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Vitamin D_3 and the immune system: maintaining the balance in health and disease

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1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active form of vitamin D_3, is a central player in Ca and bone metabolism. More recently, important immunomodulatory effects have been attributed to this hormone. By binding to its receptor, the vitamin D receptor, $1,25(OH)_2D_3$ regulates the expression of various genes and consequently affects the behaviour of different cell types within the immune system. $1,25(OH)_2D_3$ can potently inhibit pathogenic T cells and gives rise to elevated numbers of regulatory T cells via the induction of tolerogenic dendritic cells. These immunomodulatory activities of $1,25(OH)_2D_3$ have also been proven useful *in vivo*: administration of $1,25(OH)_2D_3$ in several animal models can prevent or cure different autoimmune diseases and graft rejection. To overcome the dose-limiting side effects of $1,25(OH)_2D_3$ on Ca and bone, less calcaemic structural analogues (alone or in combination with synergistically acting drugs or bone-resorption inhibitors) have been successfully used in animal models. Furthermore, as $1,25(OH)_2D_3$ also contributes to host defence against infectious agents by the induction of antimicrobial responses, this molecule might provide a new strategy to deal with drug-resistant infections. According to the pleiotropic effects of $1,25(OH)_2D_3$ in the immune system, increasing epidemiological data underline the importance of adequate vitamin D intakes in reducing the risk of several autoimmune diseases and infections such as tuberculosis.

Vitamin D_3: Immune function: Autoimmune diseases: Infections

Introduction

Sources and metabolism

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the biologically active form of vitamin D_3, is well known for its effects on mineral homeostasis and bone metabolism. This secosteroid hormone can be obtained by nutritional uptake (for example, fortified dairy products, fatty fish and their liver oils); however, UVB-mediated photosynthesis in the skin serves as the main source of vitamin D_3. Upon sunlight exposure, photolytic cleavage of 7-dihydrocholesterol in the skin results in the formation of previtamin D_3, which is subsequently converted by a spontaneous thermal isomerisation into vitamin D_3[1]. Once in the blood circulation, vitamin D_3 and its metabolites are bound to a carrier molecule, vitamin D_3 binding protein. Two subsequent hydroxylation steps are required to convert the hormone into its biologically active form[2]. The first activation step, the hydroxylation of vitamin D_3 at the carbon-25 position, occurs primarily in the liver and is catalysed by D_3-25-hydroxylase (25CYP2D25). Interestingly, 25-hydroxylase activity has also been reported to be present in kidney, parathyroid cells and keratinocytes[3–5]. The second hydroxylation step occurs predominantly in the proximal tubule cells of the kidney and is carried out by $25(OH)D_3$-1-α-hydroxylase (CYP27B1), resulting in the production of the biologically active metabolite $1,25(OH)_2D_3$. Besides its presence in kidney, 1-α-hydroxylase has also been found in other tissues such as skin, intestine, macrophage and bone, possibly allowing a local extrarenal production of high $1,25(OH)_2D_3$ levels within these tissues, without affecting serum concentrations of this hormone[6].

Regulation of vitamin D levels

Tissue availability of $1,25(OH)_2D_3$ depends on dietary intake and sun exposure, but is also influenced by the activity of the hydroxylating enzymes, as mentioned earlier. The

Abbreviations: AICD, activation-induced cell death; APC, antigen-presenting cell; CAMP, human cathelicidin antimicrobial peptide; FasL, Fas ligand; IFN, interferon; iNOS, inducible NO synthase; IU, international units; LPS, lipopolysaccharide; NOD, non-obese diabetic; $25(OH)D_3$, 25-hydroxyvitamin D_3; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; RXR, retinoid X receptor; Th, T helper; TLR, Toll-like receptor; VDR, vitamin D_3 receptor; VDRE, vitamin D_3 responsive element.

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25-hydroxylation of vitamin D_3 is poorly regulated, leading to a conversion of almost all vitamin D_3 present into 25-hydroxyvitamin D_3 ($25(OH)D_3$). Consequently, $25(OH)D_3$ is the major circulating form of vitamin D_3 and its concentration is commonly used as an indicator of vitamin D status[7]. In contrast, renal $1,25(OH)_2D_3$ production by 1-α-hydroxylase is tightly controlled by a variety of factors, including serum Ca, phosphate, parathyroid hormone and $1,25(OH)_2D_3$ itself[8]. Ultimately, $1,25(OH)_2D_3$ induces the expression of 24-hydroxylase (CYP24A1), the enzyme that catalyses the first step of $1,25(OH)_2D_3$ catabolism, eventually leading to its own degradation[2]. This negative feedback mechanism probably serves as an internal rescue to avoid excessive vitamin D_3 signalling.

Vitamin D deficiency has severe consequences for bone health: it causes rickets among children and osteomalacia in adults. Therefore, routine dietary supplementation is recommended, especially for those at risk of deficiency or in conditions of high demand such as pregnancy, lactation and early childhood[9]. Although there is still no consensus about appropriate vitamin D levels, serum concentrations of 30–50 ng $25(OH)D_3$/ml or higher are currently accepted as normal[10]. According to the current dietary reference intakes, adequate intake for children and younger adults is 5 μg (200 international units (IU))/d, whereas 10 μg (400 IU)/d is recommended for adults aged 51–70 years, and 15 μg (600 IU)/d for individuals older than 70 years of age[7]. Remarkably, these recommended daily vitamin D intakes are believed to be inappropriate: various clinical studies revealed that an intake of 12·5–25 μg (500–1000 IU) vitamin D/d is needed to maintain serum levels of 30 ng/ml, assuming that this would lead to a total supply of 95 μg (3800 IU)/d when also taking into account other sources such as tissue stores[11–13]. Therefore, an intake of 12·5–25 μg (500–1000 IU)/d might even be insufficient in certain populations such as sunlight-deprived individuals[14]. To reach the upper optimal level of 50 ng/ml, at least 25 μg (1000 IU)/d are needed and even with daily intakes of 100 μg (4000 IU) vitamin D, serum levels of $25(OH)D_3$ have been reported to remain within this physiological range[15,16]. Moreover, the formulated guidelines are based on maintaining bone health and do not take into account the non-calcaemic benefits of vitamin D_3.

