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Compound A, a Plant Origin Ligand of Glucocorticoid Receptors, Increases Regulatory T Cells and M2 Macrophages to Attenuate Experimental Autoimmune Neuritis with Reduced Side Effects

Zhiren Zhang,1,2 Zhi-Yuan Zhang,1 and Hermann J. Schluesener

Experimental autoimmune neuritis (EAN) is a helper T cell-mediated autoimmune demyelinating inflammatory disease of the peripheral nervous system (PNS) that mirrors many clinical and immunological features of the human acute inflammatory demyelinating polyradiculoneuropathies (1). Patients with inflammatory polyneuropathies receive supportive management or active treatments, like plasma exchange and i.v. Ig (2). Although these treatments are beneficial to some of the patients, only around 65% patients with Guillain-Barre syndrome (GBS) respond to plasma exchange or i.v. Ig, for example (3). Approximately 8% of GBS patients die, and up to 20% remain disabled despite modern treatment. Even in those who recover well, residual weakness and loss of motor units can usually be detected and could explain the fatigue that is a common problem (4). Therefore, it will be necessary to establish more effective therapeutic strategies for inflammatory polyneuropathies.

EAN can be actively induced by immunization with autoantigen (purified myelin, P0, or P2 peptides). Pathologically, EAN is characterized by breakdown of the blood-nerve barrier, robust accumulation of reactive T cells and macrophages in the PNS and demyelination of peripheral nerves (3). Glia activation in spinal cords of EAN rats was observed as well and is considered to relate to pain hypersensitivity in EAN (5). Reactive lymphocytes and macrophages orchestrate a robust local inflammation that causes demyelination and axon degeneration and are essential for the development of EAN.

Th cells are important for the pathogenesis of EAN, as adoptive transfer of PNS autoantigen-specific CD4+ cells can produce EAN (3, 6). Th cell polarization following autoAg stimulation is essential for the determination of type and severity of autoimmune disorders (7). EAN is considered to be a disease dominated by a pathological cellular immune system that is mainly mediated by Th1 cells and Th1 cytokines, like IFN-γ or TNF-α (3). In addition, Th17 cells were believed to contribute to the development of EAN (9).

Macrophages represent the major cell population in the inflamed PNS, serve as Ag-presenting and major effector cells of demyelination, and are therefore responsible for most of the neuropathological effects (6, 10, 11). Macrophages can cause demyelination and axon loss with the help of autoAg Ab or complement components or result in tissue damage by secretion NO or inflammatory cytokines, like IL-1β and TNF-α (10). Depletion of macrophages
or inhibition of their activity during the presymptomatic phase has been shown to prevent all clinical, electrophysiological, and histological signs of EAN (12). So, macrophages are the major effector cells responsible for pathological changes of EAN. However, it is well established that recruited macrophages can differentiate into two distinct subsets that categorized as either classically activated (M1) or alternatively activated (M2). M1 macrophages induced by LPS or inflammatory cytokines, like IFN-γ, are characterized by high expression of IL-12, IL-23, TNF-α, inducible NO synthase (iNOS), etc., and are mainly considered to cause autoimmune tissue damage or host defense to infection. M2 macrophages induced by IL4, IL13, IL10, or TGF-β, highly express anti-inflammatory molecules, such as IL-10 and TGF-β, extracellular matrix molecules, like fibronectin, and scavenger receptors, which underlie their roles in anti-inflammation and tissue repair (13, 14). Therefore, a switch of macrophage phenotype from M1 to M2 during EAN could favor the outcome of EAN.

Steroidal glucocorticoids (GCs) represent the most effective and frequently used anti-inflammatory agents for a variety of inflammatory and both Th1- and Th2-mediated immune disorders, like rheumatoid arthritis, multiple sclerosis, asthma, and others. They inhibit both T cells and APCs, like macrophages, at the level of proliferation and cytokine production (15). However, long-term application of these drugs causes significant mortality and morbidity in patients due to their side effects, including osteoporosis, muscle wasting, hypertension, GC-mediated insulin resistance, truncal obesity and fat redistribution, and inhibition of wound repair (16). Both the beneficial and detrimental effects of GCs are mediated via the GC receptor (GR) that is a ligand-activated transcription factor. Following ligand binding, GR translocates into the nucleus and binds, directly or indirectly, to regulatory elements in promoter regions of genes, resulting in transactivation or transrepression. Transactivation is mainly mediated by direct binding of ligand-activated homodimerized GR onto inducible enhancer regions of genes, whereas many concomitant side effects are mainly the consequence of its transactivating activities of its direct binding onto GREs, resulting in direct transcription of target genes of which the proteins are mostly associated with well-known endocrine side effects of GC (16–20).

