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## The Molecular and Clinical Evidence of Vitamin D Signaling as a Modulator of the Immune System: Role in Behçet's Disease

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### Running title: Immunoregulatory role of vitamin D in Behçet's disease

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### Abstract

Various tissues and cell types are the targets of vitamin D. However, the major targets of vitamin D in the immune system are monocytes/macrophages, dendritic cells (DCs), as well as B and T cells. Vitamin D plays an important role in the immune system modulation via regulating the expression of genes that generate pro-inflammatory mediators and inhibiting the proliferation of pro-inflammatory cells, both of which have been implicated in the pathophysiology of the inflammatory diseases. Recent studies have revealed the important relations between vitamin D and Behçet's disease (BD). Vitamin D function and its deficiency have been linked to a wide range of metabolic disorders including malignant, cardiovascular, infectious, neuromuscular, and autoimmune diseases. Here, we provide a brief analysis of the recent literature regarding immune-regulatory effects as well as clinical evidence of vitamin D influence on the molecular level in BD.

**Keywords:** Vitamin D, immune system, Behçet's disease, autoimmune diseases

### Introduction

BD is a chronic relapsing multi-system inflammatory disease characterized by recurrent oral aphthous ulcers, genital ulcers, skin lesions, and uveitis. There are many other organs that are

affected including vascular system, central nervous system, musculoskeletal system, epididymis, kidney, and gastrointestinal tract (1). BD often occurs between the ages of 20 and 40 (2, 3). It was most commonly observed along the ancient Silk Road, especially in the Far East and Middle East, and is the leading cause of disability in these countries. Turkey and Iran have the highest prevalence of the disease (2, 4, 5). The etiology and pathogenesis of BD are unknown. However, environmental factors—especially microbial agents (6-10), genetic (11-15) and epigenetic factors (16) role are introduced.

Vitamin D is a fat-soluble vitamin that is essential for health. Less than 10% of vitamin D is ingested through food (17); the rest is produced in the deep epidermis layer of the skin under the effect of sunlight(18). The genes involved in the regulation and synthesis of vitamin D include 25-hydroxylase (*CYP2R1*), 1 $\alpha$ -hydroxylase (*CYP27B1*), 24-hydroxylase (*CYP24A1*), and *DHCR7* (19, 20).

There are two physiologically relevant forms of vitamin D—25-hydroxyvitamin D3 [25(OH) D3] and 1 $\alpha$ , 25 dihydroxyvitamin D3 [1 $\alpha$ , 25(OH) 2D3]. Of these two types, 25 (OH) D3 is the main circulating form of vitamin D and its concentration in blood is approximately 1000 times more than that of 1 $\alpha$ , 25 (OH) 2D3. Therefore, it is the most reliable parameter to define human vitamin D status (21). The other form of vitamin D; that is, 1 $\alpha$ , 25(OH) 2D3—the biologically active form of vitamin D—acts as a pleiotropic endocrine hormone (21).

The main physiological action of 1 $\alpha$ , 25(OH) 2D3 is calcium and phosphorus metabolism; thus, it controls bone formation. Other actions of 1 $\alpha$ , 25 (OH) 2D3 include the control of immune functions, cellular growth and differentiation. All genomic activities of 1 $\alpha$ , 25 (OH) 2D3 are mediated by vitamin D receptors (VDRs) (21, 22) – the DNA-binding transcription factors of the nuclear receptor superfamily, which mediate 1 $\alpha$ , 25(OH) 2D3 signaling (23). The hormonal form of vitamin D enters the target cells and binds to the VDR in the cytoplasm(24). When 1 $\alpha$ , 25(OH) 2D3 is bounded to the VDR complex, it induces conformational changes in the VDR, increasing the receptor's affinity to the retinoid x receptor (RXR), the main partner of VDR in the lipophilic molecules (21). The VDR-vitamin D complex forms heterodimers with RXR (1,25D–VDR–RXR) and translocates to the nucleus binding to the vitamin D response elements (VDREs) in the promoters of its target genes. It then recruits a few nuclear co-activator (CoA) and co- repressor proteins to regulate the transcription of 1 $\alpha$ , 25(OH) 2D3 target genes (23, 25) (Figure1).

The result of the active contact between VDR and its partner proteins (RXR) on their binding sites of DNA, called the vitamin D response elements (VDREs), modulates the transcription of the primary target genes. Together with secondary target genes, they enable the physiological functions of 1 $\alpha$ ,25(OH)2D3 and its receptor VDR (21).

The VDR gene is maximally expressed in metabolically active tissues such as kidneys, bones, and the gastrointestinal tract, but it is found in a small to moderate amount in almost all immune cells and other cells (21, 26). The widespread expression of VDR and *CYP27B1* genes by almost all body tissues and cells provides a biological basis for the effects of vitamin D (18). The bond between 1 $\alpha$ , 25 (OH)2D and the intracellular VDR regulates over 900 genes involved in many physiological processes (27). Recent studies confirm the association between vitamin D receptor (VDR) polymorphisms and the increased incidences of autoimmune diseases (27).

Vitamin D function and its deficiency have been introduced in the etiology of autoimmune diseases (27, 28). It plays key roles in both innate and adaptive immune modulation and may confer a protective role in autoimmune diseases (29). The findings from recent studies show important relations between vitamin D and BD, suggesting that vitamin D may be a potential suppressor of the inflammatory response in BD (29). Vitamin D has recently started to be considered important for the maintenance of physiological homeostasis, and its deficiency has been linked with a wide range of chronic and inflammatory diseases, such as metabolic disorders, malignant, cardiovascular, infectious, neuromuscular, and autoimmune disorders. In this article, current data are summarized in order to give an overview of vitamin D's immunoregulatory role as well as the clinical evidence on its molecular level in BD.

