Lipocalin-2 Protein Deficiency Ameliorates Experimental Autoimmune Encephalomyelitis

THE PATHOGENIC ROLE OF LIPOCALIN-2 IN THE CENTRAL NERVOUS SYSTEM AND PERIPHERAL LYMPHOID TISSUES*

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Youngpyo Nam‡, Jong-Heon Kim‡, Minchul Seo‡1, Jae-Hong Kim‡, Myungwon Jin‡1, Sangmin Jeon‡1, Jung-wan Seo‡13, Won-Ha Lee‡9, So Jin Bing‡, Youngheun Lee‡, Won Kee Lee‡11, Dong Ho Park**†, Hyun Kook‡11, and Kyoungho Suk‡14

From the ‡Department of Pharmacology, Brain Science and Engineering Institute, and Department of Biomedical Sciences, BK21 Plus KNU Biomedical Convergence Program, Kyungpook National University School of Medicine, Daegu 700-422, Korea, †School of Life Sciences and Biotechnology, Kyungpook National University, Daegu 702-701, Korea, 1Department of Veterinary Medicine and Applied Radiological Institute, Jeju National University, Jeju 690-756, Korea, 1Center of Biostatistics, Kyungpook National University School of Medicine, Daegu 700-422, Korea, 11Department of Ophthalmology, Kyungpook National University School of Medicine, Daegu 700-422, Korea, and 14Department of Pharmacology, Chonnam National University Medical School, Gwangju 501-746, Korea

Background: The role of LCN2 in EAE is not clear.

Results: LCN2 expression increased in EAE. Lcn2 deficiency attenuated EAE symptoms and pathology. LCN2 enhanced glial expression of inflammatory mediators and peripheral encephalitogenic T cell activation in vitro and in vivo.

Conclusion: Both central and peripheral LCN2 contributed to EAE development.

Significance: LCN2 can be targeted for treatment of multiple sclerosis.

Lipocalin-2 (LCN2) plays an important role in cellular processes as diverse as cell growth, migration/invasion, differentiation, and death/survival. Furthermore, recent studies indicate that LCN2 expression and secretion by glial cells are induced by inflammatory stimuli in the central nervous system. The present study was undertaken to examine the regulation of LCN2 expression in experimental autoimmune encephalomyelitis (EAE) and to determine the role of LCN2 in the disease process. LCN2 expression was found to be strongly increased in spinal cord and secondary lymphoid tissues after EAE induction. In spinal cords astrocytes and microglia were the major cell types expressing LCN2 and its receptor 24p3R, respectively, whereas in spleens, LCN2 and 24p3R were highly expressed in neutrophils and dendritic cells, respectively. Furthermore, disease severity, inflammatory infiltration, demyelination, glial activation, the expression of inflammatory mediators, and the proliferation of MOG-specific T cells were significantly attenuated in Lcn2-deficient mice as compared with wild-type animals. Myelin oligodendrocyte glycoprotein-specific T cells in culture exhibited an increased expression of Il17a, Ifng, Rorc, and Tbet after treatment with recombinant LCN2 protein. Moreover, LCN2-treated glial cells expressed higher levels of proinflammatory cytokines, chemokines, and MMP-9. Adoptive transfer and recombinant LCN2 protein injection experiments suggested that LCN2 expression in spinal cord and peripheral immune organs contributes to EAE development. Taken together, these results imply LCN2 is a critical mediator of autoimmune inflammation and disease development in EAE and suggest that LCN2 be regarded a potential therapeutic target in multiple sclerosis.

Multiple sclerosis (MS)5 is a chronic inflammatory disease of the central nervous system (CNS) characterized by immune cell infiltration and demyelination of the brain and spinal cord (1–3). In MS, inflammatory reactions involve complex interactions between infiltrating immune cells and resident CNS cells that lead to inflammatory lesion formation, demyelination, oligodendrocyte, and axonal damage (4, 5). Experimental autoimmune encephalomyelitis (EAE) is an animal model widely used to study neuroimmunologic responses in MS. The key players in MS, which include T cells (6–8), B cells (9–11), mast cells (12, 13), macrophages (14), CNS resident glial cells (15, 16), adhesion molecules (17, 18), cytokines, and chemokines (19, 20).

5 The abbreviations used are: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium binding adapter molecule 1; Ly6G, lymphocyte antigen 6G; LCN2, lipocalin-2; MOG, myelin oligodendrocyte glycoprotein; MMP, matrix metalloproteinase; Rorc, RAR-related orphan receptor C; Tbet, T-box transcription factor.
Role of LCN2 in EAE

LCN2 (lipocalin 2, also termed 24p3) is a member of the lipocalin family, a group of small extracellular proteins with great functional diversity. LCN2 expression in CNS-resident glial cells, and adoptive transfer and LCN2 protein enhanced the activation of encephalitogenic T cells and demyelination, and the expression of inflammatory cytokines and chemokines significantly attenuated disease severity, inflammatory infiltration, and perivascular cuffing.

EXPERIMENTAL PROCEDURES

Animals—Wild-type (WT) and Lcn2-deficient mice with 4% paraformaldehyde diluted in 0.1 M PBS. Lumbar spinal cords were fixed using 4% paraformaldehyde, and neutrophils after spinal cord injury, and Lcn2-deficient mice showed reduced leukocyte infiltration and levels of proinflammatory mediators, such as MCP-1, TNF-α, IL-1β, IL-6, and inducible nitric-oxide synthase, after spinal cord injury (46), suggesting that Lcn2 deficiency may play an important role in CNS inflammation and related diseases. LCN2 is recognized by cell surface receptor 24p3R, a brain-type organic cation transporter (BOCT) (28). The expression of 24p3R has been detected in glia and neurons in the CNS, and recent studies have shown that LCN2 regulates neuronal migration, morphology (47), and excitability (48). These previous results suggest that LCN2 may play a pleiotropic role in different aspects of CNS physiology and pathology. In particular, the reported associations between LCN2 and IL-17 and chemokine expression strongly suggest its involvement in EAE, MS, and other inflammatory diseases of the CNS.

In this study, we investigated the involvement of LCN2 in the pathogenesis of EAE. LCN2 expression was induced under many inflammatory conditions, such as inflammatory bowel disease (31), psoriasis (32), rheumatoid arthritis (33), and systemic lupus erythematosus (34). Furthermore, recent studies have reported that LCN2 secretion can be induced by IL-17 (35, 36), a Th17 cell-derived cytokine that contributes to the pathogenesis of many autoimmune and inflammatory diseases. LCN2 has also been implicated in CNS inflammation, mild cognitive impairment (37), Alzheimer disease (38), amyotrophic lateral sclerosis (39), and frontotemporal lobar degeneration (40). In a previous study, we reported that LCN2 is secreted by microglia and astrocytes in culture (41) and that LCN2 induces chemokine production (42) and microglial M1 polarization in the CNS (43). Other laboratories have reported that LCN2 expression is increased in the CNS after systemic LPS injection (44) or by kainate-induced excitotoxicity (45). In addition, LCN2 is up-regulated in astrocytes, neurons, and neutrophils after spinal cord injury, and Lcn2-deficient mice showed reduced leukocyte infiltration and levels of proinflammatory mediators, such as MCP-1, TNF-α, IL-1β, IL-6, and inducible nitric-oxide synthase, after spinal cord injury (46), suggesting that Lcn2 deficiency may play an important role in CNS inflammation and related diseases. LCN2 is recognized by cell surface receptor 24p3R, a brain-type organic cation transporter (BOCT) (28). The expression of 24p3R has been detected in glia and neurons in the CNS, and recent studies have shown that LCN2 regulates neuronal migration, morphology (47), and excitability (48). These previous results suggest that LCN2 may play a pleiotropic role in different aspects of CNS physiology and pathology. In particular, the reported associations between LCN2 and IL-17 and chemokine expression strongly suggest its involvement in EAE, MS, and other inflammatory diseases of the CNS.

