

Vitamin D3 ameliorates herpes simplex virus-induced Behçet's disease-like inflammation in a mouse model through down-regulation of Toll-like receptors

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ABSTRACT

Objectives. This study was conducted to understand the role of vitamin D3 through the regulation of Toll-like receptors (TLRs) and cytokines in herpes simplex virus-induced Behçet's disease (BD)-like mice.

Methods. Serum 25-hydroxyvitamin D ($1,25(\text{OH})_2\text{D}_3$) levels were measured in BD-like mice, as well as virus injected and asymptomatic appearance BD normal mice (BDN). The frequencies of TLRs of peritoneal macrophages were compared by FACS. To determine the effect of vitamin D3 in vitro, peritoneal macrophages were isolated and then incubated with $1,25(\text{OH})_2\text{D}_3$. To identify the mechanism of improvement of BD-like symptoms induced by $1,25(\text{OH})_2\text{D}_3$, mice were orally administered $1,25(\text{OH})_2\text{D}_3$.

Results. The serum 25-hydroxyvitamin D levels in BD-like mice were $12.4 \pm 5.4 \text{ ng/ml}$, while they were $17.5 \pm 7.2 \text{ ng/ml}$ in BDN mice. The frequency of TLR2 in BD-like mice was $32.91 \pm 20.88\%$, while it was $12.73 \pm 7.67\%$ in BDN. The frequency of TLR4 was $26.09 \pm 10.20\%$ in BD-like mice and $9.72 \pm 5.30\%$ in BDN. In a 72 h culture of peritoneal macrophages in $10^{-8} \text{ M } 1,25(\text{OH})_2\text{D}_3$, the frequency of TLR2 was $25.0 \pm 2.7\%$, while it was $37.3 \pm 5.8\%$ in the control group. The frequency of TLR4 was $18.9 \pm 5.3\%$ with $1,25(\text{OH})_2\text{D}_3$, while it was $30.3 \pm 0.1\%$ in the control group. Treatment with $1,25(\text{OH})_2\text{D}_3$ improved the symptoms in six out of 11 BD-like mice and down-regulated the frequency of TLR2 and TLR4. Moreover, $1,25(\text{OH})_2\text{D}_3$ influenced Interleukin-6 and TNF-alpha expression in the sera of BD-like mice.

Conclusion. Vitamin D improved BD-like symptoms by down-regulating the expression of TLRs and pro-inflammatory cytokines in in vivo mouse models.

Introduction

Recent studies have shown the anti-inflammatory effects of vitamin D3 in Kawasaki disease (1) and psoriasis (2). Reduction of vitamin D is associated with increased renal inflammation (3) and Behçet's disease (4). Vitamin D3 treatment has been shown to decrease chemokine synthesis and monocyte trafficking (5), as well as to down-regulate Toll-like receptor (TLR) 2 and 4 of monocytes (6). The immunomodulatory effect of $1,25(\text{OH})_2\text{D}_3$ in T cell subsets has also been shown to induce a significant decrease in IFN γ and TNF- α cytokines in single cell expression by patients with Mycobacterium tuberculosis (7).

Behçet's disease (BD) is a chronic, multi-systemic disorder that has arthritic, intestinal, mucocutaneous, ocular, vascular, and central nervous system affects. BD takes a chronic course with periodic exacerbations and progressive deterioration (8, 9). The etiology of BD is unclear, but viral infection has long been postulated as one of the main factors. Since Hulusi Behçet first proposed a viral etiology (10), the viral hypothesis has been verified by detection of the virus in the saliva (11), intestinal ulcers (12), and genital ulcers (13, 14) of patients with BD. Subsequent to these findings, inoculation of the earlobes of ICR mice with herpes simplex virus (HSV) was found to result in the development of BD-like symptoms (15). Manifestations in mice after HSV inoculation include multiple symptoms such as oral ulcers, genital ulcers, skin ulcers, eye symptoms, intestinal ulcers, arthritis, and neural involvement, as well as skin crusting. The frequency of these symptoms is similar to that of patients with BD (16).

TLR-expressing cells play important roles in innate immunity in patients

Competing interests: none declared.

with inflammatory disorders. TLR-4 is expressed by macrophages in mice and humans (17) and TLR-2 is upregulated in macrophages in response to LPS (17). It has been reported that both TLR-2 and TLR-4-expressing cells accumulate in the lesions of BD patients; however, TLR-2 and TLR-4 were not detected in unaffected sites of the same tissue sample (18). TLR-2-expressing cells play a pivotal role in priming for destructive Th1-type responses at the sites of BD lesions (18). Regulation of TLR-2 and TLR-4 may be related to the symptoms of BD. Therefore, we analysed TLR-2 and TLR-4 in macrophages after administration of 1,25(OH)₂D₃.

