Immunologic Research (2019) 67:48–57
https://doi.org/10.1007/s12026-018-9033-4

ORIGINAL ARTICLE

1,25-Dihydroxyvitamin D_3 modulates T cell differentiation and impacts on the production of cytokines from Chinese Han patients with early rheumatoid arthritis

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Published online: 24 October 2018

Abstract

To study the effects of 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) on the differentiation of T cells and the levels of cytokines in patients with early rheumatoid arthritis (eRA). The levels of Th1, Th2, Th17, and Treg cells were detected with BDFACS Calibur flow cytometer. The expression of IFN- , TNF-α, IL-2, IL-4, IL-6, IL-10, IL-17, and IL-22 was examined in 54 patients with eRA using a cytometric bead array (CBA). After 72 h of incubation of PBMCs from eRA patients with 1,25$(OH)_2D_3$, the levels of IFN- , TNF-α, IL-2, IL-6, and IL-17 significantly decreased compared to those of the control. 1,25$(OH)_2D_3$ had no significantly impact on the levels of IL-4, IL-10, and IL-22. The proportion of Th17 and the ratio of Th17/Treg significantly decreased in 1,25$(OH)_2D_3$-treated groups compared to those of the control. 1,25$(OH)_2D_3$ had no significantly impact on the proportion of Th1, Th2, Treg, and the ratio of Th1/Th2. Although no statistically significant difference was observed, proportion of Th1 was decreased after 1,25$(OH)_2D_3$ treatment compared with anti-CD3/CD28 only. The present study demonstrated that 1,25$(OH)_2D_3$ inhibited the synthesis of specific cytokines: Th1 (IFN-) and Th17 (IL-17, IL-22, IL-6, TNF-α) might upregulated Th2 cytokine (IL-4), which indicated the possible immunoregulatory roles and bone-sparing effects of 1,25$(OH)_2D_3$ in eRA through modulation of the Th1 and Th17 cytokine balance.

Keywords Vitamin D · Rheumatoid arthritis · T cells · Cytokines · Chinese Han

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology, in which genetic, environmental, and immunological factors participate [1]. It can affect joints and other organs as well. T cells have been postulated to play a fundamental role in RA. Patients affected by RA have self-targeted T cells and drive the immune system to induce inflammatory reactions in the peripheral tissues. One of the first events is the activation of antigen-dependent T cells, triggering an immune response classically considered to be of the Th1 type. This activation has multiple effects, including activation and proliferation of endothelial and synovial cells, recruitment and activation of proinflammatory cells, secretion of cytokines and proteases by macrophages and fibroblast-like synovial cells, and production of autoantibodies [2]. T lymphocytes secrete cytokines that directly induce the formation and differentiation of osteoclasts [3–5].

Vitamin D is a hormone with an immunoregulatory role that induces improvement in phagocytosis and reduces major histocompatibility complex class II DR in dendritic cells, while it induces maturity in natural killer cells and $CD4^+CD25^+FOXP^3$ T cells, with the ability to mediate immune tolerance, reducing the development of autoimmune disorders [6]. It is well known that 1,25-dihydroxyvitamin D_3(1,25$(OH)_2D_3$) can inhibit the cytokines such as interleukin (IL)-1, IL-6, IL-12, and tumor necrosis factor alpha (TNF-α) [7]. However, it is unclear what is involved in the T cell differentiation and the production of cytokines.

In the present study, using early RA (eRA) patients and healthy controls peripheral blood mononuclear cells (PBMCs), we studied effects of 1,25$(OH)_2D_3$ on T cell differentiation and cytokines. Methotrexate (MTX) is a common drug used in the treatment of RA because of its role of immune

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modulation [8, 9]. Therefore, the effects of combination of $1,25(OH)_2D_3$ and MTX on T cell differentiation as well as cytokines were also investigated.

Materials and methods

Subjects Fifty-four incident or newly diagnosed RA patients were recruited from the Department of Rheumatology of the Second Hospital of Shanxi Medical University (Taiyuan, China), including 18 males and 36 females with an age between 30 to 65 years old (Table 1). They all signed the informed consents and fulfilled the American College of Rheumatology revised criteria for RA [10]. None of the patients had ever used vitamin D, glucocorticoids, immunosuppressants, or a tumor necrosis factor antagonist prior to the study. All patients had normal liver and kidney functions. Eighteen healthy volunteers were used as healthy control, and gender and age were matched to the RA patients. The study was approved by the Research Ethics Committee of the Second Hospital of Shanxi Medical University and conducted in accordance with the Declaration of Helsinki (approval number 2013ky007).

Sample collection The isolated PBMCs were obtained as reported previously [11]. Peripheral venous blood was collected from fasting subjects in the early morning. Forty-five milliliters was placed in a tube with heparin sodium anticoagulant for extracting the peripheral blood mononuclear cells (PBMCs), and the remaining for extracting serum was placed in a tube without any anticoagulant. The blood samples without anticoagulant were kept at room temperature for 30 min to allow coagulating followed by centrifuging for 15 min at 1000 rpm. After centrifugation, the supernatants (serum) were removed and stored at − 80 °C for future experiments.

Table 1 Main characteristics of RA patients ($N = 54$)

Male	18 (33.3%)
Female	36 (66.7%)
Age (years)	46 ± 14.29
Disease duration (months)	14.83 ± 12.04
Tender joint counts	9.83 ± 5.57
Swelling joint counts	4.33 ± 2.67
Pain VAS score	5.66 ± 0.77
Patient's global assessment	7.33 ± 1.66
Physician's global assessment	5.16 ± 1.52
Erythrocyte sedimentation rate (mm/h)	52.5 ± 41.82
C reactive protein (mg/L)	30.79 ± 21.86
DAS28	4.9 ± 1.25
CDAI	28.33 ± 13.91
RAPID3	10.33 ± 6.37

Values are expressed as mean ± SD

VAS visual analog scales, *DAS28* Disease Activity Score in 28 joints, *CDAI* Clinical Disease Activity Index, *RAPID3* Routine Assessment of Patient Index Data

In vitro stimulation and PBMC culture Lymphocytes were isolated by anti-$CD4^+$ mAb using prestimulation of $CD3^+CD28^+$ in PBMCs. The purity of the $CD4^+$ T cells was greater than 90%, as assessed by flow cytometry. Trypan blue staining was used to confirm that cell viability was > 95%. The cells were suspended in phenol red-free Iscove's modified Dulbecco's medium (IMDM, Gibco, USA) supplemented with 10% charcoal-treated FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin, and the cell suspension was prepared at a density of 2×10^6/mL.

