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Aurintricarboxylic Acid Ameliorates Experimental Autoimmune Encephalomyelitis by Blocking Chemokine-Mediated Pathogenic Cell Migration and Infiltration

Feifei Zhang,* Wei Wei,* Hui Chai,* and Xin Xie*,†

Multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE), are autoimmune diseases characterized by the immune-mediated demyelination and neurodegeneration of the CNS. Overactivation of CD4+ T cells, especially the Th1 and Th17 subpopulations, is thought to be the direct cause of this disease. Aurintricarboxylic acid (ATA), an inhibitor of protein-nucleic acid interaction, has been reported to block the JAK/STAT signaling pathway that is critical for Th cell differentiation. In this study, we discovered that ATA treatment significantly reduces the clinical score of EAE, but it does not directly inhibit the differentiation of Th1 and Th17 cells in vitro. ATA was found to block the chemotaxis and accumulation of dendritic cells in the spleen of EAE mice before the onset of the disease and to reduce the percentage of Th1 and Th17 cells in the spleen. Further study revealed that ATA also blocks the infiltration of pathogenic T cells into the CNS and blocks the onset of passive EAE. ATA was found to inhibit the functions of many chemokine receptors. By blocking chemokine-mediated migration of dendritic cells and pathogenic T cells, ATA alleviates the pathogenesis of EAE and might be used to treat autoimmune diseases, including multiple sclerosis. The Journal of Immunology, 2013, 190: 1017–1025.

Multiple sclerosis is an autoimmune disease characterized by the immune-mediated demyelination and neurodegeneration of the CNS (1, 2). Experimental autoimmune encephalomyelitis (EAE) is an animal model that shares many similarities with multiple sclerosis. It is generally accepted that overactivation of CD4+ T cells, especially the Th1 and Th17 subpopulations, is the direct cause of this disease (3–6). In EAE, the integrity of the blood–brain barrier (BBB) is impaired, allowing perivascular infiltration of the pathogenic T cells into the CNS, which in turn leads to the infiltration and accumulation of other immune cells and the activation of glia cells and eventually causes demyelination, axonal damage, impaired nerve conduction, and paralysis (7–9).

The activation and differentiation of T cells is orchestrated by APCs. Dendritic cells (DCs) are the professional APCs that play critical roles in the pathogenesis of EAE. Immature DCs reside in peripheral tissues, particularly at sites of interface with the environment, such as skin and mucosae. Once encountering Ags, immature DCs capture them, process them into peptides, and then load the peptides onto MHC molecules. Meanwhile, signals from pathogens or inflammatory cytokines initiate the maturation process of the DCs and further enhance their Ag presentation abilities. Mature DCs carrying Ags then migrate out of the tissues to reach secondary lymphoid organs, where they stimulate the proliferation and differentiation of T cells by direct cell–cell contact and cytokine secretion and initiate the Ag-specific immune responses (10). Many signal transduction pathways have been implicated in the development of effector T cells. Among them, the JAK/STAT signaling pathway has been found to be critical for the differentiation and function of Th1 and Th17 cells (11, 12). STAT1 and STAT4, activated through JAK2 and TYK2 following IL-2 stimulation, are involved in Th1 differentiation (3, 4), whereas STAT3, activated by IL-6 and IL-23, is essential for Th17 differentiation (13). The loss of STAT3 in CD4+ T cells prevents development of experimental autoimmune diseases (5). Drugs targeting the JAK/STAT pathway have been used to treat EAE. For example, cyclooxygenase-2 inhibitors were found to ameliorate EAE by blocking tyrosine phosphorylation of JAK2, TYK2, STAT3, and STAT4 in T cells. Berberine was also reported to inhibit Th17 differentiation through direct actions on the JAK/STAT pathway (14, 15).

Aurintricarboxylic acid (ATA) is a potent inhibitor of protein–nucleic acid interactions and thus inhibits a number of enzymes involving DNA or RNA processing (16). It is also used to inhibit protein biosynthesis in its initial stages. As an ammonium salt (known as aluminon), it is used as a reagent to estimate the aluminum in water, biological tissue, and foods (17–19). It has also been discovered to block the binding of the HIV coat protein gp120 to the CD4 molecule (20–22). More recently, a report sug-
gusted that ATA might be able to inhibit cytokine-induced JAK/STAT signaling pathways (23). This leads us to speculate that ATA might affect T cell differentiation and alleviate EAE pathogenesis.

