Secreted protein lipocalin-2 promotes microglial M1 polarization

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ABSTRACT Activated macrophages are classified into two different forms: classically activated (M1) or alternatively activated (M2) macrophages. The presence of M1/M2 phenotypic polarization has also been suggested for microglia. Here, we report that the secreted protein lipocalin 2 (LCN2) amplifies M1 polarization of activated microglia. LCN2 protein (EC50 1 μg/ml), but not glutathione S-transferase used as a control, increased the M1-related gene expression in cultured mouse microglial cells after 8–24 h. LCN2 was secreted from M1-polarized, but not M2-polarized, microglia. LCN2 inhibited phosphorylation of STAT6 in IL-4-stimulated microglia, suggesting LCN2 suppression of the canonical M2 signaling. In the lipopolysaccharide (LPS)-induced mouse neuroinflammation model, the expression of LCN2 was notably increased in microglia. Primary microglial cultures derived from LCN2-deficient mice showed a suppressed M1 response and enhanced M2 response. Mice lacking LCN2 showed a markedly reduced M1-related gene expression in microglia after LPS injection, which was consistent with the results of histological analysis. Neuroinflammation-associated impairment in motor behavior and cognitive function was also attenuated in the LCN2-deficient mice, as determined by the rotarod performance test, fatigue test, open-field test, and object recognition task. These findings suggest that LCN2 is an M1-amplifier in brain microglial cells.—Jang, E., Lee, S., Kim, J.-H., Kim, J.-H., Seo, J.-W., Lee, W.-H., Mori, K., Nakao, K., Suk, K. Secreted protein lipocalin-2 promotes microglial M1 polarization. FASEB J. 27, 1176–1190 (2013). www.fasebj.org

Key Words: neuroinflammation · central nervous system · innate immunity · brain

Macrophages mediate innate immune responses to pathogens (1) and contribute to adaptive immune responses, inflammation, and their resolution and repair (2). Microglia, which are macrophages resident in the central nervous system (CNS), play a central role in neuroinflammation in response to CNS injury or infection, and undergo rapid morphological and functional activation, which is manifest as phagocytosis and antigen presentation, as well as the production and secretion of reactive oxygen species (ROS), cytokines, and growth factors (3, 4). Activated macrophages comprise classically activated (M1) or alternatively activated (M2) macrophages (5, 6). Classical activation (M1 polarization) by lipopolysaccharide/interferon-γ (LPS/IFN-γ) or Th1 cytokines is associated with the production of proinflammatory cytokines and chemokines, such as interleukin (IL)-12, IL-23, tumor necrosis factor-α (TNF-α), CC chemokines, and inducible nitric oxide synthase (iNOS), which are involved in cytotoxicity and microbial killing (7, 8). Alternative activation (M2 polarization) by Th2 cytokines IL-4, IL-13, or IL-10 is associated with the expression of scavenger receptors and proangiogenic factors, such as mannose receptor, dectin-1, and arginase, which are involved in adaptive immunity and tissue repair and remodeling (2, 9). M2-polarized macrophages can be further divided into 3 subsets (M2a, M2b, and M2c), including M2 macrophages activated by IL-4 or IL-13 (M2a), immune complexes (ICs) plus IL-1β or LPS (M2b), and TGF-β, glucocorticoids, or IL-10 (M2c). Differential cytokine/chemokine production is a key phenotype of polarized macrophages. However, the existence of such a polarized phenotype is less clearly established for brain microglia (10–16), and little is known about its regulatory mechanisms.

Lipocalin 2 (LCN2), a member of the lipocalin family of proteins, was initially found in neutrophil granules (17) and was later described as an acute-phase
were chopped up and dissociated by mechanical disruption previously (21, 31). In brief, the forebrains of newborn mice from the brains of 2- to 3-d-old ICR mice (Samtako, Osan, mouse primary neonatal microglial cultures were prepared in DMEM supplemented with 5% heat-inactivated FBS (Invitrogen, Sigma-Aldrich). BV-2 mouse microglial cells were maintained in other chemicals, unless otherwise stated, were obtained from Sepharose 4B beads (GE Healthcare, Princeton, NJ, USA). All siderophore. The protein was purified using glutathione-

Escherichia coli-derived mouse LCN2 proteins were purchased from R&D Systems (Minneapolis, MN, USA). The bacterially expressed protein in liver (18), peritoneal cells (19), glial cells (20, 21), and several epithelial tissues (22). LCN2 has multiple functions that include regulation of cell death/survival (23), cell migration/invasion (24), cell differentiation (25), and iron delivery (19, 26, 27). LCN2 expression can be induced under diverse inflammatory conditions (28). LCN2 expression is increased under inflammatory conditions in macrophages (29) and lung epithelial cells (22). Recently, we have reported that the expression and secretion of LCN2 is increased under inflammatory conditions in brain glial cells (20, 21) and that LCN2 induces chemokine expression to promote cell migration in the CNS (30). Furthermore, the expression of LCN2 is enhanced by LPS or M1-polarizing cytokine IFN-γ in glia (20, 21). These results suggest that LCN2 may be involved in the M1 polarization of glial cells in brain. Therefore, this study was carried out to determine how LCN2 influences phenotypic polarization of microglia.

Here, we confirm that a functional dichotomy such as M1 vs. M2 phenotype also exists in brain microglia and present evidence that LCN2 plays an essential role in the M1 polarization of microglia. In particular, LCN2 increases M1-related gene expression, while inhibiting M2-related gene expression and IL-4-induced STAT6 signaling. Using a mouse neuroinflammation model, we show that LCN2 plays a critical role in the M1 polarization of microglia in vivo. Notably, in LCN2-deficient mice, a pronounced reduction is evident in M1-polarized phenotypes, such as microglial M1 gene expression and neuroinflammation-associated impairment in motor behavior and cognition.

