Delayed microglial activation associated with the resolution of neuroinflammation in a mouse model of sublethal endotoxemia-induced systemic inflammation

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ABSTRACT

Systemic inflammation affects brain functions. In our previous study in which lipopolysaccharide (LPS) was injected intraperitoneally into mice at sublethal doses, choroid plexus macrophages produced interleukin-1β and stimulated neighboring stromal cells. Activated stromal cells stimulate choroid plexus epithelial cells, and then choroid plexus epithelium-derived cytokines enter the brain parenchyma and stimulate astrocytes. Stimulated astrocytes then produce cytokines such as CCL11, CXCL10 and G-CSF and change the brain parenchymal microenvironment. However, the effects of an altered brain microenvironment on other brain cells remain to be determined. In the present study, we hypothesized that microglia are activated in response to astrocyte-induced changes in the brain microenvironment. Using the brains of mice treated with intraperitoneal LPS injection, Luminex multiplex cytokine immunoassays revealed increased hippocampal concentrations of CCL11, CXCL10 and G-CSF at 48 h after systemic LPS challenge. The concentrations of all cytokines examined returned to control levels at 72 h after LPS injection, which indicated a resolution of the neuroinflammation. Immunohistochemistry revealed that microglia were hypertrophied in mice at 48 h after systemic LPS challenge. Following isolation of microglial cells from the brain using magnetic-activated cell sorting, gene expression assays were performed with real-time reverse transcriptase-polymerase chain reaction. Isolated microglial cells exhibited much higher gene expression of the receptors for CCL11, CXCL10 and G-CSF than other brain cells. Microglial cells isolated from the brains of mice at 48 h after systemic LPS challenge exhibited the M2-like phenotype. In conclusion, microglial hypertrophy occurs following astrocytic reactions in a mouse model of sublethal endotoxemia-induced systemic inflammation, and hypertrophic microglia are polarized toward the M2-like phenotype and involved in the resolution of neuroinflammation.

1. Introduction

The central nervous system during both inflammatory and non-inflammatory conditions interacts with the peripheral immune system. One clinically important disorder in which the peripheral systemic inflammation affects brain functions is sepsis-associated encephalopathy [1–4]. Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (Sepsis 3) [5,6]. Systemic inflammatory response syndrome (SIRS), although it has moved away from the concept of Sepsis 3, is still considered as an important clinical condition [7] in which systemic immune activation perturbs brain homeostasis. Sepsis and SIRS have been modeled by treating animals with cecal ligation and puncture [8], intraperitoneal cecal slurry [9] and systemic LPS administration [10,11].

Using mice with endotoxemia induced by intraperitoneal (IP) LPS injection, we previously examined the histological architecture of the pathway through which the effects of systemic inflammation are transmitted to the brain parenchyma. Our results showed that there was stimulation of macrophages of the choroid plexus stroma and leptomeninges within 1 h after the IP injection of the endotoxin.

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(lipopolysaccharide, LPS, derived from E. coli) into mice at a dose of 3 mg/kg [12]. The macrophages produce interleukin (IL) -1 and stimulate neighboring stromal cells, especially of the choroid plexus, via the IL-1 receptor. Activated stromal cells produce early cytokines such as CC-motif ligand (CCL)2, CXC-motif ligand (CXCL)1, CXCL2 and IL-6 and stimulate choroid plexus epithelial cells via the corresponding cytokine receptors [12]. The choroid plexus epithelial cells also produce CCL2, CXCL1 and CXCL2 and stimulate stromal cells in a reciprocal manner. Simultaneously, there is entrance via the brain parenchyma, which presumably occurs through the cerebrospinal fluid (CSF), in addition to attachments of choroid plexus by the choroid plexus epithelium-deriv ed cytokines [13-15]. Astrocytes have been shown to be the major brain parenchymal cells that are targeted by choroid plexus-derived cytokines. As the astrocytes contain cytokine receptors on the endfeet, these processes are involved in the choroid plexus interactions [13]. After stimulation of the astrocytes, late cytokines such as CCL11, CXCL10 and granulocyte-colony stimulating factor (G-CSF) are subsequently produced, thereby changing the microenvironment of the brain parenchyma [14,15]. However, it remains to be clarified how such changes in the brain microenvironment affect the other cells including microglia.

We hypothesized that microglia are activated in response to astrocyte-induced changes in the brain microenvironment, which includes alterations in cytokine concentrations, and thus, exert pro-inflammatory or anti-inflammatory functions. Microglia are brain resident macrophages [16,17], and therefore are considered to be involved in a variety of immune responses exerted by the brain [18,19]. Peripheral macrophages alter their phenotype depending on the microenvironmental status: M1 polarization in which macrophages function as a pro-inflammatory driver versus M2 polarization in which macrophages function as an anti-inflammatory driver [20,21]. Alterations in the representative gene expression have been reported to differentiate between the M1 and M2 phenotypes [22,23]. Macrophages that are activated by proinflammatory stimuli then undergo an energy metabolic switch from the initial state of the cells before treatment [24]. It has been reported that microglia also undergo phenotypic alteration in response to the microenvironment and represent the M1-like versus the M2-like phenotype [25]. In the present study, using the brains of mice with sublethal endotoxemia-induced systemic inflammation, we determined the brain tissue concentrations of various cytokines at 48–72 h after systemic LPS challenge and studied the gene expression patterns of microglial cells isolated from brains at 48 h after LPS administration.