In this study, we investigated the involvement of LCN2 in the pathogenesis of EAE. LCN2 expression was increased in spinal cord, lymph nodes, and spleen during EAE. Lcn2 deficiency significantly attenuated disease severity, inflammatory infiltration, demyelination, and the expression of inflammatory cytokines and chemokines. Furthermore, recombinant LCN2 protein enhanced the activation of encephalitogenic T cells and CNS-resident glial cells, and adoptive transfer and LCN2 protein injection experiments showed that LCN2 expression in the CNS and peripheral lymphoid tissues is critically involved in the development of EAE.

EXPERIMENTAL PROCEDURES

Animals—Wild-type (WT) and Lcn2-deficient mice (KO) C57BL/6 mice obtained from Samtaco (Osan, Korea) and Dr. Shizuo Akira (Osaka University, Japan), respectively. Lcn2 KO mice were back-crossed for 8–10 generations in a C57BL/6 background to generate homozygous and heterozygous animals, as described previously (49, 50). The absence of Lcn2 in Lcn2-deficient mice was confirmed by PCR of genomic DNA. Animals used in the present study were acquired and cared for in accordance with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health.

EAE Induction—Mice (7–8 weeks) were immunized subcutaneously with 50 or 200 μg of myelin oligodendrocyte glycoprotein (MOG35–55: MEVGWYRSPFRVHLYRNGK) (GLBiochem, Shanghai, China) in 100 μl of a solution containing 50% of complete Freund’s adjuvant with 10 mg/ml heat-killed H37Ra strain Mycobacterium tuberculosis (Difco) in areas draining into axillary and inguinal lymph nodes. Pertussis toxin (List Biological Laboratories, Campbell, CA) in phosphate-buffered saline (PBS; at 200 ng/mouse) was administered intraperitoneally on days of immunization and again 48 h later. Animals were weighed and examined for disease symptoms daily. Evaluation of disease severity and other experiments were carried out in a blinded fashion. Disease severity was scored using a scale 0–5 as follows: 0 = no symptom; 1 = limp tail; 2 = weakness and incomplete paralysis of one or two hind limbs; 3 = complete hind limb paralysis; 4 = forelimb weakness or paralysis; 5 = moribund state or death.

Histological Analysis—Mice were anesthetized with diethyl ether, transcardially perfused with cold saline, and perfused with 4% paraformaldehyde diluted in 0.1 M PBS. Lumbar spinal cords were fixed using 4% paraformaldehyde, embedded in OCT compound (Tissue-Tek, Sakura Finetek, Japan) for frozen sections and then sectioned coronally at 20 μm. Sections were stained with hematoxylin and eosin (H&E) to assess inflammatory lesions. To detect LCN2 expression, demyelination, glial activation, and immune cell infiltration, sections were incubated with goat anti-LCN2 antibody (1:500 dilution; R&D Systems, Minneapolis, MN), fluoromyelin (1:300 dilution; Invitrogen), mouse anti-GFAP antibody (1:500 dilution; BD Bioscience), rabbit anti-Iba-1 antibody (1:500 dilution; WAKO Osaka, Japan), and rat anti-CD4 antibody (1:200 dilution; Serotec, Oxford, UK). Sections were visualized directly or incubated with FITC-conjugated anti-mouse, rat, rabbit, or goat IgG antibody (The Jackson Laboratory, Bar Harbor, ME) or Cy3-conjugated anti-goat IgG antibody (The Jackson Laboratory). Percentage of Lcn2- or 24p3R-expressing cells was determined by quantification of fluorescence intensities using Image J software and co-localization plug-in. For the quantification of demyelination, images of the lumbar sections were obtained, and Image J software was used to outline the demyelinated area and total white matter area. Pixel area for each sample was calculated, and the percentage of demyelination was determined by dividing the total white matter area by the total demyelinated area (three lumbar sections examined for each animal). Histological scores were evaluated using H&E-stained lumbar spinal cord sections and the following scale (51): 0 = no infiltration; 1 = mild infiltration around pial vessels; 2 = single-cell infiltration within the CNS; 3 = infiltration with mild perivascular cuffing; 4 = very intense infiltration with perivascular cuffing. For double-immunofluorescence
staining, sections were incubated with a mixture of rabbit anti-LCN2 antibody (R&D Systems) or anti-24p3R (Slc22A17) antibody (Sigma) and either mouse anti-GFAP antibody (BD Bioscience), rabbit anti-Ly6G (Gr-1) antibody (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), or hamster anti-CD11c antibody (1:200 dilution, eBioscience, San Diego, CA), or rat anti-CD45 antibody (1:200 dilution, BioLegend). Sections were then incubated with secondary antibody, washed, cover-slipped with Vectashield mounting medium (Vector Laboratories), and examined under a confocal microscope (Olympus FV1000). GFAP and Iba-1 fluorescence intensities and numbers of cells were quantified using Image J software. Pictures were taken of each PCR product was electrophoresed on a 1% agarose gel and detected under UV light. Primer sequences (Table 1).

