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Original Article

Effects of antiepileptic drugs on microglial properties

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Abstract

Purpose: To elucidate the effects of antiepileptic drugs (AEDs) on microglial cytokine production, polarization, and morphology.

Methods: MG6, an immortalized mouse microglial cell line, was stimulated with lipopolysaccharides (LPS) and the levels of pro-inflammatory cytokines were measured by enzyme linked immunosorbent assay, quantitative polymerase chain reaction (q-PCR), and intracellular staining using flow cytometry. M1 and M2 signatures of microglia after polarization were assessed using quantitative PCR and flow cytometry. Primary microglia prepared from CX3CR1-GFP mice were used to study the effects of AEDs on microglial morphology.

Results: Valproic acid (VPA) or gabapentin (GBP) augmented LPS-induced interleukin-6 (IL-6) production, while phenobarbital (PB) suppressed it. Tumor necrosis factor α (TNFα) production was enhanced by VPA, but was suppressed by PB and GBP. Levetiracetam did not alter cytokine production. It was difficult to assess the effects of water-insoluble AEDs because dimethyl sulfoxide solvent markedly suppressed IL-6 production. The mechanism of altered IL-6 production by AEDs was independent of their transcription or extracellular release. VPA augmented microglial M1 polarization. AEDs did not substantially affect the expression of microglial surface markers and had limited effect on the morphology of primary microglia.

Discussion: Although VPA increased microglial production of pro-inflammatory cytokines, partly due to augmented M1 polarization, most of the AEDs tested in the present study had neither beneficial nor adverse effects on inflammation in clinical practice.

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Introduction

Epilepsy is a brain disorder characterized by unprovoked seizures [1]. The causes of epilepsy include structural abnormalities of the brain, mutations in ion channel genes, brain injury, and metabolic disorders involving the central nervous system [2]. Although therapeutic options for epilepsy have expanded, antiepileptic drugs (AEDs) are still the mainstay of treatment. Most AEDs target ion channels and neurotransmitters [3]. However, their precise mechanisms of action are not fully understood.

Glial cells have been demonstrated to play significant roles in the pathogenesis of epilepsy [3]. In particular, microglia are immune cells in the central nervous system, which affect neuronal excitability by modulating inflammatory responses. Activated microglia decrease the seizure threshold in animal models of epilepsy by producing pro-inflammatory mediators. Based on these observations, emerging treatment strategies for epilepsy include immunomodulation.

Some AEDs have been reported to have anti-inflammatory action. However, it is largely unknown whether or how these reagents act on microglia. In the present study, we investigated the effects of AEDs on microglial properties.

Methods

Cell culture

MG6, a mouse microglial cell line immortalized by a human c-Myc gene, was purchased from RIKEN BioResource Center (Japan) [4]. MG6 cells were cultured and maintained in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml amphotericin B, 10 μg/ml insulin, and 100 μM 2-mercaptoethanol, in a humidified atmosphere with 5% CO_2.

CX3CR1-GFP mice expressing enhanced green fluorescent protein (EGFP) in monocytes, dendritic cells, natural killer (NK) cells and brain microglia, under control of the endogenous Cx3cr1 locus, were purchased from The Jackson Laboratory (Bar Harbor, Maine). Primary glial cells were prepared from CX3CR1-GFP mice on postnatal day 4. In brief, the cortices were dissected and the meninges were carefully removed. The brain tissues were dissociated into single cells by gentle shearing with scissors and pipetting, followed by enzymatic treatment for 10 min with 2.5% trypsin (Thermo Fisher Scientific, Waltham, MA) and 5 min with 400 μg/ml DNase I (Roche, Switzerland). The resulting cell suspension was seeded into a T75 flask with DMEM supplemented with 10% FBS and antibiotics-antimycotics.

All animal experiments were conducted following the national and international guidelines and the relevant national laws on the protection of animals and were approved by the Ethics Review Committee for Animal Experimentation of the Tokyo Metropolitan Institute of Medical Science (approval ID: 15029, 16015).

Chemicals

Valproic acid (VPA), phenobarbital sodium (PB), carbamazepine (CBZ) and diazepam (DZP) were purchased from Wako Pure Chemical Industries (Japan). Levetiracetam (LEV), gabapentin (GBP), topiramate (TPM), and zonisamide (ZNS) were purchased from

Tokyo Chemical Industry (Japan). VPA, LEV, PB, GBP and TPM were dissolved in Milli-Q water, and CBZ, ZNS and DZP were dissolved in dimethyl sulfoxide (DMSO).