In this study, we found ATA significantly reduces the clinical symptoms of EAE. However, the mechanism study indicated that ATA does not directly affect Th1 or Th17 differentiation. Further study revealed that ATA blocks the chemotaxis of DCs and prevents their accumulation in the spleen, and it also blocks the infiltration of pathogenic T cells into the CNS. ATA was found to block the functions of many chemokine receptors. By blocking chemokine-mediated migration of DCs and pathogenic T cells, ATA alleviates the pathogenesis of EAE.

Materials and Methods

Reagents

Plasmids encoding CCR2, CCR4, CCR5, CCR6,CCR7, CCR9, CXCR4, CXCR5, CXCR6, sphingosine-1-phosphate receptor 1 (S1P1), β-oid receptor (DOR), κ opioid receptor (KOR), β2-adrenergic receptor (β2AR), and Gα16 were purchased from the Missouri S&T cDNA Resource Center. ATA, LPS, Hoechst 33342, forskolin, glucagon-like peptide 1 (GLP-1), and G16 were purchased from the Missouri S&T cDNA Resource Center. ATA, LPS, Hoechst 33342, forskolin, glucagon-like peptide 1 (GLP-1), U50488, and isoproterenol were purchased from Sigma-Aldrich. Anti-mouse CD3 (145-2C11), anti-mouse CD28 (37.51), and anti-mouse IFN-γ (R4-6A2) mAbs were purchased from BD Pharmingen. PE-Cy7-conjugated anti-mouse CD4 (RM4-4), -CD8α (53-6.7), -allopheohycyanin-conjugated anti-mouse CD8α (53-6.7), -allophycocyanin-conjugated anti-mouse IFN-γ (XM12.1), PE-conjugated anti-mouse CD11c (N418), and -mouse CD45 (30-F11) were obtained from eBioscience. Anti-myc and anti-hemagglutinin (HA) mAb was purchased from Cell Signaling Technology. Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 488 goat anti-rat IgG, and Fluoro-4 AM were obtained from Invitrogen. Recombinant MCP-1, TARC, RANTES, MIP-3α, CD40- and CD42b-conjugated anti-mouse CD11c (N418), and anti-mouse CD45 (30-F11) were obtained from eBioscience. Anti-myc and anti-hemagglutinin (HA) mAb was purchased from Cell Signaling Technology. Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 488 goat anti-rat IgG, and Fluoro-4 AM were obtained from Invitrogen. Recombinant MCP-1, TARC, RANTES, MIP-3α, MIP-3β, TECK, SDF-1, CXCL13, and CXCL16 were obtained from PeproTech. Recombinant murine (rm)IL-12, rmIL-6, recombinant human TGF-β1, rmIL-1β, rmIL-23, and rmTNF-α were purchased from R&D Systems.

EAE induction and treatment

Female C57BL/6 mice were obtained from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. Mice (8–10 wk age) were immunized s.c. with 200 μg myelin oligodendrocyte glycoprotein (MOG)35-55 (MEVGWYRSPFSRVVHVNG; obtained from GL Biochem) emulsified in CFA (Sigma-Aldrich), which contains 5 mg/ml Mycobacterium tuberculosis (Difco Laboratories). The mice received i.p. injections with 200 ng pertussis toxin (Calbiochem) on days 0 and 2. Disease severity was assigned scores on the following scale: 0, no symptoms; 1, tail weakness; 2, hind limb weakness; 3, paraplegia; 4, paraplegia with forelimb weakness or paralysis; 5, moribund or dead. For drug treatment, mice received i.p. injections of ATA (20 mg/kg) once daily from day 3 or day 12 until the end of the study, or from day 3 to day 12. PBS was given as a vehicle control (200 μl for each mouse).

For passive transfer of EAE, donor mice were primed by immunization with MOG35-55 in CFA. Ten days later, splenocytes were harvested and cultured at 5 × 10^6/ml in RPMI 1640 for 72 h with 25 μg/ml MOG35-55 and then CD4+ T cells were isolated. Recipient mice were sublethally irradiated and treated with ATA (20 mg/kg) or PBS 24 h before transfer of 3 × 10^6 CD4+ T cells via the i.v. route. After transfer, ATA or PBS was given once daily until the end of the experiment.

Histology and immunofluorescence analysis

Mice were anesthetized and perfused with PBS followed by 4% (w/v) paraformaldehyde. Spinal cord and brain were removed and fixed in 4% (w/v) paraformaldehyde overnight. Paraffin-embedded sections of spinal cord were stained with HE&E and Luxol fast blue for analysis of inflammation or demyelination, respectively. Frozen sections of spinal cord and brain were blocked for 30 min with 5% BSA and then incubated for 1 h each with rat anti-mouse CD45 (30-F11) and Alexa Fluor 488 goat anti-rat IgG diluted in 1% BSA at room temperature, with washing steps of PBS between incubations. Cells nuclei were stained with Hoechst 33342 for 10 min at room temperature. Sections were mounted in fluorescent mounting medium (Dako).