Compound A or 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride, is a stable analog of the hydroxy phenyl aziridine precursor found in the Namibian shrub Salsola tuberculiformis Botschantzev (21). Compound A has been identified as a GR ligand without the steroidal structure, like prednisolone (Fig. 1). Interestingly, Compound A was reported to be a selective GR ligand that had the anti-inflammatory effects but lacked transactivation potential, indicating that Compound A can greatly reduce inflammation with reduced side effects compared with GCs (22). Recently, it has been reported, that Compound A efficiently attenuates arthritis of mice with reduced side effects (23). In this study, we have investigated the therapeutic effects of Compound A in EAN and its underlying mechanisms.

Materials and Methods

Animals

Male Lewis rats (8–10 wk, 170–200 g, Charles River Laboratories) were housed under a 12 h light-12 h dark cycle with free access to food and water. All animal procedures were in accordance with a protocol approved by the local Administration District Official Committee. All efforts were made to minimize the number of animals and their suffering.

EAN induction and Compound A treatment

Rats were immunized by s.c. injection at the basal part of tails with 100 μl of an inoculum containing 100 μg of synthetic neurotogenic P2 57–81 peptides (GeneScript). Because EAN is characterized with ascending paraparesis/paralysis that can impair the ability to produce hindpaw withdrawal responses during mechanical allodynia measurement EAN rats were induced with reduced amount of P2 peptide (80 μg) to induce alleviated motor deficit for analysis of mechanical allodynia. The peptide was dissolved in PBS (2 mg/ml) and then emulsified with an equal volume of CFA containing 2 mg/ml mycobacterium tuberculosis to get a final concentration of 1 mg/ml.

Neurological signs of EAN were evaluated every day as follows: 0 = normal; 1 = reduced tonus of tail; 2 = limp tail, impaired righting; 3 = absent righting; 4 = gait ataxia; 5 = mild paresis of the hind limbs; 6 = moderate paraparesis; 7 = severe paraparesis or paraplegia of the hind limbs; 8 = tetraparesis; 9 = moribund; 10 = death.

For treatment, EAN rats received intraperitoneal injection of Compound A (0.8 mg/kg or 3.2 mg/kg, Alexis Biochemicals) daily from day 9 to 14 (six rats/group). Compound A was dissolved in PBS and the same volume of PBS was given as control. The dosaging of Compound A was comparable to treatment of arthritis in mice (23).

Mechanical allodynia

Mechanical allodynia was assessed by measuring rat hind-paw withdrawal threshold (HWT) using an automatic von Frey system, namely a mechanical plantar test apparatus (Ugo Basile). HWT was tested between 11:00 and 13:00 every day post immunization. Four days before the start of measurement, rats received training sessions every day. Rats were allowed to habituate to the environment of the test room for at least 10 min first and then placed into a Perspex enclosure over a mesh floor and allowed acclimating for another 10 min. The mechanical force, which went from 0 to 50 g over a period of 15 s, was then exerted onto the middle of the hind-paws using a fine metal filament. The force triggering the withdrawal reflex was recorded automatically. Left and right hind-paws were measured five times each, with a minimum 1-min interval between stimuli and the mean values were calculated. Twelve rats were immunized and grouped on day 9 when mechanical allodynia was well established. Then one group of rats was treated by Compound A (3.2 mg/kg) once daily and another group by PBS control (six rats/group). Compound A or PBS was given to rats after assessment of mechanical allodynia.

Immunohistochemistry

To evaluate inflammatory cell infiltration and pathological changes in the PNS and spinal cords, six Compound A-treated or control EAN rats were sacrificed at day 15. Rats were deeply anesthetized with ether and perfused intracardially with 4°C, 4% paraformaldehyde in PBS. Left and right sciatic nerves and lumbar spinal cords were quickly removed and postfixed in 4% paraformaldehyde overnight at 4°C. Tissues were cut into two equally long segments, embedded in paraffin, serially sectioned (3 μm), and mounted on silan-covered slides.

After dewaxing, cross-sections were boiled (in a 600 W microwave oven) for 15 min in citrate buffer (2.1 g sodium citrate/L, pH 6). Endogenous peroxidase was inhibited with 1% H2O2 in methanol for 15 min.

FIGURE 1. Chemical structures of Compound A and prednisolone.
Sections were incubated with 10% normal pig serum (Biochrom) to block nonspecific binding of immunoglobulins and then with the following mAbs: CD3 (1/50; Serotec) for T-lymphocytes, OKX22 (1/200; Serotec) for B cells, ED1 for activated macrophages or microglia (1/100; Serotec), ED2 for anti-inflammatory macrophages (1/100; Serotec), and glial fibrillary acidic protein (GFAP) for astrocytes (glial fibrillary acidic protein; 1/500; Chemicon International). Abs binding to tissue sections was visualized with a biotinylated IgG(Fab)2 secondary Ab fragment (DakoCytomation). Subsequently, sections were incubated with a TRP-conjugated streptavidin complex (DakoCytofication), followed by development with diaminobenzidine substrate (Fluka). Finally, sections were counterstained with Maier’s Hemalum.

