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Eun-Jung Lee,* Jeong Eun Han,† Moon-Sook Woo,* Jin A. Shin,‡ Eun-Mi Park,§ Jihee Lee Kang,¶ Pyong Gon Moon,¶ Moon-Chang Baek,¶ Woo-Sung Son,‖ Young Tag Ko,† Ji Woong Choi,† and Hee-Sun Kim*

Matrix metalloproteinases (MMPs) play important roles in normal brain development and synaptic plasticity, although aberrant expression of MMPs leads to brain damage, including blood–brain barrier disruption, inflammation, demyelination, and neuronal cell death. In this article, we report that MMP-8 is upregulated in LPS-stimulated BV2 microglial cells and primary cultured microglia, and treatment of MMP-8 inhibitor (M8I) or MMP-8 short hairpin RNA suppresses proinflammatory molecules, particularly TNF-α secretion. Subsequent experiments showed that MMP-8 exhibits TNF-α–converting enzyme (TACE) activity by cleaving the prodomain of TNF-α (A74/Q75, A75/N77 residues) and, furthermore, that M8I inhibits TACE activity more efficiently than TAPI-0, a general TACE inhibitor. Biochemical analysis of the underlying anti-inflammatory mechanisms of M8I revealed that it inhibits MAPK phosphorylation, NF-κB/AP-1 activity, and reactive oxygen species production. Further support for the proinflammatory role of microglial MMP-8 was obtained from an in vivo animal model of neuroinflammatory disorder. MMP-8 is upregulated in septic conditions, particularly in microglia. Administration of M8I or MMP-8 short hairpin RNA significantly inhibits microglial activation and expression/secretion of TNF-α in brain tissue, serum, and cerebrospinal fluid of LPS-induced septic mice. These results demonstrate that MMP-8 critically mediates microglial activation by modulating TNF-α activity, which may explain neuroinflammation in septic mouse brain. *The Journal of Immunology, 2014, 193: 2384–2393.

ever, at later stages, MMPs are involved in neuronal regeneration and recovery (2, 7). Thus, inhibition of MMPs in early stages of brain injury may be a therapeutic strategy for neuronal disorders (7, 8).

Although a number of studies report the role of MMPs in normal and diseased brains, their role in microglial activation remains obscure. Our group recently reported that MMP-3 and MMP-9 play critical roles as inflammatory mediators in LPS- or α-synuclein-stimulated microglia (9, 10). We demonstrated that MMPs secreted from activated microglia bind to protease activated receptor-1 (PAR-1) and activate microglia in an autocrine or paracrine manner. We also showed that MMP-8 is induced in activated microglia, and its inhibition suppresses various proinflammatory molecules, suggesting a role for MMP-8 in microglial activation.

MMP-8, known as collagenase-2 or neutrophil collagenase, plays an important role in various inflammatory disorders, such as asthma, rheumatoid arthritis, pulmonary diseases, diabetes, and cancer (11). Considering neurologic disorders, the role of MMP-8 has been studied in bacterial meningitis and experimental autoimmune encephalomyelitis (EAE), an animal model of human MS. In bacterial meningitis, MMP-8 degrades occludin, a component of the BBB, and induces neuroinflammation (12). In EAE, MMP-8 modulates Th1/Th2 polarization, causing inflammation and demyelination (13), and knockdown of MMP-8 or administration of an MMP-8 inhibitor attenuates BBB breakdown and EAE progression. A recent study also demonstrated an increase in MMP-8 in cerebrospinal fluid (CSF) in rats with spinal cord injury, suggesting that MMP-8 is a biomarker for subacute spinal cord injury (14).

Although several recent studies reported the involvement of MMP-8 in neurologic disorders, it remains unclear whether microglial MMP-8 mediates neuroinflammation. In this article, we demonstrate a proinflammatory role for MMP-8 in vitro and in vivo neuroinflammatory conditions. Moreover, we identified modulation of TNF-α activation in activated microglia as one of the major mechanisms of MMP-8. These findings point toward...
MMP-8 as a promising target in the treatment of neuroinflammatory disorders involving microglial activation.

Materials and Methods

Animals

Male ICR (7-wk-old) and C57BL/6 (10–11-wk-old) mice were purchased from Orient Bio (Seoul, Korea), a branch of Charles River Laboratories. All animal experiments were approved by the Institutional Animal Care and Use Committee at Lee Gil Ya Cancer and Diabetes Institute, Gachon University and at the School of Medicine, Ewha Womans University.

Reagents

LPS (Escherichia coli serotype O55:B5) was obtained from Sigma-Aldrich (St. Louis, MO). MMP-8 inhibitor (M8I) was purchased from Calbiochem (La Jolla, CA). Recombinant TNF-α converting enzyme (TACE) protein was supplied by R&D Systems (Minneapolis, MN). TAPI-0 and recombinant MMP-8 protein were purchased from Enzo Life Sciences (Lausen, Switzerland), and all enzymes and chemicals for semiquantitative RT-PCR (semi-PCR) were purchased from Promega (Madison, WI). All reagents for cell culture were obtained from Invitrogen (La Jolla, CA). All other reagents were obtained from Sigma-Aldrich, unless otherwise stated.

