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Differential regulation of CD4$^+$ T helper cell responses by curcumin in experimental autoimmune encephalomyelitis

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Abstract

Nutraceuticals and phytochemicals are important regulators of human health and diseases. Curcumin is a polyphenolic phytochemical isolated from the rhizome of the plant *Curcuma longa* (turmeric) that has been traditionally used for the treatment of inflammation and wound healing for centuries. Systematic analyses have shown that curcumin exerts its beneficial effects through antioxidant, antiproliferative and anti-inflammatory properties. We and others have shown earlier that curcumin ameliorates experimental autoimmune encephalomyelitis (EAE) model for multiple sclerosis. In this study, we show that C57BL/6 mice induced to develop EAE express elevated levels of interferon (IFN) γ and interleukin (IL)-17 in the central nervous system (CNS) and lymphoid organs that decreased significantly following *in vivo* treatment with curcumin. The EAE mice also showed elevated expression of IL-12 and IL-23 that decreased after treatment with curcumin. *Ex vivo* and *in vitro* treatment with curcumin resulted in a dose-dependent decrease in the secretion of IFNγ, IL-17, IL-12 and IL-23 in culture. The inhibition of EAE by curcumin was also associated with an up-regulation of IL-10, peroxisome proliferator activated receptor γ and CD4$^+$CD25^{+-} Foxp3$^+$ Treg cells in the CNS and lymphoid organs. These findings highlight that curcumin differentially regulates CD4$^+$ T helper cell responses in EAE.

Keywords: Autoimmune disease; EAE/MS; Inflammation; Th1 cell; Nutraceuticals; Curcumin

1. Introduction

Human diets of plant origin, containing many different bioactive compounds, play important roles in regulating health and diseases. Curcumin (diferuloylmethane) is a naturally occurring yellow pigment isolated from the rhizomes of the plant *Curcuma longa* (turmeric) found in south Asia [1,2]. It is commonly used as a coloring and flavoring spice in food products. Curcumin has been traditionally used for centuries to treat inflammatory disorders and wound healing. The medicinal value of curcumin is well recognized as it has antioxidant, antitumor and anti-inflammatory properties and is under preclinical trials in the treatment of cancer and inflammation [3,4]. Recent studies have shown that curcumin inhibits inflammation in the animal models of atherosclerosis, Alzheimer's disease and arthritis [5]. The anti-inflammatory activity of curcumin was associated with its ability to inhibit proinflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-1β, IL-8, inducible nitric oxide synthase and C-reactive protein [6–8]. Although the exact mechanisms in the anti-inflammatory activity of curcumin is not fully defined, it modulates the activation of nuclear factor (NF)-κB, activator protein-1 (AP-1), mitogen-activated protein kinase and other inflammatory signaling pathways [9,10]. Curcumin also inhibits IL-12 production and T helper (Th) 1 differentiation [11,12], suggesting its significance in the treatment of autoimmune inflammatory diseases.

The immune system has evolved to discriminate self from nonself antigens, thereby protecting the host from microbial pathogens and malignant tumor. Nevertheless, a breakdown in the fundamental immune responses often results in the development of chronic infectious diseases, malignant tumors and organ-specific autoimmune diseases. Multiple sclerosis (MS) [13] is an inflammatory demyelinating disease of the central nervous system (CNS) that afflicts more than one million people worldwide [14]. A substantial percentage of MS patients develop clinical paralysis and become disabled and wheelchair bound for the rest of their lives. There is no medical treatment available so far that can cure MS [13,15]. The destruction of oligodendrocyte and myelin sheath in the CNS is the pathological hallmark of MS [16]. Although the etiology of MS is not known, it is generally viewed as an autoimmune inflammatory disease resulting from activation, expansion and homing of myelin

Abbreviations: EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PPAR, peroxisome proliferator activated receptor; qRT-PCR, quantitative reverse transcription polymerase chain reaction; Treg, regulatory T cells; ELISA, enzyme-linked immunosorbent assay.

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antigen-reactive T cells in the CNS [17–19]. Experimental autoimmune encephalomyelitis (EAE) is a $CD4^+$ T cell mediated demyelinating disease of the CNS [20]. EAE can be induced in susceptible rodents and primates by immunizing with neural antigens such as myelin basic protein (MBP), proteolipid protein and myelin oligodendrocyte glycoprotein (MOG) [21]. Clinical and pathological features of EAE show close similarity to MS; therefore, EAE has been commonly used as a model system to study the mechanism of MS pathogenesis and to test the efficacy of potential therapeutic agents for the treatment of MS.

The pathogenesis of EAE/MS is a complex process involving the activation of antigen presenting cells (APCs) such as macrophage and microglia, differentiation of neural antigen-specific Th1/Th17 cells and secretion of inflammatory cytokines in the CNS [22]. IL-12 and IL-23 produced by APCs induce the differentiation of encephalitogenic Th1 and Th17 cells and the pathogenesis of EAE/MS [23–25]. MS patients show increased expression of IL-12/IL-23 in brain lesions, cerebrospinal fluid and circulation during clinical relapses [25]. While the activation of NF-κB signaling leads to IL-12/IL23 gene expression in APCs [26], IL-12/IL23-induced activation of Jak-Stat pathway leads to Th1/Th17 differentiation in EAE/MS [27]. We and others have shown earlier that curcumin ameliorates EAE by blocking IL-12 production and IL-12 signaling through Jak-Stat pathway in T cells [28,29].

In this study, we investigated the mechanisms in the regulation of EAE by curcumin. We found that curcumin attenuates EAE by modulating Th1/Th17 differentiation in association with the inhibition of IL-12/IL-23 and up-regulation of Th2/regulatory T cell (Treg) responses, suggesting its significance in the treatment of MS and other autoimmune diseases.

2. Materials and methods

2.1. Animals

Six- to eight-week-old female C57BL/6 mice were obtained from Harlan (Indianapolis, IN, USA) or Jackson Laboratory (Bar Harbor, ME, USA) and