2. Experimental procedures

2.1. Animals

Male CS7BL/6NCrSlc (B6) mice at the age of 8 weeks were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Following acclimatization, mice were treated with IP injection of LPS derived from E. coli O55:B5 (Sigma, St. Louis, MO, USA) at a dose of 3 mg/kg, in which LPS was dissolved in physiological saline at a total volume of 7.5 mL/kg. Control mice were treated with IP injections of physiological saline at the same volume. The National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) were followed for the handling of all of the mice. The Institutional Animal Care and Use Committee of the Kyorin University Faculty of Health Sciences (Protocols 117-08-03 and 117-08-04) approved all of the experiments described here.

2.2. Tissue protein extraction and multiplex cytokine immunoassays

Using 11-week-old mice at 48 after treatment (n = 4) and 72 h after treatment (n = 4), protein extracts were prepared from the hippocampi and spleens. Mice were deeply anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) at 48 h and 72 h after the IP injection of LPS or saline. In order to reduce the contribution of blood cytokines, we used transcardial perfusion with sterile phosphate-buffered saline (PBS) to flush the blood. After the brains were rapidly removed, the hippocampi were dissected out on ice under a stereomicroscope. The spleens were also harvested. Tissues were snap frozen in liquid nitrogen and kept at −80 °C prior to protein extraction. Using a BioMasher II (Nippi, Tokyo, Japan) with 20 volumes of Tissue Protein Extraction Reagent (T-PER; Thermo Fisher Scientific, Waltham, MA, USA) containing Halt Protease Inhibitor Cocktail, EDTA-free (Thermo Fisher Scientific), we separately homogenized the spleen and one side of the hippocampus. The tissue homogenates were centrifuged at 16,000 × g for 5 min at −4 °C. The supernatants were collected as the protein extracts and kept frozen at −80 °C.

Using a multiplex immunoassay system Luminex 200 xPONENT (Luminex, Austin, TX, USA) and MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (MCYTOMAG-70 K) (Merck Millipore, Billerica, MA, USA), we measured tissue concentrations of 15 cytokines: CCL2, CCL11, CXCL1, CXCL2, CXCL10, G-CSF, interferon (IFN)-γ, IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-12, IL-17 and tumor necrosis factor (TNF)-α. All assays were run in duplicate (for spleen) or triplicate (hippocampus).

2.3. Frozen tissue section preparation and immunohistochemistry

Three-month-old B6 mice were deeply anesthetized with ketamine and xylazine at 48 h after the injection of LPS or saline (n = 3 each). After perfusion with 80 mL of 4% paraformaldehyde at a rate of 10 mL/min, blood was flushed by transcardial perfusion with PBS. Using the same fixative, heads of mice were soaked overnight at 4 °C. After each brain was removed, we made coronal slices of the cerebral cortex that coronal sections through the hippocampus were obtained. Subsequently, we then cryo-protected the tissues using 20% sucrose, followed by embedding of the samples in Tissue-Tek™ Optimal Cutting Temperature Compound (OCT, Sakura Finetek Japan, Tokyo, Japan). Finally, the samples were frozen in 100% ethyl alcohol cooled with dry ice. The frozen tissue blocks were maintained at −80 °C until use. The samples were then cut into 16-μm thick sections using a cryostat (CM3050 S; Leica, Wetzlar, Germany). Following the mounting of the sections on coated slides, a Buchi V-100 Vacuum Pump (Fisher Scientific, Leicestershire, UK) was used for the purpose of air-drying and then vacuum-drying the sections. All samples were stored at −20 °C prior to undergoing immunohistochemistry.

Prior to performing the immunohistochemical staining, the frozen sections were soaked in Tris-buffered saline with Tween 20 (TBS-T) for 10 min. Samples then underwent preincubation using a blocking solution (5% normal horse serum in TBS-T) These samples were then incubated overnight at room temperature with rabbit monoclonal antibodies for ionized-calcium binding adaptor molecule-1 (Iba-1) EPR16588 (Abcam, Cambridge, UK). Sections were subsequently incubated with reagents from the ImmPRESS HRP Anti-Rabbit (Peroxidase) Polymer Detection Kit (Vector Laboratories, Burlingame, CA, USA) at room temperature for 1 h. We visualized reactions by incubating sections with an ImmPACT DAB Substrate Kit, Peroxidase (SK-4105; Vector Laboratories). Nuclei were counterstained with hematoxylin. Sections were dehydrated with 80%, 90%, 95% and 100% ethanol sequentially, cleared with xylene and coverslipped with HSR mounting medium (Sysmex, Kobe, Japan). We examined sections using an Eclipse Ci-L light microscope (Nikon, Tokyo, Japan).

