Treatment with lysophosphatidic acid prevents microglial activation and depression-like behaviours in a murine model of neuropsychiatric systemic lupus erythematosus

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Abstract
Neuropsychiatric systemic lupus erythematosus (NPSLE) is an incurable disease characterised by neuropsychiatric symptoms, particularly depression. Novel therapeutic options for NPSLE are urgently needed. Several previous reports have suggested that both microglial activation and impaired neurogenesis may be involved in the progression of depression. In contrast, the administration of lysophosphatidic acid (LPA) ameliorates depression and anxiety. Therefore, in the present study, we determined whether treatment with LPA affects microglial activation, impaired neurogenesis, and abnormal behaviour in MRL/lpr mice. In both tail suspension test and forced swim test, the MRL/lpr mice exhibited a significant increase in total immobility time compared with MRL/+ mice. Treatment with LPA significantly suppressed the prolonged immobility time in MRL/lpr mice. In contrast, pretreatment with ki16425 (a specific antagonist of LPA receptor 1 and 3) significantly reversed the effects of LPA. Furthermore, MRL/lpr mice exhibited impairments in spatial working memory and visual cognitive memory, which were suppressed by LPA treatment. The expression levels of TMEM119, CD68, GFAP, and caspase-3 in the hippocampus and prefrontal cortex of MRL/lpr mice were significantly higher than those in MRL/+ mice. Pretreatment with ki16425 reversed LPA-mediated inhibition of microglial activation. The quantity of sodium fluorescein that leaked into the brain tissues in MRL/lpr mice were significantly higher than that in MRL/+ mice. Treatment with LPA tended to decrease the sodium fluorescein leakage. These findings suggest that treatment with LPA may regulate microglial activation, which is important in the pathogenesis of NPSLE, as well as blood-brain-barrier weakening and abnormal behaviour.

Keywords: NPSLE, microglia, LPA, neuroinflammation, depression

Introduction
Systemic lupus erythematosus (SLE) is an autoimmune disease that most commonly affects women, and the lesions are found in various organs, including skin and kidney. Neuropsychiatric systemic lupus erythematosus (NPSLE) causes neuropsychiatric symptoms, mainly depression. NPSLE is conventionally treated with corticosteroid pulse therapy, immunosuppressive drugs, and antidepressants [1]. However, the adverse effects of these drugs, such as increased susceptibility to infections and steroid psychosis, pose a significant burden on the patients. Therefore, there is an urgent need to develop novel therapeutic strategies.

MRL/lpr mice, a well-known animal model of SLE, produce autoantibodies such as anti-dsDNA and anti-Sm antibodies and develop systemic vasculitis and arthritis [2]. These mice are useful animal models for NPSLE studies because they exhibit depression-like behaviours [3]. In addition, both microglial activation [4] and blood-brain barrier (BBB) weakening [3], which may be involved in the pathogenesis of NPSLE, occur in these mice. Moreover, they demonstrate impairments in spatial working memory and visual cognitive memory [6] and exhibit hippocampal astrogliosis [7] and brain cell apoptosis [8].

Microglial activation in the prefrontal cortex and hippocampus was reported to be positively correlated with depression. In rats subjected to repetitive social defeat stress, peripherally derived monocytes [9], and microglia mutually enhance neuroinflammation [10]. Furthermore, selective serotonin and serotonin noradrenaline reuptake inhibitors exert anti-depressive effects by suppressing...
Figure 1: Effect of LPA on depression-like behaviours in MRL/lpr mice. The tail suspension tests (A and B) and forced swimming tests (C) were performed to evaluate depression-like behaviours in MRL/lpr mice. Bar graphs show the quantitative data of the total time of immobility and latency to the bout of immobility in the vehicle-treated MRL/+ group (control-vehicle; n = 12), vehicle-treated MRL/lpr group (lpr-vehicle; n = 12), or LPA-treated MRL/lpr group (lpr-LPA; n = 11). The data are expressed as mean ± SEM. *P < 0.05, **P < 0.01 versus control-vehicle; #P < 0.05 versus lpr-vehicle; ##P < 0.01 versus lpr-vehicle.

