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Vitamin D: modulator of the immune system

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1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active form of vitamin D, is known to regulate calcium and phosphorus metabolism, thus being a key-player in bone-formation. However $1,25(OH)_2D_3$ also has a physiological role beyond its well-known role in skeletal homeostasis. Here, we describe $1,25(OH)_2D_3$ as an immunomodulator targeting various immune cells, including monocytes, macrophages, dendritic cells (DCs), as well as T-lymphocytes and B-lymphocytes, hence modulating both innate and adaptive immune responses. Besides being targets, immune cells express vitamin D-activating enzymes, allowing local conversion of inactive vitamin D into $1,25(OH)_2D_3$ within the immune system. Taken together, these data indicate that $1,25(OH)_2D_3$ plays a role in maintenance of immune homeostasis. Several epidemiological studies have linked inadequate vitamin D levels to a higher susceptibility of immune-mediated disorders, including chronic infections and autoimmune diseases. This review will discuss the complex immune-regulatory effects of $1,25(OH)_2D_3$ on immune cells as well as its role in infectious and autoimmune diseases, more in particular in tuberculosis and type 1 diabetes (T1D).

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Introduction

The Nobel Prize in Chemistry in 1928 was given to Adolf Windaus on account of his work on the constitution of sterols and their connection with vitamins. The vitamin in question was vitamin D, which already had a longstanding history before Windaus studied it. The clinical disorder resulting from vitamin D deficiency during infancy and childhood has been known since the mid-17th century. In that period, the cause of rickets was unknown, but it was endemic in England in the southwest cities of Dorset and Somerset, areas that were heavily polluted by industrial smoke and smog. It was only by the beginning of the 20th century, with the description of the anti-rachitic properties of cod-liver oil and UV irradiation (2 sources of vitamin D), and the use of biochemical tests in the study of rickets, that lack of vitamin D was finally identified as the leading cause of this disease. Thereafter, vitamin D was ultimately recognized as a central regulator of calcium homeostasis and bone metabolism and nowadays it is well understood that vitamin D deficiency in adults results in poorly mineralized skeletal matrix, a symptom known as osteomalacia.

During the past decades, our knowledge of the vitamin D metabolism has greatly evolved and the discovery of vitamin D receptors (VDRs) as well as of vitamin D-activating enzymes in cell types other than those involved in mineral and bone homeostasis strongly indicates a more diverse role for vitamin D than originally accepted. Indeed, activation of these VDRs has so-called non-classical effects, which include the modulation of growth, differentiation status, and function of a variety of cells, exposing additional roles for vitamin D in the regulation of immune responses, cardiovascular processes, and in cancer prevention [1•,2]. With the growing appreciation of these non-classical effects of vitamin D and their importance for human health, the high prevalence of vitamin D insufficiency or hypovitaminosis D in many populations across the globe is a worrying awareness [3•,4•]. Vitamin D deficiency is known to cause osteoporosis and muscle weakness, increasing the risk for fractures due to falls, especially in elderly [5]. Moreover, hypovitaminosis D is associated with an increased risk of multiple malignancies, metabolic and cardiovascular diseases, and immune disorders such as autoimmune diseases and high infection rates [6–10].

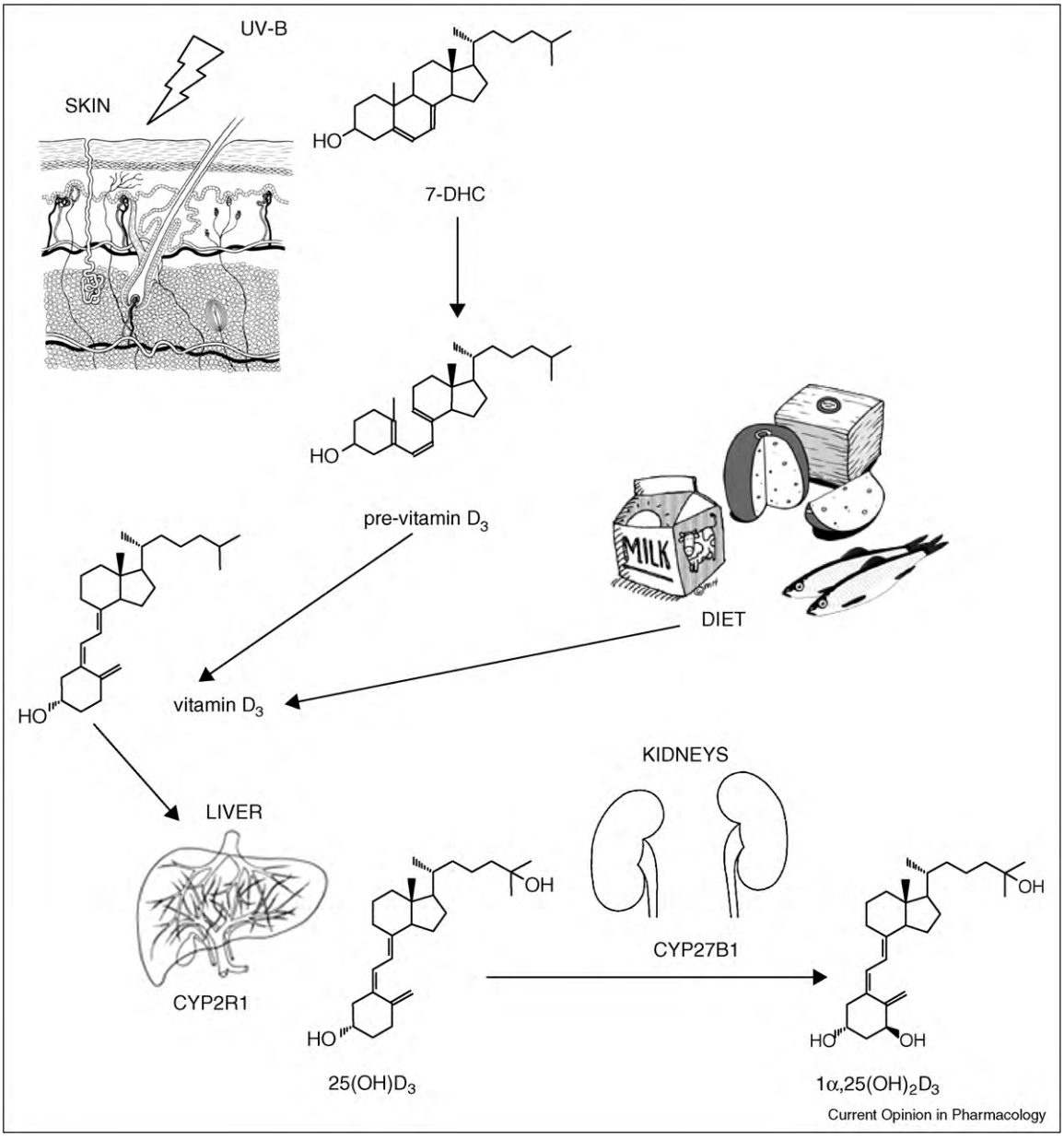
In the present review, we summarize the current knowledge on the role of vitamin D as regulator of the immune system, including its effects on a cellular level. Furthermore, we give an overview of the immunological mechanisms linking vitamin D to protection against infectious and autoimmune diseases such as tuberculosis and T1D respectively. In addition, the prospective application of vitamin D as a therapeutic agent is discussed.