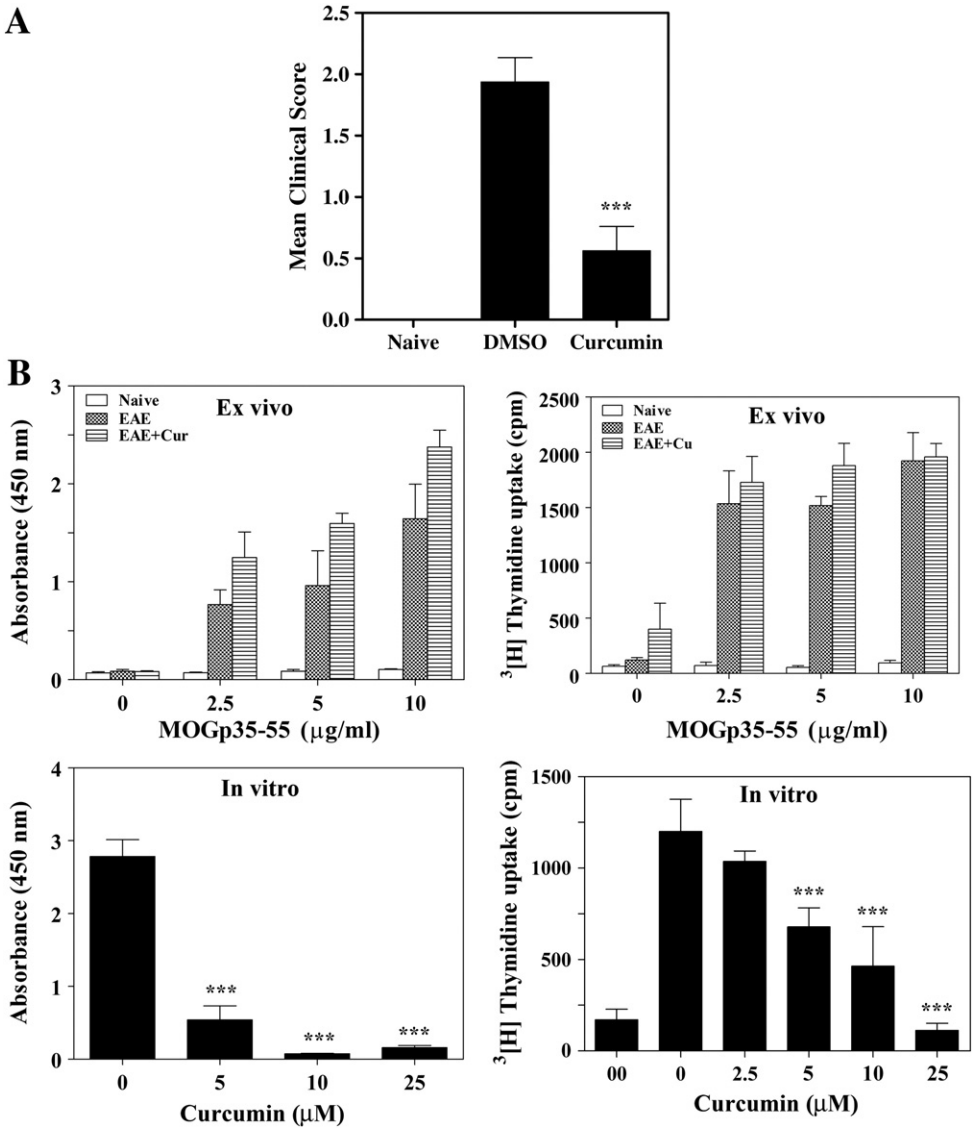


Fig. 1. Inhibition of EAE by curcumin. (A) C57BL/6 mice were induced to develop EAE by immunization with MOGp35-55 and treated with 0 or 100 μg curcumin in 25 μl DMSO every other day. The clinical signs were evaluated, and the mean clinical score (n=8) on day 14 is presented as histogram. (B) Spleen cells were isolated on day 14 from EAE mice treated with DMSO or curcumin and stimulated with MOGp35-55 antigen *ex vivo*. Spleen cells from EAE mice treated with DMSO were stimulated with 10 μg MOGp35-55 antigen in the presence of curcumin for 96 h, and the proliferation was measured by WST-1 or [^{3}H] thymidine uptake assay. The values are mean±S.D./S.E.M., and *P<.05, **P<.01 and ***P<.001 compared to medium control.

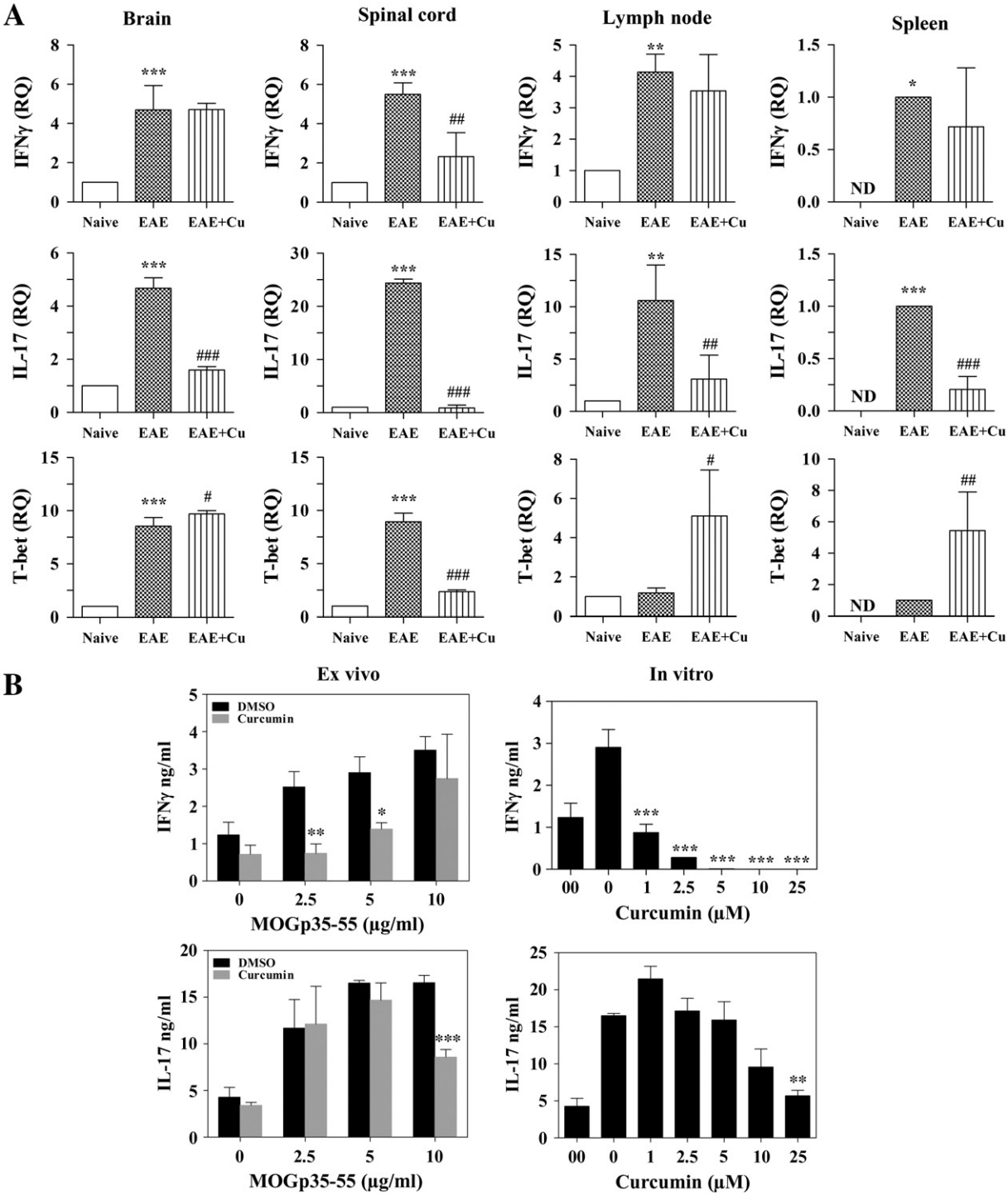
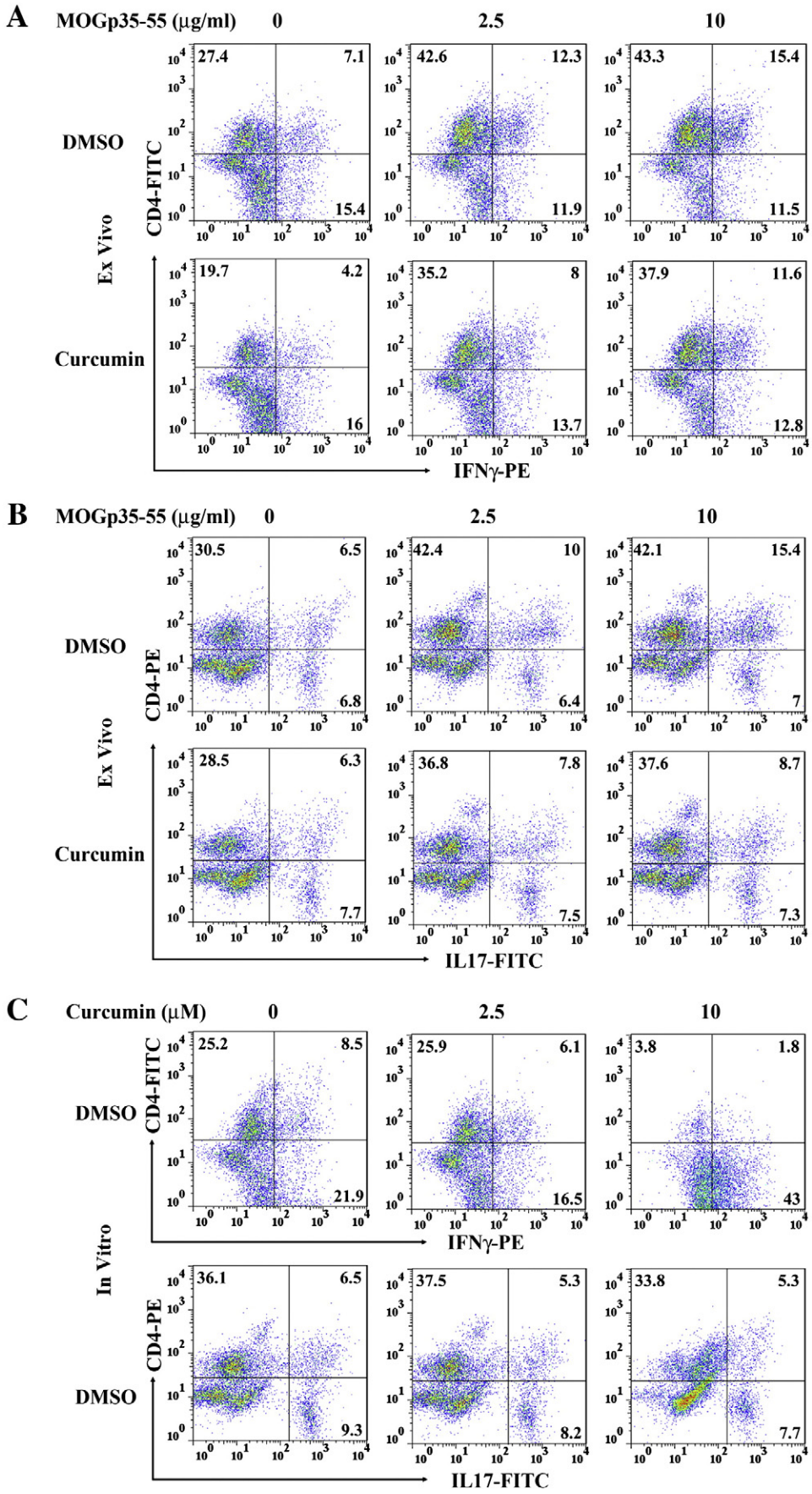