Figure 2: Effect of an LPA receptor antagonist on depression-like behaviours in LPA-treated MRL/lpr mice. The tail suspension tests (A and B) and forced swimming tests (C) were performed to evaluate depression-like behaviours in the MRL/lpr mice. Bar graphs show the quantitative data of the total time of immobility and latency to the bout of immobility in the vehicle-pretreated + vehicle-treated MRL/lpr group (lpr-vehicle-vehicle; n = 12), vehicle-pretreated + LPA-treated MRL/lpr group (lpr-vehicle-LPA; n = 12), or Ki16425-pretreated + LPA-treated MRL/lpr group (lpr-Ki16425-LPA; n = 12). The data are expressed as mean ± SEM. *P < 0.05, **P < 0.01 versus lpr-vehicle-vehicle; #P < 0.05 versus lpr-vehicle-LPA; ##P < 0.01 versus lpr-vehicle-LPA.
Impaired neurogenesis by methylazoxymethanol induces depression-like behaviour [12]. Promoting neurogenesis via Bax gene inactivation in neural progenitor cells improves depression-like behaviours. These findings suggest that both microglial activation and impaired neurogenesis are involved in the progression of depression.

Recently, lysophosphatidic acid (LPA), a lysospholipid, is involved in various physiological functions and pathological conditions as a lipid mediator [13]. The administration of LPA or LPA receptor 1 (LPAR1) agonist ameliorates depression and anxiety [14]. However, the effects of LPA on microglial activation and impaired neurogenesis have not been examined. Several studies have shown that LPA has anti-inflammatory effects, such as a shift from M1 to M2 macrophages, decreased TNFα production in dendritic cells, and increased IL-10 production [15]. In contrast, LPA promotes IL-1β production by macrophages [16]. Thus, a definitive conclusion has not yet been reached.

Therefore, in the present study, we sought to determine whether treatment with LPA affects microglial activation, impaired neurogenesis, brain cell apoptosis, hippocampal astrogliosis, depression-like behaviours, and memory impairments in MRL/lpr mice. Finally, we assessed the effect of LPA treatment on the expression of inflammatory cytokines in the hippocampus.

### Materials and methods

#### Mice

All experiments were designed and performed in compliance with the Institutional Ethical Guidelines for Animal Experiments of the National Defense Medical College (Tokorozawa, Japan), and were approved by the Animal Experiment Committee of the National Defense Medical College (Approval No. 20008).

Female MRL/+ (n = 12) and MRL/lpr mice (n = 24) (12 weeks old) were purchased from Japan SLC Inc. (Hamamatsu, Japan) and maintained under controlled light and temperature conditions. C18:1 LPA (LPA) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Ki16425 (a specific antagonist of LPA receptors 1 and 3) was purchased from Sigma–Aldrich. The MRL/lpr mice (15 weeks old) were randomized to receive a vehicle consisting of 10% dimethyl sulfoxide (DMSO) and 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (n = 12) or LPA at 1 mg/kg/day (n = 12) via intraperitoneal injection for 2 weeks. The MRL/+ mice (15 weeks old) received a vehicle consisting of 10% DMSO and 0.1% BSA in PBS (n = 12) for 2 weeks. BrdU-labelling in the hippocampus following BrdU administration is widely used to evaluate neurogenesis [17]. Therefore, we administered BrdU intraperitoneally at 10 mg/kg/day for 1 week before dissection of the brain tissues. In the next experiment, 36 MRL/lpr mice (15-weeks old) were randomly divided into three groups: (1) pretreated with vehicle (10% DMSO and 0.1% BSA in PBS) and treated with vehicle after 30 min, (2) pretreated with vehicle (10% DMSO and 0.1% BSA in PBS) and treated with LPA at 1 mg/kg/day after 30 min, (3) pretreated with Ki16425 at 10 mg/kg/day and treated with LPA at 1 mg/kg/day after 30 min. These drugs were intraperitoneally administered to mice for 2 weeks.