The calculation of the percentages of areas of immunoreactivity to areas of sciatic nerve cross-sections was described previously (24). The density of GFAP immunoreactivity was manually counted. In brief, five cross-sections from each rat were examined by light microscopy after immunostaining. Positive cells were counted under ×100 magnification using Nikon Coolscope light microscopy (Nikon). Areas of sciatic nerve cross-sections were measured using software Meta Morph Offline 7.1 (Molecular Devices).

The routine Luxol Fast Blue (LFB) staining was applied to show myelin. Histological changes between Compound A and control EAN rats were compared by an established semiquantitative method. In brief, four cross-sections from root and middle level of both sides of sciatic nerves from EAN rats were analyzed. All perivascular areas present in cross-sections were evaluated by two observers unaware of treatment, and the degree of pathological alteration was graded semiquantitatively on the following scale: 0 = normal perivascular area; 1 = mild cellular infiltration adjacent to the vessel; 2 = cellular infiltration plus demyelination in immediate proximity to the vessel; and 3 = cellular infiltration and demyelination throughout the section. Results were given as mean histological score (25).

**Tissue preparation, RNA isolation, and reverse transcription**

Following treatment, EAN rats were perfused intracardially with 4°C PBS under anesthesia and then the sciatic nerves, lumbar spinal cords, inguinal lymph nodes, and liver were quickly removed and stored in liquid nitrogen under anesthesia and then the sciatic nerves, lumbar spinal cords, inguinal lymph nodes, and liver were quickly removed and stored in liquid nitrogen until RNA isolation. Total RNA was isolated using TRIzol LS Reagent (Invitrogen) and reverse transcribed into cDNA using Quantitect Reverse Transcription Kit (Qiagen).

**Semi quantitative RT-PCR**

cDNA equivalent to 20 ng of total RNA was subjected to subsequent semiquantitative PCR analysis using following primers: PEPCK (sense, AAC TGT TGG CTG CTC GT; antisense, GAA CCT GCC GTT GAA TGC), TAT (sense, GTC TAG CTG TGG CCA AT; antisense, GTC GCC TTC CTT AGG GT), IL-4 (sense, TGA TGG GCC ATC GT; antisense, TTC CAT AGA GCA ATG ACT CC), IL-1β (sense, TGG TAT GTG ATT TGC TAC AT; antisense, TGC CAT AGA GCA ATG ACT CC). In preliminary experiments, optimal cycling conditions were established allowing amplification of each cDNA in the linear range. PCR products were separated on 1.5% agarose gels containing 10 μg/ml ethidium bromide, photographed using the UV-solo system (Whatman Biometra) and densitometric analysis was performed with the software BioDocAnalyze (Whatman Biometra). Results were calculated as levels of target mRNAs relative to those of the housekeeping gene β-actin (three samples from each group were analyzed by PCR).

**Real-time quantitative RT-PCR**

The resulting cDNA was used to measure quantitatively the expression of genes using SYBR green qPCR master mix according to the manufacturer’s protocol (Bio-Rad). Real-time measurements of gene expression were performed with a Molecular Dynamics 480 thermal cycler and 2% agarose gel containing 1 μg/ml ethidium bromide. The cDNA was amplified using the following primers:

- IL-1β (sense, TGG TAT GTG ATT TGC TAC AT; antisense, TGC CAT AGA GCA ATG ACT CC), IL-4 (sense, TGA TGG GCC ATC GT; antisense, TTC CAT AGA GCA ATG ACT CC), IL-6 (sense, GCC CTC CAG GAA CAG CTA TG; antisense, CAG AAT TGC CAT TGA ACA AC), TNF-α (sense, TGG TAT GTG ATT TGC TAC AT; antisense, CAG AAT TGC CAT TGA ACA AC), TGF-β (sense, TGG TAT GTG ATT TGC TAC AT; antisense, CAG AAT TGC CAT TGA ACA AC), IFN-γ (sense, AAA GAC AAC GAC GCC ATC AG; antisense, CTT TTC CTG TTC AGG CT; antisense, TGG TAT GTG ATT TGC TAC AT), and β-actin (sense, TGG TAT GTG ATT TGC TAC AT; antisense, CTT TTC CTG TTC AGG CT; antisense, TGG TAT GTG ATT TGC TAC AT).

Analysis was performed with the software BioDocAnalyze (Whatman Biometra). Results were calculated as levels of target mRNAs relative to those of the housekeeping gene β-actin (three samples from each group were analyzed by PCR).

