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Isoflurane preconditioning ameliorates electromagnetic pulse-induced neural damage by shifting microglia polarization toward anti-inflammatory phenotype via upregulation of SOCS1

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ABSTRACT

With the speedy technological advances during the past few decades, human exposure to the electromagnetic field (EMF) has become increasingly common. Exposure to EMF may induce neural injuries and dysfunction of various organs, likely involving neuroinflammation and activation of microglial cells. Isoflurane preconditioning (IP) is shown to provide neuroprotection in various neurological diseases by inhibiting excessive neuroinflammatory responses. Brain samples harvested from rats exposed to electromagnetic pulse (EMP) with or without IP were subjected to qPCR, Western blot assay, and immunohistochemistry to determine the expression of pro-inflammatory/anti-inflammatory microglia markers and a variety of pro- and anti-inflammatory mediators. Suppressor of cytokine signaling 1 (SOCS1) siRNA was used in cultured N9 microglia cells to examine the roles of SOCS1 in the effect of IP. In both in vivo and in vitro experiments, EMP-exposed microglia were predominantly pro-inflammatory microglia, accompanied by increased expression of pro-inflammatory cytokines and chemokines, and activation of TLR4 pathway, leading to neuronal death. IP reversed the changes induced by EMP and switched the activated microglia to an anti-inflammatory phenotype. SOCS1 siRNA abolished the beneficial effects of IP. IP ameliorates EMP-induced neural injuries by shifting microglia polarization from pro-inflammatory to anti-inflammatory phenotype via upregulation of SOCS1.

1. Introduction

With the speedy technological advances during the past few decades, it has become nearly inevitable for human beings to be exposed to the electromagnetic field (EMF). Exposure to EMF may exert negative biological impacts, including tissue damages and dysfunction of various organs [1,2]. Hence, there is a pressing need to explore for an effective approach to prevent or treat the detrimental consequences of EMF. Electromagnetic pulse (EMP), particular type of pulsing EMF, poses more complex effects on biological processes. In a prior investigation, *Jiang* and colleagues [3] using a rodent model reported that long-term exposure to EMP induced an increase of β-amyloid (Aβ) deposition and elevated oxidative stress in the hippocampus. Consistently, in Morris water maze test EMP-exposed rats exhibited impaired spatial cognition and memory in comparison to the sham-group, demonstrating the noxious effects of EMP exposure on the central nervous system (CNS).

Microglia are regarded as the resident immune cells of the CNS, constituting the macrophage population in the brain [4]. In cases of pathogen intrusion, tissue damage, or the presence of abnormal proteins or nucleotides, microglial cells go through a process known as "microglial activation", to transform from the resting state to the activated or reactive state [5]. Similar to macrophages, microglia switch between pro-inflammatory and anti-inflammatory activation phenotypes [6]. The classical pro-inflammatory activation of microglia leads to the production and release of pro-inflammatory cytokines and chemokines, and thereby mediates neuroinflammation, even causing neuronal damages or destruction of synapses. Alternatively, anti-inflammatory-skewed microglial activation is characterized by enhanced

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scavenging capability and release of anti-inflammatory cytokines and chemokines such as interleukin-10 (IL-10) and brain-derived neurotrophic factor (BDNF). Therefore, anti-inflammatory microglia is considered to be a neuro-protective phenotype that represses neuroinflammatory responses and promotes tissue repair [7]. Pervious in vitro studies revealed that EMF-treated N9 microglial cells presented characteristics of pro-inflammatory phenotype, with suppressed phagocytic ability but significantly elevated production of tumor necrosis factor-α (TNF-α), IL-6, and IL-1β [8,9]. Thus, regulation of microglia polarization possesses the potential to serve as a therapeutic strategy for interventions against neuroinflammation in the CNS.

