists induce VDR expression in osteoblasts and the VDR is functional as to the expression of its downstream target gene the receptor activator of NF- κ B ligand (*Rankl*). 1,25D3 stabilizes VDR protein within osteoblasts and sustains $\beta 2$ -adrenergic receptor agonist-induced *Rankl* expression at a high level. The specific role of *Rankl* expression in G-CSF-induced mobilization is still unclear. Here, VDR is playing a role with G-CSF in the osteoblastic niche for HSCs/HPCs regarding the bone remodelling events that are important to the release of HSCs/HPCs from the bone marrow [29].

In addition to osteoblast niches, the bone marrow environments in which HSCs develop are a mixture of cells, including mesenchymal stem cells, endothelial cells, pericytes and Schwann cells. We know that 1,25D3 affects a wide variety of mature cell types and an effect of 1,25D3 on any of the many bone marrow cell types may well in turn influence haematopoiesis. 1,25D3 and its precursor 25D3 induce osteogenic differentiation of human mesenchymal stem cells that is important to haematopoiesis as above [30]. There is limited information about the effect of vitamin D on bone marrow endothelial progenitors and the endothelial cell system per se. However, D3 supplementation of cultures improved the viability of marrow-derived endothelial progenitors and their capacity to form colonies [31]. There is still considerable scope regarding future investigation of the effect of 1,25D3 on marrow stromal cells and the subsequent impact on steady-state haematopoiesis, during stress and in disease states. For example, bone marrow pericytes are different populations of cells with different properties and they crosstalk with other niche cellular components [32], and does 1,25D3 plays a role. Similarly, 1,25D3 does affect non-myelinating and niche Schwann cells that sustain the hibernation of HSCs, by converting latent TGF- β to its active form [33].

The Direct Influence of 1,25D3 on Stem and Progenitor Cell Developmental

VDR knockout mice have relatively normal numbers of erythrocytes and white blood cells and therefore a lack of VDR does not appear to have an overt effect on normal haematopoiesis, as long as mice are kept in a pathogen-free environment [34]. The exposure of mice with inactive VDR to pathogens led to significant disturbances to the production of blood cells. The disorder observed was the appearance of chronic myeloid leukaemia-like cells, splenomegaly, granulocytosis and thrombocytosis and reduced erythropoiesis [35]. In vitro studies have revealed that 1,25D3 and its derivatives influence the progeny of HSCs and have largely

focussed on an effect on myelopoiesis [36]. We have known for many years that mouse myeloid leukaemia cells differentiate towards monocytes in response to 1,25D3 ([37] and see below), and that 1,25D3 drives normal blood monocytes to differentiate into macrophages [38]. Early in vitro studies also showed that 1,25D3 suppresses the formation of colonies by granulocyte/macrophage progenitors (CFU-GM) and instead induces colonies containing monocytes/macrophages (CFU-M) [39]. Even so, it seems that 1,25D3 and VDR are facilitating monocyte/macrophage differentiation, rather than absolutely required to sustain the generation of monocytes/macrophages during steady-state haematopoiesis because VDR knockout mice have relatively normal numbers of myeloid cells. It is important to bear in mind that majority of data have been obtained from murine models, and that not all aspects of haematopoiesis in mice apply to humans [40]. There are significant differences in the regulation of *VDR* expression between mice and humans. The *Vdr* gene in mice has an organization that is simpler than that in humans, and differences lie in the untranslated exons and their promoters [41]. In consequence, ligand-activated VDR regulates the expression of murine *Vdr*, whilst ligand-activated RAR α regulates the expression of human *VDR* rather than activated VDR [42, 43].