**Flow cytometric analysis of Foxp3+ T regulatory cells in blood**

T regulatory cells were identified as Foxp3+CD4+ cells. Blood was drawn intracardially from anesthetized rats and 100-μl blood was incubated with RPE-labeled mouse anti-CD4 Ab (1/10, Serotec) for 30 min at room temperature and then fixed with ERYTHROLYSE (Serotec) according to the manufacturer’s instructions. After washing, cells were permeabilized using the eBioScience Foxp3 Staining Buffer Set (eBioScience). FITC-labeled Foxp3 Ab (eBioScience) was used for staining according to the protocol recommended by eBioScience. For all staining, isotype controls were used. Following staining, cells were suspended in PBS and analyzed by FACSscan (BD Biosciences). Mononuclear cells were gated by forward and sideward scatter.

**Immunofluorescence analysis**

In brief, 105 RAW 264.7 macrophages in 0.4 ml RPMI 1640 were seeded onto eight-well chamber slides (Lab-Tek Chamber Slide, Nalgene Nunc) and cultured overnight. To observe the effects of Compound A on cellular localization of GR, RAW 264.7 macrophages were serum-starved in phenol red-free medium for 24 h and then treated with PBS, prednisolone (1 μM), or Compound A (10 μM) for 1 h. The immunostaining of GR was performed as described by using the anti-GR Ab (1/100, Affinity Bio-Reagent) as the primary Ab and the IgG-F(ab′)2 fragment conjugated to FITC (1/100, Abcam) as the secondary Ab. Cells were mounted in a mounting medium containing 4’,6-diamidino-2-phenylindole (Vector Laboratories), and then observed by using fluorescence microscopy. All experiments were performed in triplicates.

**Evaluation and statistical analysis**

The unpaired t test or Mann-Whitney U test was performed to compare difference between Compound A and control EAN rats (Graph Pad Prism 4.0 for windows). For all statistical analyses, significance levels were set at p < 0.05.
Results

Compound A treatment ameliorated neurological signs and neuropathic pain in EAN

EAN was induced by s.c. injection of 100 μg neuritogenic synthetic P2 peptide. Compound A or PBS (control group) was injected once daily from day 9 to 14. For PBS treatment, the first neurologic sign of EAN rats was observed on day 9 (0.167 ± 0.0.167); the neurologic severity of EAN reached maximum on day 15 (5.5 ± 0.8) and disappeared by day 20 (0 ± 0). Low-dosage Compound A treatment (0.8 mg/kg) only slightly attenuated EAN severity (Fig. 2A) compared with PBS. However, treatment with high-dosage of Compound A (3.2 mg/kg) greatly decreased neurologic severity (p < 0.05 compared with PBS control) and shortened duration of EAN (day 9 to Day 17 for Compound A-treated EAN rats and day 9 to 19 for control EAN rats) (Fig. 2A).

Neuropathic pain is a common symptom of demylinating inflammatory polyneuropathies. In this study, we examined the influence of Compound A on neuropathic pain development in EAN. Twelve rats were immunized with a reduced dose of neuritogenic synthetic P2 peptide to avoid severe motor deficit and grouped on day 9 when mechanical allodynia was well established. One group was treated by Compound A (3.2 mg/kg) once daily and another group was treated with PBS as control. Mechanical allodynia was recorded as a significant reduction of HWT in EAN rats compared with individual baselines that were obtained as mean HWT for the first 4 days following immunization. As shown in Fig. 2B, mechanical allodynia was established by day 7. Compound A or PBS was given from day 9. For the PBS-treated group, HWT decreased smoothly, indicating the steady progression of mechanical allodynia. However, for the Compound A-treated group, HWT decreased sluggishly but steadily, suggesting a slower progression of mechanical allodynia. Already after the first injection, significant differences of HWT values between the Compound A and PBS group were found and remained until the end of the observation, day 14. So, Compound A greatly slowed but did not inhibit or reverse the progression of established EAN mechanical allodynia.

Effects of Compound A treatment on histological changes, inflammatory cell accumulation, and expression of inflammation-related molecules in sciatic nerves of EAN rats

EAN rats were therapeutic-treated by Compound A or PBS as described and sacrificed on day 15 to take sciatic nerves for histological analysis (n = 4). LFB staining was used to show myelin and cell infiltration. As shown in Fig. 3A, obvious perivascular demyelination and inflammatory cell infiltration was seen in sciatic nerves of control EAN rats. Compound A significantly decreased the incidence of perivascular demyelination and inflammatory cell infiltration (Fig. 3A). Histological changes between Compound A-treated and PBS-treated EAN rats were further compared by an established semiquantitative method. In sciatic nerves, the mean histological scores were marked lower in Compound A group (0.76 ± 0.06) than control group (1.5 ± 0.05) (Fig. 3A).

Attenuation of different types of inflammatory cell infiltration in sciatic nerves of day 15 EAN rats following therapeutic Compound A
treatment was further characterized by immunohistochemistry. In PBS control EAN rats, infiltration of T cells (CD3), B cells (OX22), and macrophages (ED1) was observed and the most dominant cells were macrophages (Fig. 3A). Therapeutic Compound A treatment significantly suppressed infiltration of T cells (p < 0.05), B cells (p < 0.05), and macrophages (p < 0.05) in sciatic nerves (Fig. 3A).

