Eriocalyxin B ameliorates experimental autoimmune encephalomyelitis by suppressing Th1 and Th17 cells

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Contributed by Zhu Chen, December 22, 2012 (sent for review November 20, 2012)

Eriocalyxin B (EriB), a diterpenoid isolated from *Isodon eriocalyx*, was previously reported to have antitumor effects via multiple pathways, and these pathways are related to immune responses. In this study, we demonstrated that EriB was efficacious in experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis. Treatment with EriB led to amelioration of EAE, which correlated with reduced spinal cord inflammation and demyelination. EriB treatment abolished encephalitogenic T-cell responses to myelin oligodendrocyte glycoprotein in an adoptive transfer EAE model. The underlying mechanism of EriB-induced effects involved inhibition of T helper (Th) 1 and Th17 cell differentiation through Janus Kinase/Signal Transducer and Activator Of Transcription and Nuclear factor- κ B signaling pathways as well as elevation of reactive oxygen species. These findings indicate that EriB exerts potent antiinflammatory effects through selective modulation of pathogenic Th1 and Th17 cells by targeting critical signaling pathways. The study provides insights into the role of EriB as a unique therapeutic agent for the treatment of autoimmune diseases.

cytokines | traditional Chinese medicine

Multiple sclerosis (MS) is an autoimmune disease, pathologically characterized by multifocal inflammatory demyelination in the white matter of the central nervous system (CNS). The disease is associated with a variety of symptoms and functional deficits that result in a range of progressive impairments and disability. Recent advancements have resulted in development of treatments for slowing disease progression and alleviating associated symptoms but not a cure for MS. Accordingly, a better understanding of cellular and molecular mechanisms driving disease is necessary to address the unmet need for more effective management of MS (1, 2). Although the pathogenesis of MS has only partially been elucidated, ample evidence suggests that dysfunction of T helper (Th) cell regulation and multiple signaling events of immune responses are involved in the tissue damage in patients. Experimental autoimmune encephalomyelitis (EAE), induced by immunization with myelin antigens or adoptive transfer of myelin-specific T cells, mimics these key aspects of human MS and is, therefore, widely used as a preclinical model of human disease (3, 4).

CD4⁺ Th cells play an important role in the adaptive immune system by activating and directing other immune cells. At least four major subsets of Th cells differentiated from naïve CD4⁺ T cells and characterized by distinct cytokine profiles after have thus far been identified, i.e., Th1, Th2, Th17, and regulatory T (Treg) cells (5). Both Th1 and Th17 cells have been shown in clinical and preclinical studies to be critically involved in the pathogenesis of autoimmune diseases including MS, rheumatoid arthritis, and inflammatory bowel disease, whereas Th2 cells are implicated in asthma and allergy when aberrantly stimulated (6, 7). Encephalomyelitis in the CNS of EAE mice is caused mainly by activated microglia and infiltrating macrophages, which are

driven by pathogenic Th1 and Th17 cells (8). The differentiation of Th1 cells, which produce the signature cytokine interferon γ (IFN γ), depends on signaling through the IFN γ receptor, the interleukin (IL) 12 receptor, and their downstream transcription factors, namely, signal transducer and activator of transcription 1 (STAT1) and STAT4. IL17-producing Th17 cells arise after IL6 stimulation and subsequent activation of STAT3. IL4-secreting Th2 cell differentiation is directed by IL4, which can mediate a positive feedback loop via STAT6. All of the cytokine receptors undergo phosphorylation on their cytosolic domains by receptor-associated Janus kinases (Jaks) and therefore activate downstream STAT proteins by their phosphorylation, dimerization, and translocation to the nucleus to initiate transcriptional regulation of target genes for Th cell differentiation (9). In addition, Treg cells derived from the thymus or induced from naïve CD4⁺ T cells in the periphery are referred to as suppressor T cells characterized by expression of the transcription factor forkhead box P3 (Foxp3). Treg cells mediate immune suppression, in part, through secreting the antiinflammatory cytokine IL10 (10).