Microglial cell cultures

The immortalized mouse BV2 (15) or rat HAPI microglial cell lines (16) were grown and maintained in DMEM with 10% heat-inactivated FBS, streptomycin (10 μg/ml), and penicillin (10 U/ml) at 37°C. Primary microglial cells were cultured from the cerebral cortex of 1–2-d-old Sprague-Dawley rat pups, as previously described (17). Briefly, cortices were triturated into single cells and cultured in MEM containing 10% FBS. After 10–14 d, microglial cells were separated by shaking the culture plate for 30 min at 200 rpm. Cells were then plated on different culture plates and allowed to settle for 1 h. Nonadhesive cells were removed by washing with the same medium before experiments.

Semi-PCR and quantitative real-time PCR

Total RNA (1 μg) isolated from BV2 cells (4.5 × 10³ cells on a 6-cm dish) or brain tissue of septic mice was reverse transcribed, and synthesized cDNA was used as a template for PCR. Semi-PCR was performed on a T100 Thermal cycler (Bio-Rad) with Go Taq polymerase (Promega). Quantitative real-time PCR were performed on an MX3000P (Stratagene) with Brilliant III Ultra-fast SYBR Green mix (Agilent). The primer sets shown in Table I were used to detect specific PCR products, which were calculated as fold change relative to control after normalization to a reference gene (GAPDH and β-actin for semi-PCR and quantitative real-time PCR, respectively).

Measurement of cytokines, nitrite, and intracellular reactive oxygen species levels

Cells (1 × 10⁵ cells/well in a 48-well plate) were pretreated with M8I for 1 h and further stimulated with LPS (100 ng/ml) for 24 h. Alternatively, BV2 cells were transfected with MMP-8 short hairpin RNA (shRNA) or control shRNA, followed by LPS stimulation. Concentrations of TNF-α, IL-6, and IL-10 in conditioned medium (CM) were measured by ELISA using mAbs and procedures recommended by the supplier (Pharmingen, San Diego, CA). Accumulated nitrite in CM and intracellular accumulation of reactive oxygen species (ROS) were measured using Griess reagent (Promega) and H2DCF-DA (Invitrogen), respectively, as previously described (18).

Transduction of BV2 cells with MMP-8 shRNA

Mouse MMP-8–specific shRNA (MMP-8 Mission shRNA, 1 × 10⁶ TU/ml, pLKO.1 vector) and nontarget shRNA control (Mission pLKO.1 puro nontarget shRNA control) lentiviral transduction particles were obtained from Sigma-Aldrich. The sequence for MMP-8 shRNA is 5′-CCGGCCCTTGATGTACCCCAACTATCTCGAGAATGTGTTGACATCAACAGGTCTTTCG-3′. BV2 cells were transduced with MMP-8 shRNA lentiviral particles and incubated in serum- and antibiotic-free media. After 24 h, the media were changed to media containing serum and antibiotics, and incubation was continued for an additional 6–8 h. Then, the cells were treated with LPS, and the effects of MMP-8 shRNA on NO and TNF-α production were determined. Knockdown efficiency of shRNAs was confirmed by Western blots.

Western blot analysis

Proteins isolated from total cell lysates, CM, and brain tissue were separated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with Abs against MMP-8 (1:1000; Epitomics), IL-1β (1:200; R&D Systems), TNF-α (1:200; Abcam), MAPKs (1:1000; Cell Signaling), or β-actin (1:1000; Santa Cruz). Membranes were incubated with HRP-conjugated secondary Abs (1:1000; New England Biolabs, Beverly, MA or Santa Cruz), and protein bands were visualized using an ECL detection kit (Pierce Biotechnology or Santa Cruz). For quantification, density of specific target bands was normalized by comparing blots for actin using the ImageJ 1.37v program (National Institutes of Health, Bethesda, MD).

EMSA

Nuclear extracts from cells were prepared as previously described (19). dsDNA oligonucleotides containing the NF-κB or AP-1 consensus sequences (Promega) were end-labeled using T4 polynucleotide kinase (New England Biolabs) in the presence of γ-[32P]-ATP. Nuclear proteins (5 μg) were incubated with [32P]-labeled NF-κB or AP-1 probe on ice for 30 min and resolved on a 5% acrylamide gel.

Determination of TACE enzymatic activity

TACE activity was assayed using the SensoLyte 520 TACE activity assay kit (AnaSpec). Recombinant protein (recombinant human [rh]TACE or nMMP-8; 250 ng) with M8I or TAPI-0 was incubated with TACE substrate. TACE activity was determined by continuous detection of peptide cleavage in wells for 30–60 min using a fluorescence plate reader. TACE activity was expressed as the change in fluorescence intensity at excitation of 490 nm/emission of 520 nm.

