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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

John J. Bright,* Caigan Du,** Michael Coon,† Subramaniam Sriram,* and Stephen J. Klaus††

Experimental allergic encephalomyelitis (EAE) is an inflammatory, CD4+ Th1-mediated autoimmune disease, which serves as a model for multiple sclerosis. We examined the effect of a novel anti-inflammatory drug, lisofylline (LSF), on EAE induced either by injection of mouse spinal cord homogenate or following transfer of myelin basic protein-reactive T cells. Orally administered LSF significantly inhibited EAE in both cases, decreasing peak clinical scores by >70% and >80%, respectively. In addition, analysis of representative spinal cord sections from LSF-treated mice showed complete lack of demyelination and lymphocyte infiltration. The reduction in EAE correlated with the inhibition of Th1 differentiation by LSF in vivo, as indicated by a reduction in T cell IFN-γ production ex vivo after Ag restimulation. The inhibition of Th1 differentiation in vivo is consistent with a block in IL-12 receptor signaling, because LSF blocked IL-12-driven Th1 differentiation and T cell proliferation in vitro, yet had no effect on IL-12 secretion from APCs ex vivo or in vitro. The Journal of Immunology, 1998, 161: 7015–7022.

E xperimental allergic encephalomyelitis (EAE) is an inflammatory, autoimmune demyelinating disease of the central nervous system (CNS), which shows pathologic and clinical similarities to human multiple sclerosis (MS) and which is used as a model system to test potential therapeutic agents (1). EAE is a CD4+ Th1-type cell-mediated autoimmune disease, and proinflammatory cytokines play an important role in disease pathogenesis. High levels of Th1-type cytokines (e.g., IFN-γ) are detected in the CNS at the height of disease in both humans with MS and mice with EAE (2–4), and, conversely, neutralizing cytokine-specific Abs ameliorate disease progression in the murine model (5–7). Because Th2-type cytokines are predominantly detected in murine brain during recovery (8), and because disease is suppressed by administration of Th2-type cytokines (9, 10), the data suggest that there is a switch from a Th1-type response to a Th2-type response during remission.

IL-12 is a 70-kDa heterodimeric cytokine produced by macrophages in response to infection, LPS, CD154 (CD40 ligand), and IFN-γ (11). IL-12 is critical for the development of Th1-type cells and influences Th1 responses primarily by inducing IFN-γ production from T cells and NK cells (12). Lack of IL-12 signaling leads to almost complete absence of Th1 cells and reduced IFN-γ production (13, 14). The importance of IL-12 has been extended to the pathogenesis of Th1 cell-mediated autoimmune disease. Prevention of EAE and trinitrobenzenesulfonic acid-induced murine colitis by neutralizing Abs against IL-12 and augmentation of diabetes in NOD mice by exogenous IL-12 have suggested an important role for IL-12 in the pathogenesis of these diseases (5, 15, 16). IL-12 neutralizing Abs also inhibit Th1-mediated acute graft-vs-host disease (17).

Lisofylline (LSF), 1-(5-R-hydroxyhexyl)-3,7-dimethylxanthine, is a novel anti-inflammatory compound that abrogates release of proinflammatory cytokines during oxidative lung injury (18), reduces inflammatory cytokine release in response to cytotoxic cancer chemotherapy (19), and protects minipigs from sepsis (20). In clinical trials involving patients receiving sibling donor, HLA-matched bone marrow transplants (BMT) after ablative chemotherapy and radiation therapy, LSF recipients experienced a significant reduction in neutropenic infection and treatment-related mortality and had improved survival (21). Reduction in toxicity and improved survival correlated with lower levels of oxidized lipids in sera from the patients (22). From these and other data, a regulatory paradigm is suggested in which inflammatory cytokines induce species of oxidized lipids (23), which, in turn, leads to amplification of inflammatory cytokine secretion (24, 25). Based on the anti-inflammatory properties of LSF in multiple models of acute inflammation, we employed LSF to inhibit the induction of EAE and examined its mechanism of action in this inflammatory, Th1-mediated autoimmune disease.