Isoflurane is a commonly used volatile anesthetic with proven clinical safety. Investigations have shown that isoflurane preconditioning (IP) provided long-lasting neuroprotection after brain ischemia in adult rats [10,11]. Pretreatments with isoflurane attenuated cell death, reduced brain infarct volumes, and improved neurological functions for up to four weeks following middle cerebral arterial occlusion (MCAO) [12]. The neuroprotective functions of IP were suggested to involve inhibition of overactive microglial cells and alleviation of neuroinflammatory responses [13]. Given that neuroinflammation mediated by microglia is a common pathogenic mechanism shared by many neurological diseases including brain ischemia, neurodegenerative diseases, and EMF-induced neural damages, we aimed to examine whether IP could regulate pro-inflammatory/anti-inflammatory microglia activation phenotypes and in turn ameliorate EMP-induced neuroinflammation, and to further elucidate the underlying mechanisms.

2. Methods and materials

2.1. Materials

Male Sprague-Dawley (SD) rats (200–220 g, 6 weeks old) were purchased from the Experimental Animal Centre of the Air Force Military Medical University (Xi'an, China). The N9 microglial cell-line was generously provided by the Chinese Academy of Sciences. Iscove's modified Dulbecco's medium (IMDM) as well as fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The primary anti-iNOS, anti-Arg-1, anti-iba-1, anti-TLR4, anti-MyD88, anti-SOCS1 and anti-cleaved Caspase3 antibody were purchased from Abcam Ltd. (Cambridge, MA, USA). The primary anti-GAPDH antibody and secondary horseradish peroxidase (HRP)-conjugated antibody were obtained from Cowin Bioscience (Beijing, China). The primary Bcl-2 or Bax antibody were purchased from Santa Cruz (Dallas, TX, USA) and the primary anti-IκB-α antibody were purchased from Novus Biological (Littleton, USA).

2.2. Animals

6 weeks old rats were housed under specific pathogen-free conditions with ad libitum access to sterile rodent chow and water, and all the rats received only once EMP exposure or sham exposure. The experimental protocol was approved by the Ethics Committee for Animal Experimentation and performed in accordance to the Guidelines for Animal Experimentation of the Fourth Military Medical University. For all the experiments in the current study, 6 rats were included in each group unless otherwise indicated.

2.3. Cell culture

The N9 microglial cells were cultured in the IMDM medium containing 10% FBS, 100 μg/ml streptomycin, 2 mM glutamine, and 100 U/ml penicillin. The humidified atmosphere of the 37 °C cell culture incubator consisted of 5% CO_2 and 95% air. The medium was changed every three days. Cells received EMP exposure or sham exposure when cell density reached about 70–80%.

2.4. EMP exposure system

As previously described, an all-solid-state nanosecond generator was used [14]. Briefly, an EMF pulse was delivered through a spark gap generator and transmitted into a gigahertz transverse electromagnetic cell. This setup delivered peak intensity 400 kV/m, rise time 10 ns, pulse width 350 ns, 0.5 pps, 1 Hz electromagnetic pulse, and the total pulse repetition was 200 pulses. A Tektronix 7000B osciloscope (Tektronix, Beaverton, OR) was used to observe the pulse waveform. The rats and cells were all exposed to EMP at 400 kV/m for 200 pulses. The animals were totally exposed to EMP and the temperature measurement before and after EMP exposed showed no significant changes. During exposure, the cells were placed in the upper chamber of a PerspexTM water bath. The temperature of the medium was kept at 37 °C by heated water circulating through a lower closed compartment. During sham exposure, cells were subjected to the same conditions only without EMP exposure. Both tissues and cells were harvested 24 h after EMP treatment.

2.5. IP procedure

IP was performed 24 h prior to the EMP exposure as previously established [11]. Briefly, for IP in living animals, 2% isoflurane mixed with air and oxygen was applied to anesthetized rats via a facemask with the aid of mechanical ventilation for 30 min. Sham animals received parallel surgical procedures but were given only the air/oxygen mixture. Inhalation and exhalation were continuously monitored. After IP, rats were allowed to recover until regular ventilation was resumed and then returned to their home cage. For IP on cultured cells, 2% isoflurane vapor was delivered into an airtight chamber gassed with 95% air and 5% CO_2, and the cell cultures were maintained in the chamber at 37 °C for 30 mins. The sham group was parallel-treated but without the isoflurane. After IP, cells were returned to the regular incubator for another 24 h before EMP exposure.