Fig. 2. Inhibition of IFNγ, IL-17 and T-bet by curcumin in EAE. (A) C57BL/6 mice were treated with DMSO or curcumin following immunization with MOGp35-55. The mice were euthanized on day 14, and total RNA was extracted from the brain, spinal cord, lymph node and spleen. The expression of IFNγ, IL-17 and T-bet was analyzed by qRT-PCR using 18S as internal control. The fold change in EAE was calculated using naïve mice as control and shown as mean±S.D. $^{*}P<.05$, $^{**}P<.01$ and $^{***}P<.001$ for EAE compared to naïve and $^{\#}P<.05$, $^{\#\#}P<.01$ and $^{\#\#\#}P<.001$ for curcumin compared to DMSO-treated EAE. (B) Spleen cells were cultured with 0, 2.5, 5 and 10 μg MOGp35-55 in the absence (*ex vivo*) or in the presence of 0, 2.5 and 10 μM of curcumin (*in vitro*). After 48 h, culture supernatants were collected, and the levels of IFNγ and IL-17 were determined by ELISA. The figure is representative of three independent experiments ($^{*}P<.05$, $^{**}P<.01$ and $^{***}P<.001$).

Fig. 3. Inhibition of Th1 and Th17 cell expansion by curcumin in EAE. C57BL/6 mice induced to develop EAE were treated with 0 or 100 μg curcumin in 25 μl DMSO every other day. On day 14, the spleen cells were isolated and cultured in the presence of 0, 2.5 and 10 μg/ml of MOGp35-55. The cells were stained for intracellular IFNγ and IL-17, and the percent $CD4^{+}IFN\gamma^{+}$ (A) and $CD4^{+}IL\text{-}17^{+}$ (B) cells was analyzed by flow cytometry. (C) Spleen cells isolated from DMSO-treated EAE mice on day 14 were cultured with 10 μg/ml MOGp35-55 antigen in the presence of 0, 2.5 and 10 μM curcumin, and the percent $CD4^{+}IFN\gamma^{+}$ or $CD4^{+}IL\text{-}17^{+}$ cells was analyzed by flow cytometry. The figure is representative of three independent experiments.


A
MOGp35-55 (μg/ml)
0
2.5
10
DMSO
Curcumin
Ex Vivo
CD4-FITC
IFNγ-PE
27.4
7.1
15.4
42.6
12.3
11.9
43.3
15.4
11.5
19.7
4.2
16
35.2
8
13.7
37.9
11.6
12.8
B
MOGp35-55 (μg/ml)
0
2.5
10
DMSO
Curcumin
Ex Vivo
CD4-PE
IL17-FITC
30.5
6.5
6.8
42.4
10
6.4
42.1
15.4
7
28.5
6.3
7.7
36.8
7.8
7.5
37.6
8.7
7.3
C
Curcumin (μM)
0
2.5
10
DMSO
In Vitro
CD4-FITC
IFNγ-PE
CD4-PE
IL17-FITC
25.2
8.5
21.9
25.9
6.1
16.5
3.8
1.8
43
36.1
6.5
9.3
37.5
5.3
8.2
33.8
5.3
7.7

maintained in the animal care facility at Methodist Research Institute. All the animal protocols used in the experiments were approved by the Institutional Animal Care and Use Committee at Methodist Research Institute.