Materials and Methods

Animals and reagents

Female SJL/J mice were purchased from Clarence Reader (National Institutes of Health, Bethesda, MD), and BALB/c mice were purchased from Charles River Labs (B & K Universal, Fremont, CA); SJL/J and BALB/c mice were maintained at Vanderbilt University Medical Center and Cell Therapeutics, Inc., respectively. Anti-IL-12 (C17.5 and C15.6) were a kind gift of Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA). The Abs RA3-3A1/6.1 (anti-B220), J11d.2, and MAR18.5 (anti-rat IFN-γ) were purchased from Cytometry, Inc. (Gaithersburg, MD); MR1 (anti-IL-12) was purchased from R&D Systems (Minneapolis, MN); IA5 (anti-BALB/c) was purchased from kindly Dr. S. Faust, Cell Therapeutics, Inc., respectively. Anti-IL-12 (C17.5 and C15.6) were a kind gift of Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA). The Abs RA3-3A1/6.1 (anti-B220), J11d.2, and MAR18.5 (anti-rat IFN-γ) were purchased from Cytometry, Inc. (Gaithersburg, MD); MR1 (anti-IL-12) was purchased from R&D Systems (Minneapolis, MN); IA5 (anti-BALB/c) was purchased from kindly Dr. S. Faust, Cell Therapeutics, Inc., respectively. Anti-IL-12 (C17.5 and C15.6) were a kind gift of Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA). The Abs RA3-3A1/6.1 (anti-B220), J11d.2, and MAR18.5 (anti-rat IFN-γ) were purchased from Cytometry, Inc. (Gaithersburg, MD); MR1 (anti-IL-12) was purchased from R&D Systems (Minneapolis, MN); IA5 (anti-BALB/c) was purchased from kindly Dr. S. Faust, Cell Therapeutics, Inc., respectively. Anti-IL-12 (C17.5 and C15.6) were a kind gift of Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA). The Abs RA3-3A1/6.1 (anti-B220), J11d.2, and MAR18.5 (anti-rat IFN-γ) were purchased from Cytometry, Inc. (Gaithersburg, MD); MR1 (anti-IL-12) was purchased from R&D Systems (Minneapolis, MN); IA5 (anti-BALB/c) was purchased from kindly Dr. S. Faust, Cell Therapeutics, Inc., respectively. Anti-IL-12 (C17.5 and C15.6) were a kind gift of Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA). The Abs RA3-3A1/6.1 (anti-B220), J11d.2, and MAR18.5 (anti-rat IFN-γ) were purchased from Cytometry, Inc. (Gaithersburg, MD); MR1 (anti-IL-12) was purchased from R&D Systems (Minneapolis, MN); IA5 (anti-BALB/c) was purchased from kindly Dr. S. Faust, Cell Therapeutics, Inc., respectively. Anti-IL-12 (C17.5 and C15.6) were a kind gift of Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA). The Abs RA3-3A1/6.1 (anti-B220), J11d.2, and MAR18.5 (anti-rat IFN-γ) were purchased from Cytometry, Inc. (Gaithersburg, MD); MR1 (anti-IL-12) was purchased from R&D Systems (Minneapolis, MN); IA5 (anti-BALB/c) was purchased from kindly Dr. S. Faust, Cell Therapeutics, Inc., respectively. Anti-IL-12 (C17.5 and C15.6) were a kind gift of Dr. G. Trinchieri (Wistar Institute, Philadelphia, PA). The Abs RA3-3A1/6.1 (anti-B220), J11d.2, and MAR18.5 (anti-rat IFN-γ) were purchased from Cytometry, Inc. (Gaithersburg, MD); MR1 (anti-IL-12) was purchased from R&D Systems (Minneapolis, MN); IA5 (anti-BALB/c) was purchased from kindly Dr. S. Faust, Cell Therapeutics, Inc., respectively.

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Abbreviations used in this paper: EAE, experimental allergic encephalomyelitis; MS, multiple sclerosis; LSF, lisofylline; FTX, pentoxifylline; MSCH, mouse spinal cord homogenate; MBP, myelin basic protein; CNS, central nervous system; BMT, bone marrow transplant; LNC, lymph node cells.