Sources and metabolism of vitamin D

In humans, vitamin D can be obtained from two distinct sources, either from diet or by UV-mediated synthesis in

the epidermal layer of the skin. Therefore, by definition, vitamin D cannot be considered as a true vitamin but rather as a pro-hormone. Two forms of vitamin D can be obtained by nutritional intake: vitamin D_2 (also known as ergocalciferol) is present in fungi/yeast, while vitamin D_3 (also known as cholecalciferol) is found in foods from animal origin. Only few foods naturally contain significant amounts of vitamin D. For example cod-liver oil and oily fish are considered as rich sources, whereas butter, cream, and egg yolk contain only small amounts. Human and cow's milk, on the contrary, are poor sources of vitamin D [11]. Despite the fact that vitamin D_3 can be obtained by nutrition, the most important source of this pro-hormone is the skin, which has a great capacity to produce vitamin D_3 upon sunlight exposure. In the skin, UV rays promote photolytic cleavage of 7-dihydrocholesterol (7-DHC) into pre-vitamin D_3, which is subsequently converted by a spontaneous thermal isomerization into vitamin D_3 [12].

Figure 1



Metabolism of vitamin D_3.
Synthesis of vitamin D_3 occurs in the skin where 7-dehydrocholesterol (7-DHC) is converted to pre-vitamin D_3 in response to UV exposure. Vitamin D_3, obtained from pre-vitamin D_3 in the skin or by intestinal absorption of dietary components, binds to vitamin D-binding protein (DBP) in the circulation and is transported to the liver. Here, vitamin D_3 is hydroxylated by liver 25-hydroxylase (CYP2R1). The resulting 25(OH)D_3 is then hydroxylated in the kidney by 1-α-hydroxylase (CYP27B1), generating the active hormone 1,25$(OH)_2D_3$.

After synthesis, vitamin D and its metabolites are bound to a carrier molecule, known as the vitamin D binding protein (DBP), for systemic transport [13].

Regardless of the source of vitamin D, it needs to be hydroxylated twice in order to become biologically active [14] (Figure 1). Vitamin D is first hydroxylated in the liver at the carbon 25-position by 25-hydroxylase. Several cytochrome P450 (CYP) isoforms have been proposed to accomplish this hydroxylation step (including the mitochondrial CYP27A1 and the microsomal CYP2R1, CYP3A4, and CYP2J3), but CYP2R1 is suspected to be the high-affinity 25-hydroxylase [15]. Little is known about the regulation of these 25-hydroxylases, but since $25(OH)D_3$ generally reflects vitamin D nutritional status, this enzyme is believed to be poorly regulated. As $25(OH)D_3$ is the major circulating form of vitamin D, having a half-life of approximately 2 weeks, this metabolite is considered as the primary indicator of vitamin D status. In healthy humans, $25(OH)D_3$ is present in serum at concentrations in the range of 30–50 ng/mL. Individuals with $25(OH)D_3$ serum levels <30 ng/mL are believed to suffer from vitamin D insufficiency, while levels <15 ng/mL are proposed to define vitamin D deficiency. Severe vitamin D deficiency, with $25(OH)D_3$ concentrations <5 ng/mL, is frequently accompanied by the presence of osteomalacia and rickets [16]. Importantly, as a fat-soluble vitamin, $25(OH)D_3$ can also be stored in muscles and adipose tissue. The second hydroxylation, mainly occurring in the kidney and catalyzed by 1-α-hydroxylase (CYP27B1), generates the bioactive metabolite 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) or calcitriol (reviewed in [14]). CYP27B1-activity is positively regulated by calcium, phosphate, and their regulating hormones (calcium, parathyroid hormone (PTH), calcitonin, growth hormone (GH), and insulin-like growth factor (IGF)), while negative regulators of this enzyme include phosphate, fibroblast growth factor (FGF)-23, klotho, and $1,25(OH)_2D_3$ itself (reviewed in [14]). For instance, low serum calcium levels elicit PTH-release by the parathyroid glands, which stimulates renal CYP27B1-activity and thus $1,25(OH)_2D_3$-production. Mechanisms by which $1,25(OH)_2D_3$ corrects serum calcium levels include a reduction of renal calcium excretion, increased intestinal calcium absorption, and stimulation of osteoclast maturation to release calcium from the bones. When normal calcium levels are obtained, PTH release – and therewith the CYP27B1 activity – is switched off. Importantly, $1,25(OH)_2D_3$ limits its own activity by inducing 24-hydroxylase (CYP24) [17], which executes the first step of vitamin D catabolism and thus prevents excessive vitamin D signaling.

Mechanism of action

$1,25(OH)_2D_3$ exerts its actions by binding to the VDR, a member of the nuclear receptor superfamily (reviewed in [14]). Similarly to other members of this receptor family, VDR possesses two discrete domains, the N-terminal DNA-binding domains (DBD) and the C-terminal ligand-binding domain (LDB). High-affinity binding of $1,25(OH)_2D_3$ to the LBD induces heterodimerization of VDR with the retinoid X receptor (RXR). Subsequently, this VDR-RXR heterodimer binds to specific DNA sequence elements, so-called vitamin D responsive elements (VDREs) identified as direct repeats of PuG(G/T)TCA motifs separated by 3 bp (DR3), in the promoter-region of vitamin D responsive genes. Depending on the target gene, either co-activators or co-repressors are attracted to the complex to induce or repress RNA polymerase II-mediated gene transcription.