2.4. Morphometric analyses of Iba-1-positive cells

Sections stained by immunohistochemistry for Iba-1 prepared from mice at 48 h after the injection of LPS or saline (n = 3 each) were subjected to morphometry. Using a 20 × PlanApo λ lens (Nikon) of an Eclipse CI-L light microscope equipped with a digital camera control unit DS-Fi3/DS-L4 (Nikon), images of the CA1 region of the dorsal
hippocampus were taken with a rectangular visual field of 622 × 442 μm. Every visual field was set so that the strata pyramidale, radiatum and lacunosum-molecular are included. The images were analyzed using the Image Analysis/Measurement software WinROOF 2018 (Mitani Corporation, Tokyo, Japan). Immunopositive structures were automatically detected and binarized. Individual cells that bore multiple cytoplasmic processes and recognizable cell bodies were subjected to further analysis. If individual cells had a total area (cell body and processes) larger than 150 μm², such cells were regarded as “hypertrophic microglial cells” and counted.

2.5. Isolation of microglia using magnetic-activated cell sorting

Three-month-old B6 mice were deeply anesthetized with ketamine and xylazine at 48 h after the injection of LPS or saline (n = 4 each). Blood was flushed by transcendal perfusion with sterile PBS to expel blood cells. After the brains were rapidly removed, whole brains were dissociated by enzymatic digestion of the extracellular matrix using the Adult Brain Dissociation Kit for mice (Miltenyi Biotec, Auburn, CA, USA). The mechanical dissociation steps were performed using the gentleMACS® Octo Dissociator with Heaters (Miltenyi Biotec) according to the manufacturer’s protocol. Brain tissue dissociates were centrifuged at 400 × g for 5 min at 4 °C. Pellets were resuspended in cold Dulbecco’s phosphate-buffered saline with calcium and magnesium containing 0.5% bovine serum albumin (PB buffer). In the presence of the Debris Removal Solution of the kit, cell suspensions were centrifuged at 3000 × g for 12 min at 4 °C to discard the debris phase.

The pellets were resuspended in cold PB buffer and incubated with R-Phycoerythrin (PE)-conjugated primary monoclonal antibody for human/mouse CD11b (130–113–235, Miltenyi Biotec) and Fc Receptor Blocking Reagent (130–092–575, Miltenyi Biotec), followed by incubation with MicroBeads UltraPure conjugated to monoclonal anti-PE antibodies (130–105–639, Miltenyi Biotec). Suspended cells labeled with anti-PE MicroBeads were enriched by magnetic separation using an LS-column (Miltenyi Biotec) that was placed in a QuadroMACS® Separator (Miltenyi Biotec) according to the manufacturer’s protocol. CD11b-positive selected cells (Positive Fraction) were regarded as microglia. During the positive selection process, CD11b-negative cells were also collected (Negative Fraction). The numbers of the Positive Fraction cells and Negative Fraction cells were determined using cell counting plates (OneCell counter; Fine Plus International, Kyoto, Japan) and an inverted phase-contrast microscope ECLIPSE Ts2 (Nikon). The Positive Fraction cells and Negative Fraction cells were finally suspended in 1 mL CELLBANKER® 1 Plus (TaKaRa, Kusatsu, Japan) and stored at −80 °C prior to RNA extraction.

2.6. Real-time RT-PCR gene expression assays of isolated cells

Frozen cells were rapidly thawed at 37 °C and centrifuged at 400 × g for 5 min at 4 °C and washed with RNase-free PBS (10 × PBS Buffer, AM9624 and UltraPure™ DNase/RNase-Free Distilled Water, 19977015, Invitrogen-Thermo Fisher Scientific, Waltham, MA, USA) by centrifugation under the same conditions. Total RNA was extracted from the cell pellets using the RNAqueous™-Micro Kit (Invitrogen) according to the manufacturer’s protocols. RNA quantification was performed by photometry using NanoVue (GE Healthcare Life Sciences, Boston, MA, USA). Utilizing the individual cell suspension samples, two hundred nanograms of total RNA were extracted and then used to perform reverse transcription to cDNA via SuperScript® III Reverse Transcriptase (Invitrogen). Subsequently, using TaqMan Fast Advanced Master Mix (Applied Biosystems-Thermo Fisher Scientific) and a TaqMan primer/probe set for 18 targets (all from Applied Biosystems), real-time quantitative RT-PCR (qRT-PCR) was then performed as follows: (1) for the validation of CD11b – cell isolation: ftagm (encoding integrin alpha-M or CD11b), Mm00434455_m1; Aif1 (encoding allograft inflammatory factor 1 or Iba-1), Mm00479862_g1; (2) for the cytokine receptors: Ccr2 (encoding C-C chemokine receptor type 2), Mm01216173_m; Csf3r (encoding granulocyte colony-stimulating factor receptor), Mm00432735_m1; Mm00438259_m1; Cxcr3 (encoding C-X-C chemokine receptor type 3), Mm00438259_m1; (3) for the M1 phenotype [22, 23]: Gd68 (encoding CD86), Mm00444540_m1; Pigs2 (encoding cyclooxygenase-2), Mm00478374_m1; Nos2 (encoding nitric oxide synthase, inducible), Mm00440502_m1; (4) for the M2 phenotype [22, 23]: Mrc1 (encoding macrophage mannose receptor 1 or CD206), Mm01329362_m1; Arg1 (encoding arginase-1), Mm00475988_m1; Chl3 (encoding chitinase-like protein 3 or Ym1); Lgs3 (encoding galexin-3), Mm00802901_m1; (5) for the glycolysis enzymes: Hk2 (encoding hexokinase-2), Mm00443385_m1; Pklr (encoding pyruvate kinase PKLR), Mm00443090_m1; and (6) for the tricarboxylic acid (TCA) cycle enzymes: Aco2 (encoding aconitate hydratase, mitochondrial), Mm00475673_g1; Sdha (encoding succinate dehydrogenase flavoprotein subunit, mitochondrial), Mm01325366_m1; Cs (encoding citrate synthase, mitochondrial), Mm00466043_m1. Using a 7500 Fast Real-Time PCR System (Applied Biosystems), reactions were run in accordance with the manufacturer’s protocols. For the reference gene, Hprt (encoding hypoxanthine phosphoribosyltransferase) was used. Analysis of the relative transcript levels was performed using the ∆∆Ct method. All assays were run in duplicate or triplicate.

