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Research Article

Valproic acid attenuates inflammation in experimental autoimmune neuritis

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Abstract. Valproic acid (VPA) is a short-chain branched fatty with anti-inflammatory, neuro-protective and axon-remodeling effects. We investigated the effects of VPA in rats in which experimental autoimmune neuritis (EAN) had been induced (EAN rats). VPA (300 mg/kg, intraperitoneally) administration to EAN rats once daily immediately following immunization significantly suppressed mRNA levels of interferon-γ, tumor necrosis factor-α, interleukin (IL)-1β, IL-4, IL-6 and IL-17 in the lymph nodes of EAN rats. In peripheral blood and sciatic nerves of EAN rats, $Foxp3^+$ cells were increased but $IL\text{-}17^+$ cells were decreased during VPA treatment. Furthermore, suppressive and therapeutic treatment with VPA greatly attenuated both accumulation of macrophages, T cells and B cells, and demyelination in sciatic nerves, and greatly reduced the severity and duration of EAN. In summary, our data demonstrated that VPA could effectively suppress inflammation in EAN, suggesting that VPA could be a potent candidate for treatment of autoimmune neuropathies.

Keywords. Experimental autoimmune neuritis, valproic acid, T helper type 17 cells, regulatory T cells, Guillain-Barré syndrome.

Introduction

Experimental autoimmune neuritis (EAN) is a T cell-mediated inflammatory demyelinating disease of the peripheral nervous system (PNS) that mirrors many clinical and immunological features of the human acute inflammatory demyelinating polyradiculoneuropathy (AIDP) subtype of Guillain-Barré syndrome (GBS). Therefore, EAN has been applied widely as a model to investigate therapy and disease mechanisms of AIDP, which is the most common subtype of GBS [1]. Treatment of GBS can be divided into supportive management and active treatment, like plasma exchange or intravenous immunoglobulin [2]. While these treatments are beneficial to patients, only around 65% GBS patients respond to plasma exchange or intravenous immunoglobulin [3]. About 8% of GBS patients die, and up to 20% remain disabled. 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, new therapeutic options are needed.

EAN is characterized by breakdown of the blood-nerve barrier (BNB), robust accumulation of reactive T cells and macrophages in the PNS and demyelination of peripheral nerves. Helper T lymphocytes are important for the initiation of EAN, as adoptive transfer of PNS autoantigen-specific $CD4^+$ cells can

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induce EAN [3, 5]. T helper cell polarization following autoantigen stimulation is essential for the determination of type and severity in autoimmune disorders. EAN is considered to be a disease dominated by a pathological cellular immune system. In particular, Th1 cells and Th1 cytokines, like interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α), are important for the pathogenesis of EAN [3, 6]. However, humoral immune responses mediated by Th2 cells also contribute to the pathogenesis of GBS and EAN [5]. Recently, interleukin-17 (IL-17), which is mainly produced by Th17 cells, was found in EAN-affected sciatic nerves congregating around blood vessels, and interestingly this accumulation was temporally correlated with severity of neurological signs, suggesting a pathological contribution of IL-17$^+$ cells to the development of EAN (submitted data). In addition, in sciatic nerves of rats in which EAN had been induced (EAN rats), accumulation of foxhead box protein3$^+$ (Foxp3$^+$) T cells was not seen during the presymptomatic phase (until day 9) or during early or peak disease activity. In contrast, Foxp3$^+$ cell accumulation was regularly seen in the recovery from neurological disease, suggesting a contribution of Foxp3$^+$ cells to the resolution of EAN (submitted data). Macrophages represent the major cell population in the inflamed PNS, serve as antigen-presenting cells and major effector cells of demyelination and are therefore responsible for most of the neuropathological effects [5, 7]. In the PNS, reactive T cells, macrophages and B cells orchestrate a robust local inflammation that causes demyelination and axonal degeneration. Therefore, therapeutic compounds that can inhibit PNS inflammation would improve EAN outcome.

Valproic acid (VPA) is a short-chain branched fatty acid and can be easily delivered into the organism and cells [8]. It is an established drug in the treatment of epileptic seizures and bipolar disorder [9]. Accumulated experimental and clinical data also show that VPA could be a potent anticancer drug [10]. Further, VPA has neuroprotective and axonal remodeling [9] and anti-inflammatory effects. In a model of ischemia, VPA treatment significant decreased brain infarct volume, suppressed microglial activation, reduced the number of microglia, and inhibited other inflammatory markers in the ischemic brain [11]. *In vitro*, VPA greatly attenuated the secretion of TNF-α and IFN-γ from reactive helper T cells and monocytes [12, 13]. Furthermore, oral VPA treatment reduced disease severity in dextran sulfate-induced colitis. The macroscopic and histological reduction of disease severity was associated with a marked suppression of colonic proinflammatory cytokines, like IFN-γ and IL-6 [14]. In addition, VPA is a well-known anticonvulsive agent and its pharmacokinetics and toxicity have been well tested [15]. The chemical properties of VPA allow easy delivery to the organism and cells [8]. Thus, the proven reliability and safety of VPA suggest that it may present an effective treatment of various neurological conditions. We have investigated the therapeutic effects of VPA in EAN.

Material and methods

Animals. Male Lewis rats (8–10 weeks, 170–200 g, Charles River, Sulzfeld, Germany) 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 VPA treatment. EAN was induced as described [16]. Briefly, rats were immunized by subcutaneous injection into both hind footpads with 100 µl of an inoculum containing 100 µg synthetic neuritogenic P257–81 (GeneScript Corporation, Scotch Plains, NJ, USA). The peptide was dissolved in phosphate-buffered saline (PBS) (2 mg/ml) and then emulsified with an equal volume of complete Freund's adjuvant (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; and 10, death. For suppressive treatment, VPA (Sigma-Aldrich Chemie, Munich, Germany) was intraperitoneally injected once daily from day 0 to day 20 (six rats/group). For therapeutic treatment, six EAN rats received an intraperitoneal injection of VPA daily from day 10 to day 21 (six rats/group). During both treatments, 300 mg/kg VPA in 1 ml PBS was administered; controls received the same volume of PBS.

