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Antiasthmatic Drugs Targeting the Cysteinyl Leukotriene Receptor 1 Alleviate Central Nervous System Inflammatory Cell Infiltration and Pathogenesis of Experimental Autoimmune Encephalomyelitis

Liefeng Wang,* Changsheng Du,* Jie Lv,* Wei Wei,* Ye Cui,* and Xin Xie*,†

Cysteinyl leukotrienes (CysLTs) are potent proinflammatory mediators and are considered to play a key role in inflammatory diseases such as asthma. Antagonists targeting the receptor of CysLTs (CysLT1) are currently used as antiasthmatic drugs. CysLTs have also been implicated in other inflammatory reactions. In this study, we report that in experimental autoimmune encephalomyelitis animals, CysLT1 is upregulated in immune tissue and the spinal cord, and CysLT levels in the blood and cerebrospinal fluid are also higher than in normal mice. Two clinically used antiasthma drugs, montelukast and zafirlukast, both targeting CysLT1, effectively block the CNS infiltration of inflammatory cells and thus reduce the incidence, peak severity, and cumulative clinical scores. Further study indicated that CysLT1 signaling does not affect the differentiation of pathogenic T helper cells. It might affect the pathogenesis of experimental autoimmune encephalomyelitis by increasing the secretion of IL-17 from myelin oligodendrocyte glycoprotein-specific T cells, increasing the permeability of the blood–brain barrier and inducing chemotaxis of T cells. These effects can be blocked by CysLT1 antagonists. Our findings indicate that the antiasthmatic drugs against CysLT1 can also be used to treat multiple sclerosis. The Journal of Immunology, 2011, 187: 000–000.

Multiple sclerosis (MS) is the most common autoimmune inflammatory disease of the CNS. It is characterized by immune-mediated demyelination and neurodegeneration of the CNS (1, 2). Experimental autoimmune encephalomyelitis (EAE) is a CD4+ T cell-mediated demyelinating disease with pathological similarities to MS and is widely used as an animal model of MS (1, 2). EAE is induced in susceptible mice by immunization with whole myelin proteins, MOG peptide, or PLP peptide emulsified in CFA, or by adoptive transfer of autoreactive T cells to normal recipient mice (3-6). In EAE, the integrity of the blood–brain barrier (BBB) is impaired, allowing perivascular infiltration of T cells to normal recipient mice (3–6). In EAE, the integrity of the blood–brain barrier (BBB) is impaired, allowing perivascular infiltration of the CD4+ T cells into the CNS. This leads to the infiltration and accumulation of macrophages and dendritic cells, as well as activation of glia cells, which eventually cause demyelination, axonal damage, impaired nerve conduction, and paralysis (3, 7, 8). The mediators involved in the pathogenesis of EAE and MS are not clear.

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Abbreviations used in this article: BBB, blood–brain barrier; CSF, cerebrospinal fluid; CysLT, cysteinyl leukotriene; EAE, experimental autoimmune encephalomyelitis; EIA, enzyme immunoassay; GPCR, G protein-coupled receptor; 5-LO, 5-lipoxygenase; LT, leukotriene; LTA4H, LTA4 hydrolase; LTC4S, LTC4 synthase; MS, multiple sclerosis; TJ, tight junction; ZO, zonula occludens.

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Leukotrienes (LTs) are potent proinflammatory mediators that have established or evolving roles in a wide variety of inflammatory diseases, including asthma, allergic rhinitis, atherosclerotic cardiovascular disease, inflammatory bowel disease, and others (4-6, 9, 10). LTs are derived from arachidonic acid via the sequential actions of cytosolic phospholipase A2α, 5-lipoxygenase (5-LO) and LTA4 hydrolase (LTA4H) for LTB4, or LTC4 synthase (LTC4S) for cysteinyl LTs (CysLTs; i.e., LTC4, LTD4, and LTE4) (11). The biological effects of LTB4 are mediated via two G protein-coupled receptors (GPCRs), BLT1 and BLT2 (12). CysLTs also activate two GPCRs, namely CysLT1 and CysLT2 (13). Modifiers of the leukotriene signaling pathways, including 5-LO inhibitor (zileuton) and CysLT1 antagonists (montelukast, zafirlukast, and pranlukast), are the only orally administered class of the three most commonly prescribed classes of asthma medications (14–17).