2.2. Reagents

The 21-amino-acid peptide [MEVGWYRSPFSRVVHLYRNGK] corresponding to mouse MOGp35-55 (>96.81% pure) was obtained from Genemed Synthesis (San Antonio, TX, USA). Curcumin (>94% pure) was purchased from Sigma Chemicals (St. Louis, MO, USA). WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) was purchased from Roche Diagnostics (Indianapolis, IN, USA), and ^{3}H thymidine was obtained from GE Healthcare Bio-Sciences (Piscataway, NJ, USA). The quantitative reverse transcription polymerase chain reaction (qRT-PCR) primers for IL-12p35, IL-12p40, IL-23p19, IL-27p28, T-Bet, peroxisome proliferator-activated receptor (PPAR) γ, IL-10, IL-4, IL-17, interferon (IFN) γ, GAPDH (glyceral-dehyde-3-phosphate dehydrogenase) and ribosomal RNA 18S were purchased from Applied Biosystems (Foster City, CA, USA). The IFNγ primer set was purchased from Qiagen (Valencia, CA, USA). Recombinant mouse IL-12, IFNγ, IL-17, IL-23, IL-4 and IL-10 were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies to IFNγ, IL-17, IL-23p19, IL-4 and IL-10 were purchased from eBioscience (San Diego, CA, USA). Anti-IL-12p40 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The goat anti-mouse IL-23p19 and IL-27p28 were purchased from R&D Systems (Minneapolis, MN, USA). Biotin-conjugated antibodies specific to IL-17 was purchased from BD Pharmingen (Franklin Lakes, NJ, USA), IFNγ was from Endogen (Woburn, MA, USA), IL-12/23p40 was from Biolegend (San Diego, CA, USA), IL-12p70 was from Mabtech (Mariemont, OH, USA), and anti-IL-4 and anti-IL-10 were from eBioscience (San Diego, CA, USA). CD4-PE, IL-17A-FITC, IFNγ-PE, CD-25 APCCy7 and Foxp3-FITC were purchased from eBioscience (San Diego, CA, USA).

2.3. Induction, treatment and evaluation of EAE

To induce EAE, 6- to 8-week-old female C57BL/6 mice were immunized (subcutaneously) with 100 μg MOGp35-55 peptide in 150 μl emulsion of Complete Freund's Adjuvant (Difco, Detroit, MI, USA) at the lower dorsum on days 0 and 7. The mice also received 100 ng pertussis toxins in 100 μl phosphate-buffered saline (PBS) [intraperitoneally (ip)] on days 0 and 2. The mice were treated (ip) with 0 or 100 μg curcumin in 25 μl dimethyl sulfoxide (DMSO) every other day from the day of first immunization. Clinical signs of EAE were scored on day 14 in a blinded manner as follows; 0, normal; 0.5, stiff tail; 1, limp tail; 1.5, limp tail with inability to right; 2, paralysis of one limb; 2.5, paralysis of one limb and weakness of one other limb; 3, complete paralysis of both hind limbs; 4, moribund; 5, death. Mean clinical score was calculated by adding the clinical score for all mice in a group and then dividing by the total number of mice.

2.4. T. cell proliferation assay

The effect of curcumin on neural antigen-induced T cell proliferation was measured by WST-1 and ^{3}H thymidine uptake assays. To determine the *ex vivo* response, C57BL/6 mice induced to develop EAE were treated with 0 or 100 μg of curcumin in 25 μl DMSO every other day. The spleen cells were isolated on day 14 and cultured in RPMI medium in 96-well tissue culture plates (2×10^5/200 μl/well) with 0, 2.5, 5 and 10 μg/ml MOGp35-55 antigen. ^{3}H thymidine (0.5 μCi/well) was added at 72 h, and the cells were harvested at 96 h using a Tomtec harvester 96 (Hamden, CT, USA). ^{3}H thymidine uptake was measured using a Wallac Microbeta Trilux counter (Perkin Elmer, Waltham, MA, USA). WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) reagent was added (10 μl/well) at 90 h, and the OD at 450 nm was measured at 96 h using a titer-plate reader (Alpha Diagnostics, San Antonio, TX, USA). To determine the *in vitro* effect of curcumin, spleen cells isolated from DMSO-treated EAE mice were cultured with 5 μg/ml MOGp35-55 antigen in the presence of 0, 2.5, 5, 10 and 25 μM curcumin, and the proliferation was measured by WST-1 and ^{3}H thymidine assay.

2.5. qRT-PCR

The effect of curcumin on the mRNA expression of inflammatory cytokines was determined by qRT-PCR. C57BL/6 mice induced to develop EAE were treated with 0 or 100 μg curcumin in 25 μl DMSO every other day. To determine the *in vivo* response, brain, spinal cord, spleen and lymph node tissue samples were isolated on day 14 and analyzed directly. To determine the *ex vivo* response, spleen cells isolated on day 14 were cultured in RPMI medium (1×10^6/ml) in 12-well tissue culture plates in the presence of 5 μg/ml MOGp35-55 antigen. To determine the *in vitro* response, the spleen cells isolated on day 14 from DMSO-treated EAE mice were cultured in RPMI medium (1×10^6/ml) in 12-well plates with 5 μg/ml MOGp35-55 antigen in the presence of 0, 1, 2.5, 5, 10 and 25 μM curcumin. Total RNA was extracted from the tissues and cultured cells using TRIzol reagent according to manufacturer's instruction (Invitrogen, Madison, WI, USA). The RNA was reverse transcribed into cDNA using TaqMan reverse transcription kit and amplified using TaqMan Universal Master Mix with probe and primers in the ABI 7900 Fast Sequence Detection System. The levels of gene expression normalized to GAPDH or 18S were calculated using the relative quantification (delta delta Ct) study software (Applied Biosystems, Foster City, CA, USA), and the results are presented as arbitrary fold change compared to control.

2.6. Cytokine enzyme-linked immunosorbent assay (ELISA)

The effect of curcumin on the secretion of inflammatory cytokines was determined by ELISA. To study the *ex vivo* response, spleen cells were isolated from C57BL/6 mice treated with DMSO or curcumin on day 14 following immunization with MOGp35-55 antigen. The cells were cultured in RPMI medium (1×10^6/ml) in 12-well plates in the presence of 5 μg/ml antigen. To determine the *in vitro* response, MOGp35-55-immune spleen cells from DMSO-treated EAE mice were cultured in RPMI medium (1×10^6/ml) in 12-well plates with 5 μg/ml antigen in the presence of 0, 1, 2.5, 5, 10 and 25 μM curcumin. The culture supernatants were collected after 48 h, and the levels of IFNγ, IL-17, IL-12p70, IL-23, IL-4 and IL-10 measured by ELISA. Briefly, 96-well ELISA plates were coated with capture antibodies in 50 μl/well of 0.06 M carbonate buffer (pH 9.6) at 4°C overnight. The excess Abs were washed off, and the residual binding sites were blocked by the addition of 1% bovine serum albumin (BSA) in PBS for 1 h. The test samples (culture supernatants) and standards were added to the respective wells and incubated at 4°C overnight. Plates were washed with PBS containing 0.05% Tween-20, and biotin-conjugated detection antibodies were added. After incubation at room temperature for 1 h, the plates were washed, and avidin-alkaline phosphatase was added followed by 1 mg/ml of *p*-nitrophenyl phosphate. After 30 min of incubation at room temperature, the absorbance was read at 405 nm, and the concentrations of cytokines in the culture supernatants were calculated from the standard curve.