Immunohistochemistry. To evaluate inflammatory cell infiltration and pathological changes in the PNS and spinal cords, four VPA-treated and control EAN rats from day 16 of suppressive or therapeutic treatment were killed. Rats were deeply anesthetized with ether and perfused intracardially with cold (4°C), 4% paraformaldehyde in PBS. Left and right sciatic nerves and lumbar spinal cords were quickly removed and post-fixed in 4% paraformaldehyde overnight at 4°C. Sciatic nerves were cut into two equally long

segments, embedded in paraffin, serially sectioned (3 μm) and mounted on silane-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 % H_2O_2 in methanol for 15 min. Sections were incubated with 10 % normal pig serum (Biochrom, Berlin, Germany) to block nonspecific binding of immunoglobulins, and then with the following monoclonal antibodies: CD3 (1:50; Serotec, Oxford, UK) for T lymphocytes, OX22 (1:200; Serotec) for B cells, ED1 for activated macrophages or microglia (1:100; Serotec), IL-17 (1:100; Santa Cruz, Heidelberg, Germany) or Foxp3 (1:250; eBioscience, Frankfurt, Germany). Antibody binding to tissue sections was visualized with a biotinylated IgG $F(ab)_2$ secondary antibody fragment (DAKO, Hamburg, Germany). Subsequently, sections were incubated with a horseradish peroxidase-conjugated streptavidin complex (DAKO), followed by development with diaminobenzidine (DAB) substrate (Fluka, Neu-Ulm, Germany). Finally, sections were counterstained with Maier's hemalum.

To evaluate immunostaining data, the percentages of areas of immunoreactivity (IR) to areas of sciatic nerve cross-sections were calculated. Briefly, images of sciatic nerve cross-sections were captured under 50× magnification using Nikon Coolscope (Nikon, Düsseldorf, Germany) with fixed parameters. Images were analyzed using Image-Pro Plus (IPP; Media Cybernetics, MD, USA). Areas of IR were selected by color threshold segmentation and all parameters were fixed for all images. Areas of sciatic nerve cross-sections were manually selected. For each EAN rat, four cross-sections from root and middle levels of both sides were analyzed. Results were given as arithmetic means of percentages of areas of IR to areas of sciatic nerve cross-sections and standard errors of means (SEM).

The routine Luxol Fast Blue (LFB) staining was applied to show myelin. Histological changes between VPA-treated and control EAN rats were compared by an established semi-quantitative method. Briefly, four cross-sections from root and middle level of both sides of 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 semi-quantitatively on the following scale: 0, normal perivascular area; 1, mild cellular infiltrate adjacent to the vessel; 2, cellular infiltration plus demyelination in immediate proximity to the vessel; or 3, cellular infiltration and demyelination throughout the section. Results were given as mean histological score [17].

Flow cytometric analysis of IL-17$^+$ cells and Foxp3$^+$ cells in blood. For intracellular IL-17 detection, blood was drawn intracardially from anaesthetized rats and 100 μl blood was fixed and lysed with ERYTHRO-LYSE (Serotec) according to the manufacturer's instructions and permeabilized with 0.5 % saponin in wash buffer for 10 min at room temperature. Thereafter, cells were incubated with rabbit anti-IL-17 antibody (1:100, Santa Cruz) for 1 h at room temperature. After two washings with wash buffer, detection was performed by incubation with a FITC-labeled goat anti rabbit IgG-$F(ab)_2$ fragment (1:100, Abcam, Cambridge, UK) for 1 h at room temperature in the dark.

For detection of Foxp3 expression, fixation and permeabilization of blood cells were performed using the eBioscience Foxp3 Staining Buffer Set (eBioscience). FITC-labeled Foxp3 antibody (eBioscience) was used for staining according to the manufacturer's protocol.

For all staining, isotype controls were used. Following staining, cells were suspended in PBS and analyzed by a FACScan (Becton Dickinson, Ueberlingen, Germany). Mononuclear cells were gated by forward and sideward scatters.

Semi-quantitative RT-PCR analysis of cytokine expression in lymph nodes. Rats were perfused intracardially with 4°C PBS under anesthesia and the inguinal lymph nodes were quickly removed and stored in liquid nitrogen until RNA isolation. Total RNA was isolated from the lymph nodes using TRIzol LS Reagent (Invitrogen, Karlsruhe, Germany) and reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). cDNA equivalent to 20 ng total RNA was subjected to subsequent semi-quantified PCR analysis using primers specific for IL-1β, TNF-α, IL-6, IFN-γ, IL-4, IL-17, transforming growth factor-β (TGF-β), or the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). The primer sequences were: IL-1β (sense, TGC TGA TGT ACC AGT TGG GG; antisense, CTC CAT GAG CTT TGT ACA AG), TNF-α (sense, TGA TCG GTC CCA ACA AGG A; antisense, TGC TTG GTG GTT TGC TAC GA), IL-6 (sense, GCC CTT CAG GAA CAG CTA TG; antisense, CAG AAT TGC CAT TGC ACA AC), IFN-γ (sense, AAA GAC AAC CAG GCC ATC AG; antisense, CTT TTC CGC TTC CTT AGG CT), IL-4 (sense, TGA TGG GTC TCA GCC CCC ACC TTG C; antisense, CTT TCA GTG TTG TGA GCG TGG ACT C), IL-17 (sense, TGG ACT CTG AGC CGC ATT GA; antisense, GAC GCA TGG CGG ACA ATA GA), TGF-β (sense, TGA ACC AAG GAG ACG GAA TAC AGG; antisense, TAC TGT