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(XMG1.2) and anti-CD40 (HM40-3) were purchased from PharMingen (San Diego, CA), and anti-IFN-γ Ab (MM700) was obtained from Endo- gen (Woburn, MA). Recombinant murine IL-12 and IL-4 was purchased from Genzyme (Cambridge, MA), recombinant human IL-10 (active on murine cells) was purchased from PeproTech (Rocky Hill, NJ), guinea pig complement was purchased from Accurate Chemical (Westbury, NY), and recombinant murine IFN-γ was purchased from Biosource International (Camarillo, CA). LPS and pentoxifylline (PTX) were obtained from Sigma (St. Louis, MO).

**Induction of EAE**

Active EAE was induced by immunization of SJL/J mice with 800 μg of mouse spinal cord homogenate (MSCH) (10) in CFA on days 0 and 7. Passive EAE was induced by adoptive transfer of myelin basic protein (MBP)-sensitized T lymphocytes as follows: SJL/J mice (4- to 6-wk-old) were immunized on days 0 and 7 with 400 μg of MBP in IFA containing 50 μg/ml of H37RA. On day 14, the regional draining lymph node cells (LNC) and spleen were harvested and pooled and cultured at 4 × 10^6 cells/well in RPMI 1640 containing 10% FBS (HyClone Labs, Logan, UT), 2 mM l-glutamine, 5 × 10^-5 M 2-ME, 1% penicillin/streptomycin, and 100 μg/ml of MBP. After 4 days, viable T cell blasts were harvested by Ficoll centrifugation, washed twice in PBS, and injected i.p. into recipient mice (1.0 × 10^6 to 1.5 × 10^7 cells in 500 μl of PBS).

**Treatment of animals with LSF and development of clinical and pathologic scores**

Mice were given either LSF (2 mg/ml in PBS) or PBS twice daily by gavage needle in volumes of 0.25 or 0.5 ml/dose. Paralysis was graded as follows: 0, no paralysis; 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 state; 5, death. To assess the degree of inflammation, mice were euthanized on day 25 and perfused by intracardiac injection of 4% paraformaldehyde and 1% glutaraldehyde in PBS. Transverse sections of the cervical, upper thoracic, lower thoracic, and lumbar region of the spinal cord were stained with Luxol Fast Blue or with hematoxylin and eosin. Each spinal cord section was further subdivided into an anterior, posterior and two lateral columns, and sections displaying either lymphocyte infiltration or demyelination were assigned a score of one. Thus, each animal had a potential maximum score of 16.

**Proliferation assay**

Fifteen days after MBP immunization, PBS- or LSF-treated donor cells were cultured at 2 × 10^5/well with MBP for 72 h, and pulsed during the last 18 h with [3H]thymidine (0.5 μCi/well). [3H]Thymidine incorporation was measured on a betaplate liquid scintillation counter (Wallac, Turku, Finland).

**Cells and IL-12 secretion**

Normal macrophages were enriched from single-cell suspensions of BALB/c spleens by adherence to tissue culture plates at 37°C for 1 h. Plates were rinsed four times with PBS before harvesting the adherent cells. Normal macrophage or the murine macrophage cell line J774A.1 (American Type Culture Collection No. TIB-67; Manassas, VA) were incubated for 48 h as indicated, and the culture media were collected and assayed for IL-12 by ELISA.

**Induction of Th1 differentiation**

T cells were enriched (>95%) from RBC-depleted BALB/c splenocytes by complement-mediated lysis of B cells using rat anti-mouse B220 and J11d, followed by cross-linking with MAR185. Anti-CD3 (4 μg/ml in PBS) was immobilized to tissue culture plates by incubation for 1 h at 37°C. After the plates were washed twice in PBS, T cells (0.5 × 10^5 cells/ml) were stimulated in the presence or absence of IL-12, with or without LSF or PTX. After 7 days, equal numbers of viable cells were restimulated overnight at 5 × 10^6 cells/ml with insoluble anti-CD3, and supernatants were collected and assayed for IFN-γ.