As considered above, it is important to bear in mind that M-CSF can drive HSCs towards a myeloid fate and granulocyte/macrophage progenitors to develop towards macrophages. Does VDR cooperate with M-CSF either directly or indirectly to this effect? We do not know the answer to this question regarding the cell lineage choice by HSCs/HPCs. However, 1,25D3 does have an action on the M-CSF/M-CSF receptor system as studies using bone marrow precursor cells, collected by pre-treatment of murine bone marrow with 5-fluorouracil, showed that 1,25D3, in a dose-dependent manner, caused a rapid appearance of the M-CSF receptor in early precursor cells and receptor expression was decreased in intermediate precursors [44]. For monocytes stimulated to differentiate into dendritic cells by interleukin-4 and GM-CSF, 1,25D3 inhibited the induction of differentiation, downregulated the surface expression of the M-CSF receptor and upregulated the secretion of M-CSF [45]. In this case, it is interesting that 1,25D3 interferes with the differentiating action of GM-CSF. These findings point to cooperation between VDR and M-CSF and that the outcome appears to be dependent on the differentiation stage of cells.

In keeping with an influence of 1,25D3 on normal macrophage development is that the human early myeloid leukaemia cell lines HL60, U937, THP-1 and MOLM-13 differentiate towards macrophages when treated with 1,25D3 (reviewed in [41]). Studies of mouse models of acute myeloid leukaemia (AML) confirmed the differentiating and anti-leukaemia action of 1,25D3 [46]. Regarding the anti-leukaemia action of 1,25D3, the cell lines and acute myeloid leukaemia cells arrest their growth in G1 of cell cycle when they differentiate towards macrophages. VDR regulates transcription from genes

that are important to both of these processes by forming a dimer with the retinoic acid receptor (RXR) which binds to the VDR response element of genes. 1,25D3 regulates the expression of between 200 and 600 genes depending on the cell type and either directly or indirectly [47, 48]. Some are important to the cell cycle arrest that accompanies differentiation, for example, the cyclin-dependent kinase inhibitor 1 p21^{Cip1} (alternatively p21^{Waf1}) is a VDR target gene. Other VDR-regulated genes encode macrophage features, such as the surface protein cluster of differentiation (CD) 14 [49•, 50, 51]. VDR strongly regulates the gene that encodes the 24-hydroxylase enzyme that catabolises 1,25D3 to an inactive metabolite as its promoter contains multiple vitamin D response elements [52]. Presumably, this action of VDR modulates/curtails the exposure of cells to 1,25D3.

The human promyeloid cell line HL60 is able to differentiate towards neutrophils, in response to all-*trans* retinoic acid (ATRA), and macrophages, in response to 1,25D3. Whilst HL60 cells provide a good in vitro model system for investigations of how an individual haematopoietic precursor cell might choose between two options, we still do not have a precise understanding of the events that control decision-making by HL60 cells. Even so, ATRA and 1,25D3 appear to play a role in the process. The effect of 1,25D3 on HL60 cells is very similar to the effect on myeloid colony formation by myeloid progenitor cells and in keeping with HSCs/HPCs that have selected cell lineage but can still change their mind. When grown to a high cell density, HL60 cells spontaneously differentiate into neutrophils and therefore ATRA enhances neutrophil differentiation [50], and it seems that 1,25D3 diverts HL60 cells towards monocytes. So, how might this happen? Like VDR, the retinoic acid receptor (RAR) for ATRA forms a dimer with RXR to facilitate granulocyte differentiation and granulocytic differentiation in vitro is impaired in haematopoietic cells lacking the isotypes RAR α 1 and RAR γ [51, 53]. HL60 cells ‘choosing’ whether to differentiate towards monocytes or neutrophils might relate to competition between VDR and RAR for binding to RXR and the relative balance of activity of VDR/RXR versus that of RAR/RXR [48]. In this case, VDR and RAR do play a role in HL60 decision-making but the interaction between these two receptors is more complex than just competition. There is cooperativity between the actions of 1,25D3 and ATRA because RAR α regulates the expression of VDR. The human stem cell-like KG-1 has a high level of expression RAR α and does not respond to 1,25D3. RAR α activation (by a specific agonist) upregulates VDR expression and KG-1 cells then respond to 1,25D3 by differentiating into monocytes. The high level of inactive RAR α in KG-1 cells is suppressing VDR expression [42].