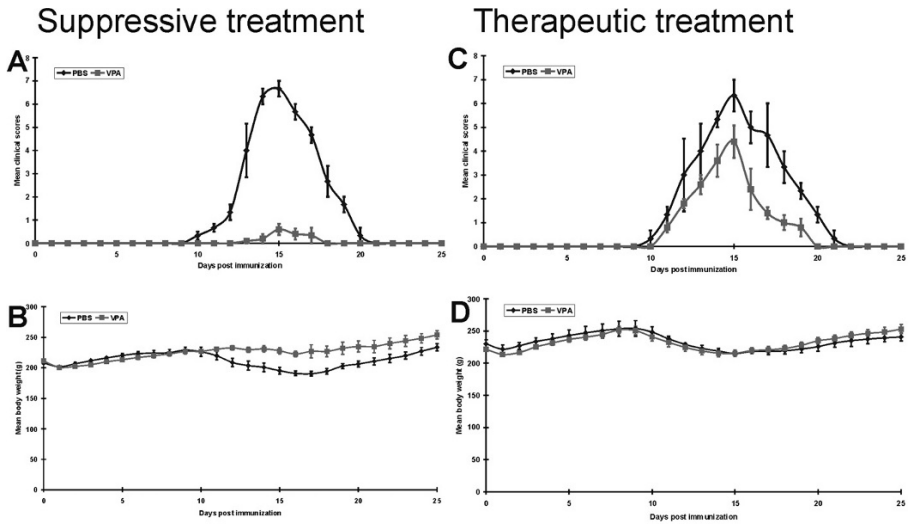

Figure 1. Suppressive and therapeutic valproic acid (VPA) treatment attenuates neurological severity and body weight loss of rats with induced experimental autoimmune neuritis (EAN rats). EAN rats (n=6) were intraperitoneally injected once daily with VPA (300 mg/kg in 1 ml PBS) or PBS (1 ml). For suppressive treatment, VPA was given from days 0 to 20. For therapeutic treatment, VPA was given from days 10 to 21. Clinical scores and body weight were measured every second day post immunization. (*A*) Suppressive VPA treatment significantly delayed onset, decreased neurological severity and shortened duration of EAN. (*B*) Suppressive VPA treatment also significantly reduced EAN induced weight loss. (*C*) Therapeutic VPA treatment decreased neurological severity of EAN and shortened EAN duration. (*D*) Therapeutic VPA treatment did not prevent EAN induced weight loss.

GTG TCC AGG CTC CAA ATG), and GAPDH (sense, AGG TCA CCC AGA GCT GAA CG; antisense, CAC CCT GTT GCT GTA GCC GTA T). 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 UVsolo system (Whatman Biometra, Goettingen, Germany) and densitometric analysis was performed with the software BioDocAnalyze (Whatman Biometra). Results were calculated as levels of target mRNAs relative to those of GAPDH (three samples from each group were analyzed by PCR).

Evaluation and statistical analysis. The unpaired t-test was performed to compare difference between VPA-treated and control EAN rats (Graph Pad Prism 4.0 for windows). For all statistical analyses, significance levels were set at $p<0.05$.

Results

Suppressive and therapeutic VPA treatment ameliorated EAN. EAN was induced by subcutaneous injection of neuritogenic synthetic P2 peptide. For suppressive treatment, VPA or PBS (control group) was injected immediately after immunization and then once daily until day 20. The first neurological sign (reduced tonus of tail) of control EAN rats was observed at day 10 (mean clinical score: 0.33±0.17). The neurological severity of EAN increased fast in the control group with a maximal score at day 15 (mean neurological score: 6.67±0.33). Thereafter, the severity of EAN slowly decreased and rats were fully recovered by day 21 (mean clinical score: 0±0). In VPA-treated EAN rats, first neurological sign were seen at day 14 (mean clinical score: 0.2±0.2), with maximal scores at day 15 (mean clinical score: 0.6±0.24) and full recovery of rats by day 18. Therefore, suppressive VPA treatment greatly delayed onset, decreased neurological severity and shortened duration of EAN (Fig. 1A).

A further feature of EAN is progressive weight loss [18]. In control and VPA-treated EAN rats, a rapid weight loss was observed immediately following immunization (day 1), which was followed by slow weight gain. Control EAN rats suffered weight loss during the period of neurological disease from day 10 to 17 post immunization, followed by slow weight gain (Fig. 1B). In contrast, no significant weight loss was apparent in EAN rats treated with VPA (Fig. 1B) during the period of neurological disease, indicating a much less severe course.

For therapeutic treatment, VPA or PBS was injected once daily from the onset to the disappearance of neurological signs (days 10–21 post immunization). As shown in Figure 1C, VPA therapeutic treatment suppressed severity of EAN from days 14 to 20 ($p<0.05$ compared to respective control) and allowed faster recovery of the rats (days 10–19 for VPA-

treated EAN rats and days 10–21 for control EAN rats). However, therapeutic VPA-treatment did not attenuate the loss of body weight in EAN rats (Fig. 1D).