A microarray analysis study indicated that 5-LO, the key enzyme in the biosynthesis of leukotrienes, is upregulated in both MS lesions and the CNS of EAE animals (18). Blocking of 5-LO with zileuton was found to delay the onset and reduce cumulative severity of EAE (19). Several studies also indicated that blocking the BLT1 signaling by genetic or pharmacological ways can suppress the recruitment of inflammatory cells into the CNS and thus the induction of EAE (12, 20). However, whether CysLTs and their receptors are involved in the pathogenesis of EAE has never been reported. In this study, we found in EAE animals that both 5-LO and CysLT1 are upregulated and CysLT levels are also increased. Inhibition of CysLT1 with both montelukast and zafirlukast attenuated the CNS infiltration of inflammatory cells and the clinical symptoms of EAE. We demonstrate that CysLTs and their receptor CysLT1 play important roles in the pathogenesis of EAE, mainly by increasing IL-17 secretion from MOG-specific T cells, modifying the permeability of the BBB, and inducing chemotaxis of pathogenic T cells.
Materials and Methods

Animals

Female C57BL/6 mice were purchased from Shanghai Laboratory Animal Center (Shanghai, China). All mice were housed in the Tongji University Animal Care Facility and were maintained in pathogen-free conditions. Mice were 8–9 wk old at the initiation of the experiment and were maintained on standard laboratory chow and water ad libitum. All experiments were approved and conducted in accordance with the guidelines of the Animal Care Committee of Tongji University.

Reagents

MOG35–55 (MEVGWYRSPFSRVHLYRNGK) was purchased from GL Biochem (Shanghai, China) with purity >95%. Montelukast was from Merck and zafirlukast was from 3B Scientific (Wuhan, China). Moloney murine leukemia virus reverse transcriptase and RNAsin RNase inhibitor were from Promega (Fitchburg, WI). SYBR Green JumpStart Taq Ready-Mix kit and sodium fluorescein were from Sigma-Aldrich (St. Louis, MO). Percoll was from GE Healthcare. FITC anti-mouse CD45, FITC anti-mouse CD8a, PE anti-mouse CD45R (B220), and an allophycocyanin anti-mouse/ rat FoxP3 staining set were purchased from eBioscience (San Diego, CA). PE-Cy7 anti-mouse CD4, PE anti-mouse IL-17a, allophycocyanin anti-mouse IFN-γ, PE anti-mouse Foxp3, and a BD Cytofix/Cytoperm kit were purchased from BD Biosciences (Franklin Lakes, NJ). A Dynal mouse CD4 cell-negative isolation kit was from Invitrogen (Carlsbad, CA). IL-17a, IFN-γ, TGF-β, and IL-4 ELISA kits were from Dakewe (Shenzhen, China).

EAE induction and drug treatment

Female C57BL/6 mice 8–9 wk age were immunized s.c. with 200 μg MOG35–55 in CFA containing heat-killed Mycobacterium tuberculosis (H37Ra strain, 5 mg/ml; BD Diagnostics). Pertussis toxin (200 ng/mouse; Calbiochem) in PBS was administered i.p. on days 0 and 2. Mice were examined daily for disease signs by researchers blinded to experimental conditions and were assigned scores on a scale of 0–5 as follows: 0, no paralysis; 1, partial paralysis of one or two hindlimbs); 3, paraplegia (complete paralysis of both hindlimbs); and 5, moribund state or death. For drug treatment, montelukast and zafirlukast were injected i.p. (10–30 mg/kg body weight in saline) once daily on days 3, 10, or 14 until the end of the study. Saline was given as a vehicle control (100 μl for each mouse).

