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Curcumin Inhibits Experimental Allergic Encephalomyelitis by Blocking IL-12 Signaling Through Janus Kinase-STAT Pathway in T Lymphocytes[1]

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Experimental allergic encephalomyelitis (EAE) is a CD4$^+$ Th1 cell-mediated inflammatory demyelinating autoimmune disease of the CNS that serves as an animal model for multiple sclerosis (MS). IL-12 is a proinflammatory cytokine that plays a crucial role in the induction of neural Ag-specific Th1 differentiation and pathogenesis of CNS demyelination in EAE and MS. Curcumin (1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is a naturally occurring polyphenolic phytochemical isolated from the rhizome of the medicinal plant *Curcuma longa*. It has profound anti-inflammatory activity and been traditionally used to treat inflammatory disorders. In this study we have examined the effect and mechanism of action of curcumin on the pathogenesis of CNS demyelination in EAE. In vivo treatment of SJL/J mice with curcumin significantly reduced the duration and clinical severity of active immunization and adoptive transfer EAE. Curcumin inhibited EAE in association with a decrease in IL-12 production from macrophage/microglial cells and differentiation of neural Ag-specific Th1 cells. In vitro treatment of activated T cells with curcumin inhibited IL-12-induced tyrosine phosphorylation of Janus kinase 2, tyrosine kinase 2, and STAT3 and STAT4 transcription factors. The inhibition of Janus kinase-STAT pathway by curcumin resulted in a decrease in IL-12-induced T cell proliferation and Th1 differentiation. These findings highlight the fact that curcumin inhibits EAE by blocking IL-12 signaling in T cells and suggest its use in the treatment of MS and other Th1 cell-mediated inflammatory diseases. *The Journal of Immunology,* 2002, 169: 6506–6513.

Multiple sclerosis (MS)[3] is an inflammatory demyelinating disease of the CNS that afflicts more than one million people worldwide (1). A substantial percentage of MS patients develop clinical paralysis and become wheelchair bound or generally disabled during their lives. There is no medical treatment available so far that can cure MS (2, 3). The destruction of oligodendrocyte and myelin sheath in the CNS is the pathological hallmark of MS (4). Although the etiology is not known, MS is generally viewed as an inflammatory autoimmune disease of the CNS resulting from activation, expansion, and homing of myelin Ag-sensitized T cells in the CNS (5–7). Experimental allergic encephalomyelitis (EAE) is a CD4$^+$ Th1 cell-mediated inflammatory autoimmune demyelinating disease of the CNS (8). EAE can be induced in many susceptible strains of rodents and primates by immunizing with whole brain homogenate or purified neural Ags such as myelin basic protein (MBP), proteolipid protein, and myelin oligodendrocyte glycoprotein (9). The antigenic epitopes of neural Ags have been mapped, and adoptive transfer of T cells reactive to these epitopes is sufficient to induce the disease (10). Many 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 CNS demyelination in EAE/MS is a complex process that involves activation of macrophage/microglial cells, differentiation of neural Ag-specific Th1 cells, and secretion of inflammatory cytokines in the CNS (11). IL-12 is a 70-kDa heterodimeric proinflammatory cytokine, produced mainly by macrophage/microglial cells in the CNS, that plays a critical role in the differentiation of encephalitogenic Th1 cells and pathogenesis of EAE and MS (12–14). MS patients showed increased expression of IL-12 in brain lesions, cerebrospinal fluid, and circulation in association with clinical relapses (15–20). The expression of IL-12 in the CNS and lymphoid organs of mice with EAE was also associated with the pathogenesis of CNS inflammation and demyelination (21–23). LPS and CD40 ligand (CD40L) are potent inducers of IL-12 gene expression that involves the activation of NF-κB signaling pathway (24, 25), whereas IL-12 signaling through Janus kinase (JAK)-STAT pathway is crucial in the induction of Th1 differentiation (26–28). We and others (29–31) have shown earlier that the inhibition of IL-12 production or IL-12 signaling prevents the differentiation of Th1 cells and pathogenesis of CNS demyelination in EAE.

Curcumin (diferuloylmethane) is a naturally occurring yellow pigment isolated from the rhizomes of the plant *Curcuma longa* (Linn), found in south Asia (32, 33). It has commonly been used as a coloring and flavoring spice in food products. Curcumin has also been traditionally used to treat many inflammatory disorders and for wound healing for centuries. The medicinal value of curcumin has been well recognized with its antioxidant, antitumor, and anti-inflammatory activities and is under preclinical trial for the treatment of cancer and inflammation (34–36). Recent studies have shown that curcumin inhibits inflammation in the animal

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[3] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental allergic encephalomyelitis; MBP, myelin basic protein; JAK, Janus kinase; MMCS, mean maximum clinical severity; MSCH, mouse spinal cord homogenate; TYK, tyrosine kinase; CD40L, CD40 ligand.

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models of atherosclerosis, Alzheimer's disease, and arthritis (37–40). The anti-inflammatory activity of curcumin was associated with its ability to inhibit the production of proinflammatory cytokines such as TNF-α, IL-1, IL-8, and inducible NO synthase (41–44). Although the exact mechanisms involved in the anti-inflammatory activity of curcumin is not fully defined, it prevents the activation of NF-κB, AP-1, and c-Jun kinase (45, 46). Recent studies have also shown that curcumin inhibits IL-12 production from macrophages and thereby prevents the differentiation of Th1 cells in vitro (47, 48). However, no previous study has examined the use of curcumin in the treatment of MS or other Th1 cell-mediated inflammatory diseases of the CNS.

In this study, we have examined the effects and mechanism of action of curcumin on the pathogenesis of CNS inflammation and demyelination in EAE. Our results showed that curcumin inhibits CNS demyelination by blocking IL-12 production, IL-12 signaling, and differentiation of neural Ag-specific Th1 cells in EAE and suggest its use in the treatment of MS and other Th1 cell-mediated inflammatory diseases.

