

ORIGINAL RESEARCH COMMUNICATION

# Methionine Sulfoxide Reductase A Negatively Controls Microglia-Mediated Neuroinflammation *via* Inhibiting ROS/MAPKs/NF- $\kappa$ B Signaling Pathways Through a Catalytic Antioxidant Function

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## Abstract

**Aims:** Oxidative burst is one of the earliest biochemical events in the inflammatory activation of microglia. Here, we investigated the potential role of methionine sulfoxide reductase A (MsrA), a key antioxidant enzyme, in the control of microglia-mediated neuroinflammation. **Results:** MsrA was detected in rat microglia and its expression was upregulated on microglial activation. Silencing of MsrA exacerbated lipopolysaccharide (LPS)-induced activation of microglia and the production of inflammatory markers, indicating that MsrA may function as an endogenous protective mechanism for limiting uncontrolled neuroinflammation. Application of exogenous MsrA by transducing Tat-rMsrA fusion protein into microglia attenuated LPS-induced neuroinflammatory events, which was indicated by an increased Iba1 (a specific microglial marker) expression and the secretion of pro-inflammatory cytokines, and this attenuation was accompanied by inhibiting multiple signaling pathways such as p38 and ERK mitogen-activated protein kinases (MAPKs) and nuclear factor kappaB (NF- $\kappa$ B). These effects were due to MsrA-mediated reactive oxygen species (ROS) elimination, which may be derived from a catalytic effect of MsrA on the reaction of methionine with ROS. Furthermore, the transduction of Tat-rMsrA fusion protein suppressed the activation of microglia and the expression of pro-inflammatory factors in a rat model of neuroinflammation *in vivo*. **Innovation:** This study provides the first direct evidence for the biological significance of MsrA in microglia-mediated neuroinflammation. **Conclusion:** Our data provide a profound insight into the role of endogenous antioxidative defense systems such as MsrA in the control of microglial function. *Antioxid. Redox Signal.* 22, 832–847.

## Introduction

**M**ICROGLIA, THE RESIDENT macrophage in the central nervous system (CNS), are the major component that contributes to the immediate response to injury, pathogen infection, or toxic insult. Although microglial activation may be beneficial to “an attack” in the brain by removing cell

debris and pathogens (19), overactivation of microglia causes excessive inflammatory responses (16), which are associated with numerous neurologic disorders, including ischemic stroke (63), CNS infections (11), and neurodegenerative diseases (38). Thus, the dissection of mechanisms that selectively shut off the pathways involved in microglial overactivation become critical in controlling neurological diseases.

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### Innovation

We demonstrate the link between methionine sulfoxide reductase A (MsrA) and the functions of microglia for the first time. We provide experimental evidence *in vitro* as well as *in vivo*, pointing to the crucial role of microglial MsrA in the anti-inflammatory actions under lipopolysaccharide-stimulated conditions. In addition, this study provides the first experimental evidence that MsrA facilitates the reaction of methionine with reactive oxygen species, which may afford the antioxidant effect that is involved in the inhibition of microglia-mediated neuroinflammation.

Microglial overactivation and reactive oxygen species (ROS) are closely interlinked. It is widely accepted that activated microglia are the major source of ROS in the CNS (3, 54). Activated microglia produce high levels of superoxide radicals and hydrogen peroxide *via* activating NADPH oxidase (NOX) (3, 47). Moreover, ROS may be an early signal triggering the induction of cytokines and participating in inflammatory signaling (8, 48), by acting as second messengers that are capable of modulating gene expression *via* activating kinase signaling, including mitogen-activated protein kinases (MAPKs) (62, 75). As a downstream signaling molecule of MAPKs, nuclear factor kappaB (NF- $\kappa$ B) is particularly sensitive to ROS, and it is central to the acquisition of pro-inflammatory phenotype (29). Thus, ROS/MAPKs/NF- $\kappa$ B signaling pathways may play a pivotal role in the switch of microglia from surveillance to an over-activated state (9, 23, 49, 61).

Microglia are also equipped with sufficient antioxidative defence mechanisms to avoid ROS-mediated cellular damage (14, 34). Although their good antioxidative potential has been revealed (20), there is much less evidence related to their participation in neuroinflammation. ROS cause reversible molecular changes and control activity of key proteins that regulate neuroinflammation, such as apoptosis signal-regulating kinase 1 (ASK1) (50), dual-specificity phosphatase 3 (DUSP3) (66), and NF- $\kappa$ B (39). Thus, endogenous antioxidants may apply brake on uncontrolled neuroinflammatory responses. Activated microglia produce high levels of superoxide radicals and the main antioxidant enzyme that is known to scavenge superoxide radical is Cu, Zn-superoxide dismutase-1 (SOD-1). It has been well documented that SOD-1 reduces lipopolysaccharide (LPS)-induced superoxide production, with concurrent increases in hydrogen peroxide, and it exerts a significant inhibition on microglia-mediated inflammatory events (8, 12, 31). Although the oxidative potential of hydrogen peroxide is much lower than superoxide radical, it also acts as a second messenger to mediate intracellular redox-sensitive signal transduction and enhance neuroinflammation (15, 21, 58). Therefore, other antioxidative defense mechanisms to control microglia status may exist.

Cyclic oxidation/reduction of methionine (Met) in proteins is an important process in scavenging ROS and preventing ROS from attack on key sites of proteins (37, 55). A variety of ROS react with Met residues to form methionine sulfoxide (MetO), followed by MetO reductases (Msrs)-mediated reduction back to Met. As a key member of Msrs, MetO reductase A (MsrA) repairs oxidative damage of pro-

teins and protects against oxidative stress in many types of cells, including neuronal cells (5, 42, 44, 72, 73). Nonetheless, the role of MsrA in neuroinflammation, particularly in the function of microglia, remains virtually unknown. The principal function of MsrA in the antioxidation is to maintain the effective content of protein-bound Met by reducing MetO timely. Recently, it has been demonstrated that MsrA also serves as a stereospecific methionine oxidase to promote oxidation of protein-bound Met (32, 33), suggesting that MsrA may facilitate the antioxidation of Met (69). In this context, we demonstrated that MsrA was functionally expressed in microglia and the MsrA-catalytic antioxidation attenuated inflammatory activation of microglia *in vitro* and neuroinflammation *in vivo*. We found that endogenous MsrA system is crucial in the limitation of uncontrolled neuroinflammation.

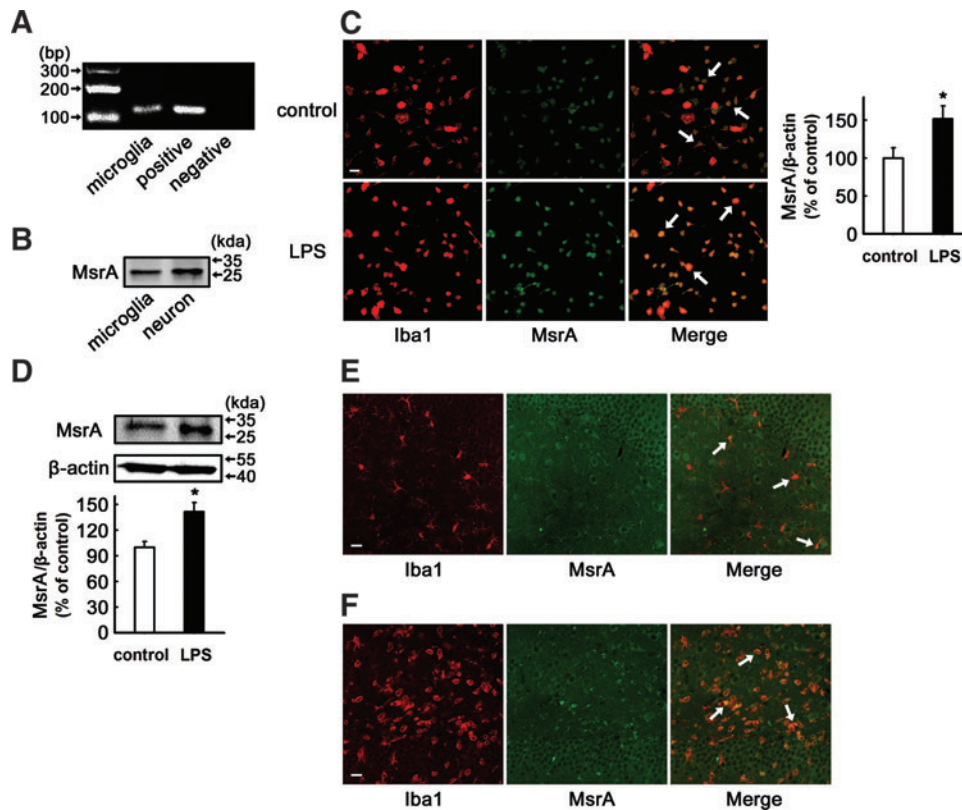
### Results

#### *MsrA is expressed in the resting and activated microglia both in vitro and in vivo*

To determine whether MsrA is present in microglia, the total RNA was extracted from primary cultured rat microglia for reverse transcription-polymerase chain reaction analysis. By using specific primers for MsrA, the unique product with predicted size of 116 bp was yielded, similar to the positive control (plasmid pcDNA3.1-rMsrA) (Fig. 1A). The expression of MsrA protein in cultured rat microglia was further detected by immunoblotting. Using neuron as a positive control, the bands in Figure 1B showed the presence of MsrA protein in primary cultured rat microglia. The expression of various molecules in microglia are known to be changed after external stimuli (56), and we therefore asked whether the MsrA expression can vary with microglial activation. To address this question, primary microglial cultures were treated with LPS (100 ng/ml) for 24 h, and the MsrA immunoreactivity was examined. Immunocytochemical (Fig. 1C) and Western blotting analysis (Fig. 1D) revealed that LPS treatment increased the endogenous expression of MsrA by  $151\% \pm 17\%$  ( $n=6$ ,  $p<0.05$  vs. control) and  $142\% \pm 11\%$  ( $n=5$ ,  $p<0.05$  vs. control), respectively. Furthermore, *in situ* brain slices (DG area from the hippocampus) also showed the co-expression of MsrA in microglia. In the normal brain tissue, the levels of MsrA in most microglial cells are relatively low, and it seemed that MsrA colocalized with only a part of Iba1-positive cells (Fig. 1E). Under LPS-stimulated condition, most of the Iba1-positive microglial cells *in situ* strongly expressed MsrA (Fig. 1F), indicating that MsrA expression in the microglia is induced by environmental stimulus.