| Genes | RT-PCR methods | GenBank™ accession no. | Primer sequences |
|-------|----------------|------------------------|------------------|
| Lipocalins Lcn2 | Traditional | NM_008491 | F: 5’-ATG GGA ATG TCA CTA CCT CCA ACC TGG TTG TC-3’<br>R: 5’-CAG CAC ACC CAT CCA TGG TCA AG-3’ |
| Lcn2 | Real time | NM_008491 | F: 5’-CCC CAT CAT TGC TCA CGT CTG TC-3’<br>R: 5’-TTC TTT TCC AGG ACC GCA TGG G-3’ |
| MMPs Mmp9 | Real time | NM_013599 | F: 5’-AAC TCT CTC TAG AGA CGA GAG AGG AG-3’<br>R: 5’-AGC TGA TGG ACT AAA AGT GCT GGA-3’ |
| Mmp2 | Real time | NM_008610 | F: 5’-CAG GGA ATG ACG ACT GGT CTG TCT-3’<br>R: 5’-ACT CAT CTT CTT AGC AGC ATC TAC-3’ |
| Cytokines Il6 | Real time | NM_008361 | F: 5’-ATG CTC CTT GGG ACT GA-3’<br>R: 5’-AGT CTT CTT GGG ACT GA-3’ |
| Il12 | Real time | NM_031168 | F: 5’-ATT CTT TAC TGC TAC CAC AAC CTG C-3’<br>R: 5’-TCC AGG TCC CAT CAG GAG AG-3’ |
| Il17a | Real time | M86672 | F: 5’-GGC CTA GCG CTT CCC TCT CAG-3’<br>R: 5’-GAG GAA CCA GAG GCT CTG TC-3’ |
| Ifng | Real time | NM_008337 | F: 5’-TTT ACT TTA AGT GCC CAA ACC ACC GCC TTTT-3’<br>R: 5’-ATG TGA GTT TGG TGC TTA TTA TCA TCA AC-3’ |
| Tnf | Real time | NM_013693 | F: 5’-ATG GGC TCC CTC TTA TAA C-3’<br>R: 5’-TTG GTG GGT TGC TAC GAC TAC-3’ |
| Chemokines Ccl2 | Real time | NM_011333 | F: 5’-TCA GCA TCC AGC GTA TAA CA-3’<br>R: 5’-AGC TCT CAT CTA CTA CTT TTT CC-3’ |
| Cxcl10 | Real time | NM_021274 | F: 5’-AGG GCC AGT TAC TAT CTA TTA C-3’<br>R: 5’-GAA CAG GAA GAA GAA GAA TTT C-3’ |
| Transcription factors Rorc | Real time | AJG32394 | F: 5’-TTT GGA ACT GCC TTC TTA CCA AG-3’<br>R: 5’-AGC TCT GTC CAT CTA CTA CTT TTT CC-3’ |
| Tbet | Real time | NM_019507 | F: 5’-ATT GCC GCC AGC CTG TCC AGC AG-3’<br>R: 5’-GGG TGG CCT CCA TCC TGG TC-3’ |
| Housekeeping genes Gapdh | Traditional | NM_008084 | F: 5’-ACC ACA GTC CAT GCC ATC AC-3’<br>R: 5’-TCC ACC CGT TCA GTC TA-3’ |
| Gapdh | Real time | NM_008084 | F: 5’-TCC ACC CGT TCA GTC TA-3’<br>R: 5’-GGG TGT CCG TCT TCA AG-3’ |

Sections were then incubated with avidin-biotin complex reagents (Vector Laboratories) for 30 min at room temperature followed by diaminobenzidine detection. The sections were mounted on gelatin-coated slides and allowed to air-dry overnight. Each section was captured using a CCD color video camera (Olympus D70) attached to a microscope (Olympus BX51) equipped with a 100× objective lens.

**Table 1**

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| Mmp2 | Real time | NM_008610 | F: 5’-CAG GGA ATG ACG ACT GGT CTG TCT-3’<br>R: 5’-ACT CAT CTT CTT AGC AGC ATC TAC-3’ |
| Cytokines Il6 | Real time | NM_008361 | F: 5’-ATG CTC CTT GGG ACT GA-3’<br>R: 5’-AGT CTT CTT GGG ACT GA-3’ |
| Il12 | Real time | NM_031168 | F: 5’-ATT CTT TAC TGC TAC CAC AAC CTG C-3’<br>R: 5’-TCC AGG TCC CAT CAG GAG AG-3’ |
| Il17a | Real time | M86672 | F: 5’-GGC CTA GCG CTT CCC TCT CAG-3’<br>R: 5’-GAG GAA CCA GAG GCT CTG TC-3’ |
| Ifng | Real time | NM_008337 | F: 5’-TTT ACT TTA AGT GCC CAA ACC ACC GCC TTTT-3’<br>R: 5’-ATG TGA GTT TGG TGC TTA TTA TCA TCA AC-3’ |
| Tnf | Real time | NM_013693 | F: 5’-ATG GGC TCC CTC TTA TAA C-3’<br>R: 5’-TTG GTG GGT TGC TAC GAC TAC-3’ |
| Chemokines Ccl2 | Real time | NM_011333 | F: 5’-TCA GCA TCC AGC GTA TAA CA-3’<br>R: 5’-AGC TCT CAT CTA CTA CTT TTT CC-3’ |
| Cxcl10 | Real time | NM_021274 | F: 5’-AGG GCC AGT TAC TAT CTA TTA C-3’<br>R: 5’-GAA CAG GAA GAA GAA GAA TTT C-3’ |
| Transcription factors Rorc | Real time | AJG32394 | F: 5’-TTT GGA ACT GCC TTC TTA CCA AG-3’<br>R: 5’-AGC TCT GTC CAT CTA CTA CTT TTT CC-3’ |
| Tbet | Real time | NM_019507 | F: 5’-ATT GCC GCC AGC CTG TCC AGC AG-3’<br>R: 5’-GGG TGG CCT CCA TCC TGG TC-3’ |
| Housekeeping genes Gapdh | Traditional | NM_008084 | F: 5’-ACC ACA GTC CAT GCC ATC AC-3’<br>R: 5’-TCC ACC CGT TCA GTC TA-3’ |
| Gapdh | Real time | NM_008084 | F: 5’-TCC ACC CGT TCA GTC TA-3’<br>R: 5’-GGG TGT CCG TCT TCA AG-3’ |

**Role of LCN2 in EAE**

Cytokines

- **MMPs**
  - **Mmp9**
  - **Mmp2**

- **Cytokines**
  - **Il6**
  - **Il12**
  - **Il17a**
  - **Ifng**
  - **Tnf**

- **Chemokines**
  - **Ccl2**
  - **Cxcl10**

- **Transcription factors**
  - **Rorc**
  - **Tbet**

- **Housekeeping genes**
  - **Gapdh**
  - **Gapdh**

Sections were then incubated with avidin-biotin complex reagents (Vector Laboratories) for 30 min at room temperature followed by diaminobenzidine detection. The sections were mounted on gelatin-coated slides and allowed to air-dry overnight. Each section was captured using a CCD color video camera (Olympus D70) attached to a microscope (Olympus BX51) equipped with a 100× objective lens.

**Traditional or Real-time Reverse Transcription-PCR—Total RNA was extracted from cells or tissues using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was conducted using Superscript II (Invitrogen) and oligo(dT) primers. PCR amplification was conducted using a DNA Engine Tetraderm Thermal Cycler (MJ Research, Waltham, MA) at an annealing temperature of 55–60 °C for 20–30 cycles using specific primer sets. To analyze PCR products, 10 μl of each PCR product was electrophoresed on 1% agarose gel and detected under UV light. Gapdh was used as an internal control. Real-time PCR was performed using the One Step SYBR® PrimeScript™ RT-PCR kit (Perfect Real-time; Takara Bio Inc., Tokyo), and detection was performed using the ABI Prism® 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). Nucleotide sequences of the primers were based on published cDNA sequences (Table 1).**
Role of LCN2 in EAE

Cell Proliferation Assay—Spleen cells were cultured at a density of 4 × 10^6 cells per well in 96-well plates in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum and 100 units/ml antibiotics. Cells were incubated with MOG35–55 peptide (10 μg/ml), proteolipid protein (10 μg/ml), or concanavalin A (5 μg/ml) (Sigma) for 48 h. Cultures were then pulsed with 1 μCi of 8-methylthymidine (specific activity 42 Ci/mmol; Amersham Biosciences) and incubated for an additional 18 h. Cells were harvested, and radioactivities were measured using a liquid scintillation spectrometer (Wallac Micro Beta TriLux, PerkinElmer Life Sciences). Results are expressed as cpm.