Materials and Methods

Animals and cells

SJL/J mice were purchased from C. Reader (National Institutes of Health, Bethesda, MD) and maintained in the animal care facility at Vanderbilt University Medical Center (Nashville, TN). Activated T cells were prepared by stimulation of spleen cells from SJL/J mice (2×10^6/ml) with 5 μg/ml Con A (Pharmacia Biotech, Uppsala, Sweden) in RPMI medium supplemented with 10% FBS (Life Technologies, Rockville, MD) at 37°C and 5% CO_2. After 3 days of culture, cells were harvested and cultured in medium containing 0.5% FBS for an additional 24 h to synchronize to G_1 phase of the cell cycle. The T cell blasts were isolated by centrifugation over Histopaque (Sigma-Aldrich, St. Louis, MO) at $1200 \times g$ for 15 min and used for experiments (49, 50). This population of cells normally contains >98% T cell blasts as measured by flow cytometry. The peritoneal macrophage was isolated from thioglycolate-stimulated SJL/J mice as described elsewhere (51, 52). The EOC-20 mouse microglial cell line (53) was a kind gift of W. Walker (St. Jude Children's Research Hospital, Memphis, TN).

Reagents

The curcumin (1,7-Bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) was purchased from Calbiochem (La Jolla, CA). Recombinant murine IL-12 and IFN-γ were purchased from R&D Systems (Minneapolis, MN). The anti-IFN-γ mAb R4-6A2 was purified from ascitic fluid collected from nude mice following transplantation of R4-6A2 hybridoma cells (no. HB 170; American Type Culture Collection, Manassas, VA). The anti-IFN-γ mAb MM700 was obtained from Endogen (Woburn, MA) and conjugated with biotin according to standard protocol. Anti-IL-12 mAb C17.8 (anti-p40) was prepared from hybridoma cells kindly provided by G. Trinchieri (Wistar Institute, Philadelphia, PA). Mouse spinal cord homogenate (MSCH) and Guinea pig MBP were prepared according to standard protocols (21, 29–31). Anti-JAK2 Ab and anti-phosphotyrosine mAb 4G10 were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-tyrosine kinase (TYK)2, anti-STAT3, and anti-STAT4 Abs were from Santa Cruz Biotechnology (Santa Cruz, CA).

Induction and treatment of EAE

To induce active EAE, 4- to 6-wk-old female SJL/J mice were immunized (s.c.) with 800 μg of MSCH in 150 μl emulsion of IFA containing 50 μg/ml H37Ra in the lower dorsum on days 0 and 7. To induce adoptive transfer EAE, 4- to 6-wk-old female SJL/J mice (donor) were immunized with 350 μg MBP in CFA on days 0 and 7. On day 14, the lymph node and spleen cells were isolated and cultured in RPMI medium (5×10^6 or 2.5×10^6 cells/ml, respectively) with 25 μg/ml MBP. After 4 days of culture, the T cell blasts were harvested and 1×10^7 cells were injected (i.p.) into naive female SJL/J mice. Mice in the test groups were treated (i.p.) with 50 or 100 μg curcumin in 25 μl DMSO on every other day from 0 to 25 days following induction of active or passive EAE. Mice in the control group received 25 μl DMSO. The clinical paralysis in active and passive EAE was graded 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 (21, 30).

Histological analysis

To assess the degree of CNS inflammation and demyelination, SJL/J mice treated with curcumin following induction of active EAE were euthanized on day 15 (at the peak of the disease) by CO_2 asphyxiation and perfused by intracardiac injection of PBS containing 4% paraformaldehyde and 1% glutaraldehyde. Five-micrometer thick transverse sections were taken from cervical, upper thoracic, lower thoracic, and lumbar regions of the spinal cord (four sections per mouse). The sections were stained with Luxol Fast Blue (E. M. Science, Cherry Hill, NJ) to assess demyelination and with H&E to asses leukocyte infiltration and inflammation. The signs of inflammation and demyelination in the anterior, posterior, and two lateral columns (four quadrants) of the spinal cord sections were scored under microscope. Each quadrant displaying the infiltration of mononuclear cells was assigned a score of one inflammation and the quadrants that showed perivascular lesion and loss of myelin staining were assigned a score of one demyelination. Thus, each animal had a potential maximum score of 16 inflammation and/or 16 demyelination, and this study represents the analysis of 15 representative mice from three different groups. The pathologic score (inflammation or demyelination) for each group was expressed as the percentage positive over the total number of quadrants examined (29–31).

T cell proliferation assay

The effect of curcumin on neural Ag- or IL-12-induced T cell proliferation was measured by [^{3}H]thymidine incorporation assay. MBP-immune spleen cells were cultured in 96-well tissue culture plates in RPMI medium (2×10^5/200 μl/well) in the presence of 0, 25, or 100 μg/ml MBP with different concentrations (0–20 μg/ml) of curcumin. [^{3}H]Thymidine (0.5 μCi/ml) was added at 72 h and the uptake of radiolabel was measured after 96 h by a Wallac beta plate scintillation counter (Wallac, Turku, Finland) (30, 31). MBP-immune spleen cells were also cultured with 0 or 25 μg/ml MBP and 2 ng/ml rIL-12 with different concentrations (0–20 μg/ml) of curcumin. [^{3}H]Thymidine (0.5 μCi/ml) was added at 72 h and the uptake of radiolabel was measured after 96 h by a Wallac beta plate scintillation counter. Con A-activated T cells were cultured in RPMI medium in 96-well tissue culture plates (1×10^5/well) with 2 ng/ml rIL-12 in the absence or presence of different concentrations of curcumin for 48 h. [^{3}H]Thymidine was added for the last 12 h and the radiolabel was measured as above (29–31).