It is known that inflammatory cytokines, like IL-1β, IL-6, IFN-γ, and IL-17, or iNOS have disease-promoting roles in EAN. Therefore, we further analyzed effects of Compound A on the mRNA level of certain inflammatory-related molecules in sciatic nerves of Compound A-treated day 15 EAN rats by real-time PCR. As shown in Fig. 3A, mRNA expression of IL-1β, IL-6, IL-12p35, IFN-γ, and iNOS in sciatic nerves of EAN rats was greatly reduced by Compound A, compared with their respective PBS controls.

Effects of Compound A treatment on glia activation and expression of inflammatory-related molecules in spinal cords of EAN rats

Although the major pathological findings of EAN are in the PNS, activation of glia (microglia and astrocytes) in spinal cords of EAN rats are known and related to mechanical allodynia (5). Lumbar spinal cords of day 15 EAN rats following therapeutic Compound A treatment were taken for immunohistochemistry. Reactive microglia were detected by ED1 immunostaining. In PBS-treated EAN rats, ED1+ cells were mainly detected in gray matter, particularly in the superficial layers of dorsal horns (Fig. 3B). Following therapeutic Compound A treatment, ED1+ spinal cells were rarely seen (Fig. 3B). Reactive astrocytes were identified by enlarged cell bodies, thicker process, and higher expression levels of GFAP. Reactive astrocytes were seen in lumbar dorsal horns of PBS-treated EAN rats (data not shown). Therapeutic Compound A treatment did not significantly alter numbers of activated, GFAP+ astrocytes (Fig. 3B). Further semiquantitative real-time PCR showed that mRNA levels of GFAP in lumbar spinal cords of EAN rats were not decreased following therapeutic Compound A treatment as compared with PBS controls (Fig. 3B). Therefore, therapeutic Compound A treatment could attenuate microglia, but not astrocyte activation in lumbar spinal cords of EAN rats.

Furthermore, mRNA expression of several pathological pain associated inflammatory cytokines was measured in lumbar spinal cords of day 15 EAN rats that were therapeutically treated with Compound A and PBS controls. As shown in Fig. 3B, mRNA expression of IL-1β and TNF-α but not IL-6 and GFAP was reduced in lumbar spinal cords following therapeutic Compound A treatment in comparison to PBS in EAN rats. *p < 0.05; **p < 0.01 compared with their respective PBS control.
Compound A by real-time PCR. As shown in Fig. 3B, mRNA expression of IL-1β and TNF-α but not of IL-6 was greatly attenuated by Compound A.

Compound A favored M2 macrophage polarization in EAN rats and in vitro

As M2 macrophages can attenuate inflammation and promote tissue repair, we analyzed whether Compound A could favor M2 polarization in sciatic nerves of EAN rats. M2 macrophages are identified by expression of CD163 that is detected by ED2 Ab. Representative microphotos show that a significant increase of ED2⁺ cells in sciatic nerves of EAN rats which was induced by Compound A (B) (in comparison to PBS (A)). Numbers of ED2⁺ cells in sciatic nerves of Compound A-treated EAN rats were significantly higher than in PBS-treated EAN rats (C, * p < 0.05 compared with their respective PBS control). D–F, Effects of Compound A on macrophage differentiation were further analyzed in vitro using murine macrophage cell line RAW 264.7 cells, which were grown in complete RPMI 1640 medium containing penicillin, streptomycin, and 10% FCS at 37°C and 5% CO₂. 10⁵ cells were seeded into 12-well cell culture plates and cultured for 24 h. Afterward, cells were stimulated with LPS for 24 h and then incubated with prednisolone (1 μM) or Compound A (5 μM) for 24 h. D, Thereafter, cells were harvested and centrifuged, and supernatants collected for the analysis of NO concentrations by a standard Griess assay. *, p < 0.05; **, p < 0.01 compared with LPS alone group. E, Total RNA from cultured cells was prepared and mRNA levels of TNF-α, iNOS, IL-1β and IL-10 was measured by real-time PCR. *, p < 0.05; **, p < 0.01 compared with their respective LPS alone group. F, Cell viability was analyzed by MTT assay.
TNF-α, iNOS, and IL-1β, but increased mRNA expression of IL-10, suggesting a polarization from M1 to M2 macrophages. Furthermore, cell viability of LPS and Compound A-treated cells was analyzed by MTT assay and no significant differences were seen among these groups (Fig. 4F). Therefore, we suggest that Compound A could promote M2 polarization at least in vitro and possibly in vivo as well.