Cytokine analysis

MG6 cells were seeded at a density of 2 × 10^4 cells/well in 96-well plates and incubated for 24 h. Cells were stimulated by 20 ng/ml lipopolysaccharides (LPS) and then treated with AEDs at indicated concentrations. The supernatants were collected for ELISA analysis. The cells were lysed for RNA isolation.

Enzyme-linked immunosorbent assay (ELISA)

Interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) in the culture supernatants were measured using BD OptEIA™ Mouse ELISA Set (BD bioscience, San Jose, CA) according to the manufacturer's instructions. Absorbance was measured at 450 nm with a 2030 ARVO™X3 Multilabel Reader (PerkinElmer, MA).

Quantitative polymerase chain reaction (q-PCR)

Total RNA was extracted from MG6 cells using RNeasy Mini Kit (QIAGEN, Hilden, Germany) and reverse transcribed to cDNA using PrimeScript™ RT Master Mix (Takara Bio, Japan). A volume of 2 μL of cDNA was mixed with primers and SYBR Premix Ex Taq™ (Takara Bio) to a final volume of 25 μL. The PCR condition was 40 cycles at 95 °C for 5 s and 60 °C for 30 s on a Thermal Cycler Dice Real Time System (Takara Bio). Threshold cycle (Ct) values were determined and normalized to the housekeeping gene *Actb*. The relative expression level of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method. The following pairs of primers (Fasmac, Japan) specific for mouse beta-actin (*Actb*), mouse IL-6 (*Il6*), mouse nitric oxide synthase 2 (*Nos2*), and mouse arginase (*Arg1*) were used: 5′-AGCCTTCCTTCTTGGGTATGG-3′ (forward) and 5′-TAGAGGTCTTTACGGATGTCAA-3′ (reverse) for *Actb*, 5′-CGTGGAAATGAGAAAAGAGTTGTGC-3 (forward) and 5′-TGTTCTTCATGTACTCCAGGTAGCT-3′ (reverse) for *IL-6*, 5′-AAAAGTCCAGCCGCACCA-3′ (forward) and 5′-ACAATCCACAACTCGCTCCA-3′ (reverse) for *Nos2*, and 5′-TGGCTTGCGAGACGTAGACC-3′ (forward) and 5′-CCTTTTCTTCTTCCCAGCA-3′ (reverse) for *Arg1*.

Flow cytometry

MG6 cells were treated with AEDs for 24 h. Cells were harvested with TrypLE™ Express (Thermo Fisher Scientific); washed with phosphate buffered saline (PBS); blocked with FC Block (1:50, 5 min, BioLegend, San Diego, CA); and stained with APC anti-mouse F4/80, PE/Cy7 anti-mouse CD45, FITC anti-mouse CD11b, and PE anti-mouse CX3CR1 antibodies (all 1:50, 30 min, BioLegend). Cells were analyzed using flow cytometry (FACS Canto II, BD). Data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Detection of intracellular cytokines by flow cytometry

MG6 cells were seeded at a density of 1 × 10^5 cells/well in 24-well plates, incubated for 24 h, stimulated by LPS, and treated with

AEDs for 6 h. A combination of LPS and brefeldin A (BFA) was used as positive control. Cells were harvested, fixed with 4% paraformaldehyde (5 min), permeabilized with Triton-X 100 (30 min, Roche), and stained with PE-conjugated anti-mouse IL-6 antibody (1:50, 30 min, BioLegend). Cells were analyzed by flow cytometry.

Microglial polarization

MG6 cells were seeded at a density of 5 × 10^4 cells/well in 24-well plates and stimulated by 20 ng/ml LPS and 20 ng/ml interferon-γ for M1 polarization or 20 ng/ml IL-4 and 20 ng/ml IL-13 for M2 polarization, in the presence or absence of AEDs for 48 h. Gene markers for M1 and M2 polarization were analyzed by q-PCR and surface markers by flow cytometry. For q-PCR, *Nos2* and *Arg1* were used as M1 and M2 markers, respectively. For flow cytometry, cells were stained by PE anti-mouse I-A/I-E antibody (M1 marker) and APC anti-mouse CD206 antibody (M2 marker) (both 1:50, 30 min, BioLegend).