This study was conducted to determine if vitamin D3 supplementation could reduce inflammation of HSV-induced BD-like symptoms in a mouse model through the regulation of TLR expression. To accomplish this, we examined the down-regulation of symptoms in BD-like mice that were administered vitamin D3. To evaluate the additive effect of vitamin D3 and colchicine, colchicine was administered to BD-like mice with or without 1,25(OH)₂D₃. A combination agent regimen was found to be more effective than a single-agent regimen for the treatment of patients with Behçet's disease (19). Colchicine is one of the medicines most frequently prescribed to patients with Behçet's disease (20). We found that increased vitamin D3 expression was related to the improvement of BD-like mice symptoms and the severity score, and that these effects occurred via regulation of TLR expression.

Materials and methods

Serum 25-hydroxyvitamin D measurement

Serum concentrations of 25-hydroxyvitamin D (25(OH)D) were assayed using a radioimmunoassay kit (Dia-Sorin, Stillwater, MN). Following extraction of 25(OH)D using donkey anti-goat (DAG) precipitating complex, the treated sample was assayed according to the equilibrium RIA procedure.

BD-like symptoms in mouse model

ICR male mice (4 to 5 week old) were

infected with HSV type 1 (1x10⁶ pfu/mL, F strain) grown in Vero cells. Virus inoculation was conducted twice with a ten day interval between treatments, and was followed by 16 weeks of observation. Animals were handled in accordance with a protocol approved by the animal care committee of Ajou University School of Medicine. Manifestations in mice after HSV inoculation involved multiple symptoms. Of the total number of HSV-injected mice, 15% developed BD-like symptoms. Symptoms in human patients including oral ulceration, genital ulceration, erythema, skin pustules, skin ulcerations, joint-arthritis, diarrhea, red eye (right, left), reduced vision (right, left), loss of balance, discolouration, and swelling of the face were selected and analysed as BD-like symptoms. Oral, genital, and other skin ulcers and eye symptoms were classified as major symptoms. Arthritis, gastrointestinal ulcers, and neurological involvement were identified as minor symptoms. Mice with ≥1 major and 1 minor symptom were classified as having BD. The score of each symptom was one and the sum of the symptoms was used to determine the severity of BD. The disappearance of symptoms or a decrease in the lesion size of more than 20% was classified as improvement. The severity of BD was followed by determination of the Behçet's disease activity index, as outlined in the BD Activity Form (www.behcet.ws/pdf/BehcetsDiseaseActivity-Form.pdf). As a control group, HSV was inoculated, but no symptomatic healthy looking mice were used (BD Normal, BDN) as previously described (15, 38).

In vivo experiments

For each BD-like mouse model, 10μg/kg 1,25 dihydroxy vitamin D (1,25(OH)₂D₃) (Ilsung Pharmaceuticals Co. LTD, Seoul, Korea), 10μg/kg 1,25(OH)₂D₃ plus 2μg/mouse colchicine, 2μg/mouse colchicine or 200μl distilled water was administered orally once per day. Treatment was conducted for ten days. For normal mice, 5, 10, 25, or 50μg/kg of 1,25(OH)₂D₃ was administered orally once per day for five or ten days. Two hours after the last administration, peritoneal macrophages

were isolated for flow cytometry and the sera were collected for ELISA.

Flow cytometric analysis of intracellular cytokines

Mouse peritoneal macrophages were washed with phosphate buffered saline (PBS; Sigma, St. Louis, MO), after which 1x10⁶ cells were incubated with 1μg of PE-labelled anti-mouse TLR4 (UT41, eBioscience, San Diego, CA, USA) or FITC-labelled anti-mouse TLR2 (6C2, eBioscience, San Diego, CA, USA) for 30min. The labelled cells were then washed with PBS and analysed using a vantage flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA).

Peritoneal macrophage isolation

After spraying the abdomen of euthanised mice with 70% alcohol, scissors and forceps were used to cut the outer skin of the peritoneum and gently pull it back to expose the inner skin lining the peritoneal cavity. Next, 5ml of ice cold PBS were injected into the peritoneal cavity using a 27 gauge needle that was slowly pushed into the peritoneum. After injection, the peritoneum was gently massaged to dislodge any attached cells into the PBS solution. The fluid was then collected in a 25 gauge needle attached to a 5ml syringe. Next, the collected fluid was deposited in tubes that were kept on ice after removing the needle from the syringe. The cell suspension was then centrifuged at 1500RPM for eight minutes, after which the supernatant was discarded and the cells were resuspended in media for counting.