The PBMCs from healthy control and eRA patients were plated in a 96-well plate at 200 μL/well and then treated with either vehicle only or the combination of anti-CD3/CD28 antibody with $1,25(OH)_2D_3$ at various concentrations (D1 = 0.1 nM; D2 = 1 nM; D3 = 100 nM), MTX at various concentrations (M1 = 0.05 μg/mL; M2 = 0.5 μg/mL; M3 = 1 μg/mL), or with the combination of $1,25(OH)_2D_3$ and MTX (D2M2 group). $1,25(OH)_2D_3$ and/or MTX treatment was performed only in anti-CD3/CD28 antibody-treated cells. For the vehicle control, no stimulant was added to the wells, which meant that anti-CD3, anti-CD28, MTX, and $1,25(OH)_2D_3$ were not added to PBMCs. The final concentration of anti-CD3 was 300 ng/mL and of anti-CD28 was 400 ng/mL. The cells were cultured in a humidified, stable-temperature incubator at 37 °C with 5% CO_2 72 h after stimulation, and the cultures were harvested by centrifuging at 2000 rpm for 8 min. The supernatants were collected and stored at − 80 °C for subsequent cytokine determination.

Measurement of cytokines in the serum and cell culture supernatant Analysis of IFN- , TNF-α, IL-2, IL-4, IL-6, IL-10, IL-17, and IL-22 was conducted using a CBA human Th1/Th2/Th17 cytokine kit (BD Co, Ltd) and a BD FACSCalibur flow cytometer. Quantity (pg/mL) of respective cytokine was calculated using CBA software.

$CD4^+$ T cell subsets analyzed by flow cytometric analysis Cells were harvested and extracellularly stained with peridinin chlorophy-II A protein-(PerCP)-Cy5.5-conjugated CD4 mAb, followed by standard intracellular staining after fixative and permeabilization with 2% paraformaldehyde and 0.5% saponin. The following mAb were used from BD Biosciences: allophycocyanin (APC)-conjugated IFN- (clone 4S.B3), APC-conjugated TNF-α (clone MAb11), APC-conjugated CD4 (clone PRA-T4), APC-conjugated CD25 (clone M-A251), and phycoerythrin (PE)-conjugated IL-4 (clone 11B11), PE-conjugated IL-17 (clone SCPL1362), and PE-conjugated FOXP[3] (clone 259D/C7). Gating strategy used for fluorescence-activated cell sorting of $CD4^+$ T cells. Singlets were defined by forward scatter-area (FCS-A) and FCS-Height. Dead cells were then excluded and lymphocytes were gated based on FCS-A and side scatter (SSC)-A. Expression of $CD4^+IFN\gamma^+$ positive Th1, $CD4^+IL\text{-}4^+$ positive Th2, $CD4^+IL17A^+$ positive Th17, and $CD4^+CD25^+FOXP3^+$ positive Treg was determined by allophycocyanin(APC)-conjugated or phycoerythrin (PE)-conjugated antibodies using flow cytometry. Quality control protocols included running known standards each day before testing samples. In addition, the laboratory is enrolled in external quality assurance testing programs with the College of American Pathologists and the United Kingdom National External Quality Assurance Service.

Statistical analyses

SPSS 13.0 software was used for data analyses. Data were presented as mean and standard deviation. All data met the conditions for a normal distribution and homogeneity of variance. To compare two groups of data, a completely randomized, independent, a two-sample *t* test was used to compare multiple groups of data, a one-way ANOVA method of square-deviation was applied, and the Student-Newman-Keuls test was used to compare data among the groups. A P value < 0.05 was considered as significant.

Results

The comparison of serum levels of cytokines in eRA patients versus healthy control We examined the expression of cytokines in the serum of eRA patients and healthy control. Overall, there was a significant increase in IL-17,IL-22, IL-6, and TNF-α of RA patients when compared with those of healthy control (Table 2). Although IFN- , IL-2, and IL-4 showed a slight increase in eRA patients, no significant difference was observed. Further, the level of IL-10 was not significantly higher compared to that in healthy control.

Cytokines were induced by anti-CD3 /CD28 in the culture supernatant of PBMCs from eRA and healthy control Anti-CD3/CD28 is the activator of T lymphocytes. Our data revealed that cultured PBMCs from freshly collected peripheral blood of eRA and healthy control responded to the stimulation of anti-CD3/CD28 very well. The healthy controls' and eRA patients' PBMCs were divided into vehicle control and anti-CD3/CD28 groups.

In both eRA and healthy control samples, 72 h after anti-CD3/CD28 stimulation, the levels of IFN- , TNF-α, IL-6, IL-17, and IL-22 were significantly elevated compared to those without stimulation ($P < 0.05$, Table 2), although the level of IL-2 and IL-10 in anti-CD3/CD28 group showed a slight increase but the differences did not reach significance ($P > 0.05$, Table 3). Interestingly, the level of IL-4 increased significantly in eRA samples ($P < 0.01$) but slightly in healthy controls ($P > 0.05$, Table 3).