Eriocalyxin B (EriB) is a diterpenoid extracted from *Isodon eriocalyx*, a perennial herb of the Labiatae family in southwest China. It has long been used in traditional Chinese medicine as an anti-inflammatory remedy (11). We have previously reported that EriB induces apoptosis of leukemia and lymphoma cells through elevating intracellular levels of reactive oxygen species (ROS) and suppressing the Nuclear factor- κ B (NF- κ B) pathway (12, 13). Here we describe studies performed in the EAE model that indicate potential therapeutic effects of EriB on inflammatory/autoimmune disorders and delineate underlying molecular mechanisms.

Results

EriB Alleviates Symptoms and Delays Disease Onset in EAE Mice. To determine the anti-inflammatory properties of EriB in autoimmune disorders, we examined its *in vivo* effects in an actively induced EAE model. For the treatment regimen, EriB administration starting from disease onset in animals (day 11 postimmunization) led to significantly decreased disease severity measured by the mean maximal clinical score compared with vehicle controls ($P = 0.037$; Fig. 1A and Table 1). In the prophylactic treatment regimen, EriB administration starting from day 0 postimmunization was even more effective in decreasing

Author contributions: B.C., Z.C., and S.-J.C. designed research; Y.L., J.-H.S., T.Z., B.-Y.W., X.L., P.L., X.Y., and Q.-L.Z. performed research; Y.L., B.C., X.-D.X., S.-D.C., and J.-P.Z. analyzed data; and Y.L., B.C., Z.C., and S.-J.C. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1222426110/-DCSupplemental.

Fig. 1. EriB ameliorates EAE via inhibiting CNS inflammation and abrogates the encephalitogenicity of T cells to transfer EAE. Mice were injected i.p. daily with vehicle (●; $n = 8$), EriB (10 mg/kg, ○; $n = 10$) or MTX (1 mg/kg, ×; $n = 5$) starting 11 d after (A) or at the day of (B) EAE induction. (C) Spinal cords from normal mice or EAE mice treated with vehicle or EriB were obtained at day 28 postimmunization (treatment protocol) and stained by H&E (Top), Luxol Fast Blue (LFB) (Middle), or immunohistochemistry for CD4 (Bottom) (magnification 400×). (D) Pathology scores of inflammation and demyelination are expressed as mean $\pm$ SD ($n = 4$). (E) The absolute number of MNCs in CNS was counted in normal, EriB-, or vehicle-treated EAE mice at day 18 postimmunization (treatment protocol) ($n = 4$). (F) Splenocytes were derived from EAE mice at day 10 postimmunization and cultured with MOG 35–55 (20 μ g/mL) in the presence of EriB (0.25 μ M) or vehicle for 3 d. These cells were transferred into irradiated (400 cGy) congenic recipients (2×10^7 per mouse). Each group consisted of four or six mice. Data are expressed as mean $\pm$ SEM * $P < 0.05$; ** $P < 0.01$.

the clinical score of EAE mice ($P = 0.002$; Fig. 1*B* and Table 1). Furthermore, the prophylactic dosing of EriB also resulted in significantly delayed disease onset ($P = 0.001$) and reduced disease incidence compared with the vehicle control (Table 1). Methotrexate (MTX), an agent commonly used in the clinical therapy for autoimmune diseases, was not as effective as EriB under either therapeutic or preventive regimens (Fig. 1*A* and *B* and Table 1). These results in a mouse model of disease suggest that EriB could provide efficacy against MS in human patients.

To evaluate the safety of EriB, hematological parameters were examined. Vehicle, EriB, or MTX was injected into normal female

C57BL/6 mice at dosages and regimens used for the treatment of EAE mice. Whereas MTX decreased red blood cell counts ($P = 0.027$) and hemoglobin levels ($P = 0.012$) compared with the vehicle control, EriB did not substantially alter the hematological parameters in mice (Fig. S1).