VDR-mediated diversion of myeloid differentiation towards macrophages has a role in pathological conditions of the bone marrow as shown for the initiation of myelofibrosis

and subsequent osteosclerosis [54]. Myelofibrosis occurs in myeloproliferative neoplasms and osteosclerosis is a complication. Investigators used a mouse model of myelofibrosis whereby VDR^{+/+} HSPC were transplanted into VDR^{-/-} mice to show that the development of myelofibrosis was dependent on macrophages. In the transplanted mice, donor-derived macrophages proliferated together with recipient myofibroblasts resulting in fibrotic tissues. Interfering with the action of VDR, such as a low vitamin D diet, and macrophage depletion prevented the occurrence of fibrosis. In this case, macrophages appear to support/influence the proliferation of collagen-producing myofibroblasts.

In addition to an effect of 1,25D3 on myelopoiesis, 1,25D3 status is important to the prenatal development of invariant natural killer T (iNKT) cells as deficiency in utero results in a significant reduction in the number of these cells. Vitamin D is required before embryonic day 13 as revealed by timed pregnancies. Later intervention with D3 or 1,25D3 did not correct the defect that is intrinsic to haematopoietic cells because vitamin D-deficient bone marrow was defective in wild-type recipients regarding the generation of iNKT cells. The investigators concluded that the reduction in iNKT cells is due to increased apoptosis of the precursors of these cells in the thymus, and that 1,25D3 controls the number of just these cells in the thymus. They also concluded that vitamin D deficiency leads to epigenetic changes in iNKT cells because of the failure to rescue the number of iNKT cells by later exposure to 1,25D3 [55]. For iNKT cells, we also see 1,25D3 playing a role in cytokine action because 1,25D3 enhances the secretion of IL-4 by iNKT cells [56].

The proposal that the block in iNKT cell development relates to epigenetic changes brings to attention whether the pleiotropic effect of 1,25D3, namely the 200 to 600 genes regulated, relates to a global effect of 1,25D3 on the epigenetic landscape. The landscape per se is important to the binding of VDR to gene loci and VDR having bound ligand interacts with the epigenome at multiple levels (reviewed in [57]). VDR interacts with coactivator and corepressor proteins that interact with chromatin modifiers and re-modellers, including histone acetyltransferases, deacetylases and methyltransferases. Regarding haematopoietic cells, VDR binds at open chromatin loci in the genome of the human leukaemia cell line THP-1 and treatment of these cells with 1,25D3 leads to a 30% increase in the number of loci that are open [58•]. The activities of histone acetyltransferases and histone methyltransferases are important to the action of liganded VDR and histone deacetylase or DNA methyltransferase inhibitors synergize to drive apoptosis of gastric cancer cells [59]. Whilst the above information is from studies of cell lines, it is likely that 1,25D3-driven changes to the epigenome are important to normal haematopoiesis.

The Influence of 1,25D3 on Immune Cells

The end cell product of ‘developmental’ haematopoiesis is a very diverse range of immune cell types each of which performs a specific role to protect the organism from the myriad of different infectious agents. Whilst the information of the effect of 1,25D3 on developmental haematopoiesis is somewhat limited, 1,25D3 does have an important influence on the effectiveness of both innate and adaptive immunity. A normal level of 1,25D3 is important to the ‘health’ of the immune system as there are effects on macrophages/monocytes, dendritic cells, T lymphocytes, B lymphocytes and iNKT cells (as above). The many effects and the potential benefits of 1,25D3 to immune function per se, and also immune reconstitution post haematopoietic stem cell transplantation, have been reviewed elsewhere [60•, 61•]. We therefore just present an overview of some of the key influences of 1,25D3 (Fig. 2).

