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Curcumin prevents experimental autoimmune encephalomyelitis by inhibiting proliferation and effector CD4+ T cell activation

J.-Q. LIU[1], Y.-Q. YAN[1], J.-T. LIU[2], Y.-R. WANG[3], X. WANG[4]

[1]Department of Neurology, Institute of Brain Science, Medical School, Shanxi Datong University, Datong, China
[2]Department of Breast and Thyroid Surgery, Dalian Central Hospital, Dalian, China
[3]Department of Cardiology, Affiliated People's Hospital, Shanxi Medical University, Taiyuan, China
[4]Department of Rheumatology, The Second Hospital of Shanxi Medical University, Taiyuan, China

Abstract. – OBJECTIVE: Multiple sclerosis (MS) has affected over 2 million people worldwide and it is thought to be initiated by the activated central nervous system (CNS). Reactive CD4+ T cells (TH1, TH17, and Treg phenotypes) are crucial to MS. The TH1 phenotype can promote major histocompatibility complex-II expression and TH17 can induce inflammatory gene expression. Curcumin, a yellow pigment, is found in turmeric rhizomes and has been reported to have various activities, such as anti-proliferative and anti-inflammatory activity. Curcumin has great potential in MS treatment. Little is known about the effect of curcumin on MS. Therefore, we investigated the effect of curcumin on MS, especially on CD4+ T cells.

MATERIALS AND METHODS: CD4+ T cells (TH1, TH17, and Treg cells) were cultured in Iscove's Modified Dulbecco's Medium (IMDM) medium. Cell proliferation was evaluated by MTT assay. The ability of individual CD4+ T cells to aggregate into viable colony clusters was assessed by clonogenic survival assay. Apoptosis of CD4+ T cells was determined by flow cytometry. The expression of Bcl-2, Bax, and active caspase-3 was detected by Western blotting. The effect of curcumin on the activation molecule was also evaluated by flow cytometry.

RESULTS: MTT assay showed that curcumin significantly inhibited CD4+ T cell viability. Furthermore, TH1, TH17, and Treg all showed a dose-dependent but not time-dependent. The results of clonogenic survival assay revealed that curcumin markedly decreased the colony formation ability of CD4+ T cells. Flow cytometry results indicated that curcumin-induced remarkable apoptosis in TH1, TH17, and Treg cells. After treatment with curcumin, the expression of Bcl-2 was decreased and that of Bax and active caspase-3 was increased. Western blotting results also showed that curcumin-induced apoptosis in CD4+ T cells. Hence, our results demonstrated that curcumin inhibited CD4+ T cell proliferation via inducing apoptosis in CD4+ T cells. Meanwhile, flow cytometry results also showed that curcumin directly inhibited CD4+ T cell activation.

CONCLUSIONS: Curcumin could inhibit CD4+ T cell proliferation and effector cell activation.

Key Words:
Curcumin, Proliferation, CD4+ T, Multiple sclerosis, Apoptosis.

Introduction

Multiple sclerosis (MS), an inflammatory demyelinating disorder of the central nervous system (CNS), has affected over 2 million people worldwide[1]. MS is dominantly diagnosed at an average age of 30 years and thought to be initiated by activated CNS[2,3]. $CD4^+$ T cells are crucial to MS. In MS, reactive $CD4^+$ T cells that acquire the TH1, TH17, and Treg phenotypes always appear at the periphery[4]. The effector for phenotype is dictated by the composition and concentration of cytokines in which they were primed. The TH1 effector develops in the presence of the cytokines, including interleukin (IL)-2 and IL-12. TH17 is driven by the cytokine, including IL-6 and transforming growth factor-beta 1 (TGF-β1). Treg cells are driven by the cytokines IL-2 and IL-10[5-7]. In MS, the TH1 phenotype is considered to activate macrophage function and promote major histocompatibility complex (MHC)-II expression. The

Corresponding Authors: Yueru Wang, Ph.D; e-mail: 13834692303@163.com
Xin Wang, Ph.D; e-mail: wxalb@163.com

TH17 phenotype is thought to induce inflammatory gene expression and enhance peripheral TH17 migration. Treg cells are thought to be related to the occurrence of autoimmune diseases and their abnormal expression can lead to autoimmune diseases[8]. Animal models of experimental autoimmune encephalomyelitis (EAE) have widely been used for MS. It is necessary to identify effective agents to inhibit cell proliferation and induce cell death in $CD4^+$ T cells for MS treatment.