EriB Treatment Reduces CNS Inflammation and Demyelination. Histological analysis of spinal cord tissue sections from healthy control mice showed intact myelin sheath and no inflammatory foci (Fig. 1C, *Left*), whereas typical demyelination and inflammation were observed in EAE mice (Fig. 1C, *Center*). EriB administration remarkably attenuated CNS demyelination and inflammation in EAE mice. In addition, the infiltration of CD4⁺ T cells was substantially decreased in response to EriB treatment (Fig. 1C, *Right*). The pathological scores of mice administered either with vehicle or EriB differed significantly ($P = 0.040$ for the infiltration score and $P = 0.020$ for the demyelination score, respectively) (Fig. 1D). Consistently, the number of mononuclear cells (MNCs) isolated from CNS of EriB-treated mice was markedly reduced compared with that of vehicle-treated mice ($P = 0.005$; Fig. 1E).

Autoreactive T Cells Are Unable to Transfer EAE After EriB Treatment. Encephalitogenic T cells obtained from EAE mice can elicit EAE via adoptive transfer into normal mice in a passive transfer model of EAE (14). We further investigated whether EriB is capable of disturbing the function of encephalitogenic T cells by adoptive transfer assay. As shown in Fig. 1F, typical EAE was established in recipient mice by transferring vehicle-treated myelin oligodendrocyte glycoprotein (MOG)-reactive T cells, with a mean maximal clinical score of 3.88 ± 0.38 , whereas MOG-reactive T cells treated with EriB failed to induce EAE in recipient mice. Results of histological analysis of spinal cord confirmed the absence of pathology and were consistent with the clinical symptoms (Fig. S2).

EriB Decreases CD4⁺ and CD11b⁺ Cell Populations and Suppresses Autoreactive Proliferation. MNCs from the spleen (splenocytes) and CNS of EAE mice were analyzed by flow cytometry for surface expression of CD4, CD8, B220, and CD11b to differentiate between T-cell, B-cell, and macrophages/microglia infiltration. The absolute numbers of these cells were reduced in CNS. More importantly, EriB treatment resulted in significantly decreased percentages of CD4⁺ T cells both in the spleen ($P = 0.006$) and CNS ($P = 0.004$) as well as a reduction of CD11b⁺ macrophages/microglia in the spleen ($P = 0.010$) and CNS ($P = 0.001$). However, the percentages of CD8⁺ T cells and B220⁺ B cells remained unaltered (Fig. 2 *A* and *B*).

In addition, splenocytes collected from EriB-treated mice showed a lower proliferative response to disease-eliciting MOG 35–55 peptide than those from vehicle-treated mice ($P < 0.001$; Fig. 2C). EriB was also proved to inhibit the proliferation of encephalitogenic T cells in response to MOG 35–55 in vitro at a concentration of 0.25 μM , whereas it had no significant suppressive effect when a nonspecific stimulus such as concanavalin A (ConA) was used (Fig. 2D). The results suggested that autoreactive T cells were more sensitive to EriB treatment.

Table 1. Clinical features of EAE in mice in the administration of vehicle, EriB, or MTX

Group	Treatment regimen		Preventive regimen		
	Incidence, %	Mean maximal score	Incidence, %	Mean maximal score	Average day of onset
Vehicle	100	3.81 ± 0.34	100	3.59 ± 0.25	12.75 ± 0.75
EriB	100	2.80 ± 0.29*	70.0	1.75 ± 0.40**	20.14 ± 1.71**
MTX	100	3.70 ± 0.56	80.0	2.10 ± 0.70*	18.00 ± 1.08**

Values are expressed as mean $\pm$ SEM * P < 0.05, ** P < 0.01 compared with vehicle control.