#### *Silencing of MsrA exacerbates LPS-induced activation of microglia and the production of pro-inflammatory factors*

To determine the role of MsrA in the microglial status, we used lentiviral short hairpin RNAs (shRNAs) against MsrA to evaluate the role of MsrA in microglia-mediated inflammatory responses (Supplementary Fig. S1A; Supplementary Data are available online at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)). Microglial activation is associated with a marked increase in Iba1 expression (74). Immunofluorescence analysis showed that neither negative control shRNA (control shRNA) nor MsrA-silencing shRNA (MsrA shRNA) alone had perceptible effects



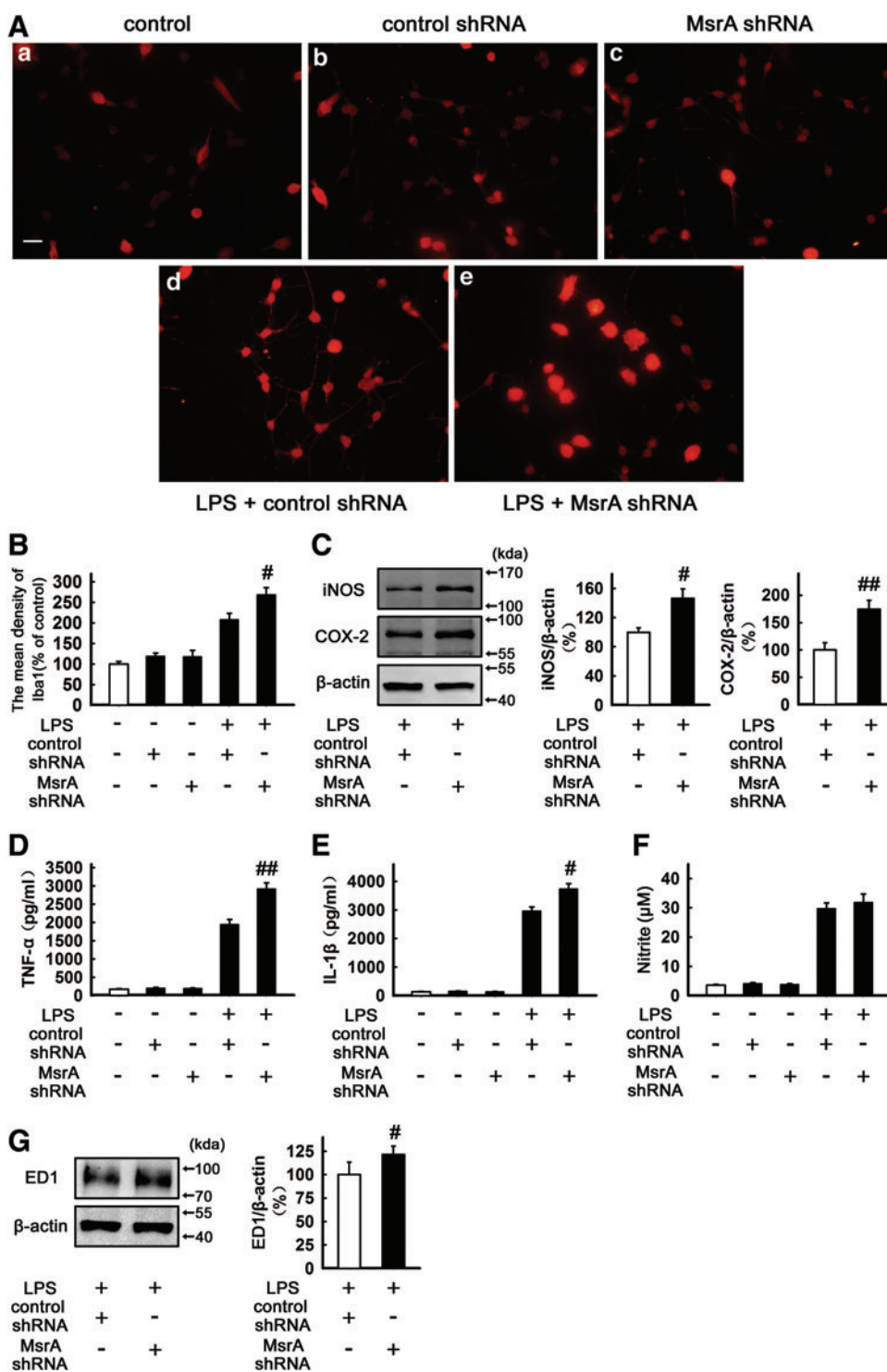
**FIG. 1. The expression of methionine sulfoxide reductase A (MsrA) in resting and activated microglia.** (A) MsrA transcript was detected in primary cultured rat microglia by reverse transcription-polymerase chain reaction (positive control, plasmid pcDNA3.1-rMsrA; negative control, ddH<sub>2</sub>O). (B) Western blotting indicated the existence of MsrA in primary cultured rat microglia (positive control, cortical neuron). (C, D) Primary rat microglia were stimulated with 100 ng/ml lipopolysaccharide (LPS) for 24 h. (C) Immunocytochemical analysis to detect the expression of MsrA (green) in microglia in the presence or absence of LPS ( $n=6$ ). Microglial cells were stained with an anti-Iba1 (a specific microglial marker, red) antibody. The mean fluorescence intensity of MsrA was determined and expressed as a relative change in comparison with the untreated cells, which was set to 100%. (D) Western blotting analysis of MsrA expression in the presence or absence of LPS ( $n=5$ ). The levels of MsrA were expressed as a relative change in comparison with the untreated cells, which was set to 100%. Data are expressed as mean  $\pm$  SEM, \* $p < 0.05$  versus untreated cells. (E, F) Immunolabeling of MsrA (green) and microglia (red) in hippocampus in *in vivo* rat brain tissues after an intrahippocampal injection of saline (E) and LPS (F) at 24 h. Arrows indicate the overlapping of MsrA with Iba1-positive microglia. Scale bars: 50  $\mu$ m. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

on the mean density of Iba1 and microglial morphology. LPS treatment (100 ng/ml, 24 h) significantly increased the Iba1 expression. As expected, the mean density of Iba1 was significantly higher in MsrA shRNA-transfected group ( $268\% \pm 18\%$ ), which displayed a more “amoeboid”-activated morphology, characterized by hypertrophic bodies, with fewer, thicker, and shorter processes, when compared with control shRNA-transfected cells ( $208\% \pm 16\%$ ) ( $n=5$ ,  $p < 0.05$  vs. LPS + control shRNA, Fig. 2A, B). Furthermore, when MsrA was silenced, LPS-induced expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) also increased by  $146\% \pm 13\%$  ( $n=5$ ,  $p < 0.05$  vs. LPS + control shRNA, Fig. 2C) and  $174\% \pm 16\%$  ( $n=5$ ,  $p < 0.01$  vs. LPS + control shRNA, Fig. 2C), respectively; whereas control shRNA or MsrA shRNA alone did not induce significant protein expression (Supplementary Fig. S1B). To assess whether MsrA knockdown aggravates the production of LPS-induced pro-inflammatory cytokines, tumor necrosis factor alpha (TNF- $\alpha$ ) and IL-1 $\beta$  were quantified by an enzyme-linked immunosorbent assay (ELISA) in the cultured primary

microglia. LPS treatment (100 ng/ml, 24 h) led to significantly increased levels of TNF- $\alpha$  ( $1947 \pm 138$  pg/ml) and IL-1 $\beta$  ( $2964 \pm 139$  pg/ml) in control shRNA-transfected microglial cultures, which were further enhanced by MsrA silencing ( $2915 \pm 165$  pg/ml and  $3732 \pm 188$  pg/ml, respectively) ( $n=5$ ,  $p < 0.01$  and  $p < 0.05$  vs. LPS + control shRNA, Fig. 2D, E). The effect of MsrA knockdown on NO production in LPS-stimulated primary microglia was also investigated. The accumulated nitrite in the culture media estimated by the Griess reaction was used as an index for NO synthesis. Although silencing of MsrA seemed to elevate LPS-induced production of nitrite, these changes were not statistically significant ( $n=5$ , Fig. 2F). Finally, we found that LPS-induced expression of ED1, a specific marker for activated microglia in LPS + MsrA shRNA group, was increased to  $121\% \pm 9\%$  of LPS + control shRNA group ( $n=5$ ,  $p < 0.05$  vs. LPS + control shRNA, Fig. 2G), whereas neither control shRNA nor MsrA shRNA alone changed the ED1 expression (Supplementary Fig. S1C). Altogether, our results evidently indicate that knockdown of MsrA renders microglia more

**FIG. 2. MsrA knockdown aggravates LPS-induced microglial activation and inflammatory responses.**

Primary microglia were transfected with control shRNA or MsrA shRNA and incubated with 100 ng/ml LPS for 24 h. (A) Immunocytochemical analysis to evaluate the mean fluorescence density of Iba1 ( $n=5$ ). Scale bars in (a–e): 50  $\mu\text{m}$ . (B) The levels of Iba1 intensity were determined and expressed as a relative change in comparison with the untreated cells, which was set to 100%. (C) Primary microglia cell lysates were subjected to Western blotting for inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) ( $n=5$ ). (D, E) Culture media of primary microglia were collected and subjected to (D) tumor necrosis factor alpha (TNF- $\alpha$ ) and (E) IL-1 $\beta$  enzyme-linked immunosorbent assay (ELISA) ( $n=5$ ). (F) NO release was measured by the Griess method (to detect nitrite,  $n=5$ ). (G) The expression of ED1 was detected by Western blotting ( $n=5$ ). The levels of ED-1, iNOS, and COX-2 were normalized to  $\beta$ -actin levels and expressed as a relative change in comparison with the LPS + control shRNA treatment. Data are expressed as mean  $\pm$  SEM, # $p < 0.05$ , ## $p < 0.01$  versus LPS + control shRNA treatment. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