Cell culture

The peritoneal macrophage cells were maintained in six well cultures in RPMI 1640 medium (supplemented with 1% antibiotics, 2mg/ml sodium bicarbonate, and 10% fetal bovine serum) for 2h under 5% CO₂ at 37°C. After the non-adherent cells were removed, 1x10⁶ adherent cells were incubated in 10% FBS/culture medium in the presence or absence of 1,25(OH)₂D₃ (Fluka 95230: Sigma-Aldrich, Switzerland) at concentrations of 10⁻⁸ M or 10⁻⁹ M for 24, 48 and 72h. Finally, the expression

of TLR2 and TLR4 was analysed by FACS and RT PCR.

ELISA

Serum was analysed using commercial ELISA kits for the detection of mouse

IL-6 and TNF- α (R&D System, Minneapolis, MN). ELISA was conducted according to the manufacturer's instructions. The means and standard deviations were calculated using ELISA values determined for each well. The

ELISA reader was a Bio-Rad model 170–6850 microplate reader, and the wavelength was 450nm.

Statistical analysis

All data shown represent the mean \pm SE. Statistical differences between the experimental groups were determined using the Student's *t*-test and Bonferroni correction. Statistical analysis was conducted using MedCalc® version 9.3.0.0.

Results

Vitamin D3 levels of the BD-like mice

Serum 25(OH)D levels were measured in Normal, BDN, and BD-like mice using a standardised method. Serum 25(OH)D levels in BD-like mice were significantly lower than in BDN mice (12.4 ± 5.4 ng/ml ($n=14$) vs. 17.5 ± 7.2 ng/ml ($n=15$), $p=0.04$) (Fig. 1A). The levels in normal mice (20.52 ± 8.59 ng/ml ($n=5$)) were also significantly higher than in BD ($p=0.04$).

Increased expression of TLR2 and TLR4 in macrophages of BD mice

To determine whether the toll-like receptor (TLR) expression differed between BD-like mice and BDN mice, peritoneal macrophages from BD-like mice and BDN mice were isolated and analysed by flow cytometry. The frequency of TLR2 positive cells was $32.91 \pm 20.88\%$ in BD-like mice ($n=5$) and $12.73 \pm 7.67\%$ in BDN mice ($n=6$) ($p=0.024$). The frequency of TLR4 positive cells was $26.09 \pm 10.20\%$ in BD-like mice ($n=5$) and $13.66 \pm 10.75\%$ in BDN mice ($n=6$) ($p=0.041$). The macrophages of BD-like mice showed higher expressions of TLR2 and TLR4 than those of BDN mice (Fig. 1B).

1,25(OH) $_2$ D $_3$ down regulated the expression of TLR2 and TLR4 in *in vitro* macrophage cultures from normal and BD mice

Macrophages were isolated from normal, BDN, and BD-like mice (Fig. 2), after which they were incubated with 10^{-9} M and 10^{-8} M $1,25(\text{OH})_2\text{D}_3$ for 24h and 72h. Treatment with $1,25(\text{OH})_2\text{D}_3$ down regulated TLR2 and TLR4 in the 10^{-9} M and 10^{-8} M $1,25(\text{OH})_2\text{D}_3$ treated groups. Specifically, the frequency of