Histopathological and immunofluorescent analysis

For analysis of CNS infiltrates, spinal cord tissues were collected after perfusion with PBS. Histological and immunofluorescent staining, mice were anesthetized and perfused with PBS (pH 7.4) followed by 4% (v/v) paraformaldehyde. Tissue samples were then fixed in 4% (w/v) paraformaldehyde overnight. Paraffin-embedded sections of spinal cord were stained with H&E or with Luxol fast blue for analysis of inflammation or demyelination, respectively. Frozen sections of spinal cord were stained with anti-mouse CD4 Ab, then with appropriate fluorescent-labeled secondary Abs.

Real-time PCR

Total RNA was extracted from spinal cord, cerebral cortex, spleen, and lymph node samples using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The RNA was subjected to reverse transcription with random hexamer primer and Moloney murine leukemia virus reverse transcriptase (Promega). Real-time PCR was conducted in a LightCycler quantitative PCR apparatus (Stratagene) using the SYBR Green JumpStart Taq Ready-Mix kit (Sigma-Aldrich). Expression values were normalized to β-actin. The primer pairs used are as follows: CysLT1 sense, 5′-CTCC-AAGGCACCAAGCAGAC-3′, antisense, 5′-TGGCAAGAAGAGGCAACACAC-3′; CysLT2 sense, 5′-CGAAGCCAGAGCCAGCATGAT-3′, antisense, 5′-GAACCCATCTAAGTACTGGTGCT-3′; IL-10 sense, 5′-CACGCATCTGGTGCTGAG-3′, antisense, 5′-CCTATGATTGTTAATGACA-3′; IL-1α sense, 5′-CCTATGAGAAAGCCTTTGGC-3′, antisense, 5′-CCTAGAGACGGTTGTTCACTCT-3′; LTC4S sense, 5′-CCCTGATGGTCTTCTACATCT-3′, antisense, 5′-GCCATGGCCACCAACAGCA-3′; β-actin sense, 5′-GGTTGATTACCTCCTCCATG-3′, antisense, 5′-CCAC-CTGTTAACAATGGCCATGTG-3′.

CysLT enzyme immunoassay

The CysLTs in brain, spinal cord, serum, and cerebrospinal fluid (CSF) were quantified by using a competitive enzyme’s immunoassay (ELISA; Cayman Chemical), according to the manufacturer’s instructions. For brain and spinal cord samples, the tissues were homogenized in ethanol and precipitated proteins were removed by centrifugation. The supernatant was dried and used for the detection. Orbital blood was collected and incubated at 4°C for 30 min and serum was collected after 10 min centrifugation at 4500 × g. CSF was collected using a glass capillary pipette. For brain and spinal cord samples, data were calculated as picograms CysLTs per milligram tissue sample and then normalized to the naive mice. For serum and CSF samples, data were calculated as picograms CysLTs per milliliter liquid and then normalized to the naive mice.

Isolation and analysis of CNS leukocyte infiltrations

Spinal cords were homogenized in ice-cold tissue grinder and filtered through a 70-μm cell strainer, and the cells were collected by centrifugation at 500 × g for 10 min at 4°C. Cells were resuspended in 8 ml 37% Percoll and centrifuged onto 4 ml 70% Percoll cushion in 15-ml tubes at 780 × g for 25 min at 25°C. Cells at the 37–70% Percoll interface were collected and subjected to flow cytometry.

CD4+ T cell separation and in vitro differentiation

Naïve CD4+ T cells were prepared by magnetic cell separation (Invitrogen) from spleens of female C57BL/6 mice 8–9 wk age. Separated cells were activated with anti-CD3 (2 μg/ml; 145-2C11, soluble; BD Pharmingen) and anti-CD28 (2 μg/ml; 37.51, soluble; BD Pharmingen) Abs and were induced to differentiate into Th1 cells by supplementation with IL-12 (10 ng/ml; PeproTech) and anti–IL-4 (10 μg/ml; 11B11; BD Pharmingen) or into inducible regulatory T cells with TGF-β1 (5 ng/ml; PeproTech), recombinant mouse IL-2 (50 U/ml; PeproTech), and anti–IFN-γ (10 μg/ml; XMG1.2; BD Pharmingen). For Th17 differentiation, cells received anti–IL-4, anti–IFN-γ, plus a Th17 mixture containing TGF-β1 (3 ng/ml), IL-6 (30 ng/ml; eBioscience), TNF (10 ng/ml; PeproTech), and IL-1β (10 ng/ml; PeproTech). Compounds at various concentrations were added with the cytokine mixture to assess their influence on T cell differentiation.

