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Impact of Vitamin D and Curcumin on CD4$^+$ and CD8$^+$ T cells Expressing CXCR3, CCR4, and CCR6 Chemokine Receptors in Patients with Relapsing-remitting Multiple Sclerosis

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ABSTRACT

Chemokines and their receptors play a central role in mediating the migration of pathogenic T cells into the central nervous system of patients with multiple sclerosis (MS). Vitamin D and curcumin are known to possess anti-inflammatory and immunomodulatory properties; however, their combined effects on T-cell chemokine receptor expression in MS remain poorly defined.

In this study, we investigated the in vitro effects of vitamin D, curcumin, and their combination on CD4$^+$ and CD8$^+$ T cells expressing CXCR3, CCR6, and CCR4 in patients with relapsing-remitting MS (RRMS). Peripheral blood mononuclear cells were collected from patients in relapse (n=10), remission (n=14), and healthy controls (n=15) and analyzed using flow cytometry.

Relapse patients exhibited elevated frequencies of CXCR3$^+$CD4$^+$ T cells compared to healthy controls, which normalized following treatment. Increased CCR6$^+$CD4$^+$ T cells and CXCR3$^+$CD8$^+$ T cells were also observed in patients, with a significant reduction achieved only after combined treatment with vitamin D and curcumin. The combined treatment further decreased the mean fluorescence intensity of CXCR3 and CCR6 on T cells in relapse patients, while vitamin D alone specifically reduced CCR4$^+$CCR6$^+$CD4$^+$ T cells, a T_H17-like subset enriched during relapse.

These findings indicate that vitamin D and curcumin, particularly in combination, modulate T-cell activity by downregulating chemokine receptor expression and may represent a promising adjunctive approach for controlling immune cell migration in MS.

Keywords: CD4-positive T-lymphocytes; Chemokine receptors; Curcumin; Multiple sclerosis; Vitamin D

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INTRODUCTION

Multiple sclerosis (MS) is one of the most prevalent autoimmune disorders affecting the central nervous system (CNS), impacting over 2.5 million people globally.[1,2] Initially, helper T (T_H)1 cells were believed to be the primary T cell subset involved in MS pathology. However, recent studies have identified other subsets, including T_H17 and cytotoxic T (T_C)17 cells, as critical contributors to CNS demyelination.[3,4] In contrast, the T_H2 population may exert a neuroprotective effect throughout the disease course.[5,6]

The migration of autoreactive T cells across the blood-brain barrier (BBB), mediated by interactions between chemokines and chemokine receptors, is considered a key process in the pathogenesis of MS. It is well-established that different T cell subsets express specific chemokine receptors.[7] For instance, the CXCR3 chemokine receptor is predominantly expressed on T_H1 cells, whereas T_H17 cells mainly express the CCR6 receptor. On the other hand, T_H2 cells express CCR4 and CCR3 as their main chemokine receptors. Notably, the CCR4 receptor is also expressed on a significant proportion of T_H17 cells, where it functions in conjunction with CCR6 to regulate their activity.[7–9] Consequently, many current MS therapies aim to downregulate the expression of these chemokine receptors.[10]

Vitamin D has demonstrated its potential as an immunomodulatory agent capable of inducing $CD4^+$ T cells to produce lower levels of interferon-γ (IFN-γ) and IL-17 and higher levels of IL-10 in relapsing-remitting MS (RRMS).[11–13] Additionally, it has been revealed that the active form of vitamin D downregulates the mRNA expression of certain chemokines in human adipocyte culture.[14] In experimental autoimmune encephalomyelitis (EAE), an animal model of MS, vitamin D treatment resulted in reduced expression of *CCL20*, *CCL22*, and *CCR4*.[8,14]

Curcumin, a bioactive compound found in turmeric, influences several aspects of cell signaling pathways involved in immune modulation, anti-tumor activities, and antioxidant responses.[15,16] Pre-treatment with curcumin before the onset of EAE has been shown to inhibit disease progression by reducing the secretion level of IL-17, IFN-γ, IL-12, and IL-23, while simultaneously upregulating $CD4^+CD25^+$*Foxp3*$^+$ regulatory T cells (Tregs) and IL-10 in lymphoid organs and CNS.[17] Curcumin has also been shown to inhibit the release of IL-6, MMP-9, and MCP-1, which are required for astrocyte activation and increased BBB permeability in MS.[18,19] Additionally, curcumin provides neuroprotection by preventing axonal degeneration by inhibiting *JNK* phosphorylation and nitric oxide (NO) release.[20] It has also been shown to protect the hippocampus from neuronal damage induced by fructose via inhibiting microglia activation and repressing the fractalkine/CXCR3 axis within the neuronal network.[21]

Despite the substantial evidence highlighting the benefits of vitamin D and curcumin in MS, the specific impact of these agents on T cells involved in MS pathogenesis, particularly those expressing particular chemokine receptors, has been largely understudied. Since T_H1 and T_H17 cells exert their pathogenic roles in MS partly through chemokine receptor–mediated migration, modulation of receptors such as CXCR3, CCR6, and CCR4 by vitamin D and curcumin may directly influence this imbalance and help restore immune regulation.[22] In the present study, we aim to investigate the in vitro effects of vitamin D and curcumin on $CD4^+$ and $CD8^+$ T cells expressing the chemokine receptors CXCR3, CCR6, and CCR4 in RRMS patients, compared to healthy controls. Furthermore, we will examine the influence of these substances on the expression pattern of these chemokine receptors.