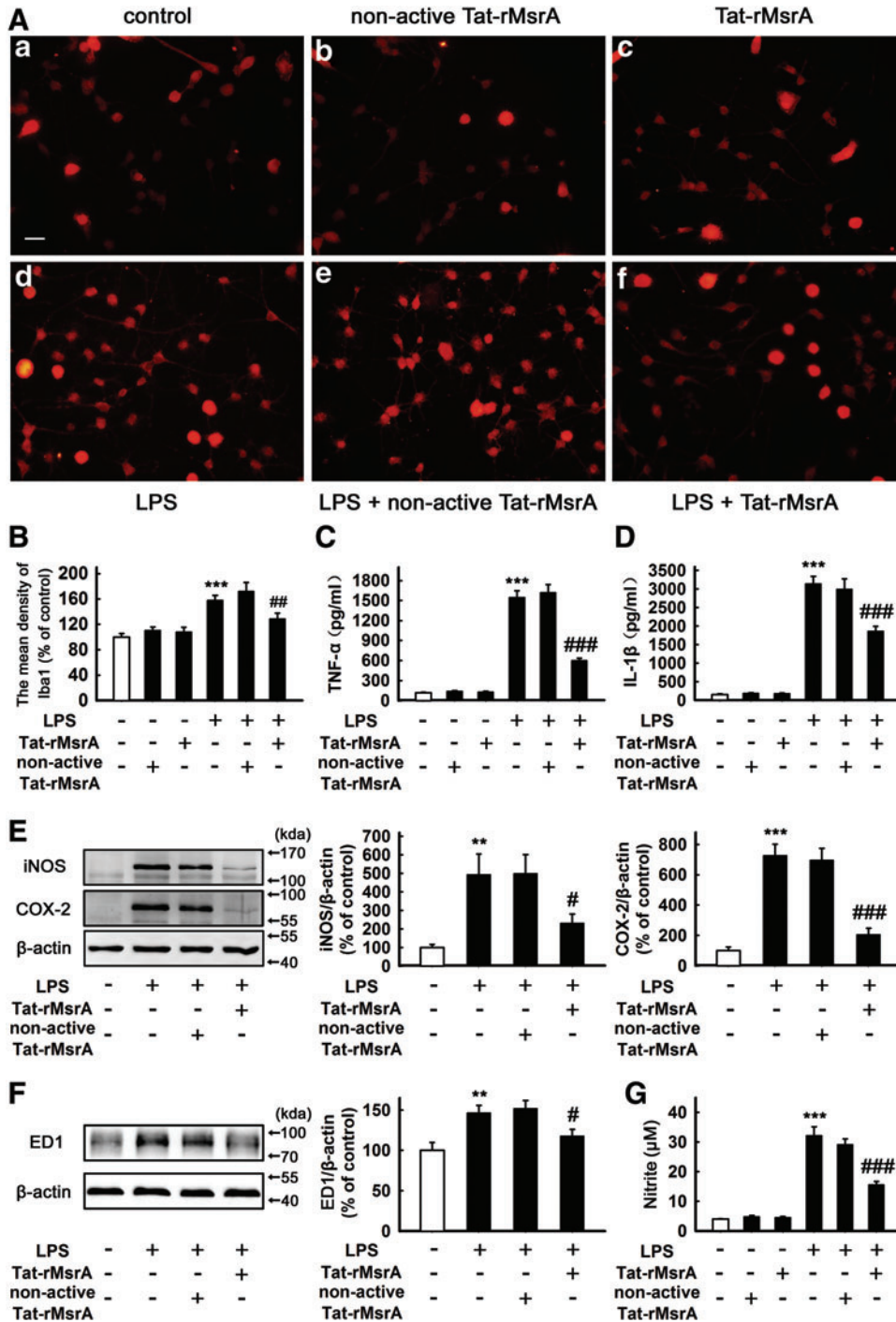


prone to activation by LPS, accompanied by higher levels of inflammatory markers.

*The transduction of Tat-rMsrA fusion protein attenuates LPS-induced activation of microglia and the production of pro-inflammatory factors*

Based on the earlier observations, we speculated that MsrA may act as an endogenous regulator to limit uncontrolled

inflammatory responses. To further confirm this point, we employed Tat-rMsrA fusion protein (Tat-rMsrA), a cell-permeable peptide that could be efficiently transduced into primary microglia, to increase the function of MsrA. As shown in Supplementary Figure S2A, Tat-rMsrA at 0.1, 0.5, and 1  $\mu\text{M}$  increased the content of MsrA in microglia in a concentration-dependent manner ( $n=4$ ,  $p < 0.001$  vs. control, Supplementary Fig. S2A). 0.5  $\mu\text{M}$  Tat-rMsrA was used in the following experiments to limit anomalous Tat effects



**FIG. 3. Tat-rMsrA alleviates LPS-induced microglial activation and inflammatory responses.** Primary microglia enriched cultures were pretreated with Tat-rMsrA or nonactive Tat-rMsrA (0.5 μM) for 1 h and then incubated with LPS (100 ng/ml) for 24 h. (A) Immunocytochemical analysis to detect the mean density of Iba1 (n=6). Scale bars in (a-f): 50 μm. (B) The mean fluorescence intensity of Iba1 was determined, and the levels of Iba1 intensity were expressed as a relative change in comparison with the untreated cells, which was set to 100%. The amounts of (C) TNF-α and (D) IL-1β secreted were measured by ELISA (n=5). (E, F) The expression of (E) iNOS, COX-2, and (F) ED1 was evaluated by Western blotting assay (n=5). The ratios of densitometry values of iNOS, COX-2, and ED1 relative to β-actin were analyzed and normalized to the untreated cells. (G) NO release was measured using the Griess reaction to detect nitrite (n=5). Data are expressed as mean ± SEM, \*\*p < 0.01, \*\*\*p < 0.001 versus untreated cells, #p < 0.05, ##p < 0.01, and ###p < 0.001 versus LPS + nonactive Tat-rMsrA treatment. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

(Supplementary Figs S2B and S3A, B). Meanwhile, non-active Tat-rMsrA that was prepared by boiling recombinant peptide at 95°C for 30 min was used as a negative control (Supplementary Fig. S2C). Pretreatment with Tat-rMsrA (0.5 μM, 1 h) attenuated LPS-mediated upregulation of Iba1 in primary microglial cells (129% ± 9%), when compared with the group pretreated with nonactive Tat-rMsrA (172% ± 14%) (n=6, p < 0.01 vs. LPS + nonactive Tat-rMsrA, Fig. 3A, B). It was also found that pretreatment with Tat-rMsrA (0.5 μM, 1 h), not nonactive Tat-rMsrA, significantly inhibited LPS-induced production of TNF-α from 1546 ± 100 pg/ml to 599 ± 38 pg/ml, and IL-1β from

3143 ± 196 pg/ml to 1857 ± 139 pg/ml, respectively (n=5, p < 0.001 vs. LPS + nonactive Tat-rMsrA, Fig. 3C, D). Similarly, LPS-induced expression of iNOS (493% ± 112%), COX-2 (727% ± 74%), and ED-1 (147% ± 9%) was obviously decreased by pretreatment with 0.5 μM Tat-rMsrA for 1 h (231% ± 50%, 205% ± 42%, and 118% ± 8%, respectively) (n=5, p < 0.05 and p < 0.001 vs. LPS + nonactive Tat-rMsrA, Fig. 3E, F). Moreover, Tat-rMsrA pretreatment (0.5 μM, 1 h) also remarkably reduced LPS-induced NO production by 46% ± 5% in primary microglia (n=5, p < 0.001 vs. LPS + nonactive Tat-rMsrA, Fig. 3G). These results suggest that supplement of exogenous MsrA inhibits

LPS-induced activation of microglia and the production of pro-inflammatory factors.