In Vitro and in Vivo Treatments with Recombinant LCN2 Protein—Recombinant mouse LCN2 protein expressed in mammalian cells was purchased from R&D Systems. For some experiments, recombinant mouse LCN2 protein expressed as a glutathione S-transferase fusion protein in E. coli BL21 strain was used (41). For in vitro treatment, draining lymph nodes and spleens were collected 10 days after EAE induction. The lymph node and spleen cells (3 × 10^6 cells/ml) were cultured in complete RPMI medium (Lonza, Walkersville, MD) containing 30 μg/ml MOG35–55 with or without 1 μg/ml of recombinant LCN2 protein. After 2 days of culture, RNA was isolated from cell pellets. Primary mixed glial cultures were prepared from the brains of neonatal mice (<2 days old). Cells were cultured in the presence of 0.01–10 μg/ml recombinant LCN2 protein for 6–24 h, and total RNA or culture media were subjected to real-time PCR, ELISA, or gelatin zymography to measure proinflammatory cytokine, chemokine, and matrix metalloproteinase levels. For in vivo LCN2 injections, recombinant LCN2 protein or its denatured form was administered intrathecally (5 and 50 ng daily from 7 to 11 days) or intraperitoneally (10 μg daily for 2 weeks) to mice after MOG immunization. Animals were weighed and examined daily for disease symptoms.

Adoptive Transfer of EAE—For adoptive transfer, WT or Lcn2-deficient mice were immunized with MOG35–55-complete Freund’s adjuvant, and pertussis toxin. Lymph nodes and spleens were collected 10 days after EAE induction, respectively; these values were chosen based on peak times for encephalitogenic immune responses in the spinal cord and peripheral lymphoid organs (52). Immunofluorescence was then used to evaluate LCN2 protein expression in spinal cords and peripheral immune organs. In spinal cords, LCN2 expression was very low in the gray matter of treatment naïve mice. However, after EAE induction, strong LCN2 expression was observed, mostly in the white matter of spinal cords (Fig. 1C). Similarly, LCN2 protein expression was also elevated in lymph nodes and spleens after EAE induction (Fig. 1D). These results led us to hypothesize that LCN2 may be involved in EAE pathogenesis.

LCN2 Expression in EAE-induced Spinal Astrocytes and Spleen Neutrophils—In our previously studies, we found Lcn2 gene expression was up-regulated and that LCN2 protein was secreted by microglia and astrocytes under inflammatory conditions (41, 42). Subsequent studies by others also demonstrated glial expression of LCN2 in the CNS (44, 46, 53). Thus, we examined whether glial cells and neurons in the spinal cord express LCN2 during EAE. Mice were immunized with MOG peptide and pertussis toxin, and spinal cords were isolated during the disease peak and chronic phases (17 and 30 days after EAE induction, respectively). The spinal cord sections were subjected to double-immunofluorescence staining with LCN2 antibody and either GFAP (an astrocyte marker), Iba-1 (a microglia/macrophage marker), or NeuN (a neuron marker) antibodies. LCN2 was highly expressed in the GFAP+ astrocytes, but not microglia or neurons, at the disease peak (Fig. 2, A–C), and astrocytes maintained a high level of LCN2 expression in the chronic phase (data not shown). The expression of LCN2 in GFAP+ astrocytes was also observed in cerebellum at the disease peak (data not shown). Because LCN2 expression was also increased in peripheral immune organs after EAE induction (Fig. 1), we next investigated the localization of LCN2 protein within spleens and lymph nodes. Spleen sections were stained with LCN2, CD4 (T cell marker), CD45 (macrophage marker), CD11c (dendritic cell marker), or Ly6G (neutrophil marker) antibodies 10 days after EAE induction, and LCN2 was found to be expressed in neutrophils, but not in T cells, macrophages, or dendritic cells, in the spleens and lymph nodes of EAE-induced mice (Fig. 2D). These findings suggest that LCN2 expressed in astrocytes and neutrophils plays an important role.
inflammatory responses in the CNS and periphery, respectively.

24p3R Expression in the Spinal Cord and Periphery during EAE—To determine whether CNS-resident cells and peripheral immune cells express LCN2 receptor 24p3R, spinal cords (17 after EAE induction) and spleen (day 10 after EAE induction) were harvested from EAE-induced mice. Immunofluorescence staining showed that astrocytes and microglia expressed high levels of 24p3R (Fig. 2E). Spinal neurons did not express 24p3R at the disease peak. In spleen tissues 24p3R was mainly expressed in CD11c+ cells (dendritic cells and activated T cells) at presymptomatic phase (day 10 after EAE induction) (Fig. 2F). In addition, 24p3R-expressing CD4+ T cells were identified in spinal cord as well (Fig. 2E). These results suggest that LCN2 protein, secreted during EAE development, may act on astrocytes, microglia, and CNS-infiltrating T cells in spinal cords and on dendritic cells and T cells in spleen.

Lcn2-deficient Mice Exhibited EAE with a Delayed Course and Reduced Severity—To investigate the potential role of LCN2 in the initiation and progression of EAE, disease onset times, susceptibilities, and severities were compared in WT and Lcn2 KO mice after injecting 50 μg (to induce mild EAE) or 200 μg (to induce severe EAE) of MOG peptide and pertussis toxin. In WT mice, severe disease symptoms, such as limping and tail and limb paralysis, were observed with a maximum EAE score of 2.75 ± 0.13. Lcn2 KO mice showed less severe symptoms (maximum EAE score, 1.66 ± 0.22) (Fig. 3A, FIGURE 1. LCN2 expression was up-regulated in EAE-induced mice. WT (n = 3) and Lcn2 KO (n = 3) mice were immunized with MOG peptide and pertussis toxin. At days 10 and 17 after EAE induction, spinal cords, lymph nodes, and spleens were removed. Total RNAs were isolated, and the mRNA levels of LCN2 were determined by traditional RT-PCR (A) and real-time PCR (B). Gapdh was used as an internal control. *, p < 0.01, naive versus EAE (Student’s t test). Results for individual animals and representative results are shown for WT and Lcn2 KO mice, respectively. Spinal cord, lymph node, and spleen tissue sections derived from naive and EAE-induced mice were stained with anti-LCN2 antibody and secondary FITC- or Cy3-conjugated anti-goat IgG antibody for immunofluorescence analysis (C and D). Scale bar = 200 μm. Results are representative of more than three independent experiments. Quantification of fluorescence intensities revealed that LCN2 expression was increased in the spinal cords, spleen, and lymph node of EAE-induced mice compared with naive mice (graphs). Results are the mean ± S.E. (n = 3). *, p < 0.01, naive versus EAE (Student’s t test).
Role of LCN2 in EAE

A

EAE (day 17)

GFAP
LCN2
GFAP/LCN2/DAPI

Ventral horn

Ventral column

Not colocalized with LCN2
Colocalized with LCN2

GFAP* fluorescence intensity (%)

Naive EAE

B

EAE (day 17)