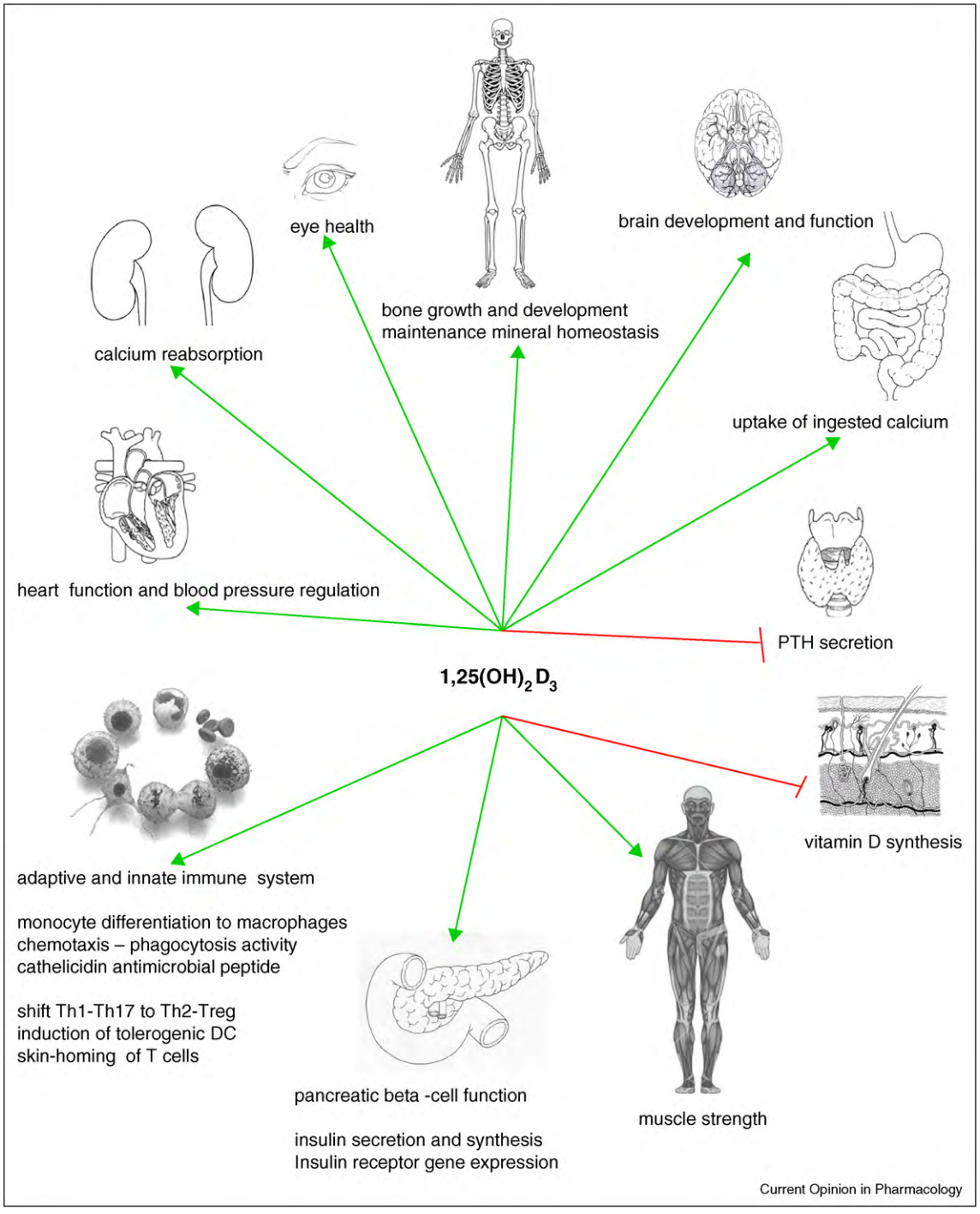
Of interest, VDRs are not only present in different tissues (Figure 2), such as bone, skin, intestine, and kidneys but also non-classical organs like brain, eyes, heart, pancreatic islets (β-cells), immune cells, muscle, adipose tissue, thyroid, parathyroid, and adrenal glands (reviewed in [1]). Importantly, many of these non-classical tissues also express vitamin D-activating enzymes, hence, allowing these non-classical actions to occur via local activation of vitamin D.

In fact, several of these vitamin D target organs are implicated in the development of diabetes (e.g. β-cells, immune cells, skeletal muscle, and adipose tissue) and its secondary complications (e.g. kidney, eyes, and heart), pointing towards therapeutic strategies for vitamin D in the prevention/intervention of type 1 diabetes (T1D) and, possibly type 2 diabetes (T2D), as well as their related health concerns.

Immune cells as targets for active vitamin D

The awareness of a role for vitamin D in the regulation of immune responses was triggered by the discovery of VDRs in almost all immune cells, including activated $CD4^+$ and $CD8^+$ T cells, B cells, neutrophils, and antigen-presenting cells (APCs), such as macrophages and dendritic cells (DCs) [18,19]. Importantly, VDR expression in some immune cells is controlled by immune signals. Whereas naïve T cells only display very low VDR levels, this receptor is abundantly present upon T cell activation [18,20]. By contrast, differentiation of monocytes, either into macrophages or DCs is accompanied by a decrease in VDR-expression, making these cells less sensitive to $1,25(OH)_2D_3$ when they mature [21,22]. Together, the high abundance of receptors for active vitamin D throughout the immune system and their regulation by immune signals argues for an important role for this hormone as a modulator of immune responses. With this finding, a new research area was founded, aiming to elucidate the immunomodulatory actions of $1,25(OH)_2D_3$, which has led to the appreciation of a plethora of effects, affecting different cellular players within the immune system (Figure 3).

Figure 2

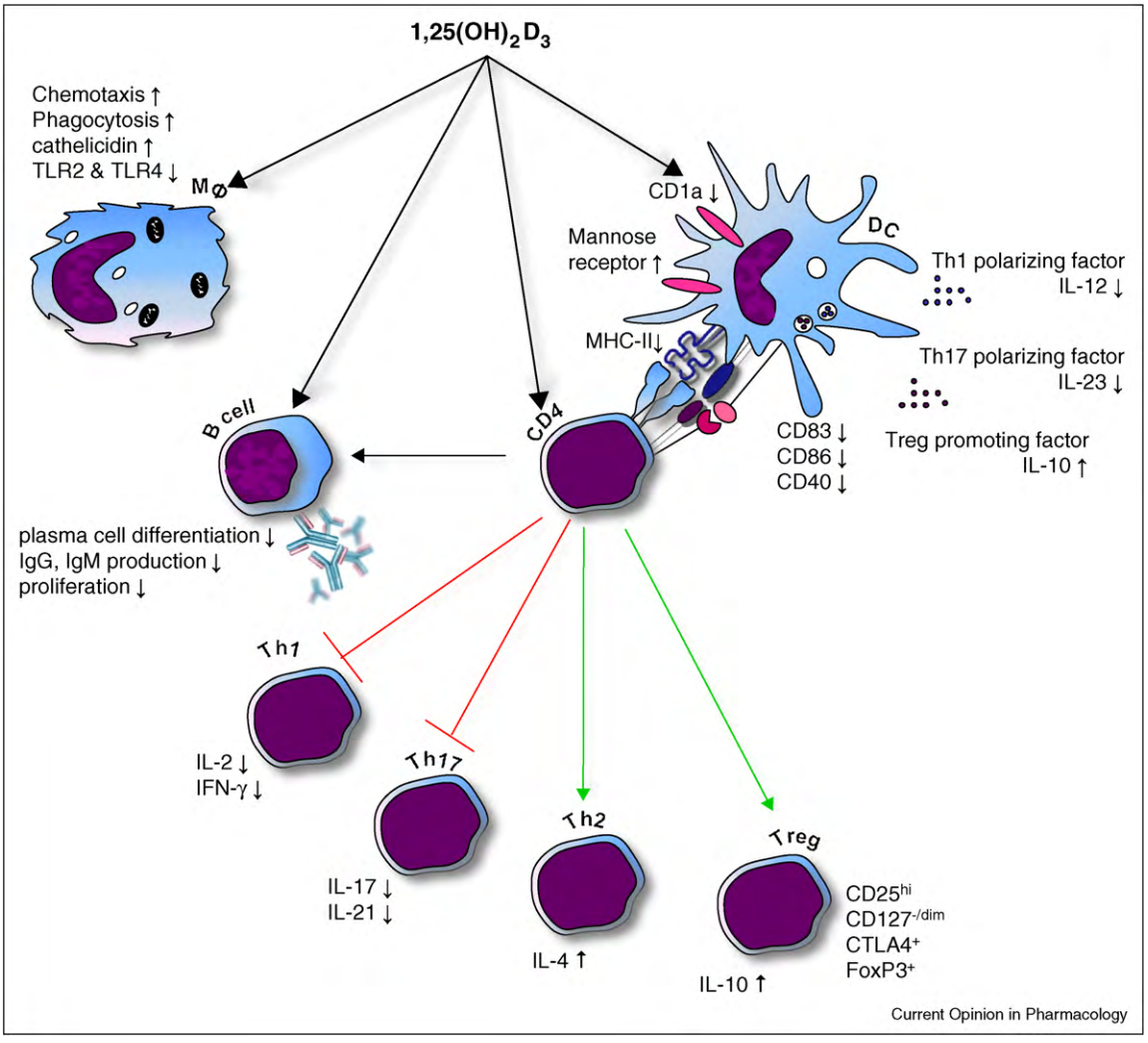


Major targets and actions of active vitamin D_3 on peripheral tissues.