MATERIALS AND METHODS

Participants

This study recruited 24 RRMS patients, diagnosed according to the McDonald criteria.[23] All patients were referred to Sina General Hospital in Tehran, Iran. Disability status for these patients was evaluated using the Expanded Disability Status Scale (EDSS). Of the RRMS patients, 10 were in the relapse phase, with inclusion criteria specifying no corticosteroid or immunomodulatory therapy for at least three months before the study. A relapse was defined as the onset of new neurological symptoms persisting for a minimum of 24 hours. Additionally, 14 patients in the remission phase, all of whom were receiving IFN-β therapy as their disease-modifying treatment and were free from relapse, were included in the study. The control group consisted of 15 healthy individuals matched by age and gender, with no familial history of autoimmune diseases. All participants, both patients and controls, were of Iranian Caucasian origin. Table 1.

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The study received ethical approval from the Ethics Committee of Tehran University of Medical Sciences under the approval number IR.TUMS.MEDICINE.REC.1396.4708, adhering to the ethical standards set forth in the Declaration of Helsinki. Written informed consent was obtained from all participants before enrollment in the study.

Cell Culture: Isolation and Treatment with Vitamin D and Curcumin

Peripheral blood mononuclear cells (PBMCs) were isolated using centrifugation on Ficoll-Hypaque gradients (Inno-train, Germany). The isolated cells were then cultured in a 24-well flat-bottom plate at a concentration of 1×10^6 cells/mL RPMI 1640 medium (GIBCO, Carlsbad, California, USA). The medium was supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 20 U/mL penicillin, and 20 µg/mL streptomycin to maintain optimal cell growth conditions.

To stimulate the cells, they were treated with purified anti-human CD3 (100 ng/mL) and anti-human CD28 (100 ng/mL) antibodies (MABTECH, Sweden). The cells were then incubated with 10 nM/mL of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2$D] (Sigma-Aldrich, USA) and/or 5 µM of curcumin (Sigma-Aldrich, USA) for 72 hours at 37°C in a 5% CO_2 environment.

The curcumin concentration was chosen based on preliminary experiments demonstrating its ability to modulate lymphocyte proliferation and its effects on cell viability across different concentrations (1, 5, 10 µM). The concentration of vitamin D used was based on previous studies that highlighted its optimal effects on immune cell modulation.[11]

Curcumin Dosage Selection

To determine the optimal concentration of curcumin for treatment, we tested three different concentrations (1, 5, 10 µM) on PBMCs at three different time points. As shown in Figure 1, after 24 and 48 hours of incubation, none of the curcumin concentrations significantly altered the proliferation of the main lymphocyte population. After 72 hours, the 1 µM concentration of curcumin still did not produce any significant change in lymphocyte proliferation compared to the control cell population (data for the control group not shown). In contrast, the 10 µM concentration of curcumin caused the main cell population to split into two groups, indicating cytotoxicity, as the increasing population in the gated area comprised dying cells. The 5 µM concentration was selected as the optimal dose because it effectively inhibited cell proliferation compared to the control group, while avoiding the cytotoxic effects observed at 10 µM. In contrast, the 1 µM concentration did not produce any measurable immunomodulatory activity, which is why it was not chosen.

Flow Cytometry

To analyze $CD4^+$ and $CD8^+$ T cells expressing chemokine receptors, the cultured cells were stained with mouse anti-human CD4-Alexa Fluor 488 (BioLegend, US), CD8-APC (BD Pharmingen, US), and CXCR3-PE.Cy5, CCR4-PE.Cy7 (BD Pharminogen, US) and CCR6-PE (eBioscience, US) monoclonal antibodies. The staining was performed in the dark at 4 °C for 30 minutes. Following incubation, the cells were washed with phosphate-buffered saline (PBS) and then analyzed using an Attune NxT flow cytometer (Thermo Fisher Scientific, USA). The acquired data were processed and analyzed using FlowJo software version 7.6.1 (Tree Star, USA) to determine the frequency and expression levels of the chemokine receptors on $CD4^+$ and $CD8^+$ T cells.

Statistical Analysis

Statistical analyses were conducted using the Statistical Package for Social Sciences (SPSS) software version 23 (SPSS Inc, Chicago, IL, USA). Data are presented as mean ± standard error of the mean (SEM). The normality of distribution for all numeric variables was assessed using the Kolmogorov–Smirnov test. Differences in the proportions of $CD4^+$ and $CD8^+$ T cells expressing specific chemokine receptors across different studied groups were analyzed using the one-way analysis of variance (ANOVA) method. Tukey's post hoc test was performed for multiple pairwise comparisons. A p value less than 0.05 was considered statistically significant.