Immunoprecipitation and Western blot analysis

The immunoprecipitation and Western blot analyses of JAK and STAT proteins were performed as described earlier (31, 49, 50). Briefly, Con A-activated T cells (2.5×10^7/lane) were pretreated with different concentrations of curcumin for 15 min and then stimulated with 2 ng/ml IL-12 at 37°C for 15 min. Cell lysates were prepared and JAK2, TYK2, STAT3, and STAT4 proteins were immunoprecipitated using specific Abs and protein A-Sepharose. The phosphoproteins in the immune complex were analyzed by 7.5% SDS-PAGE and Western blot using anti-phosphotyrosine mAb 4G10 and visualized by an ECL detection system. The blots were stripped and reprobed with specific Ab to ensure equal protein loading.

Culture for IL-12 and IFN-γ assay

MBP-immune spleen cells were cultured in 24-well plates in RPMI medium (5×10^5/ml) with 25 μg/ml MBP in the presence of different concentrations of curcumin and the culture supernatants were collected after 24 h. Peritoneal macrophage and EOC-20 microglial cells were cultured in DMEM containing 10% FBS with 50 ng/ml IFN-γ plus 1 μg/ml LPS or 2 μg/ml anti-CD40 Ab plus second Ab in the absence or presence of curcumin, and the culture supernatants were collected after 48 h (31, 52). Naive spleen T cells from SJL/J mice were enriched (>95% purity) by passing through a nylon wool column and plastic adherence. Anti-CD3 mAb (2C11, 5 μg/ml) was immobilized onto six-well tissue culture plates by incubation for 1 h at 37°C. After washing the plates with PBS, T cells were added into the wells (1×10^6 cells/ml) and cultured in the presence or absence of 2 ng/ml rIL-12 and different concentrations of curcumin. After 5 days of culture, an equal number of viable cells (5×10^5 cells/ml) were restimulated with soluble anti-CD3 mAb (2C11, 5 μg/ml), and the supernatants were collected after 36 h (30).

ELISA for IL-12 and IFN-γ

The levels of IL-12 and IFN-γ in the culture supernatants were measured by ELISA as described earlier (21, 29–31, 52). Briefly, ELISA plates were coated with 2 μg/ml anti-IL-12 mAb C17.15 or anti-IFN-γ mAb R4-6A2

capture Ab, and the residual binding sites were blocked with 3% BSA. The test samples (culture supernatants) and standards (rIL-12 or rIFN-γ) were added and incubated overnight at 4°C. The plates were washed and 0.2 μg/ml biotin-conjugated anti-IL-12 mAb, C15.6, or anti-IFN-γ mAb MM700 was added. After a 1-h incubation at room temperature, avidin-alkaline phosphatase and *p*-nitrophenyl phosphate were added and the absorbance was read at 405 nm. The levels of IL-12 and IFN-γ in the culture supernatants were calculated from the standard curve.

Results

Curcumin inhibits active immunization EAE in SJL/J mice

To test the use of curcumin in the treatment of MS and other inflammatory demyelinating diseases of the CNS, we first examined the protective effect of curcumin on the pathogenesis of active immunization EAE. SJL/J mice were treated with curcumin (i.p., 50 and 100 μg every other day) from day 0 to 25 following induction of active EAE by immunization with MSCH. All 15 mice in the DMSO-treated control group developed clinical paralysis for a mean duration of 16 days with a mean maximum clinical severity (MMCS) of 3.1 on day 14 (Fig. 1*A*). Conversely, treatment with curcumin decreased the clinical severity and duration of active EAE. The mice treated with 50 μg curcumin developed paralysis only for a mean duration of 10 days (37.5% reduction) with a MMCS of 0.8 (74.2% reduction). In contrast, treatment with 100 μg curcumin decreased the mean duration of disease to 8 days (50% reduction) with a MMCS of 0.3 (90.32% inhibition; $p < 0.001$). These results suggest that curcumin inhibits the severity and duration of clinical paralysis in active EAE.

Curcumin inhibits adoptive transfer EAE in SJL/J mice

To further study the effect of curcumin on the pathogenesis of CNS inflammation and demyelination, we examined the in vivo effect of curcumin on adoptive transfer EAE. SJL/J mice were treated with curcumin (i.p., 50 and 100 μg every other day) from days 0 to 25 following induction of passive EAE by adoptive transfer of MBP-specific T cells. All 15 mice in the DMSO-treated control group developed clinical paralysis for a duration of 16 days with a MMCS of 2.8 on day 10 (Fig. 1*B*). Treatment of mice with curcumin decreased the clinical severity of adoptive transfer EAE. Treatment with 50 μg curcumin decreased the development of paralysis only for a duration of 12 days (25% reduction) with a MMCS of 1.2 (64.3% inhibition), and that decreased further to a duration of 8 days (50% inhibition) with a MMCS of 0.3 (89.3% inhibition; $p < 0.001$) after treatment with 100 μg curcumin (Fig. 1*B*). These results suggest that curcumin inhibits the clinical severity and duration of adoptive transfer EAE.

Curcumin decreases CNS inflammation and demyelination in EAE

We have further examined the effect of curcumin on the pathogenesis of inflammation and demyelination in the CNS of mice with EAE. Spinal cord sections from mice treated with curcumin following induction of active EAE were analyzed for the infiltration of mononuclear cells (inflammation) and myelin loss (demyelination). As shown in Fig. 1*C*, the DMSO-treated control EAE mice showed profound inflammation and demyelination in the CNS that decreased following treatment with curcumin. In control group of EAE mice, 52% of spinal cord quadrants showed positive for inflammation and 48% of spinal cord quadrants showed positive for demyelination. Conversely, treatment with curcumin decreased inflammation and demyelination in the CNS. The treatment of mice with 50 and 100 μg curcumin resulted in 75.9 and 95% decreases in inflammation, respectively (Fig. 1*C*). Treatment with 50 and 100 μg curcumin also resulted in 67.7 and 90.6% decreases in CNS demyelination. These results suggest that curcumin inhibits CNS inflammation and demyelination in EAE.