Fig. 2. EriB decreases CD4⁺ and CD11b⁺ cell populations and suppresses auto-reactive proliferation. MNCs were derived from spleen or CNS in EriB or vehicle-treated EAE mice at day 18 postimmunization (treatment protocol). (A) Cells were analyzed for expression of CD4, CD8, or B220 in the lymphocyte gate and that of CD11b in total MNC gate by flow cytometry. (B) Absolute numbers and percentages of cells positively expressed with these antigens in spleen (Left) or CNS (Right) are represented ($n = 5$). (C) Proliferation was measured by [³H]thymidine incorporation of splenocytes. The stimulation index (SI) was the ratio of MOG 35–55-stimulated proliferation to spontaneous cell proliferation. (D) Splenocytes from EAE mice stimulated with MOG 35–55 (20 μ g/mL) or normal congenic mice stimulated with ConA (5 μ g/mL) were treated with EriB (0.25 μ M) in vitro and examined for proliferation after a 72-h culture. Data are expressed as mean $\pm$ SEM. ns, $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Effects of EriB on Th1 and Th17 Cell Subsets Are Mediated via the Jak/STAT Signaling Pathway. The reduction of CD4⁺ T cells prompted us to investigate which subsets among these cells were affected by EriB treatment. To this end, we analyzed the subsets of MOG-reactive CD4⁺ Th cells from EAE mice after EriB treatment. These studies revealed that EriB significantly reduced the percentage of MOG-reactive Th1 (CD4⁺/IFN γ ⁺) and Th17 (CD4⁺/IL17⁺) cells both in the spleen ($P = 0.025$ and $P = 0.044$, respectively) and CNS ($P = 0.014$ and $P = 0.013$, respectively) compared with the vehicle control. However, the relative numbers of Th2 (CD4⁺/IL4⁺) and Treg (CD4⁺/Foxp3⁺) cells showed no significant changes (Fig. 3 A and B). The Th2-polarization assay further confirmed that EriB treatment did not obviously affect its differentiation (Fig. S3). The production of both the Th1 cytokine IFN γ and the Th17 cytokine IL17 were remarkably reduced by EriB treatment ($P < 0.05$). In contrast, the levels of the Th2 cytokines IL4 and anti-inflammatory cytokine IL10, which were very low but detectable, were not affected by EriB after reactivation of splenocytes with MOG 35–55 ex vivo (Fig. 3C). In parallel, the expression of key transcription factors, *T-box expressed in T cells (T-bet)* for Th1 cells and *retinoic-acid-receptor-related orphan receptor (ROR) γ t* for Th17 cells, but not *GATA3* for Th2 or *Foxp3* for Treg was significantly reduced in the same

CD4⁺ T-cell preparations derived from EriB-treated EAE mice (Fig. 3D).

Because members of the STAT transcription factor family play a crucial role in the differentiation of Th cells (9), we examined whether the Jak/STAT intracellular signaling pathway was modulated by EriB treatment. Splenocytes isolated from EriB- or vehicle-treated EAE mice were cultured in the presence or absence of the MOG 35–55 for 24 h. As shown in Fig. 3E, increased phosphorylation levels of all STAT proteins as well as their upstream kinases Jak1/2 were induced by MOG 35–55 stimulation. Notably, the phosphorylation levels of STAT1, STAT4, STAT3, and Jak1/2 were markedly attenuated when treated with EriB, whereas phosphorylation of STAT6 was not affected by EriB treatment. When the splenic CD4⁺ T cells were directly subjected to Western blot assay, the same trends were observed (Fig. 3F). The protein levels of Foxp3 were not altered in either MOG-reactive or purified CD4⁺ T cells (Fig. 3 E and F). Furthermore, EriB was confirmed to suppress the production of IFN γ and IL17 in fresh samples from five MS patients when we detected the cytokine secreting level of their peripheral CD4⁺ T cells (Fig. 3G). Taken together, these data suggest that the therapeutic effect of EriB is a result of selective inhibition of Jak/STAT pathways in vivo.

ROS Elevation Is Essential for EriB-Induced Th1 and Th17 Cell Suppression at Disease Priming Stage.