Curcumin, a yellow pigment, is found in turmeric rhizomes. Curcumin has no cytotoxic effects on healthy individuals and exhibits various properties, including anti-proliferative, anti-oxidant, analgesic, anti-inflammatory, antiseptic activities, and so on[9]. Curcumin has also been reported to induce apoptosis in malignant cells[10]. Meanwhile, previous studies have found that MS is driven by reactive $CD4^+$ T cells (TH1, TH17, and Treg)[11,12]. Apoptosis, a programmed cell death process, is very important in embryonic development and tissue homeostasis[13]. The Bcl-2 protein family is a central regulator of apoptosis and plays a role in controlling mitochondrial membrane potential. Bcl-2 and Bax are two important members of the Bcl-2 protein family. Bcl-2 is an anti-apoptotic protein and Bax is a pro-apoptotic protein[14,15]. Caspases are a family of cysteine proteases that respond to pro-apoptotic signals. Caspase-3 is one member of the caspase family and modulates the expression of some enzymes that participate in DNA fragmentation[16,17]. It is surprising that little is known about the effect of curcumin on $CD4^+$ T cells. Curcumin has great potential in MS treatment. The activation molecule, IL-2 receptor-α chain CD25, and CD44 have been reported to be important in cell proliferation[18]. Hence, the expression of both CD25 and CD44 was explored after treatment with curcumin. In this work, we investigated the effect of curcumin on $CD4^+$ T cells and whether the effect is related to apoptosis. Meanwhile, the effect of curcumin on the expression of CD25 and CD44 was also evaluated. The results showed that curcumin significantly inhibited $CD4^+$ T cell viability and the ability of individual cells to aggregate into viable colony clusters by inducing apoptosis, and suppressed $CD4^+$ T cell activation.

Materials and Methods

Materials

Iscove's Modified Dulbecco's Medium (IMDM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Dimethylsulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hoechst 33342, lysis buffer, and bicinchoninic acid (BCA) protein kit were from Beyotime Institute of Biotechnology (Shanghai, China). The enhanced chemiluminescence (ECL) kit and Annexin V-FITC (fluorescein isothiocyanate) apoptosis detection kit were obtained from Pharmingen-Becton Dickinson (San Diego, CA, USA). The mouse $CD4^+$ T cell isolation kit was from Stem Cell Technologies (Vancouver, Canada). The plate-bound α-CD3 and soluble α-CD28 were from BD Pharmingen (San Jose, CA, USA). IL-2, IL-12, IL-6, and TGF-β1 antibodies were from PeproTech (Rocky Hill, NJ, USA). Antibodies against α-IL-4 and α-IFNγ were from BioLegend (San Diego, CA, USA). IL-23 and IL-1β were purchased from R&D Systems (Minneapolis, MN, USA).

Mice

Mice, which have a transgenic T-cell receptor for MOG_{35-55} peptide (2D2 mice) were purchased from Harlan Laboratories and fed in our animal laboratory. Female C57BL/6 mice were obtained from the National Cancer Institute (NCI). Female mice were used for experiments. All animal studies were approved by the Animal Care and Use Committee of the Shanxi Medical University of Medicine.

T Cell Activation and Culture In Vitro

Female C57BL/6 mice were fed for 6-8 weeks. Spleens were harvested and naïve $CD4^+$ T cells were isolated using the mouse $CD4^+$ T cell isolation kit. For activation, $CD4^+$ T cells were plated at a density of 2×10^5 cells/well in flat-bottomed 24-well plates. $CD4^+$ T cells were maintained in IMDM containing plate-bound α-CD3 (5 μg/mL) and soluble α-CD28 (1 μg/mL). For TH1 polarization, cells were treated with IL-2 (5 ng/mL), IL-12 (10 ng/mL), and α-IL-4 (2.5 ng/mL). For TH17 generation, cells were treated with α-IL-4 (2.5 ng/mL), α-IFNγ (2.5 ng/mL), IL-6 (20 ng/mL), TGF-β1 (2.5 ng/mL), IL-23 (10 ng/mL), and IL-1β (10 ng/mL). For Treg generation, cells were treated with TGF-β1 (10 ng/mL), α-IL-4 (2.5 ng/mL), and α-IFNγ (2.5 ng/mL).