The effects of $1,25(OH)_2D_3$ on the levels of cytokines in the culture supernatant of stimulated PBMCs To determine whether $1,25(OH)_2D_3$ affected the expression of cytokines, we tested roles of three different doses (D1, D2, and D3 groups) of $1,25(OH)_2D_3$ in anti-CD3/CD28-treated PBMCs of eRA patients and healthy volunteers. Our data revealed that 72 h after incubation, the levels of IFN- , TNF-α, IL-2, IL-6, and IL-17 in culture supernatant of those PBMCs with $1,25(OH)_2D_3$ from eRA patients significantly decreased compared with anti-CD3/CD28 stimulation only ($P < 0.05$, Table 3) (Fig. 1). However, there was no significant difference in the levels of the previously mentioned five cytokines in three different dose groups, suggesting that inhibitions were not in dose-dependent manner ($P > 0.05$, Table 3). The treatment of $1,25(OH)_2D_3$ had an increased trend on the levels of IL-4 compared to anti-CD3/CD28 group of eRA patients, but the difference did not reach significance ($P > 0.05$, Table 4). Although the level of IL-22 decreased, or that of IL-10 increased, the difference did not reach significance ($P > 0.05$, Table 4). Further, of healthy controls, the level of TNF-α significantly decreased in $1,25(OH)_2D_3$-treated groups compared with the anti-CD3/CD28 group ($P > 0.05$, Table 4). However, there was no significant difference in the other seven cytokines mentioned

Table 2 The serum levels of cytokines in RA patients ($n = 54$) versus healthy control group ($n = 18$)

	IFN	TNF	IL-2	IL-4	IL-6	IL-10	IL-17	IL-22
RA	4.48 ± 3.48	5.91 ± 2.53	2.55 ± 0.52	2.68 ± 0.36	16.63 ± 12.00	3.00 ± 0.46	42.56 ± 6.43	51.36 ± 16.52
HC	3.13 ± 0.58	2.63 ± 0.27	2.33 ± 0.75	2.66 ± 0.31	4.16 ± 2.27	3.00 ± 0.45	21.10 ± 3.22	22.13 ± 14.04
t	1.31	4.43	0.72	0.10	3.48	− 0.28	7.63	3.70
P	0.215	< 0.01	0.49	0.93	< 0.01	0.78	< 0.01	< 0.01

Results are given as pg/mL (mean ± standard deviation). Samples were analyzed at least in triplicate

HC healthy control group, *RA* rheumatoid arthritis group

Table 3 The effect of anti-CD3/CD28 stimulation on the expression of cytokines in PBMCs of RA ($n = 54$) and healthy control group ($n = 18$)

	IFN		TNF		IL-2		IL-4		IL-6		IL-10		IL-17		IL-22	
	RA	HC	RA	HC	RA	HC	RA	HC	RA	HC	RA	HC	RA	HC	RA	HC
Vehicle control	13 ± 11	9 ± 4	13 ± 5	7 ± 0.6	17 ± 28	10 ± 6	5 ± 1	5 ± 2	2884 ± 1389	153 ± 303	314 ± 672	250 ± 191	46 ± 13	25 ± 4	46 ± 22	23 ± 14
Anti-CD3/CD28	5919 ± 2704	3812 ± 2029	508 ± 92	196 ± 53	36 ± 25	17 ± 13	9 ± 5	7 ± 5	8134 ± 2164	2608 ± 233	387 ± 336	298 ± 203	607 ± 52	250 ± 18	518 ± 320	119 ± 65
t	− 7.57	− 4.59	− 18.63	− 9.60	− 1.74	− 1.21	− 3.61	− 0.88	− 7.07	− 7.06	− 0.34	− 0.29	− 36.46	− 30.36	− 5.11	− 3.56
P	< 0.01	< 0.01	< 0.01	< 0.01	0.10	0.26	< 0.01	0.40	< 0.01	< 0.01	0.740	0.32	< 0.01	< 0.01	< 0.01	< 0.01

Results are given as pg/mL (mean ± standard deviation). *P* values = vehicle control versus anti-CD3/CD28 groups

RA rheumatoid arthritis group, *HC* healthy control group

above between $1,25(OH)_2D_3$-treated groups and anti-CD3/CD28 group ($P > 0.05$, Table 4).

The levels of cytokines in the culture supernatant of stimulated PBMCs treated with MTX MTX has been demonstrated to be effective in the treatment of patients with active RA [12]. Healthy volunteers and eRA patients' PBMCs were divided into anti-CD3/CD28 group and the anti-CD3/CD28 plus three different MTX-dose-treated groups M1, M2, and M3. Of eRA patients, 72 h after incubation, the levels of TNF-α and IL-17 from the PBMCs significantly decreased in MTX-treated groups compared with anti-CD3/CD28 group ($P < 0.05$, Table 4), but this was not dose-dependent manner ($P > 0.05$, Table 4). The levels of IFN- , IL-2, IL-6, IL-22, IL-4, and IL-10 did not reach significance ($P > 0.05$, Table 4) (Fig. 1). Further, of healthy controls, the level of IL-17 significantly decreased in MTX-treated groups compared with anti-CD3/CD28 group ($P < 0.05$, Table 4) (Fig. 1). However, there was no significant difference in the other seven cytokines.

The levels of cytokines in the PBMC culture supernatant of eRA patients and healthy control after cotreatment with $1,25(OH)_2D_3$ and MTX To determine coeffect of $1,25(OH)_2D_3$ and MTX, eRA patients and healthy volunteers' PBMCs were divided into anti-CD3/CD28 group and anti-CD3/CD28 + D2/M2 group. With the stimulation of anti-CD3/CD28, the cells were cotreated with MTX (M2) and $1,25(OH)_2D_3$(D2). Our data revealed that 72 h after incubation of PBMCs from eRA patients with D2M2 as compared with anti-CD3/CD28 only, the levels of IFN- , TNF-α, IL-6, IL-17, and IL-22 significantly decreased ($P < 0.05$, Table 4) (Fig. 1), the level of IL-4 in D2/M2 group significantly increased ($P < 0.05$, Table 4), and there were no significant changes in the levels of IL-2 and IL-10 ($P > 0.05$, Table 4). Further, in healthy controls, the levels of TNF-α and IL-17 also significantly decreased in the D2/M2-treated groups compared with anti-CD3/CD28 group ($P < 0.05$, Table 4). Also, there was no significant difference in the other six cytokines between the D2/M2-treated group and anti-CD3/CD28 group ($P > 0.05$, Table 4), indicating that the alterations of those cytokines were specific for stimulated T cells of eRA.

The levels of T cell differentiation induced by anti-CD3/CD28 in the cultures of eRA PBMCs In eRA, after 72-h stimulation, the levels of Th1, Th2, Th17, and Treg in the anti-CD3/CD28 group significantly were increased compared with the vehicle control group ($P < 0.05$, Table 5) (Fig. 1); the ratio of Th1/Th2 and Th17/Treg did not reach significance ($P > 0.05$, Table 5).