Iba-1
LCN2
Iba-1/LCN2/DAPI

Ventral horn

Ventral column

Not colocalized with LCN2
Colocalized with LCN2

Iba-1* fluorescence intensity (%)

Naive EAE

C

EAE (day 17)

NeuN
LCN2
NeuN/LCN2/DAPI

Ventral horn

Not colocalized with 24p3R
Colocalized with 24p3R

NeuN* fluorescence intensity (%)

D

EAE (day 10)

CD4
LCN2
CD4/LCN2/DAPI

Spleen

CD45
LCN2
CD45/LCN2/DAPI

Not colocalized with LCN2
Colocalized with LCN2

E

EAE (day 17)

NeuN
24p3R
NeuN/24p3R/DAPI

Ventral horn

CD4
24p3R
CD4/24p3R/DAPI

Spleen

24p3R
LCN2
24p3R/LCN2/DAPI

Fluorescence intensity (%)

NeuN* Iba-1* GFAP* CD4*
of EAE. The infiltration of effector T cells is a critical process for the induction of CNS inflammation and the pathology of EAE. Thus, we compared accumulation of T cell populations in the spinal cords of WT and Lcn2 KO mice after EAE induction. CD4+ T cells influx into spinal cords was observed in WT mice, whereas CD4+ T cells were less abundant in the spinal cords of Lcn2 KO mice (Fig. 4C). These results indicate that the absence of Lcn2 leads to diminished CNS infiltration by effector T cells and reduced demyelination and subsequent axonal injury.

**Lcn2 Deficiency Diminished the mRNA Expression of Inflammatory Cytokines and Chemokines during EAE**—To determine whether Lcn2 deficiency affects the expression of proinflammatory genes in spinal cords during EAE, quantitative real-time RT-PCR was performed to assess the expression levels of inflammatory cytokines and chemokines. At day 17 after EAE induction, the mRNA expression of proinflammatory cytokines and chemokines (Il17a, Ifng, Tnf, Il12, Il6, Il11b, Ccl2, and Cxcl10) was strongly enhanced in the spinal cords of WT mice, and this enhancement was significantly less in spinal cords of Lcn2 KO mice. In particular, EAE-induced Il17a and Ifng, which are known to be produced by Th17 and Th1 cells, respectively, were expressed at significantly lower levels in the spinal cords of Lcn2 KO mice (Fig. 5A). Furthermore, EAE-induced proinflammatory cytokines (Tnf, Il6, and Il11b) and chemokines (Cxcl10) were also expressed at lower levels in Lcn2 KO mice 30 days after EAE induction, whereas Il12 was expressed at similar levels in WT and Lcn2 KO spinal cords (data not shown). These results were consistent with the histological finding that the absence of the Lcn2 gene was associated with reduced spinal cord inflammation. To determine how Lcn2 deficiency influences peripheral immune response, proinflammatory cytokine gene expression in lymph nodes and spleens were compared in WT and Lcn2 KO mice. The EAE-induced expression of Il17a, Ifng, and Il6 in secondary lymphoid tissues was found to be significantly lower in Lcn2 KO mice than in WT mice (Fig. 5B). These results indicate that Lcn2 expression is essential for the induction of inflammatory mediators in peripheral immune organs and in the CNS during EAE. In addition, we asked whether Lcn2 deficiency affects the proliferative ability of MOG-specific T cells in peripheral lymphoid tissue at disease peaks. We found that autoreactive T cells from Lcn2 KO EAE mice showed significantly lower levels of proliferation than WT EAE mice when restimulated in the presence of MOG peptide (Fig. 5C). However, no significant difference was found after restimulation with a control proteolipid protein (one of the three major myelin proteins). Next, we compared the sizes of lymph nodes and spleens of WT and Lcn2 KO mice. WT mice

**FIGURE 2** Lcn2 and 24p3R expression in spinal cords, spleens, and lymph nodes during EAE. Frozen sections of lumbar spinal cords, spleens, and lymph nodes were prepared from EAE-induced mice at peak disease severity (day 17; n = 3) or during the pre-symptomatic phase (day 10; n = 3) and subjected to immunofluorescence analysis and confocal microscopy to localize Lcn2 (A–D) and 24p3R (its receptor) (E and F) immunoreactivity in several cell types; that is, in astrocytes, microglia, neurons, T cells, macrophages, dendritic cells, and neutrophils. Arrows indicate the co-localization of Lcn2 or 24p3R and cell type-specific markers. Scale bars = 100 μm. Quantification of Lcn2 or 24p3R fluorescence intensity was carried out by determining the percent co-localization of Lcn2 or 24p3R and cell type-specific markers (graphs). Gfap, astrocyte-specific marker; Iba-1, microglial cell marker; NeuN (neuronal marker); CD4 (T cell marker); CD45 (macrophage marker); CD11c (dendritic cell marker); Ly6G (neutrophil marker). Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole; blue). Images are representative of three independent experiments.
FIGURE 3. Reduction of EAE pathology and disease severity in Lcn2-deficient mice. WT and Lcn2 KO mice were immunized with MOG peptide at doses of 200 μg (n = 12 per group) or 50 μg (n = 5 per group) with complete Freund’s adjuvant and pertussis toxin. The immunized mice were observed daily for 35 days (A, left) or 25 days (A, right) to determine mean EAE scores. Disease severity was significantly less in Lcn2 KO mice than in WT mice after injecting 200 μg of MOG (A, left), but no significant difference was found between WT and KO mice after injecting 50 μg of MOG (A, right). Results are the mean ± S.E. *, p < 0.05, WT versus Lcn2 KO mice (Mann-Whitney U test). Frozen sections of lumbar spinal cords from non-immunized naive mice or EAE-induced WT or KO mice taken at day 17 after EAE induction were stained with H&E or fluoromyelin (B). Scale bars = 200 μm. Inflammatory infiltration (B, upper) and demyelination (B, lower) were greater in WT-EAE spinal cord white matter than in non-immunized WT controls but significantly less in the spinal cords of Lcn2 KO-EAE mice than in WT-EAE animals. Inflammatory infiltration and demyelination were quantitatively assessed in the spinal cords of WT and KO mice (B graphs). The results are the mean ± S.E. (n = 5). *, p < 0.05, WT versus Lcn2 KO mouse (Student’s t test).
LCN2 was found to induce the next evaluated the effect of LCN2 on glial MMP expression. Due to blood brain barrier disruption and demyelination in MS, protein (Fig. 6). Induction, adoptive transfer experiments were performed to an autocrine or paracrine manner. Of proinflammatory mediators by activating spinal glial cells in the lymph nodes and spleens of Lcn2 KO mice.