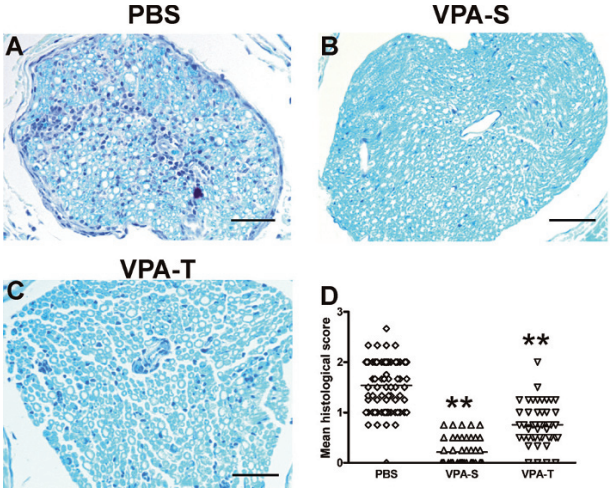

Figure 2. Suppressive and therapeutic VPA treatment suppressed histopathological alterations in sciatic nerves of EAN rats. EAN rats (n=4) were intraperitoneally injected once daily with VPA (300 mg/kg) or PBS. For suppressive treatment, VPA was given from days 0 to 15. For therapeutic treatment, VPA was given from days 10 to 15. Rats were killed on day 16 and sciatic nerves were taken for Luxol Fast Blue (LFB) staining with hematoxylin counterstaining. Representative microphotos of PBS control (*A*), suppressive VPA- (*B*) or therapeutic VPA-treated (*C*) EAN rats are shown. Scale bars for (*A, B*) are 50 μm. (*D*) Mean histological scores were calculated as described in Materials and methods. Suppressive and therapeutic VPA treatment significantly reduced mean histological scores compared to control EAN rats. ** $p<0.01$, VPA-S: suppressive VPA treatment; VPA-T: therapeutic VPA treatment.

Suppressive and therapeutic VPA treatment reduced demyelination and inflammatory cell infiltration in sciatic nerves of EAN rats. EAN rats were suppressively or therapeutically treated with VPA or PBS as described and killed on day 16 to remove sciatic nerves for histological analysis (n=4). LFB staining was used to show myelin and cell infiltration. As shown in Figure 2A, obvious perivascular demyelination and inflammatory cell infiltration was seen in sciatic nerves of control EAN rats. Suppressive VPA treatment significantly decreased the incidence of perivascular demyelination and inflammatory cell infiltration (Fig. 2B). Histological changes in sciatic nerves of control EAN rats treated by a therapeutic regimen with PBS were similar to controls of the suppressive treatment (data not shown). However, therapeutic VPA treatment greatly reduced perivascular demyelination and only a much-reduced accumulation of inflammatory cells was seen in sciatic nerves (Fig. 2C). Histological changes between VPA-treated and PBS-treated control EAN rats were further compared by an established semi-quantitative method. In sciatic nerves, the mean histological scores were markedly lower in suppressive (0.2±0.04) or therapeutic (0.76±0.06) VPA-treated than in control EAN rats (1.5±0.05) (Fig. 2D).

Attenuation of different types of inflammatory cell infiltration in sciatic nerves of day 16 EAN rats following suppressive and therapeutic VPA 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–C). Both suppressive and therapeutic VPA treatment significantly suppressed accumulation of T cell ($p<0.05$), B cell ($p<0.01$) and macrophage ($p<0.01$) infiltration (Fig. 3).

Suppressive and therapeutic VPA treatment inhibited spinal microglia activation in EAN. While the major pathological findings of EAN are in the PNS, activation and proliferation of microglia in spinal cords of EAN rats are known and related to mechanical allodynia. Reactive microglia were detected by ED1 immunostaining, which recognizes CD68, a lysosomal membrane protein. Lumbar spinal cords of day 16 EAN rats following suppressive and therapeutic VPA treatment were taken for immunohistochemistry. In PBS-treated day 16 EAN rats, $ED1^+$ cells were seen, mainly detected in gray matter, particularly in the superficial layers of dorsal horns (Fig. 4A). Following suppressive and therapeutic VPA treatment, spinal $ED1^+$ cells were rarely seen (Fig. 4B, C).

VPA altered lymph node cytokine expression profile in EAN rats. Immune responses are orchestrated by cytokines whose interplay decides the outcome of the immune responses [19]. To understand the underlying mechanisms of VPA effects in EAN, we investigated the lymph node cytokine expression profile at the mRNA level. EAN rats were suppressively treated with VPA and mRNA levels of Th1 (IL-1β, IFN-γ and TNF-α), Th2 (IL-4 and IL6, Th17, like IL-17), and T regulatory (TGF-β) cytokines were detected in lymph nodes on day 16. As shown in Figure 5, suppressive VPA treatment significantly reduced mRNA levels of IL-1β, IFN-γ, TNF-α, IL-4, IL-17 and IL-6 ($p<0.05$) but had no significant effects on TGF-β expression.

VPA decreased IL-17$^+$ and increased Foxp3$^+$ cell numbers in peripheral blood and sciatic nerves of EAN rats. IL-17 plays essential roles in a variety of autoimmune diseases, and regulatory T (Treg) cells that express Foxp3 are important in immune homeostasis. Therefore, we further analyzed effects of VPA