**ELISA for IL-12, IFN-γ, and IL-4**

IL-12 secretion was determined by a sandwich ELISA. Plates were coated with 2 μg/ml of anti-IL-12 (C17.15) and blocked with 3% BSA in PBS. After overnight incubation at 4°C, sample wells were washed with PBS/0.05% Tween 20, and incubated with biotinylated anti-IL-12 (C17.15) at 0.2 μg/ml. After 1 h at room temperature, the plates were washed, incubated for 1 h with avidin-alkaline phosphatase, and washed again before addition of 1 mg/ml of p-nitrophenyl phosphate. Absorbance was read at 405 nm. The IL-12 concentration was calculated by interpolation from a standard curve. IFN-γ production was also measured by a sandwich ELISA as described above, except for using R4-6A2 as the capture Ab and biotinylated MM700 as the second step. Total IL-12 and IFN-γ levels were assessed with InterTest-12 and InterTest-γ ELISA kit, respectively, both also Genzyme (Cambridge, MA).

**Results**

**LSF inhibits induction of active and passive EAE**

Mice were immunized with MSCH in CFA to induce active EAE, and treated with either LSF (1 mg per dose i.e., 50 mg/kg; see inset, Fig. 1) or PBS by gavage twice daily for 15 days; the summed data from two separate experiments are shown (Fig. 1). In control groups, 7 of 10 animals receiving PBS developed hind limb paralysis with a mean clinical score of 2.4 on day 20. In contrast, only 2 of 13 animals receiving LSF became paralyzed and the mean clinical score was 0.75 (p < 0.025). No significant difference in the day of onset of paralysis was seen. LSF also prevented the development of passive EAE induced by adoptive transfer of MBP-sensitized T cells. All six mice receiving PBS developed paralytic signs with a mean clinical score of 2.35 on day 9 (Fig. 2). Mice given LSF at either 0.5 or 1.0 mg/dose twice a day were...
significantly protected, and only 1 of 12 animals that received LSF developed hind limb paralysis ($p < 0.025$).

The marked differences in clinical scores for animals in the passive model were consistent with histologic studies of three randomly selected animals from each group following recovery. In mice receiving PBS, the mean pathologic scores were 6.6 and 7.2 for demyelination and infiltration, respectively, whereas no demyelination or lymphocyte infiltration into the CNS was observed in LSF-treated mice (Fig. 3, A and B).

**LSF reduces in vivo Th1 differentiation of MBP-specific T cells**

Because LSF decreased the severity of paralysis in both models of EAE, we examined the effects of LSF treatment in vivo on the potency and phenotype of MBP-reactive T cells. Mice immunized with MBP in CFA were given either LSF (0.5 mg/dose) or PBS twice daily for 14 days. On the fifteenth day, spleen and LNC were pooled and cultured in vitro with MBP for 4 days. At each day of the 4-day in vitro culture with MBP, a sample of culture supernatant was collected and tested for the amount of IFN-$\gamma$ and IL-4 produced, and after 4 days blast cells from each group were compared for their potency at adoptively transferring EAE. Duplicate cultures were also tested for proliferative capacity in response to MBP.

When compared with cells from PBS-treated mice, donor cells from LSF-treated animals were less effective at inducing passive EAE after transfer into naive hosts. In experiment 1 (Fig. 4A), all six animals that received PBS-treated donor cells developed clinical paralysis (mean clinical score = 4.5), and four died at the height of disease. In contrast, none of the mice that received cells from LSF-treated mice died, the mean maximum score was 3.8, and all animals recovered to a mean score of 1.8 by day 24. In the second experiment, the clinical severity in mice receiving PBS-treated cells was 3.25, whereas in the LSF group it was 2.0 (Fig. 4B). The reduced encephalitogenicity of MBP-specific T cells that were generated in LSF-treated mice was long lasting, as these T cells were transferred to naïve recipients without any additional drug administration. The reduction in clinical severity of EAE induced by LSF-treated donor cells vs that induced with PBS-treated donor cells was significant ($p < 0.001$) and was not due to differences in lymphocyte activation, because both populations proliferated equally well in response to MBP in vitro (Fig. 4C).