2.6. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from freshly isolated rat brain or N9 microglial cells with Trizol reagent (Invitrogen, USA) according to the provided manual. Extracted RNA (5 μg) was used for cDNA synthesis utilizing a cDNA synthesis kit (Invitrogen, USA). The PCR protocols were as follows: after initial denaturation at 95 °C for 2 min, 45 cycles of 94 °C for 5 s, 60 °C for 10 s were performed, followed by a 10 min extension at 72 °C. Relative expression levels of target genes were calculated using the Ct method. The PCR primers are summarized in Table 1. The final results were normalized to GAPDH and expressed as the fold change of control.

2.7. Western blotting

Western blotting was employed to assess the expression of iNOS, Arg1, cleaved caspase-3, Bcl-2, Bax, TLR4, MyD88, SOCS1 and IκB-α

Table 1
Primers for qRT-PCR.

Gene	Sense (5′-3′)	Anti-sense (5′-3′)
Rat iNOS	GTTTGACCAGAGGACCCAGA	GTGAGCTGGTAGGTTCCTGT
Rat Arg1	CCTATGCGTCATTTGGGTGG	TACACGATGTCCTTGGCAGA
Rat IL-1β	AGGCTGACAGACCCCAAAAG	CTCCACGGGCAAGACATAGG
Rat IL-6	CCCAACTTCCAATGCTCTCCT	AGGTTTGCCGAGTAGACCTC
Rat TNF-α	GCCCTACGGGTCATTGAGAG	TTGTTCCACAGGGGTCTTGG
Rat IL-10	CCTGCTCTTACTGGCTGGAG	TGTCCAGCTGGTCCTTCTTT
Rat TGF-β1	CCTGCAAGACCATCGACATG	TGTTGTACAAAGCGAGCACC
Rat BDNF	TGAGCCGAGCTCATCTTTGC	ATAGCGGGCGTTTCCTGAAG
Rat GAPDH	GGCAAGTTCAACGGCACAGT	ATGACATACTCAGCACCGGC

