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COX-2 Inhibitors Modulate IL-12 Signaling Through JAK-STAT Pathway Leading to Th1 Response in Experimental Allergic Encephalomyelitis

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Experimental allergic encephalomyelitis (EAE) is a Th1 cell-mediated autoimmune disease model of multiple sclerosis (MS). IL-12 plays a crucial role in the pathogenesis of EAE/MS and inhibition of IL-12 production or IL-12 signaling was effective in preventing EAE. Cyclooxygenase (COX-2) is a key enzyme promoting inflammation in rheumatoid arthritis and tumor induced angiogenesis. Recent studies have shown that COX-2 inhibitors prevent EAE, however, their mechanism of action is not fully understood. In this study, we show that *in vivo* treatment (i.p.) with 100 μg COX-2 selective inhibitors (LM01, LM08, LM11, and NS398), on every other day from day 0 to 30, significantly reduced the incidence and severity of EAE in SJL/J and C57BL/6 mice. Further analyses showed that the COX-2 inhibitors reduced neural antigen-induced IL-12 production, T cell proliferation and Th1 differentiation *ex vivo* and *in vitro*. The COX-2 inhibitors also decreased IL-12-induced T cell responses through blocking tyrosine phosphorylation of JAK2, TYK2, STAT3, and STAT4 proteins in T cells. These results demonstrate that COX-2 inhibitors ameliorate EAE in association with the modulation of IL-12 signaling through JAK-STAT pathway leading to Th1 differentiation and suggest their use in the treatment of MS and other Th1 cell-mediated autoimmune diseases.

KEY WORDS: Autoimmunity; EAE/MS; Th1 cell; cytokine signaling; COX-2.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that afflicts more than one million people worldwide (1, 2). About 30% of MS patients develop clinical paralysis and become wheel chair-bound for rest of their lives. Although the etiology of MS is not known, it is generally viewed as an autoimmune disease of the CNS (3, 4). The destruction of oligodendrocyte myelin sheath in the CNS is the pathological hallmark of MS (4). There is no medical treatment available so far that can cure MS (5, 6). Experimental allergic encephalomyelitis (EAE) is a $CD4^+$ Th1 cell-mediated inflammatory demyelinating autoimmune disease of the CNS. EAE can be induced in susceptible animals by immunization with whole brain homogenate, purified neural antigens such as myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) or adoptive transfer of neural antigen-specific T cells (7). Using a combination of selected neural antigens and animal species, it is possible to induce EAE with clinical symptoms similar to that seen in relapsing remitting or chronic progressive MS. For example, active immunization with MBP or adoptive transfer of MBP-specific T cells induces a relapsing-remitting variant of EAE in SJL/J mice. On the other hand, immunization of

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Abbreviation used: EAE, experimental allergic encephalomyelitis; MS, multiple sclerosis; MSCH, mouse spinal cord homogenate; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; CNS, central nervous system; MCS, mean clinical score; MMCS, mean maximum clinical score; AMCS, average mean clinical score; COX-2, cyclooxygenase 2; IL-12, interleukin 12; IFNγ, interferon gamma; Th1, T helper 1; JAK, janus kinase; STAT, signal transducer and activator of transcription; PBS, phosphate buffered saline; CSF, cerebrospinal fluid; CFA, complete Freund's adjuvant; LPS, lipopolysaccharide; FBS, fetal bovine serum.

C57BL/6 mice with MOG induces a chronic progressive EAE. The clinical and pathological features of EAE show close similarity to human MS and therefore 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 (7–14).

The pathogenesis of EAE/MS is a complex process involving activation of macrophage/microglial cells, differentiation of neural antigen-specific Th1 cells and secretion of inflammatory cytokines in the CNS (15, 16). Interleukin-12 (IL-12) is a 70 kD heterodimeric cytokine produced by macrophage, microglia and dendritic cells in the CNS that plays a crucial role in the differentiation of encephalitogenic Th1 cells and pathogenesis of EAE and MS (17, 18). Systematic studies in MS patients revealed an increase in the levels of IL-12 in brain lesions, cerebrospinal fluid (CSF) and blood in association with clinical relapses (19, 20). The expression of IL-12 in the CNS and lymphoid organs of mice with EAE also associates with the pathogenesis of CNS inflammation and demyelination and *in vivo* treatment with neutralizing anti-IL-12 antibodies prevents EAE (8, 10, 21, 22). LPS and CD40L are two potent inducers of IL-12 gene expression in macrophage, microglia and dendritic cells. While the activation of NF-κB pathway is required for IL-12 gene expression, IL-12-induced activation of JAK-STAT pathway leads to Th1 differentiation and pathogenesis of Th1 cell-mediated autoimmune diseases (23–26). We and others have shown earlier that the inhibition of IL-12 signaling is effective in preventing the differentiation of Th1 cells and pathogenesis of EAE (8–14). While, inflammatory cytokines such as TNFα, IL-12, and IFNγ, produced by activated immune cells in the CNS, contribute to pathogenesis of EAE and MS, anti-inflammatory cytokines such as IL-4, IL-10, and TGFβconfer recovery (27–29).

Cyclooxygenase (COX; prostaglandin H synthase; prostaglandin endoperoxidase) is a key family of enzymes in arachidonic acid metabolism that participates in the generation of prostaglandins and thromboxane (30). COX-1 is a constitutive isoform that performs housekeeping functions in gastric mucosa, kidney and platelets. In contrast, COX-2 is induced by pro-inflammatory cytokines, implying a role in the pathogenesis of inflammatory diseases (31, 32). Earlier studies have shown that the expression of COX-2 associates with the pathogenesis of inflammatory diseases including rheumatoid arthritis, Alzheimer's disease and tumor-induced angiogenesis (30–34). Moreover, treatment with non-selective COX-2 inhibitors induces protective effects on inflammatory diseases including EAE with multiple side effects (35–37). While therapeutic actions of non-steroidal anti-inflammatory drugs (NSAIDs) depend on inhibition of COX-2, the inhibition of COX-1 is responsible for their gastric and renal side effects. Recent studies have shown significant promise in developing COX-2 selective inhibitors and successfully using them in the treatment of inflammatory diseases such as, rheumatoid arthritis and tumor induced angiogenesis. These agents display reduced gastrointestinal side effects, but they exert unexpected cardiovascular side effects (30–38). Although recent studies have also shown the inhibition of EAE by COX-2 inhibitors (38), the mechanisms involved in the amelioration of EAE by COX-2 inhibitors is not fully understood. In this study, we demonstrate that COX-2 inhibitors ameliorate CNS inflammation and demyelination in association with the blockade of IL-12 signaling and Th1 differentiation in EAE.

MATERIALS AND METHODS

Animals and Cells

SJL/J and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and maintained in the animal care facility at Vanderbilt University Medical Center. Activated T cells were prepared by stimulation of spleen cells from SJL/J mice (2×10^6/mL) with 5 μg/mL of Concanavalin A (Con A, Pharmacia Biotech, Uppsala, Sweden) in RPMI medium supplemented with 10% FBS (Gibco BRL, 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 6 h to synchronize to G1 phase of the cell cycle. The T cell blasts were isolated by centrifugation over Histopaque (Sigma, St Louis, MO) at 1200 rpm for 15 min and used for experiments. This population of cells normally contains >98% T cell blasts as measured by flow cytometry (11, 39, 40).