Molecular mechanism of action: genomic v. *non-genomic actions*

Due to their lipophilic state, vitamin D metabolites can easily penetrate cell membranes and translocate to the nucleus[8,17]. In target cells, most of the known biological effects of vitamin D_3 are mediated by the binding of the ligand to its receptor, the vitamin D_3 receptor (VDR)[18]. The VDR is a ligand-dependent transcription factor, belonging to the steroid receptor superfamily. Upon ligand binding, VDR undergoes conformational changes, thereby allowing heterodimerisation with the retinoid X receptor (RXR). The RXR–VDR–ligand complex subsequently binds to vitamin D_3 responsive elements (VDRE) which are located in the promoter region of target genes. Different types of VDRE have been identified. The classical DR3-type is composed of a direct hexanucleotide repeat separated by three interspacing nucleotides[19]. Similar direct repeats with four (DR4-type) or six (DR6-type) interspacing nucleotides have been reported as well[20–22]. Another well-documented VDRE type is known as the IP9 type which comprises an inverted palindromic arrangement of two hexameric binding sites[23]. Interaction between the ligand–VDR–RXR complex and a VDRE facilitates the assembly of the transcription initiation complex by the release of co-repressors and the recruitment of nuclear receptor coactivator proteins, including members of the steroid receptor coactivator family and the vitamin D_3 receptor interacting proteins. The recruited proteins induce chromatin remodelling through intrinsic histone-modifying activities and attract key components of the transcription initiation complex to the regulated promoters. Alternatively, when ligand–VDR–RXR is recruited to an inhibitory VDRE, co-repressors are recruited and transcription of the gene is inhibited[8].

Besides its genomic actions, rapid transcription-independent events that occur within seconds or minutes upon $1,25(OH)_2D_3$ exposure have been reported in different cell types[8,24–27]. These non-genomic signalling events include changes in Ca flux and kinase activities and are thought to initiate at the membrane surface. The nature of the receptor that is responsible for these rapid actions remains controversial. The absence of rapid $1,25(OH)_2D_3$-mediated signalling events in osteoblasts lacking the VDR and the presence of the VDR in plasma membrane caveolae in different cell types both favour the idea that the VDR itself is involved in these non-genomic actions of $1,25(OH)_2D_3$[28,29]. On the other hand, the membrane-associated rapid response steroid binding protein has been proposed as a possible plasma membrane receptor for $1,25(OH)_2D_3$, since this receptor can bind the hormone and induce rapid responses[30,31].

Non-calcaemic actions of 1,25-dihydroxyvitamin D_3

The observation that the VDR is present in tissues other than bone, intestine, kidney and parathyroid glands suggests a role for VDR ligands beyond Ca and phosphate metabolism. Indeed, $1,25(OH)_2D_3$ affects the growth, differentiation status and function of several other cell types expressing VDR, including normal cells (keratinocytes of the skin, β-cells of the pancreas) as well as malignant cells (leukaemia cells, breast, prostate and colon cancer cells)[32–35]. Moreover, the discovery about two decades ago that VDR is expressed in almost all immune cells prompted the investigation of a possible role of vitamin D_3 in the immune system[36]. Ever since, many efforts have been made to elucidate the immune-modulating properties of vitamin D_3 and several observations in human, animal and *in vitro* experiments have led to the understanding that vitamin D_3 indeed plays an important role in the immune system. The findings in this research area will be discussed extensively in the present review.

1,25-Dihydroxyvitamin D_3 and the immune system

Physiological relevance of 1,25-dihydroxyvitamin D_3 in the immune system

The VDR is expressed in most cells of the immune system, including activated $CD4^+$ and $CD8^+$ T-lymphocytes and

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antigen-presenting cells (APC) such as macrophages and dendritic cells[37,38]. VDR expression has also been reported in B lymphocytes[37]; however, in a more recent analysis, this could not be confirmed[38]. We and others demonstrated the presence of 1-α-hydroxylase in macrophages, meaning that these immune cells are not only responsive to $1,25(OH)_2D_3$, but are even able to produce the hormone autonomously[39,40]. This $25(OH)D_3$-hydroxylating enzyme present in macrophages is identical to the renal form, but its expression is regulated in a completely different manner. 1-α-Hydroxylase expression in macrophages is significantly up regulated by immune signals such as interferon (IFN)-γ and lipopolysaccharide (LPS) or viral infections[40–44]. More recently, 1-α-hydroxylase expression has also been observed in dendritic cells and this phenomenon is associated with the p38 MAPK- and NF-κB-dependent maturation of these cells[45]. Importantly, and in contrast with 1-α-hydroxylase regulation in kidney, neither in macrophages, nor in dendritic cells, 1-α-hydroxylase activity is subjected to negative feedback signals deriving from $1,25(OH)_2D_3$ itself[40,45]. This observation explains the massive local production of $1,25(OH)_2D_3$ by disease-associated macrophages that is seen in patients with granulomatous diseases (sarcoidosis and tuberculosis). As it has been shown that up regulation of 1-α-hydroxylase and therefore $1,25(OH)_2D_3$ synthesis by APC occurs only at a later stage of macrophage activation, this system may act as a negative feedback loop in order to tone down inflammation[40].