MATERIALS AND METHODS

Reagents and cells

The following chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA): LPS from Escherichia coli 0111:B4 prepared by phenolic extraction and gel filtration chromatography, leflunomide (5-methylisoxazole-4-(4-trifluoromethyl) carboxamide; STAT6 inhibitor), ovalbumin (OVA), and polyvinyl pyrrolidone. IgG (Cappel, Durham, NC, USA) with OVA for 30 min at room temperature. Recombinant mouse IL-4, mouse IL-10, and mouse IFN-γ and NSO murine melanoma cell-derived mouse LCN2 proteins were purchased from R&D Systems (Minneapolis, MN, USA). The bacterially expressed recombinant mouse LCN2 protein was prepared as described previously (21). In brief, the recombinant mouse LCN2 protein was expressed as a glutathione-S-transferase (GST) fusion protein in E. coli BL21, which does not synthesize sildemore. The protein was purified using glutathione-Sepharose 4B beads (GE Healthcare, Princeton, NJ, USA). All other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich. BV-2 mouse microglial cells were maintained in DMEM supplemented with 5% heat-inactivated FBS (Invitrogen, Carlsbad, CA, USA) and gentamicin (50 µg/ml). The mouse primary neonatal microglial cultures were prepared from the brains of 2- to 3-d-old ICR mice (Samtako, Osan, Korea) or LCN2-deficient mice (see below), as described previously (21, 31). In brief, the forebrains of newborn mice were chopped up and dissociated by mechanical disruption using a nylon mesh. The cells were seeded in culture flasks.

Mixed glial cultures were established after in vitro culture for 10–14 d. Primary microglial cultures were then prepared from these mixed glial cultures by mild trypsinization as described previously with minor modifications (32). The purity of microglial cultures was >95% as determined by isoelectric B4 staining. Animals were acquired and cared for in accordance with guidelines published in the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Kyungpook National University Animal Experiment Ethics Committee.

Traditional and real-time RT-PCR

Total RNA was extracted from microglial cells cultured in 6-well plates using TRIzol reagent (Invitrogen), according to the manufacturer's protocol. Reverse transcription was conducted using Superscript II (Invitrogen) and oligo(dT) primer. Traditional PCR amplification using specific primer sets was carried out at an annealing temperature of 55–60°C for 20–30 cycles. PCR was performed using a DNA Engine Tetrad Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). For the analysis of PCR products, 10 µl of each PCR reaction was electrophoresed on 1% agarose gel and detected under ultraviolet light following ethidium bromide staining. β-Actin was used as an internal control. Real-time PCR was performed using the Perfect real-time One Step SYBR PrimeScript RT-PCR Kit (Takara Bio, Shiga, Japan), according to the manufacturer's instructions, followed by detection using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control. Nucleotide sequences of the primers were based on published cDNA sequences (Table 1).

Nitrite quantification

Cells were treated with stimuli in 96-well plates, and nitrite (NO$_3^-$) in the medium was measured to assess nitrite levels using the Griess reaction, as described previously (21).

TNF-α, CXCL10, and LCN2 ELISA

The levels of TNF-α and CXCL10 in the culture medium were measured by sandwich ELISA using a rat monoclonal anti-mouse TNF-α and a rabbit polyclonal anti-mouse TNF-α and CXCL10 antibody as the capture antibody and goat biotinylated polyclonal anti-mouse TNF-α and CXCL10 antibody as a detection antibody (ELISA development reagent; R&D Systems), respectively, as described previously (30, 33). The levels of LCN2 in the culture medium were measured by a sandwich ELISA using a goat polyclonal anti-mouse LCN2 antibody (R&D Systems) as the capture antibody, and rabbit polyclonal anti-mouse LCN2 antibody as a detection antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The polyclonal anti-mouse LCN2 antibody was detected by sequential incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Pierce, Rockford, IL, USA) and chromogenic substrates (R&D Systems). The recombinant proteins were used as a standard.

Flow cytometric analysis

Primary microglial cultures (2×10³) were pelleted, fixed, and incubated with primary antibodies [goat polyclonal anti-arginase 1 (ARG1) antibody (Novus Biologicals, Littleton, CO, USA); goat polyclonal anti-MRC1 antibody (R&D Sys-
Western blot analysis

Cells were lysed in triple-detergent lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.02% sodium azide; 0.1% sodium dodecyl sulfate (SDS); 1% Nonidet P-40; 0.5% sodium deoxycholate; and 1 mM phenylmethylsulfonfluoride). Protein concentration in cell lysates was determined by using a protein assay kit (Bio-Rad, Hercules, CA, USA). An equal amount of protein from each sample was separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybrid enhanced chemiluminescence (ECL) nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked with 5% skim milk and sequentially incubated with primary antibodies [rabbit polyclonal anti-phospho-STAT6 at Tyr641 antibody (Cell Signaling Technology, Beverly, MA, USA); rabbit polyclonal anti-total STAT6 antibody (Cell Signaling Technology, Beverly, MA, USA); goat polyclonal anti-CD11b antibody (Amersham Biosciences), followed by ECL detection (Amersham Biosciences).

Phagocytosis of apoptotic cells

As prey for engulfment, Jurkat T lymphocytes were labeled with 5-chloromethylfluorescein diacetate (CMFDA) at the density of 10⁷ cells in a 75-cm² culture flask for 30 min in serum-free medium. Apoptosis of Jurkat T lymphocytes was induced by treatment with 10⁻³ M dexamethasone for 4 h.

### Table 1. DNA sequences of the primers used for RT-PCR

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in serum-free medium (34). Primary microglial cells were seeded at the density of 10^5 cells/coverslip 18 h prior to the assay. Apoptotic Jurkat T lymphocytes were added to coverslips (ratio of Jurkat T lymphocytes to microglia, 10:1) at 37°C for 3 h. Afterward, microglial cells were washed 10 times with ice-cold PBS to remove the bound apoptotic Jurkat T lymphocytes, and then fixed with 4% formaldehyde in PBS for 10 min at room temperature and subsequently washed with PBS. Fixed cells were examined under a model BX50 fluorescence microscope (Olympus, Tokyo, Japan), and the percentage of cells internalizing particles was determined by taking the ratio of cells that internalized the particles to the total number of cells. Three random fields (>100 cells) were counted, and data were quantified as a percentage of total cells internalizing particles (35). Three nonoverlapping fields were selected and examined in each well (3 wells/experimental group).