2.7. Statistical analysis

For the tissue cytokine immunoassays of mice from the four experimental conditions (saline vs. LPS administration; 48 h vs. 72 h after treatment), the results were analyzed using two-way analysis of variance (ANOVA; main effects of time and treatment) with non-repeated measures. Post hoc tests were performed using Tukey’s procedure. For the morphometric analysis, the numbers of “hypertrophic microglial cells” were analyzed with Student’s unpaired t test. For the analyses of isolated cells, we performed real-time RT-PCR gene expression. Analyses of the relative transcript levels were undertaken using non-repeated two-way ANOVA using STATISTICA (StatSoft, Tulsa, OK, USA). In order to compare the mean ∆Ct values, we used a statistical design for which the main effects were defined as the cell fractions (CD11b-positive vs. CD11b-negative) and the treatments (saline vs. LPS administration). Post hoc tests were performed using Tukey’s procedure. P-values less than 0.05 were considered as significant in all analyses.

3. Results

3.1. Endotoxiaemia-induced changes in the hippocampal cytokine levels

Mice treated with LPS exhibited sickness behavior around 24 h after injection and body weight changes of −3.25 ± 1.03 g (mean ± SEM, n = 4) at 48 h and −3.50 ± 1.19 g (mean ± SEM, n = 4) at 72 h after injection. In contrast, saline-treated mice had body weight changes of 0 ± 0.40 g (mean change ± SEM, n = 4) at 48 h and 0.25 ± 0.25 g (mean change ± SEM, n = 4) at 72 h after injection. Since none of the LPS-treated mice died prior to experimental use in the present study, we considered that IP injection of LPS at a dose of 3 mg/kg was sublethal.

Multiplex immunoassays revealed that the hippocampal concentrations of CCL11, CXCL10 and IL-1α were significantly higher in LPS-treated mice than in saline controls at 48 h after injection (Fig. 1). At 72 h after the IP LPS injection, the concentration of each cytokine returned to its control level. The hippocampal G-CSF concentration appeared to be higher in mice at 48 h but not 72 h after LPS challenge as compared to the saline controls, although the difference was not significant. The hippocampal concentrations of CCL2, CXCL1, CXCL2, IFN-γ, IL-6, IL-10, IL-17 and TNF-α were similar between mice treated with LPS and saline (Fig. 2). The hippocampal concentration of IL-1β was rather lower in LPS-treated mice at 48 h and 72 h after injection.
compared to that in saline-treated mice (Fig. 3). Similarly, the hippocampal concentrations of IL-4 and IL-12 were lower in LPS-treated mice at 48 h and 72 h, respectively, as compared to those in saline-treated mice (Fig. 3). However, the tissue concentrations of IL-1β, IL-4 and IL-12 in LPS-treated mice were so low that the potential biological significance of these minor changes is uncertain.

Fig. 1. Endotoxemia-induced increase in the hippocampal cytokine concentrations. Tissue cytokine concentrations were determined with multiplex immunoassays. The concentrations of CCL11, CXCL10 and interleukin (IL)-1α in the hippocampus were increased significantly at 48 h after systemic LPS challenge. mean ± SEM, n = 4 per experimental group. *p < 0.05 and **p < 0.01, compared to saline control mice. Green lines indicate a comparison between 48 h and 72 h and green asterisk indicates p < 0.05 (*). The concentration of the hippocampal G-CSF appeared to be elevated at 48 h, although the difference was not significant.