Besides 1-α-hydroxylase, monocytes, macrophages and dendritic cells also express 24-hydroxylase[45,46]. The promoter of this gene comprises two VDRE, making 24-hydroxylase expression highly inducible by $1,25(OH)_2D_3$[47]. In monocytes and macrophages, however, the presence of this feedback mechanism depends on the differentiation or maturation stage of the cells. Undifferentiated monocytes are highly susceptible to $1,25(OH)_2D_3$-mediated 24-hydroxylase induction, whereas differentiated or activated macrophages are resistant. The latter is due to an interplay between IFN-γ-mediated and $1,25(OH)_2D_3$-mediated effects: STAT-1, a transcription factor involved in IFN-γ-signalling, interacts with the DNA-binding domain of the VDR, thereby prohibiting binding of the ligand–VDR–RXR complex to the 24-hydroxylase promoter and preventing $1,25(OH)_2D_3$-mediated induction of the enzyme[46]. Remarkably, 24-hydroxylase expression in dendritic cells was only observed when the cells underwent their differentiation process in the presence of $1,25(OH)_2D_3$[45].

The presence of VDR and the regulated expression of 1-α-hydroxylase and 24-hydroxylase in the immune system indicates a possible paracrine role for $1,25(OH)_2D_3$ in normal immune function. Indeed, different authors have reported an association between vitamin D deficiency and important immune defects in experimental animal models and in human subjects. In non-obese diabetic (NOD) mice (which spontaneously develop autoimmune diabetes and provide an interesting model for human type 1 diabetes because of the similar pathogenesis), vitamin D deficiency during early life results in a more aggressive manifestation of the disease with an earlier onset and a higher incidence[48,49]. This is consistent with epidemiological data showing a threefold increase in human type 1 diabetes when vitamin D deficiency was present in early life[50]. Also in animal models of other autoimmune diseases, vitamin D deficiency has been shown to accelerate disease development[51,52]. Accordingly, epidemiological studies revealed a correlation between areas with low vitamin D supplies (due to insufficient sunlight exposure time or nutritional vitamin D uptake) and incidences of different autoimmune diseases (type 1 diabetes, multiple sclerosis, inflammatory bowel disease and rheumatoid arthritis)[52–55].

Next to the correlation between vitamin D status and the prevalence of autoimmune diseases, vitamin D deficiency has also been associated with an increased susceptibility to infections such as tuberculosis[56]. A more detailed analysis of the immune system of vitamin D-deficient mice revealed defects in macrophage functions, such as chemotaxis, phagocytosis and pro-inflammatory cytokine production, all indispensable for antimicrobial activity[49,57]. In addition, a disturbed delayed-type hypersensitivity response has been reported in mice lacking vitamin D[58]. Taking together these findings, the importance of adequate vitamin D levels in normal immune function is beyond question.

In vitro *pharmacological effects of 1,25-dihydroxyvitamin D_3*

T cells. Soon after the discovery of VDR expression in activated T cells, direct effects of $1,25(OH)_2D_3$ on these cells were demonstrated[36,59–62]. In the presence of $1,25(OH)_2D_3$, the *in vitro* antigen- and lectin-stimulated proliferation and cytokine production (IL-2, IFN-γ, TNF-α) of human and murine T cells is inhibited[63–65]. Cell cycle analysis revealed that $1,25(OH)_2D_3$ blocks the transition from the G1a to the G1b phase[66]. Based on their cytokine profile, $CD4^+$ T lymphocytes can be classified as T helper (Th)1 lymphocytes (characterised by the production of IL-2, IFN-γ and involved in the elimination of intracellular pathogens) and Th2 lymphocytes (IL-4, IL-5, IL-10, IL-13 production and indispensable for the removal of extracellular organisms). Th1 cells are considered to be the key mediators in unwanted immune reactions such as autoimmune diseases and graft rejection, whereas Th2 cells influence these processes in a positive way. Importantly, Th1/Th2 differentiation is a self-perpetuating process; Th1 cells stimulate their own differentiation and inhibit the development of Th2 responses and vice versa[67]. By affecting the production of different cytokines in T cells, $1,25(OH)_2D_3$ has a serious impact on the outcome of immune reactions. Suppression of IL-2 by $1,25(OH)_2D_3$ prevents further activation and proliferation of the T cell population, since this cytokine acts as an autocrine growth factor. Remarkably, the inhibitory action of $1,25(OH)_2D_3$ on IL-2 production does not arise from a classical ligand–VDR–RXR–VDRE interaction within the promoter region of the target gene, but results from interference with nuclear factor of activated T cells–activator protein 1 complex formation and its subsequent binding to the nuclear factor of activated T cells binding site in the IL-2 promoter[68]. By inhibiting IFN-γ, the major macrophage-activating cytokine, $1,25(OH)_2D_3$ precludes antigen presentation and the recruitment of other T cells, thereby attenuating the immune reaction[69]. $1,25(OH)_2D_3$-mediated down regulation of

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IFN-γ results from binding of the ligand–VDR–RXR complex to a negative VDRE in the IFN-γ promoter. Moreover, an upstream enhancer element that is crucial for activation of the IFN-γ promoter is also involved in this effect[69]. IFN-γ is not only a macrophage-activating cytokine; it is also considered one of the driving forces behind Th1 development. In this way $1,25(OH)_2D_3$ has important effects on the Th1/Th2 balance; by suppressing IFN-γ, $1,25(OH)_2D_3$ can inhibit the generation of Th1 responses, thereby indirectly favouring the emergence of a Th2 population. Moreover, a study of Boonstra *et al.*[70] proposes also a direct role for $1,25(OH)_2D_3$ in the emergence of a Th2 phenotype, mediated through the up regulation of the Th2-specific transcription factors GATA-3 and c-maf and resulting in increased levels of IL-4, IL-5 and IL-10. Remarkably, *in vitro* immunomodulatory effects of $1,25(OH)_2D_3$ do not always correspond with the effects of the hormone observed *in vivo*. The consistent $1,25(OH)_2D_3$-mediated down regulation of IFN-γ that is seen in various *in vitro* settings has been confirmed by different *in vivo* studies, whereas no *in vivo* suppression of this cytokine upon $1,25(OH)_2D_3$ treatment could be detected by others[71–76]. Furthermore, while some studies could confirm *in vivo* a $1,25(OH)_2D_3$-induced up regulation of IL-4 and subsequent skewing towards a Th2 phenotype, others report no effect or even suppression of IL-4 by $1,25(OH)_2D_3$[76–78]. These conflicting results might be a consequence of the differences in experimental design; however, it certainly also reflects the complexity of the mechanisms underlying the immune-modulating properties of $1,25(OH)_2D_3$.