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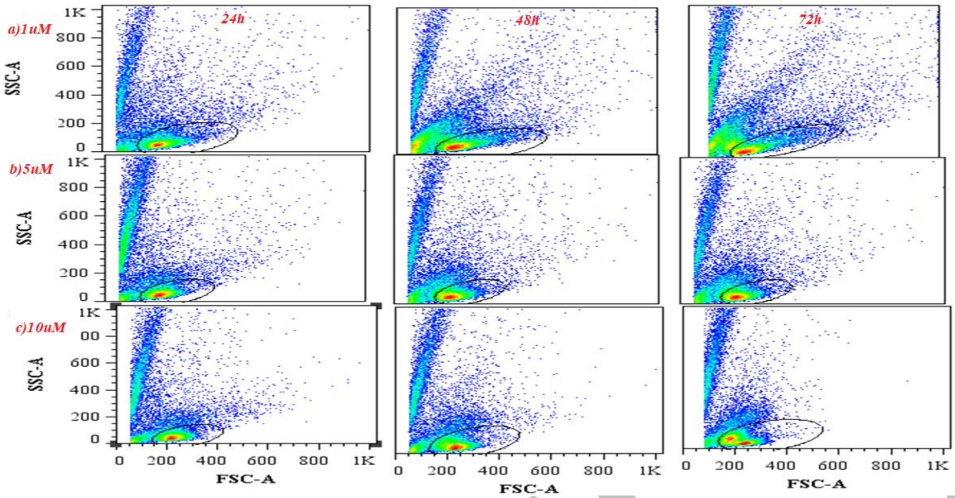

Figure 1. Curcumin Dosage Selection. The effect of different concentrations of curcumin (1, 5, 10 µM) on peripheral blood mononuclear cells (PBMCs) at three time points to determine its optimum dosage. A. Lymphocyte proliferation was not affected by 1 µM of curcumin. B. A reduction in lymphocyte proliferation was observed with 5 µM of curcumin. C. At 10 µM, curcumin induced cell death, as evidenced by dividing the main cell population into two distinct subpopulations.

RESULTS

A total of 24 MS patients and 15 healthy controls participated in this study. The demographic and clinical characteristics of the MS patients and controls are summarized in Table 1.

For each experiment, an average of 100 000 events was acquired from the cultured PBMCs. To exclude doublets, cells were initially gated based on forward scatter height versus width (FSC-H vs FSC-W). Subsequently, lymphocytes were selected from the singlet cell population, and different subsets of $CD4^+$ and $CD8^+$ T cells were identified based on the expression of chemokine receptors CXCR3, CCR6, and CCR4 (Figure 2).

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Effect of Vitamin D and Curcumin on $CXCR3^+CD4^+$ T Cells in Relapse Phase

A significantly higher frequency of $CXCR3^+CD4^+$ T cells, which are indicative of T_H1 cells, was observed in RRMS patients during the relapse phase compared to healthy controls (HCs) (p<0.05) (Figure 3). Following treatment with vitamin D, curcumin, as well as the combinations of vitamin D and curcumin, the proportion of $CXCR3^+CD4^+$ T cells in relapse patients decreased to the extent that the difference between this group and HCs was no longer statistically significant.

Table 1. Demographic and clinical characteristics of patients and controls.

Characteristics	RRMS patients		Healthy controls
	Relapse phase	Remission phase	
Number of subjects	10	14	15
Sex, female/male	6/4	8/6	8/7
Age, y	34.33±2.9	34±1.75	32.35±1.87
EDSS score	<2	<2	-
Plasmatic vitamin D, ng/mL	44±5.44	49.47±5.53	40.38±5.3

Data are presented as mean ± SEM. EDSS: Expanded Disability Status Scale; RRMS: relapsing-remitting multiple sclerosis; SEM: standard error of the mean.