Isolation of CNS infiltrates

Mice were anesthetized and perfused through the left cardiac ventricle with PBS. Spinal cords and brains were isolated and homogenized in ice-cold tissue grinders 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 were subjected to flow cytometry.

Flow cytometry

For surface staining, cells were incubated with fluorescence-labeled surface Abs against CD4 or CD11c for 30 min at 4°C. For intracellular staining of cytokines, cells were stimulated for 5 h at 37°C with PMA (50 ng/ml), ionomycin (750 ng/ml), and brefeldin A (3 μg/ml) (all from Sigma-Aldrich) and then incubated with Abs against IL-17 and IFN-γ at 4°C. A guava easyCyte 8HT flow cytometry system and guavaSoft (Millipore) were used for the analysis.

T cell isolation and in vitro differentiation

Naive CD4+ T cells were isolated from spleens of female C57BL/6 mice using the Dynal mouse CD4 cell negative isolation kit (Invitrogen). Purified cells were stimulated with anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) and were induced to differentiate into Th1 cells by supplementation of rmIL-12 (10 ng/ml) and anti-IL-4 (10 μg/ml). For Th17 differentiation, cells received anti-IFN-γ (10 μg/ml), anti-IL-4 (10 μg/ml), recombinant human TGF-β1 (2 ng/ml), rmIL-6 (30 ng/ml), rmTNF-α (10 ng/ml), rmIL-23 (10 ng/ml), and IL-1β (10 ng/ml). ATA at various concentrations was added with the cytokine mixture to assess its influence on T cell differentiation.

DC isolation and DC–T cell coculture

Single-cell suspensions of splenocytes were prepared and then incubated with MACS CD11c-conjugated MicroBeads (Miltenyi Biotec), and DCs were positively selected with MACS LS separation columns (Miltenyi Biotec). Purified CD4+ T cells (1 × 10^5/well) isolated from untreated EAE mice were labeled with carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Invitrogen) and were cocultured with MACS-purified CD11c+ DCs (3 × 10^5/well) isolated from ATA- or vehicle-treated EAE mice in the presence of MOG peptide or not. In another set of experiments, DCs and CD4+ T cells were both isolated from untreated EAE mice and cocultured in the presence of ATA (10 μM). Three days later, the proliferation of CD4+ T cells was analyzed by flow cytometry, and the cytokines (IFN-γ, IL-17, IL-6, and TNF-α) in the supernatants were measured using ELISA kits (Dakewe Biotech, Shenzhen, China).

In vivo DC homing assay

DCs were isolated from the spleen of C57BL/6 mice via MACS sorting and labeled with CFSE. The labeled DCs were then injected via the lateral tail vein into recipient mice treated with ATA (20 mg/kg) or vehicle 24 h and 1 h before the cell transfer. The percentage and number of CFSE+ DCs in the spleen of recipient mice were analyzed by FACS 24 h later.

DC apoptosis assay

MACS-purified CD11c+ DCs (2 × 10^5/well) were incubated with ATA (0–30 μM) or camptothecin (0–30 μM) for 18 h. The cells were then stained with annexin V-FITC and propidium iodide (PI). The early (annexin V+, PI−) and late apoptotic cells (annexin V+, PI+) were detected by flow cytometry.

Chemotaxis assay

In vivo chemotaxis assays were conducted with splenocytes and DCs in Transwell cell culture chambers with 5-μm polycarbonate filter pores (Corning Costar). Splenocytes obtained from naive mice were stimulated with 10 μg/ml LPS (Sigma-Aldrich) for 48 h prior to the chemotaxis assay. Cells were resuspended to a density of 1.0 × 10^6/ml in RPMI 1640 supplemented with 0.5% BSA. Various chemokines were added into the lower chamber, and cells were added into the upper chamber. ATA was supplied in both upper and lower chambers. The chambers were then incubated for 5 h at normal culture condition, and the number of cells that migrated to the lower chamber was counted with flow cytometry.