Modulation of innate immunity

Monocytes and macrophages are crucial members of the innate immune compartment, which exhibit a great ability to sense pathogen-associated molecular patterns (PAMPs) of various infectious agents by means of pattern-recognition receptors, such as Toll-like receptors (TLRs), and thus provide a first-line defense against dangerous microbial invaders. In this context, $1,25(OH)_2D_3$ has been recognized as an important mediator of innate immune responses, enhancing the antimicrobial properties of immune cells such as monocytes and macrophages.

Figure 3



The immunomodulatory effects of $1,25(OH)_2D_3$.
$1,25(OH)_2D_3$ targets different players of the innate and adaptive immune compartment. $1,25(OH)_2D_3$ stimulates innate immune responses by enhancing the chemotactic and phagocytotic responses of macrophages as well as the production of antimicrobial proteins such as cathelicidin. On the contrary, $1,25(OH)_2D_3$ also modulates adaptive immunity. At the level of the APC (like DCs), $1,25(OH)_2D_3$ inhibits the surface expression of MHC-II-complexed antigen and of co-stimulatory molecules, in addition to production of the cytokines IL-12 and IL-23, thereby indirectly shifting the polarization of T cells from a Th1 and Th17 phenotype towards a Th2 phenotype. In addition, $1,25(OH)_2D_3$ directly affects T cell responses, by inhibiting the production of Th1 cytokines (IL-2 and IFN-γ), Th17 cytokines (IL-17 and IL-21), and by stimulating Th2 cytokine production (IL-4). Moreover, $1,25(OH)_2D_3$ favors Treg cell development via modulation of DCs and by directly targeting T cells. Finally, $1,25(OH)_2D_3$ blocks plasma-cell differentiation, IgG and IgM production and B-cell proliferation.

Already since the mid-19th century $1,25(OH)_2D_3$ is known to induce anti-mycobacterial activity in human monocytes and macrophages and various mechanisms are likely to underlie these actions (reviewed in [23]). For instance, $1,25(OH)_2D_3$ was demonstrated to exert pro-differentiating effects on monocytes and monocyte-derived cell lines, as these cells acquire phenotypical features of macrophages when exposed to the hormone [24]. In addition, $1,25(OH)_2D_3$ enhances the chemotactic and phagocytic capacity of macrophages [25]. More recently, the antimicrobial actions of $1,25(OH)_2D_3$ were demonstrated to be mediated via the VDR and associated with the upregulation of cathelicidin hCAP-18 gene. Addition of the active peptide, which is cleaved from cathelicidin, could specifically counteract mycobacterial growth in culture [26]. Importantly, TLR activation of monocytes and macrophages (but not DCs) results in upregulation of VDR and other VDR-target genes and leads to the induction of cathelicidin antimicrobial peptide (CAMP) and killing of *Mycobacterium tuberculosis* [27]. The link between vitamin D-triggered antimicrobial activity in monocytes/macrophages and cathilicidin has been confirmed using siRNA inhibition of $1,25(OH)_2D_3$-induced CAMP protein production, which resulted in

increased mycobacterial growth [28••]. Besides CAMP, also the gene encoding the antimicrobial peptide, defensin β2, was identified as direct a target for $1,25(OH)_2D_3$ [29]. Exposure to $1,25(OH)_2D_3$ results in a strong induction of these peptides, directly leading to enhanced antimicrobial activity in various cell types, including myeloid cells, keratinocytes, neutrophils, and bronchial epithelial cells [29–31]. Just now, it has been demonstrated that $1,25(OH)_2D_3$ is a direct and robust inducer of expression of the gene encoding NOD2/CARD15/IBD1 in cells of monocytic and epithelial origin [32]. This pattern recognition receptor detects muramyl dipeptide (MDP), a lysosomal breakdown product of bacterial peptidoglycan common to Gram-negative and Gram-positive bacteria. MDP-induced NOD2 activation stimulates the transcription factor NF-κB, which induces expression of the defensin β2 gene [32]. Other signaling pathways have also been proposed to participate in the anti-mycobacterial activities of $1,25(OH)_2D_3$. For example, phosphatidylinositol 3-kinase was found to regulate the anti-mycobacterial activity of $1,25(OH)_2D_3$ by enhancing the generation of reactive oxygen species (ROS) in monocytes and macrophages [33]. Also regulation of inducible nitric oxide synthase (iNOS) potentially contributes to the antimicrobial effects of $1,25(OH)_2D_3$, but conflicting data have been reported here, ranging from a $1,25(OH)_2D_3$-mediated induction of iNOS expression in a human macrophage-like cell line, while others documented inhibitory actions of $1,25(OH)_2D_3$ on this enzyme [34,35]. Importantly, Yuk *et al.* have recently demonstrated the ability of vitamin D to induce autophagy and to mediate colocalization of *Mycobacterium tuberculosis* and antimicrobial peptides within autophagolysosomes, facilitating the destruction of these bacteria [36••]. Strikingly, whereas $1,25(OH)_2D_3$ promotes the antimicrobial activities of myeloid cells, this hormone also inhibits TLR2 expression and TLR4 expression on monocytes, thus inducing a state of hyporesponsiveness to PAMPS. This effect, which is most prominent after 72 h, was suggested to take place as negative feedback mechanism, preventing excessive TLR activation and inflammation at a later stage of infection [37]. Interestingly, $1,25(OH)_2D_3$ was also reported to attenuate the *Mycobacterium tuberculosis*-induced expression of matrix metalloproteinases (MMP), such as MMP-7, MMP-9 and MMP-10 by peripheral blood mononuclear cells, while inducing secretion of IL-10 and PGE_2 [38,39]. These findings represent a novel immunomodulatory role for $1,25(OH)_2D_3$ in *Mycobacterium tuberculosis* infection.