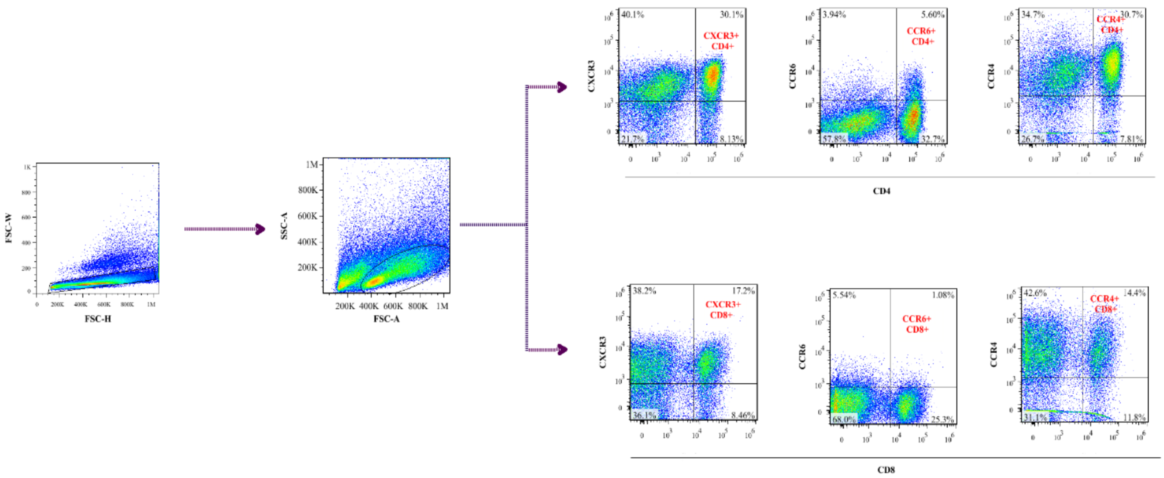

Figure 2. Representative gating strategy for CD4$^+$ and CD8$^+$ T-cell subsets based on chemokine receptor expression, CXCR3, CCR6, and CCR4. Cells were first gated for singlet cells (FCS-W vs FSC-H). Within the lymphocytes gate, CD4$^+$ and CD8$^+$ cells were analyzed for CXCR3$^+$, CCR6$^{+,}$ and CCR4$^+$ expression.

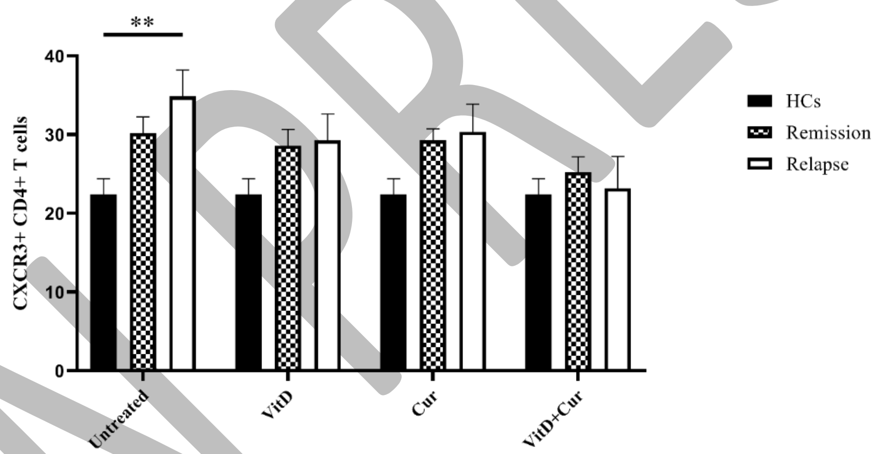

Figure 3. Effect of vitamin D and curcumin on the percentages of CD4$^+$ T cells expressing CXCR3 in different study groups. The frequency of CXCR3$^+$CD4$^+$ T cells within peripheral blood mononuclear cells isolated from relapsing-remitting multiple sclerosis patients was compared to that of healthy controls, following treatment with vitamin D and curcumin. All data are presented as mean±SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) by Tukey's Multiple Comparison Test. ****p<0.05, *****p**<0.01.**

Vitamin D alone exhibited a greater effect compared to curcumin, while the simultaneous administration of both agents led to an even greater reduction in CXCR3$^+$CD4$^+$ T cells than when either agent was applied individually (Figure 3). In contrast, remission-phase patients undergoing IFN-β therapy exhibited lower levels of CXCR3$^+$CD4$^+$ T cells compared to those in the relapse phase, but still had a higher frequency than healthy controls. However, the differences between remission patients, healthy controls, and relapse patients were not statistically significant.

Vitamin D and Curcumin Combination Decreased CCR6$^+$CD4$^+$ Cells in Patients at Relapse and Remission Phase

Relapse and remission patients had a higher frequency of CCR6$^+$CD4$^+$ cells compared to healthy controls in the untreated cells (p<0.01) (Figure 4).

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Vitamin D and curcumin alone were successful in decreasing the percentages of these cells, yet the significant differences still existed. Applying both vitamin D and curcumin together was able to decrease the amount of these cells to a level that the significant differences seen between relapse and remission groups with healthy controls were no longer there.

$CXCR3^+CD8^+$ Cells Were Decreased in Relapse and Remission Patients Following Vitamin D and Curcumin Combination Treatment

Patients in the relapse and remission phase also had more $CXCR3^+CD8^+$ T cells than healthy controls, which was statistically significant (p<0.05) (Figure 5). Just like $CCR6^+CD4^+$ cells, both Vitamin D and curcumin reduced the level of these cells in all patient groups; even though none of these reductions could eliminate the significant differences between these groups and healthy controls. Administering vitamin D and curcumin together reduced these cells in relapse and remission patients and erased the significant differences between patient groups and healthy controls.

CXCR3 and CCR6 Expression Intensity Were Modified by Vitamin D and Curcumin in Relapse Patients

To evaluate the effect of vitamin D and curcumin on chemokine receptor expression, we also analyzed the mean fluorescent intensity (MFI) of these molecules on $CD4^+$ and $CD8^+$ T cells in various experimental groups (Figure 6). As shown in Figure 7, although treatment with vitamin D, curcumin, or their combination reduced receptor expression levels within each group in almost all cases, only the reduction of CXCR3 and CCR6 expression intensity was significant in relapse patients when the combination of vitamin D and curcumin was applied (p<0.05).