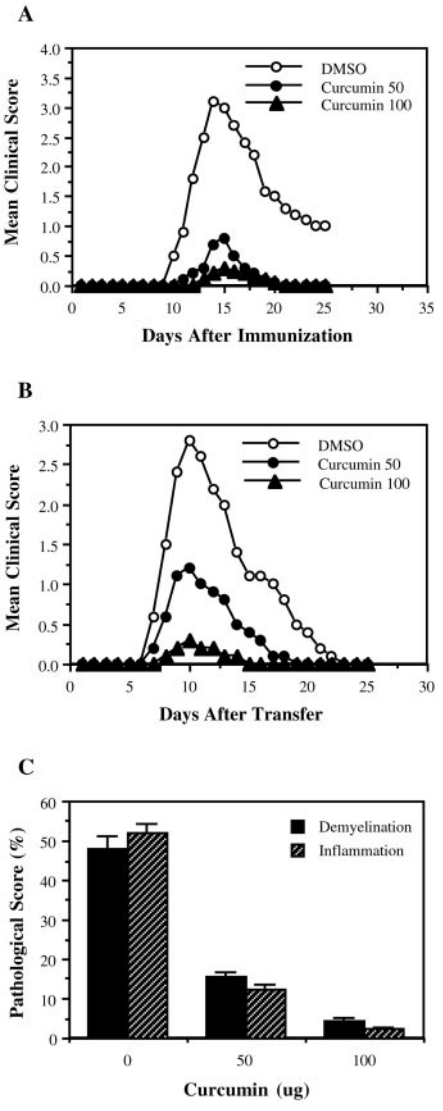

FIGURE 1. Inhibition of clinical and pathological symptoms of EAE by curcumin. *A*, Active EAE was induced in SJL/J mice by immunization with MSCH in CFA. *B*, Passive EAE was induced by adoptive transfer of MBP-sensitized T cells into recipient SJL/J mice. The mice (five per group) were treated (i.p.) with 50 or 100 μg curcumin on every other day from days 0 to 25 following induction of EAE. The clinical symptoms were scored every day and the mean clinical score of 15 mice from three different experiments is shown. Data are representatives of five different experiments. *C*, The spinal cord sections from cervical, upper thoracic, lower thoracic, and lumbar regions were prepared (four sections per mouse) and the four quadrants of every spinal cord section were scored for the presence of inflammation or demyelination. The pathological score (one for each positive quadrant) for each treatment group was expressed as a percentage over total number of quadrants examined. The average number of quadrants examined per mouse was 16; therefore, this study included the analysis of 240 spinal cord quadrants ($n = 3 \times 5$ mice)

Curcumin inhibits neural Ag-specific T cell responses

To define the mechanisms involved in the regulation of CNS demyelination by curcumin, we examined its effect on neural Ag-specific T cell responses in vitro. As shown in Fig. 2*A*, MBP-immune T cells displayed a strong proliferative response to Ag in vitro and treatment with curcumin resulted in a dose-dependent

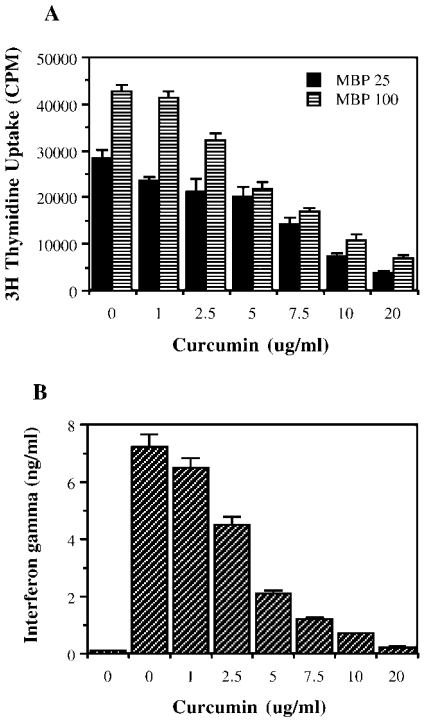

FIGURE 2. Inhibition of MBP-specific T cell responses by curcumin in vitro. *A*, MBP-immune spleen cells were stimulated in vitro with Ag in the presence of different concentrations of curcumin. [^{3}H]Thymidine was added at 72 h and the uptake of radioactivity measured after 96 h. *B*, MBP-immune spleen cells were stimulated with Ag in the presence of curcumin for 48 h. The levels of IFN-γ secreted in the culture supernatants was measured by ELISA. The values are means of triplicates at each point and the error bars represent SD. Data are representatives of five independent experiments.

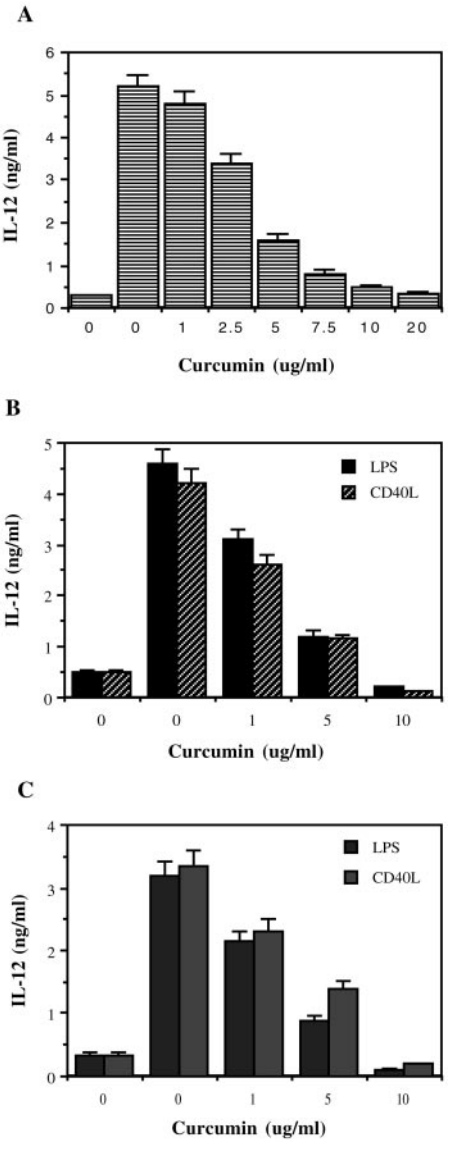

FIGURE 3. Inhibition of IL-12 production by curcumin. *A*, MBP-immune spleen cells were stimulated with 25 μg/ml MBP in the presence of curcumin for 48 h. Peritoneal macrophage (*B*) and EOC-20 microglial cells (*C*) were stimulated with LPS plus IFN-γ in the presence of curcumin for 48 h. The secretion of IL-12 in the culture supernatant was measured by ELISA. The values are means of triplicates at each point and the error bars represent SD. Data are representative of four different experiments.