The levels of T cell subsets in the cultures of PBMCs from eRA patients treated with $1,25(OH)_2D_3$, MTX, and cotreatment with $1,25(OH)_2D_3$ and MTX We next tested whether $1,25(OH)_2D_3$, MTX, or cotreatment with $1,25(OH)_2D_3$ and

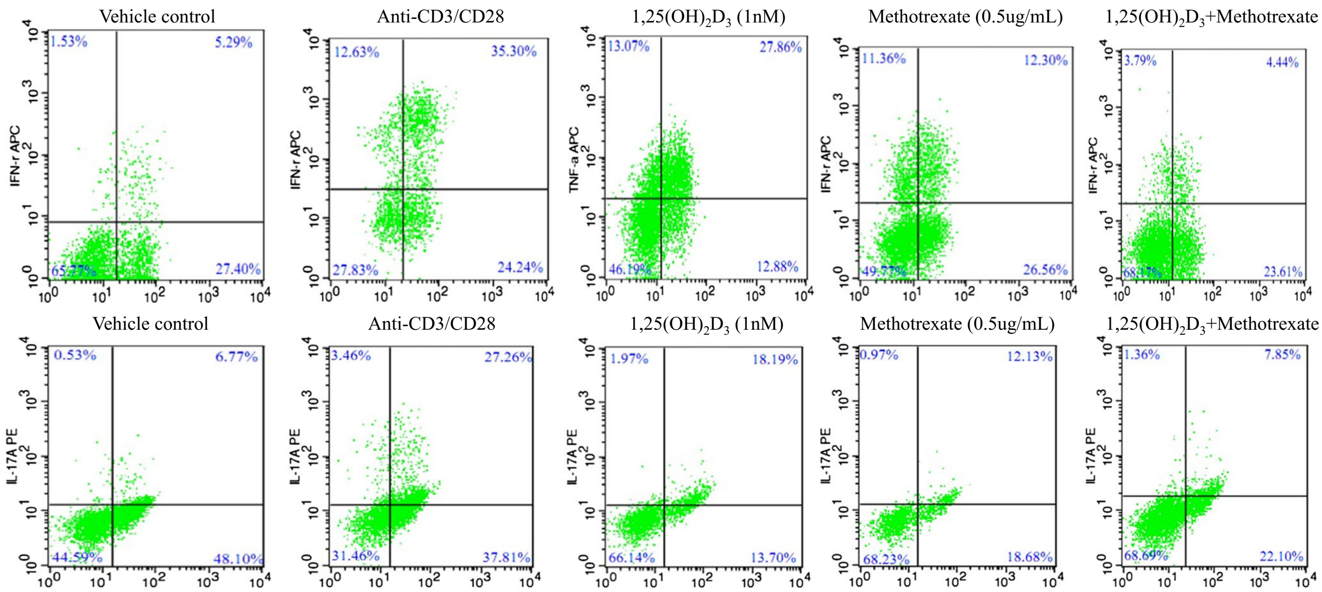

Fig. 1 Expression of CD4$^+$IFNγ^+ positive Th1 and CD4$^+$IL17A$^+$ positive Th17 in vehicle control, CD3/CD28 activated, 1,25(OH)$_2$D$_3$ (1 nM) treated, methotrexate (0.5 μg/mL) treated, and combination of 1,25(OH)$_2$D$_3$ and methotrexate groups

MTX affected T cell differentiation in anti-CD3/CD28-activated PBMCs of eRA patients at 72 h. The proportions of Th17 and the ratio of Th17/Treg significantly decreased in 1,25(OH)$_2$D$_3$-treated groups compared with anti-CD3/CD28 only ($P < 0.05$, Table 6) (Fig. 1). However, there was no significant difference among three different dose groups, indicating that the inhibitions were not in dose-dependent manner ($P > 0.05$, Table 6). Although the level of Th1 and the ratio of Th1/Th2 showed decrease, the difference did not reach significance ($P > 0.05$, Table 6) (Fig. 1). Also, there were no significant differences in the levels of Th2 and Treg ($P > 0.05$, Table 6).

The levels of Th1 and Th17 and the ratio of Th1/Th2 and Th17/Treg significantly decreased in MTX-treated groups compared with anti-CD3/CD28 group in eRA patients ($P < 0.05$, Table 6). However, the inhibitions of MTX were not in dose-dependent manner ($P > 0.05$, Table 6). The alteration of Th2 and Treg did not reach significance ($P > 0.05$, Table 6).

The levels of Th1, Th17, and the ratio of Th17/Treg significantly decreased in D2/M2-treated group compared with anti-CD3/CD28 group ($P < 0.05$, Table 6). There was no significant change in the levels of Th2, Treg, and the ratio of Th1/Th2 in D2/M2-treated group compared to those in the anti-CD3/CD28 group in eRA ($P > 0.05$, Table 6).

Discussion

RA is one of the most prevalent chronic inflammatory diseases. Cells affecting the development of generalized infiltration of the synovium are neutrophils, macrophages, T and B cells, and DCs. Furthermore, cytokines like interleukin-1α (IL-1α), IL-1β, IL-17, tumor necrosis factor-α (TNF-α), interferon- (IFN-), and IL-6, released and circulating from the inflamed joint, cause widespread articular cartilage and bone destruction [13]. Also, in recent years, many studies have been carried out on the roles of cytokines in the pathogenesis of RA [14, 15]. Cytokines active in inflammation and immunity may also play a role in physiologic and pathologic resorption of bone. In this paper, we evaluated RA patients at the very beginning of their disease, therefore permitting us to investigate early immunological alterations in RA. Consistent with this idea, our study revealed firstly a significant increase in levels of IL-17, IL-22, IL-6, and TNF-α in the peripheral blood of eRA patients compared to age- and gender-matched control subjects. Previous literature reported increased homing of IL17A, IL-22, IL-6, and TNF-α cells in the inflamed synovium in more advanced RA [16]. And our finding suggests that, during the early phase, levels of these cells might increase in the peripheral blood and migrate to the synovium in the more advanced phase.