LCN2 Treatment in Vitro Increased MOG-specific T Cell and Glial Cell Responses—To determine the effect of LCN2 on the activation of peripheral immune cells, we examined the effect of LCN2 on the expression of Il17a and Ifng in MOG-specific T cells. WT and KO mice were immunized with MOG and pertussis toxin, and lymph node and spleen cells were isolated 10 days after immunization. These cells were then cultured with MOG peptides in the presence or absence of recombinant LCN2 protein (1 μg/ml). MOG-induced Il17a and Ifng expression was significantly increased by treatment with recombinant LCN2 protein in lymph nodes and spleen cells (Fig. 6, A and B). In addition, LCN2 treatment also enhanced the expression of Rorc (encoding RORyt) and Thet (encoding T-bet), the signature transcription factors of Th17 and Th1 cells, respectively, in lymph node and spleen cells (Fig. 6, A and B). Similar results were found in WT and Lcn2 KO mice, supporting the central role of LCN2 in augmenting the proliferation and activation of encephalitogenic T cells in peripheral lymphoid tissues. We then investigated how LCN2 influences the activation of glial cells in the spinal cord. Primary glial cultures were incubated with recombinant LCN2 protein (0.01–10 μg/ml), and the expression and secretion of proinflammatory mediators was assessed. When treated with LCN2 protein, glial cultures showed dose-dependent elevation in the expression of Il1b, Tnf, Cxcl10, and Ccl2 (Fig. 6C). The production of TNF-α and CXCL10 proteins by glial cells was also significantly increased in the culture media after treatment with 1–10 μg/ml LCN2 protein (Fig. 6D). Because MMPs have been shown to contribute to blood brain barrier disruption and demyelination in MS, we next evaluated the effect of LCN2 on glial MMP expression. LCN2 was found to induce Mmp9 dose-dependently, but not Mmp2, at the mRNA and protein levels in glial cells (Fig. 6, E and F). Furthermore, the expression of cytokines, chemokines, and Mmp-9 was unaffected by denatured LCN2 protein or polymyxin B-pretreated LCN2 protein, which excluded the possibility of LPS contamination of the recombinant LCN2 protein preparation (data not shown). These results suggest that astrocyte-derived LCN2 protein promotes the expression of proinflammatory mediators by activating spinal glial cells in an autocrine or paracrine manner.

LCN2 Expression in the CNS and Periphery and EAE Development—Because LCN2 expression was highly induced in spinal astrocytes and peripheral lymphoid organs after EAE induction, adoptive transfer experiments were performed to determine the pathogenic roles of the CNS and peripheral LCN2 in EAE onset and severity. Draining lymph nodes and spleen cells were harvested from WT and Lcn2 KO mice on day 10 after EAE induction, restimulated in vitro with MOG35–55 for 3 days, and then transferred intravenously into naïve WT or Lcn2 KO mice. As was expected, the transfer of WT cells reproduced EAE in WT recipients with an incidence of 100%, a symptom onset of ~13 days, peak symptom severity on day 19 (EAE score 2.83 ± 0.16), and remission from day 20 (Fig. 7A). In contrast, WT recipient mice transferred with Lcn2 KO cells exhibited reduced disease severity (EAE score 0.50 ± 0.22 at day 19). In addition, disease onset in the KO to WT transfer group was significantly delayed as compared with that in the WT to WT control group. Interestingly, when cells from immunized WT mice were transferred to naïve Lcn2 KO mice, lesser symptoms and delayed onset (day 17.16 ± 0.90) were observed, although mean disease score was similar to that of the WT to WT control group at day 19 (Table 3). These findings show that a lack of Lcn2 in CNS and peripheral immune tissues ameliorates EAE development. To confirm the role of spinal LCN2 in EAE development, we evaluated the effect of directly injecting recombinant LCN2 protein into the spinal cords on disease symptoms. LCN2 protein (at 5 or 50 ng) was administered intrathecally daily for 5 days (days 7, 8, 9, 10, and 11) after EAE induction. Intrathecal injections of recombinant LCN2 protein were found to accelerate EAE development and to increase disease severity significantly in the acute stage but not to significantly influence peak disease severity (Fig. 7B). Mice administered with denatured LCN2 protein as a control developed EAE symptoms on day 12 after EAE induction and peak symptom severity on day 16, whereas recombinant LCN2 protein-injected mice developed EAE symptoms on day 9 and peak symptom severity on day 13 (50 ng of LCN2) or day 14 (5 ng of LCN2) (data not shown). To further examine the role of peripheral LCN2 in the EAE development, intraperitoneal injection of recombinant LCN2 protein was also done. Mice injected with LCN2 showed accelerated onset (day 10.2 ± 0.96) and increased disease severity (EAE score 2.0 ± 0.47 at day 15) compared with control group (onset day 12.0 ± 0.77, EAE score 1.2 ± 0.20). These results indicate that LCN2 expressed in both the CNS and the peripheral tissues plays a critical role during EAE development.

**DISCUSSION**

LCN2 plays an important role in diverse cellular processes including cell death, survival, migration, invasion, and differentiation. Because we issued our first report on the glial expression of LCN2 (41, 55), several studies have demonstrated the potentially important roles played by LCN2 in the physiology.

**TABLE 2**

| Mouse genotype | Incidence | Day of onset | Development of complete hind-limb paralysis | Maximum EAE score |
|----------------|-----------|--------------|--------------------------------------------|------------------|
| Lcn2 +/−       | 12/12 (100%) | 11.41 ± 0.35 | 75.0%                                       | 2.75 ± 0.13      |
| Lcn2 −/−       | 12/12 (100%) | 13.16 ± 0.42* | 8.3%                                       | 1.66 ± 0.2*      |

*p < 0.05 as compared to Lcn2 +/− animals. p values calculated by Mann-Whitney U test.

showed enlarged lymph nodes and spleens at 10 days after EAE induction, whereas Lcn2 KO mice exhibited lesser enlargements (Fig. 5D). This result was consistent with the lower expression of proinflammatory cytokines observed in the lymph nodes and spleens of Lcn2 KO mice.
and pathology of the CNS (38, 44, 47, 56). Nevertheless, its functional roles in neuroinflammation and related diseases of the CNS are far from clear, and the results of some previous studies have been contradictory. For example, Ip et al. (44) reported no significant difference between inflammatory and glial markers in WT and Lcn2 KO mice after a peripheral injection of LPS, whereas Rathore et al. (46) reported on the detrimental effects of LCN2 after spinal cord injury and on reductions in the expression of several proinflammatory chemokines and cytokines and inducible nitric-oxide synthase after spinal cord injury in Lcn2 KO mice as compared with WT animals. Furthermore, the proinflammatory effects of LCN2 are supported by our recent findings that LCN2 caused proinflammatory chemokine expression and M1 polarization of microglia. 