MTT Assay

TH0, TH1, TH17, and Treg were seeded at a density of 6000 cells/well in 96-well plates. After 24 h, cells were treated with curcumin (5, 10,

20, 40, and 60 μM) for 24 h or 40 μM curcumin for 24, 48, and 72 h. The control group was Phosphate-Buffered Saline (PBS) in IMDM. Then, MTT solution (20 μL, 0.5 mg/mL in PBS) was added to each well and the plates were incubated at 37°C for 4 h. Finally, the medium was removed and DMSO (150 μL) was added to each well for 10 min to dissolve the purple formazan crystals. Absorbance was measured at 570 nm using a microplate reader. Cell viability was determined based on the following formula: A_{570} Experiment/ A_{570} Control × 100%. All assays were performed in three independent experiments.

Clonogenic Survival Assay

TH0, TH1, TH17, and Treg cells were counted and plated in 12-well plates (3000 cells/well). After 24 h, the cells were treated with curcumin (10 μM) for 7 d. Then, cells were fixed with methyl alcohol (200 μL, -20°C) for 5 min at 4°C and stained with 0.1% crystal violet (200 μL). The plates were observed under a stereomicroscope (Olympus SZX16; Tokyo, Japan). The numbers of colonies (more than 50 cells/colony) were counted in 10 different microscopic fields. The colony formation rate was calculated based on the following formula: Colony formation rate = (Colonies/Seeded cells) × 100%.

Flow Cytometric Analysis

The apoptosis of TH0, TH1, TH17, and Treg cells induced by curcumin was determined by flow cytometry using the Annexin V-FITC and propidium iodide (PI) staining method. In brief, cells were treated with curcumin (10 and 20 μM) for 24 h. The cells were trypsinized and washed with cold PBS. Then, cells were re-suspended in binding buffer containing Annexin V-FITC (5 μL) and PI (2.5 μL) or CD25-PE (5 μL) and CD44-FITC (5 μL). The cell suspension was cultured in the dark for 30 min. Finally, cells were analyzed using the FACSort flow cytometer (Becton Dickinson, San Diego, CA, USA).

Western Blotting

The treated cells were lysed with lysis buffer containing 1% phenylmethylsulphonyl fluoride for 10 min and centrifuged at 13000 rpm to precipitate the insoluble material at 4°C for 15 min. An equal amount of protein (60 μg) was separated on 12% sodium dodecyl sulphate-polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). The membrane was blocked in Tris-Buffered Saline (TBS) containing 5% milk and Tween 20 for 1 h. Then, the membrane was incubated with anti-Bcl-2 (1:500), anti-Bax (1:500), and anti-active caspase-3 (1:500) antibodies overnight at 4°C. Next, the membrane was washed three times in TBS and then incubated with secondary antibodies (1:1000) for 1 h. Finally, the protein band was detected by ECL.

Statistical Analysis

The results were analyzed by Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA). All data were presented as the mean ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests. A p-value less than 0.05 ($p<0.05$) was considered statistically significant.

Results

Curcumin Inhibits CD4+ T Cell Proliferation

Curcumin has been reported to exhibit anti-proliferative activity[9]. To detect the effect of curcumin on $CD4^+$ T cell viability, TH0, TH1, TH17, and Treg cells were seeded in 96-well plates. Then, cells were treated with curcumin (5, 10, 20, 40, and 60 μM) for 24 h or 40 μM curcumin for 24, 48, and 72 h and stained with MTT solution. Absorbance was measured using a microplate reader. As indicated in Figure 1, curcumin treatment significantly inhibited $CD4^+$ T cell viability. Furthermore, TH1, TH17, and Treg cells all showed a dose-dependent decrease in cell viability, and Treg cells were more insensitive to the indicated concentration. The effect of curcumin on $CD4^+$ T cell viability was not time-dependent.

Meanwhile, the effect of curcumin on the ability of individual $CD4^+$ T cells to aggregate into viable colony clusters was evaluated by clonogenic survival assay. TH0, TH1, TH17, and Treg cells were treated with curcumin (10 μM) for 7 d and then stained with crystal violet. The results showed that curcumin markedly decreased the colony formation ability of $CD4^+$ T cells (Figure 2). These results demonstrate that curcumin decreases $CD4^+$ T cell proliferation.