Fig. 2. Hippocampal cytokine concentrations that were not changed after endotoxemia. The concentrations of CCL2, CXCL1, CXCL2, interferon (IFN)-γ, IL-6, IL-10, IL-17 or tumor necrosis factor (TNF)-α were not changed in the hippocampus at 48 h or 72 h after systemic LPS challenge. mean ± SEM, n = 4 per experimental group.
Fig. 3. Endotoxemia-induced decrease in the hippocampal cytokine concentrations. The concentrations of IL-1β, IL-4 and IL-12 were decreased significantly at 48 h and/or 72 h after systemic LPS challenge. mean ± SEM, n = 4 per experimental group. *p < 0.05 and **p < 0.01, compared to saline control.

Fig. 4. Endotoxemia-induced increase in the splenic cytokine concentrations. The concentrations of CCL2, CXCL1, CXCL10, G-CSF, IL-1β, IL-6, IL-10 in the spleen were increased significantly at 48 h after systemic LPS challenge. mean ± SEM, n = 4 per experimental group. *p < 0.05 and **p < 0.01, compared to saline control. Green lines indicate a comparison between 48 h and 72 h and green asterisks indicate p < 0.01 (**).
3.2. Endotoxemia-induced changes in the splenic cytokine levels

At both 48 h and 72 h after the injection, the multiplex immunoassays demonstrated that there was a significantly higher splenic concentration of CCL2 in LPS-treated mice versus the saline controls (Fig. 4). In addition, the splenic concentrations of CXCL1, CXCL10, G-CSF, IL-1β, IL-6 and IL-10 were significantly higher in LPS-treated mice than in saline controls at 48 h after injection (Fig. 4). At 72 h after the LPS challenge, the cytokine concentrations returned to control levels. The splenic concentrations of CCL11, CXCL2, IFN-γ, IL-1α, IL-4, IL-12, IL-17 and TNF-α did not differ significantly between mice treated with LPS and saline at 48 h or 72 h after injection (Fig. 5).

3.3. Immunohistochemistry and morphometry for microglial changes

Anti-Iba-1 immunohistochemistry revealed that at 48 h after LPS injection, the microglial cells in the hippocampus as well as the neocortex of mice were hypertrophied compared with those of saline-treated mice (Fig. 6A and B). Microglial cell bodies of LPS-treated mice were enlarged and accompanied by thickened cytoplasmic processes that were relatively shortened compared to the microglial cells of saline-treated mice. These changes were most easily detectable in the hippocampus, but similar microglial hypertrophy was also detectable in the neocortical regions throughout the cerebrum. Morphometric analysis indicated that the number of “hypertrophic microglial cells” was 34.6 ± 8.2 (mean ± SEM) for LPS-treated mice, whereas it was 6.6 ± 4.2 for saline control mice (Fig. 6C). Therefore, hippocampal microglia became hypertrophic at 48 h after systemic LPS challenge. The hypertrophic morphology of microglia led us to study the gene expression profile of the microglia isolated from fresh brains of mice at 48 h after treatment with LPS or saline.

3.4. Real-time RT-PCR gene expression assays to validate microglial isolation

The number of Positive Fraction cells was (4.27 ± 0.54) x 10⁵ (mean ± SEM) and that of Negative Fraction cells was (2.00 ± 0.49) x 10⁶ (mean ± SEM). The gene expression level of Itgam in the Positive Fraction isolated from the brains of saline control mice was 325 times as high as that in the Negative Fraction (p < 0.01). The expression of Itgam in the Positive Fraction isolated from the brains of LPS-treated mice was 135 times as high as that in the Negative Fraction (p < 0.01; Fig. 7). In addition, the expression of Aif1 in the Positive Fraction isolated from the brains of saline control mice was 1105 times as high as that in the Negative Fraction cells (p < 0.01; Fig. 7). Therefore, we considered that our separation of the Positive Fraction from the Negative Fraction prepared from fresh brain dissociates was successful and thus, regarded the Positive Fraction cells as microglial cells. LPS treatment-induced changes in the expression of Itgam or Aif1 in microglial cells were not significant. Flow cytometric analysis of representative Positive Fractions and Negative Fractions to show the viability and purity of isolated cells from a naive mouse is described in Supplementary Figure.

3.5. Cytokine receptor gene expression of microglia based on the real-time RT-PCR

There was a 10.3 times as high Ccr3 gene expression level in the microglial cells isolated from the brains of the saline control mice as compared to the CD11b-negative brain cells isolated from the same mice (p < 0.05; Fig. 8). The expression of Ccr3 in microglial cells isolated from the brains of LPS-treated mice was 12.0 times as high as that in...
3.6. Real-time RT-PCR gene expression assays to characterize microglial decrease compared to that of saline control mice (p < 0.05). The expression of Cd86 in microglial cells isolated from the brains of saline control mice was 200 times as high as that in CD11b-negative brain cells isolated from the same mice (p < 0.01; Fig. 8). The expression of Csf3r in microglial cells isolated from the brains of LPS-treated mice was 87.4 times as high as that in CD11b-negative brain cells (p < 0.01). The LPS treatment-induced change in the expression of microglial Ccr3 or Csf3r was not significant. The gene expression level of Cxcr3 in microglial cells isolated from the brains of saline control mice was 2.80 times as high as that in CD11b-negative brain cells isolated from the same mice (p < 0.01; Fig. 8). However, the expressions of Cxcr3 in microglial cells and CD11b-negative brain cells isolated from the brains of LPS-treated mice were comparable. Surprisingly, Cxcr3 expression in microglial cells isolated from the brains of LPS-treated mice exhibited a 0.369-fold decrease compared to that of saline control mice (p < 0.01).