*The transduction of Tat-rMsrA fusion protein inhibits LPS-induced activation of MAPKs and NF- $\kappa$ B signaling pathways in microglia*

We therefore asked the underlying mechanism of the anti-inflammatory effect of MsrA. Activation of MAPKs and NF- $\kappa$ B signaling pathways in microglia are thought to play a pivotal role in inflammatory responses by regulating those genes encoding pro-inflammatory cytokines (22, 29). First, we investigated the effect of Tat-rMsrA on the activation of LPS-induced MAPK signaling cascades, including p38, ERK, and c-Jun N-terminal kinase (JNK). Western blotting analysis indicated that the increased phosphorylation of p38 and ERK, but not JNK was significantly decreased by pretreatment with Tat-rMsrA ( $0.5 \mu\text{M}$ , 1 h) in the presence of LPS ( $n=3-5$ ,  $p<0.001$  and  $p<0.01$  vs. LPS + nonactive Tat-rMsrA, Fig. 4A–C), whereas neither Tat-rMsrA nor nonactive Tat-rMsrA alone ( $0.5 \mu\text{M}$ ) induced a perceptible change in the expression of phosphorylated-MAPKs (Supplementary Fig. S4A–C). NF- $\kappa$ B is usually kept in an inactive form in the cytoplasm through binding an inhibitory protein of the I $\kappa$ B family, which was phosphorylated and degraded under inflammatory stimulation, allowing the phosphorylation of NF- $\kappa$ B p65 subunit and the translocation of NF- $\kappa$ B dimers to the nucleus (25). As shown in Supplementary Figure S4D and E, Tat-rMsrA or nonactive Tat-rMsrA alone ( $0.5 \mu\text{M}$ ) had no significant effect on the expression of phosphorylated-I $\kappa$ B $\alpha$  (p-I $\kappa$ B $\alpha$ ) and p-p65, two widely used indicators of NF- $\kappa$ B activation. Pretreatment with Tat-rMsrA ( $0.5 \mu\text{M}$ , 1 h) significantly reduced LPS-induced phosphorylation of I $\kappa$ B $\alpha$  and p65 by  $47\% \pm 13\%$  and  $27\% \pm 4\%$ , respectively, when compared with the nonactive Tat-rMsrA pretreatment ( $n=5$ ,  $p<0.01$  vs. LPS + nonactive Tat-rMsrA, Fig. 5A, B). Moreover, we analyzed the subcellular distribution of p65. Immunocytochemical analysis revealed that stimulation with LPS (100 ng/ml, 1 h) led to NF- $\kappa$ B activation in microglia, evidenced by the translocation of the p65 subunit from the cytoplasm to the nucleus. When Tat-rMsrA

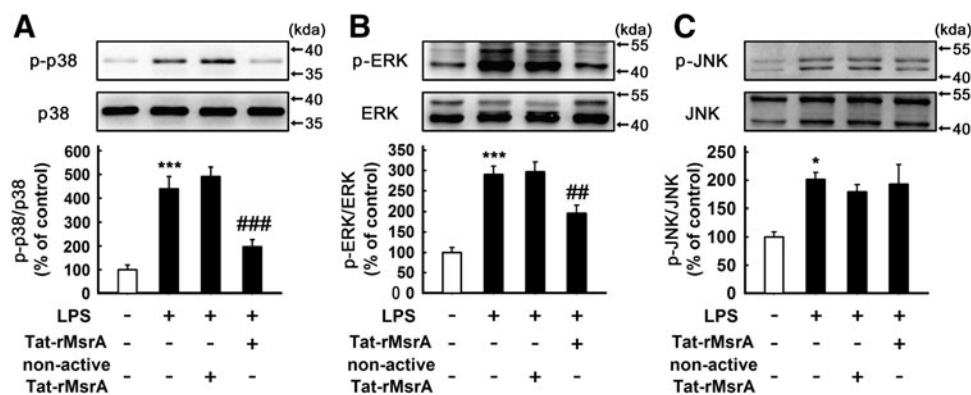
was pretreated ( $0.5 \mu\text{M}$ , 1 h), the nuclear accumulation of p65 was significantly reduced ( $n=5$ ,  $p<0.001$  vs. LPS + nonactive Tat-rMsrA, Fig. 5C). Altogether, these results demonstrate that the transduction of Tat-rMsrA fusion protein inhibits LPS-induced activation of p38, ERK, and NF- $\kappa$ B, suggesting that these signaling pathways play an essential role in the anti-inflammatory property of Tat-rMsrA in LPS-stimulated microglia.

*The transduction of Tat-rMsrA fusion protein alleviates oxidative stress during microglial activation*

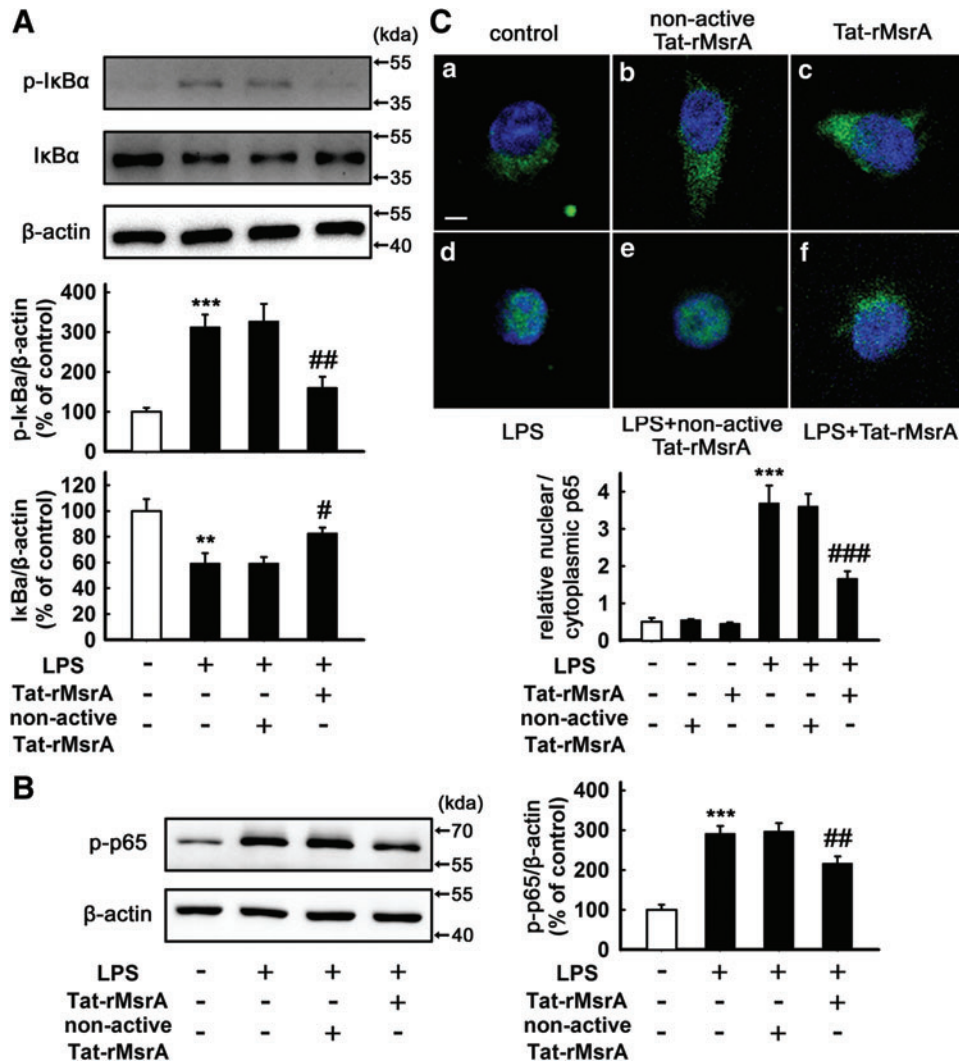
A growing number of evidence suggests that ROS can serve as signaling molecules (46, 52, 57) and are involved in the regulation of transcriptional changes through classical signaling molecules, including MAPKs and NF- $\kappa$ B (2, 51). Considering the role of MsrA in oxidative stress, we next examined whether the transduction of Tat-rMsrA was able to decrease LPS-induced production of ROS in primary cultured microglia. As shown in Figure 6A–C, LPS treatment (100 ng/ml, 30 min) increased the amounts of intracellular ROS (measured by H<sub>2</sub>DCFDA) and extracellular superoxide by  $141\% \pm 20\%$  ( $n=5$ ,  $p<0.01$  vs. control, Fig. 6A, B) and  $483\% \pm 78\%$  ( $n=5$ ,  $p<0.001$  vs. control, Fig. 6C), respectively, when compared with the untreated cells. Notably, pretreatment with Tat-rMsrA ( $0.5 \mu\text{M}$ , 1 h) reduced LPS-induced intracellular ROS production almost to the control levels ( $n=5$ ,  $p<0.05$  vs. LPS + nonactive Tat-rMsrA, Fig. 6A, B). Moreover, LPS-induced extracellular superoxide was also significantly decreased by the Tat-rMsrA pretreatment ( $294\% \pm 43\%$ ), in comparison to the nonactive Tat-rMsrA pretreatment ( $479\% \pm 67\%$ ) ( $n=5$ ,  $p<0.01$  vs. LPS + nonactive Tat-rMsrA, Fig. 6C). Thus, the transduction of Tat-rMsrA can remarkably attenuate LPS-induced oxidative stress in primary cultured microglia.

*The transduction of Tat-rMsrA fusion protein increases the free radical-scavenging property of methionine via a catalytic antioxidant in vitro*

As a reductase, how can MsrA scavenge LPS-induced ROS in microglia? One possible explanation is that MsrA



**FIG. 4. Tat-rMsrA inhibits activation of p38 and ERK MAPKs in LPS-stimulated primary microglia.** Primary microglia enriched cultures were pretreated with Tat-rMsrA or nonactive Tat-rMsrA ( $0.5 \mu\text{M}$ ) for 1 h and then incubated with LPS (100 ng/ml) for 30 min. The phosphorylation of (A) p38 ( $n=4$ ), (B) ERK ( $n=5$ ) and (C) JNK ( $n=3$ ) in the lysates was analyzed by Western blotting. The levels of phosphorylated-MAPKs were expressed as a relative change in comparison with the untreated cells, which was set to 100%. Data are expressed as mean  $\pm$  SEM, \* $p<0.05$ , \*\*\* $p<0.001$  versus untreated cells, ## $p<0.01$ , ### $p<0.001$  versus LPS + nonactive Tat-rMsrA treatment.