Mouse breeding and maintenance

LCN2-deficient mice were a gift from Dr. Shizuo Akira (Osaka University, Osaka, Japan). LCN2 wild-type (LCN2-deficient mice were a gift from Dr. Shizuo Akira (Osaka Mouse breeding and maintenance and examined in each well (3 wells/experimental group). Of cells that internalized the particles to the total number of cells. Three random fields (>100 cells) were counted, and data were quantified as a percentage of total cells internalizing particles (35). Three nonoverlapping fields were selected and examined in each well (3 wells/experimental group).

Neuroinflammation model based on intraperitoneal (i.p.) or intracerebroventricular (i.c.v.). LPS injection

Peripheral injection of LPS was done to evoke neuroinflammation in mice as described previously (37). len2^+/+ or len2^-/- mice were anesthetized using enflurane, and received an i.p. injection of saline or LPS (5 mg/kg). For the mRNA analysis, mice were anesthetized at 4 h after LPS injection and transcardially perfused with sterile diethyl pyrocarbonate (DEPC)-treated saline solution. The brains were then removed and stored at −80°C until analysis. This dose of LPS and time point (4 h) induced an optimal neuroinflammatory response at the mRNA levels in a preliminary pilot study and has been used to produce neuroinflammation in other laboratories (38). Behavioral and immunohistochemical analyses were carried out 4–24 h after LPS injection. All experiments were carried out on 12-wk-old mice (~30 g). For i.c.v. injection of LPS, mice were anesthetized by i.p. injection of a mixture of Zoletil (tiletamine/zolazepam; Virbac, Carros, France) and Rompun (xylazine; Bayer Pharma, Puteaux, France) at a dose of 30 and 10 mg/kg, respectively, and secured in a stereotactic instrument (Stoelting, Wood Dale, IL, USA). One microliter of saline, LPS (5 μg), or IL-4 (100 ng) was injected using a 5-μL Hamilton syringe (Hamilton, Reno, NV, USA) into lateral ventricles on both sides with coordinates of 0.5 mm posterior, 1 mm lateral to the bregma, and 1.75 mm below the skull surface at the point of entry. Behavior test and immunohistochemical analysis were performed 24–48 h after the i.c.v. injection.

Primary adult microglial cultures

Mouse primary adult microglial cultures were prepared as described previously, with minor modifications (39). The primary adult microglial cultures were prepared from the whole brains of 12-wk-old len2^+/+ or len2^-/- mice. Brains were homogenized in Hank’s balanced salt solution (HBSS; Gibco BRL) with collagenase/DNase (Sigma-Aldrich). Resulting homogenates were passed through a nylon cell strainer and centrifuged at 500 g for 6 min. Supernatants were removed, and cell pellets were resuspended in 37% isotonic Percoll (Amersham Biosciences) at room temperature. A discontinuous Percoll density gradient was set up as follows: 70, 37, 30, and 0% isotonic Percoll. The gradient was centrifuged for 20 min at 2000 g and microglia were collected from the interphase between the 37 and 30% Percoll layers. Microglial cells were washed and then resuspended in sterile HBSS. Each culture was generated from a single animal.

Rotarod treadmill performance

To assess LPS-induced progressive neurodegeneration, a behavioral test was performed by using a rotarod treadmill (Ugo Basile, Comerio, Italy). len2^+/+ or len2^-/- mice were administered i.p. injections of 5 mg/kg LPS or sterile saline. Mice were tested on the rotarod treadmill in three consecutive trials at 4, 12, and 24 h after LPS injection. During the training session, mice were trained on the rotarod device for 7 d before LPS injection. Before each test trial, acclimation to the device was performed by positioning the mice on the rod at 4 rpm for 1 min 3 times, and the rotating was gradually accelerated from 4 to 40 rpm. Mice that reached 40 rpm were allowed to stay on the rod for a total of 300 s, which was defined as normal ability. Latency to fall was determined at each time point after LPS injection for len2^+/+ or len2^-/- mice. Each testing session was repeated 3 times and had at least a 10-min break between trials. To further investigate the role of LCN2 in the psychomotor abnormality, the motor learning ability was also evaluated. The motor learning test evaluates whether mice show an improved performance over consecutive trials. Latency to fall was determined in a similar manner after 12 or 24 h after LPS injection without the training session. At each time point, rotarod test was run 3 times with a 1-h interval.

Fatigue test

To assess the effect of LPS injection on the volitional fatigue, mice were placed on a motorized treadmill with individual lanes separated by plastic dividers at 4 h after i.p. injection of LPS (5 mg/kg) or saline. Mice ran for 10 min at 6 m/min and then for an 8-min period, during which the speed was gradually increased to 18 m/min (40). Volitional exhaustion was defined as 10 s of consistent nonrunning at the back of the treadmill lane, where a foam sponge was placed. Electric shock was not used.

Open-field test

Open-field test was performed to assess LPS-induced depression of motility. At 24 h after i.c.v. injection of LPS, mice were placed individually at the corner of the arena (40×40×40 cm; with white acrylic walls). After a 1-min adaptation period, animal behavior was recorded for 30 min using a digital camera fixed 2 m above the floor. Total distance traveled (cm) and movement speed (cm/s) were analyzed with a video-tracking system (Smart; Panlab, Barcelona, Spain). The arena was wiped between the trials with 70% ethyl alcohol. To minimize stress levels, tests were performed under a low-illumination red light.