Since $1,25(OH)_2D_3$ is a consistent inhibitor of Th1 cytokines, in some cases even actively driving a Th2 response, it has been hypothesised that the association between vitamin D supplementation in newborns and the incidence of allergic diseases later in life – as suggested by several epidemiological studies – might be ascribed to the $1,25(OH)_2D_3$-mediated inhibition of Th1 differentiation[79–81]. To address this issue, Pichler *et al.*[82] investigated the effects of $1,25(OH)_2D_3$ on Th cell differentiation of human cord blood cells; $1,25(OH)_2D_3$ not only inhibited IL-12-stimulated IFN-γ production, but also IL-4 and IL-4-induced IL-13 expression. In light of these findings, it seems plausible to assume that the application of vitamin D during early life should not promote the development of allergies.

Also, other T cell-produced compounds are under the direct influence of $1,25(OH)_2D_3$. Granulocyte-macrophage colony-stimulating factor is suppressed by $1,25(OH)_2D_3$ in cultures of human mitogen-activated T cells and in a T cell line[83]. Fas ligand (FasL) has been identified as another important target of $1,25(OH)_2D_3$-mediated suppression in T cells[84]. Moreover, the same study reported a decreased rate of activation-induced cell death (AICD) of T cells upon *in vitro* treatment with $1,25(OH)_2D_3$. The Fas–FasL pathway regulates AICD in T lymphocytes, a fundamental mechanism for the maintenance of central and peripheral tolerance to self-antigens by the elimination of autoreactive T cells. Aberrant expression of Fas and FasL has also been implicated in the induction and regulation of organ-specific autoimmune diseases[85,86]. In type 1 diabetes, for example, pancreatic β-cells express Fas on their surface in response to IL-1β, making them susceptible to apoptosis upon interaction with FasL-bearing activated T lymphocytes[87]. Moreover, reverse signalling through FasL is believed to be essential for optimal proliferation of cytotoxic T lymphocytes[88,89]. Fas is also constitutively expressed by dendritic cells and triggering this receptor by FasL has been demonstrated to induce a phenotypical and functional maturation of dendritic cells and an increased expression of IL-1β and TNF-α. In addition, Fas-triggered dendritic cells have a Th1-driving potential[90]. Considering the various processes in which the Fas–FasL system is involved, down regulation of FasL by $1,25(OH)_2D_3$ in T cells might modulate the immune response at various levels. A discrepancy appears to exist between Cippitelli's work and the *in vivo* data available. Our group showed that $1,25(OH)_2D_3$ treatment restores the thymocyte apoptosis sensitivity in NOD mice, resulting in an enhanced elimination of self-reactive T cells in the thymus as well as in the periphery by AICD[91]. It is, however, important to realise that this study was conducted in mice already having multiple immune abnormalities, comprising a lower sensitivity to AICD in T lymphocytes[92]. Moreover, interactions between thymic dendritic cells and thymic T cells have been demonstrated to be crucial for these $1,25(OH)_2D_3$-mediated effects, possibly explaining the contrasting results with Cippitelli's *in vitro* work.

Antigen-presenting cells. APC have been shown to be central targets for $1,25(OH)_2D_3$-mediated actions[36,59,61,62]. A number of studies report that $1,25(OH)_2D_3$ exerts pro-differentiating effects on monocytes and monocyte-derived cell lines, driving them towards a macrophage-like phenotype[59]. Exposing macrophages to $1,25(OH)_2D_3$ enhances their chemotactic and phagocytic capacity, which is indispensable for their tumour cell cytotoxicity and microbacterial activity[93]. In contrast, $1,25(OH)_2D_3$ seriously impairs the antigen-presenting and T cell-stimulatory capacities of monocytes and macrophages; surface expression of MHC class II and co-stimulatory molecules, such as CD40, CD80 and CD86, is down regulated when monocytes are exposed to $1,25(OH)_2D_3$ *in vitro*[93].

Among the APC, dendritic cells play a pivotal role in initiating and regulating T cell responses. These highly specialised cells reside in an immature state in the peripheral tissues where they sample the environment and mediate antigen uptake. When dendritic cells receive a maturation signal, they migrate to the local lymph nodes where they can provide all signals necessary for full T cell activation; presentation of MHC-coupled antigen as well as expression of co-stimulatory molecules and secretion of key cytokines such as IL-12. Multiple *in vitro* studies, using either human peripheral blood monocytes or murine bone marrow cells as precursors, revealed that $1,25(OH)_2D_3$ can potently inhibit dendritic cell differentiation[94–96]. Moreover, the *in vitro* and *in vivo* maturation process of dendritic cells is seriously impaired by $1,25(OH)_2D_3$, with a decreased surface expression of MHC class II, co-stimulatory molecules (CD40, CD80, CD86) and other maturation-induced surface markers. It has even been shown that *in vitro* $1,25(OH)_2D_3$ treatment can redirect already differentiated dendritic cells towards a $CD14^+$ cell type. In addition, $1,25(OH)_2D_3$ treatment of differentiating dendritic cells disturbs their

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migratory capacity in response to inflammatory and lymph node-homing chemokines, although the expression of the cognate chemokine receptors was unaffected[96].