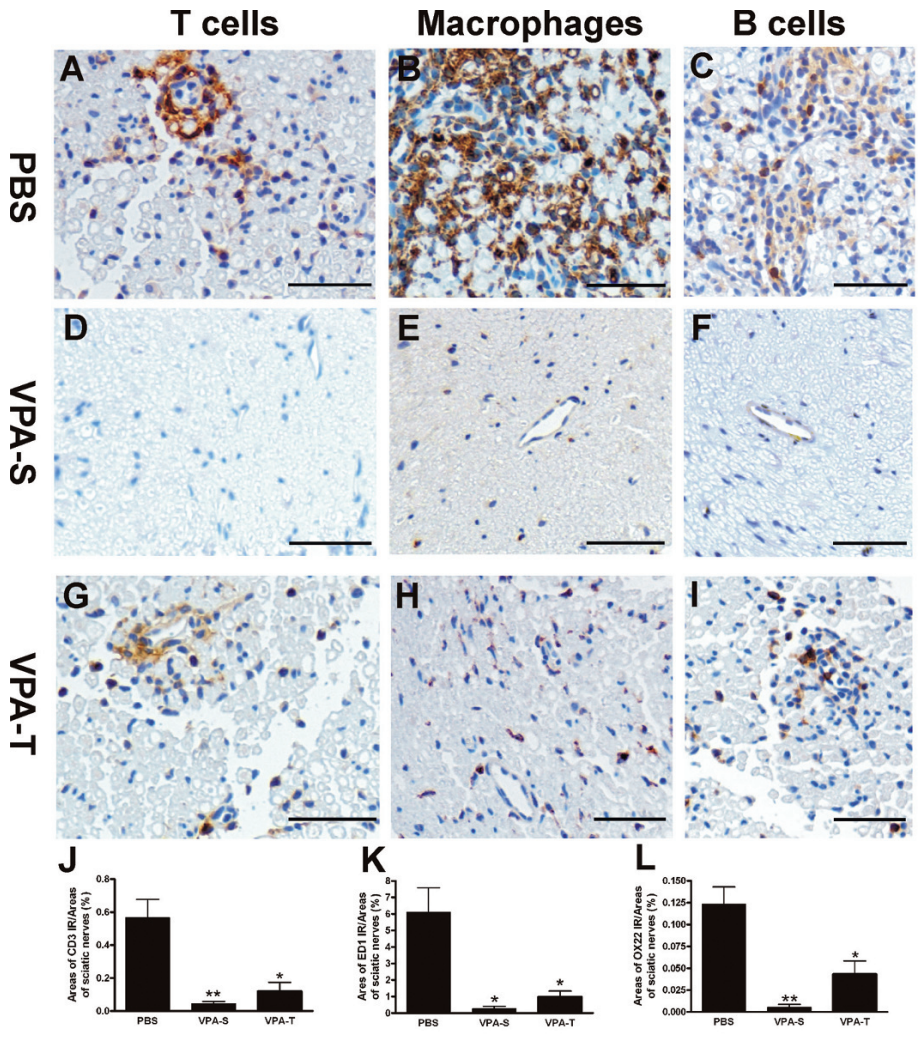

Figure 3. Suppressive and therapeutic VPA treatment inhibited T cell, B cell and macrophage infiltration into sciatic nerves of EAN rats. EAN rats (n=4) were intraperitoneally injected once daily with VPA (300 mg/kg) or PBS. For suppressive treatment, VPA was given from days 0 to 15. For therapeutic treatment, VPA was given from days 10 to 15. Rats were killed on day 16 and sciatic nerves were taken for immunohistochemical staining. (*A, D, G*) CD3 immunostaining was applied to detect T cells; (*B, E, H*) ED1 for reactive macrophages; and (C, F, I) OX22 for B cells. Representative microphotos of peripheral nerves from control (A–C), suppressive VPA- (*D–F*) or therapeutic VPA-treated (*G–I*) EAN rats are shown. Scale bar 50 µm. (*J–L*) Infiltration of T cells, macrophages and B cells in sciatic nerves was further semi-quantified as indicated in Materials and methods. The unpaired t-test was performed to compare the differences between VPA-treated and control EAN rats. Suppressive and therapeutic VPA treatment significantly reduced infiltration of T cells, macrophages and B cells to sciatic nerves. * $p<0.05$ and ** $p<0.01$ compared to PBS, VPA-S: suppressive VPA treatment, VPA-T: therapeutic VPA treatment.

on IL-17$^+$ and Foxp3$^+$ cells in EAN rats that were suppressively treated with VPA until day 16 and then killed. In peripheral blood, percentages of Foxp3$^+$ cells (Fig. 6A) increased, but percentages of IL-17$^+$ cells were reduced (Fig. 6B) following VPA treatment ($p<0.05$). Similarly, in sciatic nerves VPA treatment reduced accumulation of IL-17$^+$ cells (Fig. 6E, F), but increased infiltration of Foxp3$^+$ cells (Fig. 6C, D).

Discussion

EAN is the prime animal model for GBS and useful in investigating new therapeutic approaches. We have studied the therapeutic effects of VPA on EAN. Our findings demonstrate that VPA greatly reduced the severity and duration of EAN, and attenuated inflammation and demyelination in the peripheral nerves when injected either immediately after immunization or after the appearance of the first neurological signs. VPA treatment of EAN decreased IL-17$^+$ cells but increased Foxp3$^+$ cells in peripheral blood and sciatic nerves. Furthermore, mRNA expression of a variety of pro-inflammatory cytokines in lymph nodes of EAN rats was reduced by VPA.

Suppressive and therapeutic VPA treatment significantly improved EAN outcome and suppressed accumulation of T cells, B cells and macrophages into peripheral nerves. Pathologically, development of EAN is characterized by the infiltration of reactive leukocytes into the PNS [20]. Activated autoreactive helper T cells, which can recognize peripheral nerve autoantigens on antigen-presenting cells, are of importance for the initiation of EAN. Following activation in lymph nodes, autoreactive T cells attach to the venular endothelium of the PNS, penetrate the BNB and generate an autoimmune reaction within the PNS that orchestrates the invasion of more lymphocytes and monocytes and local inflammation and development of edema. Activated macrophages cause demyelination by direct phagocytic attack and secretion of inflammatory mediators. Depletion of macrophag-