Object recognition task

Two different objects were used: a metal cylinder (diameter 7 cm, height 10 cm) and a plastic rectangular cuboid filled with sand (5×5×10 cm). Mice were first habituated to the testing
Mice were euthanized 24–48 h after LPS injection by inhalation of an overdose of ether. Mice were subjected to intracardiac perfusion-fixation using 0.9% NaCl and 4% paraformaldehyde (PFA) dissolved in 0.1 M PBS (pH 7.4). Isolated brains were immersion fixed in 4% PFA for 72 h. For cryoprotection, the brains were incubated in 30% sucrose diluted in 0.1 M PBS for 72 h and embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura, Torrance, CA, USA) for frozen section and then cut into 12-μm-thick coronal or sagittal sections. The sections were permeabilized in 0.1% Triton X-100 and blocked with 1% BSA and 5% normal donkey serum for 1 h at room temperature. Brain sections were incubated with primary antibodies [mouse monoclonal anti-NeuN (1:500 dilution; Santa Cruz Biotechnology), mouse polyclonal anti-LCN2 (1:1000 dilution; R&D Systems), rabbit polyclonal anti-iNOS (1:200 dilution; BD Transduction Laboratories, Lexington, KY, USA), goat polyclonal anti-ARG1 (1:200 dilution; Novus Biologicals), goat polyclonal anti-Iba-1 (1:500 dilution; Abcam) and rabbit polyclonal anti-Iba-1 (1:500 dilution; Wako, Osaka, Japan) antibodies] at 4°C overnight, followed by incubation for 1 h at room temperature with secondary antibodies (Cy3-conjugated donkey anti-mouse IgG, Cy3-conjugated donkey anti-goat IgG, FITC-conjugated donkey anti-rabbit IgG, and FITC-conjugated donkey anti-rabbit IgG antibodies; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Gelatin containing DAPI was used to mount and counterstain. Tiled images of each section were captured with a CCD color video camera (Olympus D70) through a ×100 objective lens attached to a microscope (Olympus BX51).

**Statistical analyses**

Data were presented as means ± sd from ≥3 independent experiments, unless stated otherwise. Statistical comparisons between different treatments were done by either a Student's t test or 1-way ANOVA with Dunnett's multiple-comparison tests by using SPSS 14.0K software (SPSS, Chicago, IL, USA). Differences with a value of P < 0.05 were considered to be statistically significant.

**RESULTS**

**M1 vs. M2 polarization of brain microglia**

To confirm whether functional dichotomy such as M1 vs. M2 polarization is present in brain microglia, we characterized the gene expression profile of microglia treated with M1 (LPS+IFN-γ) or M2 stimuli (IL-4, IC+LPS, and IL-10). Real-time RT-PCR analysis was conducted for individual M1 (IL-12, IL-23, iNOS, TNFα, and CXCL10) or M2-related genes (Fizz1, Ym1, IL-10, Arg1, and Mrcl) in mouse primary microglial cultures (Table 2). Previously, the expression of the M1 genes, such as IL-12 or TNFα, was highly induced in LPS/IFN-γ-activated macrophages, whereas M2 genes, such as IL-10 or Arg1, were induced in IL-4-activated macrophages. Similarly, in this study, M1-related genes were highly expressed in LPS/IFN-γ-activated microglia and remained at a low level in IL-4, IC/LPS-, or IL-10-activated microglia, as determined by real-time RT-PCR (Table 2). In contrast, mRNA levels of M2-related genes were consistently higher in IL-4-activated microglia as compared with LPS/IFN-γ-activated microglia (Table 2). M2a, M2b, and M2c stimuli, however,
exerted differential effects on these M2-related genes: M2a stimulus IL-4 induced all M2 genes examined, whereas M2c stimulus IL-10 induced the expression of ARG1, MRC1, and IL-10 itself. M2b stimulus upregulated only ARG1 and MRC1 gene expression. The expression of M1- or M2-related genes was also analyzed by traditional RT-PCR in BV-2 microglial cells, where similar results were obtained (Supplemental Fig. S1A). LPS/IFN-γ-activated BV-2 microglial cells showed an increased expression of M1-related genes (IL-12, IL-23, IL-1β, IL-6, iNOS, TNF-α, and CXCL10). In contrast, IL-4, IC/LPS-, or IL-10-activated BV-2 microglial cells showed an increased expression of M2-related genes (IL-10, ARG1, MRC1, and IL-1ra) to varying degrees (Supplemental Fig. S1A). We next evaluated NO production, as well as TNF-α and CXCL10 protein secretion in primary microglial cultures (Fig. 1A) and BV-2 microglial cells (Supplemental Fig. S1B). LPS/IFN-γ-activated microglia showed a strong induction of M1-related NO production and cytokine/chemokine secretion (TNF-α and CXCL10). The results indicate differential gene expression of M1- or M2-polarized microglia and confirm the existence of phenotypic dichotomy of brain microglia. Previously, LCN2 was expressed as an acute-phase protein (20), and the expression of LCN2 was modulated by inflammatory stimuli in macrophages and glia (20–22, 29). Thus, we sought to determine how the expression of LCN2 is regulated by M1- or M2-polarizing stimuli in microglia. The expression of LCN2 was strongly enhanced by LPS/IFN-γ in primary microglial cultures (Fig. 1B) and BV-2 microglial cells (Supplemental Fig. S1C). The secretion of LCN2 was also enhanced by LPS/IFN-γ as determined by ELISA of primary microglial culture medium (Fig. 1C). The LCN2 induction was not observed following M2-related stimulation (IL-4, IC+LPS, and IL-10). The results indicate that microglial expression and secretion of LCN2 is up-regulated under M1-polarizing conditions, but not under M2 conditions.