There is an increasing appreciation that vitamin D exerts broad regulatory effects on cells of the innate and adaptive immune system. These include reducing antigen presentation through reducing the activity of dendritic cells or promoting their tolerogenic phenotype, affecting the polarization of monocytoid cells, altering B cell function, decreasing chemokine gradients, and reducing tissue-specific homing [6, 17]. We demonstrated that 1,25(OH)$_2$D$_3$ affects Th cell polarization by inhibiting Th1 (IFN-gamma production) and augmenting Th2 cell development (IL-4 production), which further shifts the T cell response toward Th2 dominance [18, 19]. A significant literature in humans also indicates that vitamin D

Table 4 The impact of 1,25(OH)$_2$D$_3$, methotrexate, and cotreatment at various concentrations on cytokines in the RA ($n = 54$) and healthy control group ($n = 18$)

	IFN		TNF		IL-2		IL-4		IL-6		IL-10		IL-17		IL-22	
	RA	HC	RA	HC	RA	HC	RA	HC	RA	HC	RA	HC	RA	HC	RA	HC
Anti-CD3/CD28	5919 ± 2704	3812 ± 2029	508 ± 92	196 ± 53	36 ± 25	17 ± 13	9 ± 5	7 ± 5	8134 ± 2164	2608 ± 233	387 ± 336	298 ± 203	607 ± 52	250 ± 18	518 ± 320	119 ± 65
D1	5064 ± 2689	7728 ± 1858	424 ± 82	169 ± 50	15 ± 12	27 ± 28	12 ± 9	7 ± 2	5513 ± 3429	3229 ± 2030	491 ± 527	526 ± 145	533 ± 47	219 ± 36	457 ± 215	108 ± 70
D2	4703 ± 2725	5592 ± 3122	382 ± 79	175 ± 26	24 ± 22	8 ± 2	15 ± 14	10 ± 7	4555 ± 3157	2602 ± 1032	475 ± 543	537 ± 216	426 ± 55	211 ± 42	467 ± 229	86 ± 53
D3	1531 ± 955	1544 ± 380	326 ± 87	129 ± 19	12 ± 11	6 ± 1	9 ± 6	8 ± 3	3748 ± 1919	3236 ± 863	737 ± 719	2029 ± 1780	319 ± 86	207 ± 28	294 ± 101	81 ± 48
F	7.72	3.48	9.78	4.64	3.80	2.26	1.04	0.40	5.80	0.50	0.90	3.63	49.51	2.11	2.16	0.56
P	< 0.01	0.61	< 0.01	< 0.01	0.02	0.11	0.39	0.76	< 0.01	0.69	0.45	0.59	< 0.01	0.13	0.11	0.64
M1	3958 ± 2594	1848 ± 810	319 ± 74	161 ± 44	15 ± 12	17 ± 13	16 ± 12	25 ± 26	5255 ± 4309	1953 ± 355	527 ± 656	253 ± 168	452 ± 50	205 ± 21	353 ± 173	144 ± 90
M2	4773 ± 2129	3985 ± 2511	292 ± 59	145 ± 24	21 ± 15	27 ± 25	11 ± 7	16 ± 11	7255 ± 4456	2177 ± 316	380 ± 289	292 ± 185	372 ± 67	188 ± 15	442 ± 299	72 ± 22
M3	3796 ± 2959	4595 ± 1182	266 ± 64	128 ± 25	22 ± 28	17 ± 12	14 ± 10	7 ± 3	4706 ± 3391	3824 ± 2479	665 ± 957	221 ± 43	315 ± 104	188 ± 41	369 ± 204	55 ± 26
F	1.66	2.72	27.03	5.48	2.04	0.49	1.59	1.93	2.31	0.86	0.57	0.30	37.82	7.00	1.05	3.03
P	0.19	0.07	< 0.01	0.07	0.122	0.69	0.21	0.16	0.09	0.06	0.64	0.82	< 0.01	< 0.01	0.38	0.06
D2M2	1130 ± 1511	3574 ± 1121	294 ± 97	151 ± 32	20 ± 15	21 ± 18	21 ± 14	11 ± 6	3465 ± 2061	2427 ± 238	753 ± 11	605 ± 91	342 ± 59	198 ± 44	260 ± 98	94 ± 48
t	5.36	0.25	5.53	2.60	1.84	− 0.40	− 2.75	− 1.31	0.54	1.28	− 1.14	− 1.38	11.76	2.540	2.68	0.75
P	< 0.01	0.81	< 0.01	0.03	0.08	0.70	0.02	0.22	< 0.01	0.23	0.27	0.06	< 0.01	0.03	0.02	0.47

Results are given as pg/mL (mean ± standard deviation). 1,25(OH)$_2$D$_3$ at various concentrations (D1 = 0.1 nM; D2 = 1 nM; D3 = 100 nM); MTX at various concentrations (M1 = 0.05 μg/mL; M2 = 0.5 μg/mL; M3 = 1 μg/mL); the combination of 1,25(OH)$_2$D$_3$ and MTX (D2M2 group)

RA rheumatoid arthritis group, *HC* healthy control group, *M* methotrexate

Table 5 The effect of anti-CD3/CD28 stimulation on T cell differentiation in the PBMCs of RA ($n = 54$) and healthy control group ($n = 18$)

	Th1 (%)		Th2 (%)		Th1/Th2		Th17 (%)		Treg (%)		Th17/Treg	
	RA	HC	RA	HC	RA	HC	RA	HC	RA	HC	RA	HC
Vehicle control	4.98 ± 1.64	3.22 ± 2.12	5.10 ± 4.38	3.49 ± 3.71	3.30 ± 3.80	0.61 ± 0.50	9.21 ± 10.66	0.72 ± 0.18	3.10 ± 0.68	17.39 ± 7.09	0.76 ± 0.89	0.05 ± 0.18
Anti-CD3/CD28	23.44 ± 13.75	31.47 ± 7.43	16.15 ± 8.29	17.85 ± 8.60	1.57 ± 0.69	2.43 ± 1.73	19.27 ± 12.09	6.98 ± 0.71	20.60 ± 12.67	17.53 ± 4.01	1.33 ± 1.10	0.41 ± 0.06
t	− 4.62	− 8.955	− 4.08	− 3.755	1.54	− 2.479	− 2.16	− 20.925	− 4.78	− 0.042	− 1.395	− 13.672
P	< 0.00	< 0.001	< 0.001	0.008	0.15	0.033	0.04	< 0.001	< 0.001	0.967	0.178	< 0.001

Results are given as mean ± standard deviation. *P* values = vehicle control versus anti-CD3/CD28 groups

RA rheumatoid arthritis group, *HC* healthy control group

increases the activity of regulatory T cells to prevent the excessive activation of autoreactive T cells [12].