Curcumin Induces Apoptosis in $CD4^+$ T Cells

To detect whether the curcumin-induced inhibition of $CD4^+$ T cell growth is related to apopto-

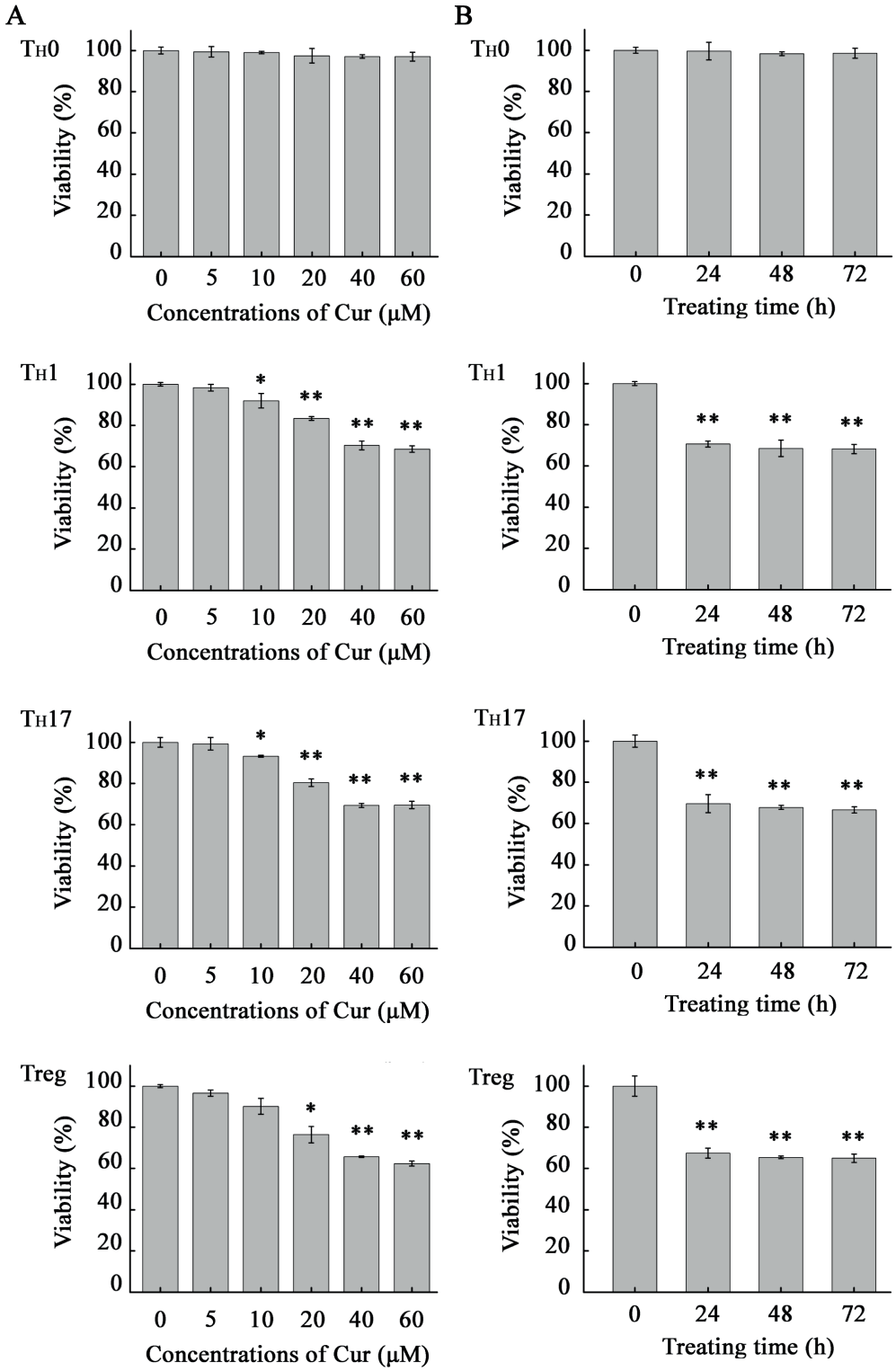

Figure 1. Curcumin inhibits $CD4^+$ T cell viability. **A,** TH0, TH1, TH17, and Treg cells were seeded in 96-well plates at a density of 6000 cells per well. After 24 h, cells were treated with curcumin (5, 10, 20, 40, 60 μM) for 24 h. The cell viability was determined by MTT assay. **B,** TH0, TH1, TH17, and Treg cells were plated in 96-well plates (6000 cells per well). After 24 h, cells were treated with curcumin (40 μM) for 24, 48, and 72 h. The cell viability was determined by MTT assay. The above data are representative of at least three independent experiments with similar results. An asterisk (*) represents a significant difference from controls (*p<0.05 and **p<0.01).

sis, TH0, TH1, TH17, and Treg cells were treated with curcumin (10 and 40 μM). Apoptosis was determined by flow cytometry using Annexin V/PI double staining. As shown in Figure 3, curcumin treatment induced remarkable apoptosis in TH1, TH17, and Treg cells. Hence, our results suggest that curcumin inhibits $CD4^+$ T cell proliferation by inducing apoptosis in $CD4^+$ T cells.