The secretion of cytokines by APC is also under the influence of $1,25(OH)_2D_3$. IL-12 production is significantly suppressed in activated macrophages and dendritic cells upon $1,25(OH)_2D_3$ treatment[97]. This effect is a consequence of the $1,25(OH)_2D_3$-mediated down regulation of NF-κB activation and subsequent binding to its NF-κB binding site in the promoter region of the p40 subunit of IL-12[98]. IL-10 is up regulated in dendritic cells upon exposure to $1,25(OH)_2D_3$[94]. When examining the influence of $1,25(OH)_2D_3$ on the expression of TNF-α by APC, conflicting data were obtained, depending on the differentiation state of the cells. On the one hand in immature cells, such as bone marrow cells, TNF-α levels are increased by $1,25(OH)_2D_3$ and a synergistic effect is observed with LPS[98]. Direct binding of the ligand–VDR–RXR complex to a VDRE in the promoter region of the TNF-α gene as well as up regulation of CD14, the co-receptor of the Toll-like receptor (TLR)4, thus enhancing the LPS-induced TLR activity, underlie these observations[98]. On the other hand, in more mature cells such as peripheral blood mononuclear cells and LPS-stimulated monocytes, TNF-α levels are decreased by $1,25(OH)_2D_3$[99,100]. This decrease of TNF-α production appears to stem (at least partially) from a $1,25(OH)_2D_3$-mediated down regulation of TLR2 and TLR4[100].

Besides cytokines, other APC-produced factors are influenced by $1,25(OH)_2D_3$. Prostaglandin E2 expression by monocytes is stimulated upon *in vitro* treatment with $1,25(OH)_2D_3$[101]. Controversial data have been obtained concerning the regulation of inducible NO synthase (iNOS) expression by $1,25(OH)_2D_3$. In a human macrophage-like cell line, an induction of iNOS expression by the hormone was observed, while other *in vitro* and *in vivo* studies report inhibitory actions of $1,25(OH)_2D_3$ on iNOS levels[102–104]. This complex relationship between iNOS and $1,25(OH)_2D_3$ still requires further investigation.

Consequences for interactions between dendritic cells and T cells. Since the main function of dendritic cells is to initiate and regulate T cell responses, the effect of $1,25(OH)_2D_3$ on dendritic cells inevitably has a major impact on T cells. Although $1,25(OH)_2D_3$ has direct effects on T cells, it is generally by this indirect way that $1,25(OH)_2D_3$ influences T cell responses. Through the suppression of dendritic cell-derived IL-12, driving the T cell differentiation towards a Th1 phenotype, and the up regulation of IL-10, thwarting this action, $1,25(OH)_2D_3$ indirectly skews the T cell differentiation towards a Th2 phenotype. Also by decreasing the surface expression of MHC II-coupled antigens and co-stimulatory molecules, $1,25(OH)_2D_3$ alters the T cell-stimulatory ability of dendritic cells. Dendritic cells lacking these (co)-stimulatory molecules become tolerogenic and give rise to regulatory T cells or even induce T cell anergy[105] (Fig. 1). This distinct $CD4^+$-regulatory T cell subset (next to $CD4^+$ Th1 and Th2 cells) is CD25-positive and is characterised by the secretion of potentially inhibitory cytokines (IL-10, transforming growth factor-β) and the ability to potently inhibit antigen-specific T cell activation. By preventing dendritic cell differentiation and maturation as well as modulating their activation and survival, $1,25(OH)_2D_3$ has

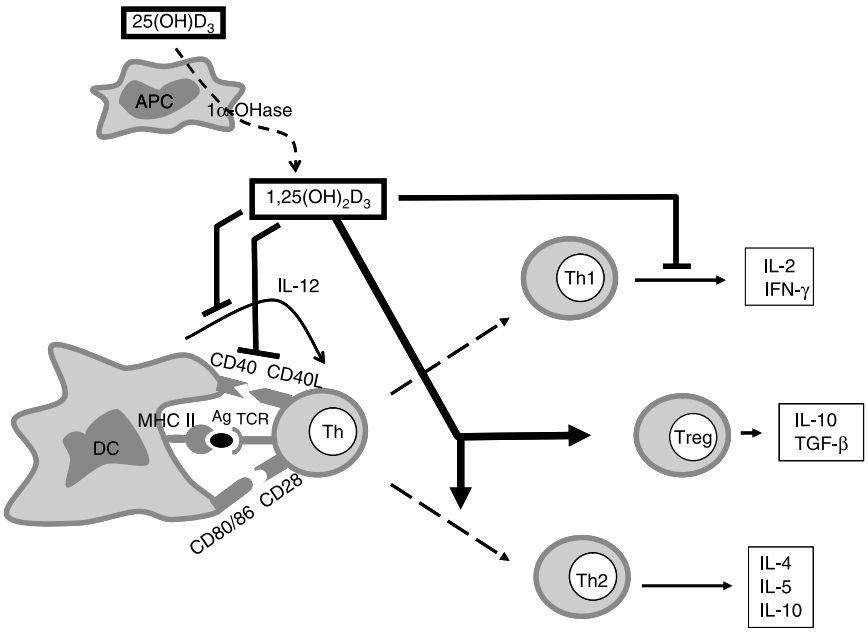

Fig. 1. The immunomodulatory effects of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). $1,25(OH)_2D_3$, locally produced within the immune system by antigen-presenting cells (APC) expressing 1-α-hydroxylase (1α-OHase), suppresses the production of T helper (Th)-1 cytokines (IL-2 and interferon (IFN)-γ), stimulates the production of Th2 cytokines (IL-4, IL-5, IL-10) and favours the emergence of regulatory T cells (Treg). In dendritic cells (DC), $1,25(OH)_2D_3$ exerts inhibitory actions on the surface expression of co-stimulatory molecules and the secretion of IL-12. $25(OH)D_3$, 25-hydroxyvitamin D_3; CD, cluster differentiation; TCR, T cell receptor; TGF, transforming growth factor.