decrease in proliferation. Stimulation of T cells with 25 μg/ml MBP increased the [^{3}H]thymidine uptake from 649 $\pm$ 89 cpm in the control background to 28,573 $\pm$ 1,795 cpm, and that decreased to 1,215 cpm (95.75% inhibition) following treatment with 20 μg/ml curcumin. Stimulation of cells with 100 μg/ml MBP induced the uptake of 48,213 $\pm$ 595 cpm [^{3}H]thymidine, and that decreased to 3,423 cpm (91.99% inhibition) following treatment with 20 μg/ml curcumin (Fig. 2*A*). We have further examined the Ag-induced secretion of IFN-γ from MBP-immune spleen cells in vitro. The spleen cells cultured with 100 μg/ml MBP produced 7.20 $\pm$ 0.27 ng/ml IFN-γ in 48 h. Treatment of cells with curcumin resulted in a dose-dependent decrease in IFN-γ production, reaching 0.25 $\pm$ 0.01 ng/ml at 20 μg/ml curcumin (96.53% inhibition, Fig. 2*B*). These results suggest that curcumin inhibits EAE by inhibiting the neural Ag-specific Th1 cell proliferation and IFN-γ production in EAE.

Curcumin inhibits IL-12 production in spleen cells, macrophages, and microglia

To determine the mechanisms involved in the curcumin regulation of neural Ag-specific Th1 responses in EAE, we examined the effect of curcumin on IL-12 production in spleen cells. As shown in Fig. 3*A*, stimulation of MBP-immune spleen cells with 100 μg/ml MBP resulted in an increase in the production of IL-12 in vitro. Treatment with curcumin decreased the neural Ag-induced IL-12 production in a dose-dependent manner with a maximum of 93.27% inhibition at 20 μg/ml (Fig. 3*A*). Because macrophages are the major producers of IL-12 in spleen, we isolated peritoneal macrophages from thioglycolate-stimulated SJL/J mice and stimulated them with 50 ng/ml IFN-γ and 1 μg/ml LPS or anti-CD40 Ab in the absence or presence of curcumin in vitro. As shown in Fig. 3*B*, treatment of macrophage with 10 μg/ml curcumin resulted in 95.44 and 97.02% inhibition of IL-12 production induced, respectively, by IFN-γ plus LPS and CD40L. Because microglial cells are the major producers of IL-12 in the CNS, we have further examined the effect of curcumin on IL-12 production in microglia. Treatment of EOC-20 mouse microglial cells with 10 μg/ml curcumin also resulted in 96.56 and 94.33% inhibition of IL-12 production induced, respectively, by IFN-γ plus LPS and CD40L in microglia (Fig. 3*C*). These results suggest that curcumin inhibits neural Ag-specific Th1 cells by blocking IL-12 production from splenic macrophages and microglia in EAE.

Curcumin inhibits IL-12-induced T cell responses

To determine the effect of curcumin on IL-12-induced responses in T cells, we first examined the effect of curcumin on the proliferative response of MBP-immune T cells in the presence of exogenous IL-12 in culture. As shown in Fig. 2*A*, curcumin inhibited the proliferation of T cells in response to MBP in vitro. However, the addition of rIL-12 failed to restore the MBP-induced proliferation of T cells from inhibition by curcumin (data not shown), suggesting that curcumin may also regulate IL-12-induced responses in T cells. To test this possibility, we have further examined the effect of curcumin on IL-12-induced proliferation of activated T cells. Stimulation of Con A-activated T cells with IL-12 resulted in a dose-dependent increase in proliferation as measured by [^{3}H]thymidine uptake assay. The cells cultured in medium alone showed a background count of 1,840 $\pm$ 126 cpm, which increased to 43,978 $\pm$ 2,186 following the addition of 2 ng/ml rIL-12. Treatment of cells with curcumin resulted in a dose-dependent decrease in IL-12-induced proliferation of T cells (Fig. 4*A*). While the cells treated with 1 μg/ml curcumin showed 50.02% inhibition, treatment with 10 μg/ml curcumin resulted in 94.41% inhibition of proliferation, suggesting that curcumin inhibits IL-12-induced proliferation of activated T cells in vitro. Finally, we have also examined the effect of curcumin on IL-12-induced Th1 differentiation in vitro. When compared with T cells stimulated with anti-CD3 alone, splenic T cells activated in the presence of IL-12 displayed a dramatic increase in Th1 differentiation, as measured by IFN-γ production upon secondary stimulation with anti-CD3 mAb. Strikingly, addition of curcumin resulted in a dose-dependent decrease in IL-12/anti-CD3-induced IFN-γ production with a maximum of 95.24% inhibition at 20 μg/ml levels (Fig. 4*B*), suggesting that curcumin inhibits IL-12-induced differentiation of Th1 cells in vitro.