Calcium mobilization assay

HEK293 or Chinese hamster ovary-K1 cells stably expressing Gα16 and various G protein–coupled receptors (GPCRs) were seeded onto 96-well plates and incubated for 24 h. Cells were loaded with 2 μM Fluo-4 AM in HBSS at 37°C for 45 min. After removal of excess dye, 50 μl HBSS containing antagonists was added. After incubation at room temperature for 10 min, 25 μl agonist of the receptor was dispensed into the wells using a FlexStation III microplate reader (Molecular Devices), and intracellular calcium was measured with a Calcium Indicator Kit (Molecular Devices).
FIGURE 1. ATA ameliorates the clinical signs of EAE. (A–C) Clinical scores of EAE mice treated with ATA or vehicle control once daily via i.p. injection from day 3 (A) or day 12 (C) until the end of the experiments, or from day 3 to day 12 (B). Data are expressed as means ± SEM (n = 6) and are representative of at least two independent experiments. *p < 0.05, **p < 0.001 (two-way ANOVA test). (D and E) H&E (D) and Luxol fast blue (E) staining of paraffin sections of spinal cords isolated from naive, vehicle, or ATA (20 mg/kg)-treated EAE mice from day 3 until the end of experiments. (F) Immunofluorescent staining of CD45+ cells in the frozen section of spinal cords isolated from naive or EAE mice treated with vehicle or ATA (20 mg/kg) from day 3 until the end of experiments. The boxed area in the middle of the image was enlarged at the lower left corner. Scale bars, 100 μm. (G–I) Quantitative analysis of the number of total infiltrates (G) in H&E-stained sections, the amount of demyelination (H) in Luxol fast blue–stained sections, and the number of CD45+ cells in immunofluorescent-stained sections presented in (D)–(F). Three mice from each group were sacrificed and 10 sections of each mouse were analyzed. Data are presented as means ± SEM. ***p < 0.001.

Results

ATA ameliorates the clinical and histological signs of EAE

Female C57BL/6 mice 8–9 wk age were immunized with MOG35–55 peptide, and ATA (10 or 20 mg/kg) or vehicle control (PBS with 0.4% DMSO) was administered i.p. once daily, starting from day 3 or day 12 until the end of the experiments or from day 3 to day 12 postimmunization. The effect of ATA on EAE development is shown in Fig. 1A–C and Table I. ATA displayed a dose-dependent inhibition of the disease severity (Fig. 1A). When given at 20 mg/kg, ATA significantly reduced the peak severity and cumulative clinical score of EAE (Fig. 1A, Table I). When given from day 3 to day 12, ATA also markedly decreased the severity of EAE at the onset and peak stage of the disease. However, the disease score started to increase after day 20, indicating that removing the drug was detrimental to the disease (Fig. 1B). More interestingly, when given after the onset of the disease (day 12 postimmunization; Fig. 1C, Table I), ATA was still able to reduce the severity of EAE, which indicates the therapeutic benefit of this drug in addition to the preventative effect.

<table>
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<th>Treatment Duration</th>
<th>Treatment Group</th>
<th>No. Sick/Total</th>
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<th>Mean Maximum Clinical Scorea</th>
<th>Cumulative Clinical Scorea</th>
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<td>0.47 ± 0.53***</td>
<td>1.87 ± 2.13***</td>
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<td>Day 3 to day 12</td>
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<td>ATA (20 mg/kg)</td>
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<td>1.27 ± 0.58</td>
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<tr>
<td>Day 12 to end</td>
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<td>ATA (20 mg/kg)</td>
<td>13/18</td>
<td>13.62 ± 2.85</td>
<td>1.1 ± 1.57***</td>
<td>7.46 ± 4.6*</td>
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*Values are means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, versus vehicle control (Student t test).

Receptor internalization assay

HEK293 cells expressing myc-CXCR4, enhanced GFP-CCR6, or HA-S1P1 were seeded onto coverslips. After overnight incubation, cells were pretreated with ATA for 10 min at 37°C and then stimulated with 30 nM ligand for 30 min in the presence of ATA. Cells were then fixed with 4% formaldehyde in PBS and permeabilized with 0.3% Triton X-100. After washing, the cells were stained with Abs against the myc or HA tags, followed by Alexa Fluor 488–conjugated secondary Ab. Fluorescent images were captured with an Olympus FV10i confocal microscope.

Statistical analysis

Data were analyzed with GraphPad Prism software (GraphPad Software). Nonlinear regression analyses were performed to generate dose-response curves and calculate IC50 values. Data are presented as means ± SEM. The statistical significance of the EAE clinical scores between treatments was analyzed with a two-way ANOVA test. EAE scores at a given date were analyzed by a Mann–Whitney U test. Other analyses were assessed by a Student t test. A p value <0.05 was considered statistically significant.
Histological analysis of the spinal cord was performed at day 21 postimmunization. Compared with vehicle, ATA treatment (20 mg/kg, day 3 to end of experiment) caused a significant decrease of leukocyte infiltration in the spinal cord (Fig. 1D, 1G). Luxol fast blue staining also revealed less extensive demyelination in ATA-treated EAE mice compared with the vehicle control group (Fig. 2).