#### Behavioural experiments

##### Tail suspension test

To evaluate depression-like behaviour, tail suspension tests were performed as described previously [18]. Mice were suspended from the edge of the desk with an adhesive tape placed approximately 5–10 mm from the tip of the tail. The suspended mouse was placed 600 mm above the floor. The total duration of immobility (i.e. head down and absence of paw movement) and latency to the first bout of immobility were measured for a period of 6 min.

##### Forced swim test

The forced swim test, which is another test used to evaluate depression-like behaviours in rodents, was performed as...
described previously [18]. The mice were individually placed in a cylinder (25 cm diameter, 46 cm depth) filled two-thirds with water (25 ± 1°C) for 6 min. The dimensions of the cylinder were selected because the mice could not escape from the cylinder or touch the bottom with their feet. Total immobility time was measured in this test. Swimming behaviour, defined as an active horizontal movement more than necessary to keep the head above the water, was measured as a parameter to assess ‘hopefulness’. Immobility, defined as no more movements than necessary to maintain balance and keep the nose above water, was measured as a parameter to assess ‘hopelessness’ as a sign of depression-like behaviours. After each test, the mice were lightly towel-dried prior to returning them to their home cages. The water in the cylinder was changed between animals.

**Y-maze test**

Spatial working memory was assessed using a Y-maze test [19]. Mice were placed in the centre of the Y-maze, and free exploration of the maze was allowed for 8 min. The total number of arms entered and the sequence in which they were entered were recorded. The percentage of correct alternations was calculated as the number of triads containing entries into all three arms divided by the maximum possible number of alternations (total number of arm entries minus 2) \times 100.

**Object recognition test**

Visual cognitive memory was assessed using an object-recognition test. A mouse was presented with two similar objects during the first session, and then one of the two objects was replaced by a new object during the second session. An hour after the first session (performed for 10 min), the second session was performed for 10 min. We evaluated the frequency taken to search for a new object as a measure of recognition memory [20].

**Immunofluorescence staining**

Following treatment, the mice were anaesthetised and transcardially perfused with ice-cold PBS and fixed by the perfusion with 4% paraformaldehyde in PBS. Brain tissues were harvested and the harvested tissues were fixed in 4% paraformaldehyde again for 24 h followed by incubation in 80% ethanol. A coronal section (1 mm thick) was cut using a mouse brain slicer (Muromachi Kikai, Tokyo, Japan). Each section was embedded in paraffin. For immunofluorescence staining, 1–3 µm thick sections were de-paraffinized with xylene and rehydrated with 80% ethanol. Staining was performed using goat anti-Iba1 (Wako, Osaka, Japan), Alexa Fluor 488 conjugated rabbit anti-CD68 (Bioss Antibodies, Woburn, MA), rabbit anti-TMEM119 (Cell Signaling...
Technology, Danvers, MA), rat anti-BrdU (Abcam, Eugene, OR), rabbit anti-NeuN (Merck Millipore, Darmstadt, Germany), rabbit anti-GFAP (Bioss Antibodies), and rabbit anti-cleaved caspase3 (Cell Signaling Technology) antibodies.

Next, the sections were incubated with Alexa Fluor 594 conjugated anti-goat IgG (Invitrogen, Waltham, MA), Alexa Fluor 594 conjugated anti-rabbit IgG (Invitrogen), or Alexa Fluor 488 conjugated anti-rat IgG (Invitrogen). Computer-assisted morphometric analysis was performed using a digital microscope controller (BZ-8000, Keyence Co., Osaka, Japan) and ImageJ software (NIH, Bethesda, MD, USA) was used for image analysis.