Autoregulatory role of LCN2 in microglial M1 polarization

The increased LCN2 may further regulate microglial polarization. To test this hypothesis, we next determined how LCN2 influences M1- or M2-related gene expression in microglia. When exposed to LCN2 protein, microglia showed an increased expression of M1-related genes (IL-12, IL-23, iNOS, TNF-α, and CXCL10) (Fig. 2). EC50 value for LCN2 protein was 1 µg/ml, based on the nitrite production in BV-2 microglial cells (Supplemental Fig. S1D, E). However, the 10 µg/ml of LCN2 was used throughout this study, as the LCN2 protein at this concentration has been previously reported to exert biological effects on microglia without the apparent cytotoxicity (21, 41). Under the inflammatory condition, this concentration of LCN2 protein has also been observed in vivo (27, 42). LCN2 had no significant effect on the expression of the M2-related genes (FIZZ1, Ym1, IL-10, ARG1, and MRC1) as evaluated by real-time PCR analysis (Supplemental Fig. S2A). The GST protein, which was purified in the process of LCN2 preparation, at the same concentration did not affect M1- or M2-related gene expression (Fig. 2, Supplemental Fig. S2A). The level of LCN2-induced M1- or M2-related gene expression was not abolished by polymyxin B treatment, ruling out the possibility of LPS contamination in the process of recombinant LCN2 preparation (Supplemental Fig. S2B, C). The LCN2 regulation of M1- or M2-related gene expression in microglia was further confirmed by using murine LCN2 protein expressed in mammalian cells (mLCN2). The mLCN2 similarly increased the expression of M1-related genes (IL-12, IL-23, iNOS, TNF-α, and CXCL10) without affecting M2-related genes (Supplemental Fig. S2D, E). The results indicate that LCN2 proteins expressed in bacteria and mammalian cells exert the same effects on the M1- or M2-related gene expression in microglia. LCN2 induction of M1-related gene expression in microglia was further...
confirmed by the overexpression or knockdown of lcn2 gene expression in BV-2 microglial cell line (Supplemental Fig. S3). BV-2 microglial cells with an increased or decreased expression of LCN2 were generated by stable transfection with sense or antisense lcn2 cDNA (20). Knockdown of endogenous LCN2 expression reduced M1 gene expression compared with LCN2 overexpression; however, M2 gene expression was not significantly affected (Supplemental Fig. S3). The results support the suggestion that both exogenous and endogenous LCN2 proteins augment M1-related gene expression in microglia. Taken together, these results suggest that microglia-derived LCN2 promotes M1 polarization of microglia in an autocrine manner.

**LCN2 inhibition of microglial M2 signaling and phenotype**

STAT6 plays a principal role in IL-4 signaling, which is associated with M2 macrophage polarization (43). To examine whether LCN2 affects IL-4-induced activation of STAT6 signaling, the phosphorylation status of STAT6 at tyrosine residue 641 was assessed. Western blot analysis revealed that IL-4 induced phosphorylation of STAT6 after 30 min stimulation, which was significantly inhibited by LCN2 in primary microglial cultures (Fig. 3A), as well as BV-2 microglial cells (Supplemental Fig. S4A). LCN2 alone had no effect on STAT6 activation. The total levels of STAT6 proteins were not affected under these conditions. Studies using pharmacological inhibitors, such as the JAK3/STAT6-specific inhibitor, leflunomide, indicated that IL-4-induced up-regulation of M2-related genes (ARG1, MRC1, IL-10, and IL-1ra) requires the STAT6 pathway in microglia (Supplemental Fig. S4B). Leflunomide treatment reduced IL-4-induced up-regulation of M2-related gene expression. The recombinant LCN2 protein similarly decreased the expression of IL-4-mediated M2-related genes (ARG1, MRC1, IL-10, and IL-1ra) in microglia, as determined by RT-PCR (Supplemental Fig. S4B). FACS analysis indicated that the expression of M2 protein following IL-4 treatment was also reduced by leflunomide and LCN2 (Fig. 3C, D). Leflunomide and recombinant LCN2 protein did not affect cell viability at the concentrations used in the current study. These data support a negative regulatory role for LCN2 in IL-4-induced STAT6 signaling and M2 gene expression in microglia. We next examined the effect of LCN2 on another representative M2 stimulus IL-10 activity in microglial phagocytosis. LCN2 also inhibited IL-10-induced phagocytic activity of microglia, as determined by uptake of apoptotic cells (Fig. 4), further supporting the inhibitory effects of LCN2 on M2-polarization of microglia. Because IL-4 did not affect microglial phagocytic activity (Fig. 4A), we did not further evaluate the effect of LCN2 in this respect. This again suggests the existence of subsets of M2-polarized microglia.

**Essential role of LCN2 in microglial M1 polarization ex vivo and in vivo**

We compared M1/M2 gene expression in primary neonatal microglia cultures prepared from len2−/− vs. len2−/− mice. Neonatal microglia cultures isolated from len2−/− and len2−/− mice were exposed to LPS/IFN-γ, IC+LPS, IL-4, or IL-10, and their M1 or M2 polarized responses were compared. In contrast to wild-type microglia, len2−/− microglia failed to show the induction of M1-related gene expression in response to LPS/IFN-γ (Fig. 5A). The expression of M2-related genes (CCL17, CCL22, IL-10, ARG1, and MRC1) was induced in response to IL-4 and IL-10 to varying degrees (Fig. 5B). Immune complex plus LPS treatment significantly increased CCL17 expression only. Compared with wild-type microglia, len2−/− microglia showed enhanced expression of M2-related genes (CCL17, CCL22, ARG1, and MRC1) following IL-4 and IL-10 treatment (Fig. 5B). We next investigated the in vivo role of LCN2 in a well-established mouse neuroinflammation model, which is dominated by M1-polarized microglia. Following i.p. injection of LPS, whole brains or primary adult microglial cultures showed an increased expression of M1-related gene (IL-12, IL-23, iNOS, TNF-α, and CXCL10) expression in len2−/− mice, compared with saline injection (Fig. 6A). LPS-injected len2−/− mice showed no such up-regulation of M1-related genes, consistent with a lack of LCN2 induction (Fig. 6A). The expression of M2-related genes (CCL17, CCL22, IL-10, ARG1, and MRC1) was not

**Figure 2. LCN2 induction of M1-related gene expression profile in microglia.** Primary microglia cultures were treated with bacterially expressed recombinant LCN2 protein (10 μg/ml) or GST protein (10 μg/ml) for 8 h. mRNA levels of M1-related genes were determined by real-time RT-PCR. GAPDH was used as an internal control. Results are means ± sd (n=3). *P < 0.05 vs. untreated control; Student’s t test.
induced by LPS or influenced by LCN2 deficiency (data not shown). These results indicate that LCN2 promotes M1 polarization of both adult and neonatal microglia. To rule out confounding effects of peripheral inflammation following i.p. injection of LPS in the results shown in Fig. 6A, we next assessed the role of LCN2 in the microglial M1/M2 polarization after the direct induction of inflammation in the CNS. *lcn2*+/+ or *lcn2*−/− mice received an i.c.v. injection of saline, LPS, or IL-4. After 24 h, the mice were euthanized, and