Curcumin Affects the Expression of Bcl-2, Bax, and Caspase-3

The Bcl-2 protein family plays an important role in the induction and control of apoptosis. Bcl-2 (an anti-apoptotic protein) and Bax (a pro-apoptotic protein) are the two important members[14]. Caspase-3 is a member of the caspase family that responds to pro-apoptotic signals and modulates the expression of some enzymes[19]. Therefore, TH0, TH1, TH17, and Treg cells were treated with curcumin (10 and 40 μM). Then, the level of Bcl-2, Bax, and caspase-3 was determined by Western blotting. As showed in Figure 4, the expression of Bcl-2 was decreased, while that of Bax and active caspase-3 was increased in $CD4^+$ T cells after treatment with curcumin. These results indicated that curcumin induces apoptosis by regulating the expression of Bcl-2, Bax, and caspase-3 in $CD4^+$ T cells.

Curcumin Suppresses $CD4^+$ T Cell Activation

The activation molecule IL-2 receptor-α chain (CD25) and CD44 are important indicators of cell proliferation[18]. To detect the relationship between the expression of these molecules and cell proliferation, TH0, TH1, TH17 and Treg cells were treated with curcumin (10 and 40 μM) similarly. The expression of CD25 and CD44 was determined by flow cytometry. Results showed that the expression levels of both CD25 and CD44 were decreased in cells after treatment with curcumin compared to those in control cells (Figure 5). These data indicated that curcumin directly suppresses $CD4^+$ T cell activation.

Discussion

MS is a chronic disease characterized by the destruction of the CNS and often the underlying axons. Its pathology has the hallmarks of an autoimmune disorder, including inflammatory re-