To assess the Th cell phenotype of donor cells from PBS- and LSF-treated mice, we analyzed supernatants from Ag-stimulated cultures for the presence of Th1- and Th2-type cytokines. Although no differences in IL-12 production were observed between cells from PBS- and LSF-treated mice (data not shown), cultures from LSF-treated mice contained significantly less IFN-$\gamma$ at each of the 4 days tested (Fig. 4D). The culture supernatants also displayed reduced IL-4 production up to day 3, although the relative inhibition of IL-4 production by ex vivo-activated T cells from LSF-treated mice was not as great as that observed for IFN-$\gamma$ (Fig. 4E).

**Effect of LSF on IL-12 secretion and signaling**

The previous experiments suggested that LSF inhibited Th1 differentiation in vivo, as shown by a reduced capacity to induce EAE and secrete IFN-$\gamma$ ex vivo in response to Ag. Because IL-12 is a key cytokine that regulates Th1 differentiation, the lack of Th1 cells could arise from a block in IL-12 secretion from APC or an inhibition of IL-12 signaling in T cells. To test the former possibility, we measured total IL-12 production from macrophages activated in vitro in the presence of LSF using the maximal concentrations detectable from patients’ sera during clinical trials and up to fourfold higher. Normal murine macrophages were induced to secrete IL-12 after activation with anti-CD40 or LPS (Fig. 5A), but this effect was not inhibited by LSF; even at concentrations of anti-CD40 inducing suboptimal IL-12 production, LSF was not inhibitory. Furthermore, LSF did not block IL-12 secretion from a murine macrophage line stimulated with LPS and IFN-$\gamma$ (Fig. 5B), whereas IL-10 completely abolished IL-12 secretion.
To determine whether LSF suppressed Th1 differentiation in vivo by blocking IL-12 signaling, we tested LSF in an IL-12-dependent in vitro Th cell differentiation assay (26). When compared with T cells stimulated with anti-CD3 alone, splenic T cells that were activated in the presence of IL-12 displayed a dramatic increase in Th1 differentiation, as measured by the production of IFN-γ upon secondary stimulation (Fig. 6A). Strikingly, the presence of LSF during T cell differentiation inhibited Th1 maturation in a dose-dependent fashion, such that the amount of IFN-γ secreted was equivalent to that of T cells activated with anti-CD3 alone, in the absence of IL-12 (Fig. 6A). A similar pattern of inhibition was seen when culture supernatants were tested for the production of other Th1 cytokines; secretion of IL-2 and TNF-α by T cells responding to IL-12 in the presence of LSF were also inhibited with respective IC50 (concentration of drug where 50% inhibition of response occurs) values of 15 μM and 20 μM (data not shown). Similar LSF concentrations also inhibited Th1 differentiation induced by addition of heat-killed Listeria monocytogenes (data not shown), which enhances Th1 development by inducing IL-12 secretion from macrophages. LSF did not affect the viability or recovery of T cells after 1 wk of culture (data not shown), consistent with its inability to block lymphocyte activation (Fig. 4C). In support of this, Th1 differentiation was also inhibited even when both LSF and IL-12 were added up to 48 h after anti-CD3 activation, indicating that LSF blocked the response to IL-12 and not the acquisition of IL-12 responsiveness (data not shown). LSF also had little effect on the low levels of IFN-γ secreted from
T cells stimulated with anti-CD3 alone, confirming it does not induce anergy or TCR desensitization (Fig. 6A). Furthermore, LSF did not augment Th2 differentiation at the expense of Th1 development, as shown above for MBP-specific T cells activated ex vivo (Fig. 4E), because the levels of IL-4 and IL-10 secretion detected in these cultures were not enhanced (data not shown).