Reagents

We have synthesized three different COX-2 inhibitors in our laboratory as described previously and used for the *in vitro* and *in vivo* experiments. They are indomethacin-β-hydroxyethylamide (NH(CH2)2OH, compound 35, LM01), indomethacin-β-phenylethylamine (NH (CH2) 2C6H5, compound 40, LM08) and indomethacin-*p*-fluorophenylamide (NHC6H4(4-F), compound 50, LM11) (41–46). LM01 exhibits an IC_{50} of 60 nM and a selectivity of >1100 against purified human COX-2. LM08 exhibits an IC_{50} of 60 nM and a selectivity >1100 for human COX-2. LM08 also inhibits COX-2 in LPS-activated RAW264.7 macrophages with an IC_{50} of 40 nM and exhibits anti-inflammatory activity in the rat

carrageenan foot pad model at 1.5 mg/kg. LM11 exhibits an IC_{50} of 65 nM and a selectivity >1000 for human COX-2. LM11 also exhibits anti-inflammatory activity in the rat carrageenan foot pad model at 0.5 mg/kg (42). The COX-2 inhibitor, *N*-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide (NS398) was purchased from Calbiochem (La Jolla, CA). Recombinant murine IL-12 and IFNγ were purchased from R&D Systems Inc. (Minneapolis, MN). The anti-IFNγ mAb, R4-6A2 was purified from ascetic fluid collected from nude mice following transplantation of R4-6A2 hybridoma cells (American Type Culture Collection # HB 170, Rockville, MD). The biotin-conjugated anti-IFNγ mAb, MM700 was obtained from Endogen (Woburn, MA). 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) was prepared by homogenization in PBS and freeze-dried under vacuum. The Guinea pig MBP and other chemicals were purchased from Sigma (St Louis, MO). Anti-JAK2 Ab and anti-phosphotyrosine mAb 4G10 were purchased from Upstate Biotechnology, Inc. (Lake placid, NY). Anti-TYK2, anti-STAT3 and anti-STAT4 Abs were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Induction and Treatment of EAE

To induce relapsing remitting EAE, 4–6-weeks old female SJL/J mice were immunized (s.c) with 800 μg of MSCH in 150 μL emulsion of incomplete Freund's adjuvant containing 50 μg/mL H37Ra in the lower dorsum on days 0 and 7. To induce chronic progressive EAE, 4–6-weeks-old female C57BL/6 mice were immunized (s.c) with 200 μg MOGp35-55 in 150 μL emulsion of incomplete Freund's adjuvant containing 50 μg/mL H37Ra in the lower dorsum on days 0 and 7. Mice in the test groups were treated (i.p.) with 100 μg COX-2 inhibitors in 25 μL DMSO on every other day from 0 to 25 days following induction of EAE. Mice in the control group received 25 μL DMSO. The clinical paralysis in EAE was graded on every day 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 (MCS) was calculated by adding every day clinical score for all mice in a group and then divided by total number of mice. Mean maximum clinical score (MMCS) was the MCS at the peak of disease. Average mean clinical score (AMCS) was calculated by adding the MCS for all days (from day 0 to 30) and then divided by 30 (8–14).

Histological Analysis

To assess the degree of CNS inflammation and demyelination, SJL/J mice treated with COX-2 inhibitors following induction of chronic EAE were euthanized on day 30 by CO_2 asphyxiation and perfused by intracardiac injection of 4% paraformaldehyde and 1% glutaraldehyde in PBS. Transverse sections (five each) taken from different levels of cervical, upper thoracic, lower thoracic, and lumbar regions of the spinal cord were stained with luxol fast blue or hematoxylin and eosin. The pathology of inflammation and demyelination in the CNS was examined under microscope in a blinded manner. The spinal cord sections were viewed as anterior, posterior and two lateral columns (four quadrants). Each quadrant displaying the infiltration of mononuclear cells or loss of myelin staining was assigned a score of one inflammation or one demyelination, respectively. Thus, each animal had a potential maximum score of 16 and this study represents the analysis of spinal cord from 10 representative mice per group. The pathologic scores for each group is expressed as percent positive over the total number of quadrants examined (8–14).

T cell Proliferation Assay

The *ex vivo* and *in vitro* effects of COX-2 inhibitors on neural antigen-induced T cell proliferation were measured by ^{3}H-thymidine incorporation assay. Four to six weeks-old female SJL/J mice were immunized with 300 μg of MBP in 150 μL emulsion of incomplete Freund's adjuvant containing 50 μg/mL of H37Ra on days 0 and 7 and treated with 25 μL DMSO or 100 μg COX-2 inhibitors on every other day from day 0 to 13. On day 14, the spleen cells were isolated from naïve and MBP-immune mice treated with DMSO or COX-2 inhibitors. To determine the *ex vivo* response, the splenic T cells were cultured in 96 well tissue culture plates in RPMI medium (2×10^5/200 μL/well) with 0, 10, 25, and 50 μg/mL MBP in the absence of COX-2 inhibitors in culture. To determine the *in vitro* response, the spleen cells from DMSO treated immune mice were cultured in 96 well tissue culture plates in RPMI medium (2×10^5/200 μL/well) with 25 μg/mL MBP in the absence or presence of different concentrations of COX-2 inhibitors in culture. ^{3}H-thymidine (0.5 μCi/mL) was added at 72 h and the uptake of radiolabel counted after 96 h by Wallac beta plate scintillation counter as a measure of proliferation (14). To test the effect of COX-2 inhibitors on IL-12-induced T cell proliferation, Con A-activated T cells were cultured in RPMI medium in 96 well tissue culture plates (1×10^5/200 μL/well) with 2 ng/mL rIL-12 in the absence or presence of different concentrations of COX-2

inhibitors (0–10 μg/mL) for 48 h. ^{3}H thymidine was added for the last 18 h and the radiolabel measured as above (8–14). To determine the reversible inhibition of IL-12-induced T cell proliferation by COX-2 inhibitor, Con A activated T cells were treated with 25 μg/mL COX-2 inhibitors at 37°C for 30 min. Then the cells were washed, cultured in fresh medium with increasing dose of IL-12 in the absence of COX-2 inhibitors and the proliferative response measured as above (8–14).

Detection of IL-12 and IFNγ by ELISA

Spleen cells were isolated from SJL/J mice on day 14 following immunization with MBP and treatment with DMSO or COX-2 inhibitors. The cells were cultured in 24 well plates in RPMI medium (5×10^5/mL) with 0, 10, 25, and 50 μg/mL MBP antigen in the absence of COX-2 inhibitors and the culture supernatants were collected after 48 h. MBP-immune spleen cells from DMSO treated mice were also cultured in 24 well plates in RPMI medium (5×10^5/mL) with 25 μg/mL MBP in the presence of different concentrations of COX-2 inhibitors (0–10 μg/mL). The culture supernatants were collected after 48 h and the levels of IL-12 and IFNγ measured by ELISA as described earlier (8–14). Briefly, ELISA plates were coated with 2 μg/mL of anti-IL-12 mAb, C17.15 or anti-IFNγ mAb, R4-6A2 captures Ab in 100 μL/well of bicarbonate buffer, pH 9.6. After overnight incubation at 4°C, excess Ab was washed off and the residual binding sites blocked by the addition of 3% BSA in PBS for 1 h. The test samples (culture supernatants) and standards (rIL-12 or rIFNγ) were added and incubated overnight at 4°C. Plates were washed with PBS containing 0.05% Tween-20 and 0.2 μg/mL of biotin conjugated anti-IL-12 mAb, C15.6 or anti-IFNγ mAb, MM700 added as detection Ab. After incubation at room temperature for 1 h, the plates were washed three times and avidin-alkaline phosphatase added followed by 1 mg/mL of *p*-nitrophenyl phosphate. After 30 min incubation at room temperature, the OD was read at 405 nm and the concentration of IL-12 and IFNγ in the culture supernatants were calculated (8–14).

Immunoprecipitation and Western Blot Analysis

The effect of COX-2 inhibitors on IL-12 signaling in T cells was examined by immunoprecipitation and Western blot analysis of JAK and STAT proteins as described earlier (11–14). Briefly, Con A activated T cells (10×10^6/lane) were pretreated with different concentrations of COX-2 inhibitors (0, 25, or 50 μg/mL) for 15 min and then stimulated with 2 ng/mL IL-12 at 37°C for 15 min. Cell lysates were prepared and the JAK2, TYK2, STAT3 and STAT4 proteins immunoprecipitated using specific antibodies and protein A Sepharose. The phosphoproteins in the immune complexes were analyzed by 7.5% SDS-PAGE and Western blot using anti-phosphotyrosine mAb 4G10 and visualized by enhanced chemiluminescence (ECL) detection system. The blots were stripped and reprobed with specific Ab to ensure equal protein loading (11–14).

Statistical Analysis

All experiments were repeated three or more times and the values are expressed as mean ± SD. The clinical and pathological scores in control and COX-2 inhibitor treated mice were analyzed using nonparametric Mann–Whitney testing. The immune responses in control and COX-2 inhibitor treated mice were analyzed by ANOVA to determine the statistical significance of the results and the values of $p < 0.05$ were considered significant.