Molecular docking analysis of TACE–TAPI-0 and TACE–M8I

The two ligands of TAPI-0 and M8I were docked into the binding pocket of TACE using the AutoDock Vina program (20). The X-ray structure of TACE (PDB code 2FVS) was used to define the binding site for molecular docking studies. Ligand files were downloaded from the PubChem database (21) and prepared in the form of a three-dimensional structure using UCSF Chimera software (22), which immediately converts the two-dimensional chemical structure into a three-dimensional structure. Energy minimization was conducted for the protein and ligand with the Amber force field (23, 24). For more accurate docking analysis of protein–ligands, another docking program, UCSF DOCK 6.5 (25–28), was used with a flexible algorithm. Before the refined docking experiment, complete energy minimization was performed for the ligands and TACE in Amber force field (23, 24). The ABPS program (29) and Coulombic surface coloring method with UCSF Chimera were used to calculate the electrostatic surface potentials of the TACE model. Electrostatic potential maps were generated by numerically solving the Poisson–Boltzmann equation based on molecular mechanics embedded in APBS.

Pro-TNF-α cleavage assay

A liquid chromatography–mass spectrometry (LC-MS)–based pro-TNF-α cleavage assay was performed to identify interactions between pro-TNF-α and TACE or MMP-8 using residues 71–82 (Ac-S 71PLAQA VRSSR82-NH2) (30). For the reaction, 2 μM pro-TNF-α was digested by 0.5 nM TACE or 1 nM MMP-8. The effects of TAPI-0 or M8I on TNF-α cleavage were also determined by digesting pro-TNF-α for 1 h with TACE (0.5 nM) or MMP-8 (1 nM) in the absence or presence of TAPI-0 (5 nM) or M8I (80 nM).

LPS-induced inflammation and administration of M8I

Systemic inflammation was induced by LPS administration (5 mg/kg, i.p.) (17) into male C57BL/6 or ICR mice. M8I (1 or 5 mg/kg, i.p.), dissolved in vehicle solution (1% DMSO and normal saline), was administered daily for 4 d before LPS treatment. Samples were obtained 3 or 6 h after LPS treatment.

Histological analysis

Mice were anesthetized and perfused with PBS (pH 7.4), followed by ice-cold 4% paraformaldehyde. Brains were removed, incubated in fixative and 30% sucrose solution, embedded in Tissue-Tek OCT. compound, and frozen on powdered dry ice. Immunohistochemistry was performed on cryostat brain sections (20 μm) to detect MMP-8, microglia, astrocytes, and TNF-α by labeling sections with rabbit anti-MMP-8 (1:100), goat anti-IBa1 (1:500; Abcam or Iba1; Abcam); mouse anti-GFAP (1:500; Sigma-Aldrich), and rabbit anti–TNF-α (1:100; Abcam) primary Abs, respectively, followed by labeling with secondary biotinylated Abs and avidin/biotin complex (Vector Labs). Signals were visualized with DAB staining. Alternatively, conjugated FITC (1:1000; Serotec, Raleigh, NC) was used as a secondary Ab. MMP-8 expression in microglia or reactive astrocytes was determined by double-immunolabeling of MMP-8 (brown) and Iba-1 or GFAP (blue-gray). In brief, tissue sections that were processed for

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MMP-8 detection (brown color) were rinsed, blocked with avidin/biotin followed by 5% normal goat serum in 0.5% Triton X-100, and incubated with goat anti-Iba1 or mouse anti-GFAP Ab. Sections were incubated with secondary biotinylated Ab, followed by avidin/biotin complex, and the signal (blue-gray) was visualized using the SG Substrate Kit (Vector Lab).

For quantification, three images were taken at 20X magnification from each section, and the mean number of immunopositive cells in each group was calculated from the three images.

**Intracerebroventricular injection of MMP-8–specific and control shRNA lentiviral particles**

Lentiviral particles (3 µl/3 min, intracerebroventricular injection) for mouse MMP-8–specific shRNA (1 × 10^6 TU/ml; Sigma-Aldrich) or nontarget shRNA control (Sigma-Aldrich) were administered into the right lateral ventricle using stereotaxic coordinates from a mouse brain atlas, as previously described (31). Sepsis induction was performed 4 d postinfection.

**M81 pharmacokinetics and brain uptake**

M81 (5 mg/kg, i.p.) was administered to ICR mice, and blood samples (30 µl) were collected from the saphenous vein at different time points (2.5, 5, 10, 15, 30, 60, 90, or 120 min) after administration. Plasma obtained by centrifugation of blood samples (2000 g, 10 min, 4°C) was analyzed for M81 content using liquid chromatography–tandem mass spectrometry (Agilent LC 1100 series).