PTX [3,7-dimethyl-1-(5-oxohexyl)xanthine], is a structural analogue of LSF containing a carbonyl in its acyl side chain, which also is antiinflammatory and inhibits induction of EAE in rats (27). Because high doses of PTX have been shown to inhibit IL-12 secretion in vitro (28), we compared the efficacy of PTX and LSF on in vitro Th1 differentiation driven by the addition of exogenous IL-12. As also shown in Fig. 6A, LSF inhibited IL-12-induced Th1 differentiation in a dose-dependent fashion, resulting in a >85% blockade at 50 μM (Fig. 6B). Th1 differentiation in the presence of LSF was approximately reduced to levels seen when T cells were stimulated with anti-CD3 alone in the absence of exogenous IL-12 (5.8 ng/ml). In contrast, equimolar concentrations of PTX had no effect on IL-12-induced Th1 differentiation. The stereochemical enantiomer of LSF, 1501S, also had no effect on Th1 maturation (data not shown).

Because LSF inhibited IL-12-driven Th1 differentiation both in vivo and in vitro, we examined whether LSF was capable of inhibiting additional IL-12-dependent responses in vitro. In addition to its effects on Th1 differentiation, IL-12 is a T cell growth factor that augments the proliferation of T cell blasts (11). Therefore, we tested, the ability of LSF to block proliferation of T cell blasts in response to IL-12. In preliminary experiments, we determined that the optimal T cell response to IL-12 occurred 3 days after T cell stimulation with anti-TCR reagents or mitogens (data not shown).

Splenic T cells stimulated for 3 days with Con A showed a proliferative response to IL-12 (Fig. 7A). T cell blasts coincubated in the presence of LSF displayed reduced proliferative responses to IL-12, resulting in a dose-dependent decrease in T cell growth, even when optimal IL-12 concentrations were used (Fig. 7B). Importantly, LSF had no inhibitory effect when T cells were activated for 3 days in the presence of LSF, i.e., during the acquisition of IL-12 responsiveness, provided the LSF was washed out before addition of IL-12 (Fig. 7B). Therefore, the presence of LSF had no effect on T cell activation or the ability to induce IL-12 responsiveness, but rather LSF blocked the ability of T cell blasts to respond to IL-12.

Although the inhibition of IL-12-induced Th1 maturation by LSF did not lead to augmented Th2 differentiation (both in vivo and in vitro), it was unclear whether LSF might block Th2 differentiation in vitro driven by exogenously added IL-4. As expected, anti-CD3-stimulated T cells coincubated with IL-4 displayed increased Th2 differentiation as compared with T cells activated with anti-CD3 alone (Fig. 6C). Addition of LSF did block IL-4-induced Th2 differentiation, yet the percent inhibition observed was less than that for Th1 differentiation driven by the addition of exogenous IL-12.

FIGURE 6. LSF inhibits in vitro Th1 differentiation induced by IL-12. A, Splenic T cells were stimulated at 5 × 10⁶/ml with insoluble anti-CD3 alone or anti-CD3 and 5 U/ml IL-12 with or without LSF at the concentrations indicated. After 7 days, equal numbers of viable cells were restimulated for 24 h with anti-CD3 without LSF, and the supernatants were collected and assayed for IFN-γ production. B, Splenic T cells were stimulated with anti-CD3 and IL-12 as above and incubated with either LSF or PTX as indicated. IFN-γ levels for T cells stimulated with anti-CD3 and no IL-12 were 5.8 ng/ml. C, Splenic T cells were stimulated as in A except cultures were given IL-4 (10 U/ml) and anti-IFN-γ (5 μg/ml).