In addition to the ability of 1,25D3 to stimulate the differentiation of monocytes to macrophages, the gene encoding the antibacterial protein cathelicidin contains a functional VDR response element and expression appears to be stimulated by the precursor of 1,25D3 namely 25D3 binding to VDR. Signalling via the toll-like 2/1 receptor induces the expression of VDR and the gene that encodes 1α-hydroxylase (CYP27B1) within monocytes [62]. Monocytes are able to differentiate into either macrophages or dendritic cells and dendritic cells can be either immunogenic or tolerogenic regarding their participation in an immune response. Overall 1,25D3 induces myeloid dendritic cells to become tolerogenic by inhibiting their maturation and therefore the expression of costimulatory molecules and IL-12 [63]. This, in turn, affects their ability to present antigens to and stimulate T cells.

1,25D3 influences the behaviour of both T lymphocytes and B lymphocytes. Early studies revealed that 1,25D3 leads

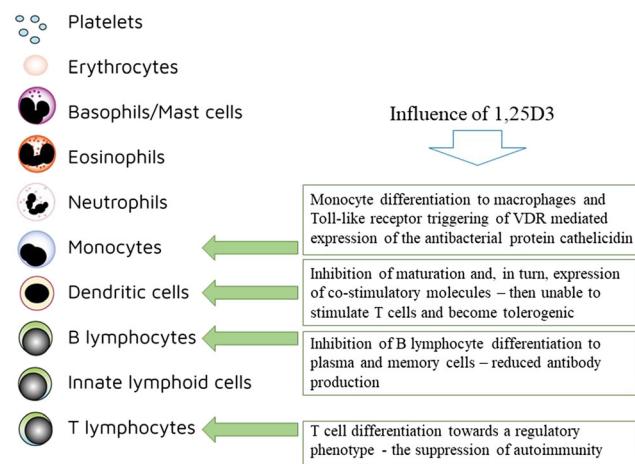


Fig. 2 Some of the influences of the most active metabolite of vitamin D (1,25D3) on immune cells. The most active metabolite of vitamin D 1α,25-dihydroxyvitamin D₃ (1,25D3) binds to the vitamin D (VDR) receptor to regulate gene expression that, in turn and for example, either drives or interferes with cell differentiation

to T lymphocytes differentiating preferentially towards a T-regulatory phenotype [64] and 1,25D3 together with steroids stimulated the production of IL-10 by CD4^{+ve}CD25^{+ve} regulatory T cells [65]. By contrast, 1,25D3 inhibits the secretion of IL-2 by CD4^{+ve} T helper 1 cells and their expansion [66]. In these instances and again, we see the action of 1,25D3 altering cytokine production to change the behaviour of immune cells. Similarly, in vitro studies have shown that 1,25D3 increases the secretion of the potent anti-inflammatory cytokine IL-10 by human dendritic cells leading to impaired activation of alloreactive T lymphocytes [63]. Indeed, 1,25D3 suppresses autoimmunity as seen for experimental autoimmune encephalomyelitis in mice [67]. 1,25D3 appears to have little effect on CD8+ve T cells [68]. Since the 1980s, we have known that 1,25D3 inhibits B lymphocyte proliferation and differentiation to plasma cells and memory cells, and consequentially decreases antibody production [68–71].

