Research paper

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Upregulation of CCL3/MIP-1alpha regulated by MAPKs and NF-kappaB mediates microglial inflammatory response in LPS-induced brain injury

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Growing evidence suggests that macrophage inflammatory protein (MIP)-1alpha (synonym CCL3) is upregulated in the neuroinflammatory processes initiated by some brain disorders, but its precise role and regulatory mechanism remain unclear. The present work aims to evaluate the role of CCL3/MIP-1alpha in lipopolysaccharide (LPS)-induced brain injury, and investigate whether the MAPKs and NF-kappaB regulate CCL3/MIP-1alpha expression. We firstly examined the patterns of CCL3/MIP-1alpha expression and phosphorylation of MAPKs in the brains of rats 6, 24, and 72 h after LPS administration. Additionally, LPS-treated rats were administered an anti-MIP-1alpha neutralizing antibody, and the microglial reaction and the expression of both cyclooxygenase-2 and inducible nitric oxide synthase (iNOS) were analyzed. We finally evaluated the effect of an inhibitor of P38 MAPK, an inhibitor of ERK1/2, or an inhibitor of NF-kappaB, on the levels of CCL3/MIP-1alpha protein and numbers of microglia in the brain. In the observation period, LPS induced CCL3/MIP-1alpha expression, which localized to OX-42-labeled microglia, leading to time-dependent increases in the phosphorylation of P38 MAPK and ERK1/2. The expression pattern of induced CCL3/MIP-1alpha was partly consistent with the phosphorylation of MAPKs (P38 MAPK, ERK1/2). Anti-MIP-1alpha attenuated microglial accumulation and the upregulation of cyclooxygenase-2 and iNOS. The inhibition of P38 MAPK, ERK1/2, or NF-kappaB signaling reduced the induced upregulation of CCL3/MIP-1alpha and the microglial accumulation. Our data suggest that upregulated CCL3/MIP-1alpha mediates the accumulation of microglia and the neuroinflammatory reaction, and its expression may be regulated by MAPKs and NF-kappaB in LPS-induced brain injury.

Key words: chemokine, MIP-1alpha, MAPK, microglia, brain injury, rat

INTRODUCTION

Inflammatory processes are involved in a broad range of neuropathological conditions, which can be simulated experimentally with lipopolysaccharide (LPS) (Henry et al. 2008, Lee et al. 2010), a polysaccharide component of the cell walls of Gram-negative bacteria. LPS causes inflammatory responses in the central nervous system (CNS), characterized by production of inflammatory cytokines, glial activation, and neuronal damage (Qin et al. 2007, Henry et al. 2008, Tanaka et al. 2011). It is used widely in experimental systems to induce neuroinflammation. Chemokines are low-molecular-weight chemotactic cytokines that mediate the accumulation of leukocytes at the sites of inflammation (Luster 1998, Charo and Ransohoff 2006). The beta-chemokines, a subfamily of chemokines, include monocyte chemoattractant protein (MCP)-1 (synonym CCL2), macrophage inflammatory protein (MIP)-1alpha (synonym CCL3), MIP-1beta (synonym CCL4), etc., and have been implicated in the inflammatory processes in the CNS (Charo and Ransohoff 2006, Lenglet et al. 2014).

The biological effects of CCL2/MCP-1 on the neuroinflammation caused by CNS diseases have been extensively investigated (Gerard and Rollins 2001, Hinojosa et al. 2011, Charo and Ransohoff 2006). The initial interest in the role of CCL3/MIP-1alpha in neuroinflammation originated from a previous observation that the intravenous administration of LPS causes a time-dependent increase in CCL3/MIP-1alpha in the rat brain, hinting at a role in the neuroinflammatory response to infection (Gourmala et al. 1999). CCL3/MIP-1alpha is upregulated in the CNS in experimental models of autoimmune encephalomyelitis (Karpus et al. 1995), traumatic brain injury (Israelsson et al. 2014), and status epilepticus (Zhu et al. 2012). The deletion

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of the CCL3/MIP-1alpha gene reduced astrocytosis and microgliosis in the hippocampus after the administration of beta-amyloid (1–40) (Passos et al. 2009). Another study, however, demonstrated that CCL3/MIP-1alpha secreted by astrocytes promotes the differentiation of embryonic neuronal cells and has neuroprotective functions (Park et al. 2009). These contradictory data prompted us explore the role of CCL3/MIP-1alpha in neuroinflammation in a model of LPS-induced brain injury.

We also examined the mechanisms regulating the induction of CCL3/MIP-1alpha in response to LPS. LPS activates many signaling molecules, including mitogen-activated protein kinases (MAPKs) (Guha and Mackman 2001, Uesugi et al. 2006) and transcription factors such as nuclear factor (NF)-kappaB and activator protein 1 (AP-1) (Guha and Mackman 2001, Diks et al. 2004). MAPKs are one of the most important kinase families, and are major intracellular signal transduction factors that regulate the production of many cytokines and chemokines in the CNS (Pearson et al. 2001, Kim and Choi 2010). In cultured microglia, activated extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and P38 MAPK regulate the expression of CCL3/MIP-1alpha after external stimulation, such as by S100B or monoclonal antibody-coated Cryptococcus neoformans immune complexes (Song et al. 2002, Soares et al. 2009). Matsuyama and others (2004) also reported that collagen induced the expression of CCL3/MIP-1alpha in human macrophages in a P38 MAPK- and NF-kappaB-dependent manner. However, a recent study showed that the induction of both CCL3/MIP-1alpha and CCL2/MCP-1 by lipoteichoic acid is regulated by ERK1/2 and JNK, rather than by the P38 MAPK signaling molecule, in murine macrophages (Park et al. 2013).

In this study, we examined the expression and role of CCL3/MIP-1alpha in the brains of rats subjected to intracerebroventricular (i.c.v.) LPS injection, and determined the roles of the MAPK and NF-kappaB signaling molecules in the upregulated expression of CCL3/MIP-1alpha. Our findings indicate that upregulated CCL3/MIP-1alpha contributes to the accumulation of microglia and mediates the inflammatory response to LPS in the brain. Furthermore, the expression of CCL3/MIP-1alpha might be regulated by the activated P38 MAPK, ERK1/2, and NF-kappaB signaling molecules. The results of this study extend our understanding of CCL3/MIP-1alpha as a therapeutic target for neuroinflammation.

METHODS

Animals

Adult wistar rats weighing 180–200 g were purchased from the Experimental Animal Center, Shandong University. They were housed in plastic cages with free access to water and food, and maintained in a temperature-controlled (22–24°C) room with a fixed 12 h light-dark cycle. All experiments were conducted in compliance with the National Institutes of Health Guidelines and the legal requirements in China. The protocol was approved by the Ethics Committee on Animal Experiments of Medical School of Shandong University. We made our efforts to reduce the number of animals used and their suffering in experiments.

Surgical preparation

Surgical Operation of rats was performed as described by Soares and others (2009), with slight adjustment from the stereotaxic coordinates of rat brain (Paxinos and Watson 2004). In brief, under anesthetized with chloral hydrate (400 mg/kg, i.p.), rats were placed in a stereotaxic apparatus to implant a stainless steel guide cannula (22-gauge, 0.8 mm OD, 10 mm length) for drug administration into the right lateral ventricle at the following stereotaxic coordinates: 1.2 mm posterior, 1.6 mm lateral, and 2.5 mm deep relative to the bregma. This cannula was fixed to the skull with stainless steel screws and dental acrylic cement. Seven days after surgery, all rats were randomly divided into the subsequent experiments.

Experimental protocols

In the first set of experiment, rats were i.c.v. injected with LPS (0.2 mg/kg BW, Escherichia coli serotype 055:B5, Sigma, USA) at a concentration of 50 mg/ml. Administration was performed through an inner cannula connected to 5 µl Hamilton microsyringe at the rate of 1 µl/min. The dose of LPS was chosen based on the optimal responses in preliminary experiments and the results reported by Tyagi and others (2010). Rats were sacrificed at 6, 24 and 72 h after administration. The control rats were injected with the equal volume of saline and sacrificed at 24 h after injection. Brains were collected for immunohistochemistry (n=6 at each time point) or dissected to collect cerebral hemispheres for enzyme-linked immunosorbent assay (ELISA) (n=4 at each time point) and Western blot (n=4 at each time point).