FIGURE 7. LSF inhibits IL-12-dependent T cell proliferation but not the ability to induce IL-12 responsiveness. A, Splenic T cells (0.5 × 10⁶/ml) were stimulated with insoluble anti-CD3 for 3 days, washed, and 1 × 10⁶ cells/well were stimulated with the indicated concentrations of IL-12 in the absence or presence of increasing concentrations of LSF. Cells were cultured for 24 h and pulsed with [³H]TdR for the last 8 h before harvesting. B, Splenic T cells were activated as described in A in the absence or presence of increasing concentrations of LSF, as indicated. After 3 days, the cells were thoroughly washed to remove LSF and stimulated with increasing amounts of IL-12 and pulsed with [³H]TdR as described above.
Discussion

This study demonstrates the potent immunoregulatory properties of LSF in a murine EAE model, which is felt to simulate human MS. LSF inhibited the clinical manifestations of EAE induced either by immunization with MSCH or by adoptively transferred encephalitogenic T cells (Figs. 1 and 2). LSF-treated mice were protected from lymphocyte infiltration into neural tissues and demyelination, as demonstrated histologically by analysis of spinal cord sections (Fig. 3). Thus, LSF blocked progression of an inflammatory, Th1-mediated autoimmune disease.

To determine how LSF ameliorates disease progression, we examined ex vivo proliferative responses of Ag-specific T cells from LSF-treated mice and their profiles of cytokine secretion. LSF administration in vivo did not inhibit the ex vivo T cell proliferative response to MBP (Fig. 4C), indicating that LSF does not induce tolerance, block Ag-specific priming, or inhibit the cellular response to subsequent Ag challenge. This is consistent with the inability of LSF to inhibit expression of T cell activation Ag, IL-2 secretion, a mitogenic response, or proliferation in response to anti-CD3 plus or minus anti-CD28 costimulation in vitro (data not shown). Furthermore, in vivo treatment with LSF did not diminish IL-12 secretion by MBP-primed APC ex vivo (data not shown), suggesting that LSF did not affect APC function and cytokine secretion.

A striking correlation emerged when we analyzed the Th cell phenotype and encephalitogenicity of donor cells from LSF-treated mice. LSF treatment in vivo reduced the amount of IFN-γ secreted by ex vivo T cells stimulated with Ag (Fig. 4D), implying that Th1 differentiation had been inhibited in vivo. LSF inhibited Th1 differentiation by blocking IL-12 signaling and not by inhibiting IL-12 secretion from APC (Fig. 5), because IL-12-dependent Th1 differentiation was suppressed in vitro (Fig. 6, A and B). We extended these observations to show that LSF abrogates IL-12-induced proliferation of anti-CD3-activated T cell blasts (Fig. 7A), but not the ability to induce IL-12 responsiveness during T cell activation (Fig. 7B). The reduced capacity to produce IFN-γ in vitro correlated with a reduced capacity to adoptively transfer EAE to susceptible, naive recipients, indicating that the inability of T cells from LSF-treated mice to transfer disease was a long-lived effect and suggesting that it stemmed from an abrogation of Th1 differentiation.

We further analyzed the functional consequences of LSF treatment by examining the Th cell response to subsequent Ag challenge. This is consistent with the reduced Th1 differentiation in vitro correlated with a reduced capacity to adoptively transfer EAE to susceptible, naive recipients, indicating that the inability of T cells from LSF-treated mice to transfer disease was a long-lived effect and suggesting that it stemmed from an abrogation of Th1 differentiation. LSF treatment in vivo reduced the amount of IFN-γ secreted by MBP-primed APC ex vivo (data not shown), suggesting that LSF did not affect APC function and cytokine secretion.

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IL-12 induces IFN-γ production by NK cells and T cells, and drives the differentiation of inflammatory Th1 cells. IL-12 was detected in early inflammatory lesions in MS brains and correlated with APC CD80 expression (3). We have shown that IL-12 expression levels in mouse brain and spinal cord correlate with the development of paralytic signs in EAE (29).

Because anti-IL-12 abrogates development of CNS demyelination and induction of EAE (5, 30), the inhibition of IL-12 signaling by LSF, which leads to reduced Th1 differentiation, is consistent with its modulatory effect on the pathobiology of this model. LSF did not appear to act by shifting the balance of Th cell differentiation toward a Th2 phenotype, which has been shown to be ameliorative in EAE (8–10). Indeed, analysis of supernatants derived from activated T cells treated with LSF in vivo (Fig. 4E) or in vitro (data not shown) did not detect enhanced secretion of IL-4.