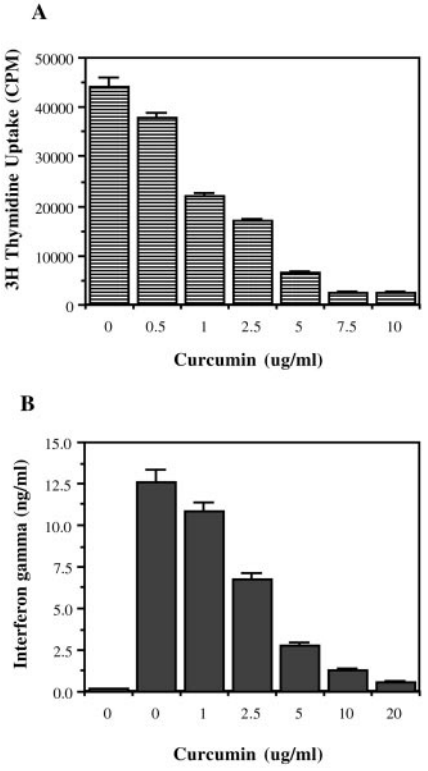

FIGURE 4. Inhibition of IL-12-induced responses by curcumin in T cells. *A*, Con A-activated T cells were stimulated with 2 ng/ml IL-12 in the presence of different concentrations of curcumin. [^{3}H]Thymidine was added at 36 h and the radioactivity was measured after 48 h. The values (cpm) are mean of triplicates at each point and the error bars represent SD. Data are representative of five experiments. *B*, Naive T cells were stimulated with anti-CD3 mAb in the presence of 2 ng/ml IL-12 and different concentrations of curcumin. After 5 days, the cells were restimulated with anti-CD3 for 24 h and the IFN-γ in the culture supernatant was measured by ELISA.

Curcumin inhibits IL-12-induced tyrosine phosphorylation of STAT3 and STAT4 in T cells

To define the mechanisms involved in the regulation of IL-12-induced T cell responses by curcumin, we have examined the effect of curcumin on the activation of STAT transcription factors in the IL-12 signaling pathway. Stimulation of Con A-activated T cells with 2 ng/ml IL-12 induced the tyrosine phosphorylation of STAT3 and STAT4 in 15 min (Fig. 5). Pretreatment of T cells with curcumin for 15 min inhibited the IL-12-induced tyrosine phosphorylation of STAT3 and STAT4. While 10 μg/ml curcumin induced partial inhibition, addition of 25 μg/ml curcumin resulted in a complete inhibition in IL-12-induced tyrosine phosphorylation of STAT3 in T cells (Fig. 5*A*). Similarly, treatment with 10 μg/ml curcumin induced partial inhibition of IL-12-induced STAT4

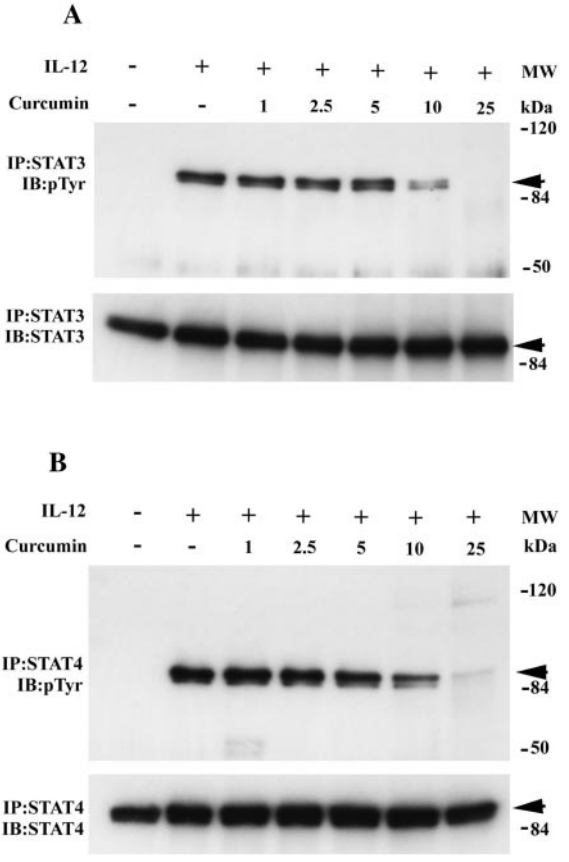

FIGURE 5. Inhibition of IL-12-induced tyrosine phosphorylation of STAT transcription factors by curcumin in T cells. Shown is Western blot analysis of STAT3 (*A*) and STAT4 (*B*) proteins immunoprecipitated from Con A-activated T cells after incubation in medium alone and with 2 ng/ml IL-12 in the absence or presence of different concentrations of curcumin at 37°C for 15 min. The cells were pretreated with curcumin for 15 min before the addition of IL-12. The STAT3 and STAT4 immune complexes were resolved on 7.5% SDS-PAGE, transferred onto nylon membrane, and probed with anti-phosphotyrosine mAb 4G10. The blots were stripped and reprobed with anti-STAT3 (*A*) or STAT4 (*B*) Ab. Arrows point to the 92-kDa STAT3 (*A*) and 89-kDa STAT4 (*B*) protein bands. Data are representative of six experiments.

phosphorylation, which was also completely inhibited by the addition of 25 μg/ml curcumin (Fig. 5*B*). These results suggest that curcumin inhibits IL-12-induced differentiation of Th1 cell by blocking the tyrosine phosphorylation of STAT3 and STAT4 transcription factors in T cells.

Curcumin inhibits IL-12-induced tyrosine phosphorylation and activation of JAK2 and TYK2 in T cells

To determine whether the inhibition of STAT proteins by curcumin was a direct effect or consequence of inhibition of upstream JAK, we examined the effect of curcumin on IL-12-induced tyrosine phosphorylation of JAK2 and TYK2 in T cells. Immunoprecipitation and Western blot analyses showed that the stimulation of Con A-activated T cells with 2 ng/ml IL-12 induced the tyrosine phosphorylation of JAK2 and TYK2 in 15 min (Fig. 6). Pretreatment of cells with curcumin reduced IL-12-induced tyrosine phosphorylation of JAK2 and TYK2 in a dose-dependent manner. While the addition of 10 μg/ml curcumin induced partial inhibition, treatment with 25 μg/ml curcumin resulted in a complete inhibition of JAK2 phosphorylation (Fig. 6*A*). Similarly, treatment with 10 μg/ml curcumin induced partial inhibition of TYK2 phosphorylation, which was also completely inhibited following addition of 25 μg/ml curcumin (Fig. 6*B*). These results suggest that curcumin inhibits IL-12-induced STAT3 and STAT4 phosphorylation by blocking tyrosine phosphorylation and activation of the upstream JAK2 and TYK2 in T cells.