In the second experiment, LPS-treated rats were i.c.v. injected with 5 ng anti-MIP-1alpha antibody (Abcam, USA) or normal immunoglobulin G (IgG) (Serotec, UK) in a volume of 1 µl sterile saline immediately and 12 h after LPS administration. The saline control rats were injected twice with the equal volume of IgG. Rats were divided into the following groups: Saline+IgG rats, LPS+IgG rats, and LPS+anti-MIP-1alpha rats. The dose of anti-MIP-1alpha

was chosen on the basis of the results of preliminary experiments and the data of Soares and colleagues (2009). Rats were anesthetized and sacrificed at 24 and 72 h after injection. Brains were collected for immunohistochemistry (n=6 per group), or dissected to collect hippocampus and prefrontal cortex for Western blot (n=4 per group).

In the third experiment, LPS-treated rats were i.c.v injected with P38 MAPK inhibitor SB203580 (0.5 mM/kg, Cell Signaling Technology, USA), ERK1/2 inhibitor PD98059 (2.5 mM/kg, Cell Signaling Technology) dissolved in 2 µl diluted dimethyl sulfoxide (DMSO) (ZSGB-BIO, China) in a range of 5–15%, or 5% NF-kappaB inhibitor pyrrolidine dithiocarbamate (PDTC) (Calbiochem, USA) in 2 µl sterile saline, 30 min prior to LPS administration. The same volume of 15% DMSO or sterile saline as a vehicle was also i.c.v. infused into LPS-treated or saline control rats. Rats were divided into the following groups: Saline+DMSO rats, LPS+DMSO rats, LPS+Saline rats, LPS+SB203580 rats, LPS+PD98059 rats, and LPS+PDTC rats. The doses of SB203580 and PD98059 were determined according to the results of previous studies by Sugino and others (2000) and Etgen and Acosta-Martinez (2003), and PDTC were determined by the results of preliminary experiments and the data of Nurmi and colleagues (2004). Rats were sacrificed at 12h for real-time polymerase chain reaction (PCR) (n=4 per group) and 24 h for immunohistochemistry (n=6 per group), Western blot (n=4 per group), or ELISA (n=4 per group) after LPS injection.

Tissue collection and preparation

Rats were anesthetized and brain tissues were collected at the indicated time-points. For molecular studies, rats were scarified and brains were rapidly removed from the skull. The dissected tissues were frozen in liquid nitrogen and stored at –80°C for later analysis. For immunohistochemical analysis, rats were perfused with 4% paraformaldehyde and brains were rapidly removed, immersed, and embedded with optimal cutting temperature compound. Coronal sections were obtained at the bregma level from 1.2 to –0.4 mm and from –2.5 to –3.8 mm, cut at a thickness of 20 µm (1-in-6 series, 120 µm apart from each other) with a sliding microtome (Leica Instruments, Germany), and collected on gelatin-coated microscope slides stored at –20°C.

ELISA assay

The brain tissues were homogenized by a tissue homogenizer in 0.1 mM phosphate-buffered solution and centrifuged at 15,000 g for 15 min at 4°C. Supernatants were harvested and stored at –80°C. Protein concentrations were measured by using a bicinchoninic acid protein assay kit (Beyotime Biotechnology, China) and the level of CCL3/MIP-1alpha was measured by ELISA kits (Peprotech, USA) according to the instructions. Results are recorded as pg of CCL3/MIP-1alpha/mg of total protein.

Immunohistochemical analysis

Serial sections were used for detection of CCL3/MIP-1alpha, ionized calcium-binding adapter molecule 1 (Iba1, also mentioned as allograft inflammatory factor 1), cyclooxygenase-2 (COX-2), phosphorylated P38 MAPK and phosphorylated ERK1/2 immunoreactivity by avidin-biotin-peroxidase methods. After rinsing, sections were blocked in 5% BSA for 1 h and incubated with specific primary antibody overnight at 4°C. The following primary antibodies were used: goat anti-MIP-1alpha (1:100, Santa Cruz Biotechnology, USA), rabbit anti-Iba1 (1:500, Wako, USA), rabbit anti- phosphorylated P38 MAPK (1:100, Cell Signaling Technology), or phosphorylated ERK1/2 (1:100, Cell Signaling Technology). Subsequently, sections were reacted with relevant biotinylated secondary antibodies (all used at 1:500, Vector Laboratories, USA) for 1 h and avidin-conjugated peroxidase complex (1:200, Vector Laboratories) for 30 min. Peroxidase reactivity was developed using 3, 3'-diaminobenzidine tablet sets (ZSGB-BIO), and the slide was counterstained, dehydrated and observed under a light microscope (Olympus, Japan).

Quantification of the immunopositive cells was analyzed by a blinded manner as described in our previous work (Zhu et al. 2012). Six coronal brain sections from each animal were observed for counting at a 200× magnification. The number of immunopositive cells was counted twice in two differently visual fields of each subfield of hippocampus and in six differently visual fields of cortex per section.

Double-labeling immunofluorescence

To identify glial cell distributions of CCL3/MIP-1alpha in the brain, double-labeling immunofluorescence was performed as our previously described (Zhu et al. 2012). After rinsing, sections were blocked in 5% BSA and 5% normal donkey serum in phosphate-buffered solution for 1 h, and incubated in primary antibody solution containing goat anti-MIP-1alpha (1:100, Santa Cruz Biotechnology) with mouse anti-glial fibrillary acidic protein (GFAP) (1:200; Chemicon/Millipore, USA) antibody, a specific marker for astrocytes, or anti-Integrin alpha M (OX-42, also mentioned as an iCb3 complement component) (1:100, Serotec, UK) antibody, a marker for activated microglia, overnight at 4°C. After three washes, sections were incubated in a mixture of Alexa 488-conjugated donkey anti-goat

antibody (1:500, Invitrogen, USA) to label CCL3/MIP-1alpha and Alexa 594-conjugated donkey anti-mouse antibody (1:500, Invitrogen) to label GFAP-positive astrocytes, or OX-42-positive microglia at room temperature for 1 h. Processed sections were coverslipped with Fluorescent Mounting Media (Beyotime Biotechnology) and examined under a laser scanning confocal microscopy with a Bio-Rad MRC 1024 system (Bio-Rad laboratories, USA). To analyze the antibody specify, both primary antibodies were omitted during double staining and no labeling was detected.

Western blot assay

The frozen tissues were homogenized with a tissue homogenizer in an ice cold lysis buffer. Supernatants were collected and protein concentrations were determined using a bicinchoninic acid protein assay kit (Beyotime Biotechnology). Thirty micrograms of protein were separated by 10% SDS-PAGE and then transferred to polyvinylidene fluoride membrane (Millipore). After blocking, the membranes were incubated with indicated primary antibodies at 4°C overnight. The primary antibodies used were as follows: rabbit anti- inducible nitric oxide synthase (iNOS) (1:500, Santa Cruz Biotechnology), and P38 MAPK, phosphorylated P38 MAPK, ERK1/2, phosphorylated ERK1/2, JNK, phosphorylated JNK, inhibitor of nuclear factor-kappaB (IkappaB) alpha, phosphorylated IkappaB alpha, NF-kappaB/p65, phosphorylated NF-kappaB/p65 (all used at 1:1000, Cell Signaling Technology). After three washes, the membranes were incubated with the conjugated IgG-horseradish peroxidase (1:1000, ZSGB-BIO) at room temperature for 2 h. Immunoreactivity was detected by an enhanced chemiluminescence kit (Millipore) and then the images were analyzed using an image analyzer (Alpha Innotech, USA). Membranes were reprobed with mouse anti-beta-actin antibody to serve as a loading control. For iNOS, protein abundance was normalized by the total content of beta-actin. For phosphorylated P38 MAPK, ERK1/2, JNK, IkappaB alpha and NF-kappaB/p65, band densities were corrected for variations in loading and normalized to the corresponding band densities for total P38 MAPK, ERK1/2, JNK, IkappaB alpha, and NF-kappaB/p65, respectively.