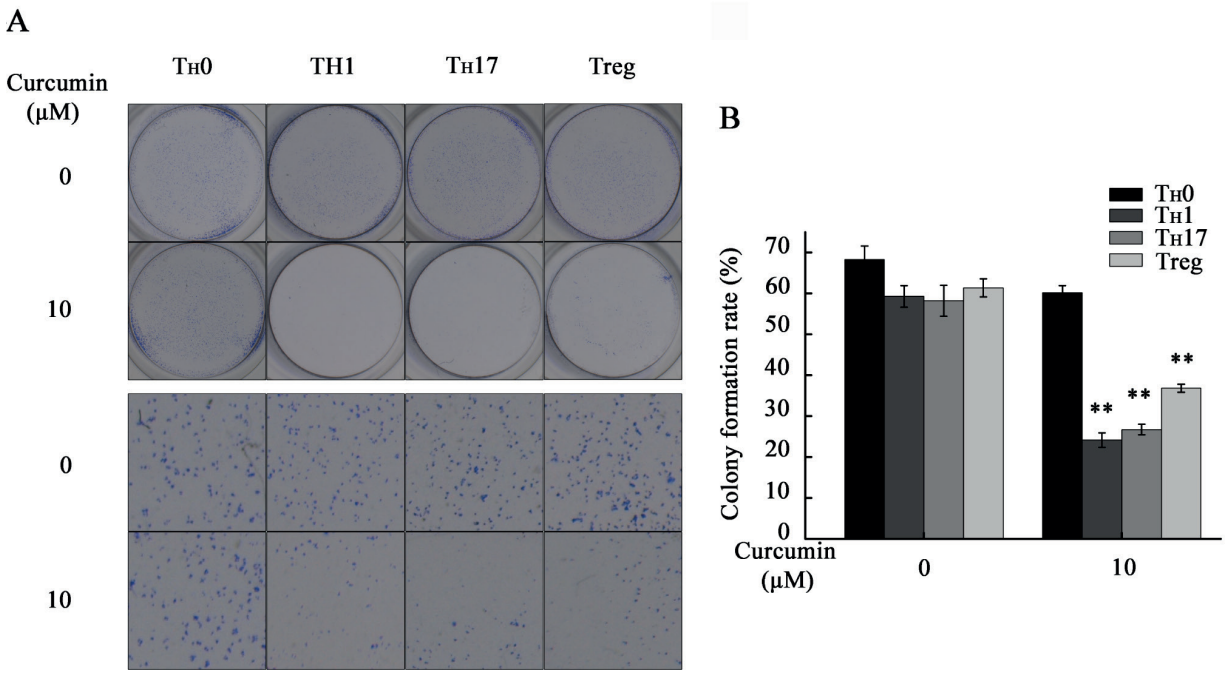

Figure 2. Curcumin inhibits $CD4^+$ T cell proliferation. **A,** TH0, TH1, TH17, and Treg cells were plated in 12-well plates (3000 cells per well). After 24 h, the cells were treated with curcumin (10 μM) for 7 d. The ability of individual cells to aggregate into viable colony clusters was detected by clonogenic survival assay. **B,** The colony formation ability was evaluated. The above blots and data are representative of at least three independent experiments with similar results. An asterisk (*) represents a significant difference from controls (*$p<0.05$ and **$p<0.01$).

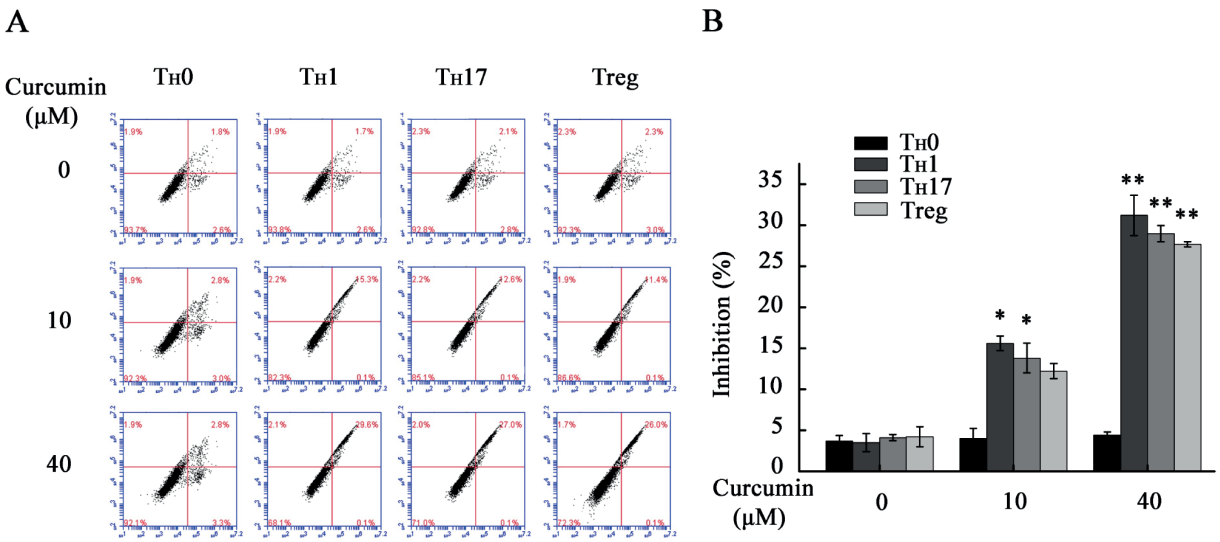

Figure 3. Curcumin induces apoptosis in CD4⁺ T cells. **A,** TH0, TH1, TH17, and Treg cells were treated with curcumin (10 and 40 μM). The cells were trypsinised, washed, and re-suspended in binding buffer containing Annexin V-Fluorescein isothiocyanate (FITC) (5 μL) and PI (2.5 μL). The cells were analyzed using a FACSort flow cytometer. **B,** Apoptosis ratios were calculated. The above blots and data are representative of at least three independent experiments with similar results. An asterisk (*) represents a significant difference from controls (*p<0.05 and **p<0.01).

sponse accompanied by leukocyte infiltration into the nervous system[2,20]. CD4⁺ T cells have been reported to be important in MS[4]. Therefore, it is necessary to identify effective and low toxicity agents for MS by inhibiting CD4⁺ T cells.

In the last decades, there has been a growing interest in curcumin. Curcumin has been reported to have various biological activities, including anti-inflammatory and antiseptic activity, as well as inhibition of cell proliferation[21,22]. Previous reports[10,23] have shown that curcumin inhibits proliferation by inducing apoptosis in malignant cells. Here, we explored whether curcumin could inhibit the proliferation of CD4⁺ T cells to prevent MS. The results indicated that curcumin markedly inhibited CD4⁺ T cell viability. Furthermore, TH1, TH17, and Treg all showed a dose-dependent but not time-dependent. Meanwhile, the effect of curcumin on the ability of individual CD4⁺ T cells to aggregate into viable colony clusters was evaluated by clonogenic survival assay. The findings revealed that curcumin remarkably decreased the colony formation ability of CD4⁺ T cells.