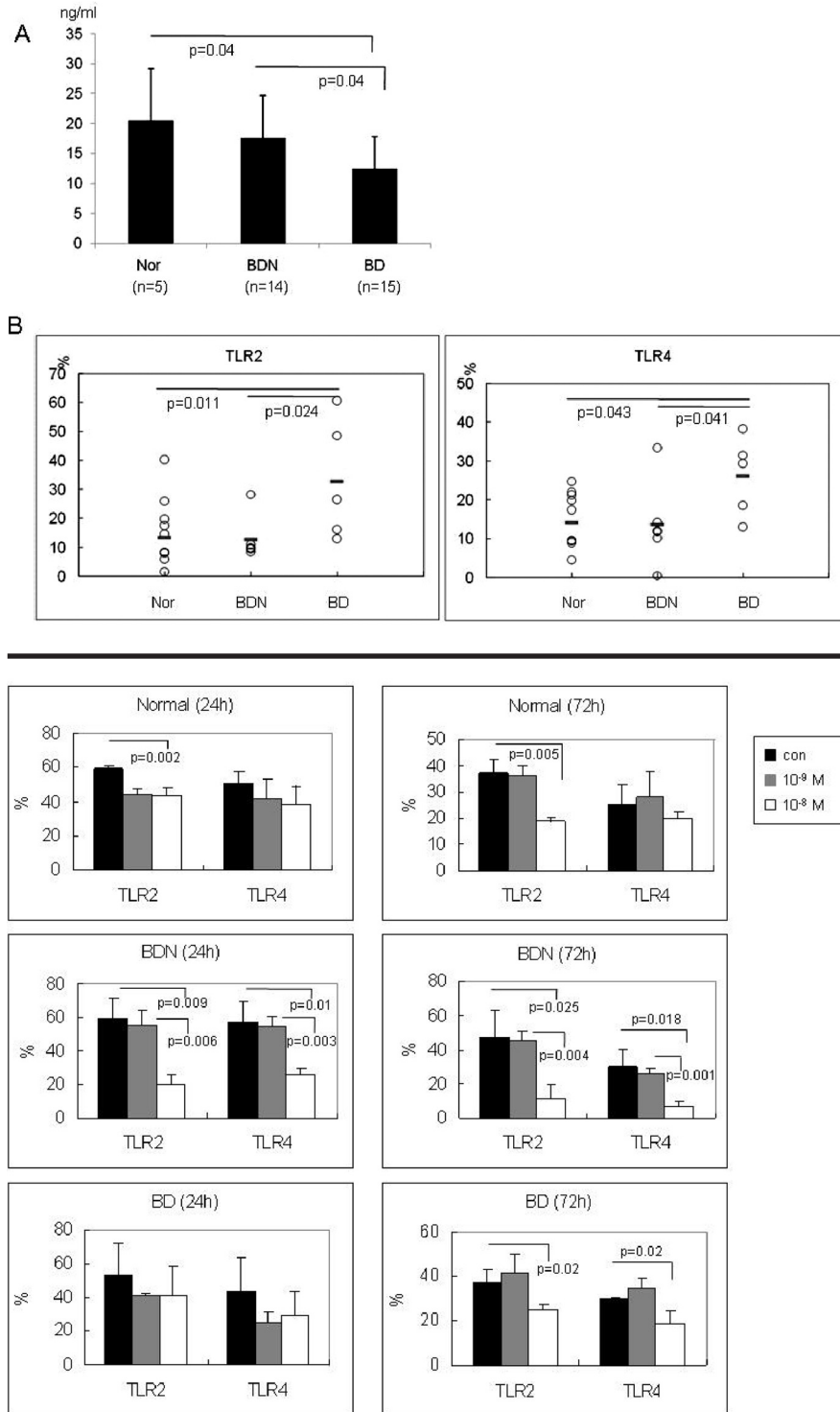


Fig 2. $1,25(\text{OH})_2\text{D}_3$ down regulated the expression of TLR2 and TLR4 in *in vitro* macrophage cultures from normal, BDN, and BD-like mice

TLR2 positive cells was $59.2 \pm 2.17\%$ in the non-treated control group and $43.6 \pm 4.8\%$ in the normal macrophage cultures that were treated with 10^{-8} M $1,25(\text{OH})_2\text{D}_3$ for 24 h ($p=0.002$). The frequency of TLR4 positive cells was $50.6 \pm 7.3\%$ in the control group and $38.8 \pm 10.1\%$ in the 10^{-8} M $1,25(\text{OH})_2\text{D}_3$ treated group of normal macrophage cultures ($p=0.06$). In macrophages from BD-like mice subjected to 72h of culture in 10^{-8} M $1,25(\text{OH})_2\text{D}_3$, the frequency of TLR2 was $25.0 \pm 2.7\%$, while it was $37.3 \pm 5.8\%$ in the control group ($p=0.02$). The level of TLR4 was $18.9 \pm 5.3\%$ in the 10^{-8} M $1,25(\text{OH})_2\text{D}_3$ treated culture, while it was $30.3 \pm 0.1\%$ in the control group ($p=0.02$). Overall, $1,25(\text{OH})_2\text{D}_3$ was found to suppress the expression of TLR2 and TLR4 in *in vitro* macrophage cultures of normal, BDN, and BD-like mice.