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been shown to give rise to tolerogenic dendritic cells *in vitro* and *in vivo*. When cultured together with these $1,25(OH)_2D_3$-modulated dendritic cells, naive or even committed autoreactive T cells showed a complete hyporesponsiveness as determined by decreased proliferation and IFN-γ secretion[94,106]. Furthermore, $1,25(OH)_2D_3$-treated dendritic cells are able to modulate the fate and function of committed autoreactive T cells via the selective and antigen-dependent induction of apoptosis[95,106].

This $1,25(OH)_2D_3$-mediated induction of regulatory T cells has also been observed *in vivo*. Treatment of NOD mice with $1,25(OH)_2D_3$ results in a restoration of regulator cells (being defective in NOD mice), preventing the spontaneous development of diabetes in treated mice as well as in untreated mice upon transfer[107,108]. Nevertheless, diabetes induction by cyclophosphamide (a drug that elicits diabetes by eliminating regulator cells) could still be prevented in these mice by $1,25(OH)_2D_3$ treatment, suggesting that the induction of a regulatory T cell population is not the only mechanism responsible for $1,25(OH)_2D_3$-induced diabetes protection[109]. Indeed, $1,25(OH)_2D_3$ also restores the apoptosis sensitivity of autoreactive T cells in NOD mice, leading to the elimination of diabetogenic T cells[109]. Recently, Decallonne *et al.*[91] identified dendritic cells as indispensable targets for $1,25(OH)_2D_3$ during the apoptosis-restorative process in the central immune system of NOD mice. Furthermore, treating NOD mice at the stage of insulitis with an analogue of $1,25(OH)_2D_3$ resulted in an arrest of disease progression while increasing the frequency of $CD4^+CD25^+$-regulatory T cells in pancreatic lymph nodes[110]. Again, the promotion of regulatory T cells by the analogue was speculated to be an indirect result of dendritic cell modulation rather than a direct T cell effect. In a murine islet transplantation model, a combined treatment of $1,25(OH)_2D_3$ and mycophenolate mofetil preventing graft rejection could generate tolerogenic dendritic cells *in vivo*, accounting for an increased regulatory T cell population in regional lymph nodes that conferred protection upon transfer[111].

Taken together, these data point towards dendritic cells as crucial players in the $1,25(OH)_2D_3$-mediated induction of regulatory T cells. However, Barrat *et al.*[112] reported that a combination of $1,25(OH)_2D_3$ and dexamethasone could induce a regulatory T cell population *in vitro* in the absence of APC. These cells produced predominantly IL-10, but no IFN-γ, IL-4 or IL-5 and could potently suppress autoimmune demyelination *in vivo* in an antigen-specific way.

In vivo *immunomodulatory properties of 1,25-dihydroxyvitamin D_3*

The in vivo *use of 1,25-dihydroxyvitamin D_3 and analogues*. The immunomodulatory effects of $1,25(OH)_2D_3$ have been confirmed *in vivo* in several animal models of autoimmune diseases and organ transplantation[36,59,62,113,114]. Yet, in accordance with the *in vitro* experiments where effects could only be demonstrated at supraphysiological concentrations, high doses of $1,25(OH)_2D_3$ have to be administered *in vivo* in order to obtain therapeutic effects. Consequently, concomitant calcaemic side effects are observed, comprising hypercalcaemia, hypercalciuria, renal calcification and increased bone resorption, preventing the clinical use of $1,25(OH)_2D_3$ as an immunomodulator. To overcome this limitation, structural analogues of $1,25(OH)_2D_3$ have been developed that show equal or even higher immunomodulatory potency than $1,25(OH)_2D_3$ itself, but lower calcaemic activity. We and others have been exploring the therapeutic potential of $1,25(OH)_2D_3$ and its analogues in NOD mice. We demonstrated that $1,25(OH)_2D_3$ and analogues prevent insulitis (the histological lesion in pancreatic islets caused by infiltrating immune cells and preceding the clinical presentation of diabetes), but also the development of overt diabetes in NOD mice when treatment is started before the onset of insulitis[107,108,115]. The beneficial effects of $1,25(OH)_2D_3$ and analogues in this model are a consequence of (a) the restoration of defective suppressor cell activity, (b) the enhanced clearance of autoreactive T cells by restoring apoptosis sensitivity as well as (c) a shift from a Th1 to a Th2 cytokine expression profile locally in the pancreas and in the pancreas-draining lymph nodes[75,109]. Moreover, $1,25(OH)_2D_3$ induces this immune shift in response to autoantigens but not to disease-irrelevant self or foreign antigens[75].

To evaluate their applicability in a more clinically relevant situation, such as the treatment of pre-diabetic patients with established insulitis, analogues of $1,25(OH)_2D_3$ were administered to NOD mice when autoimmune β-cell destruction is already taking place. Treatment with different $1,25(OH)_2D_3$ analogues, either alone or in combination with a short induction course of cyclosporin A, blocks diabetes progression in NOD mice suffering from insulitis[73,110].

Nowadays, pancreatic islet transplantation has proven to be an effective therapy in patients with type 1 diabetes[116]. However, strong immunosuppression is needed to prevent, besides allorejection, also the autoimmune destruction of transplanted islets by self-reactive memory T cells and thus diabetes recurrence. In NOD mice, treatment with a $1,25(OH)_2D_3$ analogue can prevent autoimmune diabetes recurrence after syngeneic islet transplantation[117,118].