Modulation of adaptive immunity

APCs: primary targets for $1,25(OH)_2D_3$

APCs represent an important component of the adaptive immune system. DCs, the professional APCs that capture, process, and present antigens to T cells, were recognized as central targets for $1,25(OH)_2D_3$ (reviewed in [40]). During DC-differentiation, these APCs down-regulate the monocytic marker CD14 while upregulating the DC marker CD1a. Addition of $1,25(OH)_2D_3$ completely inhibited the differentiation of $CD1a^+$ DCs, while sustaining the expression of monocytic markers [41,42]. Moreover, activation of VDR signaling pathways also inhibited DC-maturation as evidenced by decreased levels of DC markers, MHC-class II, co-stimulatory molecules (CD40, CD80, and CD86), and other maturation-induced surface markers (e.g. CD83) [41,42,43,44,45•]. Interestingly, $1,25(OH)_2D_3$ may play an important role in DC binding and capturing foreign antigens at the initiation of immune response, since this hormone upregulated mannose receptor expression, a molecule involved in antigen uptake, and this correlated with an enhanced endocytotic capacity [46]. Furthermore, $1,25(OH)_2D_3$ also modulates DC-derived cytokine and chemokine expression, by inhibiting the production of IL-12 and IL-23 (known as major cytokines driving Th1-differentiation and Th17-differentiation respectively), and enhancing the release of IL-10 (a cytokine exerting broad-spectrum anti-inflammatory activities) and the chemokine MIP-3α (also known as CCL22, a chemokine involved in the recruitment of CCR4-expressing regulatory T cells (Tregs)) [41,43–45,47–50].

Similarly to DCs, the antigen-presenting and T cell stimulatory capacities of monocytes/macrophages are reduced upon exposure to $1,25(OH)_2D_3$, as demonstrated by the reduced surface expression of MHC-II and co-stimulatory molecules, such as CD40, CD80, and CD86 [25,51]. Furthermore $1,25(OH)_2D_3$ inhibits the expression of inflammatory cytokines in monocytes, including IL-1, IL-6, TNF-α, IL-8, and IL-12 [51–53].

Interestingly, our group observed significant differences in the protein profiles of DCs being exposed to a VDR-agonist, showing major alterations in three specific protein groups, including proteins involved in protein biosynthesis/proteolysis, metabolism, and cytoskeleton structure [54]. Such alterations in cytoskeleton proteins may not only contribute to the altered trafficking capacities of $1,25(OH)_2D_3$-modulated DCs towards inflammatory and lymph node homing chemokines [44], but may also affect the formation of DC-T cell contacts. Considering their position at the interface of innate and adaptive immunity, with antigen-presentation and T cell activation as their main functions, modulation of DCs by $1,25(OH)_2D_3$ indeed has a major impact on the outcome of T cell responses. $1,25(OH)_2D_3$-modulated DCs have a reduced capacity to trigger T cell proliferation [41,55]. Moreover, $1,25(OH)_2D_3$-mediated modulation of DC-derived cytokines alters the Th-balance, by limiting inflammatory Th1 and Th17-responses, while skewing the T cell response towards a Th2-phenotype [41,43,56]. Importantly, the reduced expression of co-stimulatory molecules and the ability of DCs to produce IL-10

are recognized as tolerogenic features, enabling $1,25(OH)_2D_3$-modulated DCs to favor the development of Tregs with suppressive capacity. Indeed, the ability of VDR-agonists to enhance Treg-induction *in vitro* was observed by different groups [47,57]. In addition, VDR-agonists were also shown to enhance the suppressive capacity of Tregs [58]. Importantly, whereas immature DCs are considered to be tolerogenic, a microarray analysis by Szeles *et al.* revealed that the $1,25(OH)_2D_3$-mediated induction of DCs with Treg-inducing capacities results from the autonomous regulation of a set of genes independently from its effects on DC-differentiation and maturation [59]. Taken together, $1,25(OH)_2D_3$-mediated modulation of DCs clearly has a major impact on adaptive immune responses, as demonstrated by its ability to regulate T cell responses.

Lymphocytes as direct targets for $1,25(OH)_2D_3$

Since VDR expression in T cells is dramatically increased upon T cell activation, direct actions on T cells are likely to represent an additional or even alternative route for $1,25(OH)_2D_3$ to shape T cell responses. We demonstrated that increased VDR expression can be elicited by various T cell activation stimuli, including anti-CD3/anti-CD28, providing the 2 signals necessary for full T cell activation. Elevated VDR levels were also seen by mimicking these signals with the lectin mitogen, PHA, or by triggering more downstream T cell signaling pathways with PMA/ionomycin [20]. Nevertheless, the levels and kinetics of VDR expression seem to vary between the different activation stimuli. These findings could provide an explanation for the conflicting results that have been reported regarding the effects of $1,25(OH)_2D_3$ on T cell proliferation [60,61,62,63•], since the type of activation stimulus was not consistent throughout all these investigations. Furthermore, $1,25(OH)_2D_3$ also directly alters the cytokine profiles of T cells, by inhibiting the production of inflammatory Th1-cytokines such as IL-2 and IFN-γ, as well as the Th17-derived cytokines IL-17 and IL-21[56,63•,64,65]. Till date, the direct effects of $1,25(OH)_2D_3$ on the emergence of Th2 cytokines are less clear: some studies show that $1,25(OH)_2D_3$ favors the emergence of Th2 cells by upregulating the expression of Th2-specific transcription factors GATA-3 and c-maf and concomitant cytokines, including IL-4, whereas others contradicted these findings [64–66]. Also at the level of Treg-induction by $1,25(OH)_2D_3$, the involvement of tolerogenic DCs does not seem to be a prerequisite, as it was shown that $1,25(OH)_2D_3$, either alone or in combination with dexamethasone could induce IL-10 producing Tregs in an APC-free *in vitro* system [63•,67]. In this respect, we found that a vitamin D analog triggered the emergence of a $CD4^+CD25^{high}CD127^{low}$ Treg phenotype and selectively induced IL-10 expression within the $CD4^+$ T cell subset (Baeke *et al.*, unpublished). Interestingly, Tregs induced by $1,25(OH)_2D_3$ and dexamethasone expressed high levels of TLR9 and ligand-dependent activation of this receptor abrogated their suppressive capacity, possibly allowing this induced Treg function to be silenced when infectious agents have to be cleared [68].

Although modulation of Th responses will inevitably affect the B cell compartment, these cells are directly targeted by $1,25(OH)_2D_3$ as well. Exposing B cells to $1,25(OH)_2D_3$ inhibits their proliferation, plasma-cell differentiation and immunoglobulin secretion (IgG and IgM), memory B cell generation and induces B cell apoptosis [69]. Recently, $1,25(OH)_2D_3$ was put forward as an important regulator of lymphocyte trafficking. Active $1,25(OH)_2D_3$, imprints activated T cells and terminally differentiating B cells with skin-homing properties via induction of the skin-homing receptor CCR10 [70••,71]. By contrast, another study revealed that $1,25(OH)_2D_3$ inhibits T cell surface expression of cutaneous lymphocyte-associated antigen (CLA), another receptor directing T cells to the skin. In accordance, we observed that a vitamin D analog profoundly altered the migratory signature of human T cells, not only affecting skin-homing properties of these cells, but additionally inducing a homing receptor profile that would favor migration to inflammatory sites (Baeke *et al.*, unpublished).