The Implications to General Health and of the Immune System

From all of the above, vitamin D is vital to the immune system and its integrity and proper responsiveness are highly germane to good health and the avoidance of risk of many different diseases. As mentioned above, 1,25D3 can suppress autoimmunity and, for example, vitamin D protects against the development of immune-mediated type I diabetes mellitus [72]. A low level of vitamin D compared with healthy subjects and VDR's polymorphisms also correlates with other organ-specific (e.g. multiple sclerosis, diabetes mellitus and primary biliary cirrhosis) and systemic (e.g. systemic lupus erythematosus and rheumatoid arthritis) autoimmune diseases [73] and vitamin D supplementation has a therapeutic benefit in mouse models of autoimmunity [73•, 74•]. Mechanistic studies support the viewpoint that there is dysregulation of dendritic cells, regulatory T lymphocytes and T helper 1 cell maturation and functionality in individuals who are genetically predisposed to autoimmunity and who have a vitamin D deficiency [74•]. A low level of vitamin D leads also to other health risks that include osteoporosis, hypertension and muscle fatigue. Around one billion people are vitamin D-deficient worldwide, and to avoid the risks to health, the American Institute of Medicine recommends taking 600 IU per day of vitamin D up to the age of 70, and 800 IU if older. UK guidelines recommend vitamin D during winter to protect bone, teeth and muscle, as sunlight is insufficient.

As mentioned above, 1,25D3 drives the growth arrest and differentiation of leukaemia cell lines and the growth arrest and apoptosis of a variety of other cancer cells (reviewed in [75]). Additionally, 1,25D3 and derivatives prevent several cancers in mouse models, with increasing evidence from epidemiological studies in humans [76, 77••]. As mentioned above, 1,25D3 enhances the

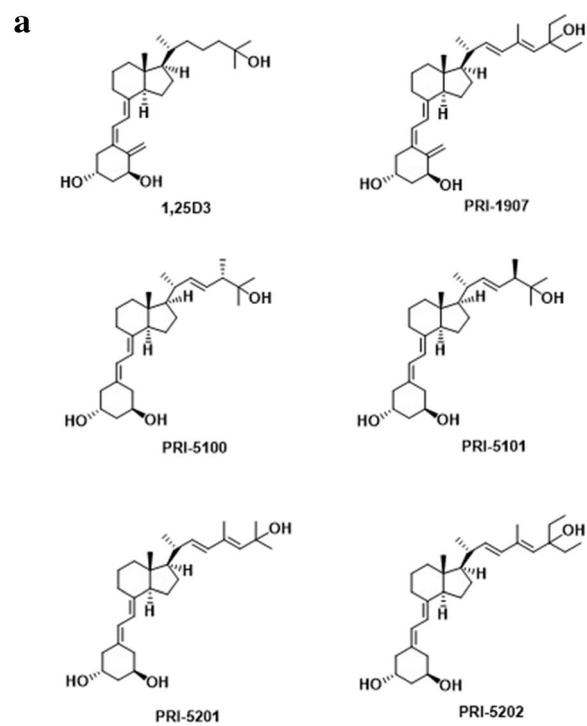
secretion of IL4 by iNKT cells [56], and these cells may play a role in immune surveillance against cancer. However and regarding the use of 1,25D3 to treat leukaemia and other cancers, it is important to bear in mind that the cancer stem cell theory states that most, if not all, cancer arise is a tissue-specific stem cell [78]. Some analogues of vitamin D are active against cancer stem cell-like cells at least regarding reducing the expression of stem cell genes by the HT-29 colorectal carcinoma cell line [79].

Led by findings from the studies of the influence of 1,25D3 on myelopoiesis, the myeloid leukaemias and myelodysplasia are perhaps the best therapeutic malignancy targets and there are trials of inecalcitol, a 1,25D3 analogue, in AML and chronic lymphocytic leukaemia and inecalcitol with decitabine in AML. There are encouraging data from trials of the use of vitamin D and its analogues in combination with chemotherapy [80, 81], but generally, the outcomes are disappointing. The fact is that calcaemic action of 1,25D3 and its analogues used so far in cancer trials with chemotherapy limits their dosage to a suboptimal level due to the risk of hypercalcemia, coma and cardiac arrest. A more potent and safer analogue of 1,25D is therefore required to allow dosage escalation to a therapeutic level. A non/weakly calcaemic analogue of 1,25D3 would also provide a better prophylactic than vitamin D regarding enhancing immune-competence as D3 hydroxylation to 1,25D3 is often impaired in older people.