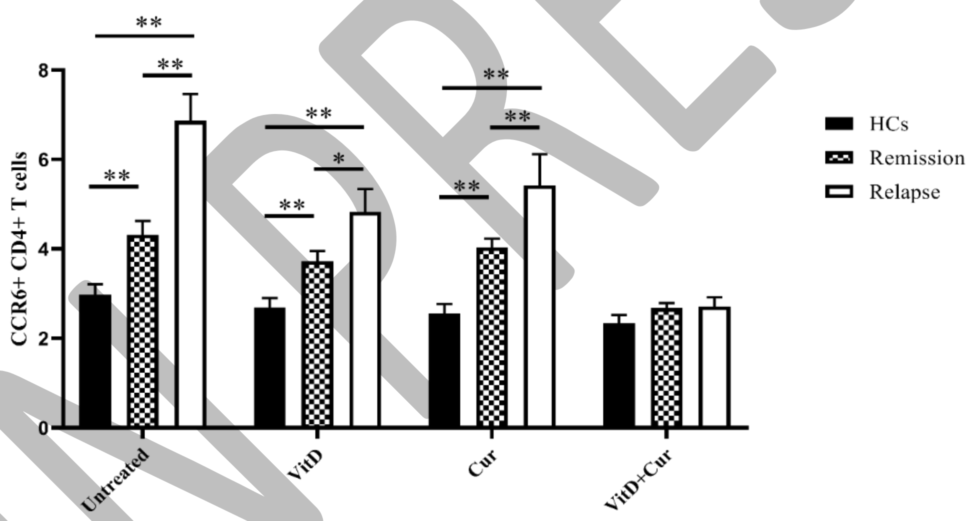

Figure 4. The effect of vitamin D (Vit D) and curcumin (Cur) on the percentages of $CD4^+$ T cells expressing CCR6 in relapsing-remitting multiple sclerosis (RRMS) patients and healthy controls. Peripheral blood mononuclear cells isolated from patients in relapse (n=10), remission (n=14), and healthy controls (n=15) were cultured and treated with vitamin D, Cur, or their combination for 72 hours. Flow cytometry analysis was performed to assess the frequency of $CCR6^+CD4^+$ T cells. Both vitamin D and curcumin alone reduced $CCR6^+CD4^+$ T cell percentages, but only the combined treatment normalized their levels to those of healthy controls. Data are shown as mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. *p<0.05, **p<0.01.

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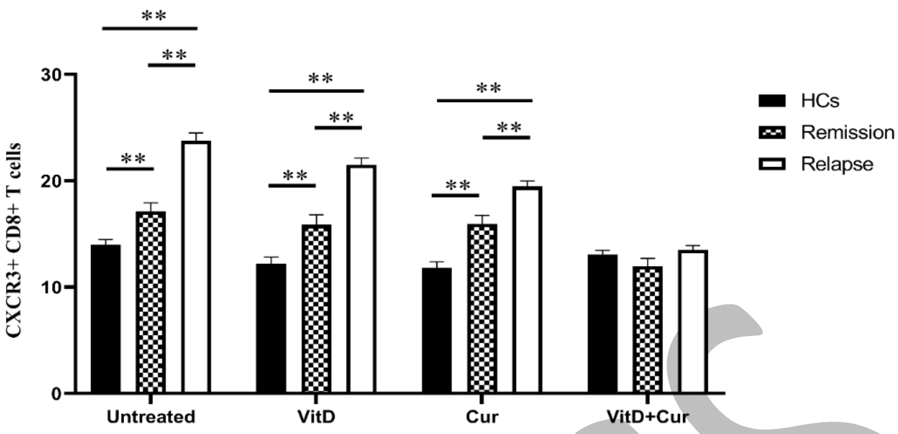

Figure 5. Effects of vitamin D (Vit D) and curcumin (Cur) on the percentages of CD8$^+$ T cells expressing CXCR3 in relapsing-remitting multiple sclerosis (RRMS) patients and healthy controls. PBMCs from relapse (n=10), remission (n=14), and control (n=15) groups were treated in vitro with vitamin D, Cur, or the combination for 72 hours. CXCR3$^+$CD8$^+$ T cells were quantified by flow cytometry. While vitamin D or curcumin alone reduced the frequency of CXCR3$^+$CD8$^+$ T cells, only the combined treatment eliminated the significant differences observed between patients and controls. Data are presented as mean ± SEM. One-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used. *p<0.05, **p<0.01.