3.6. Real-time RT-PCR gene expression assays to characterize microglial phenotype

The gene expression level of Cd86 in microglial cells isolated from the brains of saline control mice was 200 times as high as that in CD11b-negative brain cells (p < 0.01; Fig. 9), while that in microglial cells isolated from the brains of LPS-treated mice was 193 times as high as that in CD11b-negative brain cells (p < 0.01). However, the microglial Cd86 gene expression level did not differ significantly between cells isolated from the brains of LPS-treated mice and saline control mice. The gene expression level of Csf3r in CD11b-negative brain cells increased 13.5-fold (p < 0.01; Fig. 9) in cells isolated from LPS-treated mice compared to those from control mice. In contrast, the microglial Ptgs2 gene expression level did not differ significantly between cells isolated from the brains of LPS-treated mice and saline control mice. There was no significant change in the gene expression level of Nos2 in cells isolated from saline control mice and LPS-treated mice. Therefore, our data indicated there was no evidence supporting microglial polarization toward the M1 phenotype following systemic LPS injection.

The gene expression level of Mrc1 in microglial cells isolated from the brains of saline control mice was 88.5 times as high as that in CD11b-negative brain cells isolated from the same mice (p < 0.01; Fig. 10). The expression of Mrc1 in microglial cells isolated from the brains of LPS-treated mice was 43.1 times as high as that in CD11b-negative brain cells (p < 0.01). However, there was no significant change in the gene expression level of Arg1 in microglial cells isolated from saline control mice and LPS-treated mice compared to those from control mice in either microglial or CD11b-negative brain cells. The gene expression level of Aif1 in microglial cells isolated from the brains of LPS-treated mice increased 101-fold compared to saline control mice.
compared to that from saline control mice (p < 0.01; Fig. 10). There was no significant change in the Arg1 gene expression level in CD11b-negative brain cells isolated from LPS-treated mice compared to those from control mice. There was a 1245 times as high Chil3 gene expression level in microglial cells isolated from saline control mice brains as compared to that in CD11b-negative brain cells (p < 0.01; Fig. 10), while in microglial cells isolated from the brains of LPS-treated mice, it was 90.3 times higher than that found in CD11b-negative brain cells (p < 0.01). There was a 4.22-fold increase in the level in microglial cells isolated from the brains of LPS-treated mice versus the saline control mice (p < 0.05), while there was a 58.2-fold increase for the CD11b-negative brain cells isolated from LPS-treated mice as compared to that observed from control mice (p < 0.01). There was a 2.44 times as high Lgals3 gene expression level in microglial cells isolated from saline control mice versus that in CD11b-negative brain cells isolated from the same mice (p < 0.01; Fig. 10), while the level was 1.8 times higher in microglial cells isolated from LPS-treated mice brains than in CD11b-negative brain cells. The transcript levels are graphically represented with average relative normalized fold expression and error bars based on SEM of $\Delta C_T$. $n = 4$ per experimental group, * $p < 0.05$, compared to saline control. Green lines indicate comparisons between the Positive Fraction and the Negative Fraction and green asterisks indicate p < 0.01 (**).

Fig. 8. Gene expression assays of three cytokine receptors. The gene expression levels of Ccr3, Csf3r and Cxcr3 encoding the receptors for CCL11, G-CSF and CXCL10, respectively, were determined with the real-time RT-PCR. None of these cytokine receptor genes were upregulated after systemic LPS challenge in either microglial cells or CD11b-negative brain cells. Only a systemic LPS-induced change was evident in the decreased Cxcr3 expression of microglial cells. In general, the expression levels of Ccr3, Csf3r and Cxcr3 were higher in microglial cells than in CD11b-negative brain cells. The transcript levels are graphically represented with average relative normalized fold expression and error bars based on SEM of $\Delta C_T$. $n = 4$ per experimental group, * $p < 0.01$, compared to saline control. Green lines indicate comparisons between the Positive Fraction and the Negative Fraction and green asterisks indicate p < 0.01 (**).

Fig. 9. Gene expression assays of the M1 phenotype-related genes. The expression levels of M1 phenotype-related genes such as Cd86, Ptgs2 and Nos2 were determined with the real-time RT-PCR. None of the M1-related genes were upregulated after systemic LPS challenge in microglial cells. The transcript levels are graphically represented with average relative normalized fold expression and error bars based on SEM of $\Delta C_T$. $n = 4$ per experimental group, * $p < 0.01$, compared to saline control. Green lines indicate comparisons between the Positive Fraction and the Negative Fraction and green asterisks indicate p < 0.01 (**).
systemic LPS challenge.