RESULTS

COX-2 Inhibitors Ameliorate Relapsing Remitting EAE in SJL/J Mice

To study the effects of COX-2 inhibitors on autoimmune inflammation and demyelination, we first examined their protective effects on the pathogenesis of relapsing remitting EAE in SJL/J mice. SJL/J mice were treated with COX-2 inhibitors (100 μg, i.p., every other day) following induction of active EAE by immunization with MSCH. All 10 mice in the DMSO treated control group developed clinical paralysis for a mean duration of 21 days with MMCS of 2.8 on day 16 and an AMCS of 31.33 (Fig. 1A). Conversely, treatment with COX-2 inhibitors decreased the clinical severity and duration of relapsing EAE. The mice treated with LM01 showed paralysis for a mean duration of 11 days (48% inhibition) with an MMCS of 0.47 (83.2% reduction; $p < 0.01$) and an AMCS of 2.31 (92.6% inhibition). The mice treated with LM08 showed clinical paralysis for a mean duration of 1.0 day (95.2% inhibition) with an MMCS of 0.08 (97.5% reduction; $p < 0.01$) and an AMCS of 0.08 (99.7% inhibition; $p < 0.001$). Treatment of mice with LM11 also decreased the mean duration of disease to 5 days (76.2% reduction) with an MMCS of 0.33 (88.2% inhibition; $p < 0.001$) and an AMCS of 0.83 (97.4% inhibition; $p < 0.001$). Similarly, treatment with NS398 also decreased the mean duration of disease to 12 days (42.9% reduction) with an MMCS of 0.94 (66.4% inhibition; $p < 0.01$) and an AMCS of 4.32 (86.2% inhibition $p < 0.01$). These results

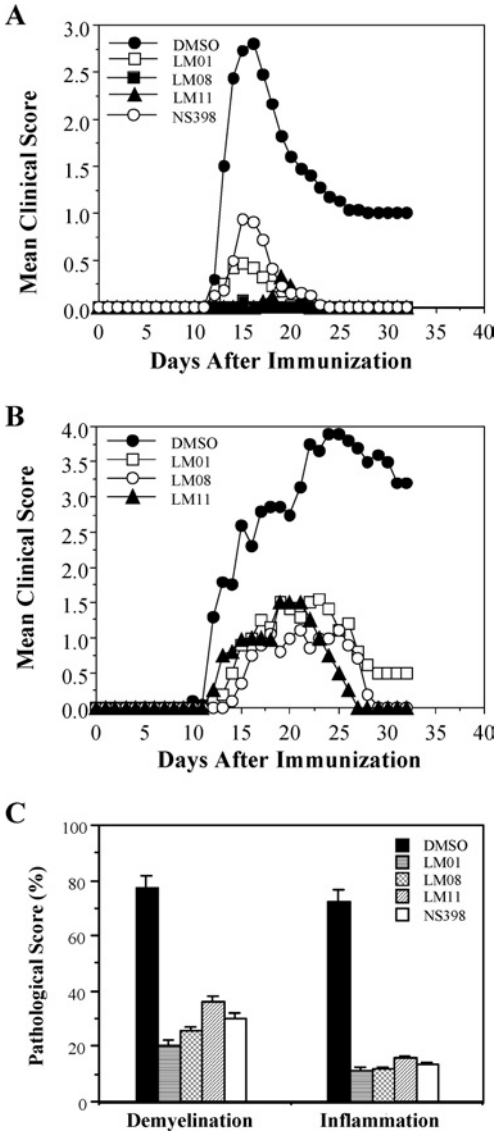

Fig. 1. Amelioration of EAE by COX-2 inhibitors. (A) Relapsing EAE was induced in SJL/J mice by immunization with MSCH in CFA. The mice (10 per group) were treated (i.p.) with 100 μg LM01, LM08, LM11 or NS398 on every other day from day 0 to 30. (B) Chronic EAE was induced in C57BL/6 mice by immunization with MOGp35-55 peptide in CFA. The mice (10 per group) were treated (i.p.) with 100 μg LM01, LM08 or LM11 on every other day from day 0 to 30. The clinical symptoms were scored every day in a blinded manner. The mean clinical score of all 10 mice per group from one experiment is shown. The figure is a representative of three independent experiments. (C) The spinal cord sections from cervical, upper thoracic, lower thoracic and lumbar regions of SJL/J mice with chronic EAE were prepared (four sections per mouse) and scored for the presence of inflammation or demyelination (one score per positive quadrant) in a blinded manner. The pathological score for each treatment group was expressed as percentage over total number of quadrants examined. The average number of quadrants examined per mouse was 16 and therefore, this study included the analysis of 160 spinal cord quadrants per group.

suggest that COX-2 inhibitors ameliorate the severity and duration of clinical paralysis in EAE.

COX-2 Inhibitors Ameliorate Chronic Progressive EAE in C57BL/6 Mice

To further study the effect of COX-2 inhibitors on autoimmune inflammation and demyelination, we then examined their protective effects on chronic progressive EAE in C57BL/6 mice. C57BL/6 mice were treated (i.p.) with 100 μg COX-2 inhibitors on every other day from day 0 to 30 following induction of EAE by immunization with MOGp35-55. All 10 mice in the DMSO treated control group developed clinical paralysis for a mean duration of 23 days with an MMCS of 3.9 on day 24 and an AMCS of 63.7 (Fig. 1B). Conversely, treatment with COX-2 inhibitors decreased the clinical severity and duration of chronic EAE. The mice treated with 100 μg LM01 showed paralysis for a mean duration of 21 days (8.7% inhibition) with an MMCS of 1.6 (58.9% reduction; $p < 0.05$) and an AMCS of 19.43 (69.5% inhibition; $p < 0.01$). Treatment with 100 μg LM08 decreased the mean duration of disease to 15 days (34.8% reduction) with an MMCS of 1.1 (71.8% inhibition; $p < 0.05$) and an AMCS of 11.78 (81.5% inhibition; $p < 0.01$). Similarly, mice treated with 100 μg LM11 showed paralysis for a mean duration of 15 days (34.8% reduction) with an MMCS of 1.5 (61.5% inhibition; $p < 0.05$) and an AMCS of 15.0 (76.4% inhibition; $p < 0.01$). These results suggest that COX-2 inhibitors reduce the severity and duration of clinical paralysis in chronic EAE.

COX-2 Inhibitors Reduce CNS Inflammation and Demyelination in EAE

We have further examined the effect of COX-2 inhibitors on inflammation and demyelination in the CNS of mice with EAE. Spinal cord sections from C57BL/6 mice treated with COX-2 inhibitors following induction of EAE were analyzed for the infiltration of mononuclear cells (inflammation) and myelin loss (demyelination). As shown in Fig. 1C, the DMSO treated control EAE mice showed profound inflammation and demyelination in the CNS that decreased following treatment with COX-2 inhibitors. The DMSO treated control mice showed 77.5% of spinal cord quadrants positive for demyelination and 72.6% of spinal cord quadrants positive for inflammation. Conversely, treatment with 100 μg of LM01, LM08, LM11, or NS398 resulted in 73.85, 66.67, 53.41, and 60.99% ($p < 0.01$) decrease in CNS demyelination, respectively. Similarly, treatments with 100 μg of LM01, LM08, LM11, and NS398 also resulted in 84.61, 84.21,

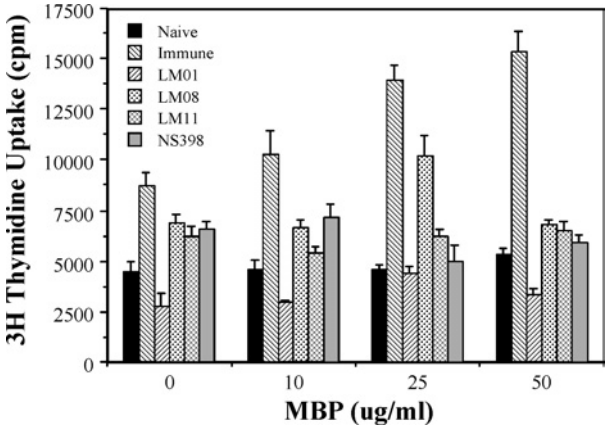

Fig. 2. Neural antigen-induced T cell proliferation in mice treated with COX-2 inhibitors *ex vivo*. Spleen cells were isolated from SJL/J mice on day 14 following immunization with MBP and treatment with 100 μg COX-2 inhibitors or DMSO vehicle alone. The cells were stimulated *in vitro* with 0, 10, 25, or 50 μg/mL MBP antigen and the *ex vivo* proliferative response measured by ^{3}H thymidine uptake assay. The values are mean of triplicates at each point and the error bars represent SD. The figures are representatives of three independent experiments.