**FIGURE 2.** ATA reduces the number of DCs and T effector cells in the spleen before the onset of the disease. (A) Spleens were isolated from EAE mice treated with vehicle or ATA (20 mg/kg, from day 3 to the end of experiments) at day 12 after immunization. The percentage of CD4+ T cells, CD11c+ DC cells, and Th1 (IFN-γ) and Th17 (IL-17) cells in the spleen were analyzed with flow cytometry. (B) Spleocytes isolated from ATA- or vehicle-treated EAE mice at day 12 after immunization were restimulated with MOG35-55 peptide for 48 h. Supernatants were analyzed for the indicated cytokines with ELISA. Data are means ± SEM (n = 6). *p < 0.05, **p < 0.01, ***p < 0.001, versus vehicle control. (C and D) Naive CD4+ T cells were purified and induced to differentiate into Th1 (C) or Th17 cells (D) in vitro in the presence of anti-CD3/CD28 Abs, various differentiation factors (DF), and indicated concentrations of ATA. Data are means ± SEM of three independent experiments. (E) Splenic DCs isolated from naive mice were incubated with ATA or camptothecin (1–30 μM) for 18 h, and apoptosis was analyzed by annexin V/PI staining. Data are representative of three independent experiments, each carried out in triplicate and presented as means ± SEM.

**FIGURE 3.** ATA blocks the chemotaxis but not other functions of DCs. (A and B) DCs were isolated, labeled with CFSE, and then injected i.v. into the ATA- or vehicle-treated recipient mice. The percentage and number of CFSE+ DCs were analyzed by FACS 24 h later. **p < 0.01. (C) DCs were isolated from EAE mice at day 12 after immunization and were induced to chemotaxis by CCL19 in the presence of 10 μM ATA or not. Data are means ± SEM of three independent experiments, each carried out in triplicate. #p < 0.01, versus no stimulation; ###p < 0.001, versus no ATA treatment. (D and E) CD4+ T cells isolated from EAE mice at day 12 after immunization were loaded with CFSE and cultured with CD11c+ DCs derived from ATA- or vehicle-treated EAE mice in the presence or absence of MOG35-55 (25 μg/ml), and the proliferation of T cells was examined by FACS (D). The supernatants of the cocultures were collected and IFN-γ, IL-17, IL-6, and TNF-α were measured with ELISA (E). (F) CD4+ T cells and CD11c+ DCs isolated from untreated EAE mice were cocultured in the presence of ATA (10 μM) and the proliferation of T cells was examined. (G–J) The supernatants of the aforementioned cocultures in (F) were collected and IFN-γ, IL-17, IL-6, and TNF-α were measured with ELISA. Data are means ± SEM from three independent experiments.
ATAs reduces the number of DCs and T effector cells in the spleen before the onset of disease

Activation, expansion, and differentiation of CD4+ T cells are thought to be prerequisites of EAE pathogenesis. Splenocytes were isolated from ATA- or vehicle-treated EAE mice right before the onset of the disease (day 12 postimmunization) and the percentages of CD4+ T cells, CD11c+ DCs, and Th1 and Th17 effector T cells were analyzed by flow cytometry. Compared with those of vehicle-treated EAE mice, the percentages of CD11c+ DCs and two major types of effector T cells in EAE (Th1 and Th17) were significantly decreased in ATA-treated EAE mice at day 12 (Fig. 2A). Consistent with this observation, cytokine production (including IFN-γ, IL-17, IL-6, and TNF-α) from splenocytes isolated from ATA-treated mice was also significantly reduced when restimulated with MOG35–55 peptide (25 μg/ml) (Fig. 2B).

To verify whether ATA directly affects Th1 or Th17 cell differentiation, naive CD4+ T cells were isolated and induced to differentiate under Th1- or Th17-polarizing conditions in the presence or absence of ATA in vitro. ATA did not seem to influence the differentiation of Th1 (Fig. 2C) or Th17 (Fig. 2D) cells at concentrations up to 30 μM in vitro. The development of Th cells is controlled largely by factors derived from APCs, such as DCs. Because ATA did not affect Th1 and Th17 differentiation in vitro, the reduction of these cells in the spleen of ATA-treated EAE mice was likely due to the reduced number of CD11c+ DCs (Fig. 2A). We then tested whether ATA induces apoptosis and thus reduces the number of DCs in vitro. As shown in Fig. 2E, camptothecin, a DNA topoisomerase I inhibitor, dose-dependently induced apoptosis in DCs. However, ATA did not have such an effect.