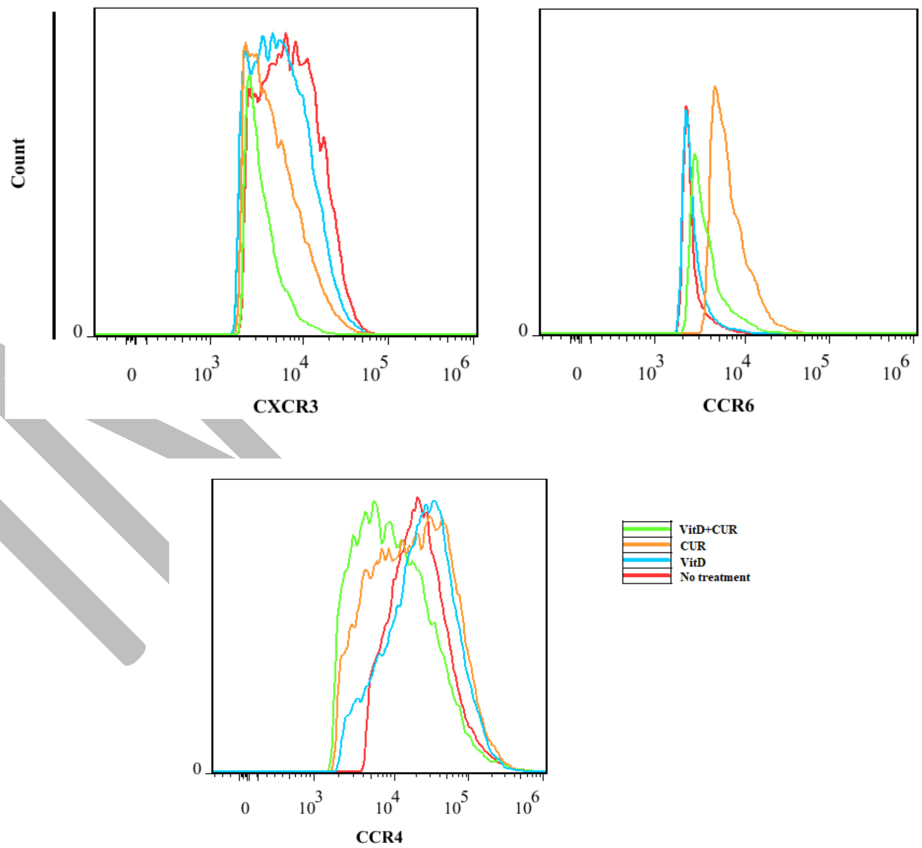

Figure 6. Mean fluorescent intensity (MFI) of chemokine receptors. The figure displays the gating strategy used for calculating changes in MFI of the chemokine receptors CXCR3, CCR6, and CCR4 following different treatments.

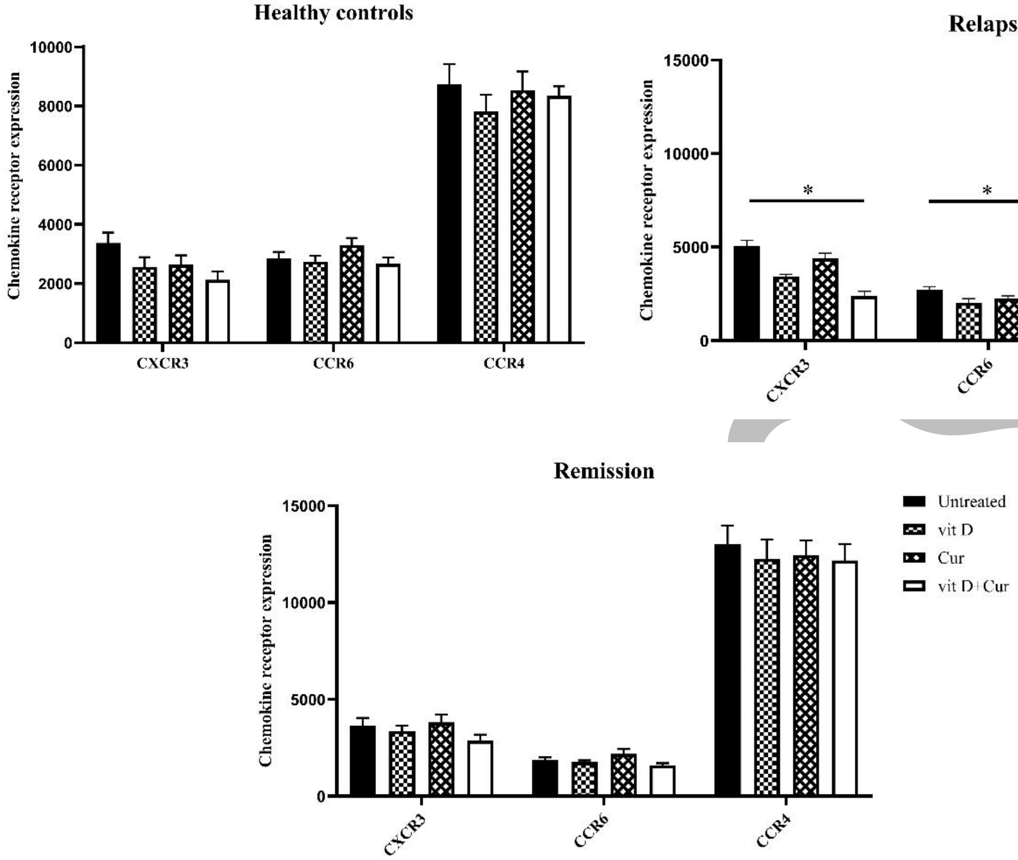

Figure 7. Changes in mean fluorescent intensity (MFI) of Chemokine Receptors following Treatments in the Studied Groups. As shown, treatment with vitamin D, curcumin, and their combination resulted in a reduction of chemokine receptor expression on T cells in nearly all cases. CXCR3 and CCR6 chemokine receptor expression intensities were significantly decreased in relapse patients undergoing vitamin D and curcumin combination treatments compared to their untreated state. The data shown here are for CD4$^+$ T cells in different groups. CD8$^+$ T cells data are not shown.