TUNEL staining

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) assay was performed using an in situ fluorescein cell death detection kit (RSD). Stained slides were analysed blindly using a fluorescence microscope and TUNEL-positive cells were counted in each section. The average values of the two sections from each mouse brain were calculated.

Assessment of BBB function

To assess BBB function, MRL/+ and MRL/lpr mice were transcardially injected with 2% sodium fluorescein (Sigma–Aldrich) in PBS. The fluorescence intensity of homogenized brain tissues was determined at excitation/emission wavelengths of 440/525 nm using a microplate reader [21].

Western blot analysis

Proteins were extracted by homogenizing hippocampal tissue with RIPA buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 1.0% Triton X-100, 0.5% Sodium deoxycholate, and 0.1% SDS). Eight mice per group were used for the western blot analysis. Total proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Co., Bedford, MA). The membranes were blocked in 5% BSA dissolved in TBST (50 mM Tris/HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween-20) for 2 h at room temperature and probed with indicated primary antibodies overnight at 4°C; the membrane was washed three times with TBST, followed by incubation with appropriate HRP-linked secondary antibodies for 2 h at room temperature; the membrane was then washed three times. Computer-assisted densitometric analysis was performed using a luminescent image analyser (LAS 3000; Fuji Film, Tokyo, Japan) and image analysis software (Image Gauge 3122; Fuji Film).
Enzyme-linked immunosorbent assay (ELISA)
Blood was collected from the MRL/+ or MRL/lpr mice and 10% EDTA (10 mg/mL) was added to the samples. Plasma was obtained by centrifugation. The level of anti-double-stranded DNA (anti-dsDNA) antibody in the plasma was measured using a commercially available ELISA kit (Fujifilm Wako Shibayagi, Gunma, Japan) according to the manufacturer’s instructions. The cut-off value of the assay was 31.3 mU/mL.

Statistical analysis
All data are expressed as mean ± SEM. The means of multiple groups were compared using one-way analysis of variance. A nonparametric test was used for data in which a normal distribution could not be obtained. Statistical significance was set at P < 0.05.

Results
Effects of LPA or an LPA receptor antagonist on depression-like behaviours in MRL/lpr mice
To determine whether the MRL/lpr mice exhibit depression-like behaviours, they were subjected to the tail suspension tests and forced swimming tests. In both tests, MRL/lpr mice exhibited a significantly higher total immobility time compared to MRL/+ mice (Fig. 1A and C). MRL/lpr mice exhibited a significant decrease in latency to the first bout of immobility compared to control mice in tail suspension test (Fig. 1B). Treatment with LPA significantly suppressed the prolonged immobility time in MRL/lpr mice (Fig. 1A and 1C) and significantly shortened the first bout of immobility compared to control mice in tail suspension test (Fig. 1B). In contrast, pretreatment with ki16425 (a specific antagonist of LPA receptors 1 and 3) significantly reversed the effects of LPA (Fig. 2A–C).

Effects of LPA on impairment of spatial working memory and visual cognitive memory in MRL/lpr mice
To assess spatial working memory and visual cognitive memory in MRL/lpr mice, they were subjected to the Y-maze and object recognition tests respectively. The correct alternations and recognition indices in MRL/lpr mice were significantly lower than those in MRL/+ mice (Fig. 3A and B). Treatment with LPA improved the decline in the correct alternation and recognition indices (Fig. 3A and B).
Effects of LPA or an LPA receptor antagonist on the expressions of Iba1, CD68, TMEM119, and GFAP in MRL/lpr mice

The expressions of Iba1 and CD68 were increased in both hippocampus and prefrontal cortex of MRL/lpr mice compared to those in MRL/+ mice (Fig. 4A–D). The increased expressions of Iba1 and CD68 in MRL/lpr mice were significantly suppressed by LPA treatment (Fig. 4A–D). In contrast, pretreatment with ki16425 significantly inhibited the suppressive effects of LPA on Iba1 and CD68 expressions (Fig. 5A–D).