Real-time PCR

Total RNA was extracted from rat brains using Trizol reagent (Invitrogen). Reverse transcription was carried out using a Reverse Transcription System (Promega, USA). Real-time PCR was performed using a LightCycler 2.0 instrument following the instructions (Roche, USA). Each reaction in a volume of 20 µl contained 2 µl cDNA, 10 µl SYBR® Premix Ex Taq™ (Takara, China), 0.4 µl of 10 µmol/L forward and reverse primers, and 7.2 µl nuclease-free water. The following primers were used *CCL3*/MIP-1alpha (GenBank accession number NM013025), forward: 5'-GCTCTGGAACGAAGTCTTCTC-3', reverse: 5'-GAAAGGCTGCTGGTCTCAAA-3; GAPDH (FQ216837), forward: 5'-CCCTTCATTGACCTCAACTACA-3', reverse: 5'-GCCAGTAGACTCCACGACATA-3'. The cycling conditions were 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 60°C for 20 s, and 65°C for 15 s. Each sample was run in triplicate. The 2(-Delta Delta ct) method was used to analyze the data. The specificity of the PCR products was assessed by Melting curve analysis.

Statistical analysis

All data were presented as means ±SD and analyzed by nonparametric test. After testing for normal distribution of variables, student's 2-tail *t*-test and one-way analysis of variance (anova) followed by the *post hoc* least significant difference test were used where appropriate. Statistical analysis was performed using SPSS 17.0 software (SPSS Inc., USA). $P<0.05$ was considered statistically significant.

RESULT

Upregulation of CCL3/MIP-1alpha in LPS-induced brain injury

ELISA and immunohistochemistry were performed to determine the pattern of CCL3/MIP-1alpha expression in the brain after treatment with LPS. The levels of CCL3/MIP-1alpha protein were scarcely detectable in samples from normal rats (0.5 ±0.3 pg/mg protein). After LPS treatment, the CCL3/MIP-1alpha levels were increased slightly in the brain tissue homogenates at 6 h (20.2±7.5 pg/mg protein) compared with that in the normal rats, reaching maximal expression at 24 h (194.5±32.7 pg/mg protein), and remained elevated at 72 h (86.1±22.6 pg/mg protein). No significant differences were observed between the saline-injected controls (0.7±0.3 pg/mg protein) and normal rats (Fig. 1A). Immunohistochemistry revealed that CCL3/MIP-1alpha immunoreactivity was barely detectable throughout the brain hemispheres of normal rats. At 6 h after LPS, a few CCL3/MIP-1alpha immunoreactive cells were present within or near the blood vessels, ventricles, or meninges. However, at 24 h, CCL3/MIP-1alpha immunoreactivity was widely disseminated throughout the brain hemispheres, especially in the hippocampus, cerebral cortex, and amygdala. At 72 h, CCL3/MIP-1alpha immunoreactivity was distributed moderately in these regions (Fig. 1B). High magnification showed immunoreactive cells with a glial

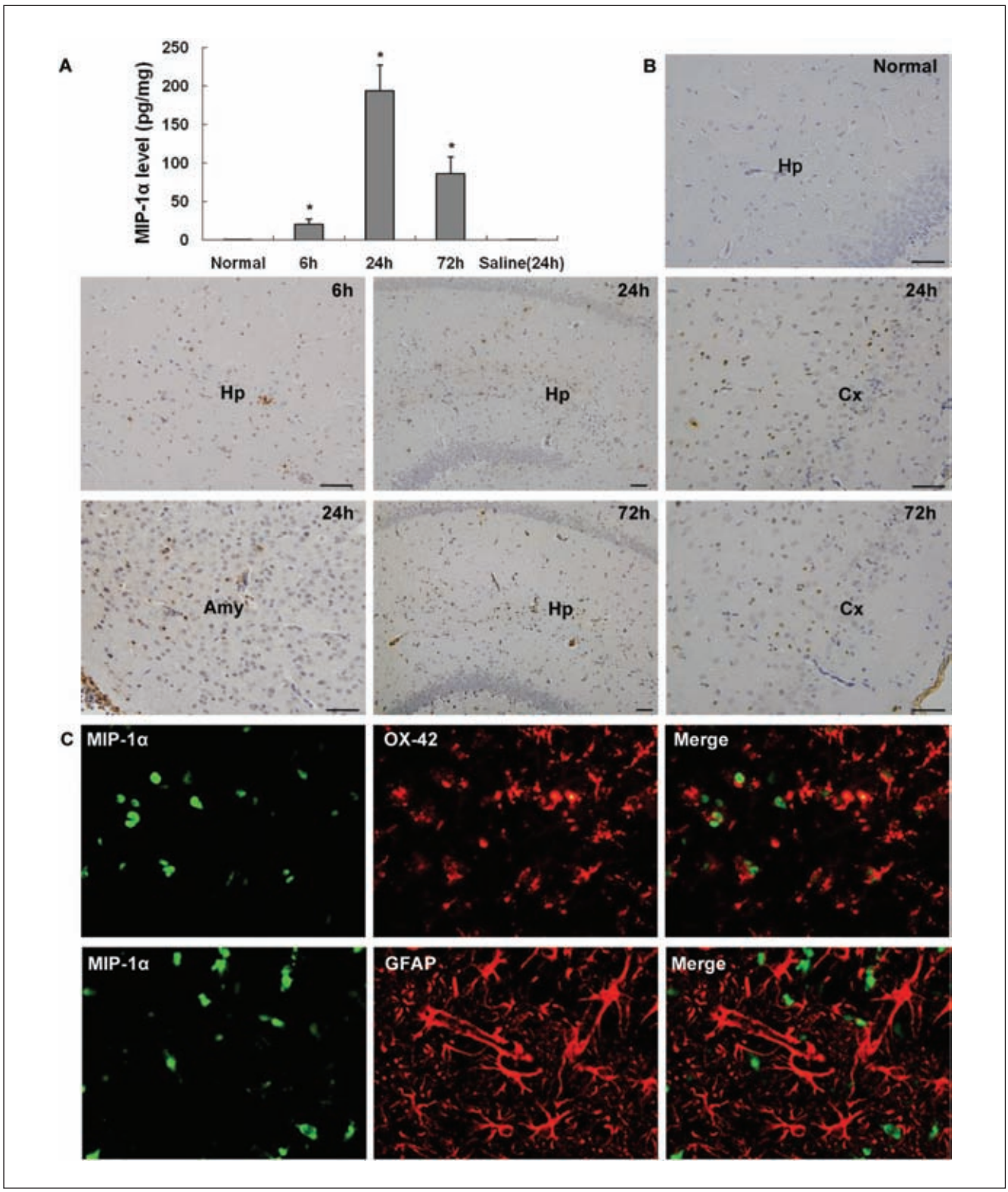

Fig. 1. Expression of CCL3/MIP-1alpha in the brain of rats. (A) Quantitative analysis by ELISA demonstrated the protein expression level of CCL3/MIP-1alpha in the brain homogenates from normal rats (Normal), LPS-treated rats at 6, 24 and 72 h (6 h, 24 h, and 72 h, respectively), and Saline-injected controls at 24 h (Saline (24h)) (*$p < 0.05$, *vs.* Normal). (B) Representative images showed CCL3/MIP-1alpha immunoreactivity in hippocampus (Hp), prefrontal cortex (Cx) or amygdala (Amy) of rats (scale bars=50 μm). (C) Double-immunofluorescence staining showed that CCL3/MIP-1alpha immunoreactivity (green) were localized to OX-42 rather than GFAP-labeled glial cells (red) in the hippocampus of rats at 24 h after LPS administration.

morphology. To determine the glial cellular sources of CCL3/MIP-1alpha, the cells were doubly immunostained for CCL3/MIP-1alpha and either OX-42 or GFAP on samples collected from rats 24 h after LPS treatment. Induced CCL3/MIP-1alpha immunoreactivity was expressed in OX-42-positive microglia rather than GFAP-labeled astrocytes (Fig. 1C).