Oral administration of $1,25(\text{OH})_2\text{D}_3$ increased the serum levels of $25(\text{OH})\text{D}$ in a dose-dependent manner
To determine if oral administration of $1,25(\text{OH})_2\text{D}_3$ can increase the serum levels of $25(\text{OH})\text{D}$, 5 $\mu\text{g/kg}$ (1x) to 50 $\mu\text{g/kg}$ (10x) $1,25(\text{OH})_2\text{D}_3$ was administered to normal mice *in vivo* each day. After five days of consecutive treatment, a significant increase in the serum levels of $25(\text{OH})\text{D}$ in 2x, 5x, and 10x treated mice was observed when compared to the control mice ($27.0 \pm 7.1\text{ng/ml}$ in 2x, $36.3 \pm 4.4\text{ng/ml}$ in 5x, and $28.3 \pm 6.1\text{ng/ml}$ in 10x; $p=0.07$, $p=0.01$, and $p=0.05$ compared to the control) (Fig. 3A). C-reactive protein (CRP) was also analysed in these mice and found to be down-regulated in 2x treated mice ($2.94 \pm 0.35\text{ng/ml}$) when compared to DW treated control mice ($5.82 \pm 0.24\text{ng/ml}$) ($p=0.00006$). In 1x treated mice, three weeks of consecutive administration led to significant downregulation of the CRP level ($5.82 \pm 0.24\text{ng/ml}$ vs. $3.95 \pm 1.74\text{ng/ml}$) ($p=0.01$).

Changes in symptoms in BD-like mice after $1,25(\text{OH})_2\text{D}_3$ administration
Ten $\mu\text{g/kg}$ (2x) of $1,25(\text{OH})_2\text{D}_3$ were orally administered to mice for 10 days to determine if $1,25(\text{OH})_2\text{D}_3$ can affect the symptoms in BD-like mice. In six out of 11 mice, the mucocuta-