The expression of TMEM119, which is specifically expressed in microglia [22], was also increased in both hippocampus and prefrontal cortex of MRL/lpr mice compared to those in MRL/+ mice (Fig. 6A–D). The increases of TMEM119 expression in MRL/lpr mice were significantly suppressed by LPA treatment (Fig. 6A–D).

The expression of GFAP, which is specifically expressed in astrocytes, in the hippocampus of MRL/lpr mice was significantly higher than that in MRL/+ mice (Fig. 7A and B). The increased expression of GFAP in MRL/lpr mice was significantly suppressed by LPA treatment (Fig. 7A and B).

Effects of LPA on the expression of BrdU in MRL/lpr mice

BrdU expression in the hippocampus of MRL/lpr mice was lower than that in MRL/+ mice (Fig. 8A and B). The treatment with LPA significantly reversed the decrease of BrdU expression in MRL/lpr mice (Fig. 8A and B).

Effects of LPA on cleaved caspase-3 expression and TUNEL staining in MRL/lpr mice

The expression of cleaved caspase-3 in both the hippocampus and prefrontal cortex of MRL/lpr mice was significantly higher than that of MRL/+ mice (Fig. 9A and B). The increased expression of cleaved caspase-3 in MRL/lpr mice was suppressed by LPA treatment (Fig. 9A and B). The average number of TUNEL-positive cells in the brain sections of MRL/lpr mice was significantly higher than those of MRL/+ mice (Fig. 9C). The increase in TUNEL-positive cells in MRL/lpr mice was significantly suppressed by LPA treatment (Fig. 9C).

Effect of LPA on BBB impairment in MRL/lpr mice

The intensity of sodium fluorescein that leaked into the brain tissues was significantly higher in MRL/lpr mice than in MRL/+ mice (Fig. 10). Treatment with LPA tended to decrease the sodium fluorescein leakage.

Effect of LPA on hippocampal expressions of IL-1β and TNF-α in MRL/lpr mice

The hippocampal expressions of IL-1β and TNF-α in MRL/lpr mice was significantly higher than that in MRL/+ mice (Fig. 11A and B). The increases in IL-1β and TNF-α level in the hippocampus of MRL/lpr mice were suppressed by LPA treatment (Fig. 11A and B).

Effect of LPA on plasma levels of anti-dsDNA antibody in MRL/lpr mice

The plasma levels of anti-dsDNA antibodies in MRL/lpr mice were significantly elevated compared with those in MRL/+ mice (Fig. 12). However, treatment with LPA did not affect plasma anti-dsDNA antibody levels in MRL/lpr mice.

Discussion

Previous studies have shown that microglial activation is involved in the development of depression. Nie et al. reported increased inflammatory cytokines and microglial activation in the brains of mice that exhibit depression-like behaviours [23]. Inflammatory cytokines have also been
Nagata et al. reported to induce neural damage and impair neurogenesis. Furthermore, astrogliosis and microglial activation have been reported to be associated with depression, neural damage, and impaired neurogenesis [24]. The MRL/lpr mice exhibit microglial activation, hippocampal astrogliosis, and brain cell apoptosis [25]. In the present study, the expressions of CD68, TMEM119 (a specific microglial marker) [22], GFAP, and cleaved caspase3 was increased in the hippocampus and prefrontal cortex of MRL/lpr mice with behavioural abnormalities. In addition, LPA treatment suppressed the expressions of CD68, TMEM119, GFAP, and cleaved caspase3 in MRL/lpr mice and improved behavioural abnormalities. In