Immunocytochemistry

Primary glial cells were seeded at a density of 1 × 10^5 cells/well in an 8-well Lab-Tek II Chamber Slide (Thermo Fisher Scientific) precoated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO) and treated with AEDs for 72 h. Cells were fixed with 4% paraformaldehyde (5 min) and mounted using Prolong Gold Antifade reagent with DAPI (Thermo Fisher Scientific) for visualization of cell nuclei. Cells were analyzed for EGFP using a laser scanning confocal microscope (FluoView FV1000, Olympus, Japan) with 488-nm laser,. Each image was saved at a resolution of 1024×1024 pixels using a 20× objective lens. Images were analyzed using the freeware ImageJ v1.51r (https://imagej.net/Fiji/Downloads). The pixel intensity for treated primary microglial cell images was thresholded and converted to binary images. Subsequently, dilation and erosion processes were performed on them and the remaining background noise and structures with autofluorescence were eliminated by size filtering (100 μm^2 to 600 μm^2). Area and circularity of each microglial cell was automatically measured using the Analyze Particle plugin.

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTS) assay

The influence of AEDs on cell viability was evaluated using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI). MG6 cells were seeded at a density of 2 × 10^4 cells/well in 96-well plates, stimulated by LPS, and treated with AEDs for 24 h. Media were replaced by MTS solution and incubated for 40 min. The absorbance was measured at 490 nm with an ARVO^{TM}X3 Reader.

Statistical analysis

Statistical analyses were performed using Statcel version 3 software (OMS, Tokorozawa, Japan). Student's t-test was used for statistical analysis of experimental replicates. A value of $p < 0.05$ was considered significant.

Results

Effects of antiepileptic drugs on microglial cytokine production

Initially, we screened eight AEDs (LEV, VPA, CBZ, ZNS, PB, TPM, GBP, and DZP) for their effects on LPS-induced IL-6 production from MG6 cells. (Fig. 1A). AED concentrations used in the study are summarized in Table 1. All AEDs tested in the experiment suppressed IL-6 production significantly when used at higher doses, and VPA and CBZ did so even at lower doses (Fig. 1A). Overall, AEDs dissolved in DMSO had a more drastic effect than those dissolved in water. Remarkably, DMSO alone significantly inhibited IL-6 production (Fig. 1A). We thus speculated that the suppressive effects of CBZ, ZNS, and DZP were mainly due to the effect of DMSO. TPM was not completely soluble in water and formed crystals, which potentially activate microglia. Therefore, we focused on the four water-soluble AEDs (LEV, VPA, PB, and GBP) in further analyses.