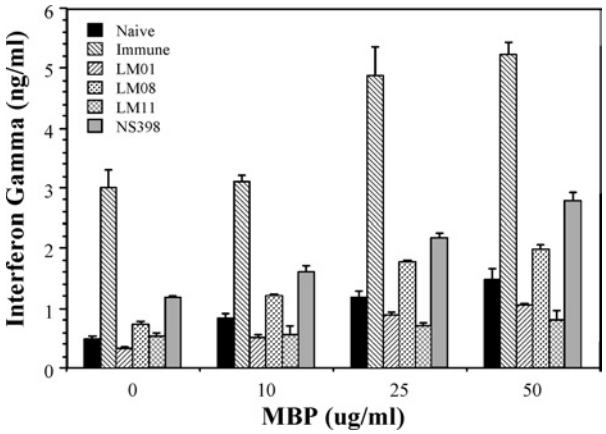

Fig. 3. Neural antigen-induced IFNγ production in mice treated with COX-2 inhibitors *ex vivo*. Spleen cells were isolated from SJL/J mice treated with COX-2 inhibitors on day 14 following immunization with MBP. The cells were stimulated *in vitro* with 0, 10, 25, or 50 μg/mL MBP antigen, the culture supernatants were collected after 48 h and the levels of IFNγ measured by ELISA. The values are mean of triplicates at each point and the error bars represent SD. The figures are representatives of three independent experiments.

77.73, and 81.38% ($p < 0.01$) decrease in CNS inflammation, respectively. These results suggest that COX-2 inhibitors reduce CNS inflammation and demyelination in EAE.

COX-2 Inhibitors Modulate Neural Antigen-Induced T Cell Proliferation Ex Vivo

To define the mechanisms in the amelioration of EAE by COX-2 inhibitors, we examined their effects on neural antigen-induced T cell proliferation *ex vivo*. As shown in Fig. 2, while the naïve spleen cells showed no increase in proliferation in response to antigen, the MBP-immune splenic T cells from DMSO treated mice displayed a dose-dependent proliferative response to the antigen. While the immune cells cultured in the absence of MBP showed a background count of 8668 ± 734 cpm, addition of 10, 25, and 50 μg/mL of MBP antigen resulted in a 1.2-, 1.6-, and 1.8-fold increase in proliferation, respectively. *In vivo* treatment with COX-2 inhibitors decreased the antigen-induced proliferation of immune spleen cells *ex vivo*. The T cell proliferative response at 50 μg/mL antigen decreased from 15312 ± 1062 cpm in DMSO control treated immune cells to 3347 ± 313 cpm (78.2% inhibition), 6825 ± 214 cpm (55.4% inhibition), 6483.3 ± 482 cpm (57.7% inhibition) and 5866.2 ± 398 cpm (61.7% inhibition) following treatment with LM01, LM08, LM11, and NS398, respectively. This decrease in *ex vivo* T cell proliferative response to antigen by COX-2 inhibitors is statistically significant ($p < 0.01$) at every concentration tested, suggesting that a decreased T cell expansion contributes to amelioration of EAE by these COX-2 inhibitors.

COX-2 Inhibitors Modulate Neural Antigen-Induced IFNγ Production in EAE Ex Vivo

We then examined the secretion of IFNγ in spleen cells from MBP-immune mice following treatment with DMSO or COX-2 inhibitors. As shown in Fig. 3, stimulation of MBP-immune spleen cells with the antigen induced a dose-dependent increase in the secretion of IFNγ in culture. Naïve spleen cells produced 1.46 ± 0.19 ng/mL IFNγ at 50 μg/ml antigen that increased to 2.99 ± 0.31, 3.12 ± 0.09, 4.87 ± 0.50, and 5.21 ± 0.24 ng/mL following stimulation of MBP-immune spleen cells with 10, 25, and 50 μg/mL antigen, respectively. Treatment with COX-2 inhibitors reduced the antigen-induced IFNγ secretion in immune spleen cells *ex vivo*. While the immune spleen cells from DMSO-treated mice stimulated with 50 μg/mL MBP antigen showed 5.21 ± 0.24 ng/mL IFNγ, that decreased to 1.05 ± 0.02 ng/mL (79.85% inhibition), 1.97 ± 0.08 (62.19% inhibition), 0.80 ± 0.17 (84.65% inhibition), and 2.79 ± 0.14 (46.45% inhibition), in spleen cells from mice treated with LM01, LM08, LM11, and NS398, respectively. These results suggest that *in vivo* treatment with COX-2 inhibitors decreased the *ex vivo* secretion of IFNγ in response to neural antigens that contributes to amelioration of EAE.

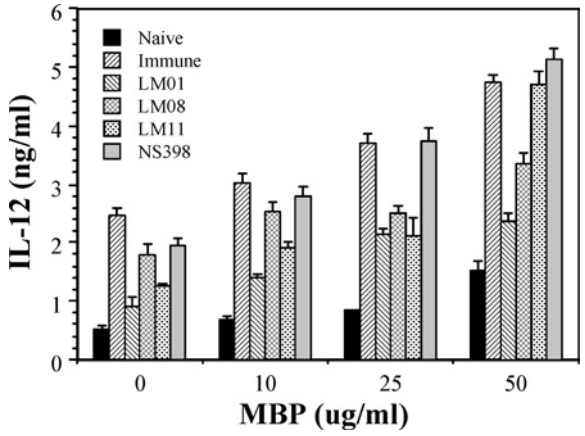

Fig. 4. Neural antigen-induced IL-12 production in mice treated with COX-2 inhibitors *ex vivo*. Spleen cells were isolated from SJL/J mice treated with COX-2 inhibitors on day 14 following immunization with MBP. The cells were stimulated *in vitro* with 0, 10, 25, or 50 μg/mL MBP antigen, the culture supernatants were collected after 48 h and the levels of IL-12 measured by ELISA. The values are mean of triplicates at each point and the error bars represent SD. The figures are representatives of three independent experiments.

COX-2 Inhibitors Modulate Neural Antigen-Induced IL-12 Production Ex Vivo

We then examined the secretion of IL-12 in spleen cells from MBP-immune mice following treatment with DMSO or COX-2 inhibitors. While, naïve spleen cells produced 1.52 ± 0.17 ng/mL IL-12 in response to 50 μg/mL antigen in culture, MBP-immune spleen cells produced 2.46 ± 0.15, 3.02 ± 0.17, 3.71 ± 0.16, and 4.78 ± 0.11 ng/mL IL-12 in response to 0, 10, 25, and 50 μg/ml antigen, respectively. *In vivo* treatment with COX-2 inhibitors decreased the secretion of IL-12 in response to antigen *ex vivo*. As shown in the Fig. 4, the immune spleen cells from mice treated with LM01, LM08, LM11, and NS398, respectively showed 2.38 ± 0.12 ng/mL (49.79% inhibition), 3.35 ± 0.19 ng/mL (29.33% inhibition), 4.70 ± 0.23 ng/mL (0.85% inhibition), and 5.13 ± 0.18 ng/mL (8.2% increase) following stimulation with 50 μg/mL MBP antigen. These results suggest that *in vivo* treatment with the LM series of COX-2 inhibitors, but not NS398, decrease the *ex vivo* secretion of IL-12 in response to neural antigen that contributes to amelioration of EAE.

COX-2 Inhibitors Reduce Neural Antigen-Induced T Cell Responses In Vitro

To further define the mechanisms in the regulation of CNS demyelination by COX-2 inhibitors, we examined its effect on neural antigen-induced T cell responses *in vitro*.