![Figure 3. LCN2 inhibition of IL-4-induced STAT6 activation and M2 gene expression in microglia.](image)

**A** Primary microglial cultures were treated with IL-4 (10 ng/ml) in the presence or absence of recombinant LCN2 protein (10 μg/ml) for 30 min. Levels of phosphorylated STAT6 (pSTAT6 at Tyr641) or total STAT6 protein were then evaluated by Western blot analysis (A). Primary microglia cultures were treated with IL-4 (10 ng/ml) in the presence or absence of recombinant LCN2 protein (10 μg/ml) for 8 h (B). mRNA levels of M1- or M2-related genes were determined by RT-PCR. β-Actin was used as an internal control. Results of densitometric analysis are shown below the gels. **C, D** Alternatively, primary microglia cultures were treated with IL-4 (10 ng/ml), recombinant LCN2 protein (10 μg/ml), or leflunomide (10 μM) alone or in combination for 24 h. ARG1 or MRC1 expression was detected by flow cytometric analysis using monoclonal antibodies specific for ARG1 or MRC1. Mean fluorescence intensity (MFI) values are also shown. Results are means ± SD (n=3). *P < 0.05; Student’s t test.

**Figure 4.** LCN2 as a negative regulator of IL-10-induced microglial phagocytosis of apoptotic cells. Primary microglia cultures were treated with LPS (100 ng/ml) plus IFN-γ (50 U/ml), IL-4 (10 ng/ml), or IL-10 (10 ng/ml) in the presence or absence of the recombinant LCN2 protein (10 μg/ml) for 24 h, and then M2-related phagocytic activity of microglia was assessed by coincubating with CMFDA-labeled apoptotic Jurkat cells for 3 h. Cells were fixed and observed by fluorescence microscopy (A) or FACS (B). **A** Top panel: representative microscopic images for each condition (×100 view). Scale bar = 25 μm. Bottom panel: Microglia that engulfed Jurkat cells were counted, and the percentage of phagocytic cells was obtained. A minimum of 100 cells/ coverslip were counted. Results are means ± SD (n=3). *P < 0.05. **B** In the FACS analysis, phagocytic activity of microglia was evaluated on the basis of the mean fluorescence intensity (MFI). Results are one representative of >3 independent experiments. Statistical comparisons between different treatments were done by a 1-way ANOVA with Dunnett’s multiple-comparison tests.
primary adult microglial cultures were prepared to evaluate the expression of M1- or M2-related genes. Expression of M1-related genes (IL-12, CXCL10, iNOS, and TNF-α) in primary adult microglial cultures was increased by i.c.v. injection of LPS compared with saline injection in wild-type animals (Fig. 6B). LPS-injected len2−/− mice showed a reduced expression of M1-related genes compared with len2+/+ mice (Fig. 6B). The expression of M2-related genes (FIZZ1, Ym1, IL-10, ARG1, and MRC1) was similarly induced by IL-4 injection in len2−/− and len2+/− mice. Absence of LCN2 did not significantly affect the IL-4-induced M2-related gene expression (Fig. 6C). These results indicate that LCN2 functions as a microglial M1 inducer in both peripheral and central inflammation models.

Role of LCN2 in M1 neuroinflammation-associated behavior impairment

To determine whether LCN2 deficiency can alter the CNS damage induced by M1-polarized microglia, psychomotor coordination of len2−/− and len2+/+ mice was first compared using the rotarod treadmill performance test and the fatigue test after i.p. injection of saline or LPS (5 mg/kg). LPS injection induced progressive motor impairment in len2+/+ animals, as determined by the average latency to fall in the rotarod test, which was significantly ameliorated in len2−/− mice (P<0.05; Fig. 7A). Motor learning ability was also tested using rotarod treadmill performance after LPS administration (Fig. 7B). Three consecutive trials (T1, T2, and T3) were performed at 1-h intervals beginning 12 or 24 h after the i.p. injection of LPS. LPS-induced psychomotor learning deficit in len2+/+ mice was significantly improved in len2−/− mice (P<0.05). Next, exhaustive fatigue test was performed following i.p. injection of LPS. LPS-induced volitional fatigue was assessed with the time of giving up running (10-s stay at the back of lane) at the speed corresponding to an intensity of 80–85% of the maximal oxygen uptake in a motorized treadmill (Fig. 7C). LPS injection significantly reduced the time to fatigue in the len2+/+ mice compared with saline injection (P<0.05). The LPS-induced reduction in the time to fatigue, however, was reversed in the len2−/− mice (P<0.05; Fig. 7C). Open-field test and object recognition task were performed after i.c.v. injection of LPS (Fig. 7D, E). LPS-injected len2+/+ mice exhibited lower locomotive activity level in the open-field test compared with saline-injected group (Fig. 7D). LCN2 deficiency ameliorated the LPS-induced reduction of total distance traveled and movement speed, implicating LCN2 in the LPS-induced locomotor deficits. When the object recognition task was performed to evaluate hippocampus-dependent memory function, LPS-injected len2+/+ mice exhibited significantly lower preference for novel object than saline-injected mice after 24 h, which was partially
reversed in \textit{len2}^{−/−} mice (Fig. 7E). We selected a 24-h delay interval based on the involvement of the hippocampus in object recognition memory (44). \textit{len2}^{+/+} mice and \textit{len2}^{−/−} mice equally explored objects, and there was no difference during the training sessions (before LPS injection; \textit{D}=0.02±0.06). Taken collectively, the data from the rotorod performance test, fatigue test, open-field test, and object recognition task indicate that LPS-induced microglial M1 polarization and subsequent neuroinflammation lead to motor and cognitive impairments, which are partially prevented by LCN2 deficiency. The results support the critical role of LCN2 in the microglial M1 polarization in vivo.