3.7. Real-time RT-PCR to study the glycogen metabolism-related genes in microglia

There was a 59.8 times as high \( Hk2 \) gene expression level in microglial cells isolated from the brains of saline control mice as compared to that in CD11b-negative brain cells (\( p < 0.01; \) Fig. 11), while the level was 40.9 times higher in microglial cells isolated from LPS-treated mice brains than in CD11b-negative brain cells (\( p < 0.01 \)). However, there was no significant change in the gene expression level of \( Hk2 \) in cells isolated from LPS-treated mice compared to those from control mice in either microglial or CD11b-negative brain cells. The gene expression levels of \( Pfkl \) (Fig. 11) and \( Pklr \) (Fig. 11) in microglial cells isolated from saline control mice were comparable to those in CD11b-negative brain cells, and those in microglial cells isolated from LPS-treated mice were comparable to those in CD11b-negative brain cells. There was no significant change in the gene expression levels of \( Pfkl \) and \( Pklr \) in cells isolated from LPS-treated mice compared to those from control mice in either microglial or CD11b-negative brain cells. Therefore, our data indicated there was no evidence supporting a microglial energy metabolic switch towards glycolysis following systemic LPS injection.

The gene expression levels of \( Aco2 \) (Fig. 11), \( Sdha \) (Fig. 11) and \( Cs \) (Fig. 11) in microglial cells isolated from the brains of saline control mice were comparable to those in CD11b-negative brain cells, and those in microglial cells isolated from LPS-treated mice were comparable to those in CD11b-negative brain cells. There were no significant changes in the gene expression levels of \( Aco2 \), \( Sdha \) or \( Cs \) in cells isolated from LPS-treated mice compared to those from control mice in either microglial or CD11b-negative brain cells. Therefore, our data indicated there was no evidence supporting a microglial energy metabolic switch away from the TCA cycle following systemic LPS injection. However, as a further investigation, protein levels and enzymatic activity in glycogen metabolism would be evaluated and performed using isolated microglia. A specified study to measure the activity and phosphorylation state of critical enzymes would contribute.
to deepening insights in the microglial responses to systemic LPS injection.

4. Discussion

4.1. Resolution of neuroinflammation following endotoxemia-induced systemic inflammation

As we reported in our previous study, brain parenchymal CCL2, CXCL1, CXCL2, IL-6 and TNF-α were “early cytokines”, since the brain concentrations of these cytokines were elevated at 4 h after systemic LPS challenge and returned to the saline control levels by 24 h [15]. Our present study indicated that there was no subsequent increase in these early cytokines up to 72 h after the systemic LPS challenge.

In contrast, we have also previously reported that since the brain concentrations of CCL11, CXCL10 and G-CSF were found to start increasing at 4 h after the systemic LPS challenge, which was maintained at these elevated levels at 24 h, these cytokines can be defined as “late cytokines” [15]. The results of our current study demonstrated that at 48 h after systemic LPS challenge, there were increases in the hippocampal concentrations of CCL11 and CXCL10, with these changes returning to the saline control level at 72 h. In addition, hippocampal G-CSF concentration appeared to increase at 48 h after systemic LPS challenge, although the difference was not significant and returned to the control level at 72 h. Thus, the concentration of the hippocampal CCL11, CXCL10 and G-CSF started to increase at 4 h after systemic LPS challenge, with the levels continuing to be elevated for up to 48 h, and the concentrations then returning to quiescent conditions by 72 h. Therefore, from the viewpoint of tissue cytokine concentrations, endotoxemia-induced hippocampal neuroinflammation was resolved by 72 h after systemic LPS challenge.

The present study indicated that the hippocampal concentration of IL-1β increased at 48 h after systemic LPS challenge. However, the increase was transient, since the hippocampal IL-1β concentration did not differ between mice treated with LPS and saline at any time other than 48 h after treatment (data not shown).

In our current results, we found that the splenic cytokine concentrations differed from those found in the brain, with these changes shown to be time-dependent. We previously reported that the splenic concentrations of CCL2, CXCL1, CXCL10, IL-1β and IL-6 were elevated at 1 and 4 h after systemic LPS challenge [14]. The present study revealed that the concentrations of these cytokines continued to be elevated at 48 h after the LPS challenge. The splenic CCL2 concentration was especially high for up to 72 h. The splenic concentration of IL-10 was shown in our previous report to be increased at 1 h after LPS challenge [14], while in the present study it was increased at 48 h after the LPS challenge. The splenic concentration of G-CSF began to increase at 4 h [14] and continued to be elevated up to 48 h after LPS challenge. The splenic CCL11 concentration increase was transient in that it did not differ between mice treated with LPS and saline at any time other than 4 h after treatment [14]. Overall features of the results from the present study and our previous ones suggest that the inflammatory milieu is resolved more quickly in the brain compared to the spleen.