**Compound A polarized a Th2 and regulatory T cell cytokine profile in lymph nodes of EAN rats**

Th cell polarization following autoAg stimulation is a central process defining the nature and severity of autoimmune disorders. Th1 cells produce IFN-γ and promote cellular immunity, which is considered to be important for EAN progression. In contrast, Th2 effector cells produce IL-4, IL-5, and IL-13 and promote humoral immunity, which can inhibit the polarization of Th1 cells. The recently identified Th17 cells produce IL-17 and are considered to participate into the progression of EAN. The lymph node is a most important place for T cell activation, differentiation, and proliferation and, therefore, we have analyzed lymph node cytokine profile at Day 15 EAN rats that were therapeutically treated by Compound A. As shown in Fig. 5A, Compound A significantly reduced the mRNA level of IFN-γ and IL-17 but increased the mRNA level of IL-4, suggesting Th2 but not Th1 and Th17 polarization of helper T cell differentiation.

As regulatory T cells are important in immune homeostasis and lymph nodes are crucial for the production of regulatory T cells, we further analyzed mRNA expression of regulatory cytokines IL-10 and TGF-β, and its unique transcription factor Foxp3 in lymph nodes of day 15 EAN rats that were therapeutic treated by Compound A. Interestingly, mRNA level of Foxp3 in lymph nodes was greatly induced following Compound A treatment. However, only IL-10, but not TGF-β mRNA level, was increased by Compound A treatment (Fig. 5A).
Compound A increased Foxp3+ regulatory T cells but decreased helper T cells in peripheral blood of EAN rats

As mRNA level of Foxp3 increased in lymph nodes following Compound A treatment, we further analyzed effects of Compound A on regulatory T cells in the peripheral blood at day 15 EAN rats that were therapeutically treated by Compound A. Regulatory T cells were identified to be Foxp3+/CD4+ in peripheral blood of Compound A-treated EAN rats, percentages of Foxp3+/CD4+ cells was significantly higher than in the PBS control group (Fig. 5B, p < 0.05). Furthermore, percentages of helper T cells (CD4+ cells) in peripheral blood were decreased by Compound A as compared with PBS (Fig. 5B, p < 0.01).

Compound A induced GR nuclear translocation but did not induce GRE-driven genes and hyperglycemia in EAN rats

GR is a ligand-activated transcription factor. In the absence of a ligand, GR is localized in the cytoplasm as a protein complex together with other chaperone molecules. Upon ligand binding, the complex dissociates and GR translocates into the nucleus and binds to regulatory elements in promoter regions of GC-responsive genes, resulting in a modulated gene transcription (28). Therefore, we analyzed GR protein subcellular localization in RAW 264.7 macrophages following Compound A treatment. As shown in Fig. 6A, upon induction with Compound A or prednisolone (a steroid ligand and positive control), GR translocated from the cytoplasm to the nucleus, indicating the activation of GR.

It is reported that Compound A is a selective GR modifier, that has the anti-inflammatory effects but lacks transactivation potential (22). Therefore, GRE-driven gene transcription was analyzed in livers of Compound A- or prednisolone-treated EAN rats. EAN rats were therapeutically treated by Compound A (3.2 mg/kg), prednisolone (10 mg/kg), or PBS and the mRNA levels of tyrosine aminotransferase (TAT) and phosphoenolpyruvate carboxykinase (PEPCK) were semiquantified by RT-PCR. TAT is the first enzyme in the catabolic pathway of tyrosine and one of the key gluconeogenic enzymes, predominantly expressed in hepatic parenchyma cells. The enzyme activity is directly correlated with the rate of TAT gene transcription (29). PEPCK is essential in glucose homeostasis and catalyzes the reversible rate-controlling step of gluconeogenesis, i.e., the conversion of oxaloacetate to phosphoenolpyruvate (30). PEPCK activity is principally controlled at the level of gene expression and is sensitive to a number of hormones (31). Both TAT and PEPCK gene contain GREs and are transactivated following GR activation (16). As shown in Fig. 6B, higher mRNA levels of TAT were induced following prednisolone treatment in liver of EAN rats compared with PBS. But mRNA levels of TAT and PEPCK in liver of EAN rats following Compound A treatment were significantly lower than of the prednisolone control group. *p < 0.05 compared with Compound A group. C, Compound A did not increase plasma glucose level in EAN rats. EAN rats were treated by Compound A (3.2 mg/kg), prednisolone (10 mg/kg), or PBS from day 9 to 14 and sacrificed at day 15. The plasma was taken for glucose measurement. *, p < 0.05; **, p < 0.01 compared with prednisolone group.

Discussion

EAN is the prime animal model for inflammatory demyelinating polyneuropathies and useful in investigating new therapeutic approaches. In this study, we have studied the therapeutic effects of Compound A on EAN. Our findings demonstrate that Compound A greatly attenuated inflammation in the peripheral nerves and spinal cords of EAN rats, and reduced paraparesis and neuropathic pain in EAN. Compound A treatment favored the macrophage switch to an anti-inflammatory M2 type in EAN and in vitro. Furthermore, in lymph nodes, Compound A attenuated Th1 and Th17
cytokines but increased Th2 and regulatory T cell cytokines. In peripheral blood, Compound A increased regulatory T cells. In addition, Compound A treatment did not induce GRE-driven genes and hyperglycemia in EAN rats compared with prednisolone.