Because a delayed onset of disease score was present in response to the prophylactic EriB treatment regimen, a key question was how EriB represses the immune response during the priming of the immune system. Subsequent studies revealed that MOG-reactive CD4⁺ Th1 and Th17 cells began to expand before the clinical symptom appeared. EriB administration led to an over fourfold reduction in the percentage of Th17 cells compared with the vehicle control ($P = 0.006$), whereas the Th1 subset was moderately affected ($P = 0.026$; Fig. 4A). Diterpenoids exert their biological activity, at least in part, via elevating levels of intracellular ROS (15, 16). To investigate whether this mechanism is involved in EriB-induced immunoregulation, we analyzed ROS levels in MOG-reactive CD4⁺ T cells. As shown in Fig. 4B, EriB treatment caused an ~ 1.5 -fold elevation of the ROS levels, which could be abolished by cotreatment with *N*-acetylcysteine (NAC), a ROS scavenger.

To gain insight into the underlying mechanisms of EriB-mediated modulation of Th1 and Th17 cell differentiation, purified naïve CD4⁺ T cells were cultured in the presence or absence of EriB under Th1- or Th17-polarization conditions. We found EriB-mediated inhibition of Th17 differentiation could be abolished by NAC; however, NAC alone had no substantial effects (Fig. 4C). Correspondingly, the mRNA levels of *IL17a/f*, as well as phosphorylated-STAT3 level decreased by EriB treatment, were antagonized by NAC coinubation (Fig. 4D). By contrast, Th1 cell differentiation was almost not affected (Fig. 4E). The influence of EriB on cell proliferation of predifferentiated Th1 and Th17 subsets was subsequently analyzed. The results indicated that EriB treatment remarkably decreased the percentage of CD4⁺IFN γ ⁺ cells in MOG-reactive lymphocytes, consistent with the lower percentages of these cells incorporating BrdU. Similar results were obtained when MOG-reactive Th17 cells were treated, indicating that the proliferation of both Th1 and Th17 cells could be suppressed by EriB. Notably, NAC could also reverse EriB-induced suppression of Th1 and Th17 cell expansion (Fig. 4 F and G).

EriB Inhibits NF- κ B Signaling and Decreases NF- κ B-Regulated Gene Expression.

It has been shown that the NF- κ B pathway is involved in the inflammatory process of EAE (17–19), and EriB could block NF- κ B activation in some tumor cells (11, 12). NF- κ B signaling and multiple other intracellular pathways have been implicated in the pathogenesis of autoimmune diseases, which led us to hypothesize that EriB interferes with immune-regulating mechanisms in general.

Fig. 3. Th1 and Th17 cell subsets are selectively reduced by EriB through Jak/STAT signaling pathway. Splenocytes or CNS MNCs of EriB- or vehicle-treated EAE mice at day 18 postimmunization (treatment protocol) were isolated. (A) Subsets of Th1/Th17, Th2, and Treg cells in CD4⁺ gate were analyzed by intracellular staining of IFN γ , IL17, IL4, and Foxp3, following stimulation with MOG 35–55 (20 μ g/mL) for 24 h. (B) Percentages of cells positive expression with these antigens in spleen (Upper) or CNS (Lower) are expressed as mean $\pm$ SEM ($n = 4$). (C) Supernatants derived from splenocytes reactivated with MOG 35–55 (20 μ g/mL) for 48 h were analyzed for the level of indicated cytokines (mean $\pm$ SEM; $n = 6$). (D) mRNA levels of *T-bet*, *ROR γ t*, *GATA3*, and *Foxp3* genes from the same CD4⁺ T-cell preparations were analyzed by real-time PCR. (E) Splenocytes were cultured in the absence or presence of MOG 35–55 (20 μ g/mL) for 24 h and analyzed by Western blot assay for the expression or phosphorylation level of indicated proteins. (F) Purified CD4⁺ T cells from splenocytes were directly analyzed by Western blot assay for the expression or phosphorylation level of indicated proteins. (G) Purified CD4⁺ T cells from MS patient samples ($n = 5$) were treated with EriB (0.25 μ M) under Th1 or Th17 culture conditions for 48 h. Supernatants were measured for IFN γ and IL17 production. Data are representative of two independent experiments. * $P < 0.05$; ** $P < 0.01$.