### **Anti-inflammatory signaling of vitamin D in immune cells**

It has become evident that vitamin D inhibits monocyte/macrophage pro-inflammatory cytokine production like IL-6 or TNF- $\alpha$  in monocytes via the inhibition of p38 MAP kinase (30). Zhang et al., in a study identified that a dose of vitamin D stimulated the upregulation of MAPK phosphatase-1 (MKP1), which dephosphorylated p38 and inhibited phosphorylation and inactivation of p38 and cytokines and IL-6 and TNF- $\alpha$  production in LPS-stimulated human monocytes (30).  $1\alpha, 25(\text{OH})_2\text{D}_3$  induces MAPK phosphatase-1 (MKP1), which can dephosphorylate p38 and inactivate p38 MAP kinase and therefore inhibit gene expression and protein release of pro-inflammatory mediators in monocytes (30, 31) (Figure1).

A comparable mechanism was found in prostate cells where the induction of Mitogen-Activated Protein Kinase Phosphatase 5 (MKP5) by  $1\alpha, 25(\text{OH})_2\text{D}_3$  was responsible for down-regulation of IL-6 mRNA expression (32).  $1\alpha, 25(\text{OH})_2\text{D}_3$  increases MKP5 transcription by inducing VDR/RXR to bind with a VDRE in the MKP5 promoter.

In this indirect modulation of signaling cascades,  $1\alpha, 25(\text{OH})_2\text{D}_3$  and its receptor complex VDR/RXR can interact with other transcription factors such as NF- $\kappa$ B, the nuclear factor of activated T-cells (NFAT) or the glucocorticoid receptor which leads to anti-inflammatory effects (31).

NF- $\kappa$ B is a ubiquitous transcription factor, which is involved in many immune-related genes such as inflammatory gene expression. The activation of VDR inhibits NF- $\kappa$ B activation and signaling by means of interacting with a subunit of NF- $\kappa$ B, I $\kappa$ B, resulting in the cytosol (33). After various cell stress stimuli, I $\kappa$ B is phosphorylated and then ubiquitinated, which leads to the proteasomal degradation of the I $\kappa$ B protein. Thus, NF- $\kappa$ B is released and translocated into the nucleus where it activates the transcription of pro-inflammatory cytokines, anti-apoptotic factors as well as enzymes involved in the generation of pro-inflammatory mediators such as cyclo-oxygenase-2 (COX-2) (31, 33, 34).

It has been shown that  $1\alpha, 25(\text{OH})_2\text{D}_3$  down-regulates NF- $\kappa$ B levels in lymphocytes(35). The analog of vitamin D TX527 also prevents NF- $\kappa$ B activation in monocytes (36). Moreover, it has been shown that,  $1\alpha, 25(\text{OH})_2\text{D}_3$  or its analogs could reduce MCP-1 and IL-6 expression via inhibiting the NF- $\kappa$ B signaling in human macrophages (37, 38). The interference between vitamin D signaling and DNA binding of NF- $\kappa$ B has been found (39). The study showed that  $1\alpha, 25(\text{OH})_2\text{D}_3$  inhibits NF- $\kappa$ B activity but the absence of the translocation of the subunits of p50 and p65 in human MRC-5 fibroblasts suggests that  $1\alpha, 25(\text{OH})_2\text{D}_3$  may regulate the expression of cellular factors which contribute to the

reduced DNA binding of NF- $\kappa$ B (39). Therefore, it seems that vitamin D is able to inhibit NF- $\kappa$ B activation, as well DNA binding.

Another interesting target for the anti-inflammatory signaling of vitamin D is NFAT. This transcription factor is activated by the protein phosphatase calcineurin that dephosphorylates NFAT. After dephosphorylating, NFAT translocates into the nucleus and interacts with a variety of other transcription factors and transcriptional activation of pro-inflammatory genes such as interleukin 2 (IL-2) and COX-2 (31). It was shown that VDR-RXR heterodimers bind to an NFAT binding site of interleukin 2 promoter and inhibit NFAT activity in T-cells (40). Similar data were obtained for interleukin17 (IL-17) in inflammatory CD4+ T cells where  $1\alpha, 25(\text{OH}) 2\text{D}_3$  blocked NFAT activity which contributed to the repression of IL-17A expression by vitamin D (31, 41).

Non-coding microRNAs (miRNAs) are a class of short RNAs that regulate the post-transcriptional expression of genes by translation repression or mRNA degradation (42). Among various microRNAs, miR155, the miRNA mostly restricted to immune cells, is of particular interest (43). MiRNA-155 forms cytokine signaling via down-regulating the suppressor of cytokine signaling (SOCS) proteins involved in the inhibition of inflammation (44). Members of the SOCS family play an essential role in immunological homeostasis (45). Recently, a novel regulatory mechanism for vitamin D has been identified which is responsible to control the innate immunity. It was demonstrated that vitamin D/VDR signaling reduces LPS- induced inflammation by increasing the negative feedback regulation and targeting the miR-155-SOCS1 (suppressor of cytokine signaling 1) pathway (46) resulting in inhibiting the pro-inflammatory pathways of cytokines such as TNF- $\alpha$ , IL-6, and IFN- $\gamma$  (47). Vitamin D receptor (VDR)-dependent signaling limits an inflammatory response by targeting the miRNA155-SOCS1 axis (48).

Toll-like receptors (TLRs) are mainly expressed on antigen-presenting cells, such as macrophages or DCs, and their signaling activates APCs to provoke the innate immunity and establish adaptive immunity. Chen et al. report that  $1\alpha, 25(\text{OH}) 2\text{D}_3$  can control TLR signaling by exciting SOCS1 through the down-regulation of miR-155 in macrophages, which provide a negative feedback regulatory mechanism for vitamin D/VDR signaling to control innate immunity (Figure1) (38, 46, 48).

### **The effects of vitamin D on immune cells in BD**

Vitamin D is a well-known immune modulator of both innate and adaptive immunities (48). A number of studies suggest that vitamin D can increase the innate immune response, whereas it can inhibit the adaptive immune response (49). The innate immune systems modification could critically be involved in the pathogenesis of BD (49). However, the exact mechanisms and kind of stimuli involved in the *in vivo* activation of the immune system of BD patients is unclear. Several studies suggested that vitamin D deficiency could lead to immune malfunctioning (50). Defects in vitamin D metabolism may contribute to the inflammatory process in active BD patients (51).