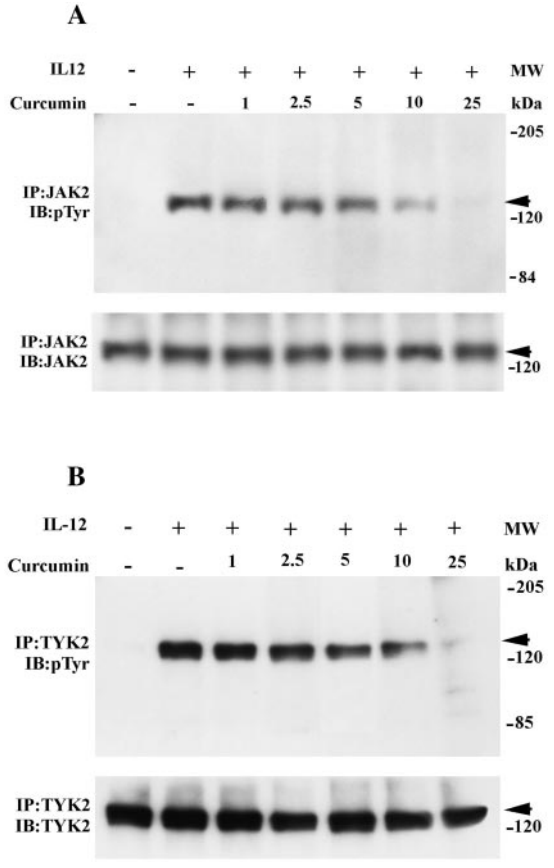

FIGURE 6. Inhibition of IL-12-induced tyrosine phosphorylation of JAK by curcumin in T cells. Shown is Western blot analysis of Con A-activated T cells incubated in medium alone and with IL-12 in the absence or presence of different concentrations of curcumin at 37°C for 15 min. The cells were pretreated with curcumin for 15 min before the induction of IL-12 signaling. The JAK2 (*A*) and TYK2 (*B*) immunoprecipitates were resolved on 7.5% SDS-PAGE, transferred onto a nylon membrane, and probed with anti-phosphotyrosine mAb 4G10. The blots were stripped and reprobed with anti-JAK2 (*A*) or anti-TYK2 (*B*) Ab. Arrows point to 130-kDa JAK2 and 135-kDa TYK2 protein bands. Data are representative of six experiments.

Discussion

In this study, we have shown that curcumin is a potent regulator of IL-12 production, IL-12 signaling, Th1 differentiation, and pathogenesis of EAE in SJL/J mice. In vivo treatment of mice with curcumin decreased the clinical and pathological symptoms of active and passive EAE. The inhibition of EAE by curcumin was associated with a decrease in IL-12 production and differentiation of neural Ag-specific Th1 cells in vivo. In vitro treatment of activated T cells with curcumin inhibited IL-12-induced activation of JAK-STAT pathway, suggesting the blockade of IL-12 signaling as the molecular mechanism in the regulation of CNS demyelination by curcumin.

The pathogenesis of EAE/MS is a complex process involving the activation of macrophage/microglial cells and differentiation of neural Ag-specific Th1 cells. The proinflammatory cytokines produced by immune cells determine the final outcome of the disease (11–14). Using the EAE model of MS, we have shown earlier that in vivo treatment with lisofylline or tyrphostin inhibits the pathogenesis of CNS inflammation and demyelination in active immunization and adoptive transfer models of EAE in SJL/J mice (30, 31). In this study, we have used the EAE model to test the potential therapeutic use of curcumin in the treatment of MS. The in vivo treatment of SJL/J mice with curcumin reduced the duration and clinical severity of active immunization and adoptive transfer models of EAE. The inhibition of clinical paralysis by curcumin was associated with a decrease in inflammation and demyelination in the CNS. Earlier studies have shown that curcumin inhibits inflammatory disease models of atherosclerosis, arthritis, and Alzheimer's disease (54–58). Those studies attributed the blockade of macrophage activation and secretion of proinflammatory cytokines and chemokines as the mechanism of inhibition of the inflammatory diseases by curcumin.

Among the many proinflammatory cytokines, IL-12 is the critical one involved in the pathogenesis of CNS demyelination in EAE and MS. Earlier studies have shown that the inhibition of IL-12 production or IL-12 signaling was effective in preventing the clinical and pathological symptoms of EAE (30, 31). In this study, we have confirmed the recent reports that curcumin inhibits LPS-induced production of IL-12 in macrophage cells in vitro (47, 48). The inhibition of IL-12 production observed in this study in MBP-immune spleen cells in response to neural Ags suggests that the inhibition of IL-12 is a mechanism of regulation of EAE by curcumin. Although the exact mechanisms involved in the regulation of IL-12 gene expression by curcumin in EAE is not known, in view of the pivotal role played by NF-κB in IL-12 gene expression (24, 25), the blockade of NF-κB signaling pathway may be a molecular mechanism in the regulation of IL-12 by curcumin in EAE.