CCR4$^+$CCR6$^+$CD4$^+$ T Cell Levels Decrease in Relapsing Patients Following Vitamin D Treatment

CD4$^+$ T cells co-expressing CCR4 and CCR6 receptors play a critical role in the pathogenesis of various diseases, including acting as targets for HIV-1 infection. These cells are known to produce cytokines and transcription factors associated with T$_H$17 cells.[24] In this study, we examined the prevalence of CCR4$^+$CCR6$^+$ cells among CD4$^+$ and CD8$^+$ T cells to determine whether this subset of T cells differs between MS patients and healthy controls (Figure 8A). Relapsing MS patients exhibited significantly higher levels of CD4$^+$CCR4$^+$CCR6$^+$ T cells compared to those in remission and healthy controls (p<0.05) (Figure 8B). As shown in Figure 8B, following treatment with vitamin D, curcumin, and the combination of vitamin D and curcumin, a marked reduction in the proportion of CCR4$^+$CCR6$^+$CD4$^+$ T cells was observed in relapsing patients compared to both the remission group and healthy controls (p<0.01). These reductions eliminated the previously observed significant difference between relapsing patients and controls. In contrast, no significant differences were observed in the frequency of CD8$^+$CCR4$^+$CCR6$^+$ T cells between MS patients and healthy controls.

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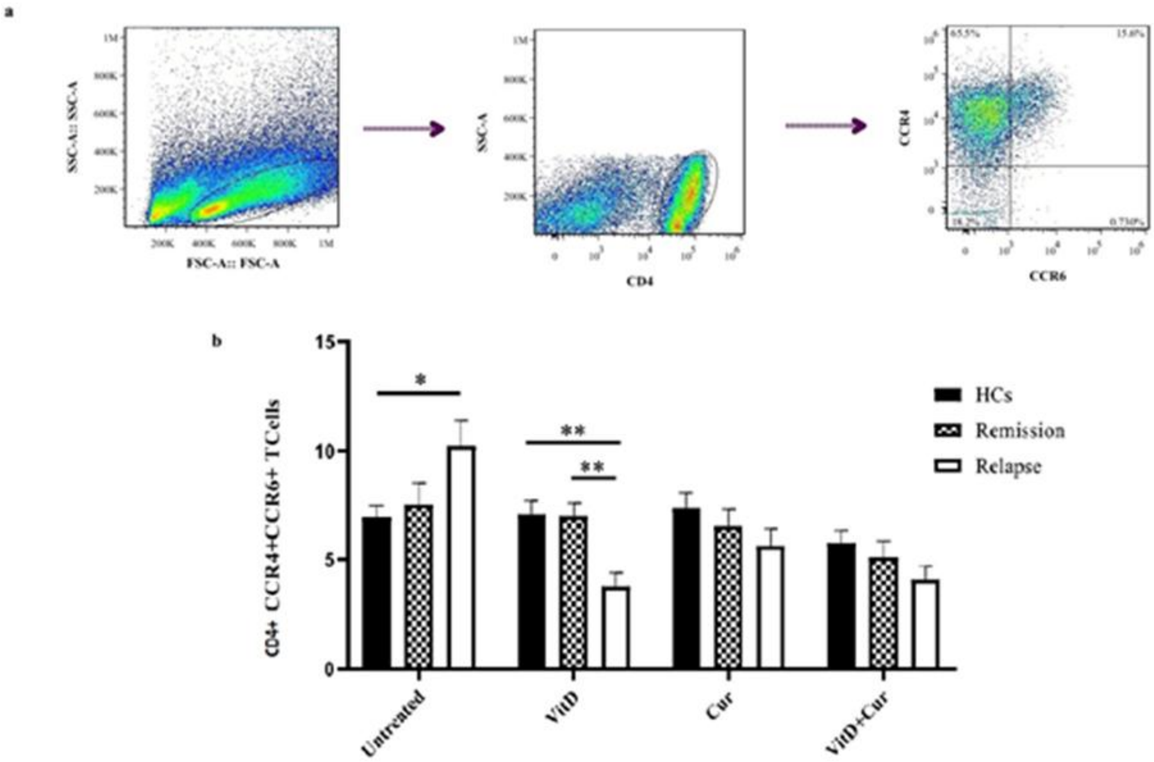

Figure 8. Effects of Vitamin D and Curcumin on the Percentage of CCR4⁺CCR6⁺CD4⁺ T Cells in the Studied Groups. A. Flow cytometric characterization of CCR4⁺CCR6⁺ T cells. B. Comparison of CCR4⁺CCR6⁺ T cells in different groups following various treatments. Data are presented as mean ± SEM and analyzed using one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test. *p<0.05, **p<0.01.