Vitamin D through VDRs can regulate the proliferation, differentiation, and function of immune cells, both directly and indirectly (50). The effects of VDR and its ligands  $1\alpha, 25(\text{OH}) 2\text{D}_3$  on immune cells are dependent on two biological phenomena namely the expression of VDR on target cells and production of  $1\alpha$ -hydroxylase (Vitamin D-activating

enzyme) by individual immune cell types, leading to the local production of the active form of vitamin D (18). VDRs have been identified on nearly all cells of the immune system including T cells, B cells, neutrophils, macrophages and dendritic cells (DCs). By modulating the maturational state of DCs cells, vitamin D probably has a great impact on the immune system (52). It has been shown that the expression pattern of VDRs in resting monocytes and dendritic cells are intracellular rather than in resting T and B-lymphocytes that express little to no VDR. However, the expression of VDR in T cells is increased 5 fold upon T cell activation (27, 53).

The effects of vitamin D on innate immunity includes increasing the chemotaxis and phagocytic capabilities of immune cells and activating the transcription of antimicrobial peptides, such as defensin B (54) and cathelicidin (55). However, it has been shown that the activated monocytes in the presence of 1,25(OH)<sub>2</sub>D led to a reduced production of TNF- $\alpha$ , IL-1 $\alpha$  and IL-6, and an increased IL-10 cytokines (table1)(56).

The inflammatory macrophages are characterized by releasing high levels of pro-inflammatory cytokines that contribute to tissue damage (57). It has been reported that serum factor(s) of patients with BD are able to induce classical pro-inflammatory macrophages activation (58) suggesting that serum factor(s) might be responsible for inflammatory changes in BD.

In addition, vitamin D can affect the adaptive immunity in many ways. The useful effects of vitamin D on adaptive immunity include the induction of tolerogenic DCs, decreased IL-12 secretion, and increased IL-10 secretion in APCs. Additionally, it could be able to change the mode of T cell immune response by shifting Th1 to Th2 cells and/or reducing of the Th17 response (51).

Although DCs, or professional APCs, are apparently considered as immune-stimulatory cells, they could also be available as a tolerogenic DCs that are characterized by immune-regulatory properties in a VDR-dependent manner (38). DCs play an important role in peripheral T-cell tolerance by inducing T cell anergy (energy?) or unresponsiveness to self-antigens (25, 59). Moreover, they mainly exert their role by developing DCs with tolerogenic properties that are able to produce anti-inflammatory cytokines such as IL-10(24).

The molecular mechanisms of the tolerogenic properties of DCs by 1 $\alpha$ , 25(OH)<sub>2</sub>D<sub>3</sub> include the inhibition of the differentiation, maturation, and immunostimulatory capacity of human DCs, reduction of surface expression of MHC class II and costimulatory molecules (CD40, CD80, and CD86), up-regulation of ILT3 (inhibitory immunoglobulin-like transcript 3), and increasing secretion of IL-10 and CCL22 (chemokine ligand 22) (Table1) (38) (Figure1). IL-10 was significantly correlated with vitamin D level in active BD (49).

The effects of 1 $\alpha$ , 25(OH)<sub>2</sub>D<sub>3</sub> as inhibitors of T cells proliferation and the secretion of select cytokines have been described (60). The vitamin D signaling in T-cells is dependent on the stimulation of T-cell antigen-receptor (TCR) signaling (38). Expression of VDR can be induced by TCR signaling via the alternative signal transducers of p38 MAP kinases pathway (38). 1 $\alpha$ , 25(OH)<sub>2</sub>D<sub>3</sub> binds to the VDR/RXR complex, leading to the inhibition of the p38MAP kinase pathway via induction of MKP1, which can dephosphorylate p38 and therefore inhibit pro-inflammatory cytokines gene expression such as IFN- $\gamma$ , IL-6 and TNF- $\alpha$ . It can also inhibit DCs maturation, Th1 and Th17 differentiation, and promotion of the development of Treg cells (Figure1).

Studies also show that  $1\alpha,25(\text{OH})_2\text{D}_3$  preferentially suppresses the secretion of IL-12, INF- $\gamma$ , and TNF- $\alpha$  but increases the production of IL-4, IL-5, and IL-10, leading to the shifting of immune response from Th1 to Th2 (Table1) (40, 61).

The Th1/Th2 balance in BD is used to indicate the inflammatory status of the T-cell compartment. This ratio is commonly used to describe the balance in the immune system between pro-inflammatory IFN- $\gamma$  + Th1 cells and anti-inflammatory IL-4 + Th2 cells. The IFN- $\gamma$  /IL-4 ratio correlated inversely with serum 25(OH) D levels (47). Smolders et al. reported that vitamin D skews the T-cell compartment from a Th1 towards a Th2 phenotype, and high 25 (OH) D levels appear to be associated with a less pro-inflammatory T-cell compartment(62). The exact role of vitamin D as a regulator of Th17 cells has not been completely clarified. However, a number of animal model studies of the gastrointestinal inflammatory disease colitis (63), autoimmune uveitis (64) and inflammatory bowel disease (65), have revealed that treatment with  $1\alpha,25(\text{OH})_2\text{D}_3$  decreases the expression of IL-17, (63, 66), whereas loss of  $1\alpha,25(\text{OH})_2\text{D}_3$  by inhibition of CYP27B1 enzyme leads to enhanced levels of this cytokine (63, 67). Vitamin D inhibits the expression of IL-6, one of the cytokines which stimulates Th17 cell genesis (24, 68). Recently, Tian et al. reported that addition of vitamin D significantly inhibited Th17 cell differentiation both in BD patients and normal controls (69). Regulatory T cells (Tregs) are known as a potent suppressor of T-cell proliferation. It has been observed that there is a straightforward relationship between serum levels of 25(OH) D and the percentage of Treg cells ( $r = 0.640$ ;  $p = 0.0024$ ). A significant positive correlation was also observed with IL-10 levels in active BD ( $r = 0.640$ ;  $p = 0.0024$ ) (51). Treatment of tolerogenic DCs with  $1\alpha, 25(\text{OH})_2\text{D}_3$  or its analogues can induce CD4+/CD25 Tregs that are able to stop the development of autoimmunity. Barrat et al. (67) report that  $1\alpha, 25(\text{OH})_2\text{D}_3$ , in conjunction with glucocorticoids, strongly stimulates the generation of IL-10-producing CD4+/CD25 Tregs. Later studies show that  $1\alpha,25(\text{OH})_2\text{D}_3$  alone can induce Tregs (70). It seems that, the presence of vitamin D favored to the Treg production rather than other inflammatory effector T cells and this is considered as a convincing approach through which vitamin D affects the adaptive immunity.(63).