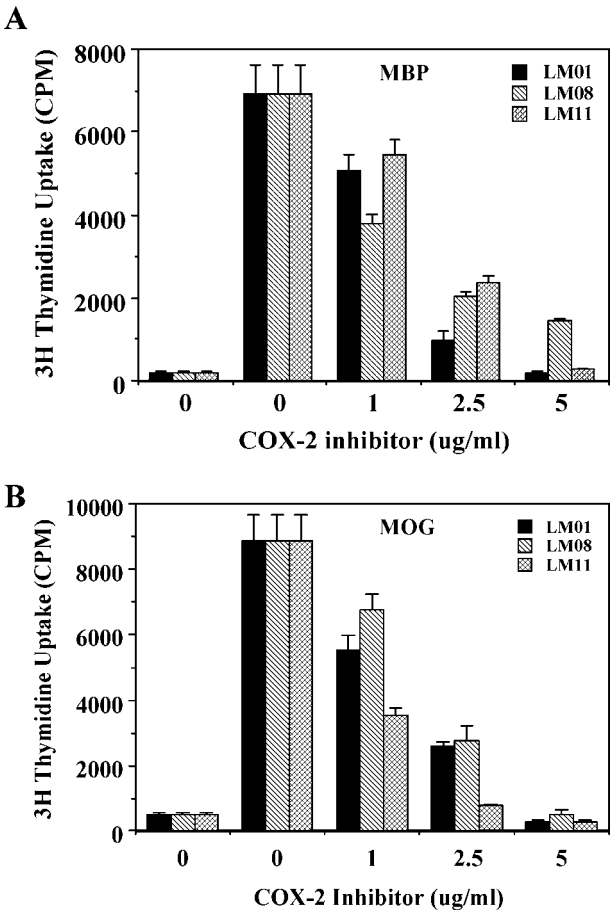

Fig. 5. Inhibition of neural antigen induced T cell responses by COX-2 inhibitors *in vitro*. (A) MBP-immune spleen cells from SJL/J mice were stimulated *in vitro* with 25 μg/mL MBP in the presence of 0, 1, 2.5, and 5 μg/mL LM01, LM08 and LM11. ^{3}H-thymidine was added at 72 h and the uptake of radioactivity measured after 96 h as an index of proliferation. (B) MOG-p35-55-immune spleen cells from C57BL/6 mice were stimulated with antigen in the presence of 0, 1, 2.5, and 5 μg/mL LM01, LM08 and LM11. The ^{3}H-thymidine was added at 72 h and the uptake of radioactivity measured after 96 h as an index of proliferation. The values are mean of triplicates at each point and the error bars represent SD. The figures are representatives of four independent experiments.

As shown in Fig. 5, spleen cells from mice immunized with MBP or MOGp35-55 antigen displayed a strong proliferative response to the respective antigens and *in vitro* treatment with COX-2 inhibitors resulted in a dose-dependent decrease in proliferation. Stimulation of MBP-immune spleen cells with 25 μg/mL MBP increased the ^{3}H thymidine uptake from 201 ± 45 in the control background to 6914 ± 679 cpm, whereas, treatment with 1, 2.5, and 5 μg/mL LM01 respectively resulted 26.6, 85.6, and 97.2% decrease in MBP-induced proliferation *in vitro*. Similar treatment with 1, 2.5, and 5 μg/mL LM08 resulted

in 45.13, 70.7, and 79.4% decrease in proliferation. Treatment with LM11 also resulted in 20.7, 65.7, and 95.5% inhibition at 1, 2.5, and 5 μg/mL, respectively (Fig. 5A). Further analyses showed that COX-2 inhibitors also decreased the MOGp35-induced proliferation in immune spleen cells from C57BL/6 mice. Stimulation of T cells with 25 μg/mL MOGp35-55 increased the ^{3}H thymidine uptake from 502 $\pm$ 40 in the control background to 8881 $\pm$ 782 following stimulation with 25 μg/mL MOGp35-55 antigen, whereas, treatment with 1, 2.5, and 5 μg/mL LM01, respectively resulted 37.9, 71.0, and 96.9% decrease in antigen-induced proliferation *in vitro*. Similar treatment with 1, 2.5, and 5 μg/mL LM08 resulted in 24.2, 68.9, and 94.3% decrease in proliferation. Furthermore, treatment with LM11, resulted in 60.4, 91.2, and 96.8% inhibition at 1, 2.5, and 5 μg/mL, respectively (Fig. 5B). These results suggest that COX-2 inhibitors decrease neural antigen-induced T cell proliferation in culture.

COX-2 Inhibitors Decrease IL-12-Induced T Cell Responses In Vitro

To determine the effect of COX-2 inhibitors on IL-12-induced responses in T cells, we examined the effect of COX-2 inhibitors on IL-12-induced proliferation of activated T cells in culture. 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, whereas, treatment with COX-2 inhibitors resulted in a dose-dependent decrease in IL-12-induced proliferation (Fig. 6A, B). Cells cultured in medium alone showed a background count of 9162 $\pm$ 887 cpm and that increased to 58854 $\pm$ 3990 (6.4-fold increase) with the addition of 2 ng/mL rIL-12 (Fig. 6B). The cells treated with 1, 2.5, 5, and 10 μg/mL LM01 showed 20.6, 39.7, 71.6, and 100% inhibition, similar treatment with LM08 resulted in 4.1, 41.5, 55.7, and 83.3% inhibition and LM11 showed 2.9, 7.3, 59.5, and 79.8% inhibition of proliferation, respectively. A similar treatment with 1, 2.5, 5, and 10 μg/mL NS398 also resulted in 27.1, 53.7, 60.4, and 79.2% inhibition of IL-12-induced T cell proliferation *in vitro*. We have further examined the reversibility of IL-12-induced proliferation inhibited by COX-2 inhibitors in T cells. As shown in Fig. 6B, Con A activated T cells treated with 25 μg/mL COX-2 inhibitors for 30 min displayed a dose-dependent proliferative response to IL-12 as measured by ^{3}H thymidine uptake assay comparable to that seen in DMSO treated control cells in culture. These results suggest that COX-2 inhibitors induce a reversible inhibition of IL-12-induced proliferation in activated T cells *in vitro*.

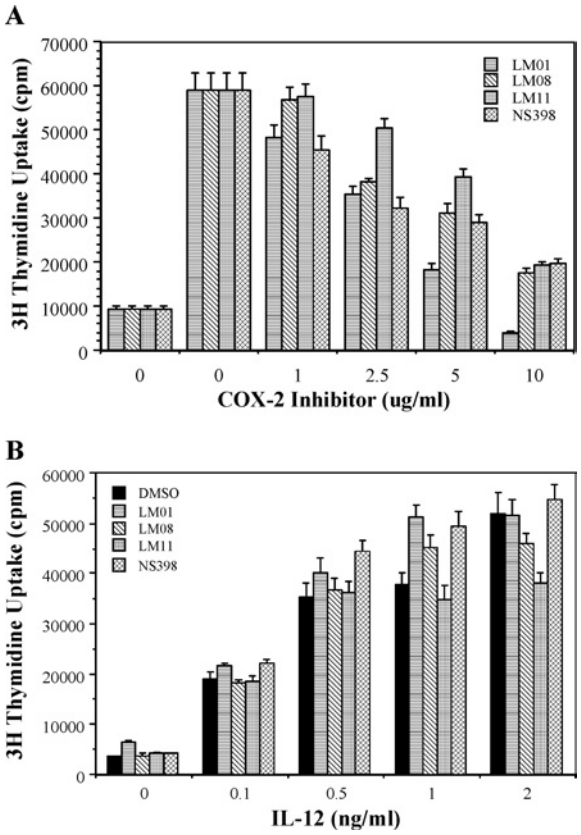

Fig. 6. Inhibition of IL-12-induced T cell proliferation by COX-2 inhibitors. (A) Con A-activated spleen T cells from SJL/J mice were stimulated with 2 ng/mL IL-12 in the presence of different concentrations of COX-2 inhibitors, LM01, LM08, LM11, and NS398. (B) Con A activated T cells were cultured with 25 μg/mL of COX-2 inhibitors, LM01, LM08, LM11 and NS398 for 30 min. The cells were washed and cultured in fresh medium with increasing dose of IL-12 in the absence of COX-2 inhibitors. ^{3}H-thymidine was added at 36 h and the radioactivity measured after 48 h. The values are mean of triplicates at each point and the error bars represent SD. The figures are representatives of five independent experiments.