**Immunohistochemical analysis of LCN2 expression and microglial polarization**

Following i.p. or i.c.v. injection of LPS and behavioral tests, immunohistochemical evaluation was performed in the \textit{len2}^{+/+} and \textit{len2}^{−/−} mice (Figs. 8 and 9). Neurons and microglia were observed in the cerebral cortex, hippocampus, and cerebellum corresponding to brain regions related with cognition, memory, learning, and motor functions. Peripheral LPS injection evoked microglial activation in the cortex, hippocampus, and cerebellum of \textit{len2}^{+/+} mice with severe hippocampal destruction, as judged by Iba-1 and NeuN staining (Fig. 8). In the brain of \textit{len2}^{−/−} mice, however, LPS-induced microglial activation and hippocampal damages were significantly diminished. These results are in agreement with those of the behavioral tests. Similarly, following the i.c.v. injection of LPS or IL-4, microglial polarization and LCN2 expression correlated with the results obtained from cultured microglial cells and behavioral test (Fig. 9). LCN2 expression was highly induced by LPS injection in brain cortex and was partly colocalized with Iba-1 staining, indicating that microglia expressed LCN2 (Fig. 9A). Expression of M1 marker iNOS was also induced by LPS in microglia. These phenotypic changes of microglia were greatly reduced in \textit{len2}^{−/−} mice. Brain injection of LPS also induced a strong microglial activation and hippocampal injury as determined by Iba-1 and DAPI staining (Fig. 9B), which was consistent with previous studies (45, 46). In the LCN2-deficient mice, however, microglial activation and hippocampal injury were significantly attenuated compared with wild-type mice. IL-4 injection induced microglial expression of M2 marker ARG1 in cortex, which was not significantly influenced by LCN2 deficiency (Fig. 9C). These histological results further support the critical role of LCN2 in the microglial M1 polarization in vivo.

**DISCUSSION**

Macrophage polarization is remarkably plastic and allows the generation of different populations of cells with distinct, and sometimes opposite, effector functions (47). The phenotype and functional activities of macrophages can be modulated by a number of cytokines and microbial products, and form a continuum
with polarization toward either M1 or M2 macrophages at the extremes. M1- and M2-type cells express different levels of cell surface markers, adhesion molecules, scavenger receptors, chemokines, cytokines, and their receptors. In the present study, we confirm that the M1/M2 polarized phenotypes are also applicable to microglia, macrophages resident in the CNS. Our results indicate that LCN2 is secreted by M1-polarized

**Figure 7.** Alleviation of LPS-induced motor and memory impairment in LCN2-knockout (KO) mice. Wild-type (WT; lcn2+/+; n=5) or LCN2-KO mice (lcn2−/−; n=7) were administered i.p. injections of LPS (5 mg/kg) and subjected to rotarod performance test (A, B) or fatigue test (C). Average latency to fall for each animal group is shown as the mean ± sd. All trials were performed 3 times for each animal. Motor learning ability was also compared (B). Three trials with 1-h intervals (T1-T3) were performed at 12 and 24 h after LPS injection. LPS injection significantly reduced the time to fatigue in the lcn2+/+ mice compared with saline injection (P<0.05); however, this reduction in the time to fatigue was partially abolised in the LPS-injected lcn2−/− mice. (C). WT (lcn2+/+; n=3) or LCN2-KO mice (lcn2−/−; n=3) were administered i.c.v. injections of saline or LPS (5 µg) and subjected to open-field test (D) or object recognition task (E). Total distance traveled and movement speed was reduced in lcn2+/+ mice 24 h after LPS injection. The motor impairment was alleviated in lcn2−/− mice. Black lines represent overall pathway of animal movements in the arena, which was obtained from a video-tracking system (shown in the middle). After 24 h, LPS-injected lcn2−/− mice showed significantly lower novel object preference than saline-injected mice. The LPS-induced cognitive deficit was attenuated in LCN2-KO mice. The relative exploration time during the test session was defined as the discrimination index [DI = (tN − tF) / (tN + tF); tN, time spent on novel object; tF, time spent on familiar object]. Data are presented as means ± se. ∗P < 0.05 vs. KO mice (A, B, D) or as indicated (C, E); Student’s t test.

**Figure 8.** LCN2 contributes to microglial activation and hippocampal destruction in the neuroinflammation model. At 24 h after i.p. injection of either saline or LPS (5 mg/kg), mouse brain sections were stained with antibodies against Iba1 (microglia, green), NeuN (neuron, red), or DAPI (nucleus, blue). LPS injection induced microglial activation in cortex, hippocampus, and cerebellum of lcn2+/+ mice. Particularly, LPS administration inflicted a serious injury to dentate gyrus region of the hippocampus in lps−/− mice. In the LPS-administered lcn2−/− mice, the microglial activation and hippocampal injury were significantly reduced. Scale bar = 200 µm. Results are one representative of >3 independent experiments.
microglial cells and acts as a key regulator of microglial M1 polarization in vivo and in vitro (Fig. 10). This conclusion was supported by the data obtained from cultured microglial cells, mouse neuroinflammation models, and their behavioral and histological analyses.

M1 macrophage polarization is associated with inflammation and tissue destruction, whereas M2 macrophages have an anti-inflammatory phenotype that is associated with wound repair and angiogenesis. In the CNS, it has been previously suggested that the M1-like phenotypes may be associated with microglia contacting human immunodeficiency virus (HIV) proteins (48) or TLR-3/4 ligands (49). The observations that increased M1 polarization was also associated with increased TNF-α in plasma and brain specimens in HIV-associated dementia and Alzheimer’s disease suggests that M1 polarization may play an important role in the pathophysiology of these diseases (50). These reports point to the possibility that M1 polarization of microglia is associated with the initiation and perpetuation of neuroinflammation, while M2 polarization of microglia is involved in the resolution of neuroinflammation (51). Macrophages undergoing M2 polarization show an enhanced phagocytic activity toward β-amyloid (52). M2 phenotype-related phagocytosis is also critical for the engulfment and degradation of invaded pathogens and apoptotic cells (53). Previously, M2-related cytokine IL-10 increased the phagocytic capacity of macrophages toward apoptotic cells or pathogens (8, 54), and higher activity of M2 macrophages was associated with debris scavenging and tissue remodeling (55). In agreement with these previous findings in macrophages, M1-polarizing LCN2 inhibited IL-10-induced phagocytic activity of microglia in this study, demonstrating the negative regulatory role for LCN2 in microglial M2-related activity. Recently, LCN2 has been proposed as a novel component of proinflammatory signaling in Alzheimer’s disease showing an increased expression in patients’ postmortem brain tissues. Plasma levels of LCN2 protein were also increased in patients with mild cognitive impairment and multiple sclerosis (56, 57). These results support the M1-polarizing activity of LCN2 under various neuroinflammatory conditions.