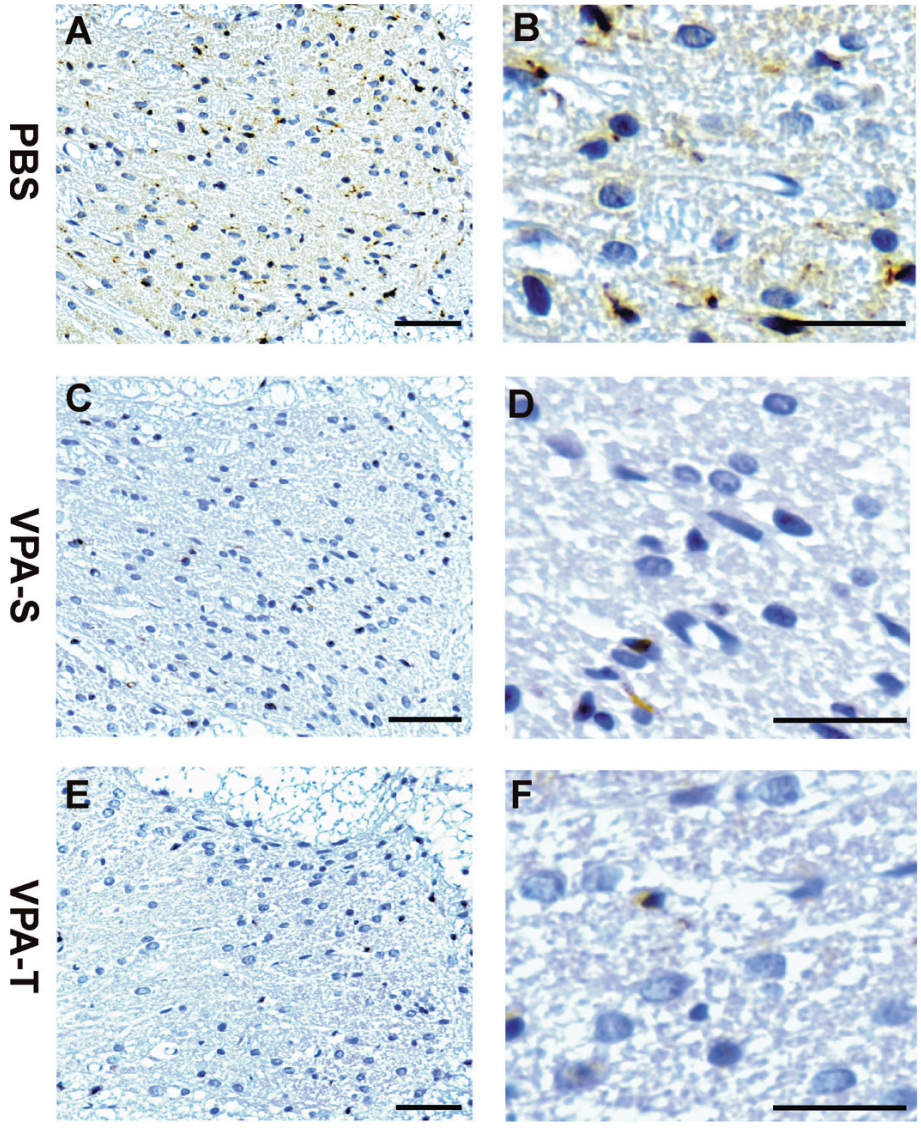

Figure 4. Suppressive and therapeutic VPA treatment attenuated microglia activation in lumbar spinal cords of EAN rats. EAN rats (n=4) were intraperitoneally injected once daily with VPA (300 mg/kg) or PBS. For suppressive treatment, VPA was given from days 0 to 15. For therapeutic treatment, VPA was given from days 10 to 15. Rats were killed on day 16 and lumbar spinal cords were taken for immunohistochemical staining. ED1 immunostaining was used for detection of reactive microglia. Representative microphotos of lumbar spinal dorsal horns for control (*A, B*), suppressive VPA- (*C, D*) or therapeutic VPA-treated (*E, F*) EAN rats are shown. Scale bars (*A, C, E*) 50 μm, and (*B, D, F*) 25 μm. VPA-S: suppressive VPA treatment, VPA-T: therapeutic VPA treatment.

es and inhibition of their activity have been shown to suppress the development of EAN [7]. Therefore, VPA reduced accumulation of inflammatory cells in PNS, attenuating local inflammation and demyelination to favor EAN outcome.

In lymph nodes of EAN rats, VPA treatment suppressed mRNA expression of a variety of cytokines that are important in regulating inflammation and immunity. Pro-inflammatory cytokines, like IFN-γ, TNF-α, IL-1β, IL-6 and IL-17, have disease-promoting roles in EAN and their expression was attenuated by VPA. 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 BNB permeability, inducing MHC class II expression on macrophages and cultured Schwann cells. TNF-α is mainly produced by macrophages, T cells and Schwann cells in EAN and can activate macrophages and Schwann cells and increase BNB permeability to contribute to EAN development. IL-1β is considered to participate in the initiation of autoimmune response in EAN. IL-6 is one of the major mediators of the immune response. The effects of IL-6 in EAN include B cell stimulation, induction of acute phase response and disturbance of BNB integrity [6, 21, 22]. While VPA was reported to induce plasma IL-6 levels in healthy male humans [23], IL-6 expression was reduced by VPA in EAN rats. IL-17 is produced by Th17 cells. It stimulates production of IL-6, nitric oxide and prostaglandin E2 to amplifies local inflammation, mediates chemotaxis of neutrophils and monocytes to sites of inflammation and augments the induction of co-stimulatory molecules such as ICAM-1 to support T cell activation [24, 25]. IL-4 promotes differentiation of Th2 cells that are essential