We next assessed the dose-response relationship between AEDs and LPS-induced

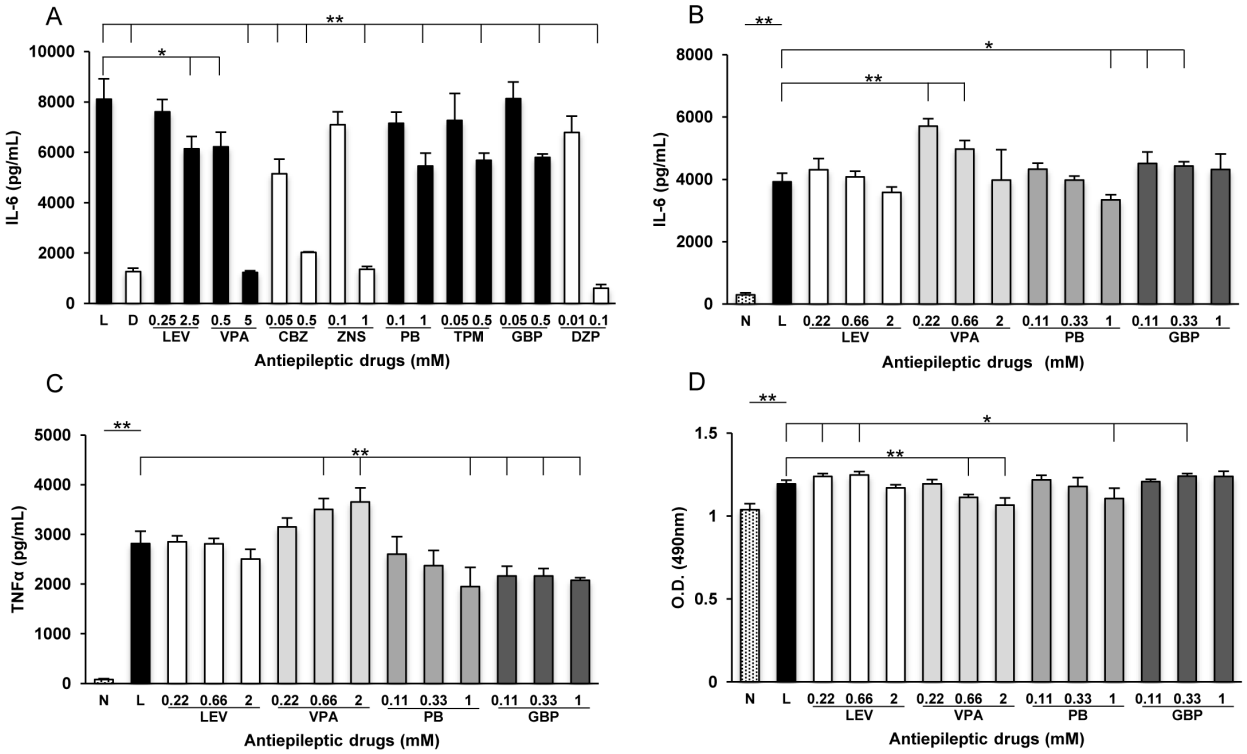

Figure 1. Effects of antiepileptic drugs on microglial cytokine production. (A) Screening of antiepileptic drugs (AEDs) that affect microglial cytokine production. Lipopolysaccharide (LPS)-stimulated MG6 cells were treated with AEDs at indicated concentrations for 24 h and interleukin-6 (IL-6) level in culture supernatant was measured using enzyme linked immunosorbent assay (ELISA) (n = 3). Empty bars show AEDs dissolved in DMSO and filled bars show AEDs dissolved in water. (B, C) Cytokine production from MG6 cells co-treated with LPS and AEDs. IL-6 level (B; n = 4) and tumor necrosis factor α (TNF α) level (C; n = 4) were measured by ELISA. Cells were treated for 24 h in (B) and 6 h in (C). (D) Viability of MG6 cells treated by LPS and/or AEDs for 24 h (n = 4) estimated by MTS-Assay. LPS-treated group (L; without AEDs) was used as a control in all experiments. N; untreated, D; DMSO (without AEDs), LEV; levetiracetam, VPA; valproic acid, CBZ; carbamazepine, ZNS; zonisamide, PB; phenobarbital, TPM; topiramate, GBP; gabapentin, DZP; diazepam. Graphs show mean ± SEM. *P < 0.05, **P < 0.01.

Table 1. Antiepileptic drug concentrations

Antiepileptic drugs	Therapeutic concentrations (μM)	Experimental concentrations (mM)
Levetiracetam	70.5-270.3	0.22-2.5
Valproic acid	300.8-601.7	0.22-5
Phenobarbital	39.3-157.4	0.1-1
Gabapentin	11.7-116.8	0.05-1
Carbamazepine	16.9-50.8	0.05-0.5
Topiramate	14.7-58.9	0.05-0.5
Zonisamide	47.1-188.5	0.1-1
Diazepam	0.35-3.5	0.01-1

cytokine production from MG6 cells. VPA (0.22 - 0.66 mM) or GBP (0.11 - 0.33 mM) augmented, while PB (1 mM) suppressed IL-6 production (Fig. 1B). TNFα production was enhanced by VPA at higher doses and was suppressed by PB (1 mM) or GBP (at all doses) (Fig. 1C). In most cases, cytokine production decreased as the doses of AEDs increased, except for the effect of VPA on TNFα production (Fig. 1B, C). We presumed that these dose-dependent decreases of cytokine production were due to the cytotoxic effect of AEDs at higher doses. Supporting this assumption, most AEDs (except GBP) reduced the viability of MG6 cells after LPS stimulation in a dose-dependent manner and the differences were significant for LEV, VPA, and PB (Fig. 1D)