ATA blocks chemotaxis but not other functions of DCs

It is known that DCs capture Ags in peripheral tissues and process and load them onto MHC molecules. DCs then migrate into secondary lymphoid organs where they become competent to present Ags to naïve T cells, stimulating these cells to differentiate into Th1 or Th17 cells, and thus initiating Ag-specific immune responses (10). Because ATA did not induce apoptosis in DCs, it was likely blocking the accumulation of DCs in the secondary lymphoid tissues. In the in vivo DC homing assay, CFSE-labeled DCs were transferred into recipient mice via the i.v. route. ATA (20 mg/kg) treatment significantly inhibited the migration of DCs into the spleen of recipient animals (Fig. 3A, 3B). Using a Transwell assay, we studied the effect of ATA on the chemotaxis of DCs. CCR7 is highly expressed in mature DCs and is the major chemokine receptor that mediates DCs accumulation (24, 25). ATA (10 μM) was found to almost completely inhibit CCL19 (the ligand of CCR7)-induced chemotaxis of DCs isolated from the spleen of untreated EAE mice at day 12 postimmunization (Fig. 3C).

We then evaluated whether ATA could directly affect DC-stimulated CD4+ T cell growth and cytokine secretion. CD4+ T cells were isolated from vehicle-treated EAE mice, labeled with CFSE, and cocultured with CD11c+ DCs purified from ATA- or vehicle-treated EAE mice in the presence of MOG35–55 peptide (25 μg/ml) or not. Proliferation of CD4+ T cells (Fig. 3D) and cytokine production (Fig. 3E) were measured with FACS and ELISA, respectively. The function of DCs isolated from ATA-treated EAE mice did not differ from those isolated from vehicle-treated EAE mice. We then directly added ATA (10 μM) into the coculture of CFSE-labeled CD4+ T cells and DCs isolated from untreated EAE mice in the presence of MOG35–55 peptide, and the proliferation of CD4+ T cells (Fig. 3F) and the cytokine production was measured (Fig. 3G–J). Coculture of DCs and CD4+ T cells significantly enhanced the proliferation of CD4+ T cells and the production of cytokines. Restimulation with MOG further enhanced these phenomena. However, ATA did not affect CD4+ T cells growth or cytokine secretion even when directly added into the DC–T cell coculture system. Thus, ATA seems to exert its protective effect by blocking the chemotaxis and accumulation of DCs in the spleen and thus reduces DC-stimulated T cell activation and differentiation.

ATA prevents infiltration of pathogenic lymphocytes into the CNS

Because ATA blocks DC migration, it was natural to ask whether ATA could block T cell migration and infiltration into the CNS, another critical step in EAE pathogenesis. The CNS infiltrates were isolated and quantified by flow cytometry analysis at day 18 postimmunization. The absolute numbers of CD4+ T cells and the major pathogenic Th1 (IFN-γ+) and Th17 (IL-17+) cells were significantly decreased by ATA treatment (Fig. 4A). The choroid plexus forms part of the BBB that EAE-initiating T cells must pass through to induce the disease. It has been reported that the CCL20/CCR6-mediated infiltration of Th17 cells triggers the initiation of EAE (26). CD45+ cells in CCR6-deficient mice fail to cross the BBB but accumulate in the choroid plexus parenchyma, and thus lead to a less severe form of EAE (26). We therefore
checked whether ATA treatment also leads to the accumulation of CD45+ cells in the choroid plexus parenchyma. Indeed, as shown in Fig. 4B and 4C, the choroid plexus parenchyma of ATA-treated EAE mice had an increased accumulation of CD45+ cells (arrows) when compared with that of vehicle-treated EAE mice, indicating that ATA blocks the infiltration process.

To rule out the effect of ATA on DC-stimulated T cell development and to directly assess whether ATA could block pathogenic T cell infiltration, passive EAE was induced by adoptive transfer of MOG-restimulated CD4+ T cells isolated from MOG-immunized donor mice. Notably, ATA (20 mg/kg)-treated mice were completely resistant to the development of passive EAE (Fig. 5A). ATA was not toxic to T cells in vivo, as the percentage of CD4+ T cells and Th1 and Th17 cells in the circulating blood did not alter after ATA treatment (Fig. 5B). Histological analysis of the spinal cord was performed at day 18 after transfer. Compared with vehicle, ATA treatment led to a significant decrease of leukocyte infiltration (Fig. 5C, 5F) and less extensive demyelination (Fig. 5D, 5G) in the spinal cord compared with the vehicle control group. Immunofluorescent staining with anti-CD4 Ab also confirmed that ATA treatment reduced the number of CD4+ T cells in the spinal cord (Fig. 5E, 5H). Taken together, these data support the idea that ATA blocks the infiltration of pathogenic T cells into the CNS and thus ameliorate the severity of EAE.