Our previous study also revealed that IL-1β was produced by macrophages of various organs immediately after systemic LPS challenge [12]. Overall, the findings for the intracranial tissues showed that while IL-1β could not be detected in the brain parenchyma, it could be detected in macrophages of the choroid plexus and leptomeninges. In the present study, IL-1β was not detectable in the hippocampus up to 72 h after LPS challenge. The present data thus indicated that brain parenchymal microglia do not respond by producing IL-1β in our sepsis model, which does not mean, however, that microglia do not respond at all to endotoxemia-induced systemic inflammation.

4.2. Microglial hypertrophic changes in response to systemic inflammation

Microglia especially in the hippocampus underwent hypertrophic changes in mice at 48 h after systemic LPS challenge. As we have previously reported, the intracranial cells that initially respond to systemic inflammation were choroid plexus macrophages but not brain parenchymal microglia [12]. The choroid plexus macrophages produced IL-1β to initiate cytokine-mediated reactions at 1–4 h following LPS challenges. IL-1 receptor-bearing choroid plexus cells were stimulated to produce cytokines (CCL2, CXCL1, CXCL2 and IL-6). Since astrocytes had receptors for these cytokines, astrocytic cytoplasmic processes including endfeet were thickened at 4 h after LPS with an enhanced expression of vimentin [14]. Compared to such preceding responses by astrocytes, microglial cells did not exhibit hypertrophy. When astrocytes started to produce CCL11, CXCL10 and G-CSF at 4–24 h after LPS, morphological changes of microglia were still not evident. Microglia isolated from the brains of mice at 48 h after LPS or saline injection exhibited higher gene expression levels of Ccr3 (receptor for CCL11), Cxcr3 (receptor for CXCL10) and Csf3r (receptor for G-CSF) as compared to that for the other brain cells, regardless of the presence or absence of endotoxemia. Therefore, the increases of the CCL11, CXCL10 and G-CSF in the hippocampus of LPS-treated mice at 4–48 h after systemic LPS challenge are considered to stimulate microglia, which leads to delayed hypertrophic changes.

4.3. Microglial phenotyping based on the gene expression profile

Macrophages have been shown to play distinct roles in the initiation and resolution of inflammation and are classified into M1 (pro-inflammatory) or M2 (anti-inflammatory) phenotypes [21,26]. While there is often a shift in cells from oxidative phosphorylation to aerobic glycolysis for energy production that occurs during M1 polarization, in contrast, during the M2 polarization, the energy metabolic state continues as oxidative phosphorylation [27,28]. It has also been demonstrated that microglia can be classified into two phenotypes as well, M1-like or M2-like [25,29].

Within the microglial cells isolated from the brains of LPS-treated mice at 48 h after injection, our present study found that there was an increased expression of M2 polarization-associated genes such as Arg1, Chi3l3 and Lgals3 [22]. In contrast, there was no significant increase in the expression of M1 polarization-associated genes such as Cdb6, Ptgs2 or Nos2 [22] in microglial cells isolated from the brains of LPS-treated mice at 48 h after injection. In addition, microglial cells isolated from the brains of LPS-treated mice at 48 h after injection did not exhibit any switch in glycogen energy metabolism. Therefore, microglia in LPS-treated mice underwent hypertrophic changes at 48 h after the initiation of endotoxemia accompanied by a polarization toward the M2-like phenotype. These changes indicated that endotoxemia-induced pro-inflammatory cytokines of microglia are oriented toward anti-inflammatory ones and designed to resolve neuroinflammation. This is consistent with our present data that showed the hippocampal concentrations of all 15 cytokines examined returned to quiescent conditions by 72 h after the initiation of endotoxemia. Hippocampal concentrations of pro-inflammatory cytokines such as IL-1β and IL-12 were found to show decreases at 72 h after the initiation of endotoxemia. Considering that the splenic CCL2 concentration was still elevated in LPS-treated mice even at 72 h after the initiation of endotoxemia, the brain may possess a capacity to resolve tissue inflammatory state more quickly than the spleen. Whether or not the microglial changes in response to sublethal endotoxemia-induced systemic inflammation that were discovered during our present study have potential long-term effects on brain functions will need to be further examined in a subsequent study.

4.4. Conclusion

Microglial hypertrophy occurs following astrocytic reaction in a...
sepsis of sublethal endotoxemia-induced systemic inflammation. Astrocyte-derived cytokines activate microglia to polarize toward the M2-like phenotype. Hypertrophic microglia in our endotoxemia model are involved in the resolution of neuroinflammation.

Author roles

AS designed the whole study, carried out experiments, analyzed the data and drafted the manuscript. MM carried out the initial pilot experiments to start up the entire project. SA prepared samples and performed multiplex cytokine immunoassays. HA prepared samples and performed immunohistochemistry. AO analyzed the data and provided fruitful discussion. SHI took part in designing the study, coordinated the project, and analyzed the data. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.06.015.

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