**In DCs,  $1\alpha, 25(\text{OH})_2\text{D}_3$ -VDR /VDR signaling in the nucleus, leading to a tolerogenic DC phenotype, described by reduced expression of MHC-II, CD40, CD80, CD86, enhanced expression of ILT-3, and increased secretion of IL-10 and CCL22, which results in the induction of Treg cells.**

**Vitamin D/VDR signaling can control TLRs signaling through down-regulation of miR-155-SOCS1 pathway and inhibits the pro-inflammatory cytokines such as TNF- $\alpha$ , IL-6, and IFN- $\gamma$ .**

**In T-cells,  $1\alpha, 25(\text{OH})_2\text{D}_3$  binds to the VDR/RXR complex, leading to Inhibition of the p38MAPkinase pathway via induction of MKP1, which can dephosphorylate p38 and therefore inhibit pro-inflammatory cytokines gene expression such as IFN- $\gamma$ , IL-6 and TNF- $\alpha$ . It can also inhibit DCs maturation, Th1 and Th17 differentiation, and promotion of the development of Treg cells.**

Abbreviations: CCL22, chemokine (C-C motif) ligand 22; ILT3, inhibitory immunoglobulin-like transcript3; MKP1, MAPK phosphatase-1; MAPK, mitogen-activated protein kinase; SOCS1, suppressor of cytokine signaling 1.

### **Immune cells importance in development of BD**

T cells hypersensitivity to different types of antigens plays a critical role in the pathogenesis of BD (71). However, the exact mechanisms by which the immune system contributes to the immunopathogenesis of BD are not clear yet. Activated neutrophils via secreted cytokines and chemokines from APCs and T cells secrete some cytokines, which lead to the stimulation of Th1 cells.

### **Th17 and regulatory T cells roles in patients with BD**

T lymphocytes are believed to play a central role in the immunopathogenesis of BD (71). Several studies show that polarization of CD4<sup>+</sup> T cells into Th1 cells and their cytokines, such as IFN- $\gamma$  and IL-12, play a central role in the pathogenesis of BD (72). However, the novel T-cell subsets Th9, Th17, Th22, and regulatory T (Treg) cells and their cytokines are undisputedly involved in inflammatory disorders (73).

The regulatory role of IL-21 in Th17/Treg cells balance in BD-associated inflammation is demonstrated by Geri et al. (74)(71). IL-21-driven Th17 pathway might be unique to patients with BD and distinguishable from the other inflammatory disorders (75). A noticeable increase in Th17 (but not Th1) cell numbers and a decreased frequency of CD4<sup>+</sup> forkhead box protein 3-positive Treg cells in the peripheral blood of patients with active BD have been reported (Table1)(75).

CCL20 and CXCL8 are potent chemoattractant for Th17 and inflammatory neutrophils cells, respectively, and are strongly expressed within the choroid plexus from patients with BD. Geri et al. demonstrate that IL-21 is produced by central memory CD4<sup>+</sup>T cells and correlate with the Th17 response and decrease forkhead box protein 3 expression. It has been suggested that IL-21 is involved both in Th17 differentiation and chemoattractant as well as neutrophils cells chemoattractant in central nervous system inflammatory lesions of patients with BD (75). Further stimulation of CD4<sup>+</sup> T cells with IL-21 increases Th17 and Th1 differentiation and decreases Treg cell frequency (75). IL-17A synergizes with TNF- $\alpha$  in induction of CXCL8 and CCL20 and might allow the recruitment of neutrophils and Th17 cells within the target tissues in patients with BD. The reinforcing role of Th17 cells as a major pathogenic subset has important implications for BD pathogenesis (76). Chi et al. demonstrate that the production of IL-23 and IL-17 by PBMCs is upregulated in patients with BD (77).

Apparently, Tregs can regulate immune response independently on the specific antigen recognition and the dysregulation of Tregs-mediated function has been considered relevant in several autoimmune diseases. However, the contribution of these cells to the pathogenesis of human autoimmune diseases is considered to be limited. Recently, Hamazaoui et al. reported that CD25+CD4<sup>+</sup> Treg cells were increased in both the peripheral blood and the cerebrospinal fluid of BD patients only in the active phase, as compared with BD in remission and healthy controls. They proposed that it was not clear whether the increased Tregs had an immunopathogenic or therapeutic properties. (78, 79).

### **Gamma Delta ( $\gamma\delta$ ) T Cells function in BD**

Gamma Delta T cells are a minor population of T cells expressing TCR  $\gamma\delta$  chain. These cells play a significant role in overall T cell function (80).  $\gamma\delta$  T cells interact with dendritic cells (DCs) to regulate their function and mutually promote mutual maturation. Activated  $\gamma\delta$  T cells can also produce high levels of IFN- $\gamma$ , TNF- $\alpha$ , Granzymes, and IL-17 reflecting their role in the effector phase of immune response as well as having a regulatory role (81).