Previous reports identified that intraperitoneally injected $1,25(OH)_2D_3$ potentiated the anti-inflammatory effect of dexamethasone (DEX) and changed a Th1/Th17 to a Th2 and regulatory T cell profile in Th1-mediated colitis in mice [20]. The negative correlation between serum $1,25(OH)_2D_3$ levels and disease activity in RA patients suggests the involvement of vitamin D in the pathogenesis of RA [21–25]. Whether $1,25(OH)_2D_3$ can modulate T cell differentiation in RA patients is currently unknown. Our study demonstrated that the level of Th17 and Th17/Treg ratio significantly decreased in $1,25(OH)_2D_3$-treated groups compared with anti-CD3/CD28 group, although there was no significant dose-dependent effect. In contrast, $1,25(OH)_2D_3$ treatment did not have a significant effect on the level of Th1, Th2, and Treg or the ratio of Th1/Th2. Although the difference did not reach statistical significance, $1,25(OH)_2D_3$ treatment reduced the level of Th1 or the ratio of Th1/Th2 as Th1. Because $1,25(OH)_2D_3$ treatment did decrease the levels of Th1-related cytokines, a reasonable explanation is that $1,25(OH)_2D_3$ inhibits the cytokine production of Th1 cells more dramatically than the proliferation of the cells.

MTX is one of the commonly used DMARDs, and its mode of action is not fully established. A potential mechanism for the remission of joint inflammation may be attributable to diminished cytokine production [8, 9]. Our data confirmed the effect of MTX in different T cell subtypes, consistent with previous studies showed an effect of MTX on activated T cells in vitro. Moreover, a higher MTX dose did not lead to a greater effect on the level of Th1 and Th17 or the Th1/Th2 and Th17/Treg ratio from patients' PBMCs. Cotreatment with $1,25(OH)_2D_3$ and MTX reduced Th1 and Th17 levels and the Th17/Treg ratio; however, this reduction was not significant compared to MTX alone, consistent with $1,25(OH)_2D_3$ that showed nonsignificant improvement in efficacy outcomes compared to placebo in patients with active RA receiving stable doses of MTX [26]. As noted, our data were obtained in vitro, while the other in vivo. Buondonno et al. [16] reported that a single dose of cholecalcipherol (300,000 IU) combined with MTX significantly ameliorates patients general health. This may be due to the different study setting since we used different doses, indicating that further investigations were needed to determine the optimal dosage for both drugs.

To date, various biological agents targeting major pro-inflammatory cytokines upregulated in RA, such as TNF-α, IL-6, and IL-1, have been established, and each of these therapies promotes significant reduction of disease activity [27–29]. However, some populations of RA patients still suffer from continuous inflammation and joint problems [30]. Thus, crucial targets to inhibit disease activity in these cases have been sought. Hajjaj-Hassouni N carried out that absence of supplementation on vitamin D is related to higher

Table 6 The impact of 1,25(OH)$_2$D$_3$, methotrexate, and cotreatment at various concentrations on T-cells in RA ($n = 54$) and healthy control group($n = 18$)

	Th1 (%)		Th2 (%)		Th1/Th2		Th17 (%)		Treg (%)		Th17/Treg	
	RA	HC	RA	HC	RA	HC	RA	HC	RA	HC	RA	HC
Anti-CD3/CD28	23.44 ± 13.75	31.47 ± 7.43	16.15 ± 8.29	17.85 ± 8.60	1.57 ± 0.69	2.43 ± 1.73	19.27 ± 12.09	6.98 ± 0.71	20.60 ± 12.67	17.53 ± 4.01	1.33 ± 1.10	0.41 ± 0.06
D1	17.02 ± 13.10	27.80 ± 8.16	12.90 ± 5.58	22.44 ± 15.63	1.39 ± 1.08	2.08 ± 1.55	15.27 ± 8.49	5.88 ± 0.50	24.22 ± 12.32	17.68 ± 6.17	0.82 ± 0.65	0.37 ± 0.15
D2	16.60 ± 13.33	29.20 ± 7.50	17.39 ± 12.11	11.24 ± 9.15	1.17 ± 0.94	1.39 ± 1.13	12.29 ± 7.01	5.30 ± 0.70	23.99 ± 12.16	17.64 ± 3.74	0.64 ± 0.47	0.31 ± 0.08
D3	12.97 ± 11.61	28.41 ± 5.03	17.89 ± 14.29	16.92 ± 13.88	1.42 ± 2.00	2.91 ± 2.00	8.60 ± 4.93	5.53 ± 0.32	20.79 ± 5.48	21.09 ± 4.43	0.44 ± 0.23	0.27 ± 0.04
F	1.35	0.305	0.54	0.852	0.20	0.924	3.37	9.95	0.38	0.823	3.60	2.753
P	0.27	0.821	0.66	0.482	0.90	0.447	0.03	0.01	0.77	0.049	0.02	0.069
M1	13.20 ± 10.47	22.47 ± 7.12	20.12 ± 10.34	15.67 ± 6.90	0.75 ± 0.49	1.89 ± 1.19	12.00 ± 5.16	5.49 ± 0.32	17.95 ± 6.20	11.07 ± 5.82	0.79 ± 0.61	0.73 ± 0.54
M2	12.65 ± 6.50	20.89 ± 9.63	22.58 ± 14.38	5.17 ± 6.19	0.68 ± 0.32	14.28 ± 10.41	10.50 ± 5.26	4.97 ± 0.69	22.33 ± 14.79	11.56 ± 5.44	0.63 ± 0.48	0.57 ± 0.35
M3	11.49 ± 5.329	20.03 ± 1.75	21.14 ± 11.29	8.95 ± 5.97	0.62 ± 0.20	3.98 ± 3.53	7.75 ± 4.09	4.51 ± 0.58	19.71 ± 7.86	11.11 ± 5.72	0.43 ± 0.23	0.66 ± 0.58
F	3.99	3.276	0.72	4.236	11.06	6.499	5.35	19.539	0.33	2.124	3.72	0.598
P	0.01	0.042	0.55	0.018	< 0.01	0.003	< 0.01	< 0.01	0.80	0.129	0.01	0.624
D2M2	9.59 ± 8.78	15.03 ± 5.53	16.69 ± 14.93	6.63 ± 5.14	2.91 ± 4.14	1.01 ± 0.94	9.21 ± 4.88	4.48 ± 0.52	20.83 ± 7.70	11.11 ± 5.97	0.47 ± 0.27	0.73 ± 0.67
t	2.94	4.348	− 0.11	2.744	− 1.11	1.768	2.67	6.047	− 0.05	2.188	2.59	− 1.171
P	< 0.01	0.001	0.91	0.021	0.29	0.108	0.02	< 0.01	0.96	0.054	0.02	0.293