Messenger RNA expression and intracellular level of IL-6

To clarify the mechanism of AEDs' effect on cytokine production, we examined mRNA expression and extracellular release of IL-6. Unexpectedly, VPA significantly reduced mRNA level of IL-6 under LPS stimulation (Fig. 2A). In intracellular cytokine staining, although brefeldin A increased the level of intracellular IL-6, none of the AEDs tested

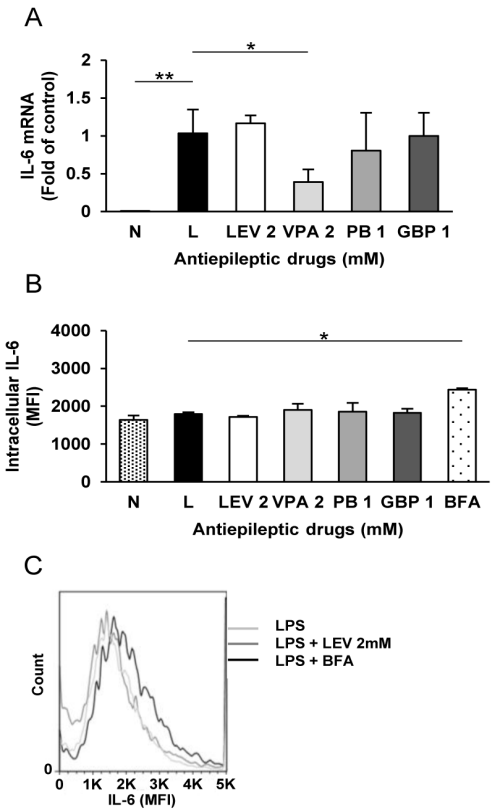

Figure 2. mRNA expression and intracellular level of IL-6. (A) LPS-stimulated MG6 cells were treated with AEDs at indicated concentrations for 6 h. IL-6 mRNA level was quantified by quantitative PCR (n = 3). LPS-treated group (L) was used as a control. (B, C) Intracellular IL-6 level measured by flow cytometry (B; n = 3). LPS-treated group (L) was used as a control, and LPS plus brefeldin A group (BFA) as a positive control. Representative flow cytometric histogram was shown in (C). N; untreated. Graphs show mean ± SEM. *P < 0.05, **P < 0.01.

changed the IL-6 level.

The results suggested that these AEDs altered IL-6 production via mechanisms independent of its transcription or extracellular release.

Changes in microglial surface markers after AED treatment

Next, we studied the effect of AEDs on microglial surface marker expression. LEV decreased the mean fluorescence intensity