We then focused on the NF- κ B pathway and analyzed the levels of inhibitor of NF- κ B (I κ B) α , p65, and phosphorylated p65 in splenocytes isolated from normal, vehicle-, or EriB-treated EAE mice by Western blot assay. These studies revealed a lower expression of I κ B α and increased phosphorylation of p65 in EAE mice compared with normal mice. Both I κ B α levels and p65 phosphorylation in EAE mice were restored to control levels upon EriB administration (Fig. 5A). Consistent with the modified NF- κ B activity, expression levels of NF- κ B-regulated gene products including IL6, IL12, tumor necrosis factor (TNF) α , and IL2 were significantly reduced in splenocytes derived from the EriB-treated mice compared with those from the vehicle group (Fig. 5B). These results suggested that EriB blocks NF- κ B signaling via regulation of I κ B α expression and subsequently reduced NF- κ B-regulated gene products.

Discussion

Our previous work has demonstrated that EriB could block TNF-induced NF- κ B activation by inhibiting I κ B degradation in Kasumi-1 cells, an acute myeloid leukemia cell line. EriB also changed the intracellular redox status through elevating ROS, which might further modulate redox-sensitive signaling pathways and transcription factors including NF- κ B (12). The role of EriB on modulating NF- κ B and ROS pathways was also described in lymphoma and many other tumor cells (11, 13, 20). Constitutive activation of the NF- κ B has been observed in many inflammatory and autoimmune responses (17, 18). In vivo administration of a peptide corresponding to NF- κ B essential modifier (NEMO)-binding domain that blocked NF- κ B activation protected mice from EAE (21). ROS-promoting substances such as phytol have been shown to improve autoimmune arthritis in animal models (22). The patients with defective ROS production often suffer from multiple autoimmune disorders (23). Accordingly, reduced

ROS-production capacity in animal models results in a higher susceptibility to arthritis and EAE (24). Although evidence shows that both NF- κ B and ROS play important roles in autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and MS, little is known regarding the effects of EriB on the immune diseases. This study uniquely shows that EriB can provide protection against the self-reactive immune response in EAE mice via multiple mechanisms. EriB treatment not only attenuated the disease severity but also delayed the onset and incidence of EAE in the prevention regimen.

EAE is an animal model that recapitulates several aspects of human MS including activation of myelin-reactive Th1 and Th17 cells, which are drivers of the autoreactive response, especially the activation of macrophages/microglia, which destroys CNS structures and results in progressive paralysis (8). Both myelin-reactive Th1 and Th17 subsets are capable of eliciting clinical signs of disease after being adoptively transferred into normal mice (7, 25). The production of IFN γ and IL17 is significantly increased in MS patients as well as in EAE mice (26–28). Therefore, searching for specific agents targeting these subsets has clinical significance.

In the present study, EriB treatment significantly reduced the percentage of MOG-reactive Th1 and Th17 cells at the peak of disease, whereas the relative numbers of Th2 and Treg cells were not affected. The selectivity of EriB for pathogenic Th1 and Th17 cells is conferred by its effect on the Jak/STAT family members as EriB inhibited phosphorylation of STAT1 and STAT4 for Th1 and STAT3 for Th17 cell differentiation, but not Th2-inducing STAT6 phosphorylation. Moreover, when given immediately after EAE induction (in the prophylactic dosing regimen), EriB delayed disease onset, suggesting that EriB may affect the early phase of immune responses, especially Th17