The potential interaction of neutrophils, monocytes, and DCs with  $\gamma\delta$  T cells may explain the determined inflammatory symptoms of BD (81). The  $\gamma\delta$  T cells respond the triggers including bacterial antigens or HSPs and keep active interaction with neutrophils, monocytes, and DCs cells. Gamma delta T cells recognize the bacterial product (HMB-PP), or HSPs, start



contacting with monocytes, and produce pro-inflammatory cytokines including TNF- $\alpha$ . As a result, local  $\gamma\delta$  T cells increase and release chemokines such as CXCL8 (IL8) that then recruit more neutrophils to the infection site. In addition,  $\gamma\delta$  T cells play a key role in the function of the neutrophils by providing survival and activation signals to newly recruited neutrophils and monocytes. Besides, activated  $\gamma\delta$  T cells present antigen to DCs and thus initiate Th1, Th2, and Th17 differentiation and proliferation (Table1)(81). Parlakgul Gunes et al. report that the number of  $\gamma\delta$  T cells does not increase in active BD patients (82).

### **Natural killer (NK) cells function in BD**

NK cells are characterized by quickly killing target cells and modulating the adaptive immune responses through cytokine and chemokine secretion. NK cells play a central role in the pathogenesis of autoimmune diseases (83). Nearly, 90% of these cells are CD56<sup>dim</sup> CD16<sup>+</sup> capable of killing and antibody dependent cell cytotoxicity (84, 85). The other 10% are CD56<sup>bright</sup> CD16<sup>-</sup> that are weakly cytotoxic. NK cells play an important role in the pathogenesis of BD by expression of stimulatory and inhibitory receptors such as Killer immunoglobulin-like receptors (KIR) and NKG2D (84). Inhibitory KIR (KIR3DL1) inhibits cell-mediated cytotoxicity via interaction with HLA-B\*51 (84). This inhibitory effect is balanced by stimulatory interactions via NKG2D and MICA (MHC class-I polypeptide-related sequence A) (86). It is argued that CD94/NKG2 work in conjunction with KIR by responding to changes in HLA expression and can also transduce both stimulatory and inhibitory signals (87).

Inhibitory CD94/NKG2A and stimulatory CD94/NKG2C receptors are expressed on natural killer, CD4, and CD8 T cells and recognize non-classical HLA-E. Peptides bind to HLA-E and function as modulators of NK cell cytotoxic activity and cytokine production(88).

The association between CD94/NKG2A, CD94/NKG2C receptors, and their ligand HLA-E polymorphisms in patients with BD has been found (Table1) (88). In active BD, an increase of the stimulatory receptor NKG2C and CD16 were observed (82).

Saruhan-Direskeneli et al. found that there was an increase in the expression of CD94 on CD56<sup>+</sup>CD16<sup>+</sup> and CD56<sup>+</sup>CD3<sup>+</sup> NK cells in patients with BD (89)., They also demonstrated that both *CD94* (c.-134\*T) and *NKG2A* (c.-4258\*C, c.338-90\*G) polymorphisms were associated with a reduced risk of BD, and this effect was enhanced upon the combination with expression of HLA-E\*0101(84).

IL-15 and IL-15R $\alpha$  are essential for normal development of NK cell proliferation (84). Elevated levels of IL-15 have been reported in serum, cerebrospinal fluid, and aqueous humor from patients with BD (90, 91). Production of cytokines such as IL-10 and IL-15 contribute to abnormal NK cell function leading to extended inflammation and further unpleasant episodes. The effect of altered levels of IL-15 and the IL-15R $\alpha$  complex is known to influence NK cell proliferation, thus suggesting a possible pathway for NK cell control in the inflammatory diseases (84).

A number of studies have shown that the functions of NK cells are compromised in BD patients. Examples of this impairments are reduced levels of IL-12R $\beta$ 2 mRNA in NK cells in patients with active BD, compared to inactive patients or healthy controls that have been documented by Yamaguchi et al. (92). Furthermore, isolated NK cells from the Broncho-alveolar lavage (BAL) of patients with pulmonary BD showed that the expression of IL-2/IL-

15R $\beta$  (CD122) was significantly reduced. CD122 is shared by the IL-15 receptor, which is partly responsible for cytotoxicity of NK cells (93). Until now, the effects of vitamin D on NK cell function in autoimmune diseases remain unclear. Vitamin D does not seem to affect the number or frequency of NK cells, but may alter their cytotoxic function (83).

### **Natural killer T (NKT) cells role in the pathogenesis of BD**

The precise role of CD1d-dependent natural killer-like T (NKT) cells in the pathogenesis of BD remains unknown. NKT cells increased in cerebrospinal fluid (CSF) from neuro-BD patients' samples. An increased expression of IFN- $\gamma$ -production has also been reported in CSF-NKT cells from neuro-BD patients (Table1) (94).

iNKT (invariant NKT) cells are a unique population of T cells, which play important roles in immune regulation, tumor surveillance, and host defense against pathogens (95). In experimental models as well as in patients with an autoimmune disease, such as, Type 1 diabetes mellitus, multiple sclerosis, and systemic lupus erythematosus, a decline has been reported in the number of iNKT cells (96). Experimental findings show that the expression of the VDR is required for the normal development and function of iNKT cells (95). It seems that the VDR and the 1,25(OH) $_2$ D ligand have a different role in regulating iNKT cell numbers and function (83). Data from VDR knock out (KO) mice show that iNKT cells have difficulties in the development of cell numbers and proliferative capacity in the absence of VDR (97). Stimulated splenocytes of VDR KO mice with  $\alpha$ -Galactosylceramide ( $\alpha$ GalCer) (NKT ligand) produce less IFN- $\gamma$  and IL-4(97). 1,25(OH) $_2$ D seems to play a role in the development of mouse iNKT cells and using vitamin D supplementation in wild type (WT) mice increases the production IFN- $\gamma$  and IL-4 by iNKT cells (97). Vitamin D supplementation in case of vitamin D deficiency can increase the number and function of iNKT cells (83).

### **VDR gene polymorphisms in BD**

The VDR-encoding gene which is located on chromosome12q13.11 contains nine exons, eight introns, and more than 470 single nucleotide polymorphisms (SNPs) (12). The four most common SNPs in the VDR gene are *BsmI* and *Apal* (both located in intron 8) and *FokI* and *TaqI* (both located in the starting codon) (25). A number of these SNPs has a great impact on immunomodulatory actions of vitamin D (12). The VDR polymorphisms have frequently been studied in various autoimmune disorders. But only few studies were performed in regard to BD (12). Several studies have found a strong association between the two most studied VDR gene polymorphisms—*FokI* and *BsmI*—with susceptibility to BD (98, 99).