Effect of anti-MIP-1alpha antibody on the microglial reaction in LPS-induced brain injury

To determine the role of CCL3/MIP-1alpha in the microglial reaction in the brains of rats after treatment with LPS, we evaluated the effects of an anti-MIP-1alpha antibody on the number and morphology of microglia in two brain areas, the hippocampus and prefrontal cortex.

Immunohistochemistry revealed that Iba1--immunopositive microglia were scattered throughout the hippocampus and prefrontal cortex in the Saline+IgG rats, displaying a typical ramified shape with small somata and thin processes. In the LPS+IgG rats, Iba1 immunoreactivity was significantly increased 24 h after LPS treatment, and remained elevated at 72 h. However, in the LPS+anti-MIP-1alpha rats, the neutralizing antibody clearly inhibited the number of Iba1-immunopositive microglia in both brain regions compared with that in the corresponding LPS+IgG rats (Figs 2A–2C). Assessing the morphological changes in microglia is a reasonable way to evaluate the status of microglial activation in response to LPS, as reported by Hwang and colleagues (2010). A high-magnification analysis showed that the activated microglia had adopted an amoeboid morphology with enlarged cell

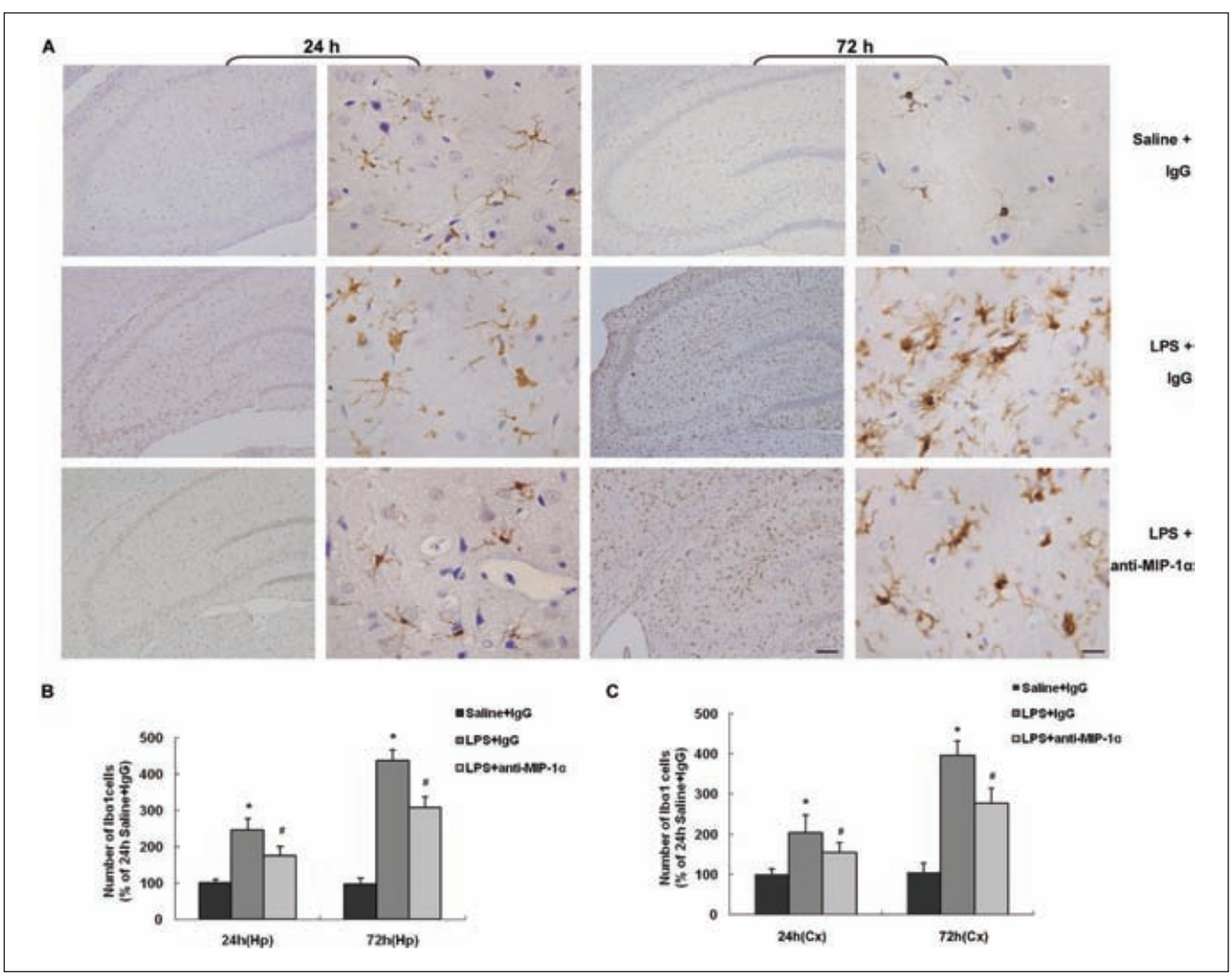

Fig. 2. Microglial reactions in LPS-induced brain injury. (A) Representative images showed the immunoreactivity of Iba1-labeled microglia and cellular morphology in the hippocampus of rats at 24 h (24 h) and 72 h (72 h) after LPS administration (scale bars=100 μm in column 1 and 3, 20 μm in column 2 and 4). Counting analysis showed the number of Iba1 immunoreactive microglia in hippocampus (Hp) (B) and prefrontal cortex (Cx) (C) of rats (*p<0.05, compared with corresponding Saline+IgG rats, #P<0.05, compared with corresponding LPS+IgG rats).

bodies and coarse processes at 24 h and 72 h after LPS treatment. The application of the anti-MIP-α neutralizing antibody did not alter the morphology of the amoeboid microglia in the brain compared with that in the corresponding LPS+IgG rats (Fig. 2A, column 2 and 4). These results mean that induced CCL3/MIP-1alpha may contribute to the accumulation rather than activation of microglia.

Effect of anti-MIP-1alpha antibody on the inflammatory response to LPS-induced brain injury

To determine the effects of CCL3/MIP-1alpha on the inflammatory response to LPS, the expression of COX-2 and iNOS was examined in the hippocampus and prefrontal cortex of rats 24 h after administration using immunohistochemistry and western blot, respectively. COX-2 immunoreactivity was significantly higher in both brain areas of the LPS+IgG rats compared with its faint expression in the Saline+IgG rats. However, COX-2 immunoreactivity was clearly blocked by treatment with the anti-MIP-α antibody (Fig. 3A). A similar change was also observed in the level of iNOS protein, which was significantly lower in the LPS+anti-MIP-α rats than in the LPS+IgG rats (Fig. 3B).

Activation of MAPKs in the LPS-induced brain injury

To determine the potential links between MAPKs and CCL3/MIP-1alpha, we evaluated the expression of phosphorylated P38 MAPK, ERK1/2, and JNK in the brain with western blot. The levels of P38 MAPK phosphorylation were slightly elevated in the brain tissue homogenates at 6 h after LPS treatment, and remained high thereafter. Similarly, the phosphorylation of ERK1/2 was dramatically upregulated during the observation period after treatment with LPS. No significant differences were detected in the level of phosphorylated JNK between normal rats and LPS-treated rats (Fig. 4A).