In this investigation, therapeutic Compound A treatment significantly improved EAN outcome and suppressed accumulation of immune cells and inflammatory molecules in peripheral nerves of EAN. Pathological development of EAN is characterized by the infiltration of reactive leukocytes into the PNS (32). Activated autoreactive helper T cells are of importance for the initiation of EAN (3). Activated macrophages cause demyelination by direct phagocytic attack and secretion of inflammatory mediators (10–12). In peripheral nerves of EAN rats, cytokines are produced and released by many cell types and regulate inflammation and immunity. Proinflammatory cytokines, like IFN-γ, IL-1β, and IL-17, have disease-promoting roles in EAN and their expression was attenuated by Compound A. IFN-γ augments both inflammation and subsequent immune responses in EAN by activation of macrophages to release oxygen radicals, promoting T cell and macrophage homing to the PNS, enhancing blood-nerve barrier permeability, and inducing MHC class II expression on macrophages and cultured Schwann cells. IL-1β is considered to participate in the initiation of autoimmune response in EAN. IL-17 is produced by Th17 cells and stimulates production of IL-6, NO, and prostaglandin E2 to amplify local inflammation; mediates chemotaxis of neutrophils and monocytes to sites of inflammation; and augments the induction of costimulatory molecules such as ICAM-1 to support T cell activation (33, 34). IL-12 is important for Th cell differentiation into Th1 phenotype and production of IFN-γ by Th1 cells (35). Similar to inflammatory cytokines, iNOS is also important for the pathogenesis of EAN. iNOS functions to produce NO, which possesses proinflammatory properties, including vasodilatation, edema formation, and cytotoxicity and mediates cytokine-dependent processes that can result in tissue destruction (36). In EAN, up-regulation of iNOS was reported to be particularly related to pathogenesis of PNS cell-mediated demyelination and even to axonal damage (37). Therefore, Compound A attenuated accumulation of inflammatory cells and expression of inflammatory-related molecules in PNS could reduce local inflammation and demyelination to favor EAN outcome.

Neuropathic pain, caused by lesion or inflammation of the nervous system, is a common symptom of human chronic inflammatory demyelinating polyneuropathies (38). In our study, therapy by Compound A after the establishment of mechanical allodynia greatly slow but could not reverse the progression of mechanical allodynia. In addition, suppression of microglia but not astrocyte activation, and attenuation of TNF-α and IL-1β, but not IL-6 expression, in lumbar spinal cord was observed. In the CNS, particularly in spinal cords, glia activation plays an essential role in the mediation of neuropathic pain. In spinal cord, microglia and astrocytes are activated in response to peripheral nerve injury to secrete proinflammatory cytokines, like IL-6, TNF-α, and IL-1β, which contribute to central sensitization of neuropathic pain (39, 40). In EAN, the major pathological changes are seen in PNS but spinal glia activation and expression of inflammatory molecules have been described and are due to peripheral nerve injury and nonspecific infiltration of reactive T cells and macrophages (5, 41). In spinal cords, both microglia and astrocytes contribute to the development of neuropathic pain in a variety of models, however their roles are different (42). Reactive microglia are considered to be important for initiation of neuropathic pain but astrocytes for maintenance and progression of neuropathic pain (43). We observed that Compound A reduced microglia activation but did not attenuate astrocyte activation, which may partly explain why Compound A could not reverse established neuropathic pain in EAN. Furthermore, the mRNA level of IL-6 in spinal cords of EAN were not significantly changed after Compound A treatment, which may contribute to the persistence of mechanical allodynia in EAN as well.

Helper T cell differentiation, which can differentiate from naïve T cells by different cytokines and by the induction of independent gene programs, is the central process defining the nature of the developing immune responses (44). Th1 cells produce large quantities of IFN-γ, whereas Th2 cells produce IL-4, IL-5, and IL-13. Th1 cells elicit delayed-type hypersensitivity responses, activate macrophages, and are highly effective in clearing intracellular pathogens or causing tissue damage. Th2 cells, in contrast, are particularly important for the production of IgE and eosinophilic inflammation and may suppress cell-mediated immunity (45). Th17 are a distinct Th cell subset, mainly producing IL-17 and IL-17F, and they have an important role in host defense against infection, by recruiting neutrophils and macrophages, and are also key players in autoimmune and inflammatory diseases (34). EAN is mainly considered to be a Th1-mediated autoimmune disorder (3) and recently it has been shown that Th17 cells participate in the progression of EAN (8). We observed that Compound A reduced IFN-γ and IL-17 but increase IL-4 mRNA levels in lymph nodes of EAN rats, indicating that Compound A could inhibit Th1 and Th17 polarization to promote Th2 differentiation to suppress autoimmune response in EAN, favoring a positive outcome.