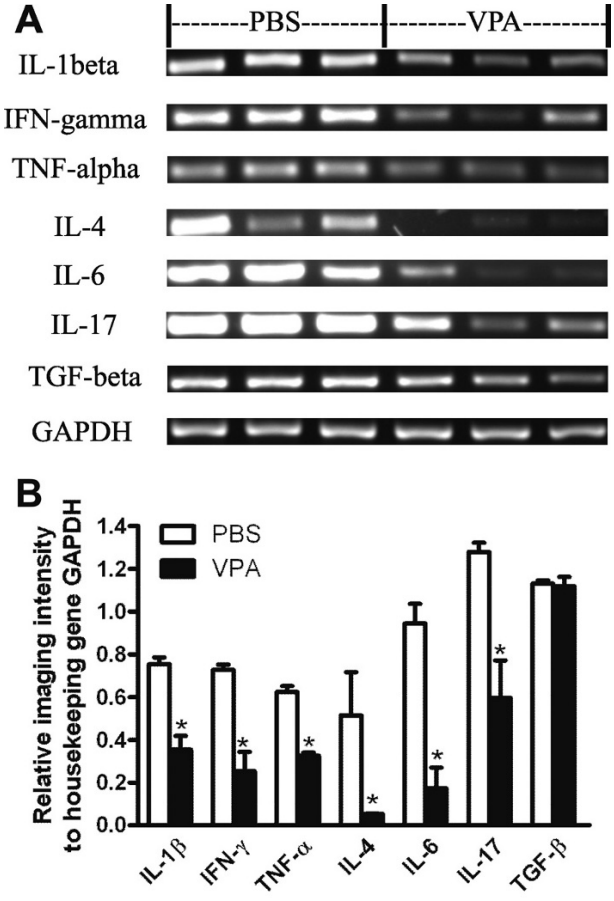

Figure 5. VPA suppressed mRNA expression of IL-1β, IFN-γ, TNF-α, IL-4, IL-6 and IL-17, but not of TGF-β in lymph nodes of EAN rats. VPA (300 mg/kg) or PBS was given once daily from days 0 to 15 to EAN rats (n=3). Rats were killed on day 16, mRNA of lymph nodes from both groups was isolated and relative expression levels of IL-1β, IFN-γ, TNF-α, IL-4, IL-6, IL-17 and TGF-β were analyzed by RT-PCR. (*A*) Photos of gel electrophoresis with PCR products showed obviously reduced mRNA levels of IL-1β, IFN-γ, TNF-α, IL-4, IL-6 and IL-17 but not TGF-β after treatment by VPA. (*B*) Bar graph shows semi-quantified results of imaging intensities relative to the housekeeping gene GAPDH. The unpaired t-test was performed to compare the differences. * $p<0.05$ and ** $p<0.01$ compared to their respective controls.

to humoral immunity, which is considered to contribute to the pathogenesis of EAN [5, 26]. The spectrum of cytokines produced during an immune response could shape the outcome of the response. In EAN, the balance between pro- and anti-inflammatory cytokines may determine the outcome. VPA reduced expression of pro-inflammatory cytokines, like IFN-γ, TNF-α, IL-1β, IL-6 and IL-17 and might thereby abrogate pathological cytokine production to terminate the inflammatory autoimmune response in EAN. One striking feature of our finding was that the anti-inflammatory effects of VPA are associated with the reduction of IL-17$^+$ cells and induction of Foxp3$^+$ cells. After antigenic stimulation, naive helper T cells proliferate and differentiate into various effector subsets characterized by the production of distinct cytokines with distinct effector functions [27]. Besides the classic Th1 and Th2 cells, a third helper T cell subtype, Th17 cells, has been recently identified [24]. Th17 cells are characterized by secretion of IL-17 and IL-17F and have been shown to play a crucial role in the host defense against of extracellular pathogens and the induction of autoimmune tissue injuries and inflammation. Pathological contributions of Th17 cells to various autoimmune and inflammatory diseases, including rheumatoid arthritis, multiple sclerosis, asthma, lupus, allograft rejection and psoriasis, have been proven [24]. In EAN, a robust accumulation of IL-17$^+$ cells in sciatic nerves was observed and temporally correlated with the severity of EAN (submitted data). IL-6 is known to be important for the differentiation of Th17 cells from naive helper T cells [28]. Following VPA treatment, mRNA expression of IL-6 in lymph nodes was significantly reduced. In parallel with this, IL-17 expression in lymph nodes, peripheral blood and sciatic nerves was found to decrease.

In addition, VPA was also found to increase Foxp3$^+$ cells in peripheral blood and sciatic nerves of EAN. The transcription factor Foxp3 is a specific marker for Treg cells; it is highly expressed in Treg cells and necessary for Treg cell development and function [29]. Treg cells suppress the activation of the immune system and thereby prevent excessive inflammation and/or autoimmunity, resulting in peripheral tolerance and immune homeostasis [30]. Evidence has accumulated that Treg cells can attenuate innate and adaptive immune responses in experimental animal models of autoimmunity including arthritis, colitis, diabetes, autoimmune encephalomyelitis, lupus, gastritis, oophoritis, prostatitis, and thyroiditis [31]. In EAN, our previous data have shown that the accumulation of Foxp3$^+$ cells to sciatic nerves may contribute to the termination of autoimmune neuritis (submitted data). Therefore, VPA-induced increase of Foxp3$^+$ cells in sciatic nerves and peripheral blood could alleviate inflammation in EAN.

In this investigation, we studied VPA effects on certain immune cells but it is important to keep in mind that VPA can also affect other immune cells, like nature killer (NK) cells, dendritic cells (DCs) and endothelial cells. It has been reported that VPA could inhibit NF-κB activation in NK cells to prevent NK cell activating receptor expression and NK cell function, resulting in reduced cytotoxicity [32]. In addition, VPA was found to affect DC differentiation and to revert these antigen-presenting cells to a less stimulatory mode through interference with signaling pathways that are directly involved in immune re-