We also mapped the distributions of phosphorylated P38 MAPK and ERK1/2 with immunohistochemistry. In normal rats, only a few cells immunoreactive for phosphorylated P38 MAPK were scattered in the brain cortex and vessel walls. At 6 h after LPS, phosphorylated P38 MAPK immunoreactivity was faintly detected in the hippocampal alveus and peripheral cortex. Thereafter,

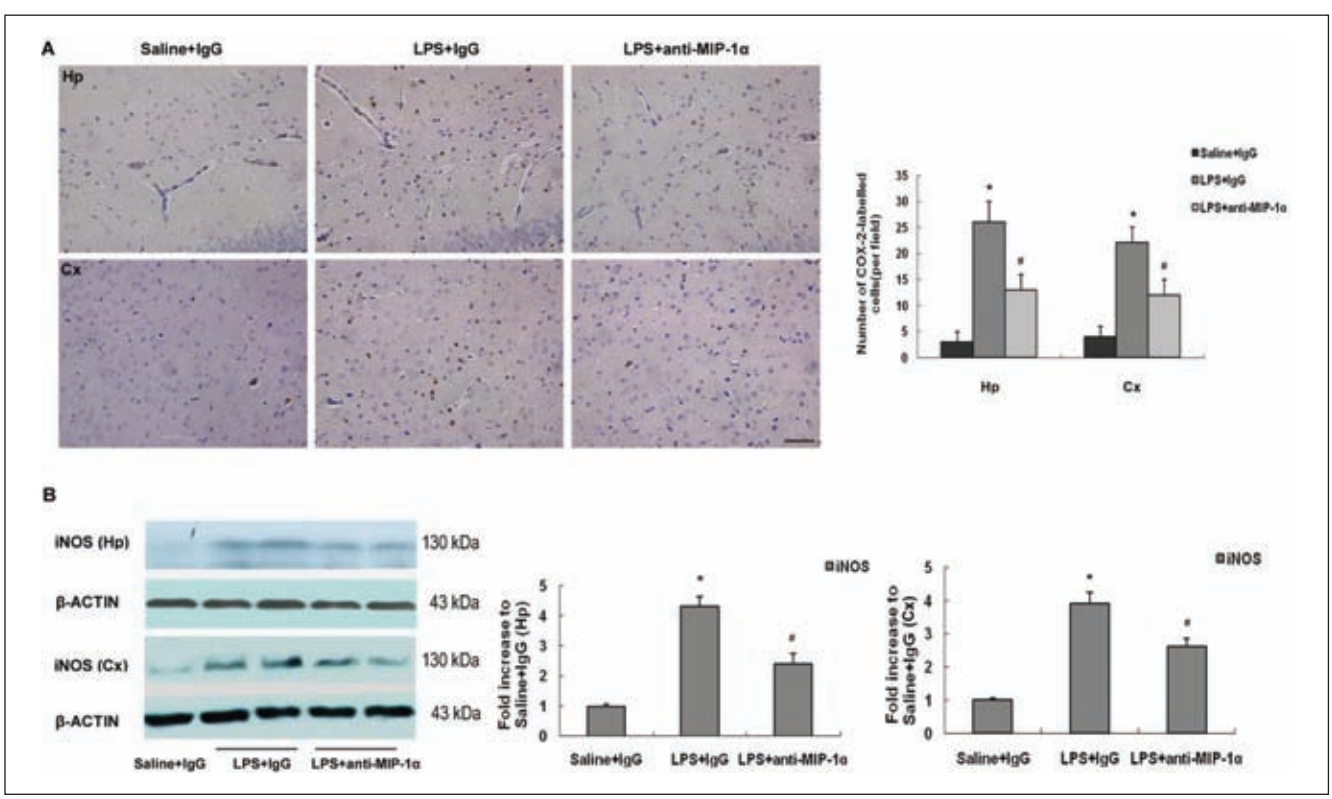

Fig. 3. Effect of anti-MIP-1alpha antibody on COX-2 and iNOS expression in the brain of rats. (A) Representative images and histogram showed COX-2 immunoreactivity in hippocampus (Hp) and prefrontal cortex (Cx) of rats (scale bar=100 μm; *p<0.05, compared with corresponding Saline+IgG rats; #p<0.05, compared with corresponding LPS+IgG rats). (B) Western blot and histograms showed the protein expression level of iNOS in the hippocampus (Hp) and prefrontal cortex (Cx) of rats (*p<0.05, *vs.* Saline+IgG; #p<0.05, *vs.* LPS+IgG).

a significant increase in P38 MAPK phosphorylation was observed throughout the brain parenchyma and ventricular system at 24 h and 72 h (Figs 4B, 4C). Unlike phosphorylated P38 MAPK, immunoreactivity for phosphorylated ERK1/2 in the normal rat brain was mainly localized to large-body neuronal cells in the peripheral cortex and endothelium-like cells in the vessel walls. After LPS treatment, phosphorylated ERK1/2 was progressively expressed by neuronal cells in the cerebral cortex and by glia-shaped cells in the hippocampus and cortex (Figs 4B, 4D). These results suggest the immunostaining for both phosphorylated P38 MAPK and phosphorylated ERK1/2 was partly coincidental with the distribution of upregulated CCL3/MIP-1alpha.

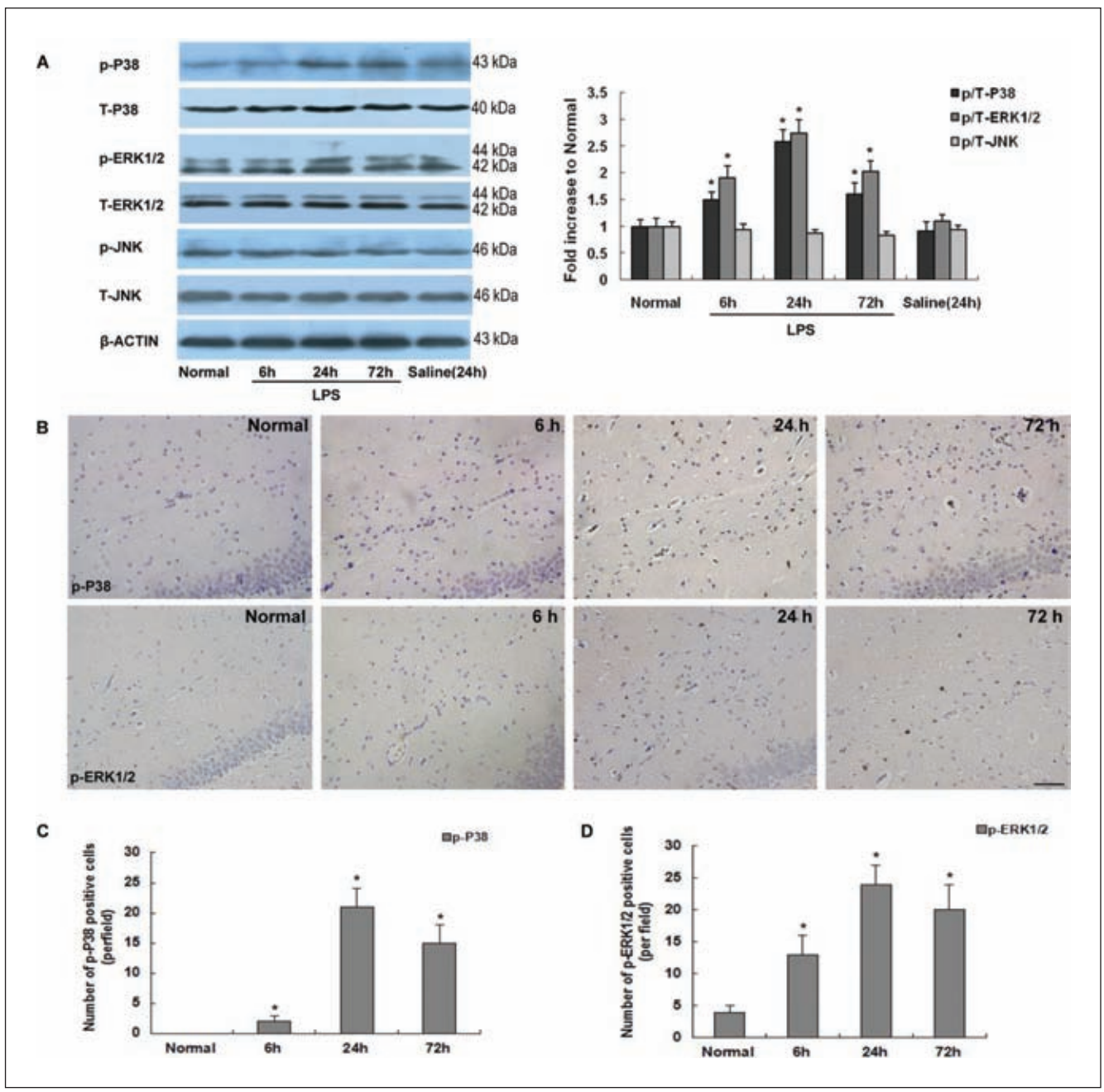

Fig. 4. Expression of MAPKs in the brain of rats. (A) Western blot and histogram showed the protein expression levels of phosphorylated (p-) and total (T-) forms of P38 MAPK, ERK1/2 and JNK in the brain from normal rats (Normal), LPS-treated rats at 6, 24 and 72 h (6, 24 and 72 h, respectively), and Saline-injected controls at 24 h (Saline (24 h)) (*p<0.05, compared with corresponding normal rats). (B) Representative images showed the immunoreactivity of phosphorylated (p-) P38 MAPK and ERK1/2 in the hippocampus of rats (scale bar=100 μm). Counting analysis showed the numbers of phosphorylated (p-) P38 MAPK (C) and ERK1/2 (D) immunoreactive cells in hippocampus of rats (*p<0.05, *vs.* Normal).