The capillary depletion method (32), modified for mice (33), was performed to determine the amount of M81 in the brain. At different time points (30, 60, or 120 min) after administration of M81 (5 mg/kg, i.p.), mice were sacrificed after blood samples were collected from the right carotid artery, and brains were removed. Brain tissue was homogenized (10 strokes) in 0.8 ml HEPES buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM CaCl_2, 1 mM MgSO_4, 1 mM NaH_2PO_4, and 10 mM D-glucose [pH 7.4]). After adding 1.6 ml 26% dextran solution to the homogenates, another 2.2 ml homogenate was centrifuged as previously reported (32). An aliquot of 100 µl homogenate (whole homogenate) was sampled, and another 2.2 ml homogenate was centrifuged (5400 × g, 10 min, 4°C) and divided into a brain parenchymal fraction (supernatant) and capillary fraction (pellet). M81 concentration (C_p, [ng/g] for brain or C_p, [ng/ml] for plasma) was determined using liquid chromatography–mass spectrometry, and pharmacokinetic parameters were determined by fitting plasma concentration–time data to the first-order equation using WinNonlin 2.1 (Pharsight, Mountain View, CA). M81 concentration was converted to the percentage of injected dose (%ID/g for brain, %ID/ml for plasma) after organ distribution values were corrected for corresponding vascular volumes using the equation

\[
\%ID/g = \left[ \frac{V_p(t) - V_0}{V_0} \right] C_p(t),
\]

where \(V_p(t)\) = (ng/g brain)/(ng/ml plasma) at time t, \(V_0\) = plasma volume of the brain (assumed to be 9.3 µl/g), and \(C_p(t)\) = plasma concentration (%ID/ml) at time t.

**Statistical analysis**

Unless otherwise stated, all experiments were performed with triplicate samples and repeated at least three times. Data are presented as mean ± SEM, and statistical comparisons among groups were performed using one-way ANOVA, followed by the Newman–Keuls post hoc test or t test. Statistical significance was accepted for p values < 0.05.

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**Results**

**MMP-8 is upregulated in LPS-stimulated microglia, and its inhibition suppresses proinflammatory molecules**

To determine whether MMP-8 is induced in activated microglia, cells were exposed to LPS and processed for semi-PCR using primers indicated (Table I) and Western blot analyses. Upregulation of MMP-8 mRNA and protein was observed in activated BV2 microglia (Fig. 1A, 1B). In particular, the more apparent upregulation of MMP-8 in CM compared with lysates from LPS-exposed cells (Fig. 1B) indicates a facilitation of MMP-8 secretion from activated microglia. This upregulation and increased secretion of MMP-8 were recapitulated in LPS-stimulated primary microglia (Fig. 1C, 1D).

To investigate the functional roles of MMP-8 in microglial activation, changes in neuroinflammatory responses were assessed by guest on April 21, 2017 http://www.jimmunol.org/ Downloaded from

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**Table I. Primer sets used for semi-PCR and quantitative real-time PCR**

<table>
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<th>Species</th>
<th>Gene</th>
<th>Forward Primer (5′→3′)</th>
<th>Reverse Primer (5′→3′)</th>
<th>Size (bp)</th>
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<td>Mouse</td>
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<td>CTTGATGAAGATGATCCTACAAAT</td>
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<td>iNOS</td>
<td>CAAAGATGTGGAGCAGAGAGC</td>
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<td>IL-6</td>
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<td>CCGCTTACTCTGCTTGAGA</td>
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<tr>
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<td>IL-10</td>
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<td>GCTTTTTTGACCTTTCTGGTTCTT</td>
<td>490</td>
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<tr>
<td></td>
<td>MMP-8α</td>
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<td>CTTGACGAAAGTCTGGAGAGAG</td>
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<tr>
<td></td>
<td>β-actinb</td>
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<td>TTCTGATTTCTTTGCTCTGAA</td>
<td>158</td>
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<tr>
<td>Rat</td>
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<td>TCAACTCTTCTTGCTGCTGCTC</td>
<td>317</td>
</tr>
<tr>
<td></td>
<td>GAPDH</td>
<td>GTGCTTCTAGTATGCTGTTGAGCTCTG</td>
<td>ACAGTCTCAGTGGACAGTTGA</td>
<td>292</td>
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*Primers used for semi-PCR and quantitative real-time PCR.