COX-2 Inhibitors Decrease IL-12-Induced Tyrosine Phosphorylation of STAT 3 and STAT 4 in T Cells

To define the mechanisms involved in the regulation of IL-12-induced T cell responses by COX-2 inhibitors, we examined the effect of COX-2 inhibitors on IL-12 signaling in T cells. Con A activated T cells (10×10^6) were stimulated with 2 ng/mL rIL-12 in the absence or presence of 25 and 50 μg/mL COX-2 inhibitors for 15 min. As shown in Fig. 7, immunoprecipitation and Western blot analyses showed that IL-12 induced the tyrosine phosphorylation of STAT3 and STAT4 in 15 min in activated T cells. Pretreatment of T cells with COX-2 inhibitors for 15 min resulted in a dose-dependent decrease in IL-12-induced tyrosine phosphorylation of STAT3

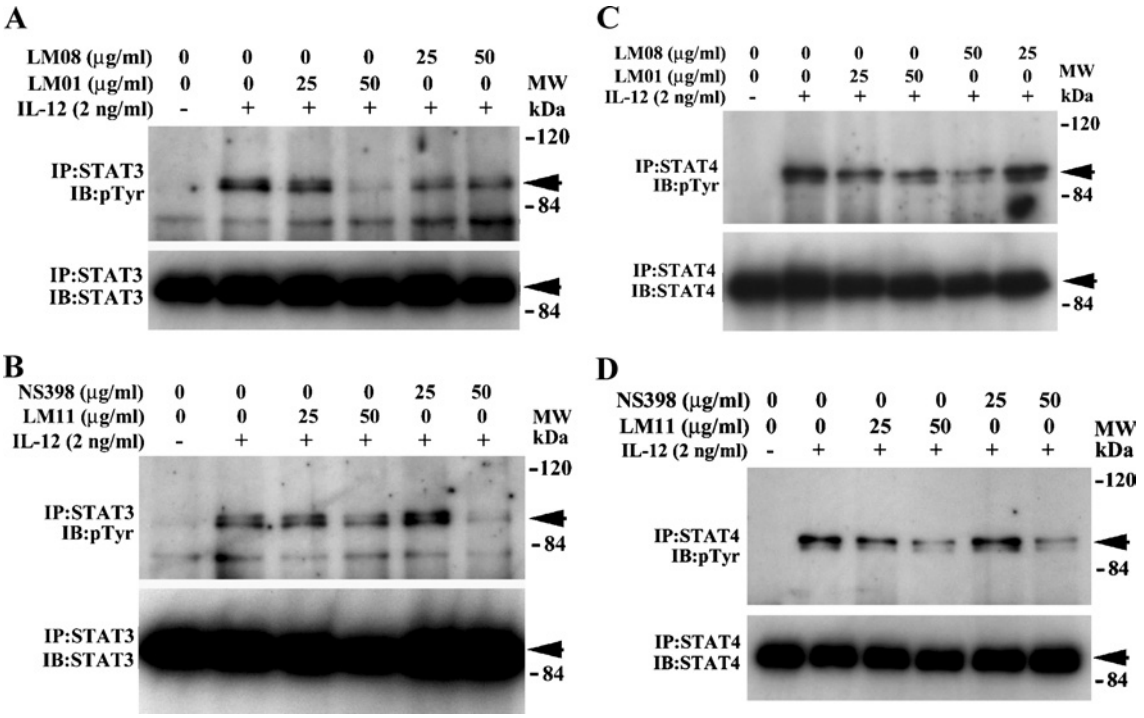

Fig. 7. Inhibition of IL-12-induced tyrosine phosphorylation of STAT proteins by COX-2 inhibitors in T cells. Western blot analysis of STAT3 (A, B) and STAT4 (C, D) proteins immunoprecipitated from Con A-activated splenic T cells from SJL/J mice after incubation in medium alone and with 2 ng/mL IL-12 in the presence of 0, 25, and 50 μg/mL COX-2 inhibitors at 37°C for 15 min. The cells were pretreated with COX-2 inhibitors for 15 min prior to 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 striped and reprobed with anti-STAT3 (A, B) or STAT4 (C, D) antibody. Arrows point to the 92 kDa STAT3 (A) and 89 kDa STAT4 (B) protein bands. The figures are representatives of six experiments.

(Fig. 7A, B) and STAT4 (Fig. 7C, D) proteins. While 25 μg/mL COX-2 inhibitors induced a partial inhibition, addition of 50 μg/mL COX-2 inhibitors resulted in almost complete inhibition of IL-12-induced tyrosine phosphorylation of STAT3 and STAT4 in T cells. Reprobing the blots with STAT3 or STAT4 antibody showed that this decrease was not due to changes in total protein. These results suggest that COX-2 inhibitors modulate Th1 differentiation by blocking IL-12-induced tyrosine phosphorylation of STAT3 and STAT4 in T cells.

COX-2 Inhibitors Decrease IL-12-Induced Tyrosine Phosphorylation of JAK2 and TYK2 in T Cells

To determine whether the inhibition of STAT3 and STAT4 phosphorylation by COX-2 inhibitors was a direct effect or consequent to inhibition of the upstream kinases, we examined the effect of COX-2 inhibitors on IL-12-induced tyrosine phosphorylation of JAK kinases in T cells. As shown in Fig. 8, 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 kinases in 15 min. Pretreatment of T cells with COX-2 inhibitors for 15 min resulted in the inhibition of IL-12-induced tyrosine phosphorylation of JAK2 (Fig. 8A, B) and TYK2 (Fig. 8C, D) kinases in a dose-dependent manner. While 25 μg/mL COX-2 inhibitors induced a partial inhibition, treatment with 50 μg/mL COX-2 inhibitors resulted in an almost complete inhibition of JAK2 and TYK2 phosphorylation. Reprobing the blots with JAK2 or TYK2 antibody showed no change in total protein. These results suggest that COX-2 inhibitors block tyrosine phosphorylation of the upstream JAK2 and TYK2 kinases, there by blocking IL-12-induced phosphorylation of STAT3 and STAT4 in T cells.

DISCUSSION

EAE is an important animal model system to study the mechanism of MS pathogenesis and to test the use of therapeutic agents for the treatment of MS. Using the EAE model of MS, we have shown earlier that *in vivo*

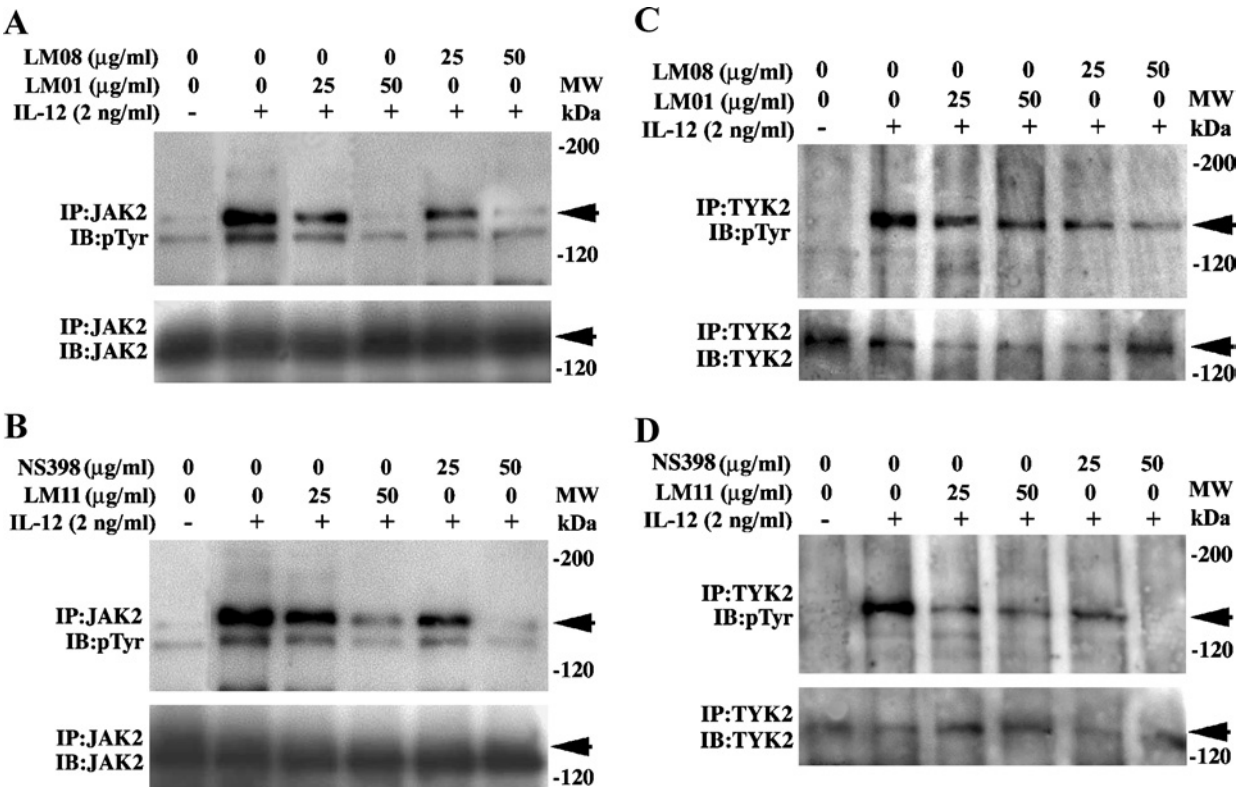

Fig. 8. Inhibition of IL-12-induced tyrosine phosphorylation of JAK kinases by COX-2 inhibitors in T cells. Western blot analysis of Con A-activated T cells incubated in medium alone and with 2 ng/mL IL-12 in the presence of 0, 25, and 50 μg/mL COX-2 inhibitors at 37°C for 15 min. The cells were pretreated with COX-2 inhibitors for 15 min prior to induction of IL-12 signaling. The JAK2 (A, B) and TYK2 (C, D) immunoprecipitates were resolved on 7.5% SDS-PAGE, transferred onto nylon membrane and probed with anti-phosphotyrosine mAb, 4G10. The blots were striped and reprobed with anti-JAK2 (A) or anti-TYK2 (B) antibody. Arrows point to 130 kDa JAK2 and 135 kDa TYK2 protein bands. The figure is a representative of five experiments.