The direct effect of VDR polymorphisms in BD population compared to RA patients and healthy controls have been studied by Karray EF et al. (98) and Tizaoui K et al. (100) In these studies one 131 BD patients, 108 RA patients and 152 healthy controls were genotyped for the VDR *FokI*, *BsmI*, *Apal* and *TaqI* polymorphisms. The results of these studies show that the *FokI* polymorphism alleles and genotype were significantly more common in the RA and BD groups than the healthy controls. The *FokI* F allele and F/F genotype of the VDR gene were significantly associated with BD in Tunisian patients but *BsmI* was not (Table.2) (98, 99). According to clinical manifestations in BD, *FokI* polymorphism was significantly

associated with the presence of vascular manifestations ( $p = 0.006$ ). No significant associations were found between the *BsmI* polymorphism and RA or BD.

A significant association between *TaqI* polymorphism and BD was observed. In addition, analysis of the genotypic distribution of *ApaI* polymorphism did not show any significant differences between BD patients and healthy controls. *TaqI* and *ApaI* polymorphisms might be modestly implicated in BD pathogenesis. In contrast, *TaqI* and *ApaI* seemed not to be implicated in RA pathogenesis (99). The association of VDR *FokI* polymorphism with susceptibility to BD in Tunisian population was similar to reported studies in other inflammatory/autoimmune diseases such as RA (101). An association between the VDR *f* allele and *ff* genotype in the Iranian Azari population-BD patients has been reported by S Kolahi et al. (Table.2)(12).

### Genetics of Behçet's disease

The cause of BD is still unknown. The first evidence for a genetic background of BD arose in 1982, when Ohno et al. published their results suggesting that *HLA-B\*51* was associated with BD in Japanese patients (84, 102). Genome-wide association studies (GWAS) investigating genetic associations in BD have supported the strong association of MHC-related allele and BD (84, 103).

Various other genes have also been implicated in BD genetic susceptibility including the MHC locus, C-C motif chemokine receptor1 (*CCR1*), *STAT4*, killer cell lectin-like receptor subfamily C (*KLRC4*), and endoplasmic reticulum aminopeptidase1 (*ERAP1*) (103). Noticeably, all these genes are involved in cytotoxicity (104). Furthermore, polymorphisms of transporters associated with antigen processing (TAP) loci (TAP1 Val-333/Asp-637) were completely lacking among Spanish BD patients compared with healthy controls, proposing that the TAP polymorphisms may indicate some importance in BD progress (105). Recently, several variants in non-HLA genes were found to be significantly associated with susceptibility to BD—e.g. three SNPs located in *IL23R* and one SNP in *IL10* (103, 106, 107). According to HLA region genotyping, three regions that have strong association with BD are: single-nucleotide polymorphisms (SNPs) in the HLA-A region, the HLA-B and the HLA-C region (106). The most common HLA-B alleles involved are: *HLA-B5* or *HLA-B51* (hereafter indicated as *HLA-B51/B5*), *HLA-B52*, *HLA-B57*, *HLA-B5101*, and *HLA-B5108* alleles, with the prevalence of *HLA-B51* in Italy, Germany, and Asia, *HLA-B52* in Israel, *HLA-B57* in England, and *HLA-B5101* and *HLA-B5108* in the countries along the ancient Silk Road (4, 108). It is believed that BD is associated with *HLA-B5* or *HLA-B51* (*HLA-B51/B5*)—more specifically with *HLA-B51* (109). An increased presence of *HLA-B51* has also also been reported in patients with *familial* forms compared to patients with sporadic forms (4, 110).

Carla Maldini et al. investigated the relationships between BD clinical features and *HLA-B51/B5* in a meta-analysis and show that in BD patients, *HLA-B51/B5* presence predominates in *males* and is associated with a moderately higher prevalence of *genetic background* and *clinical BD phenotype* such as genital ulcer, ocular, and skin manifestations (111). Another meta-analysis by de Menthon et al. (109) on 4,800 BD patients and 16,289 controls, indicate a strong association between *HLA-B51/B5* and BD. This association across

populations of various ethnicities provides further support for the contribution of *HLA-B51/B5* as a primary and causal risk determinant for BD (Table 2).

A more recent meta-analysis show that subjects with *HLA-B51/B5* have an increased risk of developing BD compared to non-carriers of *HLA-B51/B5* (4, 109). Montes-Cano et al. (107), in a multi-centric study in the Spanish population, showed that *HLA-B51*, *HLA-B57* was found to be a risk factors in BD, whereas *B35* was found to be protective. Various other studied alleles of *HLA-A* and *B* could be a risk (*A02* and *A24*) or protective (*A03* and *B58*) factor and other non-*HLA* genes, such as *IL23R* and *IL-10*, also play a role in the susceptibility to BD (Table 2.) (106).

### **Effects of vitamin D on the expression of TLR in BD**

It is now well established that the pathogen-associated molecular patterns activate pathogen-recognition receptors, such as TLR, in the immune cells. These receptors are mainly expressed on the antigen-presenting cells, such as macrophages or dendritic cells. Immune system uses these signaling accomplishments to provoke innate immunity and then provide appropriate alarm for conducting the adaptive immunity. However, a number of different TLRs need to be considered (112).

It has recently been reported that the expression of TLRs were enhanced in BD patients, resulting in an excess production of Th1 cytokine(113). Do et al., (114) in an *in vitro* study, indicate that the TLRs are naturally up-regulated in BD. They also show that the levels of vitamin D were inversely correlated with the expressions of TLR2, TLR4.

Dickie et al. show that vitamin D suppresses the expression of TLR2 and TLR4 protein in human monocytes in a time- and dose-dependent manner. Additionally, down-regulation of TLR9 expression in monocytes has been reported by vitamin D supplementation (114, 115). Xiaoli Liu et al. indicate that a higher expression of TLR2, TLR3, TLR4, and TLR8 at the mRNA and protein level was observed in active ocular Behçet's Disease (OBD) patients as compared to healthy control groups (Table3) (116).