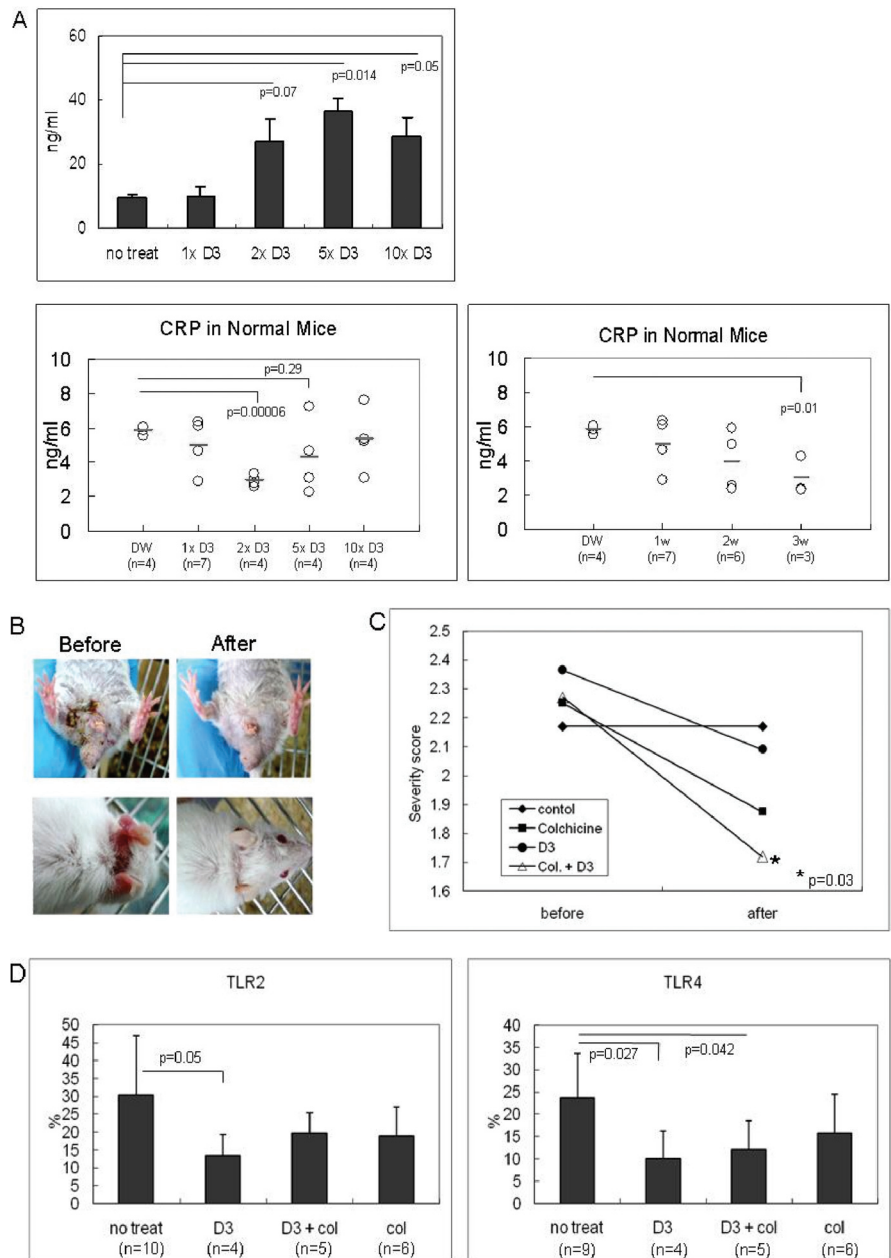


Fig 3. Oral administration of $1,25(\text{OH})_2\text{D}_3$ up-regulated the serum levels of $25(\text{OH})\text{D}$ and down-regulated CRP (A), ameliorated the BD-like mice symptoms (B), decreased the BD severity score (C), and down-regulated TLRs (D). A. 1x (5 $\mu\text{g/kg/day}$), 2x, 5x, and 10x $1,25(\text{OH})_2\text{D}_3$ was orally administered to mice for five consecutive days, after which the serum was analysed for $25(\text{OH})\text{D}$. C-reactive protein (CRP) was also analysed in these mice. B. 2x $1,25(\text{OH})_2\text{D}_3$ was orally administered to mice for ten consecutive days. The treated mice showed reduced BD-like symptoms. C. To compare the effect of $1,25(\text{OH})_2\text{D}_3$ and colchicine, and to evaluate the additive effect of $1,25(\text{OH})_2\text{D}_3$ and colchicine, 2 μg of colchicines/mouse was administered with or without $1,25(\text{OH})_2\text{D}_3$ to BD-like mice. The severity scores were compared before and after drug administration. D. After ten consecutive days of oral administration of $1,25(\text{OH})_2\text{D}_3$ to BD-like mice, peritoneal macrophages were isolated and analysed by flow cytometry for TLR2 and TLR4. Treatment with $1,25(\text{OH})_2\text{D}_3$ led to significant down-regulation of TLR2 and TLR4 in peritoneal macrophages of BD-like mice.

neous symptoms were improved (Fig. 3B) and the severity score changed from 2.36 ± 0.4 to 2.09 ± 0.8 ($p=0.4$); however, this change was not statistically significant (Fig. 3C). To compare the effects of $1,25(\text{OH})_2\text{D}_3$ and colchi-

cine and to determine the additive effect of $1,25(\text{OH})_2\text{D}_3$ and colchicine, 2 μg /mouse colchicine was administered to BD-like mice ($n=8$) with ($n=11$) or without $1,25(\text{OH})_2\text{D}_3$. Colchicine treatment changed the severity score from

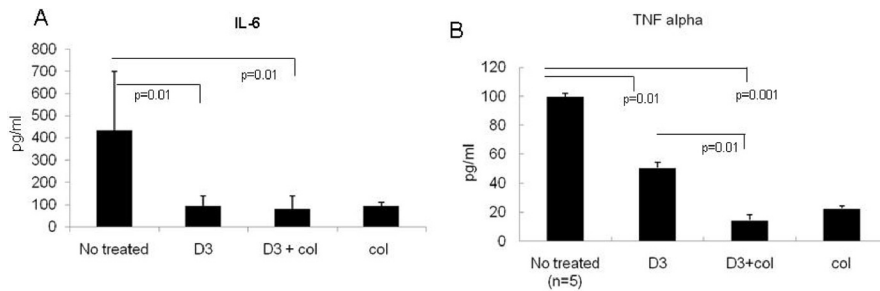


Fig 4. Serum level of IL-6 (A) and TNF- α (B) in 1,25(OH) $_2$ D $_3$ administered BD-like mice. After 10 consecutive days of oral administration of 1,25(OH) $_2$ D $_3$ to BD-like mice, the serum levels of IL-6 and TNF- α were analysed in BD-like mice by ELISA. IL-6 and TNF- α were significantly down-regulated in response to the treatment.

2.25 \pm 0.5 to 1.88 \pm 0.9 ($p=0.28$), while treatment with colchicine combined with 1,25(OH) $_2$ D $_3$ resulted in a change in severity from 2.27 \pm 0.5 to 1.72 \pm 0.7 ($p=0.03$). Combined treatment with colchicine and 1,25(OH) $_2$ D $_3$ was most effective and led to a significant decrease in the severity score (Fig. 3C).