Although the pathogenesis of MS and EAE depends on activation of CD4⁺ T cells, innate immune cells also play important roles in disease progression. Among them, dendritic cells (DCs) as professional antigen-presenting cells (APCs) have potent capacity to prime naïve T cells and activate autoreactive response (33). Skarica et al. reported that inhibition of DCs diminished Th1 and Th17 responses in EAE mice (34). Therefore, we investigated the effect of EriB on bone marrow-derived dendritic cells (BMDCs) in vitro, which share a set of common features as naturally derived DCs. The results demonstrated that neither specific surface marker CD11c nor costimulatory molecule CD86 on BMDCs was significantly altered by EriB treatment (Fig. S4). Besides the direct effects on Th cells, EriB administration disturbed the cytokine microenvironment, which governs the differentiation and activation of Th cells. This effect may be mediated, at least in part, by the inhibition of the NF- κ B signaling pathway, which is critically involved in the production of proinflammatory cytokines. IL12, a cytokine regulated by NF- κ B in innate immune cells, initiates IFN γ production and Th1 differentiation from naïve CD4⁺ T cells. Similarly, IL6, which induces Th17 cell development in the presence of TGF β , is also a NF- κ B-regulated gene product. In addition, NF- κ B can activate the transcription of IL2, which plays an important role in Th cell growth and survival. Moreover, some Th1- and Th17-secreted cytokines, such as TNF and IL17, which we have tested, can in turn stimulate NF- κ B activity. Such cytokine-mediated positive feedback loops based on NF- κ B are highly active in expanding inflammatory response (9, 35). Interestingly, EriB treatment suppressed abnormal NF- κ B activation in EAE mice through the decreasing phosphorylation of p65 and increasing level of I κ B. The modification of NF- κ B activity might exert profound effects, such as the blockade of cytokine-mediated feedback loops in T-cell fate determination.

Taken together, EriB not only disturbs the priming of pathogenic Th1 and Th17 cells at the early stage of EAE by elevating

intracellular level of ROS, but also suppresses these subsets at the peak of disease via inhibition of both Jak/STAT and NF- κ B pathways. As the pathogenesis of autoimmune diseases often involves a complicated network of multiple factors, compounds from traditional Chinese medicine such as EriB, which exhibit multiple influences against autoimmune inflammation, might need more attention. An efficacy/side-effect profile of EriB in EAE mice, superior to MTX, clearly demonstrates a potential for therapeutic intervention in MS and possibly in other autoimmune and inflammatory diseases.

Materials and Methods

Methods and associated references are in *SI Materials and Methods*. The EAE model was treated with EriB. The severity of EAE is estimated by clinical score and histopathological examination. Splenocytes and CNS MNCs of mice were used for analyzing cell population, detecting cytokine levels, and examining the status of Th cell polarization and proliferation by flow cytometry. Western blot assay was performed to detect the activation of Jak/STAT and NF- κ B signaling pathways; real-time PCR was used to measure the mRNA levels of key transcription factors and cytokines. ROS generation was determined by the increase of 2',7'-dichlorofluorescein diacetate (DCFDA) fluorescence.

ACKNOWLEDGMENTS. The authors thank Dr. Han-Dong Sun (Kunming Institute of Botany, Chinese Academy of Sciences) for providing pure EriB powder; Dr. Yu-Hong Xu and Xiao-Hui Wei (School of Pharmacy, Shanghai Jiao Tong University) for preparing EriB stock solution; Xiang-qin Weng, Wu Zhang, and Yan Zhao for technical assistances in flow cytometry and histology analysis; and Dr. Wei-Dong Le (Institute of Neurology, Ruijin Hospital; and Department of Neurology, Baylor College of Medicine) for valuable comments on the manuscript. This work was supported by the National Natural Science Foundation of China (81270619 and 30830119), the Research Special Fund of the Ministry of Health (201202003), National High Tech Program for Biotechnology Grant 863 (2012AA02A505), the Mega Project of the Ministry of Science and Technology (2013ZX09303302 and 2009ZX09103-431), and the Science and Technology Commission Foundation of Shanghai (09dz1974500).

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