Figure 8: Effect of LPA on BrdU expression in the hippocampus of MRL/lpr mice. Representative images of immunofluorescence staining of NeuN (red) and BrdU (green) in sub-granular zone of hippocampus (A) in vehicle-treated MRL/+ (control-vehicle), vehicle-treated MRL/lpr (lpr-vehicle), and LPA-treated MRL/lpr groups (lpr-LPA). White bar = 50 μm. The bar graph shows the quantitative analysis of BrdU-positive rates in the sub-granular zone of the hippocampus (B) in vehicle-treated MRL/+ group (control-vehicle; n = 6), vehicle-treated MRL/lpr group (lpr-vehicle; n = 4), or LPA-treated MRL/lpr group (lpr-LPA; n = 6). The data are expressed as mean ± SEM. *P < 0.05 versus control-vehicle, ###P < 0.01 versus lpr-vehicle.
Figure 9: Effect of LPA on cleaved caspase3 expression and TUNEL staining in MRL/lpr mice. Representative images of immunofluorescence staining of cleaved caspase3 (red) in the sub-granular zone of hippocampus and prefrontal cortex (A) in vehicle-treated MRL/+ (control-vehicle), vehicle-treated MRL/lpr (lpr-vehicle), or LPA-treated MRL/lpr (lpr-LPA) groups. White bar = 100 μm. Bar graphs show quantitative analysis of cleaved caspase3 positive rates in the sub-granular zone of the hippocampus and prefrontal cortex (B) in vehicle-treated MRL/+ group (control-vehicle; n = 5), vehicle-treated MRL/lpr group (lpr-vehicle; n = 6), or LPA-treated MRL/lpr group (lpr-LPA; n = 5), and the average value of TUNEL positive cells in the two sections containing prefrontal cortex (C) in vehicle-treated MRL/+ group (control-vehicle; n = 5), vehicle-treated MRL/lpr group (lpr-vehicle; n = 5), or LPA-treated MRL/lpr group (lpr-LPA; n = 5). The data are expressed as mean ± SEM. **P < 0.01 versus control-vehicle; #P<0.05, ##P<0.01 versus lpr-vehicle.
neuroinflammation cannot be ruled out. The possibility that invading macrophages may enhance phagocytes [29]. Although the results of TMEM119 indicate and many reports have described them as microglia/macro-
include macrophages that invade the BBB due to disruption, lpr mice. Moreover, Iba1 and CD68 positive cells may also affect on microglial activation and BBB impairment in MRL/lpr mice. They also reported that LPA tended to decrease the sodium fluorescein leakage. In addition, Mathew . reported that LPA suppressed the extravascular migration of macrophages and that these phenomena may be mediated by reactive oxygen species. In addition, Mathew . reported that LPAR5 stimulation suppresses B-cell activation and antibody production [15]. Anti-dsDNA antibodies are useful markers for assessing organ damage and disease severity in SLE, such as lupus nephritis, but they do not always correlate with disease severity in NPSLE [33]. In the present study, plasma anti-dsDNA antibody levels in MRL/lpr mice were also significantly higher than those in control mice; however, treatment with LPA did not affect plasma anti-dsDNA antibody levels in MRL/lpr mice. Autoantibodies, such as anti-ribosomal P and anti-U1RNP antibodies are associated with the onset and progression of NPSLE [34, 35]. Whether treatment with LPA or LPA receptor agonists affects the levels of these autoantibodies should be examined in future studies. In addition, we need to consider the possibility that intraperitoneal administration of LPA may affect systemic immunity or inflammation in MRL/lpr mice. Thus, whether treatment with LPA affects immune cells such as lymphocytes and macrophages, as well as other organs, should also be clarified. Our results suggest that treatment with LPA may improve depression-like behaviours in MRL/lpr mice by suppressing microglia activation and BBB vulnerability. Therefore, LPA treatment may have a beneficial effect on depressive symptoms in NPSLE patients.

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Conflict of interests

The authors declare no competing of interest.

Author contributions


Data Availability

The data underlying this article are available in the article.

The animal research adheres to the ARRIVE guidelines

All experiments were designed and performed in compliance with the Institutional Ethical Guidelines for Animal Experiments of the National Defense Medical College (Tokorozawa, Japan).

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References