Flow cytometry

Splenocytes or CNS infiltrates were incubated for 5 h at 37°C with PMA (50 ng/ml; Sigma-Aldrich), ionomycin (750 ng/ml; Sigma-Aldrich), and brefeldin A (1.0 μg/ml; Sigma-Aldrich). Surface markers were stained with relevant Abs. After surface staining, cells were resuspended in Fixation/Permeabilization solution (Cytofix/Cytoperm kit; BD Pharmingen) and intracellular cytokine staining was done according to the manufacturer’s protocol. For Foxp3 staining, cells were not stimulated with PMA and ionomycin; instead, a Foxp3 staining buffer set was used following the manufacturer’s instructions (eBioscience). Guava easyCyte 8HT system and GuavaSoft software were used for the analysis.

ELISA of cytokines from MOG-specific cells

Naïve or EAE mice treated with vehicle or montelukast were sacrificed on day 10 postimmunization. The spleens were isolated and lysed with RBC lysis buffer. The remaining leukocytes were collected via centrifugation at 1000 × g for 3 min and then resuspended in RPMI 1640 supplemented with 10% FBS. Leukocytes were seeded onto 96-well plates at a density of 2 ×10^5/well/100 μl and restimulated with MOG35–55 (20 μg/ml) at 37°C for 48 h. The supernatants were collected and subjected to ELISA kits (Dakewe) according to the manufacturer’s instructions.

In vivo BBB permeability assessment

In vivo BBB permeability was assessed using a previously described method in which sodium fluorescein was used as a tracer molecule (21). In brief, mice received 100 μl 10% sodium fluorescein in PBS via i.p. injection. Forty-five minutes later, cardiac blood was collected and the animals were perfused with PBS containing 1000 U/I heparin. Brain and spinal cord were weighed and homogenized in 1.5 ml cold 7.5% TCA and centrifuged for 10 min at 10,000 × g to remove insoluble precipitates. Serum was diluted 1:10 in sterile PBS prior to an additional 1:10 dilution in 7.5% TCA. Following the addition of 0.25 ml i 5 N NaOH, the fluorescence of a 100 μl supernatant sample was determined using a FluXStation microplate reader ( Molecular Devices) with excitation wavelength at 485 nm and emission at 530 nm. Standards (0.064–100 μg/ml) were used to calculate the sodium fluorescein content of the samples. Fluorescent value of the CNS was first normalized to the fluorescent value of the blood of the same animal and then normalized to the naive control animals.

In vitro BBB assay

In vitro coculture BBB assay (22, 23) was performed using the 12-mm Transwells with 0.4-μm pore size (Corning). Inserts were coated with laminin (Sigma-Aldrich) for 30 min. Mouse brain microvascular endo-
CysLT1 antagonists ameliorate clinical symptoms and CNS infiltration in EAE

To further assess the involvement of CysLTs signaling in EAE pathogenesis, montelukast and zafirlukast, two CysLT1 receptor antagonists currently marketed as antiasthma drugs, were used to treat MOG-immunized EAE animals. Drugs were given once daily i.p. from days 3 (Fig. 2A, 2B), 10 (Fig. 2C), or 14 (Fig. 2D) postimmunization until the end of the experiments, whereas the control mice were injected with 0.9% saline. Montelukast displayed dose-dependent inhibition of EAE severity when given from both days 3 and 10 (Fig. 2A, 2C) postimmunization. Zafirlukast (30 mg/kg) also significantly ameliorated the severity of EAE (Fig. 2B, 2C). More interestingly, when given after the onset of the disease (Fig. 2D), montelukast (30 mg/kg) was still able to reduce the severity of EAE, which indicated the therapeutic benefit of this drug in addition to the preventative effect.