*Primers used for quantitative real-time PCR.
in LPS-stimulated BV2 cells using M8I, a specific MMP-8 inhibitor (34). We confirmed that M8I efficiently inhibited LPS-induced MMP-8 activity but not MMP-3 or MMP-9 activities in BV2 cells (Supplemental Fig. 1). Pretreatment with M8I significantly inhibited the production of proinflammatory molecules TNF-α, IL-6, and NO and upregulated the anti-inflammatory cytokine IL-10 (Fig. 2A). Among these molecules, the inhibition of TNF-α production was most prominent (IC_{50}, 1 μM; Fig. 2A). These results suggest that MMP-8 acts as an inflammatory mediator, with a particular effect on TNF-α production in activated microglia. In support of this possibility, MMP-8 knockdown by MMP-8 shRNA (Fig. 2B) suppressed TNF-α and NO production in LPS-stimulated BV2 cells (Fig. 2C). These specific roles for MMP-8 were confirmed by M8I treatment in LPS-stimulated rat primary microglia (Fig. 2D) and HAPI microglial cell lines (Fig. 2E).

To determine whether M8I effects are linked to transcriptional regulation, we examined changes in mRNA levels of inducible NO synthase (iNOS) and cytokines. M8I suppressed expression of iNOS and IL-6 mRNA and increased expression of IL-10 mRNA, but it did not alter expression of TNF-α mRNA (Fig. 2F). These results indicate that M8I modulates iNOS, IL-6, and IL-10 expression at the transcriptional level, but it modulates TNF-α expression at the posttranscriptional level.

**M8I reduces TNF-α secretion via inhibition of TACE activity in microglia**

Because M8I most efficiently inhibited TNF-α production, we further investigated the underlying mechanisms of this effect. Western blot showed that M8I suppressed the secretion of the active form of TNF-α, but it only slightly increased or did not alter pro–TNF-α levels in BV2 cells (Fig. 3A, 3B) and primary microglia (Fig. 3C, 3D). TNF-α ELISA also showed that M8I blocked the release of TNF-α in a concentration-dependent manner (Fig. 3E). These results demonstrate that reduced TNF-α productivity by MMP-8 inhibition (Figs. 2A–E, 3A–E) is a consequence of reduced TNF-α secretion.
TNF-α is produced as a proform (26 kDa) and secreted as an active form (17 kDa) after cleavage of its prodomain by TACE (3). To further dissect the mechanism of the effect of M8I on TNF-α secretion, we examined whether M8I inhibits TACE activity using recombinant proteins. M8I inhibited TACE activity of rhTACE more efficiently than TAPI-0, a general TACE inhibitor (Fig. 3F). Interestingly, we found that rhMMP-8 itself has TACE activity (Fig. 3F). Taken together, these results demonstrate that MMP-8 itself has TACE activity and that M8I is a strong inhibitor of TACE activity.

The efficient inhibition of TACE activity by M8I was supported by molecular docking analysis. M8I bound to the catalytic domain of TACE like TAPI-0 with similar docking scores (Fig. 4). M8I interacted with TACE deep inside positively charged binding pockets, leading to a complex with greater structural stability (Fig. 4A–D). Moreover, two hydrogen bonds were found between M8I and TACE (190His.TACE-Hε2···O2-M8I, 200His.TACE-Hε2···O2-M8I) (Fig. 4E), which presumably contribute to the stabilization of the complex as a result of the strong binding force.

MMP-8 cleaves the prodomain of TNF-α with one more cleavage site than TACE

To address whether active MMP-8 directly cleaves the prodomain of TNF-α, we performed a TNF-α cleavage assay using LC-MS analysis. Both rhTACE and rhMMP-8 cleaved the A76/V77 residue, a conventional cleavage site in the N-terminal propeptide of TNF-α (Fig. 5). Interestingly, an additional cleavage site (A74/Q75) was identified with rhMMP-8 (Fig. 5B–D). To confirm that the cleavage reaction was specifically induced by MMP-8, M8I was added to the reaction mixture. As expected, no meaningful products were produced (Fig. 5D). These results indicate that MMP-8 has TACE activity by cleaving two specific sequences of pro–TNF-α.

MMP-8 regulates signaling molecules responsible for microglial activation

Several signaling molecules are linked to microglial activation, including MAPKs (JNK, ERK1/2, and p38), NF-κB, AP-1, and ROS (35–38). To determine whether MMP-8 modulates the activation or production of these molecules, biochemical analyses were performed using M8I in activated microglia. M8I inhibited the phosphorylation of three types of MAPKs (Fig. 6A) and the DNA-binding activities of NF-κB and AP-1 (Fig. 6B). Furthermore, M8I significantly reduced intracellular levels of ROS, which are early signaling inducers of microglial activation (39) (Fig. 6C, 6D). The results suggest that MMP-8 mediates LPS-induced inflammatory reactions via modulating upstream-signaling molecules.