2.7. Intracellular cytokine and Treg analysis by flow cytometry

The effect of curcumin on the differentiation of Th1, Th17 and Treg cells was analyzed by flow cytometry. Spleen cells were isolated from C57BL/6 mice treated with DMSO or curcumin on day 14 following immunization with MOGp35-55 and cultured in RPMI medium (1×10^6/ml) in 12-well plates with 5 μg/ml antigen for 36 h. For intracellular cytokine staining, GolgiStop (monensin) was added for the last 4 h to prevent cytokine secretion. The cells were washed, fixed and permeabilized using BD Cytofix/Cytoperm solution (BD Bioscience Pharmingen, San Diego, CA, USA) at room temperature for 15 min. Samples were washed and incubated in BD Perm/Wash buffer with anti-IFNγ-PE and anti-IL-17-FITC antibodies (1:50) at 4°C for 30 min. For Treg analysis, spleen cells were washed and stained with anti-CD4-APC and anti-CD25-PE antibodies (1:50) at 4°C for 30 min. The cells were then washed and incubated in BD Perm/Wash buffer with anti-Foxp3-FITC antibody (1:50) at 4°C for 30 min. The samples were washed, suspended in PBS with 0.1% BSA and acquired using an LSR-II flow cytometry instrument (BD Biosciences, Franklin Lakes, NJ, USA). The data were analyzed using FlowJo 8.2.6 software (Ashland, OR, USA).

2.8. Statistical analysis

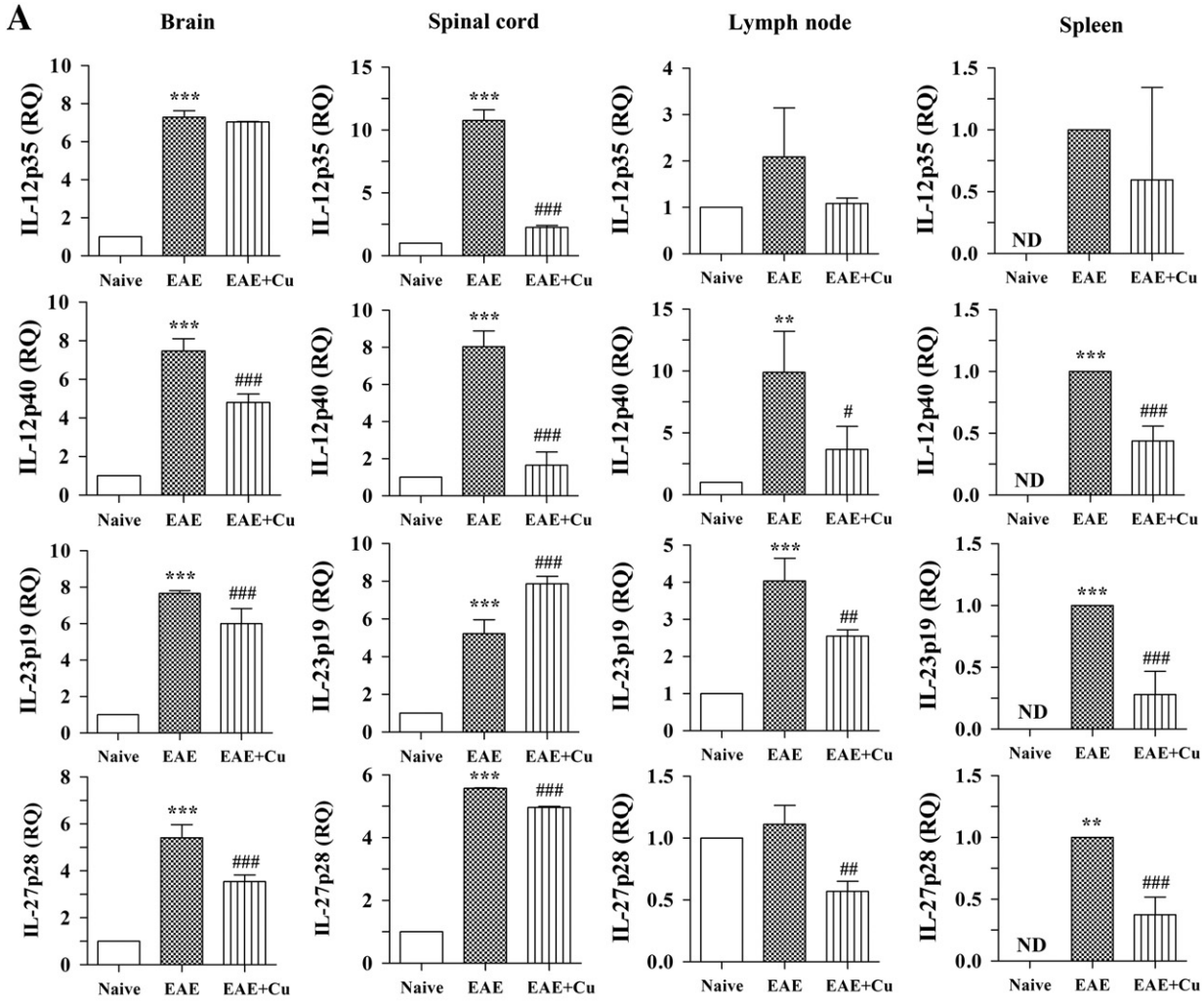
The values are mean±S.D./S.E.M., and the data were analyzed by one-way analysis of variance using the GraphPad Prism 5.0 software. The significance of the data is expressed as $^{*/\#}(P<.05)$, $^{**/\#\#}(P<.01)$ and $^{***/\#\#\#}(P<.001)$ in the figures.

3. Results

3.1. Curcumin ameliorates EAE without inhibiting spleen cell proliferation ex vivo

To determine the mechanisms in the regulation of EAE by curcumin, we induced EAE in C57BL/6 mice by immunization with MOGp35-55 antigen. As shown in Fig. 1A, the DMSO-treated control mice developed EAE with a mean clinical score reaching 1.95 on day 14. Interestingly, *in vivo* treatment with 100 μg curcumin resulted in the attenuation of EAE, reaching a mean clinical score of 0.6 (69%