FIGURE 4. Reduction of EAE-induced glial activation and T cell infiltration in Lcn2-deficient mice. Activation of astrocytes (A) and microglia (B) at day 17 after EAE induction was assessed by immunofluorescence staining using anti-GFAP and anti-Iba-1, respectively, in lumbar spinal cord sections obtained from treatment naïve or EAE-induced WT or KO mice. Astrocytes and microglia were found to be markedly more activated in EAE-induced WT mice than in naïve controls but to be less activated in EAE-induced KO mice than in WT-EAE animals. In addition, lumbar spinal cord sections obtained from treatment naïve or EAE-induced WT or Lcn2 KO mice at 17 days after EAE induction were stained with anti-CD4 antibody for immunofluorescence analysis (C). Scale bar = 200 μm. Quantification of fluorescence intensities or cell numbers revealed that glial activation and T cell infiltration were less in the spinal cords of Lcn2 KO mice than in WT mice (graphs). Results are the mean ± S.E. (n = 5) and were analyzed by two-way ANOVA. Genotype x disease significance levels (p value): GFAP ventral column 0.010, dorsal column 0.004, ventral horn 0.015, Iba-1 ventral column 0.000, dorsal column 0.001, ventral horn 0.00, CD4 infiltration 0.000. *, p < 0.05, WT EAE versus KO EAE in the same spinal cord region (one-way ANOVA with Bonferroni’s post-hoc test). 

and pathology of the CNS (38, 44, 47, 56). Nevertheless, its functional roles in neuroinflammation and related diseases of the CNS are far from clear, and the results of some previous studies have been contradictory. For example, Ip et al. (44) reported no significant difference between inflammatory and glial markers in WT and Lcn2 KO mice after a peripheral injection of LPS, whereas Rathore et al. (46) reported on the detrimental effects of LCN2 after spinal cord injury and on reductions in the expression of several proinflammatory chemokines and cytokines and inducible nitric-oxide synthase after spinal cord injury in Lcn2 KO mice as compared with WT animals. Furthermore, the proinflammatory effects of LCN2 are supported by our recent findings that LCN2 caused proinflammatory chemokine expression and M1 polarization of micro-
glia (43). LCN2 has also been proposed as a novel component of proinflammatory signaling in Alzheimer disease (38).

In the present study we report that LCN2 plays a pivotal role in EAE based on the following results: 1) genetic ablation of Lcn2 significantly attenuated disease severity and reduced inflammatory infiltration, glial activation, inflammatory cytokine/chemokine expression, demyelination, and autoreactive T cell proliferation; 2) LCN2 treatment in vitro up-regulated the expression of IL-17 and IFN-γ in MOG-specific T cells and the expression of pro-inflammatory cytokines, chemokines, and neurotoxic molecules in glial cells; 3) intrathecal administration of LCN2 protein during EAE induction caused a dose-dependent shortening of disease onset; 4) adoptive transfer of EAE from WT to KO mice and from KO to WT mice reduced disease severity as compared with WT to WT transfer, indicating that LCN2 expressed in the CNS and peripheral lymphoid tissues plays an important role in EAE development. Furthermore, our results on the localization of LCN2 and its receptor in different cell types indicate that glia-derived LCN2 acts on multiple target cells in the CNS, whereas neutrophil-derived LCN2 acts on dendritic cells in the periphery (Fig. 8).

The present study shows that LCN2 expression is highly up-regulated in spinal astrocytes after EAE induction and that Lcn2 deficiency reduces inflammatory activation of glial cells in the EAE spinal cord and concomitantly attenuates inflammation, and these findings are in line with our previous findings that LCN2 promotes the classical proinflammatory activation of

**FIGURE 5.** Reduced expression of proinflammatory cytokines and chemokines and the proliferation of autoimmune T cells in Lcn2 KO mice after EAE induction. WT and Lcn2 KO mice were immunized with MOG and pertussis toxin. Spinal cords (at day 17 after EAE induction) (A), draining lymph nodes, and spleens (at day 10 after EAE induction) (B) were removed, total RNAs were isolated, and the gene expression of inflammatory cytokines and chemokines was determined by real-time PCR. The results shown are the means ± S.E. (n = 5) and were analyzed by two-way ANOVA. Genotype x disease significance levels (p value); spinal cord Il17a 0.001, Ifng 0.004, Tnf 0.002, Il12 0.003, Il6 0.032, Il1b 0.021, Ccl2 0.041, Cxcl10 0.029, lymph nodes and spleen Il17a 0.014, Ifng 0.014, Il6 0.042, *, p < 0.05; **, p < 0.01 (one-way ANOVA with Bonferroni’s post-hoc test). Alternatively, spleen cells from WT and KO mice immunized with MOG and pertussis toxin were cultured in medium alone (control) or in the presence of MOG35–55 or proteolipid protein (PLP138–151) peptides (C). T cell responses were determined by measuring [3H]thymidine incorporation. The results are the mean ± S.E. (n = 3) and analyzed by two-way ANOVA. Genotype x treatment significance levels: 0.000. **, p < 0.01, WT versus Lcn2 KO mice (one-way ANOVA with Bonferroni’s post-hoc test). Photographs of the spleens and draining lymph nodes of WT and Lcn2 KO mice at 10 days after EAE induction are shown (D).
microglia (M1 polarization) (43) and astrocytes (41). Previous studies have shown LCN2 induction is associated with many inflammatory conditions in the CNS and peripheral tissues (31, 33, 34, 37, 38, 44, 50), and we have also observed that LCN2 levels in CSF are higher in MS patients than in healthy control subjects (data not shown). The above-mentioned results show that astrocytes and LCN2 play central roles in CNS inflammation during EAE.

Proinflammatory cytokines play central roles in the pathogenesis of MS and EAE via immune system activation and the

FIGURE 6. Effect of LCN2 on proinflammatory gene expression in encephalitogenic T cells and glial cells. At day 10 after EAE induction in WT (A) and Lcn2 KO mice (B), lymph node and spleen cells (3 × 10^6 cells per well) were treated with MOG (30 μg/ml) in the presence or absence of recombinant LCN2 protein (1 μg/ml). Total RNA was isolated after 48 h of MOG re-stimulation. Levels of Il17a, Ifng, Rorc, and Tbet mRNA were determined by real-time PCR. Data were normalized versus Gapdh, and the results shown are the means ± S.E. (n = 4). *, p < 0.05 versus control; #, p < 0.05 between the indicated groups (one-way ANOVA).

C–F, primary mixed glial cultures were treated with LPS (100 ng/ml) alone, with LPS and IFN-γ (50 units/ml), or with recombinant LCN2 protein (0.01–10 μg/ml) for 6 h, and total RNA was isolated. The mRNA levels of cytokines (Il1b and Tnf), chemokines (Cxcl10 and Ccl2) (C), and Mmp2 and Mmp9 (E) were determined by real-time RT-PCR. Amounts of TNF-α and CXCL10 protein (D) or MMP proteins (F) in culture media were measured by specific ELISA or gelatin zymography, respectively, 24 h after stimulation. mRNA data were normalized versus Gapdh. Results are the means ± S.E. (n = 3). *, p < 0.05 versus untreated controls (one-way ANOVA).
recruitment of leukocytes to inflammatory regions (5). In the present study we observed that levels of proinflammatory cytokines, such as, IL-17A, IFN-γ, IL-1β, TNF-α, and IL-6, were markedly lower in Lcn2 KO mice than in WT animals. IL-17A and IFN-γ, which are produced by Th17 cells and Th1 cells, respectively, play crucial roles in the development and progression of EAE (58). In the CNS, the astrocytic expression of MHC class II molecules is induced by IFN-γ (59) and modulated by TNF-α (60), suggesting that astrocytes are capable of activating T cells via myelin antigen presentation. Astrocytes are also critical in IL-17-Act1-mediated immune cell recruitment during autoimmune-induced inflammation of the CNS (15), which explains the relation between reductions in these proinflammatory cytokine levels in Lcn2 KO mice and reduced demyelination and inflammatory responses in spinal cords.