Immune cells as local producers of vitamin D: physiological role for vitamin D in immune regulation

Local vitamin D metabolism within the immune system

Considering the short half-life time of bioactive $1,25(OH)_2D_3$ (4–6 h) and the supraphysiological concentrations of $1,25(OH)_2D_3$ required to modulate the behavior of immune cells, it is rather unlikely that its immunomodulatory actions would depend on systemic levels of the hormone. In this context, the discovery of expression of vitamin D metabolizing enzymes in various target cells of $1,25(OH)_2D_3$, comprising the majority of immune cells, caused a major breakthrough in understanding the non-classical actions of $1,25(OH)_2D_3$.

Various immune cells, including macrophages, DCs, and even B-lymphocytes and T-lymphocytes were found to express CYP27B1, while DCs also express CYP2R1 [21,69,70••,72,73]. The theory of local vitamin D metabolism within the immune system is further supported by the demonstration that all these cell types are capable to convert $25(OH)D_3$ into bioactive $1,25(OH)_2D_3$, allowing them to respond not only to the active vitamin D metabolite, but also to its precursors [21,27,69,70••]. Importantly, regulation of CYP27B1 in immune cells is remarkably different from the renal counterpart, since its expression is controlled by immune signals. For example, CYP27B1 expression by monocytes/macrophages is strongly upregulated by IFN-γ, the TLR4-ligand LPS, ligands triggering the TLR2/1-complex such as the 19 kDa lipoprotein of *Mycobacterium tuberculosis*,

and viral infections [27,73–75]. In DCs, CYP27B1 levels increase during maturation of these cells [21], while in T-lymphocytes and B-lymphocytes, expression of this enzyme is dramatically enhanced upon activation of the cells [62,69,76]. In further contrast with renal CYP27B1, expression of this enzyme in macrophages and DCs is not suppressed by $1,25(OH)_2D_3$ itself [21,73], offering an explanation for the massive local production of $1,25(OH)_2D_3$ by disease-associated macrophages in patients with granulomatous diseases. However, the ability of $1,25(OH)_2D_3$ to trigger CYP24 in immune cells is likely to serve as a negative feedback loop. Importantly, the susceptibility of monocytes and macrophages to this $1,25(OH)_2D_3$-mediated induction of CYP24 depends on the differentiation/maturation stage of the cells, as undifferentiated monocytes are highly sensitive to $1,25(OH)_2D_3$-mediated CYP24 induction, whereas differentiated/activated macrophages are not [22].

Together, local processing of vitamin D precursors into the active ligand represents an important mechanism by which immune cells can reach the supraphysiological levels of $1,25(OH)_2D_3$ needed to shape immune responses, without affecting systemic levels of this hormone. Therefore, the presence of VDRs and vitamin D metabolizing enzymes and their regulation by immune signals provide strong evidence for an autocrine and/or paracrine role for $1,25(OH)_2D_3$ in normal immune physiology.

Impaired vitamin D signaling: implications for the immune system

There are relates dated already from ancient Greece describing sunlight's curative properties. Hippocrates himself most certainly used sunlight for the treatment of diseases. Much later, modern medicine reacquired interest in the healing properties of sunlight; it is described that Finsen in 1893 could cure systemic lupus erythematosus (SLE) by using direct light after filtering out the heat rays. Moreover, in 1903 Rollier started a sunlight treatment of surgical tuberculosis in Switzerland. With the discovery of extrarenal activation pathways of vitamin D, such as the local $1,25(OH)_2D_3$ production in the immune system and the appreciation of the wide array of non-classical effects of this hormone, the mechanisms underlying the beneficial properties of sunlight are largely explained. Importantly, these findings clearly emphasize the necessity of adequate vitamin D levels to maintain bone health, but also to ensure optimal immune function. With this knowledge, the increasing incidence of insufficient vitamin D levels in many populations across the globe is obviously a worrying trend.

Multiple groups have reported a correlation between vitamin D deficiency and susceptibility to respiratory infections, especially in the context of infection by *Mycobacterium tuberculosis* and Gram-negative bacteria (reviewed in [77]). For example, a higher susceptibility to tuberculosis is seen in subjects with relatively low serum vitamin D levels, including elderly, uremic patients, and dark-skinned people [78]. An explanation for the observed association between vitamin D status and infection rates was – at least partly – provided by Liu *et al.*, recognizing that $1,25(OH)_2D_3$-mediated induction of CAMP represents an integral component of human TLR-mediated immune responses [27]. More specifically, TLR2/1-triggering of human monocytes resulted in a selective induction of VDR and CYP27B1 expression, increasing the ability of these cells to convert $25(OH)D_3$ into active $1,25(OH)_2D_3$, and thus to produce CAMP [27]. Remarkably, induction of CAMP is not the only mechanism linking vitamin D signaling to TLR-mediated antimicrobial responses: TLR-induced expression of another antimicrobial peptide, defensin β4, by monocytes requires the convergence of both VDR-activation pathways and IL1β-activation pathways [79].

In addition to its effects on innate immune responses (as described above), a growing amount of data strongly supports the proposed role of vitamin D as a regulator of adaptive immune responses. Different epidemiological studies report an inverse correlation between vitamin D status and the incidence of autoimmune diseases, such as T1D, SLE, multiple sclerosis (MS), inflammatory bowel disease (IBD), and rheumatoid arthritis (RA) [80–84]. For example, a considerable percentage of the population living in more northern areas of the northern hemisphere (and thus receiving less UV radiation) is vitamin D deficient and this deficiency positively correlated with higher incidences of autoimmune diseases. Also, similar to the serum levels of $25(OH)D_3$, the onset and exacerbations of different autoimmune diseases have been documented to vary with seasonality. Furthermore, patients suffering from different autoimmune diseases such as MS, SLE, RA, and T1D display lower serum $25(OH)D_3$ levels in comparison to healthy individuals. In the context of T1D, a Finnish birth cohort study revealed a three-fold increased disease incidence in individuals that were vitamin D deficient during early life [85••].

The proposed relation between circulating vitamin D levels and immune function is further confirmed by various experimental studies, investigating the consequences of impaired vitamin D signaling on immune function. Disease progression following infection with *Mycobacterium tuberculosis* was severely aggravated when mice were rendered vitamin D deficient [86]. Importantly, a lack of vitamin D in mice was found to result in impaired macrophage function and proinflammatory cytokine production, being essential for their antimicrobial activity [87].

Also in animal models of various autoimmune diseases, vitamin D deficiency profoundly affects disease incidence and severity (reviewed in [80]). For example, in non-obese diabetic (NOD) mice (a mouse model spontaneously developing T1D with a pathogenesis similar to human disease), vitamin D deficiency during early life resulted in more aggressive disease manifestation and a higher incidence [87]. At the cellular level, vitamin D deficient NOD mice displayed decreased numbers of $CD8^+$ T cells in the thymus, but increased numbers of immature $CD4^+CD8^+$ T cells, possibly pointing towards a T cell maturation defect. In addition, compared to their vitamin D sufficient counterparts, reduced numbers of $CD4^+CD62L^+$ Treg cells were detected, both in the thymus and in the periphery of these mice, suggesting a defect in the maintenance of tolerance [87].