treatment with lisofylline, tyrphostin AG490, PPARγ agonists, curcumin or quercetin inhibits the pathogenesis of CNS inflammation and demyelination in SJL/J mice (8–14). In this study, we found that *in vivo* treatment with COX-2 selective inhibitors reduce the clinical severity and duration of EAE in SJL/J and C57BL/6 mice. The inhibition of clinical paralysis by COX-2 inhibitors was associated with a decrease in the pathology of inflammation and demyelination in the CNS. Thus, consistent with recent reports (37, 38), we have also found that 100 μg dose (5 mg/kg body weight) of COX-2 selective inhibitors ameliorate the clinical and pathological symptoms of EAE, suggesting their use in the treatment of MS. Although the expression of COX-2 associates with the pathogenesis of EAE and treatment with COX-2 inhibitors ameliorate the disease, exact mechanisms in the regulation of EAE by COX-2 inhibitors was not known.

The pathogenesis of EAE/MS is a complex process involving activation of macrophage/microglial cells and differentiation of neural antigen-specific Th1 cells in which the proinflammatory cytokines influence the outcome of the disease (15). Among the many proinflammatory cytokines, IL-12 plays a crucial role in the pathogenesis of CNS demyelination in EAE and MS (18). We and others have shown earlier that the inhibition of IL-12 production or IL-12 signaling was effective in preventing the clinical and pathological symptoms of EAE (8–14, 22). In this study, we found that *in vivo* treatment with COX-2 inhibitors reduced the neural antigen-induced proliferation and secretion of IL-12 and IFNγ *ex vivo*. In view of the pivotal role played by IL-12 in the differentiation of Th1 cells (17), it is likely that the inhibition of IL-12 production is a mechanism by which the COX-2 inhibitors regulate Th1 differentiation, T cell proliferation and IFNγ production in EAE. Although the exact mechanisms in the regulation of IL-12 gene expression by COX-2 inhibitors in EAE are not known, the inhibition of NF-κB by COX-2 inhibitors may be sufficient to block IL-12 production in EAE (23).

To further understand the mechanisms in the regulation of CNS inflammation and demyelination by COX-2 inhibitors, we examined their direct effects on neural antigen-induced T cell responses. Consistent with earlier reports (35), we found that *in vitro* treatment of MBP-immune spleen cells with COX-2 inhibitors resulted in a significant decrease in neural antigen-induced T cell proliferation in culture. While, the activation of T cell receptor (TCR) drives resting naive T cells from G0 to G1 phase of the cell cycle (47), signaling through IL-2 or IL-12 receptor is required for the transition from G1 to S/G2/M phase of the cell cycle (47–51). IL-12 also plays a crucial role in the induction of cell cycle progression in activated T cells and in particular Th1 cells (48–51). However, the inhibition of IL-12-induced T cell proliferation by COX-2 inhibitors *in vitro* suggests their inhibitory effects on IL-12-induced responses in T cells.

To determine the molecular mechanisms on the inhibition of IL-12-induced T cell responses, we examined the effect of COX-2 inhibitors 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 kinases leading to the activation of STAT3 and STAT4 transcription factors (25, 26). Interestingly, in this study, we report for the first time that treatment of activated T cells with COX-2 inhibitors blocked IL-12-induced tyrosine phosphorylation of STAT3 and STAT4 proteins in culture. The COX-2 inhibitors also inhibit IL-12-induced activation of JAK2 and TYK2 kinases in T cells, suggesting that the inhibition of STAT3 and STAT4 proteins was consequent to the blockade of upstream JAK kinases in IL-12 signaling pathway. This is consistent with our earlier reports showing inhibition of STAT3 and STAT4 phosphorylation following the blockade of IL-12-induced tyrosine phosphorylation of JAK2 and TYK2 kinases by AG490, TGFβ, PPARγ, Curcumin and Quercetin (11–14, 39, 40). The blockade of JAK-STAT pathway by COX-2 inhibitors results in a decrease in IL-12-induced proliferation and Th1 differentiation in 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* (11–14, 52). The inhibition of JAK kinase and STAT proteins by COX-2 inhibitors observed in this study suggests that JAK-STAT pathway be the molecular target for COX-2 inhibitors to regulate neural antigen-induced Th1 cell responses and pathogenesis of CNS inflammation and demyelination in EAE and perhaps MS.

The inhibition of IL-12 signaling and Th1 differentiation by COX-2 inhibitors observed in this study implies that the blockade of JAK-STAT pathway be an important mechanism by which COX-2 inhibitors regulate immune and inflammatory responses. Although the exact mechanisms by which COX-2 inhibitors modulate IL-12 signaling is not known, it is likely that COX-2 inhibitors may directly interact with and block the tyrosine phosphorylation of JAK and STAT signaling proteins, thereby impairing neural antigen-specific Th1 differentiation in EAE. Alternatively, COX-2 inhibitors may block COX-2 and other associated proteins that in turn modulate IL-12 signaling through the JAK-STAT pathway and Th1 differentiation in EAE. In conclusion, this study highlights the fact that COX-2 inhibitors ameliorate EAE by blocking IL-12 signaling leading to the differentiation of neural antigen-specific Th1 cells and suggests its use in the treatment of MS and other Th1 cell-mediated inflammatory autoimmune disease.

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REFERENCES

1. Martin R, McFarland HF, McFarlin DE: Immunology of demyelinating disease. Ann Rev Immunol 10:153–169, 1992
2. Dean G: How many people in the world have MS? Neuroepidemiology 13:1–7, 1994
3. Sayetta RB: Theories of the etiology of multiple sclerosis: A critical review. J Clin Lab Immunol 21:55–70, 1986
4. Steinman LR, Martin C, Bernard P, Conlon, Oksenberg JR: Multiple sclerosis: Deeper understanding of its pathogenesis reveals new targets for therapy. Annu Rev Neurosci 25:491–505, 2002
5. Donoghue S, Greenlees C: Drugs in development for the treatment of multiple sclerosis: Antigen non-Specific therapies, an update. Expert Opin Investig Drugs 9:167–171, 2000
6. Clegg A, Bryant J: Immunomodulatory drugs for multiple sclerosis: A systematic review of clinical and cost effectiveness. Expert Opin Pharmacother 2:623–639, 2001
7. Gold R, Hartung HP, Toyka KV: Animal models for autoimmune demyelinating disorders of the nervous system. Mol Med Today 6:88–91, 2000
8. Bright JJ, Musuro, BF, Du C, Sriram S: Expression of IL-12 in CNS and lymphoid organs of mice with experimental allergic encephalomyelitis. J Neuroimmunol 82:22–30, 1998
9. Bright JJ, Du C, Coon M, Sriram S, Klaus SJ: Prevention of experimental allergic encephalomyelitis via inhibition of IL-12 signaling and IL-12-mediated Th1 differentiation: An effect of the novel anti-inflammatory drug lisofylline. J Immunol 161:7015–7022, 1998