Analogues of Vitamin D that Are Selective in their Action

There are several metabolites of vitamin D and hundreds of analogues and the modifications to generate analogues provided important information about structure-activity relationships. A major advance in the field of vitamin D research was the development of analogues of vitamin D that separate the differentiating/anticancer and calcaemic actions of 1,25D3. We elucidated some rules to achieving this endeavour. A β-chair conformation of the A-ring is essential for differentiating activity, as are hydrogen bonds and the (24E) side-chain geometry. CD ring modification and a *closely*-carbonyl cluster at the side-chain diminish calcaemic action. A rigid and straight (24E) side chain confers resistance to catabolism by the enzyme CYP24A1 (reviewed in [82••]). Unquestionably, agents with increased differentiating/anticancer potency and with minimal calcaemic action provide a perfect tool for scientific studies of haematopoiesis and a refined therapeutic, as to the avoidance of the calcaemic action of 1,25D3. Figure 3 shows some of the analogues of vitamin Ds generated at the Pharmaceutical Research Institute in Warsaw and potencies regarding their differentiating and calcaemic actions. Some of the analogues have minimal

residual calcaemic action, for example, PRI-5202. Importantly, some of the analogues are substantially more potent as differentiating agents as to their action on myeloid leukaemia cell lines than the parental hormone 1,25D3, including PRI-5202 that has a reduced calcaemic action. The low calcaemic analogues are also effective against AML cells in vitro and in animal models of disease [83•].



b

	Calcaemic action Ca^{2+} (serum) mg dL^{-1}	YDR binding $\text{IC}_{50} \text{nM}^2$	Differentiation $\text{EC}_{50} \text{nM}^2$
1,25D3	16.3	2	0.5
PRI-5201 ¹	14.2	1	0.03
PRI-5101 ¹	13.8	0.5	1
PRI-5100 ¹	13.1	0.6	1
PRI-5202 ¹	12.3	4	0.02
PRI-1907 ¹	11.4	6	0.06
solvent	10.9		

Fig. 3 Structures of leading analogues of $1\alpha,25$ -dihydroxyvitamin D₂ and their activities. The analogues separated the differentiating and calcaemic actions of 1,25D3 and some are highly potent differentiating agents and very weakly calcaemic, for example, PRI-5202. The EC₅₀ is for differentiation of the promyeloid cell line HL60 to macrophages. We treated mice with 0.3 $\mu\text{g/kg}$ of 1,25D3 or analogues of $1\alpha,25$ -dihydroxyvitamin D₂ every other day for 3 weeks and measured blood calcium on day 21. The calcaemic action is graded weak (green) to high as for 1,25D3 (red). VDR, vitamin D receptor, IC₅₀ is the binding affinity for VDR; EC₅₀ is the concentration from dose response curves that resulted in 50% of maximum differentiation

Unfinished Business Regarding the Action of 1,25D3 on Haematopoietic Cells

There are a number of unresolved issues regarding our understanding of the mode of action of 1,25D3 during ‘developmental’ haematopoiesis and the influences on the immune cell types. As mentioned above, 1,25D3 promotes monocyte/macrophage as opposed to neutrophil development of normal myeloid progenitors and the promyeloid cell line HL60. However, we now know that the decision to develop along the monocyte/macrophage pathway can occur within HSCs, and does the influence of 1,25D3 extend to these cells and this process? The data obtained using human CD34+ve haematopoietic progenitors indicate that physiological levels of 1,25D3 downregulate CD34 antigen and increase the number of monocyte-committed progenitors [84].