Histological examination of spinal cord was performed on day 17 postimmunization. Compared to vehicle treatment, montelukast caused a dramatic decrease of leukocyte infiltration in spinal cord (Fig. 2E, 2H). Luxol fast blue staining showed multiple widespread areas of myelin damage in white matter region of the spinal cord in saline-treated EAE mice, whereas in montelukast-treated animals, such demyelination was greatly reduced (Fig. 2E, 2F). The CNS leukocyte infiltration was further analyzed by in situ immunofluorescent staining of the spinal cord sections. Consistent with the results of H&E staining, montelukast treatment reduced the number of CD4+ T cells (Fig. 2G, 2J) in the spinal cord sections of EAE mice.

Leukocyte infiltration of the CNS was also quantified by flow cytometry analysis on day 17 postimmunization. Results again confirmed that both the total CNS infiltrations (Fig. 3A) and the CD4+ T cells accumulated in CNS (Fig. 3B, 3C) were decreased by montelukast treatment. Th17 and Th1 are the main pathogenic CD4+ T effector cells in EAE (24). After montelukast treatment, both the percentage and absolute number of Th1 cells were significantly reduced (Fig. 3D, 2F). Although the percentage of Th17 cells in the CD4+ CNS infiltrates was not changed, the absolute number of Th17 cells was significantly decreased (Fig. 3D, 2F). Taken together, these data indicate that blocking CysLT1 signaling by antagonists significantly reduces EAE severity accompanied by decreased CNS inflammation and demyelination.
CysLT1 signaling does not influence T cell differentiation both in vivo and in vitro

Next we examined whether CysLT1 signaling is involved in T cell differentiation. In EAE animals, treatment with montelukast did not significantly change the percentage of total leukocytes (CD45+ cells), CD4+ T cells, CD8+ T cells, and B cells in both spleen and blood (Fig. 4A, 4C). We found no differences in the frequency of Th1, Th17, or regulatory T cells in the CD4+ population between montelukast-treated mice and saline-treated mice (Fig. 4B, 4D). In vitro differentiation was performed to further examine whether CysLTs or the antagonist montelukast influence Th1, Th17, or regulatory T cell differentiation. Naive CD4+ T cells were prepared by magnetic cell sorting from spleens of female C57BL/6 mice 8–9 wk of age. Sorted cells were activated with anti-CD3 and anti-CD28
FIGURE 2. CysLT1 receptor antagonists alleviate pathogenesis of EAE. EAE was induced in female C57BL/6 mice by immunization with MOG35–55. Drugs were given once daily via i.p. injection from days 3 (A, B), 10 (C), or 14 (D) until the end of the study and clinical scores were recorded. Control groups were treated with 0.9% saline injections. Data represent means ± SEM. ###p < 0.001 (two-way ANOVA test), *p < 0.05, **p < 0.01 versus vehicle control (Mann–Whitney U test).

E, H&E staining (left panels, original magnification ×10; middle and right panels, original magnification ×40) and (F) Luxol fast blue staining of paraffin sections of spinal cords isolated from naive-, vehicle-, or montelukast- (10 mg/kg, starting from day 3) treated EAE mice on day 17 after immunization (left panels, original magnification ×10; middle and right panels, original magnification ×20). G, Immunofluorescent staining of CD4+ T cells in the frozen section of spinal cords isolated from naive-, vehicle-, or montelukast- (10 mg/kg, starting from day 3) treated EAE mice on day 17 postimmunization (left panels, original magnification ×10; right panels, original magnification ×40). Boxed areas in left columns in E–G are presented enlarged at the right side. H–J, Quantitative analysis of the numbers of total infiltrates and CD4+ T cells and the amount of demyelination presented in E–G. Data are represented as means ± SEM. Three animals from each group were sacrificed and 20 sections of the spinal cord of each animal were analyzed. ***p < 0.001 versus wild type. **p < 0.01 versus vehicle control (Student t test).
Abs and then induced to differentiate into Th1, Th17, or regulatory T cells by supplementation with differentiation factors and various concentrations of LTD4 and montelukast. Cells were harvested 3 d later and intracellular staining for IFN-γ, IL-17, or Foxp3 was performed. Upon FACS analysis, we found that both LTD4 and montelukast did not influence the differentiation of Th1 (Fig. 4E), Th17 (Fig. 4F), or regulatory T cells (Fig. 4H) in vitro.