10. Bright JJ, Rodriguez M, Sriram S: Differential influence of interleukin-12 in the pathogenesis of autoimmune and virus-induced CNS demyelination. J Virol 73:1637–1639, 1999
11. Bright JJ, Du C, Sriram S: Tyrphostin B42 inhibits IL-12-induced tyrosine phosphorylation and activation of Janus kinase-2 and prevents experimental allergic encephalomyelitis. J Immunol 162:6255–6262, 1999
12. Natarajan C, Bright JJ: Peroxisome proliferator-activated receptor-gamma agonists inhibit experimental allergic encephalomyelitis by blocking IL-12 production, IL-12 signaling and Th1 differentiation. Genes Immun 3:59–70, 2002
13. Natarajan C, Bright JJ: Curcumin inhibits experimental allergic encephalomyelitis by blocking IL-12 signaling through JAK-STAT pathway in T cells and differentiation of neural antigen specific Th1 cells. J Immunol 169:6506–6513, 2002
14. Muthian G, Bright JJ: Quercetin ameliorates experimental allergic encephalomyelitis by blocking IL-12 signaling through JAK-STAT pathway in T lymphocyte. J Clin Immunol 24:541–551, 2003
15. Brosnan CF, Raine CS: Mechanisms of immune injury in multiple sclerosis. Brain Pathol 6:243–257, 1996
16. Bradl M, Linington C: Animal models of demyelination. Brain Pathol 6:303–311, 1996
17. Trinchieri G: IL-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen specific adaptive immunity. Ann Rev Immunol 13:251–276, 1995
18. Trembleau ST, Germann MK, Gately Adorini L: The role of IL-12 in the induction of organ-specific autoimmune diseases. Immunol Today 16:383–386, 1995
19. Nicoletti F, Patti F, Cocuzza C, Zaccone P, Nicoletti A, Marco Di R, Reggio A: Elevated Serum levels of IL-12 in chronic progressive multiple sclerosis. J Neuroimmunol 70:87–90, 1996
20. Balashov KE, Smith DR, Khoury SJ, Hafler DA, Weiner HL: Increased IL-12 production in progressive multiple sclerosis: induction by activated CD4+ T cells via CD40 ligand. Proc Natl Acad Sci USA. 94:599–603, 1997
21. Pagenstecher A, Lassmann S, Carson MJ, Kincaid CL, Stalde AK, Campbell IL: Astrocyte-targeted expression of IL-12 induces active cellular immune responses in the central nervous system and modulates experimental allergic encephalomyelitis. J Immunol 164:4481–4492, 2002
22. Leonard JP, Waldburger KE, Goldman SJ: Prevention of EAE by antibodies against interleukin-12. J Exp Med 181:381–385, 1995
23. Murphy TL, Cleveland MG, Kulesza P, Magram J, Murphy KM: Regulation of interleukin 12 p40 expression through an NF-kappa B half-site. Mol Cell Biol 15:5258–5267, 1995
24. Darnell JE Jr, Kerr IM, Stark GR: JAK-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264:1415–1421, 1994
25. Jacobson NG, Szabo SJ, Weber-Nordt RM, Zhong Z, Schreiber RD, Darnell JE Jr., Murphy KM : Interleukin-12 signaling in Th1 cells involves tyrosine phosphorylation of Stat3 and Stat4. J Exp Med 181:1755–1762, 1995
26. Bacon CM, EM Petricoin 3rd, Ortaldo JR, Rees RC, Larner AC, Johnston JA, O'Shea JJ: Interleukin 12 induces tyrosine phosphorylation and activation of STAT4 in human lymphocytes. Proc Natl Acad Sci USA. 92:7307–7311, 1995
27. Liblau RS, Singer SM, McDevitt HO: Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. Immunol Today 16:34–38, 1995
28. Conboy IM, DeKruyff RH, Tate KM, Cao ZA, Moore TA, Umetsu DT, Jones PP: Novel genetic regulation of T helper 1 (Th1)/Th2 cytokine production and encephalitogenicity in inbred mouse strains. J Exp Med 185:439–451, 1997
29. Segal BM, Dwyer BK, Shevach EM: An interleukin (IL)-10/IL-12 immunoregulatory circuit controls susceptibility to autoimmune disease. J Exp Med 187:537–546, 1998
30. Vane JR, Bakhle YS, Botting RM: Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol 38:97–120, 1998
31. Seibert K, Masferrer J, Zhang Y, Gregory S, Olson G, Hauser S, Leahy K, Perkins W, Isakson P: Mediation of inflammation by cyclooxygenase-2. Agents Actions Suppl 46:41–50, 1995
32. Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, VanDePutte LBA, Lipsky PE: Cyclooxygenase in biology and disease. FASEB J 12:1063–1073, 1998
33. Tsujii M, Kawano S, Tsuji S, Sawaoka H, Hori M, DuBois RN: Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell 93:705–716, 1998
34. Lipsky PE, Isakson PC: Outcome of specific COX-2 inhibition in rheumatoid arthritis. J Rheumatol 24: 9–14, 1997
35. Misko TP, Trotter JL, Cross AH: Mediation of inflammation by encephalitogenic cells: IFN, iNOS and COX-2. J Neuroimmunol 61:195–204, 1995
36. Fretland DJ: Potential role of prostaglandins and Leukotrienes in multiple sclerosis and experimental allergic encephalomyelitis. Protaglandins Leukot. Essent. Fatty Acids 45:249–257, 1992
37. Miyamoto K, Oka N, Kawasaki T, Satoi H, Akiguchi I, Kimura J: The effect of cyclooxygenase-2 inhibitor on experimental allergic neuritis. Neuroreport 9:2331–2334, 1998
38. Moon C, Ahn M, Jee Y, Heo S, Kim S, Kim H, Sim KB, Koh CS, Shin YG, Shin T: Sodium salicylate-induced amelioration of experimental autoimmune encephalomyelitis in Lewis rats is associated with the suppression of inducible nitric oxide synthase and cyclooxygenases. Neurosci Lett. 12;356:123–126, 2004
39. Bright JJ, Kerr LD, Sriram S: TGF-beta inhibits IL-2-induced tyrosine phosphorylation and activation of JAK1 and STAT5 in T lymphocytes. J Immunol 159:175–183, 1997
40. Bright JJ, Sriram S: TGF-beta inhibits IL-12-induced activation of JAK-STAT pathway in T lymphocytes. J Immunol 161:1772–1777, 1998
41. Kalgutkar AS, Crews BC, Rowlinson SW, Marnett AB, Kozak KR, Remmel RP, Marnett LJ: Biochemically based design of cyclooxygenase-2 (COX-2) inhibitors: facile conversion of nonsteroidal antiinflammatory drugs to potent and highly selective COX-2 inhibitors. Proc Natl Acad Sci USA 97:925–930, 2000
42. Kalgutkar AS, Marnett AB, Crews BC, Remmel RP, Marnett LJ: Ester and amide derivatives of the nonsteroidal antiinflammatory drug, indomethacin, as selective cyclooxygenase-2 inhibitors. J Med Chem 43:2860–2870, 2000
43. Marnett LJ, Kalgulkar AS: Design of selective inhibitors of cyclooxygenase-2 as nonnuclcerogenic anti-inflammatory agents. Curr opin Chem Biol 2: 482– 490, 1998
44. Marnett LJ: Forum:reactive species of peroxynitrite. Chem Res Toxicol 11: 709, 1998
45. Marnett LJ, Kalgutkar AS: Cyclooxygenase 2 inhibitors: Discovery, selectivity and the future. Trends Pharmacol Sci 20:465–469, 1999
46. Marnett LJ, Rowlinson SW, Goodwin DC, Kalgutkar AS, Lanzo CA: Arachidonic acid oxygenation by COX-1 and COX-2. Mechanisms of catalysis and inhibition. J Biol Chem 274:22903–22906, 1999
47. Crabtree GR: Contingent genetic regulatory events in T lymphocyte activation. Science 243:355–357, 1989
48. Gately MK, Desai BB, Wolitzky AG, Quinn PM, Dwyer CM, Podlaski FJ, Familletti PC, Sinigaglia F, Chizonnite R, Gubler U: Regulation of human lymphocyte proliferation by a heterodimeric

cytokine, IL-12 (cytotoxic lymphocyte maturation factor). J Immunol 147:874–882, 1991
49. Bertagnolli MM, Lin BY, Young D, Herrmann SH: IL-12 augments antigen-dependent proliferation of activated T lymphocytes. J Immunol 149:3778–3783, 1992
50. Yanagida T, Kato T, Igarashi O, Inoue T, Nariuchi H: Second signal activity of IL-12 on the proliferation and IL-2R expression of T helper cell-1 clone. J Immunol 152:4919–4928, 1994
51. Kennedy MK, Picha KS, Shanebeck KD, Anderson DM, Grabstein KH: Interleukin-12 regulates the proliferation of Th1, but not Th2 or Th0, clones. Eur J Immunol 24:2271–2278, 1994
52. Thierfelder WE, van DeursenJM, Yamamoto K, Tripp RA, Sarawar SR, Carson RT, Sangster MY, Vignali DA, Doherty PC, Grosveld GC, Ihle JN: Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. Nature 382:171–174, 1996