Many other examples of animal autoimmune disease models exist in which treatment with $1,25(OH)_2D_3$ or its analogues prevents disease or attenuates disease progression, including systemic lupus erythematosus, experimental autoimmune encephalomyelitis, collagen-induced arthritis, inflammatory bowel disease and Heymann nephritis[119–124]. Even a few clinical trials have already demonstrated disease-improving effects of $1,25(OH)_2D_3$ analogues in patients suffering from multiple sclerosis or rheumatoid arthritis[125,126]. In addition, $1,25(OH)_2D_3$ and its analogues are effective tools for the prevention of allograft rejection. They can prolong the survival of heart, aorta, kidney, liver, small bowel, pancreatic islet and skin allografts[111,127–131]. When using standard immunosuppressants (such as cyclosporin A, FK506, rapamycin, mycophenolate mofetil and glucocorticoids), several problems arise, such as severe long-term toxicity and opportunistic infections. Interestingly, a sustained resistance to opportunistic infections is observed upon treatment with $1,25(OH)_2D_3$ or analogues[132]. Moreover, none of the

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standard drugs is able to prevent chronic rejection, a phenomenon caused by immunological and non-immunological factors and characterised by perivascular inflammation, fibrosis and vascular narrowing due to smooth muscle cell proliferation. In an aortic allograft model, which is used to mimic the vascular lesions seen in human chronic allograft rejection, treatment with a $1,25(OH)_2D_3$ analogue prevents chronic aortic allograft rejection and attenuates vascular damage[133,134]. In addition, $1,25(OH)_2D_3$ or analogue treatment can overcome post-transplant bone loss, a side effect of several immunosuppressants[132,135]. Moreover, since chronic administration of standard immunosuppressants promotes the development of post-transplant malignancies, the antiproliferative (hence tumour-suppressive) capacity of $1,25(OH)_2D_3$ and its analogues provides an additional asset for their use in allotransplantation[136]. In light of these findings, it is presumable that addition of $1,25(OH)_2D_3$ analogues to standard clinical immunosuppressive regimens may significantly improve long-term graft and patient survival.

Combined immunotherapy. Throughout the years, research has led to the successful development of $1,25(OH)_2D_3$ analogues with stronger immunomodulatory potential combined with fewer calcaemic side effects compared with the parent molecule. Notwithstanding, researchers have not succeeded to date in creating analogues of $1,25(OH)_2D_3$ that are completely free from calcaemic side effects. As a consequence, other strategies have to be used to circumvent the toxic effects of the current VDR agonists. One method to further increase the clinical applicability of $1,25(OH)_2D_3$ analogues is based on the principle of drug synergism, a phenomenon in which two or more individual agents acting together create an effect that is stronger than the simple sum of the effects generated by each agent independently. By combining synergistically acting drugs, the doses of the individual agents can be reduced to a subtherapeutical level, thereby reducing the toxic effects of each drug. *In vitro*, the combination of $1,25(OH)_2D_3$ or an analogue with standard immunosuppressants resulted in the synergistic inhibition of phytohaemagglutinin-stimulated T cell proliferation[137–140]. Particularly *in vivo*, in numerous animal models of autoimmune diseases and of allotransplantation, synergism between $1,25(OH)_2D_3$ or its analogues and standard immunosuppressants has been observed[62]. In a model of syngeneic islet transplantation in NOD mice, better protection from autoimmune diabetes recurrence and less toxicity were obtained with combinations of $1,25(OH)_2D_3$ analogues and standard immunosuppressants (cyclosporin A, IFN-β) as with analogue monotherapy[117,118,141]. For combinations with cyclosporin A, graft survival even lasted after withdrawal of therapy, suggesting a re-induction of self-tolerance[117,118]. Again, an immune shift from Th1 towards Th2 locally in the transplanted islets could be observed. Taken together, these findings point to VDR ligands as valuable dose-reducing agents for classical immunosuppressants in the treatment of several autoimmune disorders and/or clinical transplantation. Differences in cooperativity can, however, be noted for different combinations. While evaluating synergism between $1,25(OH)_2D_3$ or analogues and a panel of immunosuppressants, Van Etten *et al.*[140] observed the strongest synergism between VDR ligands and the calcineurin inhibitors (cyclosporin A, FK506) and rapamycin *in vitro* and *in vivo*. These data have to be taken into account in order to design clinically valuable combination protocols.

A broader therapeutic window can also be pursued by directly counteracting the side effects that are associated with the *in vivo* use of $1,25(OH)_2D_3$ and its analogues. Bisphosphonates are inhibitors of osteoclast activity, thus preventing bone loss. They are generally used in several conditions that are accompanied with aberrant levels of bone turnover, such as bone metastases associated with breast cancer or multiple myeloma, tumour-induced hypercalcaemia and Paget's disease of bone. Our group demonstrated that the bone-directed side effects of a $1,25(OH)_2D_3$ analogue in a model of experimental autoimmune encephalomyelitis can be completely abolished by adding the bisphosphonate pamidronate to the treatment protocol while leaving the protective effects of the analogue unaffected[142]. Interestingly, not only the analogue-induced accelerated bone turnover was prevented, but also a remarkable growth of bone mass and mineral content was induced. Combining such less-calcaemic $1,25(OH)_2D_3$ analogues with bisphosphonates or other inhibitors of bone resorption might be a promising strategy to treat various immune disorders in human subjects without affecting bone.

1,25-Dihydroxyvitamin D_3 and infection. Exposing monocytes and macrophages to $1,25(OH)_2D_3$ improves their chemotactic and phagocytotic capacity, both features that are indispensable for their tumour cell cytotoxicity and microbacterial activity[93]. Monocytes and macrophages are, next to their role as APC in the stimulation of T cell-mediated immune responses, key players in mounting innate immune responses against various infectious agents, including bacteria, viruses, fungi and parasites. They rapidly detect dangerous microbial invaders by means of their pattern-recognition receptors (for example, TLR) and subsequently produce antimicrobial peptides such as defensins and cathelicidins in order to repel enemies[143,144]. In addition to their anti-infective activities, antimicrobial peptides also contribute to processes such as chemotaxis, wound repair and local angiogenesis[145,146]. Lately, Wang *et al.*[147] demonstrated that $1,25(OH)_2D_3$ induces the expression of human cathelicidin antimicrobial peptide (CAMP) in isolated human monocytes, keratinocytes, neutrophils and different human cell lines, directly resulting in enhanced antimicrobial activity. They identified consensus VDRE sequences in the promoter of the CAMP gene and observed a synergistic effect with LPS in neutrophils. Shortly thereafter, the induction of CAMP by $1,25(OH)_2D_3$ and three of its analogues has also been observed in other cell types such as acute myeloid leukaemia and colon cancer cell lines, bone marrow-derived macrophages and bone marrow cells[148].