Next we examined whether montelukast can influence the production of cytokines from splenocytes upon MOG restimulation (25, 26). Naive or EAE mice treated with vehicle or montelukast (10 mg/kg) were sacrificed on day 10 postimmunization. Splenocytes were isolated and restimulated with MOG35–55 (20 μg/ml) at 37˚C for 48 h. The supernatants were collected and the amounts of IL-17a, IFN-γ, IL-4, and TGF-β were determined using specific ELISA kits. As shown in Fig. 4H, montelukast significantly decreased the production of IL-17a. In contrast, the levels of IFN-γ, TGF-β, and IL-4 (Fig. 4I, 4J) were not significantly affected by montelukast. Taken together, these results indicate that CysLT1 signaling is not responsible for the differentiation or proliferation of inflammatory T cells or regulatory T cells, but might affect the cytokine production of MOG-specific Th17 cells.

**Montelukast alleviates LTD4-induced BBB disruption**

Our results showed that leukocyte infiltration was suppressed by montelukast treatment, but meanwhile no significant differences in T cell populations were found in the peripheral immune tissues. Based on these observations, we speculate that montelukast might influence the leukocyte infiltration process. Because there is a clear correlation between the permeability of BBB and the severity of clinical signs of EAE (7), and CysLTs has also been reported to increase microvascular permeability (27, 28), we wondered whether CysLT1 activation leads to increased permeability of BBB in EAE. Sodium fluorescein has been used as a tracer to check the integrity of BBB (7). Compared to normal mice, sodium fluorescein leaked extensively from peripheral blood into the spinal cord (9.3-fold) and brain (1.95-fold) in EAE mice on day 14 after immunization, whereas montelukast treatment significantly reduced sodium fluorescein leakage from periphery blood into the spinal cord (Fig. 5A).

Then we tested the effect of CysLTs on BBB with an in vitro coculture model with bEnd.3 mouse brain microvascular endothelial cells and C6 rat glioma cells in Transwells as previously...
Montelukast treatment does not affect T cell differentiation, but reduces IL-17 production from MOG-specific cells. Leukocytes were isolated from EAE animals treated with montelukast (10 mg/kg) or vehicle control on day 10 postimmunization and analyzed with flow cytometry. A and C, Surface staining of CD45+ cells, CD4+ T cells, CD8+ T cells, and B cells; B and D, Th1, Th17, and regulatory T cells were analyzed by intracellular staining of IFN-γ, IL-17, and Foxp3, respectively, within the CD4+ gate. E–G, Naive CD4+ T cells isolated from spleen of 8- to 9-wk-old mice were induced to differentiate into Th1 (E), Th17 (F), or regulatory T cells (G) in vitro in the presence of various concentrations of LTD4 or montelukast. Data are represented as means ± SEM of three independent experiments. H–K, Splenocyte from naive and EAE mice treated with vehicle or montelukast were re-stimulated with MOG35–55 for 48 h and the supernatants were collected for IL-17a, IFN-γ, IL-4, and TGF-β detection by ELISA. Data are represented as means ± SEM of three independent experiments. *p < 0.05, **p < 0.01 versus naive control, ***p < 0.001 versus naive control (Student t test).

Montelukast blocks LTD4-induced leukocyte chemotaxis

The permeability of the BBB is one of the important factors that influence leukocyte infiltration. Chemotaxis also plays an important role in this process. Blockade of leukocyte chemotaxis has been reported to alleviate EAE (31, 32). It has been reported that CysLTs induce chemotactic activity in eosinophils (33) and monocytes (34). We wondered whether CysLTs also induce the chemotaxis of pathogenic T cells in EAE. Using the Transwell chemotaxis assay, we found that LTD4 induced a significant, dose-dependent chemotaxis in splenocytes isolated from MOG-EAE mice on day 10 postimmunization. The bell-shaped dose–response curve of LTD4 is typically seen with many chemoattractants, with the highest response at 100 nM (Fig. 6A). As expected, montelukast treatment diminished the LTD4-induced chemotaxis in splenocytes (Fig. 6A). More interestingly, surface staining of the splenocytes that migrated into the lower chamber showed that LTD4 had a stronger chemotactic effect in CD4+ T cells compared with the whole splenocytes (2.7-versus 1.6-fold). Furthermore, montelukast treatment almost completely inhibited the migration of CD4+ T cells toward LTD4 (Fig. 6B). These results correlate well with our observations that the CysLT1 receptor was upregulated in spleen and blood, and the CysLTs were greatly increased in the CSF of EAE mice. These data indicate that CysLTs-induced chemotaxis of leukocytes, especially CD4+ T cells, may contribute to the CNS infiltration and eventually the pathogenesis of EAE.