MMP-8 mediates neuroinflammation in the septic mouse brain

To identify whether MMP-8 is upregulated in the mouse brain with LPS-induced sepsis, a well-known neuroinflammatory disorder, mRNA and protein levels of MMP-8 were determined 3 h after LPS challenge. LPS induced upregulation of MMP-8 mRNA and protein (Fig. 7A, 7B) in the brain, with MMP-8 induction particularly prominent in microglia (Fig. 7B) and reactive astrocytes (Supplemental Fig. 2).
Next, we determined whether M8I distributes within the brain. Concentration–time profiles of M8I content in plasma following its injection were obtained using pharmacokinetic analysis (Supplementary Fig. 3A, 3C). Starting from $139 \pm 90.1$ ng/ml at 2.5 min, a $C_{\text{max}}$ of $430 \pm 82.6$ ng/ml was reached at a $T_{\text{max}}$ of 10 min, which corresponds to 0.235% of injected dose per ml (%ID/ml). After this peak, the concentration slowly decreased to 0.005 %ID/ml at 120 min. The amount of M8I in the brain, expressed as volume of distribution ($V_d$, ml/ml) in whole-brain homogenates, initially increased and then decreased (Supplemental Fig. 3B). $V_d$ in the supernatant and pellets varied accordingly, but $V_d$ in the supernatant was higher than that in pellets at all time points with a relatively constant ratio, indicating rapid equilibrium between the parenchyma and capillaries. The percentage of injected dose of M8I/g of brain tissue (%ID/g) reached 0.0143 at 60 min. These findings suggest that a large quantity of M8I crossed the BBB and entered the brain during the initial distribution phase and then was eliminated from the brain at a rate slower than that of plasma.

To determine whether MMP-8 acts as observed in cultured cells, mice were injected with M8I for 4 d before LPS treatment, followed by assessment of microglial activation and TNF-$\alpha$ production 3 h after LPS treatment. Microglial activation was first assessed by immunoreactivity of Iba1, a marker of activated microglia (40). The number of Iba1$^+$ cells with a densely stained round shape, indicative of activated cells, was increased in the inflamed cortex of LPS-treated mice compared with control mice. However, M8I (5 mg/kg) significantly reduced the number of Iba1$^+$ cells (Fig. 7C). Next, TNF-$\alpha$ productivity was assessed by ELISA in serum and CSF. LPS markedly increased TNF-$\alpha$ levels in both serum and CSF, and this effect was significantly inhibited by M8I (Fig. 7D). Also, increases in TNF-$\alpha$ and IL-1$\beta$ protein levels in the LPS-treated brain were significantly reduced by M8I (5 mg/kg) (Fig. 7E). These results demonstrate a necessary role of MMP-8 in modulating microglial activation and TNF-$\alpha$ production in the septic mouse brain.

This identified role of MMP-8 was confirmed by genetic knockdown of MMP-8 in the brain using a lentiviral system. Because MMP-8 is induced by LPS stimulation, we checked knockdown efficiency in the LPS-injected mouse brain. Intracerebroventricular injection of shRNA lentivirus reduced MMP-8 expression by $\sim 60\%$, as assessed by quantitative real-time PCR (Supplemental Fig. 4). This knockdown of MMP-8 significantly reduced microglial activation and TNF-$\alpha$ expression in the septic mouse brain (Fig. 7F).

**Discussion**

To our knowledge, this is the first study to demonstrate the role of MMP-8 in neuroinflammatory conditions in vitro and in vivo. MMP-8 was increased in activated microglia, and specific inhibition of MMP-8 reduced inflammatory responses in cultured microglia,
as well as septic mouse brains. Mechanistic studies using in vitro systems showed that MMP-8 exhibited TACE activity by cleaving the prodomain of TNF-α. M8I inhibited this TACE activity and also suppressed proinflammatory signaling molecules, such as MAPKs, NF-κB/AP-1, and ROS, in LPS-stimulated microglia. Thus, our results provide evidence that MMP-8 is an important proinflammatory mediator in activated microglia and that its modulation has therapeutic benefits for neuroinflammatory disorders.

MMP-8 mediates inflammatory processes and is expressed in many cell types at sites of inflammation, such as neutrophils, macrophages, plasma cells, granulocytes, and epithelial choroid plexus cells (41, 42). In this study, we identified microglia as an additional locus of MMP-8 expression. Microglia are major immune cells in the brain that play important roles in neuroinflammation, such as phagocytosis and the production of various cytokines and chemokines (43–45). Microglia activated by LPS exposure robustly expressed MMP-8, and neuroinflammatory responses were prevented by MMP-8 genetic knockdown or pharmacological inhibition, supporting the direct involvement of MMP-8 in inflammatory responses in activated microglia. The identified role of MMP-8 in cultured microglia was reaffirmed in an animal model of sepsis, which is characterized by neuroinflammation. In septic conditions, MMP-8 was upregulated in microglia at inflammatory brain sites, and its inhibition reduced neuroinflammation. These results point toward microglia as cellular targets in the treatment of CNS disorders, such as MS, spinal cord injury, and traumatic brain injury, all of which involve upregulation of MMP-8 as a pathological feature (13, 14, 46).