Fig. 4. Inhibition of IL-12 family cytokines by curcumin. (A) C57BL/6 mice were treated with DMSO or curcumin following immunization with MOGp35-55. The mice were euthanized on day 14, and total RNA was extracted from the brain, spinal cord, lymph node and spleen. The expression of IL-12p35, IL-12p40, IL-23p19 and IL-27 was analyzed by qRT-PCR using 18S as internal control. The fold change in EAE was calculated using naïve mice as control and shown as mean±S.E.M. $^*P<.05$, $^{**}P<.01$ and $^{***}P<.001$ for EAE compared to naïve and $^{\#}P<.05$, $^{\#\#}P<.01$ and $^{\#\#\#}P<.001$ for curcumin compared to DMSO. (B) Spleen cells were cultured with 0, 2.5, 5 and 10 μg MOGp35-55 antigen in the absence (*ex vivo*) or presence of 0, 2.5 and 10 μM of curcumin (*in vitro*). After 48 h, culture supernatants were collected, and the levels of IL-12p70 and IL-23 were determined by ELISA. The figure is representative of three independent experiments ($^*P<.05$, $^{**}P<.01$ and $^{***}P<.001$).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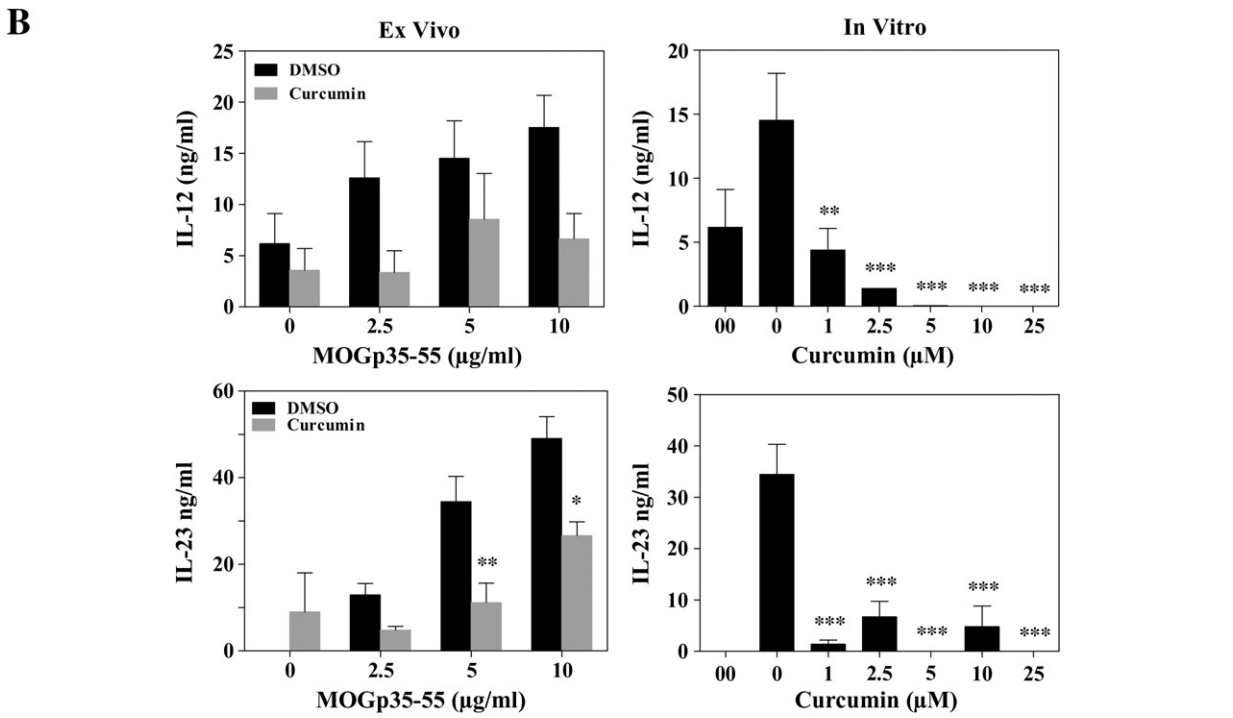
Brain
Spinal cord
Lymph node
Spleen
IL-12p35 (RQ)
IL-12p40 (RQ)
IL-23p19 (RQ)
IL-27p28 (RQ)
Naive
EAE
EAE+Cu
ND


B
Ex Vivo
In Vitro
DMSO
Curcumin
IL-12 (ng/ml)
IL-23 ng/ml
MOGp35-55 (µg/ml)
Curcumin (µM)

inhibition) on day 14. To study the mechanisms in the regulation of EAE by curcumin, we examined neural antigen-induced T cell proliferation in culture. As shown in Fig. 1B, spleen cells isolated from C57BL/6 mice on day 14 following immunization with MOGp35-55 showed a dose-dependent increase in viability and proliferation in response to antigen *ex vivo*. When compared to DMSO control, spleen cells from EAE mice treated with curcumin showed no significant decrease, rather a trend towards an increase, in antigen-induced proliferation and survival *ex vivo*. However, *in vitro* treatment of spleen cells from DMSO-treated EAE mice with curcumin resulted in a dose-dependent inhibition of antigen-induced T cell proliferation and survival in culture (Fig. 1B). These results suggest that curcumin ameliorates EAE without affecting the expansion of neural antigen-specific T cells in C57BL/6 mice.

3.2. Curcumin inhibits IFNγ and IL-17 expression in the CNS and lymphoid organs of EAE mice

To understand the mechanisms in the regulation of EAE by curcumin, we then examined the expression of IFNγ and IL-17 in the target organ. As shown in Fig. 2A, C57BL/6 mice induced to develop EAE showed a significant increase in the transcription of IL-17 in the brain and spinal cord compared to naïve mice. Interestingly, *in vivo* treatment with curcumin resulted in a significant decrease in the expression of IL-17 mRNA in the CNS. EAE mice also showed elevated expression of IFNγ and T-bet in the CNS that decreased in the spinal cord but not in the brain following treatment with curcumin. Further analyses showed that EAE mice expressed elevated IFNγ and IL-17 mRNA in lymph node and spleen compared to naïve mice. However, *in vivo* treatment with curcumin resulted in a significant inhibition of IL-17 with a partial inhibition of IFNγ in the lymphoid organs. The EAE mice also showed a mild increase in T-bet expression that was further increased by curcumin in lymphoid organs.

To understand the effect of curcumin on neural antigen-specific Th1 and Th17 responses, we analyzed the secretion of IL-17 and IFNγ in culture. As shown in Fig. 2B, *in vitro* culture of MOGp35-55-immune spleen cells with antigen resulted in the secretion of IFNγ and IL-17 in a dose-dependent manner (Fig. 2B). When compared to DMSO-treated control, the spleen cells from EAE mice treated with curcumin showed significant decrease in the secretion of IFNγ and IL-17 in culture *ex vivo*. *In vitro* treatment of MOGp35-55-immune spleen cells with curcumin also resulted in a dose-dependent decrease in IFNγ and IL-17 secretion in response to antigen in culture (Fig. 2B).

To further study the effect of curcumin on Th1 and Th17 responses, we examined the effects of curcumin on the expansion of IFNγ- and IL-17-producing spleen cells in EAE. As shown in Fig. 3, flow cytometry analyses showed a dose-dependent increase in $CD4^{+}$-$IFN\gamma^{+}$ (A) and $CD4^{+}IL\text{-}17^{+}$ (B) T cells from DMSO-treated EAE mice following stimulation with MOGp35-55 antigen in culture. Interestingly, *in vivo* treatment with curcumin resulted in a significant decrease in $CD4^{+}IFN\gamma^{+}$ ($P<.01$) and $CD4^{+}IL\text{-}17^{+}$ ($P<.001$) T cells in response to MOGp35-55 antigen compared to DMSO controls (Fig. 3A, B). *In vitro* culture of spleen cells from DMSO -reated EAE mice with 10 μg MOGp35-55 antigen in the presence of curcumin also resulted in a dose-dependent inhibition of $CD4^{+}IFN\gamma^{+}$ and $CD4^{+}IL\text{-}17^{+}$ T cells in culture ($P<.001$, Fig. 3B). These results suggest that curcumin inhibits Th1/Th17 responses in EAE.