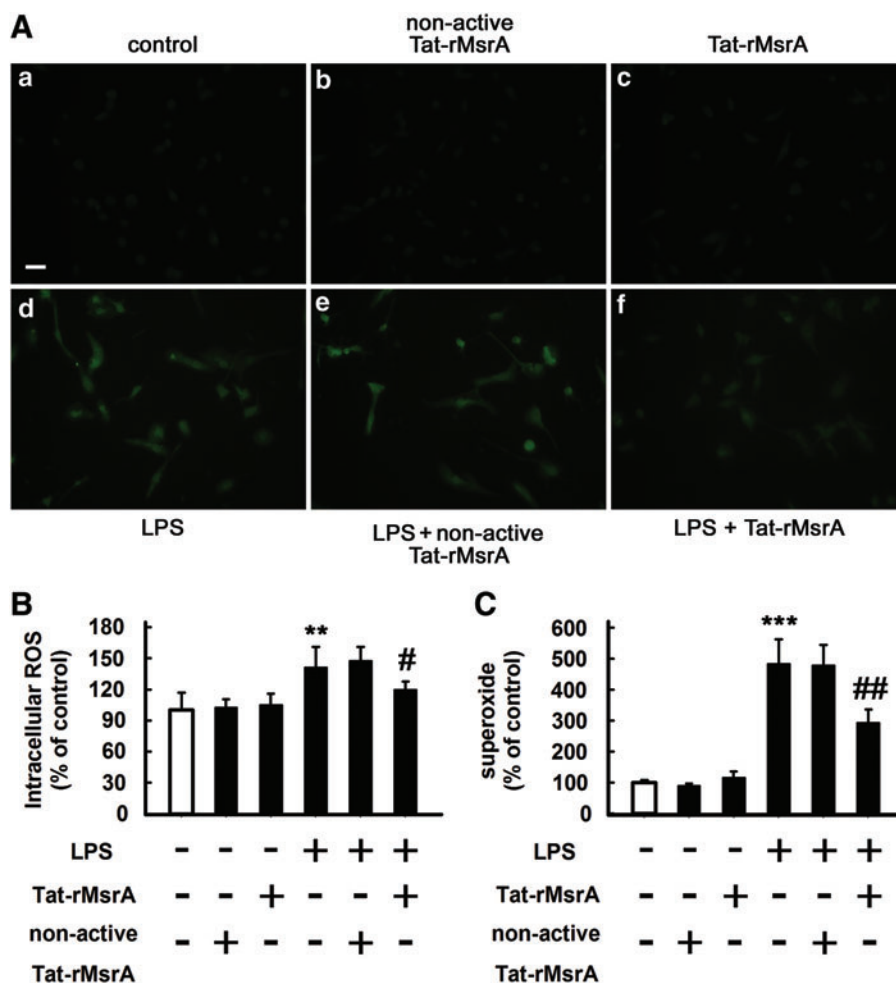


**FIG. 5. Tat-rMsrA suppresses activation of NF- $\kappa$ B in LPS-stimulated primary microglia.** (A, B) Primary microglia enriched cultures were pretreated with Tat-rMsrA or nonactive Tat-rMsrA ( $0.5 \mu\text{M}$ ) for 1 h and then incubated with LPS ( $100 \text{ ng/ml}$ ) for 5 min. The whole-cell lysates were analyzed by Western blotting assay. The ratios of densitometry values of (A) p-I $\kappa$ B $\alpha$ , I $\kappa$ B $\alpha$ , and (B) p-p65 relative to  $\beta$ -actin were analyzed and normalized to the untreated cells ( $n=5$ ). Data are expressed as mean  $\pm$  SEM. (C) Immunocytochemical assay of p65 (green) translocation from the cytoplasm to the nucleus in primary microglia (1 h post stimulus) in response to the (a) DMEM-F12; (b) nonactive Tat-rMsrA alone ( $0.5 \mu\text{M}$ ); (c) Tat-rMsrA alone ( $0.5 \mu\text{M}$ ); (d) LPS; (e) LPS + nonactive Tat-rMsrA pretreatment ( $0.5 \mu\text{M}$ , 1 h); and (f) LPS + Tat-rMsrA pretreatment ( $0.5 \mu\text{M}$ , 1 h) ( $n=5$ ). The hoehchst stained nuclei are shown in blue. Scale bars in (a-f):  $10 \mu\text{m}$ . Quantification of the translocation of p65 from the cytoplasm to the nucleus is expressed as mean  $\pm$  SEM of the nuclear/cytoplasmic p65 fluorescence intensity. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus untreated cells, # $p < 0.05$ , ## $p < 0.01$ , and ### $p < 0.001$  versus LPS + nonactive Tat-rMsrA treatment. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

serves as a stereospecific Met oxidase (33) and facilitates the reaction of Met with ROS. To confirm this hypothesis, we studied the antioxidant effect of Met in the presence of Tat-rMsrA or nonactive Tat-rMsrA. The hydroxyl radicals ( $\text{OH}\cdot$ ) were produced by the Fenton reaction, and the levels of  $\text{OH}\cdot$  were evaluated by ESR spin-trapping using N-tert-butyl- $\alpha$ -phenylnitron (PBN). As indicated in Figure 7A, treatment with  $1 \text{ mM}$  Met, but not  $0.1 \text{ mM}$  Met, significantly decreased the signal intensity of PBN-OH adducts ( $n=11$ ,  $p < 0.001$  vs. control, Fig. 7A), which was not changed by Tat-rMsrA or nonactive Tat-rMsrA alone ( $1 \mu\text{M}$ ) (Supplementary Fig. S5A). However, co-treatment of  $0.1 \text{ mM}$  Met and Tat-rMsrA

( $1 \mu\text{M}$ ) decreased the PBN-OH signals by  $23\% \pm 6\%$ , when compared with the group co-treated with  $0.1 \text{ mM}$  Met and nonactive Tat-rMsrA ( $n=5$ ,  $p < 0.05$  vs.  $0.1 \text{ mM}$  Met + nonactive Tat-rMsrA, Fig. 7B), which strongly suggested that Tat-rMsrA increased Met-mediated reduction of  $\text{OH}\cdot$ . Similar results were obtained by luminol-chemiluminescence assay. It was shown that  $\text{H}_2\text{O}_2$ -induced luminescence was significantly inhibited by both  $1$  and  $10 \text{ mM}$  Met, but not by  $0.1 \text{ mM}$  Met ( $n=6$ ,  $p < 0.001$  vs. control, Fig. 7C). Therefore, we next determined the inhibitory effect of  $0.1 \text{ mM}$  Met on the luminescence intensity in the presence of Tat-rMsrA or nonactive Tat-rMsrA. Neither Tat-rMsrA nor nonactive

**FIG. 6. Tat-rMsrA attenuates oxidative stress during microglial activation.** Primary microglia enriched cultures were pretreated with Tat-rMsrA or nonactive Tat-rMsrA ( $0.5 \mu\text{M}$ , 1 h) and then incubated with LPS ( $100 \text{ ng/ml}$ ) for 30 min. (A) The levels of intracellular ROS were detected with  $\text{H}_2\text{DCFDA}$  ( $n=5$ ). Scale bars in (a–f):  $50 \mu\text{m}$ . (B) The fluorescence intensity of DCF was determined. (C) The release of superoxide was determined immediately by measuring the SOD-inhibitable reduction of WST-1 ( $n=5$ ). Results were expressed as a percentage of control. Data are expressed as mean  $\pm$  SEM.  $**p < 0.01$ ,  $***p < 0.001$  versus untreated cells,  $\#p < 0.05$ ,  $\#\#p < 0.01$  versus LPS + nonactive Tat-rMsrA treatment. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)



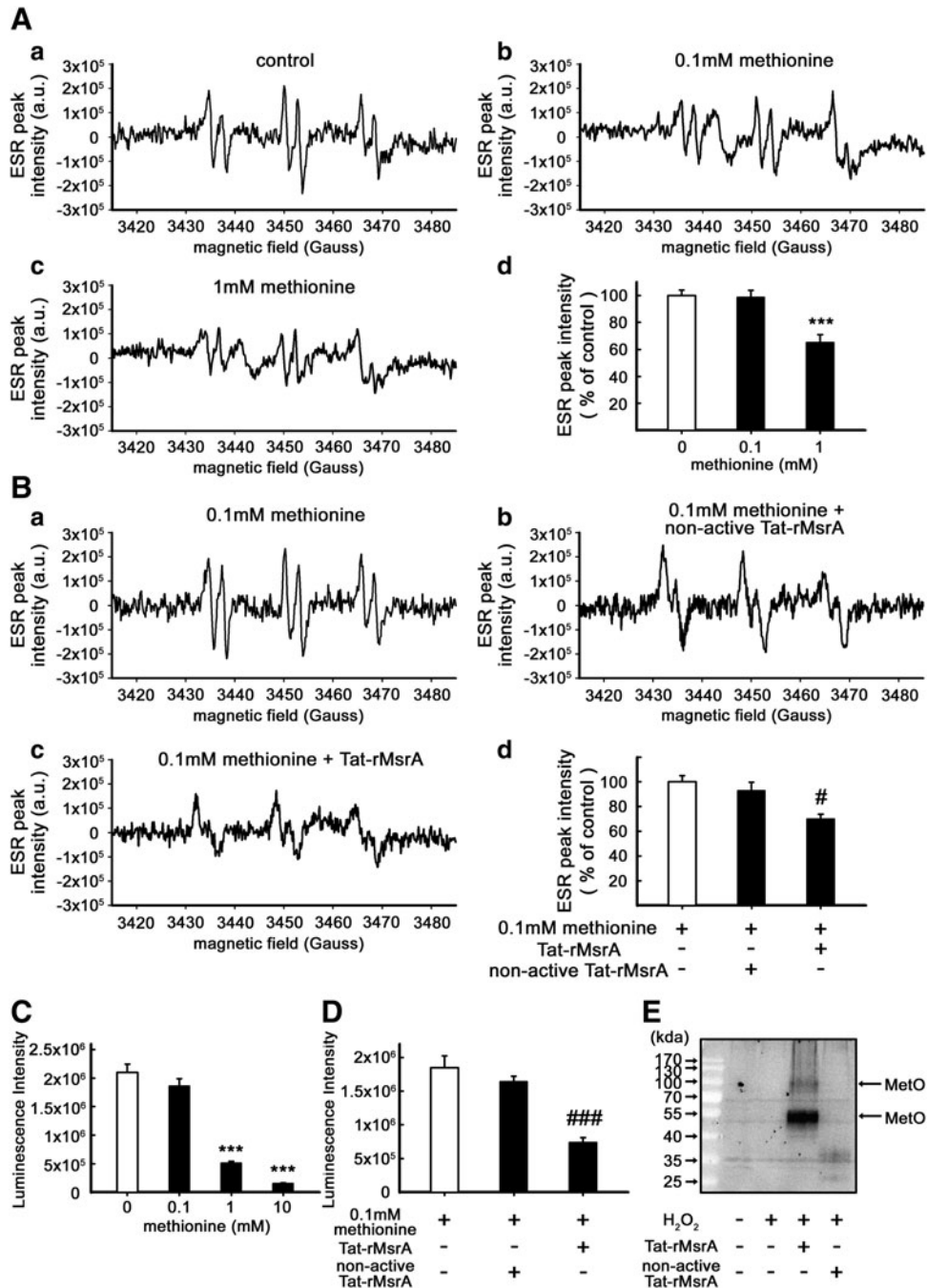
Tat-rMsrA alone ( $1 \mu\text{M}$ ) had a perceptible effect on the luminescence intensity (Supplementary Fig. S5B). Notably, co-treatment with  $0.1 \text{ mM}$  Met and Tat-rMsrA ( $1 \mu\text{M}$ ), but not nonactive Tat-rMsrA, remarkably reduced the luminescence signal intensity by  $54\% \pm 5\%$  ( $n=5$ ,  $p < 0.001$  vs.  $0.1 \text{ mM}$  Met + nonactive Tat-rMsrA, Fig. 7D). Furthermore, Western blotting analysis for detection of protein-bound MetO revealed that Met residues in primary cultured microglial lysates (boiled) were not oxidized to MetO in the presence of  $0.5 \text{ mM}$   $\text{H}_2\text{O}_2$  alone. However, when the primary microglial lysates (boiled) were co-treated with  $0.5 \text{ mM}$   $\text{H}_2\text{O}_2$  and Tat-rMsrA ( $1 \mu\text{M}$ ) for 40 min, the protein-bound MetO of 100- and 50-kDa protein obviously increased ( $n=5$ , Fig. 7E). Taken together, these results demonstrate that MsrA displays free radical-scavenging activity by catalyzing the oxidation reaction of ROS from Met or Met residues of proteins to MetO *in vitro*.