**FIGURE 5.** TNF-α cleavage assay using LC-MS analysis. (A–D) The 12-residue peptide covering pro–TNF-α cleavage sites was incubated with active rhMMP-8 or rhTACE and analyzed by LC-MS. Cleavage profiles of pro–TNF-α by TACE (A) and MMP-8 (B). (C) Summary of pro–TNF-α sites cleaved by TACE and MMP-8. (D) Summary of enzyme activities of TACE and MMP-8 by comparing cleaved sequences of pro–TNF-α based on LC-MS data.

**FIGURE 6.** MMP-8 modulates MAPK signaling, NF-κB/AP-1 DNA-binding activities, and ROS production. (A) Effect of M8I on the phosphorylation of three types of MAPKs was determined by Western blot 30 min after LPS treatment. Representative blot (left panel) and quantification (right panel). (B) Effect of M8I on NF-κB or AP-1 DNA-binding activity was determined by EMSA 3 h after LPS treatment. DNA–NF-κB and DNA–AP-1 protein complexes are identified. The autoradiography of the gel is representative of three independent experiments (upper panel), and the quantification data (lower panel) are mean ± SEM of three independent experiments. (C and D) BV2 microglia were treated with M8I for 1 h and then stimulated with LPS for 16 h. Effect of M8I on intracellular ROS levels was measured by the DCF-DA method. (C) Values for fluorescence intensity are expressed as fold increase relative to control. (D) Representative image of DCF-derived fluorescence (green). Original magnification ×200. n = 3–4/group. *p < 0.05 versus LPS-treated cells.
FIGURE 7. MMP-8 is critical for neuroinflammation induced by systemic LPS administration. (A and B) Changes in MMP-8 mRNA and protein expression were determined in LPS (3 h)–induced septic brains. (A) MMP-8 mRNA expression, as determined by quantitative real-time PCR. Representative gel showing quantitative real-time PCR products (upper panel). Vehicle (veh), n = 3; LPS, n = 5. (B) Representative images of MMP-8+ cells and double-positive cells (MMP-8, brown; Iba-1, blue-gray), as determined by immunohistochemistry (upper panels). Arrows indicate double-immunopositive cells. Scale bars, 50 μm. Quantification of data (lower panel). n = 4/group. (C–E) Effects of M8I on microglial activation and TNF-α production were determined in LPS (3 h)–induced septic mice. (C) Representative images for immunofluorescence labeling for Iba1 (green) (upper panels) Scale bar, 20 μm. Quantification of data in the cortex (lower panel). n = 3 per group. (D) TNF-α levels in serum and CSF, as measured by ELISA (n = 3/group). (E) Changes in TNF-α and IL-1β protein levels in the mouse cortex. Representative Western blots (upper panel) and quantification of data (lower panels) (n = 3/group). (F) Effects of MMP-8 shRNA lentivirus on microglial activation and TNF-α expression were determined by immunohistochemistry in LPS (3 h)–induced septic mice. Representative images (left panels) and quantification of data (right panels). Brown DAB staining. Scale bars, 50 μm. Control (con), n = 4/group; LPS (veh), n = 3 for Iba-1 and n = 5 for TNF-α; LPS+control shRNA, n = 3/group; LPS+MMP-8 shRNA, n = 4/group. *p < 0.05 versus control mice (A and B), versus LPS-treated mice (C–E), or versus LPS+control shRNA–treated mice (F).
The in vivo effects of M8I and MMP-8 shRNA might not be confined to microglia. Rather, other cell types, such as infiltrated neutrophils and monocytes and reactive astrocytes, could also be involved. In particular, reactive astrocytes are observed in LPS-treated cortices and express MMP-8. However, we detected no clear differences in the presence of infiltrating neutrophils and monocytes in our in vivo model (data not shown). Although MMP-8–producing neutrophils and monocytes enter the CNS in response to neuroinflammatory stimuli, it seems that the infiltration of these cell types occurs rarely at early time points (i.e., 3 h) after LPS treatment. This notion is in line with several reports showing that microglial activation is a main event at early stages of LPS-induced neuroinflammation (47–49). Further studies are necessary to identify the specific cell types that express MMP-8 and are important for neuroinflammation in the LPS-treated brain.

The site of action of M8I seems to be inside of the CNS. In this study, we demonstrated that M8I is distributed into the brain parenchyma, which indicates a significant transport of M8I across the BBB into the brain. It might be technically possible that M8I remains associated with the capillaries upon generation of the homogenates because mice were not perfused prior to homogenization. However, this may not affect the observed BBB permeability of M8I in this study. We did not perform the vascular washout to correct the contamination of the parenchymal fraction during brain homogenization. Instead, we did all homogenization processes on ice in <1 min to minimize the dissociation of capillary-associated M8I as reported previously, where no measurable dissociation was detectable even without vascular washing out (32).