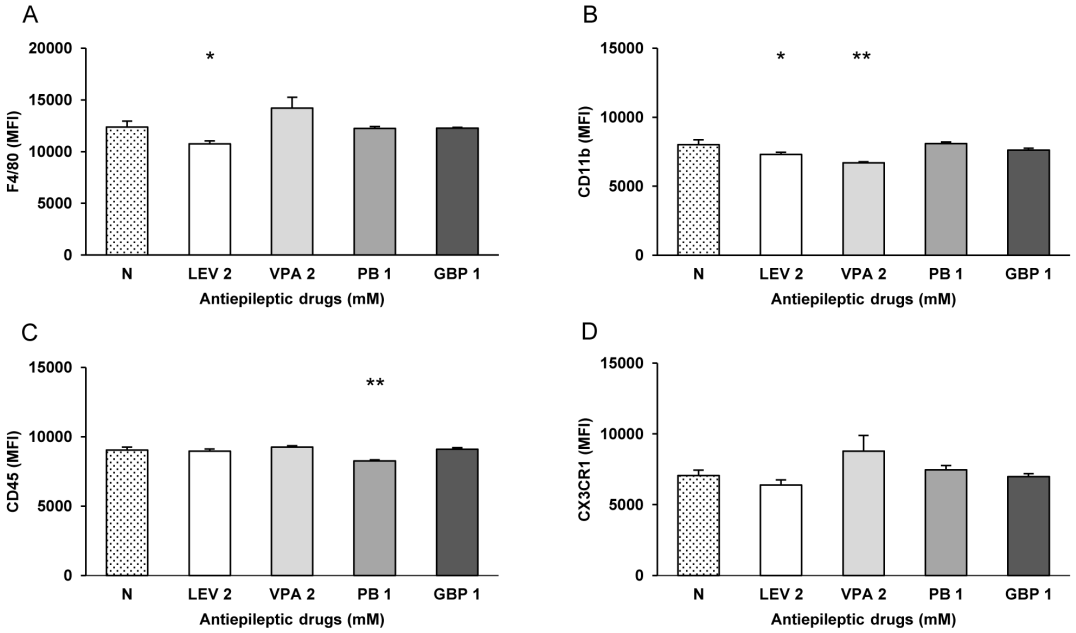

Figure 3. Changes in microglial surface markers after AED treatment. (A-D) MG6 cells were treated with AEDs for 24 h. The mean fluorescence intensities (MFI) of F4/80 (A), CD11b (B), CD45 (C), and CX3CRI (D) were quantified by flow cytometry (n = 3). Untreated group (N) was used as a control. Graphs show mean ± SEM. *P < 0.05, **P < 0.01.

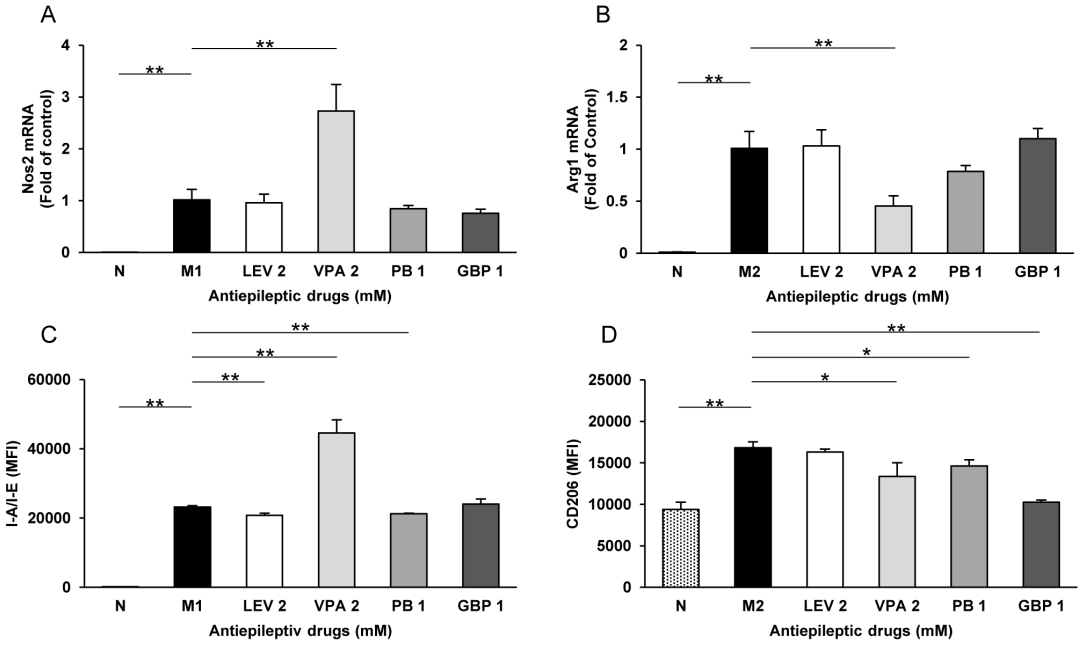

Figure 4. Effects of AEDs on microglial polarization. (A-D) MG6 cells were treated by LPS and interferon-γ for M1 polarization, or by IL-4 and IL-13 for M2 polarization, with or without AEDs for 48 h. (A, B) *Nos2* mRNA level as an M1 marker (A; n = 3), and *Arg1* mRNA level as an M2 marker (B; n = 3) were quantified by quantitative PCR. (C, D) MFI of I-A/I-E as an M1 marker (C; n = 3), and CD206 as an M2 marker (D; n = 3) were measured by flow cytometry. M1/M2 treated groups (without AEDs, filled bars) were used as a control. N; untreated. Graphs show mean ± SEM. *P < 0.05, **P < 0.01.

(MFI) of F4/80 (Fig. 3A). LEV and VPA decreased the MFI of CD11b (Fig. 3B). PB decreased the MFI of CD45 (Fig. 3C). None of the AEDs changed the MFI of CX3CR1 (Fig. 1D). GBP did not change the expression level of any of the surface markers (Fig. 3A-D). On the whole, AEDs did not substantially affect the expression of microglial surface markers.