*The transduction of Tat-rMsrA fusion protein inhibits LPS-induced microglial activation and neuroinflammation in vivo*

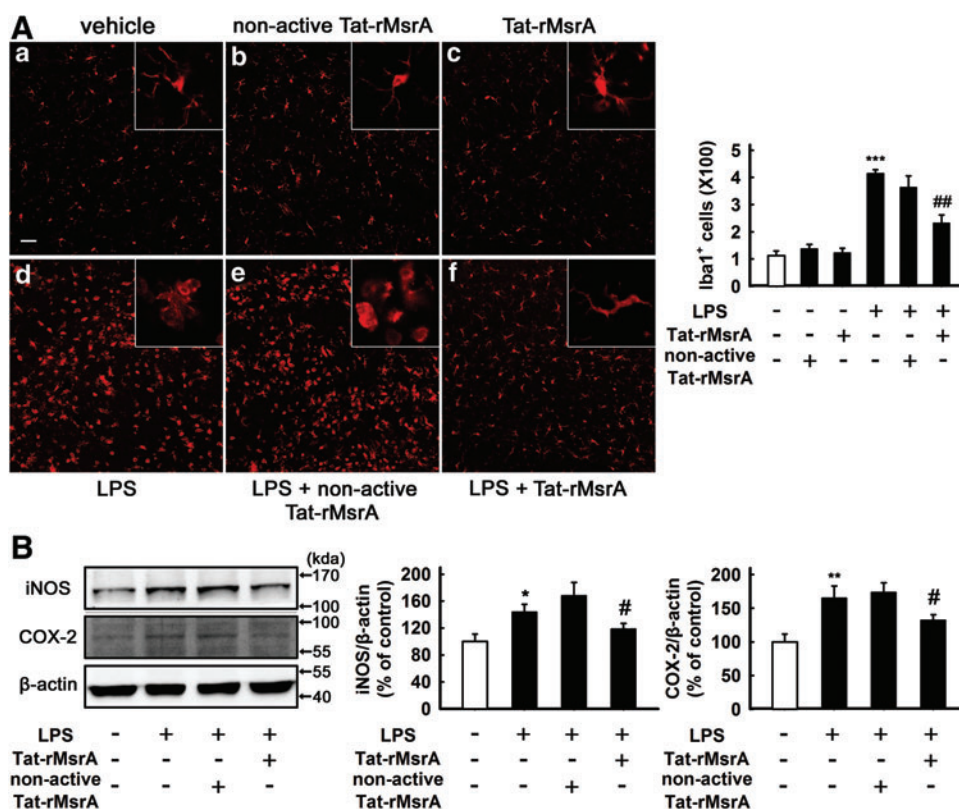
It has been demonstrated that microglia are mainly distributed in the gray matter with highest concentration in the hippocampus (30). The effect of Tat-rMsrA on microglia-mediated neuroinflammation was further confirmed by using

a rat model of acute neuroinflammation, in which LPS ( $2 \mu\text{l}$ ,  $5 \text{ mg/ml}$ ) was directly injected into the dentate gyrus of hippocampus (1). As shown in Figure 8A, neither Tat-rMsrA nor nonactive Tat-rMsrA alone ( $2 \mu\text{l}$ ,  $3 \text{ mg/ml}$ ) significantly altered the number and morphology of Iba1-positive microglia. Notably, at 24 h after LPS injection, microglia in the dentate gyrus displayed remarkably enhanced Iba1 immunoreactivity and an “activated” morphology with hypertrophic cell bodies, and thickened and shortened processes. Preadministration of Tat-rMsrA ( $2 \mu\text{l}$ ,  $3 \text{ mg/ml}$ ) at 24 h before LPS injection significantly reduced the number of Iba1-positive microglia, and the majority of microglial cells were reverted to the “resting” state that displayed a ramified, extensively branched morphology ( $n=5$ ,  $p < 0.01$  vs. LPS + nonactive Tat-rMsrA, Fig. 8A). Furthermore, Western blotting analysis revealed that Tat-rMsrA or nonactive Tat-rMsrA alone ( $2 \mu\text{l}$ ,  $3 \text{ mg/ml}$ ) had no perceptible effect on the expression of pro-inflammatory factors such as iNOS and COX-2 (Supplementary Fig. S3C). However, LPS-induced increased levels of iNOS and COX-2 were evidently reduced by the Tat-rMsrA pretreatment ( $2 \mu\text{l}$ ,  $3 \text{ mg/ml}$ ) ( $119\% \pm 8\%$  and  $132\% \pm 8\%$ , respectively), when compared with the nonactive Tat-rMsrA pretreatment ( $168\% \pm 20\%$  and  $174\% \pm 14\%$ , respectively) ( $n=6$ ,  $p < 0.05$  vs. LPS + nonactive Tat-rMsrA, Fig. 8B). Taken together, these observations suggest that the upregulation of MsrA expression suppresses





**FIG. 7. Tat-rMsrA increases the free radical-scavenging property of methionine via a catalytic antioxidation *in vitro*.** (A) ESR spectra of the spin adduct of OH<sup>•</sup> radical observed during the reaction of 0.5 mM H<sub>2</sub>O<sub>2</sub> containing 30 mM PBN and 0.2 mM FeSO<sub>4</sub> with 0.1 or 1 mM methionine (Met). (a) Control, containing H<sub>2</sub>O<sub>2</sub>, FeSO<sub>4</sub>, and PBS; (b) As in (a) in the presence of 0.1 mM Met; (c) As in (a) in the presence of 1 mM Met; (d) The levels of ESR peak intensity were expressed as a relative change in comparison with the untreated control, which was set to 100% (*n* = 11). (B) The effect of 0.1 mM Met on the signal intensity of PBN-OH adducts in the presence of Tat-rMsrA or nonactive Tat-rMsrA was evaluated under the same experimental conditions (*n* = 5). (a) Control, containing H<sub>2</sub>O<sub>2</sub>, FeSO<sub>4</sub>, PBS, and 0.1 mM Met; (b) As in (a) in the presence of nonactive Tat-rMsrA (1 μM); (c) As in (a) in the presence of Tat-rMsrA (1 μM); (d) The levels of ESR peak intensity were expressed as a relative change in comparison with the 0.1 mM Met treatment, which was set to 100%. (C) Concentration-dependent effects of Met (0.1, 1, and 10 mM) on luminol-enhanced chemiluminescence were assessed (*n* = 6). (D) Inhibitory effect of 0.1 mM Met on luminol-enhanced chemiluminescence in the presence of Tat-rMsrA or nonactive Tat-rMsrA (1 μM) was determined (*n* = 5). (E) Lysates of primary microglia were treated with 0.5 mM H<sub>2</sub>O<sub>2</sub> in the presence of Tat-rMsrA or nonactive Tat-rMsrA (1 μM). The expression of methionine sulfoxide (MetO) was determined by Western blotting (*n* = 5). Data are expressed as mean ± SEM. \*\*\**p* < 0.001 versus control, #*p* < 0.05, ###*p* < 0.001 versus 0.1 mM Met + nonactive Tat-rMsrA treatment.



**FIG. 8. Tat-rMsrA suppresses the activation of microglia and the production of pro-inflammatory factors in a rat model of acute neuroinflammation.** SD rats were injected with 2  $\mu$ l saline or LPS (5 mg/ml) into the dentate gyrus of hippocampus for 24 h in the presence of Tat-rMsrA or nonactive Tat-rMsrA, which were preadministered into the hippocampus (2  $\mu$ l, 3 mg/ml, 24 h before LPS injection). (A) Iba1 immunoreactivity was evaluated by immunofluorescence study, and the number of Iba1<sup>+</sup> microglia was counted ( $n=5$ ). Insets are images at high magnification. Scale bars in (a–f): 100  $\mu$ m. (B) The levels of iNOS and COX-2 were normalized to  $\beta$ -actin levels and expressed as a relative change in comparison with the untreated slices, which was set to 100% ( $n=6$ ). Data are expressed as mean  $\pm$  SEM, \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  versus untreated slices, # $p < 0.05$ , ## $p < 0.01$  versus LPS + nonactive Tat-rMsrA treatment. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

microglial activation and the production of pro-inflammatory cytokines during LPS-induced inflammation *in vivo*.