In a previous study we showed that LCN2 secreted by glial cells up-regulates the expression of CXCL10 in the CNS under inflammatory conditions (42), and CCL2 has been shown to be expressed primarily by reactive astrocytes under inflammatory conditions (61–63). Moreover, both of these chemokines are known to play central roles in the inflammatory recruitment of leukocytes and other cell types. In the present study, we found that Lcn2 deletion reduced the expression of CXCL10 and CCL2 in the CNS, which in turn may have impaired the infiltration of inflammatory cells into the CNS. Furthermore, Lcn2 deletion reduced the influx of CD4+ T cells into spinal cord parenchyma. We also found that LCN2 induced Mmp9 at the mRNA and protein levels in cultured glial cells. Interestingly, MMP-9 is up-regulated in MS and in other inflammatory neurodegenerative diseases, and its production has been associated with blood brain barrier damage in EAE model (64). Furthermore, it has been reported MMP-9 levels are elevated at around the time of EAE onset and that the MMP inhibitor GM6001 blocks blood brain barrier breakdown and attenuates disease severity (65). In addition it has been suggested that the interaction between LCN2 and MMP-9 enhances MMP-9 activity by blocking MMP-9 auto-degradation (66, 67). Moreover, MMP-9 is known to be neurotoxic and has been previously associated with neuronal death (68). Interestingly, in an EAE model, genetic ablation of TIMP-1 (tissue inhibitor of metalloproteinase 1, a natural inhibitor of the MMP) was found to increase disease severity (69). Taken collectively, our data suggest that the diminished expression of CXCL10, CCL2, and Mmp9 in Lcn2-deficient glial cells probably reduces CNS infiltration by inflammatory cells and demyelination of the EAE spinal cord.

Neutrophils play a key role in the immune system by participating in inflammatory responses and adaptive immunity (70). Neutrophils contribute to adaptive immune responses by secreting cytokines to induce the activation of B and T cells (71, 72). Neutrophils are also involved in the differentiation and proliferation of naïve T cells by inducing the activation of antigen presenting cells, such as macrophages and dendritic cells (73). In the present study we identified neutrophils as the major cellular source of LCN2 in peripheral lymphoid tissues after EAE induction. These results suggest that LCN2 secreted by neutrophils may be involved in peripheral immune responses.

### TABLE 3

**EAE progression after adoptive transfer**

| Adoptive transfer | Incidence | Day of onset | Development of complete hind-limb paralysis | Maximum EAE score |
|-------------------|-----------|-------------|-------------------------------------------|-------------------|
| WT to WT          | 6/6 (100%)| 13.33 ± 0.21| 83%                                       | 2.83 ± 0.16       |
| WT to KO          | 5/6 (83%) | 17.16 ± 0.90*| 50%                                       | 2.33 ± 0.49*      |
| KO to WT          | 4/6 (66%) | 19.00 ± 0.77*| 0%                                        | 1.00 ± 0.36*      |

*p < 0.05 as compared to the WT to WT transfer group. p values calculated by Mann-Whitney U test.
during EAE. In the present study the expression of the LCN2 receptor, 24p3R, was detected in dendritic cells of the EAE spleen, which suggested that neutrophil-derived LCN2 acts on dendritic cells to influence the antigen presentation process in peripheral lymphoid organs during EAE. This finding is consistent with the previous observation of 24p3R expression in different tissues, such as, spleen, kidney, liver, and lung (28). We also found that the degree of T cell proliferation was lower after the in vitro MOG re-stimulation of Lcn2 KO spleen cells compared with WT cells. In addition, LCN2 increased the mRNA expression of Rorc and Tbet in MOG-restimulated lymph node cells or spleen cells. Rorc and Tbet are transcription factors that are associated with the polarizations of Th17 and Th1 cells, respectively, which are critical steps in production of specific cytokines and in the differentiation of naïve T cells to effector T cells (3). Thus, LCN2 secreted by neutrophils in the peripheral immune system appears to be involved in the differentiation, proliferation, and activation of T cells under EAE conditions by directly acting on T cells or by indirectly acting on dendritic cells and other antigen presenting cells.

During the preparation of this manuscript, two studies on the role of LCN2 in EAE development were published. Having previously demonstrated the detrimental effects of LCN2 in a spinal cord injury model (46), the same research group reported...
that Lcn2 KO mice displayed a more severe EAE phenotype than WT animals (74). In another study by Marques et al. (75) on the EAE brain, LCN2 expression was found to be increased in EAE, particularly in brain regions typically affected in patients with MS. In the study by Berard et al. (74), immunization of C57BL/6 mice with 50 μg of MOG peptide induced both chronic and relapsing-remitting EAE. In this study, although the percentage of animals that developed chronic disease was higher for Lcn2 KO mice than for the WT, the percentages of animals that died or were sacrificed due to disease severity were not significantly different (74). Furthermore, mean disease onset time and mean EAE score at disease peak for chronic EAE animals were not significantly different between WT and KO mice. However, disease severity was significantly different only for mice with relapsing-remitting characteristics. During the initial stage of the present study, 200 μg of MOG peptide was injected to induce EAE, and Lcn2 KO mice showed a less severe disease phenotype and had lower mean EAE scores than WT animals. Later, we injected animals with 50 μg of MOG peptide to induce a mild disease condition, in which the disease severities of WT and Lcn2 KO mice were similar, although it should be added that Lcn2 deficiency reduced mean EAE scores. Accordingly, our results support the notion that LCN2 plays a pathogenic role in EAE but that its role in the disease process may be less important under mild conditions, which is at odds with the previously reported protective role of LCN2 in EAE. We do not have a clear explanation for this discrepancy. Nonetheless, the study by Berard et al. (74) showed a significant up-regulation of LCN2 in the spinal cord throughout EAE, which is consistent with our observations. Marques et al. (75) also reported LCN2 levels were increased in the EAE brain and that neutrophil and astrocyte LCN2 expression was observed during the onset and relapse phases in the brains of proteolipid protein-induced SJL mouse model. Furthermore, these authors also found natalizumab (an integrin inhibitor) down-regulated the expression of LCN2 and ameliorated disease severity and that LCN2 CSF levels were correlated with EAE progression (75). These findings of Marques et al. (75) concur with the findings of the present study. Indirect evidence also indicates that LCN2 is potentially involved in the development of EAE, as upstream or downstream signaling events associated with LCN2 have been implicated in EAE development. The complement components C3 and C5 are important for the recruitments of inflammatory cells under physiological conditions and in several acute and chronic inflammatory diseases (76, 77). In particular, component C3 mediates LCN2 expression in the spleen-resident immune cells of mice. In C3-deficient mice, EAE severity was attenuated and the infiltrations of macrophages and T cells into CNS parenchyma were reduced (78, 79). LCN2 expression is controlled by IL-6-gp130 signaling pathway during the early inflammatory response (80), and EAE severity was reported to be reduced in gp130 KO mice (81). These previous reports and the present study suggest that LCN2 has a proinflammatory disease-promoting effect during EAE development.

In summary, our findings show LCN2 plays a critical role in inflammatory responses involving reactive astrocytes within the CNS and in the activation of T cells in peripheral immune tissues during EAE development and progression. Accordingly, we advocate that LCN2 be regarded as a novel target for the diagnosis and treatment of MS.

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