The mode of action of 1,25D3 to the differentiation per se of haematopoietic cells is still in need of further clarification. Many investigators hold the viewpoint that this biological action is solely via ‘a genomic effect’, namely the 1,25D/VDR-driven regulation of the gene transcription that is required for cell differentiation, for example CD14 expression and macrophage differentiation. However and in addition to this, 1,25D elicits rapid intracellular signals leading to the activation of the RAS/RAF/ERK-MAP kinase signalling pathway. The activation of this pathway within seconds or minutes means that the signals are not attributable to VDR-driven regulation of gene expression since transcription requires at least a few hours. The rapid intracellular signals are important to 1,25D3-driven differentiation of the promyeloid cell line NB4 and nuclear events are not required [85]. At present, the nature of a membrane receptor that might be responsible for the rapid response of cells to 1,25D3 is unclear. A small proportion of a cell’s canonical VDR is at the cell membrane [86] but this is not the sole reason for the 1,25D3-provoked rapid signals because they occur when investigators treated cells from VDR^{-/-} mice 1,25D3 [87]. Other putative membrane receptors include the Membrane Associated Rapid Response Steroid-binding protein (MARRS) [88], megalin and cubilin ([89], reviewed in [90]). Megalin and cubilin transport vitamin D, complexed to its serum binding protein. Receptor endocytosis can generate rapid intracellular signals via the Rab5/PI3-kinase pathway [91]. However and from knockout studies, we have excluded the involvement of megalin and cubilin as to the differentiating potency and selectivity of our analogues of 1,25D3 [92]. Even so, an interplay between genomic events and rapid intracellular signalling is likely to be important to how 1,25D3 brings about a change to the status of developing and mature haematopoietic cells.

Perhaps we might exclude the calcaemic action of 1,25D3 as having a major role in the differentiating action of 1,25D3 because analogues that are weakly calcaemic are highly potent

differentiating agents (see Fig. 3). However, this leaves us with another problem because the potent differentiating analogues do not always bind to the ligand-binding domain of VDR with higher affinity (see Fig. 3 and [93]). A possibility that we favour is that our new analogues bind to a particular pocket of the VDR ligand-binding domain leading to a particular conformational change that drives a selective change to the behaviour of cells. Whether there is a separate ligand-binding domain of VDR for the calcaemic action of 1,25D3 is also an uncertainty. The jury is still out on these matters because the lack of availability of the full-length VDR crystal structure hampers proper investigation. The use of truncated VDR for crystallography has led to discrepancies between affinity measurements and docking information.

Concluding Remarks

There has been a resurgence of interest in 1,25D3 and much is unrelated to its classical and longstanding roles in regulating bone health regarding calcium/phosphate homeostasis. Most types of cells express VDR, and 1,25D3 and VDR regulate the conduct of, in particular, monocyte/macrophage and iNKT cell development and the functionality of many of the immune end cells. We often think of this in terms of the regulation of gene expression either directly or indirectly by activated nuclear VDR. However, 1,25D3 and analogues of 1,25D3 have other interesting actions that are general as to an effect on different cell types. These include influences on the production/action of the haematopoietic cytokines that are essential to the development and function of the blood cells. In fact, in many of the above examples of changes to the status or functionality of blood cells, there is a link between the action of 1,25D3 and a cytokine/cytokine receptor. In addition, some analogues influence chromatin structure and the epigenetic landscape [57] and regulate the expression of micro RNAs [94] that might fine-tune the action of the cytokine receptors.

It is most reasonable to suggest that the level of intake of vitamin D that various agencies and doctors have recommended is important to not only the health of the haematopoietic cell system but also to that of the organism as a whole. Important attributes to this interest and tools to providing a better understanding of the actions of vitamin D are the analogues of 1,25D3 that are weakly calcaemic. There is also the prospect of applying the rules elucidated so far to eliminate the calcaemic action from 1,25D3 whilst retaining highly potent differentiating/anticancer actions. The use of existing and newer analogues of 1,25D3, which are even more selective and efficacious, may well lead to the prevention of the onset of significant health problems, immune cell related or otherwise, and provide a useful adjunct to cancer treatment. Primary 1,25D3 target genes allow the evaluation of subjects who might benefit from supplementation of vitamin D and there are appropriate transcriptomic biomarkers, for example,

upregulation of the genes encoding CD14 and thrombomodulin [48].

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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