Apoptosis, a programmed cell death process, is very important in regulating cell proliferation[13,24]. Next, we investigated whether the curcumin-induced inhibition of CD4⁺ T cell growth is related to apoptosis by flow cytometry. The results indicated that curcumin-induced remarkable apoptosis in TH1, TH17, and Treg cells. Bcl-2 is an anti-apoptotic protein and Bax is a pro-apoptotic protein. Caspases are a family of cysteine proteases, which respond to pro-apoptotic signals, and caspase-3 is an important member of this family. Therefore, the expression of Bcl-2, Bax, and active caspase-3 was determined by Western blotting. The findings showed that curcumin decreased the expression of Bcl-2, but increased that of Bax and active caspase-3 in CD4⁺ T cells. These data indicated that curcumin inhibits CD4⁺ T cell proliferation by inducing apoptosis in CD4⁺ T cells.

The activation molecule IL-2 receptor-α chain (CD25) and CD44 have been reported to be involved in the upregulation of cell proliferation by T cells[25]. The inhibition of cell division in the presence of curcumin was explored. Flow cytometry results also showed that curcumin decreased the expression of both CD25 and CD44. Compared with the control group, other groups showed significant suppression of cell activation in a dose-dependent manner after treatment with curcumin. These results showed that curcumin could directly inhibit CD4⁺ T cell activation.

Conclusions

Curcumin inhibits CD4⁺ T cell viability in a dose- and time-dependent manner by inducing