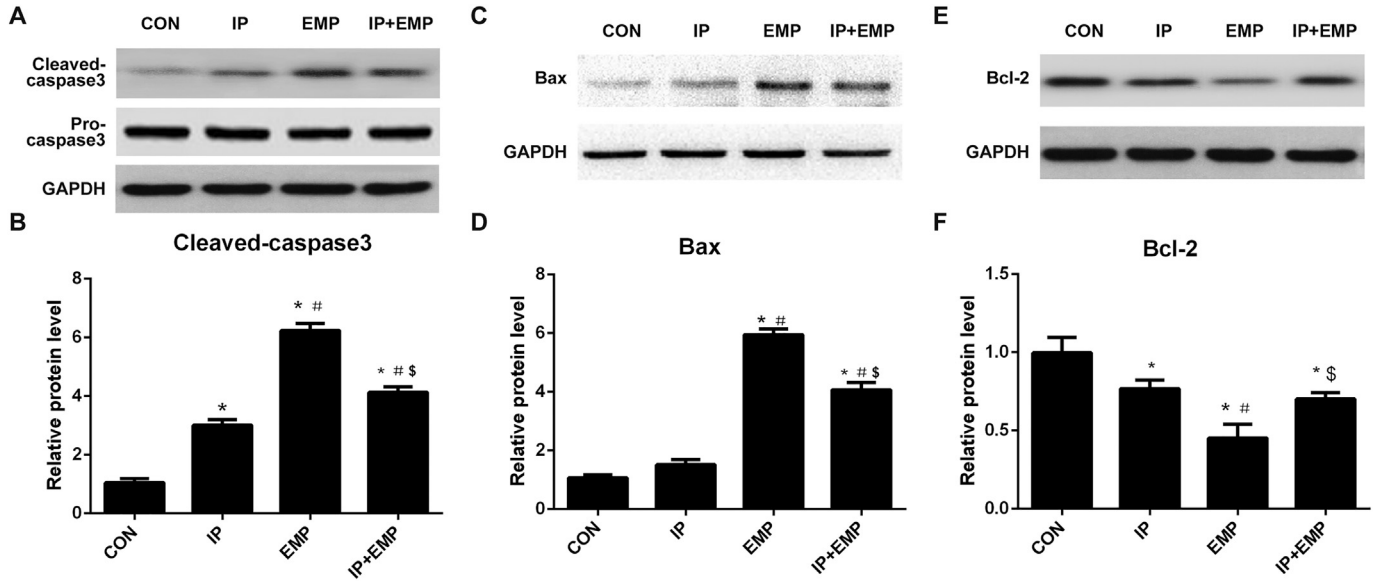

Fig. 1. EMP exacerbated apoptosis of rat cortical neurons which was partially prevented by IP. (A, C and E) Protein levels of cleaved caspase-3, pro-caspase-3, bax, and Bcl-2 revealed by western blot assay ($n = 6$), respectively. (B, D and F) Quantitative presentation of results in A, C and E, respectively. The relative protein level was normalized to the intensity of respective GAPDH and expressed as fold of control (control = 1). *$p < 0.05$ vs. Con group, #$p < 0.05$ vs. IP group, \$ $p < 0.05$ vs. EMP group.

protein in the rat cerebral cortex or N9 cells as described previously. In brief, rats were decapitated under deep anesthesia, and their cortical tissues at bregma + 1.5 mm were immediately collected. Total protein was prepared on ice with an extraction kit (KeyGen, Nanjing, China). The blots were probed with a rabbit antibody against cleaved caspase-3 (17 kDa) (1:200; Abcam, Cambridge, MA, USA), rabbit antibody against iNOS (1:500; Abcam, Cambridge, MA, USA), rabbit antibody against Arg1 (1:1000; Abcam, Cambridge, MA, USA), rabbit antibody against TLR4 (1:100; Abcam, Cambridge, MA, USA), rabbit antibody against MyD88 (1:200; Abcam, Cambridge, MA, USA), rabbit antibody against SOCS1 (1:1000; Abcam, Cambridge, MA, USA), mouse monoclonal antibodies against IκB-α (1:500, Novus Biologicals), and with mouse monoclonal antibodies against Bcl-2 or Bax (1:1000, Santa Cruz, Dallas, TX, USA) and GAPDH (1:1000; Cowin Bioscience). Secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody or goat anti-mouse antibody (1:5000; Cowin Bioscience) was used. The signal was quantified by densitometry by an immunoblotting detection system (Alpha Innotech, USA), and the relative protein levels were presented as the ratio of the optical density of the target protein bands to that of GAPDH.

2.8. Immunofluorescent staining

The deeply-anesthetized animal was transcardially fixed with 4% paraformaldehyde, and the brain was immediately removed, post-fixed, and dehydrated. Cortical tissues at the level of bregma + 1.5 mm were embedded in paraffin and sectioned at 5 μm thickness. After deparaffinization, sections were subjected to antigen retrieval in Tris-buffered saline containing 20 μg/ml proteinase K for 10 min followed by 10-min in distilled water containing 3% H_2O_2, then treated with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and 2% normal goat serum for 24 h. Next, sections were incubated with antibody against iNOS (1:500; Abcam, Cambridge, MA, USA), rabbit antibody against Arg1 (1:1000; Abcam, Cambridge, MA, USA), mouse antibody against iba-1 (1:1000; Abcam, Cambridge, MA, USA). After three washes with PBS, the sections were subjected to incubation with the appropriate fluorescent secondary antibody (1:500) and co-staining with DAPI (Beyotime Institute of Biotechnology, Shanghai, China). Finally, images were acquired using an Olympus laser scanning microscope (Olympus Corp., Shinjuku, Tokyo, Japan).

2.9. siRNA transfection

SOCS1 siRNA was designed and prepared by GenePharma Ltd. (Shanghai, China). Commercially available transfection kit was utilized to transfect 100 nM control or SOCS1 siRNA into N9 microglial cells seeded in 6-well plate (5×10^5 cells per well) following the manufacturer's instructions. 24 h later, cells were harvested, and the expression of SOCS1 was assessed using RT-PCR or Western blot assay.