### **Vitamin D deficiency and BD**

Investigations in Mediterranean countries, the Middle East, and the Far East indicate an inverse relationship between vitamin D and BD (117). On the other hand, higher levels of vitamin D correlate with lower levels of disease activity (29, 118, 119). A majority of previous studies report a low level of 25(OH) D3 in BD patients compared to controls. However, none of these studies show similar results. Several studies shed light on the reverse relationship between BD activity and vitamin D deficiency (114, 120, 121). Another study reveals a lower level of vitamin D in active BD cases compared with silent BD cases and control group (122).

Hamzaoui et al. found a lower serum level of 25(OH) D3 in active BD patients compared to inactive BD patients and healthy controls. In addition, they show that disease activity is associated with lower vitamin D serum levels in active BD patients (51, 118).

In a study on patients with BD, S. S Ganeb et al. show that serum levels of 25(OH) D3 are significantly lower in BD patients compared to controls. Some associations have also been found between vitamin D levels and age, vitamin D and ESR and vitamin D and CRP in BD patients (119, 123, 124).

Do et al. have found that the serum vitamin D levels are inversely correlated with the serum CRP and the ESR levels in BD (114). Significantly, low levels of 25(OH) D3 in BD patients has also been reported by Karatay et al. (Table3) (117).

Khabbazi et al. show that 25(OH) D3 values in patients with BD are significantly lower than those of healthy controls. Deficiency of 25(OH) D3 is more common in the BD group than in the control group (120). Moreover, no correlation has been found between the major symptoms of BD and 25(OH) D3 values. (120).

## Conclusion

Vitamin D—with pluripotent immunomodulation and anti-inflammatory effects—plays a critical role in physiological conditions and autoimmunity and its deficiency is linked to chronic inflammatory diseases. The regulatory mechanisms involved in the effect of vitamin D through VDRs and its role in auto-inflammatory diseases are still under debate; more and larger studies on the molecular level are needed to determine how vitamin D affects immune cells and gene expression under inflammatory conditions.

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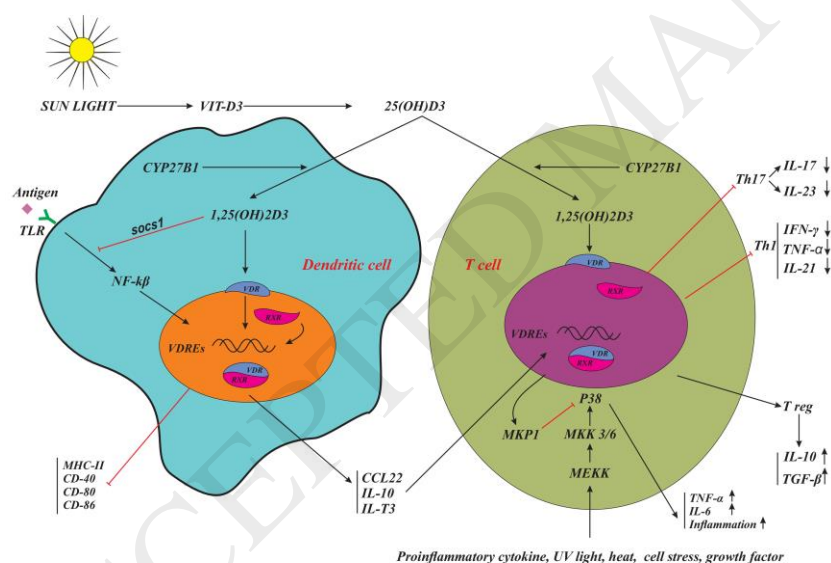
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## Figures

**Figure1: Anti-inflammatory signaling of vitamin D through which vitamin D-regulated dendritic cells (DCs) and T-cells function (modified source(38)).**



**Table1. The effects of Vitamin D and the importance of immune cells in the development of BD**

Immune cells	Subset	Immune cells role in BD	Effects mediated by 1α, 25(OH)2D3	References
Monocytes/ macrophage	-	Chemotactic and phagocytic capacity Induction cathelicidins IL-1β, IL-6, TNF-α↑	Chemotactic and phagocytic capacity ↑ Induction cathelicidins ↑ In vitro: IL-1a, IL-6, TNF-α, ↓ IL-10 ↑ NF-κB signaling activation ↓ p38 MAPK pathway ↓ IL-12, IL-23 ↓	(54) (54) (55,56) (38) (31) (30) (31)

DCs	-	Antigen presentation IL-12 ↓ IL-10↑	Tolerogenic properties↑ Cell maturation and differentiation↓ IL-12 ↓, IL-10↑ IL-1, IL-6, TNF- $\alpha$ , INF- $\gamma$ ↓ IL-4, IL-5↑ ILT3, CCL22↑ ↓CD40, ↓CD80, ↓CD86, ↓MHC-II,	(24) (24) (24, 49) (40,59) (40,59) (38) (38)
T helper cells (CD4+ T cells)	Th1	Th1↑ IL-23 ↑	IL-12, INF- $\gamma$ , TNF- $\alpha$ ↓ IL-4, IL-5, and IL-10↑ IL-17, IL-21, IL-23 ↓	(70) (75)
		INF- $\gamma$ (Th1) /IL-4(Th2) ratio↑	INF- $\gamma$ (Th1) /IL-4(Th2) ratio↓	(47)
		Th2	Th2↓ INF- $\gamma$ (Th1) /IL-4(Th2) ratio↓	IL- 4↑ INF- $\gamma$ (Th1) /IL-4(Th2) ratio↓
	Th17	Th17↑ IL-21↑ Th17/Treg ratio ↑	Th17↓ Th17/Treg ratio ↓	(63) (74)
	Tregs	Tregs ↑ IL- 10↑	Tregs ↑ IL- 10↑	(74)(51) (79,80)
B-cells	-	-	differentiation and maturation ↓ B-cells function ↓	(15, 43)
NK cells	-	NKG2C ↑, NKG2D ↓ CD16 ↑, CD94↑ IL-15↑ IL-2/IL-15R $\beta$ (CD122), IL-12R $\beta$ 2↓	Cytotoxicity ↑	(88) (89,82) (84,91,92,93) (94)
NK T (NKT) cells	iNKT	INF- $\gamma$ ↑	INF- $\gamma$ ↑ in WT mice INF- $\gamma$ , IL- 4↑ number and function of iNKT cells ↑	(94) (97)
$\gamma\delta$ T cells	-	CXCL8 (IL8) ↑ Th1/Th2/Th17↑, INF- $\gamma$ ↑, TNF- $\alpha$ ↑	-	(81,82)