In contrast to the effects of vitamin D deficiency, abrogation of VDR in mice has yielded conflicting results in different autoimmune disease settings. In models of IBD, absence of the VDR results in an exacerbation of the disease [88–90], whereas $VDR^{-/-}$ mice on a high calcium diet are less susceptible to develop experimental autoimmune encephalomyelitis (EAE) [91]. In the case of T1D, a study by Zeits *et al.* showed that $VDR^{-/-}$ mice presented higher blood glucose levels and lower levels of circulating insulin [92], whereas VDR abrogation in diabetes-prone NOD mice did not reveal major alterations in glucose tolerance or diabetes incidence [93,94]. Nevertheless, $VDR^{-/-}$ NOD mice displayed a defect in the number of $CD4^+CD25^+$ Tregs, in addition to decreased numbers of $TCR\alpha/\beta^+CD4^-CD8^-$ NKT cells. Also, the thymus and lymph nodes of these mice contained less mature DCs, possibly contributing to an impaired elimination of diabetogenic T cells [94]. The observed discrepancies might be partially explained by differences in the genetic background of the mice. Another explanation could be the involvement of possible compensatory mechanisms when VDR is completely abrogated, which further highlights the complexicity of vitamin D signaling.

Therapeutic perspectives

Vitamin D supplementation

Local vitamin D metabolism allows immune cells to modulate immune responses autonomously when regulation is required, but optimal functioning of this autocrine and/or paracrine circuit crucially depends on the availability of circulating $25(OH)D_3$. The exact levels of circulating $25(OH)D_3$ needed to meet the requirements of vitamin D sufficiency are still a matter of debate, especially in the light of the non-classical effects of vitamin D. Nevertheless, it is generally accepted that vitamin D insufficiency or even deficiency are highly prevalent in many populations across the globe. Therefore, vitamin D supplementation represents an attractive strategy to ensure sufficient $25(OH)D_3$ levels for adequate immune function, thereby eliminating one of the proposed risk factors that may underlie disorders such as chronic infections and autoimmunity.

A recent randomized control trial concluded that administration of vitamin D supplements is associated with decrease in overall mortality rates [95•]. With specific regard to the immune conditions, the use of vitamin D supplements was found to improve tuberculosis outcome. For example, vitamin D supplementation in Indonesian pulmonary tuberculosis patients has reported more rapid sputum clearance of acid-fast bacilli and radiological improvement [96]. By contrast, a more recent study reported that vitamin D supplementation did not improve the clinical outcome among tuberculosis-patients [97], although it is possible that the dose used was not sufficient (100,000 IU of vitamin D or placebo at inclusion and again 5 and 8 months after the start of treatment). Recently, Yamshchikov *et al.* published a review on the different vitamin D trials in treatment or prevention of tuberculosis, as well as influenza, and viral upper respiratory tract illnesses [98]. Although there was substantial heterogeneity in baseline patient demographics, sample size, and vitamin D strategies, the strongest evidence supported a role for vitamin D in the medical care of patients, especially those with vitamin D deficiency, in infectious disease settings.

Supplementation studies have also been conducted in the context of autoimmune diseases. With regard to T1D, distinct studies have found that supplementation with regular vitamin D in early life is associated with a lower risk of disease onset. In 1999, the results of a large-scale study sponsored by the European Community were published: the Concerted Action on the Epidemiology and Prevention of Diabetes showed a 33% reduction of T1D in children who received vitamin D supplementation early in life [99]. In accordance with these results, Hypponen *et al.* also found that the risk of T1D development was significantly reduced when high doses of vitamin D supplementation (up to 2000 IU/d) were given during infancy [85••]. Furthermore, a meta-analysis of data from 4 case–control studies and one cohort-study support the beneficial effects of vitamin D in T1D prevention, since infants receiving vitamin D supplementation showed a 29% reduction of disease onset [100]. Overall, these studies suggest that vitamin D-mediated diabetes protection may be dose-dependent, with individuals receiving higher amounts of vitamin D having a lower risk of developing T1D. On the contrary, some studies did not find a correlation between T1D prevention and vitamin D supplementation. In Norway, intake of cod-liver oil by children <1 year did not result in significant effects on T1D prevention, though there was a tendency for a negative association between cod-liver oil intake and diabetes development [101]. More recently a study in Sweden with 1–2.5 year-old children, who

received vitamin D supplementation, could not find a correlation between supplementation and development of diabetes-related auto-antibodies [102]. However, despite the fact that some studies failed to show an association between the reduction T1D risk and vitamin D supplementation during infancy, none of them were associated with an increased risk.

Although promising results were obtained in a few clinical trials, there is still a lack of non-biased large-cohort studies that can sustain the proposed benefits of vitamin D supplementation for optimal immune function. Small sample sizes, short follow-up duration and lack of control groups constitute major limitations of the reported studies. In addition, different doses of vitamin D have been applied, and the initial vitamin D status of the individuals included was not always known, making it unclear whether the administered vitamin D supplements restored existing deficiencies or augmented circulating vitamin D in already sufficient individuals.

In mice, administration of 1000 IU of regular vitamin D_3 (intraperitoneally) in early life, to vitamin D sufficient NOD mice did not prevent diabetes development, though pancreatic insulin content was higher in treated mice compared to controls [103]. Possibly, higher doses, different route of administration or a longer time-frame of supplementation with regular vitamin D may be required to observe prevention. However, prolonged intake of high doses of vitamin D can be toxic and shows symptoms such as nausea/vomiting, fatigue, anorexia, polyuria/polydipsia, nocturia, and even chronic kidney failure [104].

Active vitamin D therapy

In spite of displaying optimal circulating vitamin D levels, the ability to metabolize vitamin D may vary between individuals and may therefore even contribute to the risk of an individual to develop immune abnormalities, as suggested in the case of certain gene polymorphisms in the vitamin D metabolizing enzymes. For example, mutations in the CYP2R1 gene, as well as in the CYP27B1 gene, impairing their enzymatic activity have been described and polymorphisms in these genes have been proposed to be associated with T1D susceptibility [105,106•]. Therefore, instead of relying on the ability of the immune system to process vitamin D autonomously, immune cells could also directly be targeted and modulated by applying active vitamin D therapy. Many studies yielded beneficial results using active vitamin D to prevent and/or intervene in different autoimmune disease models, including T1D, SLE, EAE, collagen-induced arthritis, IBD, and autoimmune prostatitis (reviewed in [107•]). Unfortunately, clinical application of $1,25(OH)_2D_3$ is obstructed by toxicity issues, since the supraphysiological doses needed to modulate immune responses elicit concomitant calcemic side effects. To overcome this limitation, structural analogs of $1,25(OH)_2D_3$ are being designed that have reduced calcemic effects with similar immunoregulatory activity.