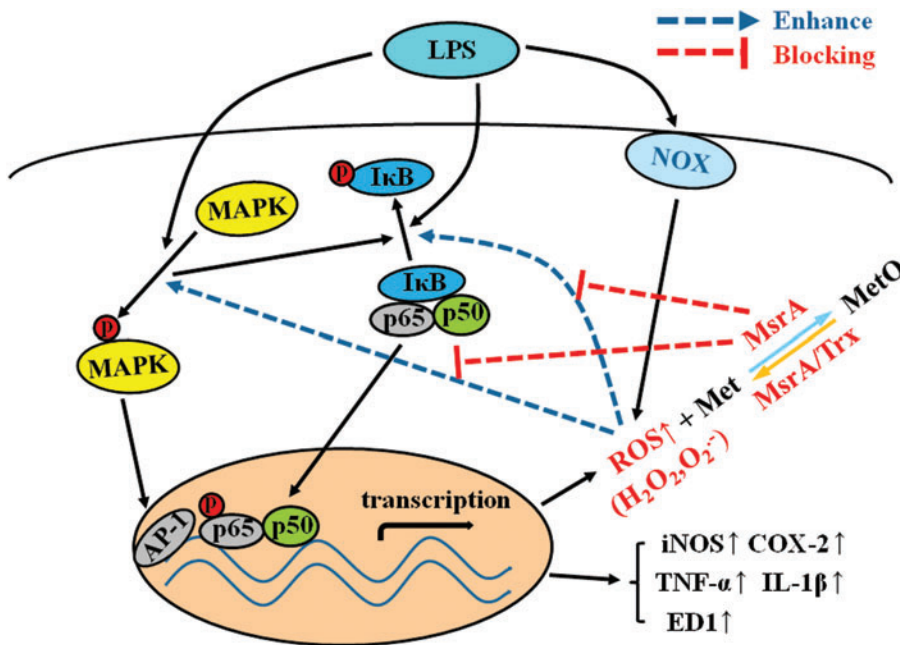
## Discussion

In this study, we found that microglial MsrA plays an anti-inflammatory role in LPS-stimulated conditions by a mechanism involving the inhibition of LPS-induced activation of p38, ERK, and NF- $\kappa$ B. These effects were associated with MsrA-mediated ROS elimination in microglia, which may be derived from a catalytic effect of MsrA on the reaction of Met with ROS (Fig. 9). Our results support the notion that endogenous antioxidants may serve as a “brake” for uncontrolled neuroinflammation.

Activated microglia are an important source for production of ROS in the CNS (3, 54, 68), and ROS also have been reported as a pathological situation that aggravates microglial response by modulating their activity (9, 23, 49, 61). Microglial cells contain sufficient antioxidative defense mechanisms to avoid ROS-mediated cellular damage (14, 20, 24, 34). However, there is much less evidence related to their participation in the function of microglia. It has been demonstrated that both SOD-1 and peroxiredoxin I inhibit microglial activation and exert significant anti-inflammatory

effects (6, 10, 24, 29). Here, we found that SOD-1 rapidly transforms superoxide into hydrogen peroxide, which also acts as a second messenger to mediate signal transduction and regulate microglial status (15, 21, 58). Therefore, other synergistical antioxidant mechanisms to control neuroinflammation may exist.

A recent study has reported that peroxiredoxin I, an ROS/p38 MAPK-dependent endogenous antioxidant, inhibits NF- $\kappa$ B function and microglial activation (26). We found that MsrA was an inducible antioxidant under LPS-stimulated conditions in rat microglia. In the normal brain tissue, the levels of MsrA in most Iba1-positive cells are relatively low. In the cultured microglia, nearly all the Iba1-immunopositive cells expressed MsrA, which may be related with environmental stimulus during primary microglia culture, such as trypsinization or trituration. Silencing of MsrA accelerated LPS-induced changes in the mean density of Iba1 and morphology of cultured microglial cells and the production of inflammatory markers, including iNOS, COX-2, TNF- $\alpha$ , IL-1 $\beta$ , and ED-1. However, MsrA silencing had little effect on subsequent NO secretion in LPS-stimulated microglia. Under LPS-stimulated condition, most reports have demonstrated that iNOS and subsequent NO production is pro-inflammatory (4). The insignificant increase in nitrite production after MsrA



**FIG. 9. Possible mechanisms by which MsrA inhibits LPS-induced microglial activation and inflammatory responses.** LPS initiates the activation of NOX and the production of ROS. ROS can function as “signaling molecules” and modulate large numbers of transcriptional changes through classical signaling molecules, including MAPKs and NF- $\kappa$ B, which lead to microglial overactivation and the release of proinflammatory factors. MsrA scavenges ROS through catalyzing the reaction of ROS with Met residues in proteins, thereby suppressing the self-perpetuating vicious cycle of “ROS-MAPKs-NF $\kappa$ B” signaling and microglia-mediated neuroinflammation. To see this illustration in color, the reader is referred to the web version of this article at [www.liebertpub.com/ars](http://www.liebertpub.com/ars)

knockdown under LPS-stimulated condition may be caused by the fact that MsrA silencing induced a compensatory increase in other endogenous antioxidants such as thioredoxin that can scavenge RNS. Supplement of exogenous MsrA by transducing Tat-rMsrA fusion protein attenuated LPS-induced neuroinflammatory events, including an increased expression of Iba1 and the production of pro-inflammatory cytokines, such as iNOS, COX-2, TNF- $\alpha$ , IL-1 $\beta$ , and NO. Furthermore, injection of Tat-rMsrA fusion protein suppressed microglial activation and the expression of pro-inflammatory factors in a rat model of neuroinflammation *in vivo*. Considering that only activated microglia cells strongly expressed COX-2 and iNOS, the reduced expression of COX-2 and iNOS is mainly due to the inhibition of microglia activation by MsrA. Our results clearly demonstrate that MsrA brakes LPS-induced uncontrolled neuroinflammation in the microglia.

There is increasing evidence that ROS serves as the messenger molecule to mediate cellular signaling pathways (46, 52, 57). In microglial cells, ROS may be an early signal triggering the induction of cytokines and participating in inflammatory signaling (8, 48). For instance, ROS control activity of key proteins that regulate the activation of MAPK signaling cascades, such as ASK1 (50) and DUSP3 (66). Furthermore, LPS-mediated ROS generation is closely associated with the activation of NF- $\kappa$ B (17), a redox-sensitive transcription factor that governs the production of neuroinflammatory mediators, such as TNF- $\alpha$ , ILs, and NOS. The results obtained in this work indicated that the exogenous supplement of MsrA by transduction peptide significantly decreased LPS-induced phosphorylation of p38 and ERK MAPKs in cultured microglial cells. In addition, Tat-rMsrA also inhibited LPS-induced phosphorylation of I $\kappa$ B $\alpha$  and subsequent translocation of p65, a key process that initiates the expression of inflammatory genes. It has been reported that methionine oxidation activates a transcription factor named HypT in bacterias and regulates gene expression in response to oxidative stress (13). Thus, the nuclear expres-

sion of MsrA may serve as a potential regulator in the control of gene expression in response to redox status. Although other mechanisms may exist, current results demonstrate that p38, ERK, and NF- $\kappa$ B are the major inhibitory signaling pathways managed by the transduction of exogenous MsrA.

MsrA has historically been considered a reductase, not an ROS scavenger (41, 59). In spite of its impressive cytoprotection and antioxidation (5, 42, 44, 72, 73), the precise mechanisms and fundamental chemical principles for MsrA-mediated antioxidation remain largely unclear. In this study, the transduction of exogenous MsrA was able to decrease LPS-induced production of ROS in primary microglia. How can MsrA scavenge ROS? It is generally considered that MsrA-catalytic antioxidation depends on the Met-centered redox cycle (40, 65), which is mainly due to the antioxidant activity of protein-bound Met residues. MsrA may exhibit antioxidation *via* maintaining an effective concentration of Met by reducing MetO timely. Interestingly, our previous study has indicated that under oxidative stress, more than 80% of protein-bound Met residues are kept in a reduced form (36), indicating that there a sufficient store of Met exists. Moreover, we have found that after inhibition of MsrA function by DMSO (27), the antioxidation of Met (1–5 mM) on CHO cells is largely abolished (69), suggesting that Met is not merely an antioxidant. Recently, it has been demonstrated that MsrA also serves as a stereospecific methionine oxidase (32, 33), which may facilitate the reaction of Met with ROS. This work provided the first experimental evidence that MsrA facilitated the reaction of Met with ROS, which may afford the antioxidant effect. In Fenton reaction system, both ESR spin-trapping and luminol-chemiluminescence assay revealed that MsrA facilitated the Met (0.1 mM)-mediated scavenging effect on hydroxyl radicals. Moreover, we found that protein-bound Met of primary microglia lysates (boiled) could not be oxidized to MetO by 0.5 mM  $\text{H}_2\text{O}_2$  alone. Interestingly, when the same lysates were co-treated with 0.5 mM  $\text{H}_2\text{O}_2$  and active MsrA (1  $\mu$ M), the MetO levels of 100- and 50-kDa protein obviously increased. Taken together,

our results demonstrate that MsrA displays free radical-scavenging activity by catalyzing the reaction of ROS with Met residues.