Our initial data obtained from cultured microglial cells indicated that LCN2 potentiates the M1 phenotype of microglia, while suppressing the M2 phenotype in vitro. Having shown that LCN2 amplifies M1 polarization of cultured microglial cells, we attempted to demonstrate a critical role of LCN2 in microglial M1 polarization in vivo using mouse neuroinflammation models with i.p. or i.c.v. injection of LPS. Peripheral injection of LPS induces proinflammatory cytokine expression profiles in the CNS (37). Injection of LPS into rodent brain also results in the activation of microglia and inflammatory responses typically found in neuroinflammatory and neurodegenerative diseases (45, 58). Activated microglia have been identified...
in a broad spectrum of neurodegenerative disorders, including Alzheimer’s disease (59), Parkinson’s disease (60), amyotrophic lateral sclerosis (61), multiple sclerosis (62), and inherited photoreceptor dystrophies (63). In the current study, systemic or brain injection of LPS was done to induce M1-polarized microglial activation, brain inflammation, and subsequent behavioral deficits. LPS injection markedly increased the expression of M1-related genes in the brain microglia, indicating the successful induction of microglial M1 polarization and neuroinflammation. In \( \text{lc} n \text{C2}^{-/-} \) mice, however, LPS-induced M1-related gene expression and neuroinflammation-associated motor/cognitive impairments were markedly reduced, suggesting the critical role of LCN2 in microglial M1 polarization \( \text{in vivo} \).

STAT1 and STAT6 play a major role in transmitting polarizing signals to the nucleus (64) and have distinct roles in macrophage polarization. STAT1 is activated in response to M1 macrophage-polarizing cytokines (LPS/IFN-\( \gamma \)), whereas STAT6 is selectively activated by M2 macrophage-polarizing cytokines (IL-4 and IL-13) (43). Here, we show that LCN2 inhibition of IL-4-induced M2-related gene expression in microglia is mediated by the blockade of JAK3/STAT6 pathway. Previously, Stat6\(^{-/-}\) mice showed a suppressed M2 macrophage phenotype, with lower levels of ARG1 expression and higher levels of NOS2/nitric oxide (65). Consistently, in this study, LCN2 inhibition of STAT6 signaling reduced IL-4-induced M2-related gene (ARG1, MRC1, IL-10, and IL-1ra) expression in microglia. Our results suggest that the JAK3/STAT6 signaling pathways be a target of the LCN2 actions in microglia (Fig. 10). However, how exactly LCN2 promotes M1 polarization of microglia remains to be determined. We have previously demonstrated that LCN2 is a chemokine inducer in the CNS; it acts on microglia to induce chemokine expression, thereby recruiting inflammatory cells into the scene (30). Thus, LCN2 may amplify M1 polarization of microglia through chemokine expression and ensuing inflammatory cell recruitment. In astrocytes, LCN2 has been shown to activate NF-kB to induce chemokine expression, NO production, and other proinflammatory phenotypes (30). Because both astrocytes and microglia express LCN2 receptors, they may similarly respond to LCN2. Therefore, LCN2 seems to promote M1 polarization of microglia through multiple mechanisms: direct induction of M1-related gene expression in microglia; recruitment of inflammatory cells \( \text{via} \) chemokine induction; and suppression of M2 signaling pathways.

Role of LCN2 in the CNS inflammation has been previously investigated using LCN2-knockout (KO) mice reporting conflicting results (56, 66, 67). In the study by Ip et al. (66), peripheral injection of LPS induced LCN2 expression in brain, consistent with the results of this study. However, host response to LPS showed similar changes in wild-type and LCN2-KO mice. LCN2 deficiency did not significantly affect the level of various cytokine and chemokine mRNAs. These results are in contrast with our previous and current findings that chemokine CXCL10 expression (30) and other M1-related proinflammatory cytokine and chemokine expression were significantly lower in LCN2-KO mice compared with wild-type mice (this study). Currently, we do not have a clear explanation for this discrepancy. It should be noted, however, that there was a slight reduction in several chemokines and TNF-\( \alpha \) expression in LCN2-KO mice following LPS exposure in the study by Ip et al. (66). The study by Rathore et al. (67) reported a decrease in the expression of proinflammatory chemokines and cytokines as well as iNOS after spinal cord contusion injury in LCN2-deficient mice compared with wild-type animals. LCN2-deficient mice also showed significantly better locomotor recovery compared with wild-type mice after spinal cord contusion injury. These results are in accordance with our findings. More recently, Berard et al. (56) reported that LCN2 is significantly upregulated in the spinal cord throughout experimental allergic encephalomyelitis (EAE), an animal model of multiple sclerosis. That study suggested that LCN2 is a modulator of EAE and multiple sclerosis pathogenesis. Further studies using other inflammation or injury models will clarify the role of LCN2 in the CNS inflammation. In the present study, nevertheless, both systemic and brain
LPS injection models were used to demonstrate that LCN2 supports M1 activity and that it can skew M2 microglia toward an M1 phenotype. Following the i.p., i.c.v., or intracortical injection of LPS, chemokine CXCL10 and other M1-related genes was also markedly reduced in LCN2-deficient mice in our previous (30) and present studies, suggesting that LCN2, indeed, polarizes microglia toward an M1 phenotype in both peripheral and central inflammation models.

In summary, we present evidence that the secreted protein LCN2 amplifies M1-polarized phenotypes of microglial cells. LCN2 not only augments M1-related gene expression, but also inhibits M2-related gene expression, IL-4-induced STAT6 signaling, and IL-10-induced phagocytic activity. More important, mice lacking LCN2 exhibit reduced M1-polarized phenotypes, such as microglial M1 gene expression and neuroinflammation-associated impairments in motor behavior and cognition following LPS exposure. These results clearly establish the essential role of LCN2 as a protein required for the M1 polarization of microglia.

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