Results are given as mean ± standard deviation. 1,25(OH)$_2$D$_3$ at various concentrations (D1 = 0.1 nM; D2 = 1 nM; D3 = 100 nM); MTX at various concentrations (M1 = 0.05 μg/mL; M2 = 0.5 μg/mL; M3 = 1 μg/mL); the combination of 1,25(OH)$_2$D$_3$ and MTX (D2M2 group)

HC healthy control group, *M* methotrexate prevalence of vitamin D deficiency in a COMORA study from 15 countries [31]. Despite some controversies [32], the majority of reports reinforce the idea that the therapeutic value for the treatment of autoimmunity diseases [33]. A possible explanation for this observation could be precisely the 1,25$(OH)_2D_3$ screening and supplementation strategies employed both at the clinic but also at national level, minimizing the risk of 1,25$(OH)_2D_3$ deficiency, even though there is no consensus concerning the amount that should be indicated.

In summary, RA is a Th1- or a Th17-associated disorder. As an immunomodulatory drug, 1,25$(OH)_2D_3$ can be used in disease prevention without causing systemic imunosuppression. The present study demonstrates that 1,25$(OH)_2D_3$ inhibits T lymphocyte production particularly in Th1 cells and Th17 cells, which further shifts the T cell response toward Th2 and Treg dominance by PBMCs with early RA. Our observations in vitro have demonstrated that 1,25$(OH)_2D_3$ inhibits the synthesis of Th1 cytokines IFN- , Th17 cytokines IL-17, IL-22, IL-6, and TNF-α.

The balance between proinflammatory and anti-inflammatory cytokines may be more important with regard to the systemic effects of cytokines on bone formation by osteoblasts than the effects on single cytokines. Based on the above research, our data indicate that the possible immunoregulatory role and bone-sparing effects of 1,25$(OH)_2D_3$ in RA through modulation of the Th1/Th17 and Th2 cytokine balance. Thus, a better appreciation of the effects of vitamin D on the continued disease activity of RA might lead to future inexpensive therapies that could contribute to the treatment of RA beyond its effects on bone and calcium metabolism. This finding would need to be further confirmed with larger numbers.

Acknowledgments All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be submitted for publication, and took responsibility for the integrity of the data and the accuracy of the data analysis. Study conception and design: Hong-yan Wen. Acquisition of data: Hong-yan Wen, Jing Luo, Xiao-feng Li, Dan-dan Wei, and Yang Liu. Analysis and interpretation of data: Hong-yan Wen. Manuscript preparation: Hong-yan Wen. Statistical analysis: Hong-yan Wen. The authors thank Dr. Alexis Ogdie for reviewing the manuscript.

Funding This study was supported by research grants from the National Nature Science Foundation of China (no.81301532/H0603), Fund Program for the Scientific Activities of Selected Returned Overseas Professionals in Shanxi Province-2017, the Shanxi Science and Technology Research Projects of China (no. 201603D321074), and the Shanxi Scholarship Council of China (2017-119).

Compliance with ethical standards

The study was approved by the Research Ethics Committee of the Second Hospital of Shanxi Medical University and conducted in accordance with the Declaration of Helsinki (approval number 2013ky007).

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

References

1. Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. Lancet. 2016;388:2023–38.
2. Wen H, Baker JF. Vitamin D immunoregulation, and rheumatoid arthritis. J Clin Rheumatol. 2011;17:102–7.
3. Horwood NJ, Kartsogiannis V, Quinn JM, Romas E, Martin TJ, Gillespie MT. Activated T lymphocytes support osteoclast formation in vitro. Biochem Biophys Res Commun. 1999;265:144–50.
4. Choi Y, Woo KM, Ko SH, Lee YJ, Park SJ, Kim HM, et al. Osteoclastogenesis is enhanced by activated B cells but suppressed by activated CD8(+) T cells. Eur J Immunol. 2001;31:2179–88.
5. Dai SM, Matsuno H, Nakamura H, Nishioka K, Yudoh K. Interleukin-18 enhances monocyte tumor necrosis factor alpha and interleukin-1beta production induced by direct contact with T lymphocytes: implications in rheumatoid arthritis. Arthritis Rheum. 2004;50:432–43.
6. Rosen Y, Daich J, Soliman I, Brathwaite E, Shoenfeld Y. Vitamin D and autoimmunity. Scand J Rheumatol. 2016;45:439–47.
7. Gardner DH, Jeffery LE, Soskic B, Briggs Z, Hou TZ, Raza K, et al. 1,25(OH)2D3 promotes the efficacy of CD28 costimulation blockade by abatacept. J Immunol. 2015;195:2657–65.
8. Jekic B, Vejnovic D, Milic V, Maksimovic N, Damnjanovic T, Bunjevacki V, et al. Association of 63/91 length polymorphism in the DHFR gene major promoter with toxicity of methotrexate in patients with rheumatoid arthritis. Pharmacogenomics. 2016;17:1687–91.
9. Kremer JM, Lawrence DA, Hamilton R, McInnes IB. Long-term study of the impact of methotrexate on serum cytokines and lymphocyte subsets in patients with active rheumatoid arthritis: correlation with pharmacokinetic measures. RMD Open. 2016;2:e000287.
10. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 1988;31:315–24.
11. Kim KW, Kim HR, Park JY, Park JS, Oh HJ, Woo YJ, et al. Interleukin-22 promotes osteoclastogenesis in rheumatoid arthritis through induction of RANKL in human synovial fibroblasts. Arthritis Rheum. 2012;64:1015–23.
12. Luo J, Wen H, Guo H, Cai Q, Li S, Li X. 1,25-dihydroxyvitamin D3 inhibits the RANKL pathway and impacts on the production of pathway-associated cytokines in early rheumatoid arthritis. Biomed Res Int. 2013;2013:101805.
13. Janssen KM, Westra J, Chalan P, Boots AM, de Smit MJ, van Winkelhoff AJ, et al. Regulatory CD4+ T-cell subsets and anti-citrullinated protein antibody repertoire: potential biomarkers for arthritis development in seropositive arthralgia patients? PLoS One. 2016;11:e0162101.
14. Kokkonen H, Soderstrom I, Rocklov J, Hallmans G, Lejon K, Rantapaa Dahlqvist S. Up-regulation of cytokines and chemokines predates the onset of rheumatoid arthritis. Arthritis Rheum. 2010;62:383–91.
15. Wang W, Shao S, Jiao Z, Guo M, Xu H, Wang S. The Th17/Treg imbalance and cytokine environment in peripheral blood of patients with rheumatoid arthritis. Rheumatol Int. 2012;32:887–93.
16. Buondonno I, Rovera G, Sassi F, Rigoni MM, Lomater C, Parisi S, et al. Vitamin D and immunomodulation in early rheumatoid arthritis: a randomized double-blind placebo-controlled study. PLoS One. 2017;12:e0178463.
17. Ishikawa LL, Colavite PM, Fraga-Silva TF, Mimura LA, Franca TG, Zorzella-Pezavento SF, et al. Vitamin D deficiency and rheumatoid arthritis. Clin Rev Allergy Immunol. 2017;52:373–388.