To further define the mechanisms involved in the curcumin regulation of CNS demyelination, we examined the direct effect of curcumin on neural Ag-specific T cells. In vitro treatment of MBP-immune spleen cells with curcumin resulted in a significant decrease in the neural Ag-specific T cell proliferation and IFN-γ production. The induction of T cell proliferation is a multistep process that requires two independent signals (59). The primary signal (signal 1) is initiated through TCR that drives resting naive T cells from G_0 to G_1 phase of the cell cycle. In contrast, the secondary signal (signal 2) can be delivered through IL-2 and/or IL-12 receptors that are required for the transition of activated T cells from G_1 to S/G_2/M phase of the cell cycle (60–63). IL-12 plays a critical role in the induction of cell cycle progression in

activated T cells and, in particular, Th1 cells (62–64). Earlier studies have shown that curcumin inhibits Ag (signal 1)-induced T cell proliferation by inhibiting the activation of AP-1 and CD28 signaling (65). The inhibition of T cell proliferation was also attributed to a decrease in IL-12 production from Ag-presenting macrophages after pretreatment with curcumin (24, 25). This finding was further supported by restoration of Ag-induced T cell proliferation by the addition of exogenous rIL-12 to curcumin-pretreated macrophages (24, 25). However, in this study we observed that the addition of rIL-12 in the presence of curcumin in spleen cell cultures failed to restore the proliferation of neural Ag-specific T cells in vitro, suggesting that curcumin also induce a direct inhibitory effect on IL-12-induced responses in T cells.

To further determine the mechanisms involved in the inhibition of IL-12-induced T cell responses, we examined the effect of curcumin on IL-12 signaling and its consequence to T cell proliferation and Th1 differentiation in vitro. Signaling through its receptor, IL-12 induces tyrosine phosphorylation and activation of JAK2 and TYK2, leading to activation of STAT3 and STAT4 proteins (27, 28). Interestingly, we report here for the first time that treatment of activated T cells with curcumin blocked IL-12-induced tyrosine phosphorylation of STAT3 and STAT4 transcription factors in T cells. Curcumin also inhibited IL-12-induced activation of JAK2 and TYK2 in T cells, suggesting that the inhibition of STAT3 and STAT4 may be consequent to the blockade of upstream JAK. This is also consistent with our earlier report showing the inhibition of STAT3 and STAT4 phosphorylation following the blockade of IL-12-induced tyrosine phosphorylation of JAK2 and TYK2 by TGF-β (49). However, the recent observation on the inhibition of oncostatin M-induced phosphorylation of STAT1 in chondrocytes without affecting the activation of JAK by curcumin suggested the possible direct effect of curcumin on STAT protein in T cells (66). The blockade of JAK-STAT pathway by curcumin resulted in a decrease in IL-12-induced proliferation and Th1 differentiation in activated T cells. Earlier studies have shown that the targeted disruption or pharmacological inhibition of STAT4 activity was sufficient to block the IL-12-induced differentiation of Th1 cells in vitro and in vivo (27–31, 67). The inhibition of JAK and STAT proteins by curcumin observed in this study suggests that JAK-STAT pathway be the molecular target for curcumin regulation of neural Ag-specific Th1 cells and pathogenesis of inflammation and demyelination in EAE (Fig. 7).

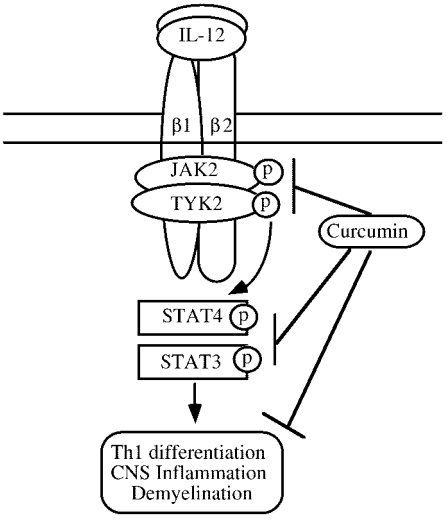

FIGURE 7. Curcumin regulation of IL-12 signaling in CNS demyelination. IL-12 induces tyrosine phosphorylation and activation of JAK-2, TYK2, STAT3, and STAT4, which are critical in the differentiation of neural Ag-specific Th1 cells and pathogenesis of CNS inflammation and demyelination. Treatment of cells with curcumin blocks IL-12-induced activation of JAK2, TYK2, STAT3, and STAT4 in T cells. The blockade of the JAK-STAT pathway by curcumin results in an inhibition of IL-12-induced differentiation of neural Ag-specific Th1 cells and pathogenesis of CNS inflammation and demyelination.

The intracellular targets involved in the anti-inflammatory actions of curcumin are not well defined. Earlier studies have shown that curcumin inhibits proinflammatory molecules such as IL-12, inducible NO synthase, and cyclooxygenase-2 by blocking the activation of NF-κB signaling pathway in macrophages (37–48). The inhibition of IL-12 signaling through JAK-STAT pathway in T cells and IL-12-induced differentiation of Th1 cells by curcumin observed in this study imply that the blockade of JAK-STAT signaling is an important mechanism by which curcumin regulates inflammation. Although the exact mechanisms involved in the curcumin regulation of JAK-STAT pathway in EAE are not known, it is likely that curcumin may directly interact with and block the tyrosine phosphorylation and activation of JAK and STAT transcription factors and interfere with the nuclear translocation and DNA binding activity of STAT proteins in T cells. Alternatively, curcumin may activate a phosphatase or other unknown proteins that in turn inhibit IL-12 signaling through JAK-STAT pathway in T cells. At this point we do not know whether the blockade of JAK-STAT pathway by curcumin is specific to IL-12 signaling alone or common to other cytokine signaling pathways as well. Our future experiments are directed to addressing these issues. IFN-β, which has been considered a therapeutic agent for MS, inhibits the induction of CD40-mediated IL-12 production from APC and differentiation of encephalitogenic Th1 cells (68–72). The inhibition of IL-12 production and IL-12 signaling in EAE by curcumin observed in this study places curcumin as a potent therapeutic agent with a complementary and perhaps synergistic effect with IFN-β in the treatment of MS. In conclusion, this study highlights the fact that curcumin inhibits EAE by blocking IL-12 signaling leading to the differentiation of neural Ag-specific Th1 cells and suggests its use in the treatment of MS and other Th1 cell-mediated inflammatory diseases.

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