DISCUSSION

Multiple sclerosis is a demyelinating disorder characterized by an aberrant immune response, wherein helper T lymphocytes invade the CNS, leading to chronic inflammation. Autoreactive T cells that escape the negative selection process in the thymus and enter the peripheral circulation can migrate to the CNS upon recognizing specific antigens.[25] In most cases, these CNS antigens, or structurally similar ones, drive the differentiation of T lymphocytes into T_H1 and T_H17 subsets, both of which are implicated in MS pathogenesis.[26] One important mechanism facilitating the migration of these lymphocytes involves chemokines and their corresponding receptors.[27] Inhibition of inflammatory cell infiltration, enhancement of remyelination, and modulation of the inflammatory cascade may be achievable by targeting chemokines and their receptors.[28,29] Recent comprehensive reviews further emphasize the crucial roles of chemokines and their receptors in MS pathogenesis and their potential as therapeutic targets.[28,30] The present study demonstrated that vitamin D and Curcumin alone effectively reduced $CXCR3^+CD4^+$ T cells in patients experiencing a relapse, bringing their levels closer to those observed in healthy controls. Additionally, curcumin, when combined with vitamin D, resulted in a more pronounced reduction in $CD4^+CXCR3^+$ T cells in both relapsing and remission patients. These cells are considered critical contributors to the pathogenesis of neurodegenerative diseases, especially MS and its animal model, EAE pathogenesis.[31] The remission patients were under Betaferon therapy might be the reason why we didn't see a significant difference in $CD4^+CXCR3^+$ T cells between remission patients and healthy controls; as one of the major mechanisms through which Betaferon applies its effects is through promoting the shift of T cells from T_H1 to T_H2 and downregulating chemokine receptors like *CCR5,* which are also expressed by T_H1 cells.[32–34] Otherwise, a higher percentage of $CCR6^+CD4^+$ (considered as T_H17) and $CXCR3^+CD8^+$ (T_C1) cells were observed in relapse and remission patients compared to healthy controls, and vitamin D and curcumin together decreased the frequency of these cells. Even though the combination of vitamin D and curcumin could have this significant reduction effect, the application of each of these agents alone had no effect as significant as the combination effect. A plausible explanation for why vitamin D and curcumin alone could not have as much effect just like they had on $CXCR3^+CD4^+$ T cells is that the total frequency of these cells, as $CXCR3^+CD4^+$ T cells, is much more abundant than $CD4^+CCR6^+$ and $CD8^+CXCR3^+$ T-cells, and this higher number of cells is easier to manipulate than less abundant cells. CXCR3 and CCR6 chemokine receptors expression intensity defined by MFI in relapse patients decreased significantly while using the combination of vitamin D and curcumin and this can again come from the higher expression of these chemokines in relapse patients in untreated state and that applying anti-inflammatory agents like vitamin D and curcumin can reduce the expression of these chemokine receptors and as a result, less inflammation in MS patients' peripheral blood and CNS.

Another subset of T cells, $CCR4^+CCR6^+CD4^+$ T cells, which are classified as T_H17 cells based on their cytokine and transcription factor profiles,[24] was also assessed. We are the first to measure the frequency of $CD4^+$ and $CD8^+$ T cells co-expressing CCR4 and CCR6 in MS patients. Our findings revealed that relapsing patients had significantly higher levels of $CD4^+CCR4^+CCR6^+$ T cells compared to those in remission and healthy controls. Vitamin D treatment successfully reduced these cell levels in relapsing patients, even below those of remission patients and healthy controls. Curcumin and the combination of vitamin D and curcumin could decrease these cells as well, although the reduction effects were not as much as the reduction seen while using vitamin D. This stronger effect of vitamin D on $CCR4^+CCR6^+CD4^+$ T cells may be due to its more pronounced influence on T_H17 differentiation pathways, including suppression of T_H17-associated cytokines and transcription factors, compared to curcumin.[35,36] $CD8^+$ T cells expressing the same chemokine receptors didn't show significant differences between groups. A limitation of this study is the relatively small sample size, which may restrict the generalizability of the findings and warrants confirmation in larger patient cohorts.

One potential mechanism by which vitamin D and curcumin alleviate MS symptoms could be the downregulation of chemokine receptors, particularly those on $CD4^+$ and $CD8^+$ T cells, which are instrumental in exacerbating the disease state. Together, these findings suggest that vitamin D and curcumin, particularly in combination, may represent a promising adjunct therapeutic strategy for modulating pathogenic T-cell responses in MS, warranting further validation in clinical studies.

STATEMENT OF ETHICS

The research was approved by Tehran University of Medical Sciences' Ethics Committee (IR.TUMS.MEDICINE.REC.1396.4708) and conducted in accordance with the Declaration of Helsinki. All participants provided written informed consent prior to blood sampling and participation in the study.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

The data supporting the findings of this study are available from the corresponding author, MI, upon reasonable request.

AI ASSISTANCE DISCLOSURE

The authors declare that no artificial intelligence

(AI) tools or technologies were used in the design of the study, data collection, data analysis, or preparation of this manuscript.

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