3.3. Curcumin inhibits the expression of IL-12 family cytokines in the CNS and lymphoid organs of EAE mice

To further determine the mechanisms in the regulation of Th1 and Th17 responses, we examined the effect of curcumin on APC-derived IL-12 family cytokines in EAE. As shown in Fig. 4, EAE mice showed a significant increase in the transcription of IL-12p35, IL-12p40, IL-23p19 and IL-27 in the brain, spinal cord, lymph node and spleen compared to naïve mice. Interestingly, *in vivo* treatment with curcumin resulted in a significant decrease in the transcription of IL-12 family cytokines in the CNS and lymphoid organs (Fig. 4A). Moreover, culture of MOGp35-55-immune spleen cells with antigen induced the secretion of IL-12 and IL-23 in a dose-dependent manner (Fig. 4B). When compared to DMSO-treated control, the spleen cells from mice exposed to curcumin showed a partial inhibition of IL-12 with significant inhibition of IL-23 secretion in culture *ex vivo*. *In vitro* treatment of MOGp35-55-immune spleen cells with curcumin also resulted in a dose-dependent decrease in antigen-induced secretion of IL-12 and IL-23 in culture (Fig. 4B). These results suggest that curcumin also inhibits Th1/Th17 responses by modulating APC-derived IL-12 family cytokines in EAE.

3.4. Curcumin augments Th2 and Treg response in EAE

To determine the induction of anti-inflammatory responses, we examined the effects of curcumin on the transcription of IL-4 and IL-10 in EAE. As shown in Fig. 5, EAE mice showed an increase in the transcription of IL-4 and IL-10 in the brain and spleen with a partial increase in lymph node and mild decrease in spinal cord compared to naïve mice. Interestingly, *in vivo* treatment with curcumin resulted in a significant increase in the transcription of IL-4 and IL-10 in both CNS and lymphoid organs (Fig. 5A). The EAE mice also showed an increase in the expression of PPARγ in the CNS and lymphoid organs that increased further following treatment with curcumin (Fig. 5A). Similarly, *in vitro* culture of MOGp35-55-immune spleen cells with antigen induced the secretion of IL-4 and IL-10 in culture. But when compared to DMSO-treated control mice, spleen cells from mice treated with curcumin (*ex vivo*) showed partial reduction of IL-4 with a significant increase in the secretion of IL-10 in culture (Fig. 5B). *In vitro* treatment of MOGp35-55-immune spleen cells with curcumin also resulted in a significant decrease in antigen-induced secretion of IL-4 while increasing IL-10 in culture (Fig. 5B). Further analyses showed that *in vivo* treatment with curcumin induced significant increase in the percentage of $CD25^{+}Foxp3^{+}$ Treg cells in the spleen (9.1%) compared to 7.1% in DMSO-treated EAE mice ($P<.001$, Fig. 5C). These results suggest that curcumin augments Th2/Treg responses in EAE.

4. Discussion

The beneficial effect of curcumin in inflammation has been shown in the animal models of atherosclerosis, arthritis, sepsis, colitis, Alzheimer's disease and multiple sclerosis [30–34]. In this study, we determined the mechanisms in the regulation of EAE model of MS by curcumin. The pathogenesis of EAE/MS is a complex process involving activation of immune cells, secretion of inflammatory cytokines and differentiation of neural antigen-specific Th1/Th17 cells. IFNγ and IL-17 are two critical mediators of CNS inflammation in EAE and MS [28]. We have shown earlier that curcumin inhibits EAE by modulating neural antigen-specific Th1 cells in SJL/J mice [35]. In this study, we found that curcumin attenuates EAE in C57BL/6 mice by inhibiting the expression of IFNγ and IL-17 in the CNS. The reduction in Th1/Th17 responses in the target organ could be due to transcriptional repression of IFNγ/IL-17 or decrease in the infiltration of Th1/Th17 cells into the CNS. In addition, the inhibition of Th1 and Th17 differentiation in the lymphoid organs could also affect the source of effector cells in EAE. We found that curcumin inhibits the expression of IFNγ and IL-17 in lymphoid organs, suggesting the regulation of effector cells at the initiation of EAE. Moreover, *in vitro* treatment with curcumin induced a significant decrease in the secretion of IFNγ and IL-17 in neural antigen-specific T cells, suggesting direct inhibition of T cell responses by curcumin in EAE. These findings are