Use of active VDR-agonists in counteracting T1D

Focusing primarily on T1D models, administration of $1,25(OH)_2D_3$ or its analogs has been documented to inhibit insulitis and disease or delay the onset of T1D in NOD mice [108–112]. Some possible mechanisms, explaining the observed disease reduction, have been proposed, suggesting that $1,25(OH)_2D_3$ and analogs act both at a central and peripheral level. $1,25(OH)_2D_3$ was found to affect thymic differentiation of DCs and T cells. For instance, administration of $1,25(OH)_2D_3$ to pre-diabetic NOD mice (known to have defective thymic selection and increased numbers of apoptosis-resistant effector T cells) increased the deletion of T-lymphocytes in the thymus and reduced the number of apoptosis-resistant T cells [113]. The same study demonstrated that this T cell deletion was DC dependent and that $1,25(OH)_2D_3$ induced thymic DC differentiation, modulating DCs into a more pronounced lymphoid phenotype and upregulating CD86 (a co-stimulatory molecule of DCs). In addition, $1,25(OH)_2D_3$- or analog treatment is proposed to increase the number of Tregs, which are likely to suppress effector T cells and to halt β-cell destruction. Indeed, work by Gregori *et al.* showed that administration of a $1,25(OH)_2D_3$ analog resulted in decreased Th1 cell infiltration in the pancreas and increased $CD4^+CD25^+$ Tregs in the pancreatic lymph nodes of treated mice [111]. Furthermore, $1,25(OH)_2D_3$-treated mice displayed a Th1-Th2 shift in the pancreas and pancreas-draining lymph nodes [114]. Importantly, as pancreatic β-cells also express VDRs, other β-cell dependent effects further contribute to the improved disease outcome, including a $1,25(OH)_2D_3$-mediated inhibition of chemokine expression by islet cells, which appears to block T cell infiltration and to arrest insulitis [115], as well as an enhanced insulin synthesis and secretion as observed in islets isolated from healthy rats [116].

Importantly, since T1D only becomes clinically overt after destruction of the majority of β-cells, the ability of $1,25(OH)_2D_3$ and analogs to intervene at a later stage and to revert ongoing autoimmunity has been investigated as well. Here, structural analogs of vitamin D could successfully block progression of insulitis in pre-diabetic NOD mice, along with preventing recurrence of autoimmune diabetes in NOD mice after syngeneic islet transplantation, when combined with other immunomodulating agents [111,117–119]. Importantly, islet or β-cell transplantation is an attractive strategy to achieve exogenous insulin independence, especially when the remaining β-cell mass is insufficient to supply the body with appropriate insulin concentrations. However, even if the autoimmune process can be halted, most grafts are derived

from allogeneic donors, therefore requiring long-term immune suppression. The use of immunosuppressive drugs on a long-term basis, however may pose a bigger threat to the patient than the detrimental effects of continuous insulin therapy. In order for islet transplantation to become a widespread procedure, immunosuppressive regimens need to be improved to obtain long-term graft survival. Work by Adorini and co-workers showed that $1,25(OH)_2D_3$ together with mycophenolate mofetil (an immune suppressant agent) was able to reduce graft rejection in an allogeneic islet transplantation model, possibly by induction of Treg cells [120]. Furthermore, our group demonstrated that a combination of the KH1060 analog with cyclosporine could prevent early graft failure and delay graft rejection of xenogeneic islets transplanted in diabetic NOD mice [121].

In humans, several clinical trials have successfully used active vitamin D and analogs for the treatment of psoriasis, a chronic skin disorder suspected to have an autoimmune origin [122,123]. A few clinical trials have also demonstrated positive effects of $1,25(OH)_2D_3$ in the treatment of autoimmune diseases, such as MS and RA [124,125]. For instance, a small pilot study in patients with relapsing-remitting MS showed that the exacerbation rate of treated patients was lower than baseline (23%), but patients presented mild-side effects [124]. By contrast, intervention studies with active vitamin D in T1D did not yield any satisfactory results yet. A trial in which 70 newly-onset diabetic children (mean age 13.6 years) were given a small dose (0.25 μg) of $1,25(OH)_2D$ or nicotinamide, showed that insulin requirements decreased in the first months of treatment in the group receiving $1,25(OH)_2D$; however the treatment did not have a long-lasting effect and there was also no improvement of C-peptide levels [126]. Recently, in a pilot study, patients with latent autoimmune diabetes in adults (LADA—a subtype of T1D in which the clinical manifestation begins and progresses slowly in adulthood) who were given a vitamin D analog in addition to insulin treatment sustained better β-cell function compared to patients treated with insulin alone [127].

Use of active VDR-agonists in counteracting tuberculosis

Patients with pulmonary tuberculosis have increased $1,25(OH)_2D_3$ levels, especially within the first 2 weeks following infection, which is postulated to lead to a downregulation of VDR levels. [128]. This latter could lead to impaired VDR-mediated immunity against *Mycobacterium tuberculosis*. Defective VDR signaling might also result in increased inflammation due to the increased expression of inflammatory cytokines. Therefore, addition of $1,25(OH)_2D_3$ might lead to increased expression of CAMP that could enhance the immunity against tuberculosis. Just now, Eisenhut commented on the negative clinical outcome of a trial with supplements of $25(OH)D_3$ in patients with tuberculosis by mentioning that it might be extremely important to give $1,25(OH)_2D_3$ and not $25(OH)D_3$ to achieve an effect on immunity against tuberculosis [129]. Moreover he commented that dose regimes need to avoid high doses leading to immune suppression associated with plasma levels greater than 10 nmol/L to optimize its effect against *Mycobacterium tuberculosis* infection.

Together, therapy with hypocalcemic vitamin D analogs offers an appealing strategy to prevent autoimmunity, especially in individuals at risk, or even to intervene in ongoing autoimmunity. Further safety studies on these non-calcemic analogs of $1,25(OH)_2D_3$ in therapeutic settings are required. However, as unwanted side effects are still not completely eliminated, the search for non-toxic vitamin D analogs continues. Alternatively, combination therapies with other immunomodulatory agents might provide a solution to circumvent the dose-related side effects of these agents.

Conclusions

With the discovery of VDRs and vitamin D-metabolizing enzymes in extra-renal tissues, our knowledge of the role of vitamin D has greatly evolved. Besides its well established function as central regulator of mineral and bone homeostasis, $1,25(OH)_2D_3$, the active vitamin D metabolite, has been rediscovered as a modulator of growth, differentiation status and function of a variety of cells, including cells of the immune system. At the cellular level, $1,25(OH)_2D_3$ has been demonstrated to exert a plethora of effects, thereby targeting both the innate and adaptive immune compartment. Importantly, immune cells are not only targets for active vitamin D, but are able to activate this hormone in a local fashion, arguing for an autocrine or paracrine role for this hormone within the immune system. From this perspective, the increasing amount of data linking inadequate vitamin D levels to immune anomalies, such as increased infection rates and autoimmunity, is of great concern. On the basis of their widespread immunomodulatory actions, VDR-agonists, and especially hypocalcemic vitamin D analogs, are plausible candidates for the prevention and/or treatment of infections such as tuberculosis and several autoimmune disorders. Alternatively, as optimal functioning of the vitamin D autocrine and/or paracrine circuit crucially depends on adequate vitamin D levels, administration of vitamin D supplements could offer an appealing strategy to counteract the worldwide phenomenon of hypovitaminosis, thereby reducing the risk to develop infections or autoimmune diseases.

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