Discussion

As an organ-specific autoimmune disease, MS is manifested by chronic inflammatory demyelination of the CNS and is one of the foremost causes of nontraumatic neurologic disability in young adults (35). Because of limited understanding of the pathogenesis of MS, there are still many difficulties in MS therapy and new therapeutic targets are needed to be identified (36). GPCRs are one of the biggest receptor superfamilies with more than one thousand members, and they broadly participate in biological processes and are involved in many human diseases (37). Because of their important physiological roles and cell surface localization, GPCRs are the most druggable targets on the market (38). Recently, fingolimod (FTY720), a sphingosine-1-phosphate receptor agonist, has been approved by the U.S. Food and Drug Administration as the first oral, first-line treatment for relapsing MS. It outperformed the established first-line therapy IFN-β1a in a 1-y, double-blind, double-dummy phase III study (known as TRANSFORMS) (39). Many other GPCRs have also been reported to participate in the pathogenesis of MS, including PGE2 receptors EP2 and EP4 (40),
AngII type 1a receptor (41), kinin receptor B1 (31) and many chemokine receptors (42, 43). In this study, we report that in EAE mice, CysLTs signaling components, including the receptor and key synthetic enzymes of the ligands, were upregulated. Two CysLT1 antagonists, montelukast and zafirlukast, could effectively reduce the disease severity.

CysLTs are produced primarily by mature leukocytes, including eosinophils, basophils, macrophages, and mast cells. CysLTs play key roles in asthma, allergic rhinitis, and other respiratory disease. Recent reports have indicated that LTs are also involved in MS and EAE pathogenesis, based on the fact that blocking CPLA2 or 5-LO, two key enzymes for LT synthesis, delayed EAE onset and reduced the disease severity. However, these studies also showed that blocking 5-LO did not reduce the production of IFN-γ or IL-17 (19). These observations are consistent with our results that blocking CysLT1 can ameliorate EAE, but does not influence Th1 or Th17 cell differentiation. In contrast, BLT1, the receptor for LTB4, might be necessary for Th1/Th17 development, since BLT1<sup>−/−</sup> mice showed the delayed onset and milder symptoms of EAE and reduced levels of Th1 and Th17 cytokines (12).

Although blocking CysLT1 with montelukast did not affect T cell differentiation, we did find that the drug significantly reduced Th1 and Th17 infiltration into the CNS of EAE animals. We therefore hypothesized that CysLT1 signaling must be influencing the T cell infiltration process. Chemotaxis plays important roles in T cell accumulation in inflammatory responses (44). In EAE mice, effector T cells are primed in peripheral lymph nodes and must migrate into CNS to initiate tissue inflammation, so effector T cell chemotaxis is an important aspect of CNS infiltration. Chemokines and their receptors are well known to play roles in T cell infiltration and EAE development. For example, TAK-779, a CCR5 antagonist, decreased EAE severity by reducing inflammatory cell migration into the CNS, without affecting T cell function (45). Blocking CXCR3 by a specific Ab also inhibited T cell migration and thus the development of EAE in an adoptive transfer model (42). CCR6 was also reported to be required for the entry of Th17 cells into the CNS and the initiation of EAE (46). In our study, we found that LTD<sub>4</sub> showed a dose-dependent

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**FIGURE 5.** CysLT1 antagonist prevents LTD<sub>4</sub>-induced permeability increase in BBB. A, Montelukast protects BBB leakage in MOG-EAE mice. The BBB permeability of naive control mice and MOG-EAE mice on day14 postimmunization treated with saline or montelukast (10 mg/kg) was measured by sodium fluorescein uptake into the CNS as described in Materials and Methods. Data are represented as means ± SEM (n = 3). ***p < 0.001 versus naive control. 