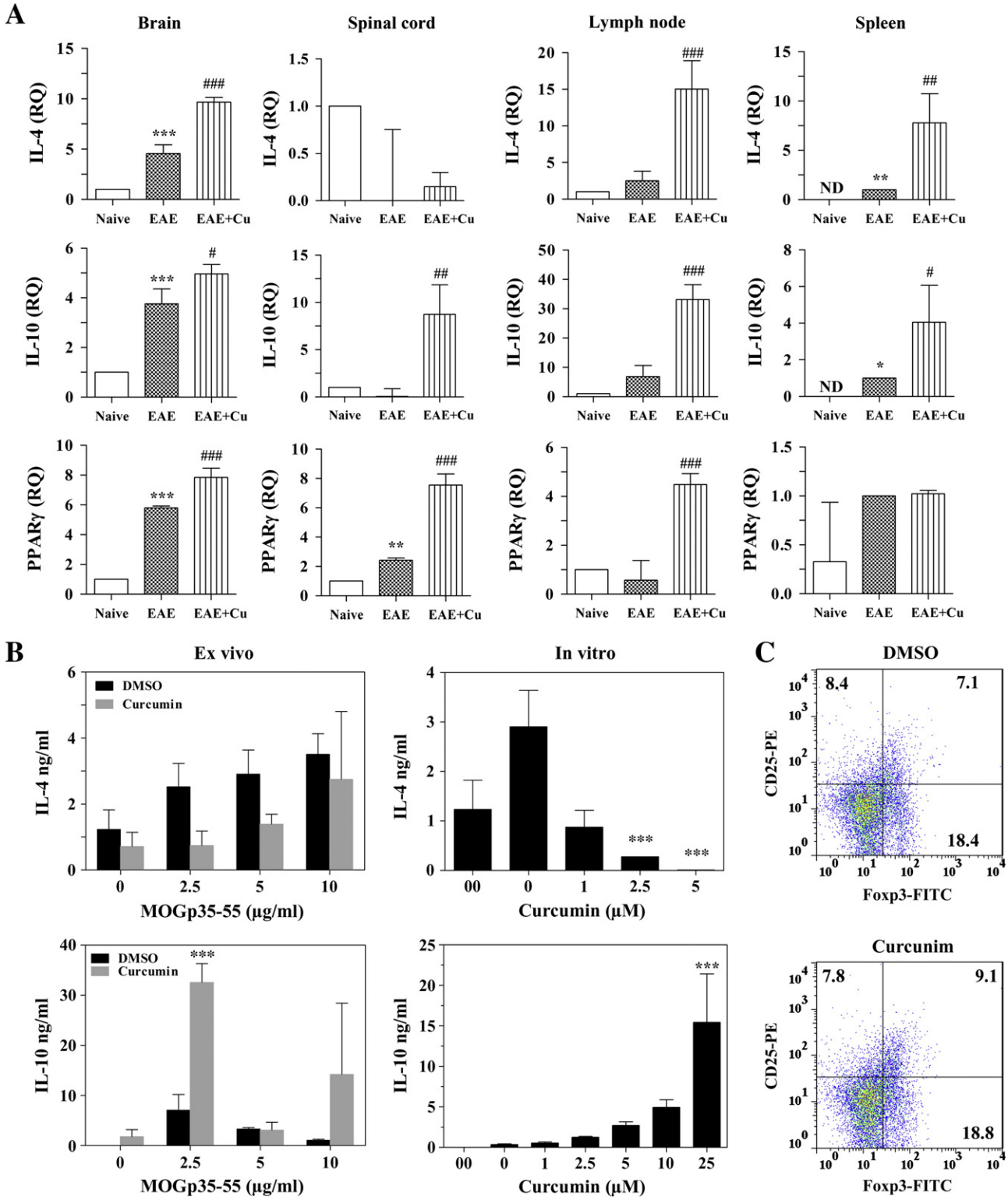


Fig. 5. Regulation of IL-4, IL-10 and Tregs by curcumin in EAE. (A) C57BL/6 mice were treated with DMSO or curcumin following immunization with MOGp35-55. The mice were euthanized on day 14, and total RNA was extracted from the brain, spinal cord, lymph node and spleen. The expression of IL-4, IL-10 and PPARγ was analyzed by qRT-PCR using 18S as internal control. The fold change in EAE was calculated using naïve mice as control and shown as mean±S.E.M. *P<.05, **P<.01 and ***P<.001 for EAE compared to naïve and $^{\#}P$<.05, $^{\#\#}P$<.01 and $^{\#\#\#}P$<.001 for curcumin compared to DMSO-treated EAE. (B) Spleen cells were cultured with 0, 2.5, 5 and 10 μg MOGp35-55 in the absence (*ex vivo*) or with 5 μg/ml antigen in the presence of 0, 2.5 and 10 μM of curcumin (*in vitro*). After 48 h, culture supernatants were collected, and the levels of IL-4 and IL-10 were determined by ELISA. (C) Spleen cells were isolated from DMSO or curcumin-treated EAE mice on day 14 and stained with anti-CD25-PE and anti-Foxp3-FITC antibodies. The percentage of Tregs was analyzed by flow cytometry, and the figure is representative of three independent experiments (*P<.05, **P<.01 and ***P<.001).

consistent with our earlier report on the inhibition of IL-12 signaling and neural antigen-specific Th1 differentiation by curcumin in EAE. This is also in agreement with earlier reports on the inhibition of T cell proliferation and IL-17 expression in MBP-reactive lymphocyte [36] and a shift from Th1 to Th2 response in cultured human peripheral blood mononuclear cells [37]. Thus, curcumin inhibits EAE in association with the inhibition of neural antigen-specific Th1 and Th17 cell differentiation.

IL-12 and IL-23 produced by APCs play critical roles in the differentiation of Th1 and Th17 cells, respectively, in EAE/MS [23,29,38]. We and others have shown earlier that targeted disruption or pharmacological inhibition of IL-12 or IL-23 was sufficient to prevent Th1/Th17 differentiation and pathogenesis of EAE [24,29]. Curcumin is known to inhibit IL-12 expression in cultured macrophages and microglia, and we have shown earlier that curcumin inhibits Th1 response by decreasing IL-12 production in neural antigen-sensitized spleen cells in culture [12,35]. In this study, we found that *in vivo* treatment with curcumin inhibits the expression of IL-12 and IL-23 in the CNS and lymphoid organs of EAE mice. Spleen cells from EAE mice treated *in vivo* or *in vitro* with curcumin also showed significant decrease in the secretion of IL-12 and IL-23 in culture. This is consistent with earlier report on the inhibition of IFNγ expression in antigen-primed $CD4^+$ T cells with macrophages by curcumin and its restoration following addition of recombinant IL-12 in culture [12]. Other studies have also shown that curcumin inhibits the expression of CD80, CD86, and MHC class II and secretion of inflammatory cytokines in inflammatory disease models [35]. However, our findings highlight that curcumin modulates Th1/Th17 responses by acting directly on T cells and indirectly by attenuating IL-12/IL-23 production by APCs in EAE.

Th2 and Treg cells are critical regulators of Th1 and Th17 responses in EAE/MS [39]. In this study, we found that *in vivo* treatment with curcumin increased the expression of IL-4 and IL-10 in the CNS of mice with EAE. Curcumin also increased the $CD4^+CD25^+$-$Foxp3^+$ Treg cells in EAE, suggesting the involvement of multiple complex mechanisms involving Th2/Treg cells in the regulation of EAE by curcumin. This is consistent with earlier reports on elevated serum levels of IgE and IL-4 in latex-exposed mice and increased Th2 differentiation in chronic colitis by curcumin [40]. Curcumin-exposed dendritic cells also induce the differentiation of naïve $CD4^+$ T cells into IL-10-producing $CD4^+CD25^+Foxp3^+$ regulatory T cells [41]. Thus, there is an interplay between many different helper T cell subsets in the regulation of EAE by curcumin. This is similar to the severe loss of effector and memory T cells, down-regulation of Th1 and up-regulation of Th2 responses with a decrease in the proliferation of effector T cells in tumors that was reversed by curcumin [42]. Moreover, $CD4^+$ and $CD8^+$ T cells from EAE mice express elevated levels of TLR4 and TLR9 that was attenuated following treatment with curcumin [43]. While the activation of many inflammatory signaling pathways may converge on the differentiation of Th1/Th17 cells, selective inhibition of these inflammatory signaling networks by curcumin would attenuate Th1/Th17 responses in EAE [44].

There are many dietary components that can influence the development and function of the immune system. Among them, curcumin remains as a potent, nontoxic, bioactive agent known for centuries as a household remedy for many ailments, but its low aqueous solubility and poor bioavailability dampen therapeutic application in human. The recent development of curcumin derivatives, liposome conjugates and nanoparticles with increased solubility and bioavailability has strengthened its promise for clinical applications. Moreover, the mechanisms by which curcumin gets transported in living cells across plasma membrane and into the brain across the blood–brain barrier are other areas of intense investigation. Interestingly, recent studies have shown that the PPARγ can function as a nuclear receptor for curcumin in different cell types [45]. We and others have shown earlier that PPARγ agonists inhibit EAE by modulating Th1/Th17 responses [46]. We have also shown that PPARγ-deficient heterozygous mice and those treated with PPARγ antagonists develop exacerbated EAE [47]. In this study, we found that *in vivo* treatment with curcumin elevated the expression of PPARγ in the CNS and lymphoid organs of mice with EAE, suggesting its association with the regulation of Th1/Th17 responses in EAE. This is consistent with an earlier study showing the activation of PPARγ by curcumin in sepsis that was blocked by PPARγ antagonist, suggesting the PPARγ-dependent actions of curcumin *in vivo* [45]. Curcumin also enhanced the expression and nuclear translocation of PPARγ (peroxisome proliferator-activated receptor) in lipopolysaccharide (LPS)-activated macrophages in culture, further suggesting the involvement of PPARγ in mediating the anti-inflammatory actions of curcumin [45]. Although a direct causal link between IL-10 and Th1/Th17 responses is not shown in this study, the presence of PPARγ response element in IL-10 gene promoter and its activation-associated increase in the production of IL-10 by PPARγ agonists in dendritic cells and $CD4^+$ T cells suggest that PPARγ/IL-10 axis would play a critical role in the regulation of Th1/Th17 responses in EAE [48]. Our future studies will further investigate the role of PPARγ and IL-10 in the regulation of Th1/Th17 responses by curcumin in EAE/MS and other inflammatory diseases.

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