Our findings indicate that the neuroinflammatory actions of MMP-8 in microglia are due, in part, to its direct action on the proinflammatory cytokine TNF-α. Many cell types that contribute to inflammation, such as microglia, produce and secrete TNF-α, which, in turn, mediates tissue damage in various disorders, such as rheumatoid arthritis, ulcerative colitis, diabetes, atherosclerosis, Alzheimer’s disease, MS, and stroke (50, 51). An important regulator of TNF-α activity is TACE (3), which cleaves the 26-kDa membrane-bound protein (pro–TNF-α) to form a 17-kDa soluble cytokine. In this study, MMP-8 was revealed to have slightly different TACE activity: MMP-8 cleaves a predominant of TNF-α at the Aα9/11Q5 residue, in addition to the Aα9/11V7 residue, a conventional cleavage site of TACE. Notably, MMP-8 inhibition primarily affected the secretion of TNF-α via inhibition of TACE activity, which may be an important consideration for therapeutic strategies. The development of agents that can control TACE activity and, hence, TACE (3) has been a major interest of pharmaceutical companies (52, 53). Therefore, the dual inhibition of MMP-8 and TACE activity by M8I may contribute to its strong anti-inflammatory and neuroprotective effects.

We found that M8I suppressed intracellular signaling molecules, such as MAPKs, NF-κB/AP-1, and ROS, which are largely involved in proinflammatory gene expression. Previous studies reported that soluble TNF-α binds to cell surface receptors on immune cells and evokes MAPK signaling and NF-κB/AP-1 activation, leading to cytokine production and inflammatory responses in an autocrine and/or paracrine fashion (3, 54, 55). Thus, the inhibition of soluble TNF-α by M8I may lead to the blockade of inflammatory signaling. Another possible mechanism of M8I is PAR-1 inhibition. Our previous study showed that MMP-8 activates PAR-1 on the cell surface of microglia and induces inflammatory reactions (9). Thus, the concurrent inhibition of TNF-α and PAR-1 by M8I may block proinflammatory signaling in activated microglia. Several studies reported that MMP-8 is increased in the serum of sepsis patients and experimental animals and that inhibition of MMP-8 improves outcome, suggesting that MMP-8 is an important diagnostic marker for sepsis (34, 56). Furthermore, a recent report highlighted the functional importance of MMP-8 in neuroinflammation using genetic nulls for MMP-8 in a sepsis model, showing that MMP-8 in epithelial choroid plexus cells causes disruption of the blood–CSF barrier and results in brain inflammation (42). In this study, we identified additional meaningful events in sepsis, including a new locus for MMP-8 upregulation and mechanisms underlying the modulation of neuroinflammation. As observed in cultured microglia, MMP-8 was significantly upregulated in septic conditions, especially in microglia, and its pharmacological or genetic inhibition prevented microglial activation and downregulated TNF-α levels in serum, CSF, and brain tissue. Moreover, the specific inhibition of MMP-8 suppressed microglial activation and TNF-α expression in activated microglia. These results suggest that MMP-8 is a valuable diagnostic marker and therapeutic target for sepsis-related neuroinflammation. Furthermore, in light of the current findings, it is tempting to speculate that MMP-8 might be a therapeutic target for various neuroinflammatory disorders that are accompanied by microglial activation.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Fig. 1: Effect of MMP-8 inhibitor (M8I) on LPS-induced MMP-3, -8 and -9 activities. BV2 cells were treated with each inhibitor for 1 h before stimulation with LPS. Twenty four hours later, the supernatants were collected to measure MMP activity using the SensoLyte® 520 MMP assay system (AnaSpec, San Jose, CA, USA). *P< 0.05; significantly different from LPS-treated samples.
Supplemental Fig. 2: Reactive astrocytes also express MMP-8 in the cortex of LPS-stimulated mice. (A) Representative images for immunolabeling with anti-GFAP on brain sections from control and LPS-treated mice. (B) Representative images of double-positive cells (MMP-8, brown; GFAP, blue-gray), as determined by immunohistochemistry. Arrows indicate double-immunopositive cells. Scale bar, 50 µm.
Supplemental Fig. 3: M8I crosses the BBB and distributes to the brain. (A) The concentration-time profiles of M8I contents in plasma following M8I injection (i.p., 5 mg/kg). $C_{pl}$: concentration in plasma. n=4. (B) The amounts of M8I in brain homogenates (H), supernatants (S), and pellet (P) at different time points after M8I injection (i.p., 5 mg/kg). $V_d$: volume of distribution. n=4 per time point. (C) Summary table for pharmacokinetic parameters derived from the curve shown in (A).
Supplemental Fig. 4: MMP-8 shRNA lentivirus induces MMP-8 downregulation in the LPS-stimulated mouse brain. Knockdown efficiency was determined by qRT-PCR. *$P<0.05$ vs. LPS + control shRNA-treated mice.