The induction of antimicrobial activity by $1,25(OH)_2D_3$ may at least in part explain the beneficial effects of UVB on host resistance to infections. It is, for example, an established fact that sun exposure can improve or even

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cure disease in most tuberculosis-infected individuals. Accordingly, a higher susceptibility to tuberculosis infections is seen in subjects with relatively low serum vitamin D levels, such as the elderly, uraemic patients and dark-skinned individuals[56]. Moreover, $1,25(OH)_2D_3$ is known to protect cultured human monocytes and macrophages against tubercle bacilli[149,150]. Recently, by means of microarray studies, Liu *et al.*[151] tried to explain why TLR2/1-activated human monocytes and macrophages can reduce the viability of intracellular *Mycobacterium tuberculosis* whereas human monocyte-derived dendritic cells can not. Rather by coincidence, their study provided data that made it possible to elucidate (at least one of) the mechanisms underlying the antimicrobial properties of sunlight. In the TLR2/1-activated monocytes, a selective up regulation of VDR and 1-α-hydroxylase was seen. Moreover, the TLR2/1-activated monocytes also expressed CAMP, but only when $25(OH)D_3$ was present in the medium. Interestingly, in the presence of serum from African-Americans, TLR2/1-activated monocytes produced lower levels of CAMP than when exposed to serum from Caucasians. This can be explained by the lower circulating $25(OH)D_3$ levels in African-Americans' serum due to a higher melatonin content in their skin and the consequently lower $25(OH)D_3$ synthesis upon UVB exposure. Addition of $25(OH)D_3$ to the African-American serum could indeed restore the impaired CAMP production. These data provide evidence for a model in which triggering of TLR results in the conversion of $25(OH)D_3$ into active $1,25(OH)_2D_3$, the induction of CAMP and eventually the initiation of an antimicrobial response. Inappropriate $25(OH)D_3$ levels impair this $1,25(OH)_2D_3$-dependent antimicrobial response and sunlight exposure might, by raising the $25(OH)D_3$ levels in circulation, contribute to the adequate functioning of this system. Based on these data, vitamin D supplementation might be a considerable strategy to prevent tuberculosis in individuals at risk because of their inadequate $25(OH)D_3$ levels.

Remarkably, while participating in TLR-induced antimicrobial responses, $1,25(OH)_2D_3$ suppresses the expression of TLR2 and TLR4 mRNA and protein in human monocytes by a VDR-dependent mechanism[100]. Although CD14 (the co-receptor of TLR4) is markedly increased in the $1,25(OH)_2D_3$-treated monocytes, TLR triggering results in an impaired inflammatory response. Since this observed down regulation of TLR is most prominent after 72 h, this might represent a negative feedback mechanism to prevent excessive TLR activation, which gives rise to sepsis, and shut down the inflammatory response at a later stage of infection.

Altogether, $1,25(OH)_2D_3$ has interesting qualities that might be of clinical relevance with regard to innate immune responses. Extrinsic manipulation of CAMP by $1,25(OH)_2D_3$ treatment might offer a novel strategy to deal with the overwhelming problem of drug-resistant bacteria, since these pathogens have difficulties developing resistance against antimicrobial peptides. Induction of CAMP by $1,25(OH)_2D_3$ might also be opportune in situations of sepsis and to promote wound healing while preventing infections, for example after burn or surgery. Evidence for the beneficial effects of CAMP under these circumstances has been provided by various *in vitro* experiments and animal studies[152–155].

Conclusions

Intensive research during the last decades has shed new light on the biological functions of vitamin D_3. Beyond its well-known role in Ca and bone homeostasis, important immunomodulatory effects have been attributed to the activated form of vitamin D_3, $1,25(OH)_2D_3$. Within the immune system, $1,25(OH)_2D_3$ targets both APC and T cells. VDR ligands can inhibit pathogenic T cells and induce tolerogenic dendritic cells, which are likely to give rise to increased numbers of regulatory T cells. Therefore, $1,25(OH)_2D_3$ is a very plausible candidate in the treatment of several autoimmune disorders and graft rejection after transplantation. Nevertheless, developing effective strategies to avoid the calcaemic side effects of this hormone still remains a major challenge. In this respect, structural analogues of $1,25(OH)_2D_3$ have been designed, showing reduced calcaemic effects together with equal or even stronger immunomodulating capacities compared with the parent molecule. Combining $1,25(OH)_2D_3$ or a structural analogue with synergistically acting immunosuppressants allows the application of both drugs at subtherapeutical doses, thereby avoiding toxicity, whereas addition of bone-resorption inhibitors to the treatment protocols provides a method to directly counteract the detrimental bone effects of high doses of the hormone. All these strategies to overcome the dose-limiting side effects of $1,25(OH)_2D_3$ have been proven very effective in various animal models of autoimmune diseases and graft rejection. In addition, besides having the capacity to interfere with autoimmune responses and the process of graft rejection, $1,25(OH)_2D_3$ is also able to fight infections by the induction of antimicrobial responses. Based on this quality, the use of $1,25(OH)_2D_3$ might be a very appealing method to deal with the problem of drug-resistant infections.

In conclusion, the immunomodulatory effects of $1,25(OH)_2D_3$ extend to different branches and cell types of the immune system. Importantly, the use of $1,25(OH)_2D_3$ and analogues in different animal models shows very promising results and favours their possible application in various clinical settings.

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