ATA inhibits the function of many chemokine receptors but not other GPCRs

Chemokine receptors are GPCRs that play important roles in cell migration and infiltration, and they have also been implicated in the pathogenesis of EAE (27). Our data suggested that ATA blocks the movement of DCs and T cells, indicating it might exerts its effect via chemokine receptors. First, we tested the effect of ATA in CCL19/CCR7-, CCL20/CCR6-, or SDF-1/CXCR4-induced chemotaxis of splenocytes. ATA dose-dependently blocked the migration of splenocytes toward CCL19, CCL20, or SDF-1, with IC50s at 0.24, 3.36, and 11.46 μM, respectively (Fig. 6A, Table II). The S1P1 receptor plays critical role in mediating the egress of lymphocytes from secondary lymphoid tissues, but ATA did not seem to affect S1P1-mediated chemotaxis (Fig. 6A, Table II). We then tested whether ATA can block chemokine receptor–mediated calcium response in HEK293 or Chinese hamster ovary cells expressing various chemokine receptors (CCR2, CCR4, CCR5, CCR6, CCR7, CCR9, CXCR4, CXCR5, and CXCR6) and Gα16, a promiscuous G protein commonly used to couple GPCRs toward the calcium response pathway (28). Interestingly, ATA dose-dependently blocked the calcium response mediated by most of the chemokine receptors we tested, with the only exception being CCR2 (Fig 6B, Table II). However, for other receptors that we have tested, in-
including S1P1, DOR, KOR, GLP-1R, and β2AR. ATA did not show a significant blocking effect (Fig. 6B, Table II).

Receptor internalization is a common phenomenon of GPCR after ligand stimulation (29). To explore the effects of ATA on receptor internalization, HEK293 cells expressing tagged CXCR4, CCR6, CCR7, CXCR4, S1P1, KOR, or β2AR and Go16 were loaded with Fluo-4 AM, preincubated with various concentrations of ATA for 10 min, and then stimulated with proper ligands (30 nM). Calcium responses were recorded. Data are representative of three independent experiments, each carried out in triplicate and presented as means ± SEM. (C) Cells expressing tagged CXCR4, CCR6, or S1P1 were stimulated with proper ligands in the presence of ATA, and the localization of receptors was visualized with immunofluorescent staining. Arrowheads indicate the receptors on the membrane; arrows indicate the internalized receptors. Scale bar, 50 μm.

### Table II. ATA activity on GPCRs

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Calcium Assay</th>
<th>Chemotaxis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (μM)a</td>
<td>Max. Inhibition (% Control)b</td>
</tr>
<tr>
<td>CCR2</td>
<td>&gt;100</td>
<td>16.65 ± 16.03</td>
</tr>
<tr>
<td>CCR4</td>
<td>14.30 ± 4.24</td>
<td>100.01 ± 7.62</td>
</tr>
<tr>
<td>CCR5</td>
<td>3.49 ± 0.65</td>
<td>100.44 ± 1.47</td>
</tr>
<tr>
<td>CCR6</td>
<td>4.12 ± 0.40</td>
<td>99.82 ± 0.46</td>
</tr>
<tr>
<td>CCR7</td>
<td>2.46 ± 0.77</td>
<td>97.89 ± 3.77</td>
</tr>
<tr>
<td>CCR9</td>
<td>0.75 ± 0.20</td>
<td>98.99 ± 0.58</td>
</tr>
<tr>
<td>CXCR4</td>
<td>19.90 ± 6.00</td>
<td>97.41 ± 0.95</td>
</tr>
<tr>
<td>CXCR5</td>
<td>5.74 ± 0.96</td>
<td>98.71 ± 1.04</td>
</tr>
<tr>
<td>CXCR6</td>
<td>43.40 ± 1.90</td>
<td>98.11 ± 0.17</td>
</tr>
<tr>
<td>S1P1</td>
<td>&gt;100</td>
<td>-3.63 ± 2.64</td>
</tr>
<tr>
<td>GLP-1R</td>
<td>&gt;100</td>
<td>29.37 ± 16</td>
</tr>
<tr>
<td>KOR</td>
<td>&gt;100</td>
<td>-1.63 ± 5.41</td>
</tr>
<tr>
<td>DOR</td>
<td>&gt;100</td>
<td>-6.72 ± 3.90</td>
</tr>
<tr>
<td>β2AR</td>
<td>&gt;100</td>
<td>0.73 ± 5.40</td>
</tr>
</tbody>
</table>

aValues are reported as means ± SEM for three independent experiments. NA, Not available.
which in turn regulate diverse signal transduction pathways, including intracellular calcium, MAPKs, phospholipase Cβ, PI3K, and Ras and Rho GTPases (31). These signal transductions are thought to be responsible for cell movement beyond immune cell trafficking.