One interesting finding of our investigation is that the anti-inflammatory effects of Compound A are associated with the induction of Foxp3+ regulatory T cells. Compound A significantly increased Foxp3 expression in lymph nodes and Foxp3+CD4+ cells in blood of EAN rats. The transcription factor Foxp3 is accepted as a specific marker for regulatory T cells and to be essential to their development and function (46). Regulatory T cells suppress the activation of the immune system and thereby prevent excessive inflammation and/or autoimmunity, resulting in peripheral tolerance and immune homeostasis (47). Evidence has accumulated that regulatory T cells can attenuate innate and adaptive immune responses in experimental animal models of autoimmunity, including arthritis, colitis, diabetes, encephalomyelitis, lupus, gastritis, oophoritis, prostatitis, and thyroiditis (48). In EAN, our previous data have shown that Foxp3+ cells may contribute to the termination of autoimmune neuritis (9). Therefore, Compound A induced increase of Foxp3+ regulatory T cells in peripheral blood could alleviate inflammation in EAN. However, regulatory T cells are not an homogeneous cell type and other regulatory T cell populations have been described (49). In our study, Compound A treatment only increased IL-10, but not TGF-β expression, which points to the IL-10-producing, type 1 regulatory T cells that are characterized by high production of IL-10, variable levels of TGF-β and inducible expression of Foxp3. Type 1 regulatory T cells mainly exert their suppressive activity through IL-10 and can be induced by immunosuppressive drugs, like dexamethasone (50). Although Compound A increased numbers of Foxp3+ regulatory T cells, the inhibitory activity of these induced regulatory T cells are not clear. However, Chen et al. (51) reported that dexamethasone, a classic glucocorticoid receptor ligand, expanded Foxp3+ regulatory T cells and remained their immune suppressive functions, suggesting the induced Foxp3+ regulatory T cells by the novel glucocorticoid receptor ligand Compound A in EAN may still maintain their inhibitory ability.

Another interesting feature of our finding is that the anti-inflammatory effects of Compound A are associated with the polarization toward M2 type macrophages. Compound A treatment significantly increased numbers of M2 macrophages (ED2+ cells)
in sciatic nerves of EAN rats. Further, Compound A switched the M1 phenotype of macrophages to a M2 type in vitro. Similarly to helper T cells, resident macrophages or recruited monocytes differentiate into two distinct subsets in response to cytokines. M1 macrophages mainly function to promote inflammation and tissue damage. On the contrary, M2 macrophages exert an anti-inflammatory and tissue repair effects (13, 14). In our in vitro study showed that Compound A had direct effects on macrophage differentiation. Treating LPS-induced M1 macrophages with Compound A significantly reduced mRNA of inflammatory molecules and induced anti-inflammatory cytokine IL-10 mRNA. Therefore, in EAN Compound A could directly and indirectly polarize M2 macrophage differentiation to favor EAN outcome.

Our study here and findings from other groups showed that Compound A could induce GR nuclear translocation, suggesting that Compound A is a GR ligand (22, 23). At present, GR ligands are still the most effective drugs for inflammatory disorders. However, their application is limited by their side effects associated with chronic use (17). In our study, mRNA level of hepatic enzymes PEPCK and TAT, which are important for gluconeogenesis, in Compound A-treated EAN rats was much lower than prednisolone-treated EAN rats. It has been reported that Compound A could bind to and inhibit dimerization of GR (23). It is widely hypothesized that the desired anti-inflammatory effects of GCs are mainly caused by the interaction of GR as a monomer with transcription factors that drive proinflammatory gene expression, including NF-κB, whereas dimerization of GR is involved in the direct binding onto GRE, resulting in direct transcription of target genes of which the proteins are mostly associated with the well-known endocrine side effects of GC (17). The promoter of hepatic gluconeogenic enzymes PEPCK and TAT contain regulatory sequences enabling activation by GCs. Following GCs application, PEPCK and TAT are up-regulated and the de novo synthesis of glucose is increased, resulting into hyperglycemia (52, 53). Therefore, application of Compound A in EAN rats did not induce transcription of GC transacted genes and so may induce less side effects, like hyperglycemia.

In summary, we have studied the effects of Compound A in EAN, an animal model of human inflammatory demyelinating polyneuropathies. Our data demonstrate that Compound A greatly attenuated inflammation in the peripheral nerves and spinal cords of EAN rats, and reduced paraparesis and neuropathic pain in EAN rats. Further, Application of Compound A attenuated Th1 and Th17 cytokines, of EAN rats, and reduced paraparesis and neuropathic pain in attenuated inflammation in the peripheral nerves and spinal cords polyneuropathies. Our data demonstrate that Compound A greatly dissocated compound of plant origin for inflammatory gene repression.

Disclosures
The authors have no financial conflict of interest.

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