TLRs in BD-like mice were down-regulated after 1,25(OH) $_2$ D $_3$ administration

After 10 consecutive days of oral administration of 10 μ g/kg 1,25(OH) $_2$ D $_3$ to BD-like mice, peritoneal macrophages were isolated and analysed by flow cytometry for TLR2 and TLR4. The frequency of TLR2 positive cells was 30.51 \pm 16.44% in non-treated controls ($n=10$) and 13.45 \pm 5.99% in 1,25(OH) $_2$ D $_3$ treated mice ($n=4$) ($p=0.05$). The frequency of TLR2 positive cells in the colchicine treated group was 18.87 \pm 8.17% ($n=6$), while it was 19.8 \pm 5.57% ($n=5$) in the combined colchicine and 1,25(OH) $_2$ D $_3$ group. The frequency of TLR4 positive cells was 23.82 \pm 9.74% in the control group ($n=9$), 10.19 \pm 6.09% in 1,25(OH) $_2$ D $_3$ treated mice ($n=4$), 15.91 \pm 8.6% in the colchicine treated group ($n=6$), and 12.21 \pm 6.27% in mice that were treated with a combination of colchicine and 1,25(OH) $_2$ D $_3$ ($n=5$) (Fig. 3D). Treatment with 1,25(OH) $_2$ D $_3$ led to significant down-regulation of TLR2 and TLR4 in peritoneal macrophages of BD-like mice. Combined treatment with colchicine and 1,25(OH) $_2$ D $_3$ did not show additive efficiency when compared to treatment with 1,25(OH) $_2$ D $_3$ alone with respect to the down-regulation of TLR2 and TLR4.

IL-6 and TNF- α ELISA in 1,25(OH) $_2$ D $_3$ treated BD mice

After ten consecutive days of oral administration of 1,25(OH) $_2$ D $_3$ to BD-like mice, serum levels of IL-6 and TNF- α were analysed. The serum IL-6 levels were 94.1 \pm 45.8ng/ml in the 1,25(OH) $_2$ D $_3$ administered group and 78.9 \pm 60.1ng/ml in the group treated with a combination of colchicine and 1,25(OH) $_2$ D $_3$, while it was 434.3 \pm 94.1ng/ml in the non-treated group (non-treated vs. 1,25(OH) $_2$ D $_3$, $p=0.01$; non-treated vs. combination, $p=0.007$) (Fig. 4A). Serum TNF- α levels were 50.5 \pm 2.5pg/ml in 1,25(OH) $_2$ D $_3$ treated BD mice and 22.2 \pm 2.8 pg/ml in mice treated with a combination of colchicine and 1,25(OH) $_2$ D $_3$, while it was 100.1 \pm 5.1 pg/ml in the untreated group (non treated vs. 1,25(OH) $_2$ D $_3$, $p=0.01$; non treated vs. combination, $p=0.01$; 1,25(OH) $_2$ D $_3$ vs. combination, $p=0.08$) (Fig. 4B). The serum TNF- α levels were down-regulated by oral administration of 1,25(OH) $_2$ D $_3$ and combined treatment with colchicine and 1,25(OH) $_2$ D $_3$. Combination treatment revealed an additive effect that down-regulated the serum levels of TNF- α when compared to 1,25(OH) $_2$ D $_3$ administration, and this was correlated with a decrease in the severity score of BD-like mice.

Discussion

It is well known that 1,25(OH) $_2$ D $_3$ modulates lymphocyte and macrophage functions (29, 30). In this study, we demonstrated the effects of 1,25(OH) $_2$ D $_3$ on the immune system by modulating TLR2 and TLR4 expression. The serum levels of 25(OH)D in BD-like mice were significantly lower

than in BDN mice. In BD patients, Do *et al.* found lower serum 25(OH)D levels in active BD patients than inactive BD patients and healthy controls (4). They also found that monocytes of active BD showed higher expressions of TLR2 and TLR4 than those of controls. In the present study, the frequencies of TLR2 and TLR4 in peritoneal macrophages were significantly higher in BD-like mice than in BDN mice. Nara *et al.* found that TLR2 was upregulated in intestinal tissues of active BD patients (18) and Kirino *et al.* found that TLR4 was upregulated in the PBMC of active BD patients (31). The use of TLR 2 and TLR 4 as possible proinflammatory markers (32) of T helper type 1 has been suggested (33). In the present experiment, 1,25(OH) $_2$ D $_3$ down regulated the expression of TLR2 and TLR4 in *in vitro* macrophage cultures from normal and BD-like mice. Additionally, 1,25(OH) $_2$ D $_3$ significantly down-regulated TLR2 and TLR4 expression in a dose-dependent manner in monocytes from human BD patients that were exposed to increasing concentrations of 1,25(OH) $_2$ D $_3$ ranging from 10 11 to 10 7 M (4). Sadeghi *et al.* also reported that vitamin D3 down-regulated monocyte TLR expression (6). Oral administration of 1,25(OH) $_2$ D $_3$ increased the serum level of 25(OH)D in a dose dependent manner in several disease groups. After ingestion of 3mg of vitamin D for 1 month, patients in an anticonvulsant therapy group showed a significant ($p<0.0001$) increase in 25(OH)D levels (34). 25(OH)D levels were also significantly higher in the treatment group in northern-dwelling patients with chronic kidney disease (35).