Effects of AEDs on microglial polarization

Since microglial polarization to M1 or M2 phenotypes is significantly associated with their cytokine production, we tested whether AEDs affect M1/M2 polarization of microglia. VPA significantly increased mRNA level of *Nos2* under M1-biased condition (Fig. 4A) and conversely decreased mRNA level of *Arg1* under M2-biased condition (Fig. 4B). Similarly, in the presence of VPA, the MFI of I-A/I-E (M1 marker) was increased (Fig. 4C) and CD206 (M2 marker) was decreased (Fig. 4D) under M1- and M2- biased conditions, respectively. Both LEV and PB decreased I-A/I-E expression (Fig. 4C) while both PB and GBP decreased CD206 expression (Fig. 4D). These results indicated that VPA augmented microglial M1 polarization.

Morphological changes of primary microglial cells after AED treatment

Finally, we focused on the morphological changes of microglia after AED treatment. Most microglia showed ramified shape with fine branched processes (Fig. 5A). There was no significant difference in area of primary microglia after AED treatment (Fig. 5B). LEV slightly but significantly increased the circularity of primary microglia (Fig. 5C). These results indicated that AEDs had limited effect on the morphology of primary microglia.

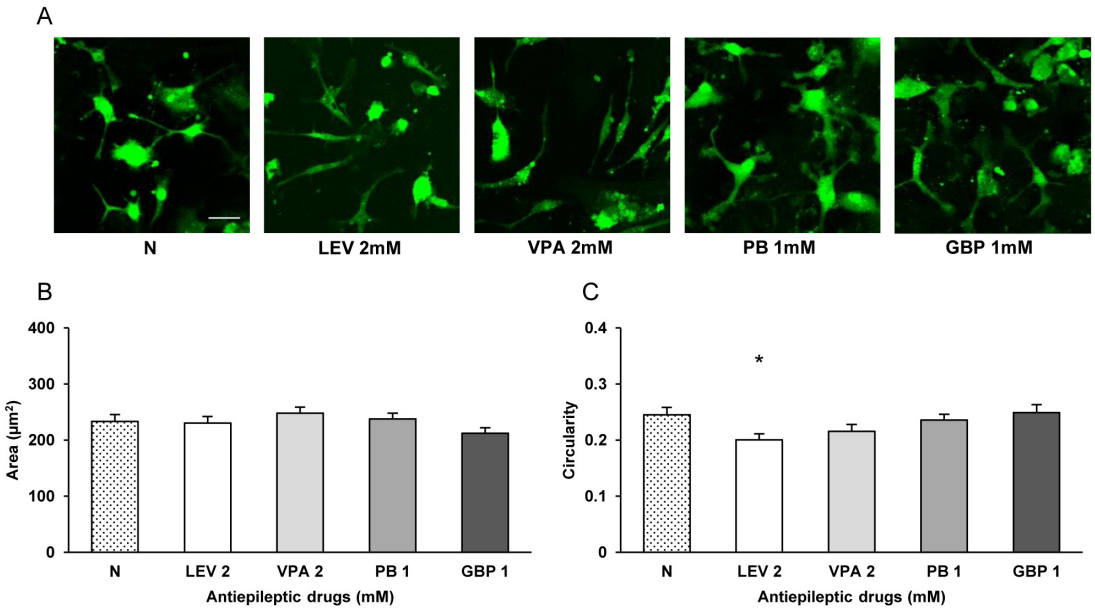

Figure 5. Morphological changes of primary microglial cells after AED treatment. (A) Primary glial cells prepared from CX3CR1-GFP mice were treated with AEDs for 72 h. Microglia were shown in green on confocal microscopic images. Scale bars; 20 μm. (B.C) Area (B) and circularity (C) of microglia in six images from each group were analyzed by ImageJ software. Untreated group (N) was used as a control. Graphs show mean ± SEM. *P < 0.05, **P < 0.01.

Discussion

In the present study, we demonstrated that VPA augmented LPS-induced production of IL-6 and TNFα by inducing microglial M1 polarization. LEV, PB, and GBP modified cytokine production slightly. Based on the analyses of microglial morphology and surface markers, it is unlikely that the AEDs tested change the property of microglia in the absence of LPS stimulation.