Inhibition of P38 MAPK and ERK1/2, CCL3/MIP-1alpha expression, and the microglial reaction

To determine the roles of P38 MAPK and ERK1/2 in CCL3/MIP-1alpha expression in LPS-induced brain injury, rats were pretreated with the P38 MAPK inhibitor SB203580 or the ERK1/2 inhibitor PD98059. As shown in Fig. 5A, CCL3/MIP-1alpha mRNA was increased significantly in the LPS+DMSO rats compared with that in the Saline+DMSO rats. The expression of the CCL3/MIP-1alpha gene was clearly lower in the brain tissues of the both the LPS+SB203580 and LPS+PD98059 rats than in those of the LPS+DMSO rats. An ELISA was used to examine the CCL3/MIP-1alpha concentrations in homogenates of the hippocampi and prefrontal cortices from each group 24 h after LPS treatment. The levels of CCL3/MIP-1alpha protein were clearly higher in both regions in the LPS+DMSO rats (206.5±31.4 and 189.6±28.5 pg/mg protein, hippocampus and prefrontal cortex, respectively) than in the Saline+DMSO rats (0.7±0.4 and 0.5±0.3 pg/mg protein, hippocampus and prefrontal cortex, respectively). However, the inhibition of P38 MAPK reduced the LPS-induced upregulation of CCL3/MIP-1alpha (130.5±25.1 and 122.3±26.8 pg/mg protein, hippocampus and prefrontal cortex in LPS+SB203580 rats) compared to the level in the LPS+DMSO rats. Similarly, the upregulated expression of CCL3/MIP-1alpha was also reduced in response to ERK1/2 inhibition in the LPS+PD98059 rats (142.1±21.7 and 118.7±29.4 pg/mg protein, hippocampus and prefrontal cortex, respectively) compared with that in the LPS+DMSO rats (Figs 5B, 5C). A study of the effects of MAPKs on Iba1 immunoreactivity revealed that the number of Iba1-positive cells was clearly lower in the brains of both the LPS+SB203580 and LPS+PD98059 rats than in the LPS+DMSO rats (Figs 5D, 5E).

Activation of NF-kappaB, and CCL3/MIP-1alpha expression and microglial accumulation

We investigated the activation of NF-kappaB by evaluating the phosphorylation of both IkappaB alpha and

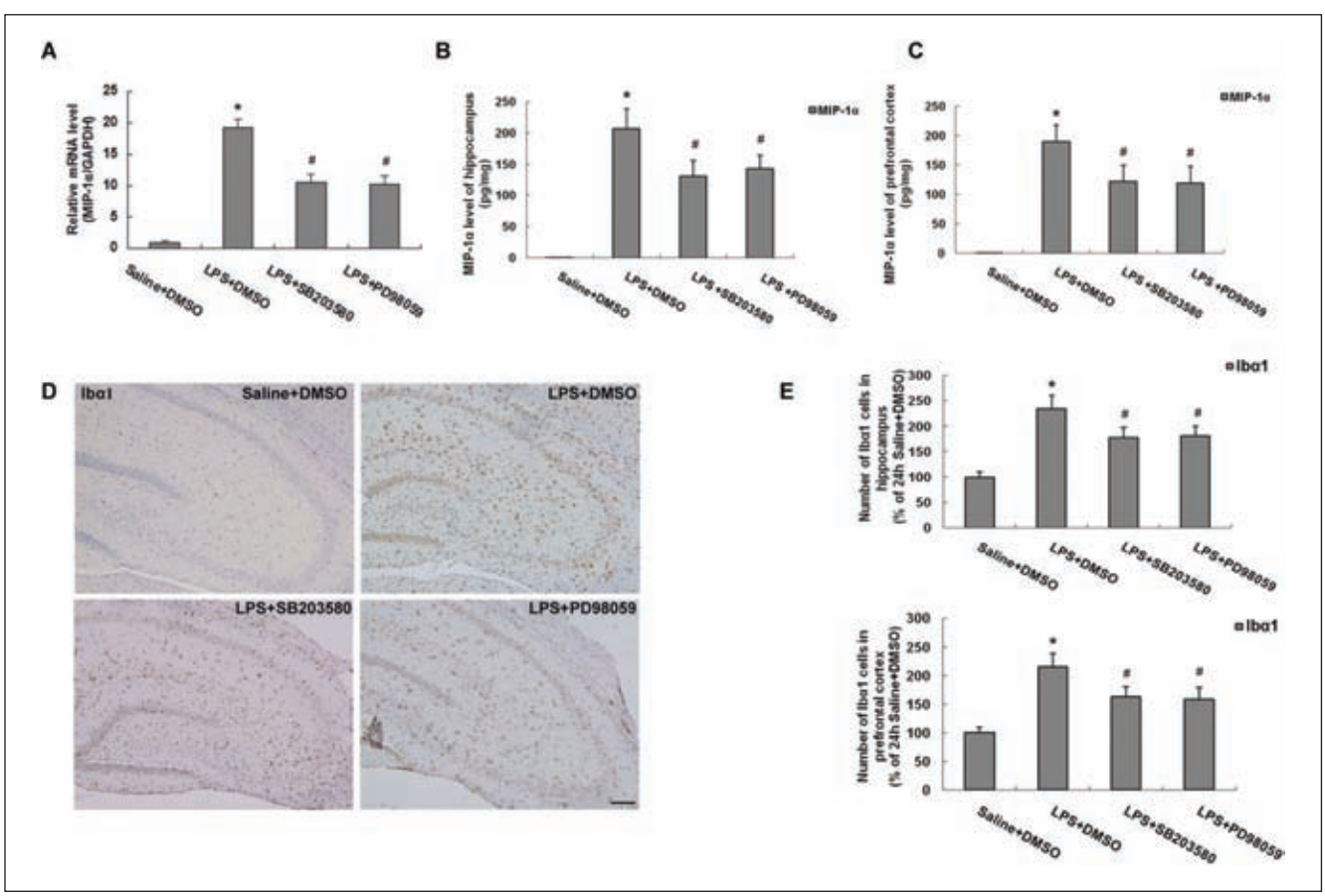

Fig. 5. Effects of inhibitors of P38 MAPK and ERK1/2 on CCL3/MIP-1alpha expression and Iba1 immunoreactivity. (A) Quantitative analysis by real-time PCR showed the CCL3/MIP-1alpha mRNA expression in the brain tissues of rats, normalized to the levels of GAPDH (*p<0.05, *vs.* Saline+DMSO, #p<0.05, *vs.* LPS+DMSO). Quantitative analysis by ELISA showed the protein expression level of CCL3/MIP-1alpha in hippocampus (B) and prefrontal cortex (C) of rats (*p<0.05, *vs.* Saline+DMSO; #p<0.05, *vs.* LPS+DMSO). (D) Representative images showed Iba1 immunoreactivity in the hippocampus of rats (scale bar=100 μm). (E) Counting analysis showed the number of Iba1 immunoreactive cells in hippocampus and prefrontal cortex of rats (*p<0.05, *vs.* Saline+DMSO; #p<0.05, *vs.* LPS+DMSO).