Until now, the role of MsrA in the inflammation remains largely unknown. A recent study has elucidated that deletion of MsrA enhances renal inflammatory responses under I/R conditions (25). Our current findings demonstrate the link between MsrA and microglia-mediated neuroinflammation for the first time. It is widely accepted that microglia can be driven to adopt two phenotypes, that is, pro-inflammatory (M1) and anti-inflammatory (M2) phenotype (48). Our results revealed that MsrA prevents microglial cells from activating into M1 phenotype. An interesting question that remains open is whether MsrA promotes activation of microglia cells into M2 phenotype. The role of MsrA in microglial M2 activation requires further investigation.

Imbalance of redox homeostasis plays a key pathological role in the process of neurodegenerative diseases and neuroinflammation. To maintain redox homeostasis, vitagenes network, an emerging concept that includes a group of genes involved in preserving cellular homeostasis during stressful conditions, operates actively to preserve neuron survival and serves as an integrated mechanism (6, 7, 10, 60). Vitagenes encode for cytoprotective proteins such as heat shock proteins, the thioredoxin, and the sirtuin protein systems and their expression can be activated by oxidative stress or dietary antioxidants, such as polyphenols. Thus, MsrA may act as a new member in the vitagenes network for its cytoprotection and inducible expression during stressful conditions. Previous studies have revealed that enhancing MsrA function may be beneficial in Parkinson's disease (PD) and Alzheimer's disease (AD) *via* scavenging ROS and repairing methionine sulfoxide (35, 43, 64). Taken together, our results suggest that MsrA negatively controls microglia-mediated neuroinflammation, and pharmacological enhancement of MsrA function may serve as a reasonable therapeutic strategy aimed at reducing neurodegeneration in the clinic. There are at least three ways to enhance MsrA function: (i) supplement of its substrates, including L-Met and S-methyl-L-cysteine (SMLC) (64, 67); (ii) upregulation of endogenous MsrA expression (70); and (iii) transduction of exogenous MsrA using novel drug delivery systems such as cell-penetrating peptides. Their clinical applicability requires further investigation.

## Materials and Methods

### Animals

Adult male Sprague–Dawley (SD) rats weighing 200–250 g were obtained from the Experimental Animals of Tongji Medical College, Huazhong University of Science and Technology. The rats were housed individually on a controlled 12:12 light cycle at a constant temperature ( $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ) with free access to water and food, and allowed to acclimate a week. The use of animals for experimental procedures was conducted in accordance with the Declaration of Helsinki and with the Guide for Care and Use of Laboratory Animals as adopted and promulgated by the United National Institutes of Health. The experimental procedures were approved by the Animal Welfare Committee of Huazhong University of Science & Technology.

### Microglial culture

Microglial cells were isolated from the mixed cultures of cerebrocortical cells from postnatal day 1–3 SD rats, as previously described, with some modification (53). In brief, rats were decapitated, and the cortices were removed and trypsinized with 0.125% trypsin (Amresco, Solon, OH) for 10 min at  $37^{\circ}\text{C}$ . After trituration and centrifugation at 120 g for 8 min, the cells were resuspended and plated on culture flasks, which were precoated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO). The cells were then cultured in DMEM-F12 (Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine, and 100 U/ml penicillin-streptomycin (Sigma-Aldrich). After 24 h, the medium was changed into fresh DMEM-F12 and replaced every 3–4 days *in vitro*. The cultures were maintained at  $37^{\circ}\text{C}$  in a humidified 5%  $\text{CO}_2$  atmosphere incubator. The confluency was achieved after 9–11 days *in vitro*. High enriched microglial cells were isolated from the mixed culture by mild trypsinization. The mixed glial cultures were incubated with a trypsin solution (0.25% trypsin and 1 mM EDTA) diluted 1:3 in DMEM-F12 for 30–60 min, which resulted in detachment of an upper layer of astrocytes in one piece, while the microglia remained attached to the bottom of the culture flask. The purity of microglia was confirmed by immunofluorescence with an anti-Iba1 antibody (98%) (Abcam, Cambridge, United Kingdom).

### Construction of rat MsrA shRNA lentiviral expression vector

A third generation of self-inactivating lentivirus vector (GeneChem, Shanghai, China) containing a CMV-driven GFP reporter and a U6 promoter upstream of the cloning sites (Age I and EcoR I) was used for cloning small hairpin RNAs (shRNAs). The target sequence for rat MsrA was 5'-AGC ACGTCAGCTTTGAGGA-3', and for control scrambled shRNA it was 5'-TTCTCCGAACGTGTCACGT-3'. Microglial cells were infected with lentivirus at a multiplicity of infection of 50 for 8 h. Then, the medium was replaced with fresh complete medium. After 72 h, cells were observed under fluorescence microscopy to confirm that more than 80% of cells were GFP positive.

### Preparation of Tat-rMsrA

See details in the Supplementary Methods section.

### Analysis of Tat-rMsrA activity

The activity of recombinant Tat-rMsrA was rapidly monitored by detecting both MetO-reducing activity and methyl sulfoxide-dependent oxidation of dithiothreitol (DTT) (71). The details are provided in the Supplementary Methods section.

### Measurements of TNF- $\alpha$ and IL-1 $\beta$ levels by ELISA

The levels of TNF- $\alpha$  and IL-1 $\beta$  were quantified in microglial supernatants using the TNF- $\alpha$  and IL-1 $\beta$  ELISA kits (Boster, Wuhan, China) according to the manufacturer's instructions.

### Nitrite quantification

The production of NO was evaluated by indirectly measuring the accumulated levels of nitrite in the culture supernatants (18), a stable metabolite of NO, using the Griess reagent (Beyotime, Shanghai, China) according to the manufacturer's instructions.

### Electron spin resonance measurement

PBN was used as a free radical trapper. Electron spin resonance (ESR) signals were detected with a Bruker e-scan ESR spectrometer (Burker, Karlsruhe, Germany). As a standard of the reactant of OH• with PBN, we produced hydroxyl radical (OH•) by the Fenton reaction in the mixture of 0.5 mM H<sub>2</sub>O<sub>2</sub> and 0.2 mM FeSO<sub>4</sub> in the presence of 30 mM PBN. The spin traps, methionine (0.1 or 1 mM), and Tat-rMsrA/nonactive Tat-rMsrA (1 μM) were added before the Fe (II) and H<sub>2</sub>O<sub>2</sub>. Samples (20 μl) were loaded into a quartz tube, and the ESR spectra were recorded at room temperature.

The ESR microwave power was set to 4.88 mW. The modulation frequency was 9.76 GHz. The time constant was 20.48 ms. The conversion time was 20.48 ms, and a sweep time of 10.49 s was used. Each sample was scanned for a total of 20 times. A sweep width of 70 G was used for experiments with PBN. The receiver gain was set to 3.17 × 10<sup>3</sup>. Simulation and fitting of the ESR spectra were performed using the Bruker WinEPR program.

### Methionine sulfoxide assay

Detection of methionine sulfoxide was performed with the methionine sulfoxide immunoblotting kit (Cayman, Ann Arbor, MI) according to the manufacturer's protocol (28, 45). The details are provided in the Supplementary Methods section.

### Measurements of intracellular ROS

See details in the Supplementary Methods section.

### Superoxide assay

See details in the Supplementary Methods section.

### Luminol-enhanced chemiluminescence assay

See details in the Supplementary Methods section.

### Reverse transcription-polymerase chain reaction

See details in the Supplementary Methods section.

### Western blotting

See details in the Supplementary Methods section.

### Immunolabeling

See details in the Supplementary Methods section.

### Rat model of acute neuroinflammation

LPS was injected into the dentate gyrus of hippocampus to evoke neuroinflammation in rats, as previously described, with minor modification (1). The details are provided in the Supplementary Methods section.

### Statistics

All experiments were performed at least thrice with similar results. Data from experiments were analyzed with the statistical program SPSS 18.0 software (SPSS, Chicago, IL). Comparison between two groups was evaluated by an unpaired and two-sided Student's *t*-test. ANOVAs and *post hoc* tests (Fisher's LSD) were used to analyze differences in different treatment groups. Data are presented as mean ± SEM. Differences between experimental conditions were considered statistically significant when *p* < 0.05.

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### Author Disclosure Statement

No competing financial interests exist.

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#### Abbreviations Used

AD = Alzheimer's disease  
 ASK1 = apoptosis signal-regulating kinase 1  
 CNS = central nervous system  
 COX-2 = cyclooxygenase-2  
 DCF = 2', 7'-dichlorofluorescein  
 DCFH = 2', 7'-dichlorodihydrofluorescein  
 DUSP3 = dual-specificity phosphatase 3  
 ELISA = enzyme-linked immunosorbent assay  
 ERK = extracellular signal-regulated kinase  
 ESR = electron spin resonance  
 GFP = green fluorescent protein  
 H<sub>2</sub>DCFDA = 2', 7'-dichlorodihydrofluorescein diacetate  
 IL-1 $\beta$  = interleukin-1 beta  
 iNOS = inducible nitric oxide synthase  
 JNK = c-Jun N-terminal kinase  
 LPS = lipopolysaccharide  
 MAPKs = mitogen-activated protein kinases  
 Met = methionine  
 MetO = methionine sulfoxide  
 MsrA = methionine sulfoxide reductase A  
 Msrs = methionine sulfoxide reductases  
 NF- $\kappa$ B = nuclear factor kappaB  
 NOX = NADPH oxidase  
 OH $\cdot$  = hydroxyl radicals  
 PBN = N-tert-butyl- $\alpha$ -phenylnitron  
 PD = Parkinson's disease  
 ROS = reactive oxygen species  
 SD rats = Sprague Dawley rats  
 shRNAs = short hairpin RNAs  
 SOD = superoxide dismutase  
 SOD-1 = Cu, Zn-superoxide dismutase-1  
 Tat-rMsrA = Tat-rMsrA fusion protein  
 TNF- $\alpha$  = tumor necrosis factor alpha