2.10. Statistical analysis

SPSS 13.0 (SPSS Inc., Chicago, IL) was used for statistical analysis. Results were expressed as means ± standard deviation (S.D). Results were compared by one-factor analysis of variance (ANOVA), in conjunction with Tukey's Multiple Comparison Test. P Values of < 0.05 were considered statistically significant.

3. Results

3.1. EMP exacerbated apoptosis of rat cortical neurons which was partially prevented by IP

In cortical tissues obtained from EMP-exposed rats, with or without IP, the protein levels of cleaved caspase-3 and Bax were significantly higher than those of control or IP-treated animals ($p < 0.05$). However, IP markedly attenuated the increase of cleaved caspase-3 (without affecting pro-caspase-3) and Bax induced by EMP (IP + EMP vs. EMP; $p < 0.05$) (Fig. 1A–D). On the other hand, the protein level of Bcl-2 in the cortices of EMP-exposed mice were reduced compared to the con or IP group ($p < 0.05$), and this effect was reversed by IP (IP + EMP vs. EMP; $p < 0.05$) (Fig. 1E and F).

3.2. Microglial cells in the brain of EMP-exposed rat presented markers of pro-inflammatory microglia, which were switched to anti-inflammatory phenotype by IP

We assessed the activation phenotypes of microglial cells in the

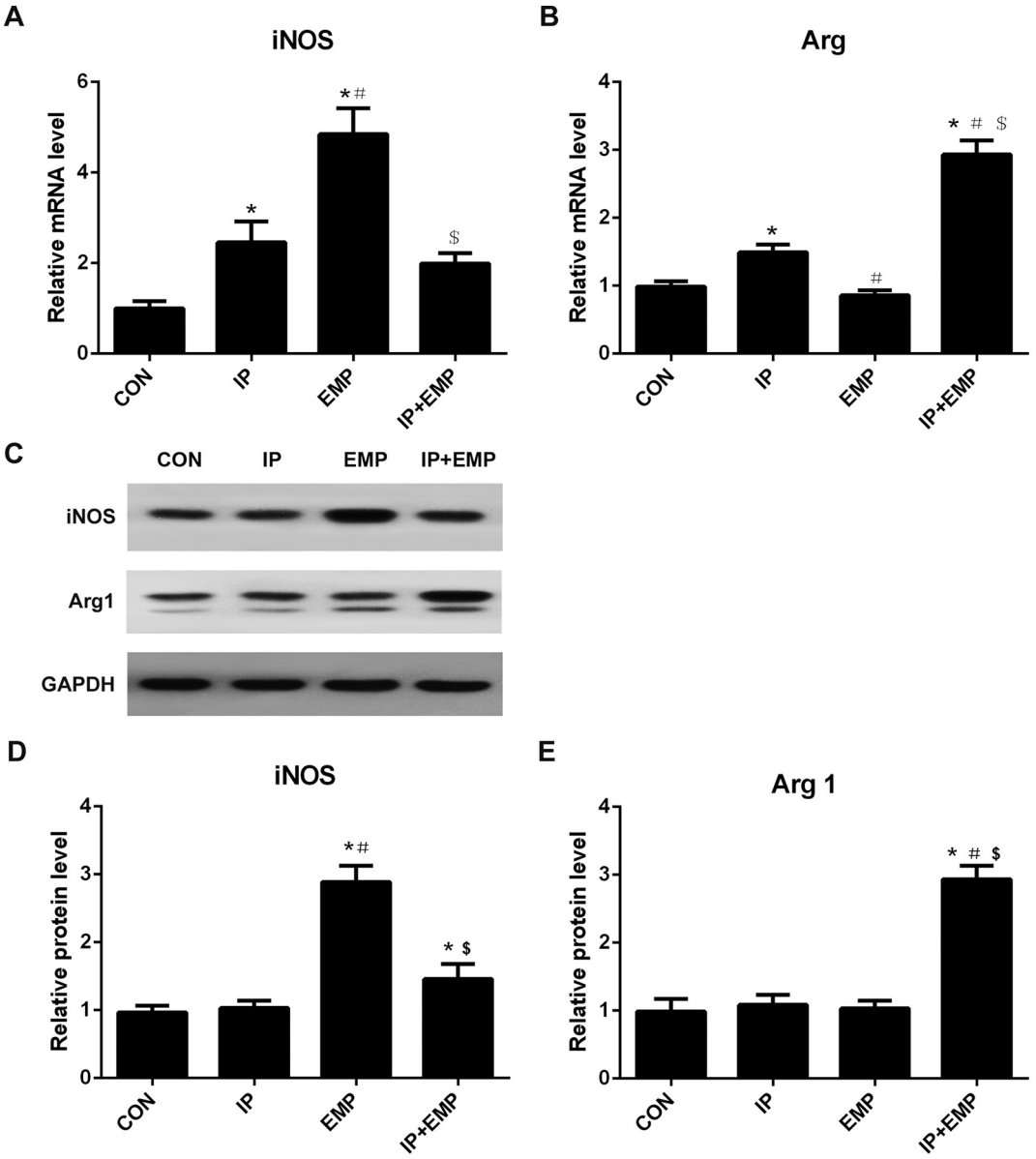

Fig. 2. Switch in the pro-inflammatory/anti-inflammatory microglia activation phenotypes following EMP with or without IP in rat cortices. (A and B) Relative mRNA levels of iNOS (pro-inflammatory microglia marker) and Arg1 (anti-inflammatory microglia marker) revealed by qPCR ($n = 6$). (C-E) Protein levels of iNOS and Arg1 examined by Western blot assay (n = 6). The relative protein level was normalized to the intensity of respective GAPDH and expressed as fold of control (control = 1). *$p < 0.05$ vs. Con group, #$p < 0.05$ vs. IP group, \$ $p < 0.05$ vs. EMP group.