NF-kappaB/p65 subunit in the hippocampus of rats 24 h after LPS administration. As shown in Fig. 6A, the protein levels of phosphorylated IkappaB alpha and NF-kappaB/p65 were clearly higher in the LPS rats than in the Saline rats.

To determine whether NF-kappaB affects CCL3/MIP-1alpha expression, we performed an experiment with the NF-kappaB inhibitor PDTC, administered by i.c.v. injection. At the dose used, PDTC successfully inhibited LPS-induced NF-kappaB/p65 phosphorylation (Fig. 6B). NF-kappaB inhibition also caused reductions in CCL3/MIP-1alpha mRNA and protein levels (192.6±30.8 *vs.* 130.2±26.5 pg/mg protein, LPS+Saline rats *vs.* LPS+PDTC rats) and in the number of Iba1-immunoreactive cells in the hippocampi of the LPS+PDTC rats compared with those in the LPS+Saline rats (Figs 6C–6E). NF-kappaB may also be a signaling molecule downstream from the MAPKs (P38 MAPK and ERK1/2) in LPS-induced brain injury, because the levels of phosphorylated NF-kappaB/p65 were significantly lower in the hippocampi of the LPS+SB203580 and LPS+PD98059 rats than in those of the LPS+DMSO rats (Fig. 7).

DISCUSSION

In this study, we have shown that i.c.v. administration of LPS caused a marked increase in CCL3/MIP-1alpha protein in the rat brain 6–72 h after treatment. Further studies, using an anti-MIP-1alpha neutralizing antibody, demonstrated that CCL3/MIP-1alpha affects microglial accumulation and the expression of COX-2 and iNOS during LPS-induced brain injury. We also showed that LPS

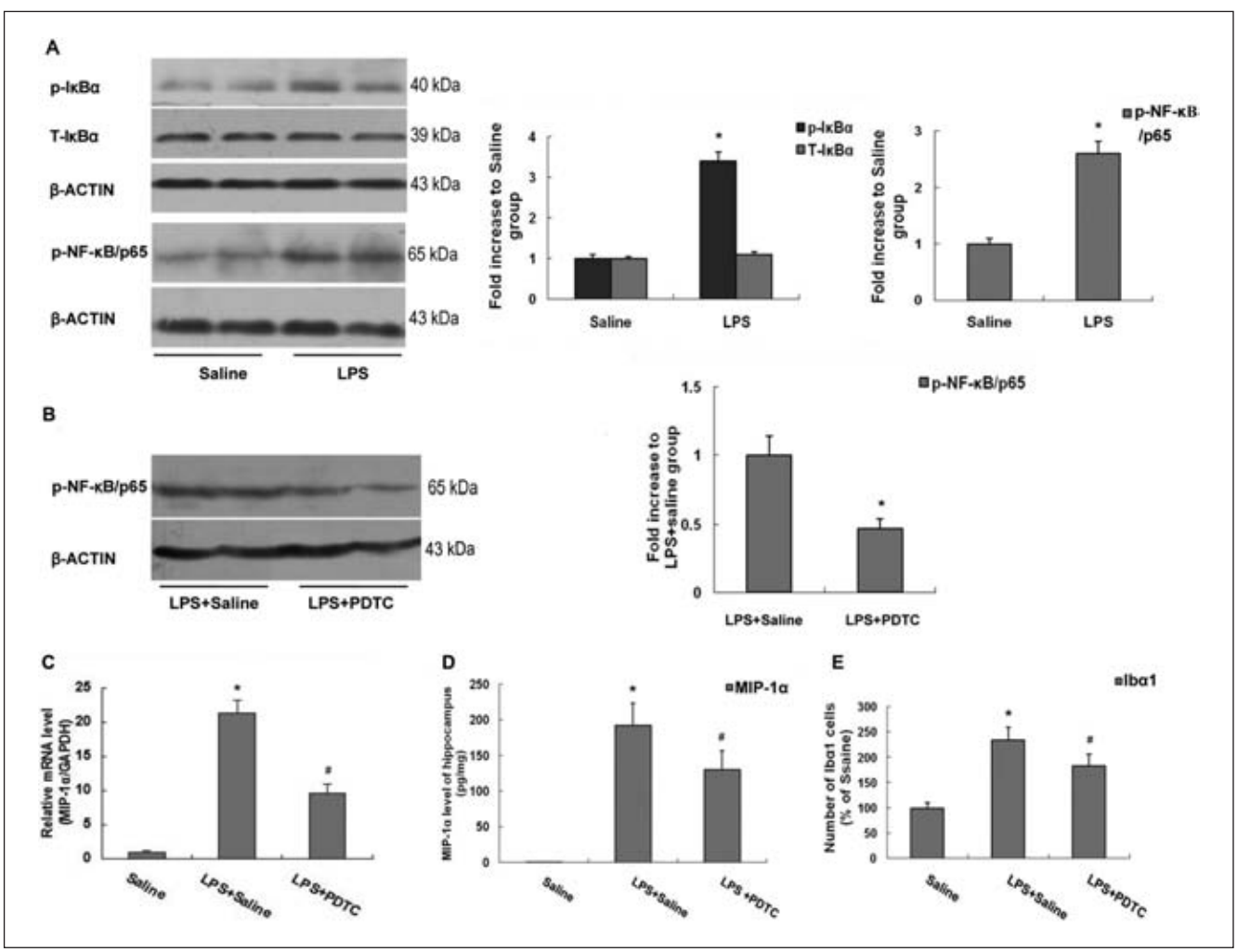

Fig. 6. NF-kappaB activation, and effect of NF-kappaB inhibitor on NF-kappaB/p65 phosphorylation, CCL3/MIP-1alpha and Iba1 expression in the brain of rats. (A) Western blot and histograms showed phosphorylated (p-) and total (T-) IkappaB alpha and phosphorylated (p-) NF-kappaB/p65 in the hippocampus of rats (*p<0.05, compared with corresponding Saline rats). (B) Western blot was performed to measure the expression of phosphorylated (p-) NF-kappaB/p65 in the hippocampus (*p<0.05). (C) Quantitative analysis by real-time PCR showed the CCL3/MIP-1alpha mRNA expression in the hippocampus of rats, normalized to the levels of GAPDH (*p<0.05, *vs.* Saline, #p<0.05, *vs.* LPS+Saline). Histograms showed the level of CCL3/MIP-1alpha protein by ELISA (D) and number of Iba1 immunoreactive cells (E) in hippocampus of rats (*p<0.05, *vs.* Saline; #p<0.05, *vs.* LPS+Saline).

induced the phosphorylation of MAPKs in the observation period, and increased the phosphorylation of NF-kappaB/p65. Moreover, the inhibition of P38 MAPK, ERK1/2, or NF-kappaB reduced the upregulation of CCL3/MIP-1alpha and microglial accumulation. Therefore, in LPS-induced brain injury, upregulated CCL3/MIP-1alpha mediates the accumulation of microglia and the neuroinflammatory response, and CCL3/MIP-1alpha may be induced under the control of the MAPK and NF-kappaB signaling molecules.

CCL3/MIP-1alpha, a vital member of the beta-chemokine subfamily, has been implicated in the inflammatory processes of various neuropathological conditions, including Alzheimer's disease (Passos et al. 2009), cerebral ischemia (Wang et al. 2008), and LPS-induced endotoxemia (Gourmala et al. 1999, Raghavendra Rao et al. 2003). CCL3/MIP-1alpha may facilitate the chemotaxis of microglia and monocytes through CCR1, CCR4 and CCR5 receptors (Wang et al. 2008, Passos et al. 2009). In this study, we observed a significant increase of CCL3/MIP-1alpha protein in the cerebral hemispheres 24–72 h after the administration of LPS, which is consistent with a previous report by Gourmala and others (1999), who demonstrated that a pyrogenic dose of LPS caused a time-dependent increase in CCL3/MIP-1alpha expression, which peaked 12–16 h after the intravenous administration of LPS. We also investigated the biological function of CCL3/MIP-1alpha in the neuroinflammation caused by LPS in pretreating animals with an anti-MIP-1alpha antibody, which reduced the number of activated microglial cells, but did not modify the morphology of the microglia. Such results suggest that CCL3/MIP-1alpha is responsible for the accumulation rather than the activation of microglia.