**FIGURE 6.** Montelukast inhibits immune cell chemotaxis induced by LTD<sub>4</sub>. A, Splenocytes from day 10 postimmunization EAE mice were added to the upper chamber of the Transwells, and various concentrations of LTD<sub>4</sub> were added to the lower chamber. For blocking assays, 1 μM montelukast was presented in both upper and lower chambers, and 100 nM LTD<sub>4</sub> was added to the lower chamber. One and a half hours later, the cells in the lower chamber were counted with flow cytometry. B, Cells that migrated to the lower chamber were reanalyzed with surface staining of CD4 and flow cytometry. The migration index of CD4<sup>+</sup> T cells is presented. Data are from three independent experiments (means ± SEM). ***p < 0.001, versus vehicle control, **p < 0.01, ***p < 0.001 versus LTD<sub>4</sub> (10<sup>−7</sup> M) treatment group (Student t test).
chemotactic activity on splenocytes from MOG-EAE mice. More interestingly, LTD₄ had a stronger chemotactic effect in CD4⁺ T cells compared with the whole splenocytes. These results correlate well with our observations that the CysLT₁ receptor was upregulated in immune tissues, and the CysLTs were greatly increased in the CSF of EAE mice. Montelukast can block LTD₄-induced T cell migration, which may contribute to its therapeutic effect in EAE.

Another important fact that influences the infiltration process is the permeability of the BBB. There is a clear correlation between the permeability of BBB and the severity of clinical signs of EAE (21). It has been reported that a high level of CysLTs is correlated with increased vascular permeability in the initial phase of zymosan-induced peritonitis (47). Another more recent study revealed that CysLT₁ antagonists, including montelukast, can inhibit tumor metastasis by inhibiting capillary permeability (27). We therefore thought that CysLT₁ signaling might also contribute to the disruption of BBB at the onset of EAE, and this could be blocked by montelukast. Indeed, in our in vivo study showed that the BBB permeability was increased significantly in EAE animals, especially in the spinal cord, which is consistent with previous reports (7), and montelukast treatment prevented the permeability increase in EAE mice. Using the in vitro BBB assay, we directly demonstrated that LTD₄ could increase the permeability of BBB and this phenomenon could be blocked by montelukast.

 Tight junctions (TJs) between endothelial cells of CNS capillaries are the most important structural elements of the BBB (48). TJs are composed of a branching network of sealing strands, which are fibril-like proteinaceous structure embedded in the lipid bilayer. TJ strands in apposing membranes associate with each other to eliminate the intercellular space, therefore preventing free passage of materials. The interplay between integral membrane proteins, claudins, and cytoplasmic plaque proteins, ZO-1/ZO-2, is critical for TJ stability (49). Targeted disruption of the ZO-1 gene and RNA interference knockdown of ZO-2 protein in mouse epithelial cells resulted in deficient TJ formation (50). We also found that LTD₄ treatment led to a significant reduction of ZO-1 protein in bEnd.3 mouse brain endothelial cells. This may contribute to the increased BBB permeability in EAE, since we found increased levels of CysLTs in both CSF and serum of the EAE animals. Treatment with montelukast prevented the loss of ZO-1 protein and thus protected the integrity of BBB. However, how LTD₄ mediates the reduction of ZO-1 remains to be elucidated. Previous studies indicated that activation of PKC and p38 signaling pathways may lead to ZO-1 disruption and increase of BBB permeability (51, 52). Other reports also revealed that endogenous CysLTs can increase in EAE mice. Montelukast treatment prevented the permeability increase in EAE mice. Using the in vitro BBB assay, we directly demonstrated that LTD₄ could increase the permeability of BBB and this phenomenon could be blocked by montelukast.

Disclosures
The authors have no financial conflicts of interest.

References