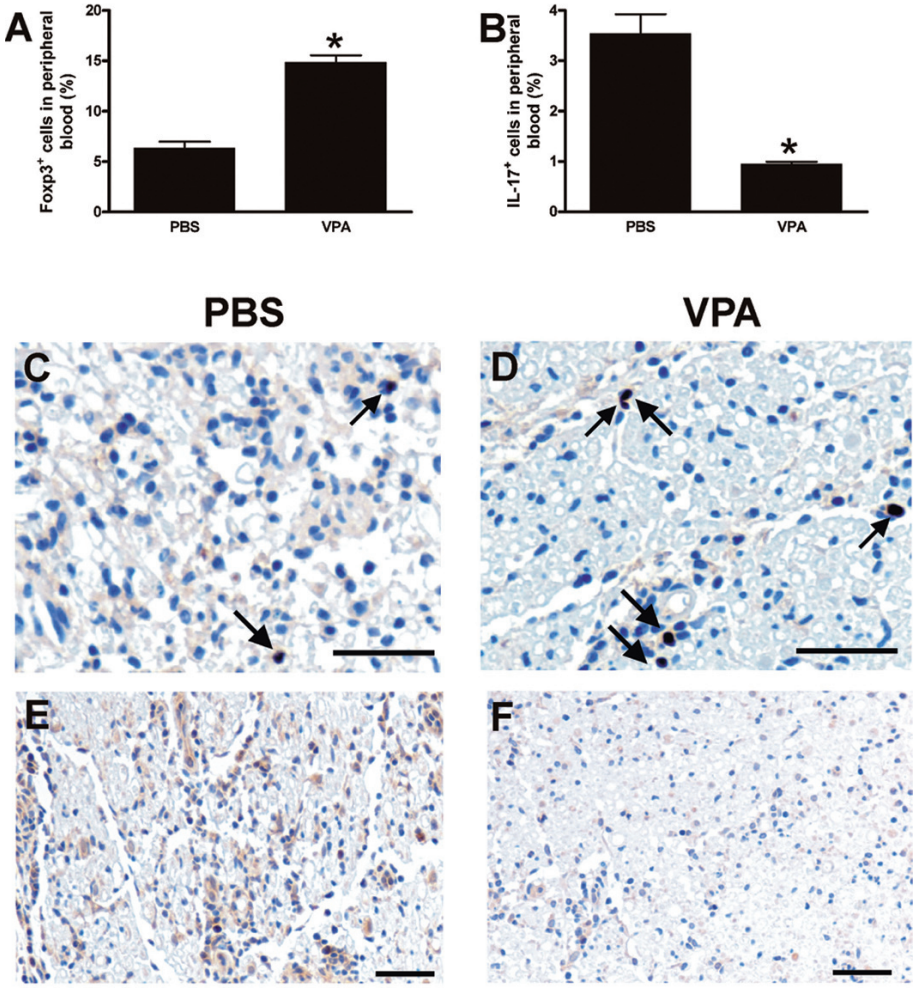

Figure 6. VPA induced Foxp3$^+$ cells but reduced IL-17$^+$ cells in peripheral blood and sciatic nerves of EAN rats. VPA (300 mg/kg) or PBS was given once daily from days 0 to 15 to EAN rats (n=3). Rats were killed on day 16. Blood was drawn intracardially under anesthesia for analysis of Foxp3$^+$ cells and IL-17$^+$ cells by FACS as described in Materials and methods. Further, sciatic nerves were taken to detect accumulation of Foxp3$^+$ and IL-17$^+$ cells by immunohistochemistry. (*A, B*) Bar graph shows that VPA treatment increased Foxp3$^+$ cells (*A*) but decreased IL-17$^+$ cells (*B*) in peripheral blood of EAN rats. Representative microphotos shows that VPA treatment increased Foxp3$^+$ cells (*C, D*), but decreased IL-17$^+$ cells (*E, F*) in sciatic nerves of EAN rats. Scale bars 50 μm.

sponses and in DC biology, including NF-κB and the IRFs [33]. Furthermore, VPA can reduce expression of tissue factor, which is the critical initiator of coagulation and also participates in the processes of inflammation, angiogenesis, metastasis and cell migration, in endothelial cells and monocytes [34].

VPA is a well-known anti-epileptic drug and a potent anti-cancer candidate by interfering with multiple regulatory mechanisms, like histone deacetylase (HDAC), GSKα and β, Akt, the ERK pathway, the phosphoinositol pathway, the tricarboxylic acid cycle, and the OXPHOS system [35]. Recently VPA has been found to have anti-inflammatory effects that are probably due to HDAC inhibitory activity [11–14, 36]. VPA is known to directly inhibit HDAC activity and cause a hyperacetylation of histones [37]. It is becoming clear that chromatin structure remodeling *via* changes in the levels of histone acetylation is a key mechanism underlying the regulation of gene expression. The modification of histone N-terminal tails by acetylation or deacetylation is catalyzed by histone acetyltransferase and HDAC, respectively, which can alter the interaction between histones and DNA and thus regulate gene expression [38]. HDAC inhibitors are known to attenuate inflammation and are effective in several inflammatory models, like EAE, asthma and allergic diseases [39–41]. Inhibition of HDAC has been found to increase the numbers and activity of Foxp3$^+$ regulatory cells [42]. Further, it is established that helper T cell differentiation and a variety of related cytokine expression accompany histone acetylation pattern remodeling [43, 44]. HDAC inhibitors effectively reduced the expression of pro-inflammatory cytokines, IFN-γ, TNF-α, IL-1β, IL-12 and IL-6 [45, 46]. IL-17 and IL-17F expression is also related to changes in histone acetylation [47]. Therefore, VPA may function through the HDAC inhibitory activity to attenuate inflammation in EAN. In summary, we have studied the effects of VPA in EAN, an animal model of human autoimmune neuropathy. Our data demonstrate that suppressive and therapeutic VPA treatment greatly reduced severity and duration of EAN, and attenuated inflammation and demyelination in the peripheral nerves. VPA

treatment reduced IL-17$^+$ cells but induced Foxp3$^+$ cells in peripheral blood and sciatic nerves. Furthermore, mRNA expression of a variety of pro-inflammatory cytokines in lymph nodes of EAN rats was reduced by VPA. Therefore, this investigation suggests that VPA could be a promising option in the therapy of human inflammatory neuropathies.

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