After Ag encounter in the peripheral tissues, immature DCs start to mature in the presence of pathogens or inflammatory cytokines. Ag carrying mature DCs must migrate out of the peripheral tissues to reach secondary lymphoid organs, where they stimulate the proliferation and differentiation of T cells (10, 32). During maturation, DCs undergo changes in their expression patterns of chemokine receptors. CCR7, which determines the accumulation of Ag-loaded mature DCs in T cell–rich areas, is upregulated (25–33–36). A recent study also suggested that CXCR4- and CCR7-mediated signaling might cooperatively regulate the migration of DCs into the splenic white pulp (37). Inhibiting the migration of DCs has been used to treat immune diseases. For example, cyclosporin A, a widely used immunosuppressant for organ transplantation, allergic disorders, autoimmune diseases, and acute inflammation (38), partially exerts its effects by blocking PGE₂ production from DCs in response to LPS, which in turn leads to the altered expression of chemokine receptors and impaired migratory capacity in DCs (39). From our results, although ATA nonselectively blocks many chemokine receptors, it is most effective in blocking CCL19/CCR7-mediated chemotaxis of DCs. This suggested that blocking homing of DCs might be an effective way to treat autoimmune diseases.

The infiltration of leukocytes into the CNS is another essential step in the neuropathogenesis of EAE, which is also critically regulated by chemokine receptors. Leukocyte extravasation from the bloodstream is a multistep process. An important step in this cascade is the binding of chemokines displayed on the vascular endothelial cell surface to chemokine receptors on circulating leukocytes, initiating intracellular signaling that leads to integrin activation, leukocyte arrest, and extravasation (40). Recent evidence indicated that the IL-17–expressing Th17 cells are the most important pathogenic T cells in EAE (41–43). Th17 cells preferentially express the chemokine receptors CCR6, and the ligand CCL20 is highly expressed in epithelial cells of the choroid plexus of healthy and EAE mice (26). Reboldi et al. (26) had reported that CCR6-Th17 cells, which entered the CNS through epithelial cells of the choroid plexus, are very important for the initiation of EAE. Knocking out CCR6 prevented Th17 cell infiltration and resulted in a diminished form of EAE (26). We also demonstrated that ATA blocks CCL20/CCR6-mediated chemotaxis of splenocytes and reduces the infiltration of pathogenic T cells into the CNS.

Many other chemokine receptors have also been reported in the pathogenesis of EAE. The fact that ATA is a nonselective inhibitor of many chemokine receptors might contribute to its therapeutic effect in EAE. Interestingly, ATA has been reported to inhibit HIV entry by blocking the binding of CD4 to the viral envelope glycoprotein gp120 (20, 44–46). It is well know that CCR5 and CXCR4 are coreceptors of HIV infection (47–50). Our data suggested that ATA might also prevent HIV entry by blocking CCR5 and CXCR4.

We initially wanted to test ATA on EAE because it has been reported to inhibit the JAK/STAT pathway, one of the major signaling pathways that regulates Th1 and Th17 cell differentiation (11, 12). Although very effective in ameliorating EAE, ATA was demonstrated to have no effect on T cell differentiation. Instead, it was found to inhibit the functions of many chemokine receptors and to block the homing of DCs and the infiltration of pathogenic T cells into CNS, and thus to alleviate EAE. The interesting question of why ATA blocks many chemokine receptors but not other GPCRs remains elusive. Because most chemokine receptors couple to Gα₁o, which is also the main G protein that mediates KOR and S1P1 function, it is obvious that ATA did not act at the G protein level. Thus, we think that ATA is an antagonist that directly binds to chemokine receptors and inhibits their interaction with chemokines. How does ATA nonselectively bind to chemokine receptors? We can only speculate that chemokine receptors might share certain structural similarities that allow the binding of ATA, and more structural insight will be needed to fully understand this phenomenon.

Disclosures
The authors have no financial conflicts of interest.

References