18. Mattner F, Smiroldo S, Galbiati F, Muller M, Di Lucia P, Poliani PL, et al. Inhibition of Th1 development and treatment of chronic-relapsing experimental allergic encephalomyelitis by a non-hypercalcemic analogue of 1,25-dihydroxyvitamin D(3). Eur J Immunol. 2000;30:498–508.
19. Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, O'Garra A. 1alpha,25-Dihydroxyvitamin d3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. J Immunol. 2001;167:4974–80.
20. Daniel C, Sartory NA, Zahn N, Radeke HH, Stein JM. Immune modulatory treatment of trinitrobenzene sulfonic acid colitis with calcitriol is associated with a change of a T helper (Th) 1/Th17 to a Th2 and regulatory T cell profile. J Pharmacol Exp Ther. 2008;324: 23–33.
21. Cutolo M, Otsa K, Laas K, Yprus M, Lehtme R, Secchi ME, et al. Circannual vitamin d serum levels and disease activity in rheumatoid arthritis: northern versus southern Europe. Clin Exp Rheumatol. 2006;24:702–4.
22. Haque UJ, Bartlett SJ. Relationships among vitamin D, disease activity, pain and disability in rheumatoid arthritis. Clin Exp Rheumatol. 2010;28:745–7.
23. Hong Q, Xu J, Xu S, Lian L, Zhang M, Ding C. Associations between serum 25-hydroxyvitamin D and disease activity, inflammatory cytokines and bone loss in patients with rheumatoid arthritis. Rheumatology (Oxford). 2014;53:1994–2001.
24. Sharma R, Saigal R, Goyal L, Mital P, Yadav RN, Meena PD, et al. Estimation of vitamin D levels in rheumatoid arthritis patients and its correlation with the disease activity. J Assoc Physicians India. 2014;62:678–81.
25. Rajaee E, Ghorbani A, Mowla K, Zakerkish M, Mohebi M, Dargahi-Malamir M. The relationship between serum level of vitamin D3 and the severity of new onset rheumatoid arthritis activity. J Clin Diagn Res. 2017;11:OC28–30.
26. Salesi M, Farajzadegan Z. Efficacy of vitamin D in patients with active rheumatoid arthritis receiving methotrexate therapy. Rheumatol Int. 2012;32:2129–33.
27. Mori T, Miyamoto T, Yoshida H, Asakawa M, Kawasumi M, Kobayashi T, et al. IL-1beta and TNFalpha-initiated IL-6-STAT3 pathway is critical in mediating inflammatory cytokines and RANKL expression in inflammatory arthritis. Int Immunol. 2011;23:701–12.
28. Choy EH, Isenberg DA, Garrood T, Farrow S, Ioannou Y, Bird H, et al. Therapeutic benefit of blocking interleukin-6 activity with an anti-interleukin-6 receptor monoclonal antibody in rheumatoid arthritis: a randomized, double-blind, placebo-controlled, dose-escalation trial. Arthritis Rheum. 2002;46:3143–50.
29. Jiang Y, Genant HK, Watt I, Cobby M, Bresnihan B, Aitchison R, et al. A multicenter, double-blind, dose-ranging, randomized, placebo-controlled study of recombinant human interleukin-1 receptor antagonist in patients with rheumatoid arthritis: radiologic progression and correlation of Genant and Larsen scores. Arthritis Rheum. 2000;43:1001–9.
30. Buch MH, Bingham SJ, Bryer D, Emery P. Long-term infliximab treatment in rheumatoid arthritis: subsequent outcome of initial responders. Rheumatology (Oxford). 2007;46:1153–6.
31. Hajjaj-Hassouni N, Mawani N, Allali F, Rkain H, Hassouni K, Hmamouchi I, et al. Evaluation of vitamin D status in rheumatoid arthritis and its association with disease activity across 15 countries: “the COMORA study”. Int J Rheumatol. 2017;2017:5491676.
32. Dankers W, Colin EM, van Hamburg JP, Lubberts E. Vitamin D in Autoimmunity: Molecular mechanisms and therapeutic potential. Front Immunol. 2016;7:697.
33. Franco AS, Freitas TQ, Bernardo WM, Pereira RMR. Vitamin D supplementation and disease activity in patients with immune-mediated rheumatic diseases: a systematic review and meta-analysis. Medicine (Baltimore). 2017;96:e7024.