Several reports have examined the effects of AEDs on inflammatory and immune responses. LEV had an anti-inflammatory effect against interleukin-1β (IL-1β) and prostaglandin E2 in animal models of epilepsy [5, 6]. However, LEV showed only minor anti-inflammatory effects that were not sufficient to ameliorate the disease course of experimental autoimmune encephalomyelitis [7]. Although VPA inhibited LPS-induced pro-inflammatory cytokine production, it significantly enhanced LPS-induced release of prostaglandins [8, 9]. These observations suggest that AEDs have pleiotropic roles in regulating immune function.

From the clinical point of view, evidence has suggested the involvement of inflammatory response in the pathomechanism of epilepsy. Autoimmune epilepsy represents a small but potentially treatable form of epilepsy, associated with acquired autoimmune etiology [10]. Febrile infection-related epilepsy syndrome is characterized by intrathecal overproduction of pro-inflammatory cytokines and chemokines [11]. In these conditions, AEDs that potentially suppress inflammation and immune activation may be potentially beneficial.

Microglial cells are a major source of inflammatory mediators in the CNS. We therefore investigated the effect of AEDs on cytokine production after LPS stimulation of microglia. We found that VPA increased the production of both IL-6 and TNFα. In contrast, VPA suppressed IL-6 mRNA expression in response to LPS. The reason for these conflicting data is unclear. GBP enhanced IL-6 production slightly but reduced TNFα production significantly. Although LEV and PB decreased the production of both IL-6 and TNFα in a dose-dependent manner, the differences were not significant except for TNFα after PB treatment at the highest concentration. IL-1β, another pro-inflammatory cytokine, has been implicated in the pathomechanism of epilepsy [12]. However, it was difficult to obtain stable data when we measured IL-1β. Since extracellular IL-1β release is significantly influenced by cell death, we speculated that the fluctuated data were due to the cytotoxic effects of AEDs at higher concentrations.

Since LEV binds to the synaptic vesicle protein SV2A that is involved in vesicle exocytosis [13], we speculated that AEDs including LEV might affect vesicular transport of inflammatory cytokines. However, LEV, VPA, PB, and GBP did not increase the intracellular level of IL-6. Therefore, it is unlikely that the change in IL-6 production is relevant to vesicular transport of cytokines.

We also demonstrated that AEDs affect microglial polarization. Microglia as well as macrophages polarize toward M1 or M2 phenotypes in response to different microenvironments [14]. M1 phenotype, characterized by prominent expression of nitric oxide synthase, major histocompatibility complex-II molecules (I-A/I-E) and pro-inflammatory

cytokines, is associated with inflammatory response; while M2 phenotype, characterized by arginase and scavenger receptors (CD206), is associated with resolution of inflammation and tissue repair. It is noteworthy that VPA augmented M1 signature and attenuated M2 signature, suggesting that VPA promotes M1 polarization. These data are consistent with increased LPS-induced production of IL-6 and TNFα after VPA treatment.

Finally, we examined whether AEDs alter microglial morphology. Resting microglia present ramified shape with branched processes, but activated microglia transform into amoeboid shape [15]. Although LEV increased the circularity of microglia slightly, other AEDs had no effect on microglial morphology.

Seizures often occur in the context of inflammatory neurological diseases. It is thus important to consider the pro- or anti-inflammatory effects of AEDs for the management of inflammation-related seizures or epilepsies. Our data suggest that VPA should be avoided when inflammation is supposed to play deleterious roles in the pathogenesis of seizures. Importantly, concentrations of AEDs, except for VPA, at which significant changes were observed in cytokine production and microglial polarization were much higher than their therapeutic concentrations (Table 1). Therefore, it is unlikely that most of the AEDs tested in the present study would have either a beneficial or an adverse effect on inflammation in clinical practice.

The present study has several limitations. First, we were unable to study water insoluble AEDs due to the significant influence of DMSO solvent on cytokine production. Second, this *in vitro* study is not always applicable to clinical practice and thus the effect of AEDs on *in vivo* inflammation needs to be clarified. However, studying the pro- and anti-inflammatory properties of AEDs is expected to provide important information for treatment strategies for epilepsy associated with inflammatory diseases.

In conclusion, VPA increased microglial production of pro-inflammatory cytokines, partly due to augmented M1 polarization. However, other AEDs tested in the present study had limited effects on microglial property.

Acknowledgements

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Conflict of interest

The authors declare that they have no conflict of interest.

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