It is well known that monocytes and macrophages constitutively express high levels of TLR2 and TLR4. TLR plays a major role in the initiation of protective immune responses; however, the extensive release of TLR-triggered pro-inflammatory mediators may harm the host organism as it becomes clinically overt in cases of sepsis or autoimmune disease disorders. The influence of 1,25(OH) $_2$ D $_3$ on TLR2 and TLR4 expression on human monocytes has also been reported (6). Do *et al.* reported that there is a trend toward lower serum

25(OH)D levels in active BD patients when compared with controls. A higher prevalence of critically low 25(OH)D levels among active BD patients suggests vitamin D deficiency as a possible risk factor for disease activation and dysregulated inflammatory status. In the present study, 1,25(OH)₂D₃ administration to BD-like mice resulted in down-regulation of TLRs, improvement of BD-like symptoms, and a decreased severity score. Moreover, 1,25(OH)₂D₃ administration reduced the severity of HSV induced inflammation in a BD-like mouse model.

Colchicine is commonly prescribed to treat mucocutaneous diseases including BD (36). To observe the additive effect of colchicine and 1,25(OH)₂D₃ on regulation of TLR, combined treatment with colchicine and 1,25(OH)₂D₃ was administered to BD-like mice. Treatment of mice with 1,25(OH)₂D₃ resulted in a 40% reduction in TLR2 and a 24% reduction in TLR4 when compared to the distilled water treated control group, but this difference was only significant in the case of TLR2. Combination treatment did not have an additive effect on the down-regulation of TLR.

Interleukin-6 (IL-6) is a multifunctional cytokine secreted by lymphoid cells (T cells, B cells) and many nonlymphoid cells, including macrophages, bone marrow stromal cells, fibroblasts, keratinocytes, mesangium cells, astrocytes, and endothelial cells, that is also involved in regulation of the immune response and inflammation systems (21-24). IL-6 is highly elevated in the culture supernatants of peripheral blood mononuclear cells (PBMC) of patients with active BD (25), as well as in the cerebrospinal fluid of patients with neuro-BD (26) and BD with ocular involvement (27). Tumor necrosis factor- α (TNF- α) is a potent paracrine and endocrine mediator of inflammatory and immune functions. TNF- α over expression has been implicated in Behçet's disease (28). Overproduction of IL-6 has been shown to play a role in BD. Treatment with leflunomide and colchicine can result in a significant reduction of IL-6 in patients with a mucocutaneous type of BD (37). Knockdown of IL-6 by siRNA improved the BD-like mice symptoms in mice

(38). In the present study, 1,25(OH)₂D₃ administration led to significant down-regulation of the serum level of IL-6 in BD-like mice. TNF- α over expression has also been implicated in BD (39). TNF- α down-regulation was found to decrease symptoms in BD patients (40-43) and a BD-like mice model (9). BD-like mice treated with 1,25(OH)₂D₃ showed down-regulated TNF- α serum levels. Taken together, these findings and those of previous studies indicate that the down-regulation of IL-6 and TNF- α may be involved in the reduction of symptoms in BD-like mice and in patients.

The results of the present study demonstrated that the serum levels of 25(OH)D in BD-like mice were lower than in BDN, while TLR2 and TLR4 were higher in BD-like mice than in BDN. Additionally, 1,25(OH)₂D₃ administration increased the serum level of 25(OH)D in normal mice and decreased TLR expression in primary macrophages isolated from 1,25(OH)₂D₃ treated BD-like mice. Moreover, 1,25(OH)₂D₃ administration led to down-regulation of the serum level of IL-6 and TNF- α in BD-like mice, improved the symptoms in BD-like mice, and decreased the severity score. Taken together, the results of this study indicate that 1,25(OH)₂D₃ administration may be useful as a complementary therapeutic option for the treatment of inflammatory diseases, including BD.

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