Consistent with previous studies (Gourmala et al. 1999), double immunostaining demonstrated that CCL3/MIP-1alpha immunoreactivity is expressed by OX-42-labeled microglia but not by GFAP-labeled astrocytes. These data

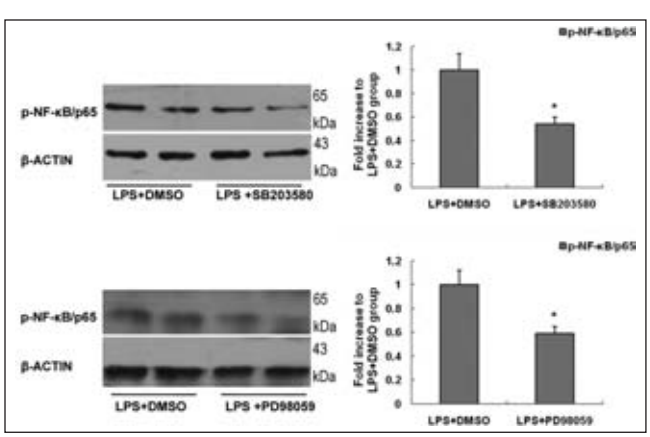

Fig. 7. Effects of inhibitors of P38 MAPK and ERK1/2 on NF-kappaB/p65 phosphorylation. Western blot was performed to measure the protein expression level of phosphorylated (p-) NF-kappaB/p65 in the hippocampus of rats at 24 h after LPS administration (*p<0.05).

indicate that upregulated CCL3/MIP-1alpha may act in an autoregulatory way to mediate the microglial reaction in LPS-induced brain injury. However, CCL3/MIP-1alpha has also been detected in neurons and astrocytes in the rodent brain under other pathological conditions (Wang et al. 2008, Xu et al. 2009). These inconsistent results can be partly explained by the differences in the species, ages, or types of injury investigated. Additionally, OX-42 antibody could recognize blood-derived monocytes and macrophages as well, so some OX-42-positive cells may stand for infiltrated leukocytes derived from blood. Our results also show that the expression of two proinflammatory factors, COX-2 and iNOS, was reduced by pretreatment with the anti-MIP-1alpha antibody. In a mouse model of Alzheimer's disease, investigators found that CCL3/MIP-1alpha prompted the production of COX-2 and iNOS after the administration of beta-amyloid (1–40) by regulating AP-1 and NF-kappaB (Passos et al. 2009). These findings suggest that CCL3/MIP-1alpha contributes to the inflammatory response in some neuropathological conditions, and that the production of cerebral cytokines is not necessarily associated with the morphological signs of microglial activation (Hanisch 2002). However, a recent report demonstrated that CCL3/MIP-1alpha failed to stimulate nitric oxide secretion in rat microglial cultures (Skuljec et al. 2011). These contradictory data emphasize the need to thoroughly investigate the role of CCL3/MIP-1alpha in regulating the production of COX-2 and iNOS by glial cells.

MAPKs are serine/threonine kinases, mainly including the P38 MAPK, ERK, and c-JNK families, that are widely expressed in many tissues, including the CNS. However, there is limited information on the pattern of MAPK expression in the brain after LPS treatment or bacterial infection (Davis et al. 2000, Ferrer et al. 2001). Our results show that the i.c.v. administration of LPS induced a time-dependent increase in phosphorylated P38 MAPK and ERK1/2, from 6 h to 72 h after treatment. Interestingly, we detected no significant differences in JNK phosphorylation among the groups studied. This finding may be ascribed to the time-course of our observations in the present study, because these phenomena have been detected in rats with traumatic brain injury (Raghupathi et al. 2003). A further immunohistochemical analysis showed that the distribution of phosphorylated P38 MAPK and ERK1/2 in glia-shaped cells was to some extent consistent with CCL3/MIP-1alpha immunostaining, indicating the potential involvement of the MAPK (P38 MAPK and ERK1/2) pathways in the induction of CCL3/MIP-1alpha by LPS. Several *in vitro* studies have verified this assumption (Song et al. 2002, Bianchi et al. 2011). However, considering the paucity of direct *in vivo* evidence, we conducted an experiment in which rats were pretreated with SB203580 or PD98059 before LPS-induced brain injury. The inhibition of P38 MAPK or ERK1/2 reduced the upregulation of CCL3/

MIP-1alpha expression and the accumulation of activated microglia. To our knowledge, this is the first *in vivo* study to confirm a causative role for P38 MAPK and ERK1/2 in signaling events that mediate CCL3/MIP-1alpha expression in microglia after the administration of LPS. In previous study using primary microglial cultures, activated P2Y6 and P2X7 receptors were shown to cause the microglial production of CCL3 (Kataoka et al. 2009, Kim et al. 2011). Moreover, considering several findings showing phosphorylation of p38MAPK and ERK1/2 in response to activation of P2Y6 and P2X7 (Morioka et al. 2013, Kim et al. 2015), it is also speculated MAPKs signaling play an important role in production of CCL3 from microglia.

NF-kappaB is an ubiquitously expressed transcription factor that regulates the expression of genes involved in a wide range of neuropathological processes, including neuroinflammation, trauma, and hypoxia (Nurmi et al. 2004, van Loo et al. 2006). The level of phosphorylation of the NF-kappaB inhibitory protein IkappaB in whole-cell extracts can be used as an index of the activity of the NF-kappaB pathway (Hoffmann et al. 2002). In accord with previous studies (Wang et al. 2009, Mao et al. 2012), we demonstrated that LPS caused a significant increase in the phosphorylation of IkappaB alpha in the hippocampus 24 h after treatment. Although the phosphorylation of the NF-kappaB p65 subunit is also crucial for full NF-kappaB activation, this activation can be incomplete contact with IkappaB alpha phosphorylation and degradation (Madrid et al. 2001). Our study further showed that LPS induced the phosphorylation of the NF-kappaB/p65 subunit 24 h after injury. Inhibition of NF-kappaB by pretreatment with PDTC not only reduced the level of NF-kappaB/p65 phosphorylation but prevented CCL3/MIP-1alpha expression and microglial accumulation. These results confirm the involvement of NF-kappaB in the induction of CCL3/MIP-1alpha expression after treatment with LPS, and hint at the neuroprotective effect of inhibiting NF-kappaB activation.

However, published data have shown that NF-kappaB may have dual roles in some neuropathological processes, and its actual roles in many CNS disorders remain controversial (van den Tweel et al. 2006, Duckworth et al. 2006). The neuroprotective effect of inhibiting NF-kappaB activation demonstrated here could be ascribed to our choice of the therapeutic window for PDTC treatment. In neonatal hypoxic–ischemic models, early NF-kappaB activation causes brain damage 3–6 h after hypoxia–ischemia, but late NF-kappaB activation provides endogenous neuroprotection and upregulates the expression of antiapoptotic molecules (Nijboer et al. 2008). The prolonged period of observation in the present study may have allowed fuller information on the role of NF-kappaB in brain damage to be collected. Furthermore, the inhibition of both P38 MAPK and ERK1/2 reduced the upregulated phosphorylation of NF-kappaB/p65 after LPS treatment, implicating NF-kappaB as a downstream molecule of the MAPKs or at least a cross-link between them. Given the limitations of pharmacological inhibitors and the complexity of the *in vivo* situation, further studies are required to provide greater insight into the interactions between MAPKs and NF-kappaB and the upstream regulators of MAPKs and NF-kappaB signaling to mediate CCL3/MIP-1alpha expression in both *in vivo* and *in vitro* experiments.

This study demonstrates that CCL3/MIP-1alpha is significantly increased in the brains of rats after the administration of LPS, and its upregulation is conducive to the accumulation of microglia and the production of both COX-2 and iNOS. The induction of CCL3/MIP-1alpha is also regulated, at least in part, by the MAPK and NF-kappaB signaling molecules. These data may allow the development of therapeutic strategies to control the neuroinflammatory reaction after brain injury by targeting CCL3/MIP-α and its upstream signaling molecules.

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