Several studies document a correlation between the presence of Th1 cytokines in MS and in experimental models of CNS inflammation (2, 4, 6, 7, 31). Although IFN-γ is the prototypical cytokine associated with Th1 responses, the role of IFN-γ in EAE and MS is equivocal. CSF-derived T cells from MS patients are Th1 cells that secrete enhanced levels of IFN-γ (56, 57), and IFN-γ dosing worsens disease progression (32). However, IFN-γ knockout mice remain susceptible to EAE (33), anti-IFN-γ may exacerbate disease (34), and in certain cases IFN-γ administration is protective (35). These conflicting results may relate to the effects of IFN-γ during development, its differential inflammatory effects systemically and locally (36), or a compensatory mechanism that leads to overexpression of other Th1 cytokines which affect EAE pathogenesis (7). Indeed, organ-specific overexpression of IFN-γ leads to spontaneous CNS demyelination, confirming that IFN-γ is a candidate cytokine for affecting MS pathogenesis (37). It was recently shown that IFN-γ knockout mice remain susceptible to EAE via an IL-12-dependent mechanism that antagonizes IL-10 production (30). Our data suggest that LSF affects this immunoregulatory circuit and may provide protection as a prophylactic in Th1-mediated autoimmune disease by short-circuiting an inflammatory cascade initiated by IL-12.

The etiology of autoimmune demyelinating disease may directly relate to IFN-γ production, because IFN-γ exacerbates inflammatory responses in several ways. These include enhanced adhesion of lymphocytes to CNS endothelium (38), increased permeability of the blood-brain barrier (31, 39), and augmented MHC expression and Ag presentation (40–43). Thus, IFN-γ coordinately promotes lymphocyte access to the CNS and presentation of autoantigen by astrocytes and microglia, which are normally MHC class II negative.

IFN-γ is also the most potent cytokine for up-regulation of inducible nitric oxide synthase (iNOS) in macrophages (44). In this regard, iNOS levels and enzyme activity correspond to disease activity in EAE (45) and correlate with recent findings linking the levels of peroxynitrites with severity of EAE and MS (46). It is noteworthy that inflammatory cytokines also induce lipid peroxidation (23), because peroxidized lipids are evident in MS patients (47, 48) and the levels of peroxidized lipids in patients undergoing treatment regimens for BMT are abrogated by LSF (22).

The ability of LSF to inhibit IL-12-induced Th1 differentiation and, to a lesser extent, IL-4-induced Th2 differentiation in vitro (Fig. 6C) was not entirely unexpected, because LSF was not developed as a specific inhibitor of IL-12 signaling pathways. LSF may act as an inhibitor of STAT activation, because the induction of STAT 4 and STAT 5 tyrosine phosphorylation in response to IL-12 and IL-2, respectively, are blocked by LSF pretreatment (52–54). Furthermore, concentrations of PTX routinely used in vitro to inhibit proinflammatory cytokine secretion or other biologic responses are usually in vast excess of those tolerated in human patients during clinical trials. Indeed, administration of PTX in a pilot study of MS patients showed no evidence for a decrease of Th1 function or Th1-derived cytokines in stimulated peripheral blood cells (55). In
the present study, LSF inhibited Th1 differentiation in response to IL-12 (Fig. 6b) at concentrations comparable to those detected in clinical trial patients undergoing BMT (C_{max} = 19 μM); in contrast, PTX was ineffective. Furthermore, the dose of LSF used to inhibit EAE in mice (25 or 50 mg/kg) is comparable to that used in human clinical trials (3 mg/kg) based upon a calculated 15% oral bioavailability of LSF in mice (data not shown). With regard to inhibition of IL-12 signaling, LSF was also selectively inhibitory when compared with its stereospecific S-enantiomer (data not shown), suggesting that the efficacy of LSF requires the R-chiral alcohol within the alkyl side chain, a determinant of the pharmacophore. Taken together, the data suggest that an orally active LSF analogue may provide a novel therapeutic approach to Th1-mediated diseases such as MS.

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References