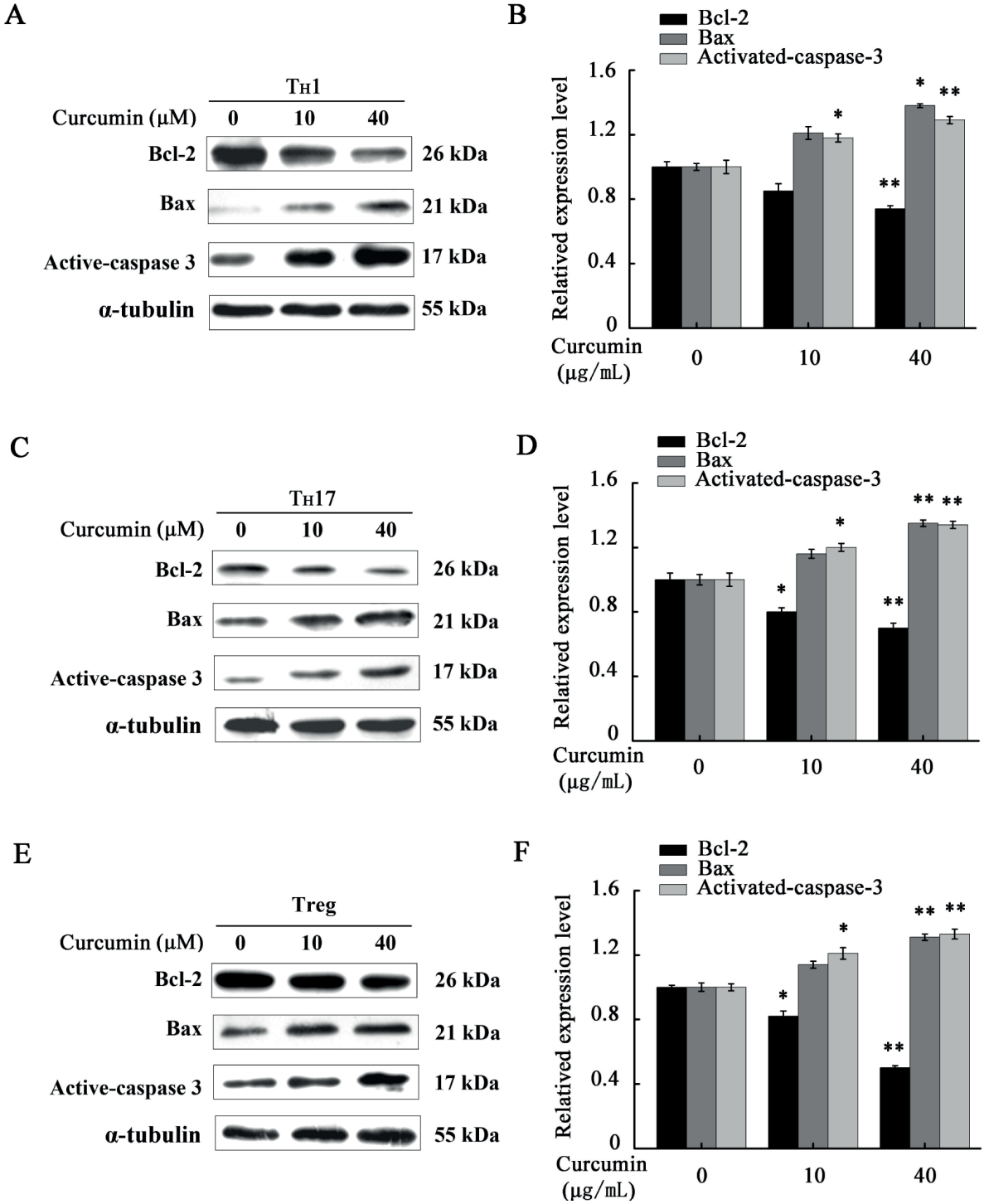

Figure 4. Curcumin affects the expression of Bcl-2, Bax, and caspase-3. TH0, TH1, TH17, and Treg cells were treated with curcumin (10 and 40 μM). **A-C,** and **E,** Western blotting was performed to measure the protein levels of Bcl-2, Bax, and caspase-3. **B-D,** and **F,** The greyscale scans of left Western blot lines. The above blots and data are representative of at least three independent experiments with similar results. An asterisk (*) represents a significant difference from controls (*p<0.05 and **p<0.01).

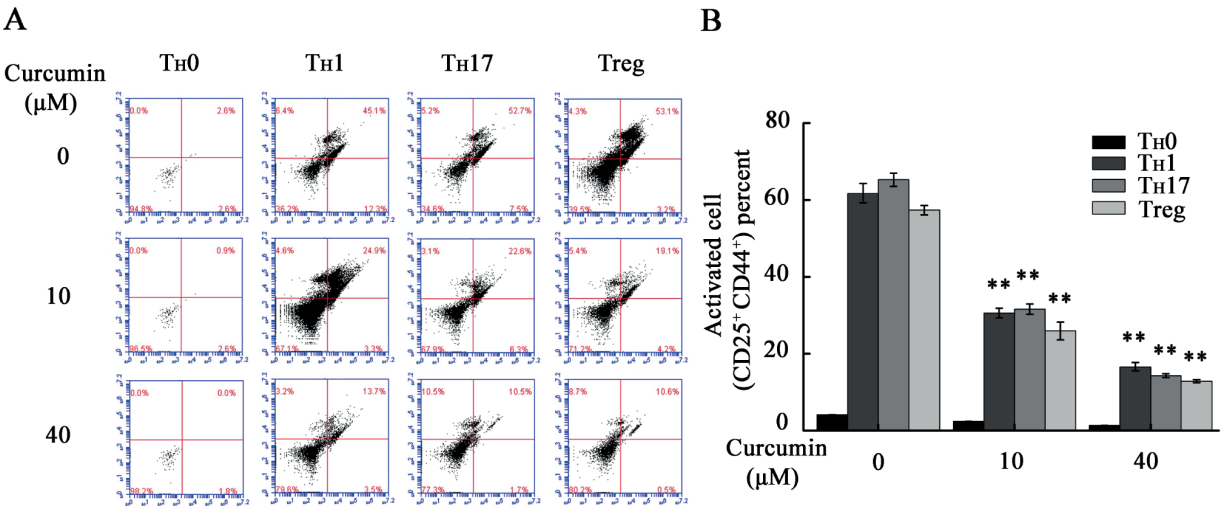

Figure 5. Curcumin suppresses $CD4^+$ T cell activation. **A,** TH0, TH1, TH17, and Treg cells were treated with curcumin (10 and 40 μM). The cells were trypsinised, washed, and re-suspended in binding buffer containing CD25-PE (5 μL) and CD44-FITC (5 μL). The cells were analysed using a FACSort flow cytometer. **B,** Apoptosis ratios obtained from flow cytometry analysis are shown in the bar graph. The above blots and data are representative of at least three independent experiments with similar results. An asterisk (*) represents a significant difference from controls (*p<0.05 and **p<0.01).

apoptosis, and decreases the expression of CD25 and CD44, which are involved in cell proliferation. These results indicated that curcumin could prevent EAE by inhibiting cell proliferation and effector $CD4^+$ T cell activation.

Conflicts of interest

The authors declare no conflicts of interest.

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