Abbreviations, CCL: chemokine (C–C motive) ligand; ILT3: immunoglobulin-like transcript 3; IFN: interferon; IL: interleukin; ILT: Immunoglobulin-like transcript; MHC: Major Histocompatibility Complex; TNF: tumor necrosis factor;  $\gamma\delta$ : Gamma Delta; CXCL8 (IL8): chemokine (CXCL8); MAPK: mitogen-activated protein kinase; iNKT : Invariant Natural killer T cells; NKG2C: Natural killer (G2C, G2A, G2D); WT: wild type

Table 2. Summary of clinical and genetical studies evaluating in BD

Source	Study design	objective	Population (cases)	Main outcome(s)
Karray EF et al. (98, 100) 2011 Tunisia	descriptive analytical case-control study	To investigate the VDR gene polymorphisms in patients with BD and RA diseases in Tunisia	108 patients with RA, 131 patients with BD, and 152 controls	<i>FokI</i> F allele and <i>F/F</i> genotype association with BD <i>FokI</i> F allele association with RA
Kolahi <i>et al.</i> (12) 2014 IRAN	Cross sectional study	To investigate the association and clinical manifestation of VDR polymorphisms with susceptibility to BD in Iranian Azari population	50 Iranian Azari patients with BD and 50 matched healthy controls	<i>FokI</i> F allele and <i>F/F</i> genotype association with BD
Montes-Cano et al. (106, 107) 2013 Spain	A multicentric case-control study	To investigate the HLA and non-HLA genes in BD	304 BD patients and 313 ethnically matched controls	HLA-B51, HLA-B57 as a risk factor in BD Protective role of B35 in BD Non-HLA genes, such as IL23R and IL-10 role in the susceptibility to BD
Carla Maldini et al. (111) 2012, France	Meta-analyses study	To investigate the relationships between BD clinical features and HLA-B51 or HLA-B5 (HLA-B51/B5) status	74 study populations	HLA-B51/B5 presence predominates in <i>males</i> and is associated with moderately higher prevalence of <i>genetic background</i> and <i>clinical BD phenotype</i> such as genital ulcer, ocular, and skin signs.
de Menthon et al. (109) 2009 France	Meta-analyses study	To investigate the genetic effect of the <i>HLA-B51/B5</i> allele on the risk of developing BD	4,800 patients with BD and 16,289 controls	A strong association between BD and <i>HLA-B51/B5</i> Its stability across populations of various ethnicities Primary and causal risk factor for BD

Abbreviations, HLA: Human Leucocyte Antigen; RA: Rheumatoid Arthritis

**Table 3.** Summary of clinical studies evaluating of relationship between vitamin D and BD

Source	Study design	Objective	Population (cases)	Main outcome(s)
<b>J. E. Do et al. (114) 2008 South Korea</b>	Descriptive analytical case-control	To investigate the association between the TLR expression and vitamin D levels in BD	41 BD patients and (19 newly diagnosed psoriasis patients and 15 healthy control ) as the control groups	Higher expressions of TLR2 and 4 in BD compared to controls. Lower level of 25(OH)D in active BD. 25 (OH)D levels inversely correlated with the expressions of TLR2, TLR4 in BD.
<b>Karatay et al. (117) 2011 Turkey</b>	Case- control	To investigated the serum 25(OH)D levels of patients with DB	32 patients with BD and 31 matched healthy controls	25(OH)D level in BD significantly lower than the control. No correlation between vitamin D levels and age, disease duration, ESR, and CRP levels.
<b>Hamzaoui et al. (51, 118) 2013 Tunisia</b>	Descriptive analytical case-control	To investigate the relationship between serum vitamin D concentrations and disease activity in patients with BD.	102 BD patients in active stage and 22 with RA and 30 with MS as control diseases	Lower vitamin D levels in active BD in comparison with silent BD and control. INF- $\gamma$ /IL-4 ratio (Th1/Th2), inversely correlated with 25(OH)D levels. Significant negative correlation between the age of active BD patients, CRP and ESR with vitamin D value
<b>S.S Ganeb et al. (119) 2013 Egypt</b>	Case- control	To investigate the vitamin D levels in patients with BD	42 patients with BD and 41 matched healthy controls	Significantly lower levels of vitamin D in BD patients compared to controls. Associations between vitamin D levels and age, ESR and CRP in BD.
<b>Xiaoli Liu et al. (116) 2013 china</b>	Case- control	To investigate the role of TLRs 2,3,4 and 8 in pathogenesis of ocular BD (OBD)	16 patients with OBD and 16 healthy controls	Higher expression of mRNA and protein level of TLR2, TLR3, TLR4, and TLR8 in active OBD patients as compared with controls. Higher expression of TLRs may be involved in the pathogenesis of BD.
<b>Khabbazi et al, (120) 2014 IRAN</b>	Cross sectional study	To characterize the status of vitamin D in patients with active BD and the relationship between vitamin D levels and BD activity.	48 patients with BD and 47 age- and sex-matched healthy controls	25(OH)D level in BD were significantly lower than the control. Insufficiency and deficiency of 25(OH)D in the BD group was more common than the control. No correlation was found between the major symptoms of BD and 25 (OH) D values.

Abbreviations, TLR: Toll-like receptors; ESR: Erythrocyte Sedimentation Rate; CRP: C Reactive Protein; OBD: ocular Behçet's Disease