cortices of EMP-exposed rats with or without IP. In comparison to the control group, the expression of iNOS, a marker for pro-inflammatory microglia, was elevated at both mRNA and protein levels ($p < 0.05$, Fig. 2A, C and D) in the EMP group; whereas the expression of Arg1, a marker for anti-inflammatory microglia, was downregulated at the mRNA level ($p < 0.05$, Fig. 2B, C and E). More importantly, IP lowered the transcript and protein levels of iNOS (IP + EMP vs. EMP, $p < 0.05$, Fig. 2A, C and D), but significantly elevated the mRNA and protein levels of Arg1 (IP + EMP vs EMP, $p < 0.05$, Fig. 2B, C and E).

Further, we utilized immunofluorescent staining to examine the changes in the expression of iNOS and Arg1, markers for pro-inflammatory or anti-inflammatory microglia respectively, in the brain sections of EMP-exposed rats. In EMP-exposed animals compare to those of the control group, the co-staining of iNOS (red) and iba-1 (marker for microglial cells, green) were greatly increased, and such increase was nearly completely reversed in the IP + EMP group (Fig. 3A). On the contrary, the co-localization of Arg1 and iba-1 was lower in the EMP group and substantially increased by IP (IP + EMP vs. EMP, Fig. 3B).

3.3. Effects of EMP and IP on the activity of TLR4/MyD88/IκB-α pathway and various inflammatory molecular mediators

In light of our previous results that IP induced a switch from pro-inflammatory pro-inflammatory microglia phenotype to an anti-inflammatory anti-inflammatory phenotype following EMP exposure, we examined the expression levels of several essential pro- or anti-inflammatory cytokines and chemokines produced by microglia after EMP and/or IP. In comparison to the control group, mRNA levels of TNF-α, IL-1β, and IL-6, all associated with pro-inflammatory microglia, were significantly elevated after EMP exposure, and IP blocked or strongly attenuated the EMP-induced upregulation of these pro-inflammatory mediators (Fig. 4A–C). Meanwhile, the expression levels of anti-inflammatory mediators associated with anti-inflammatory

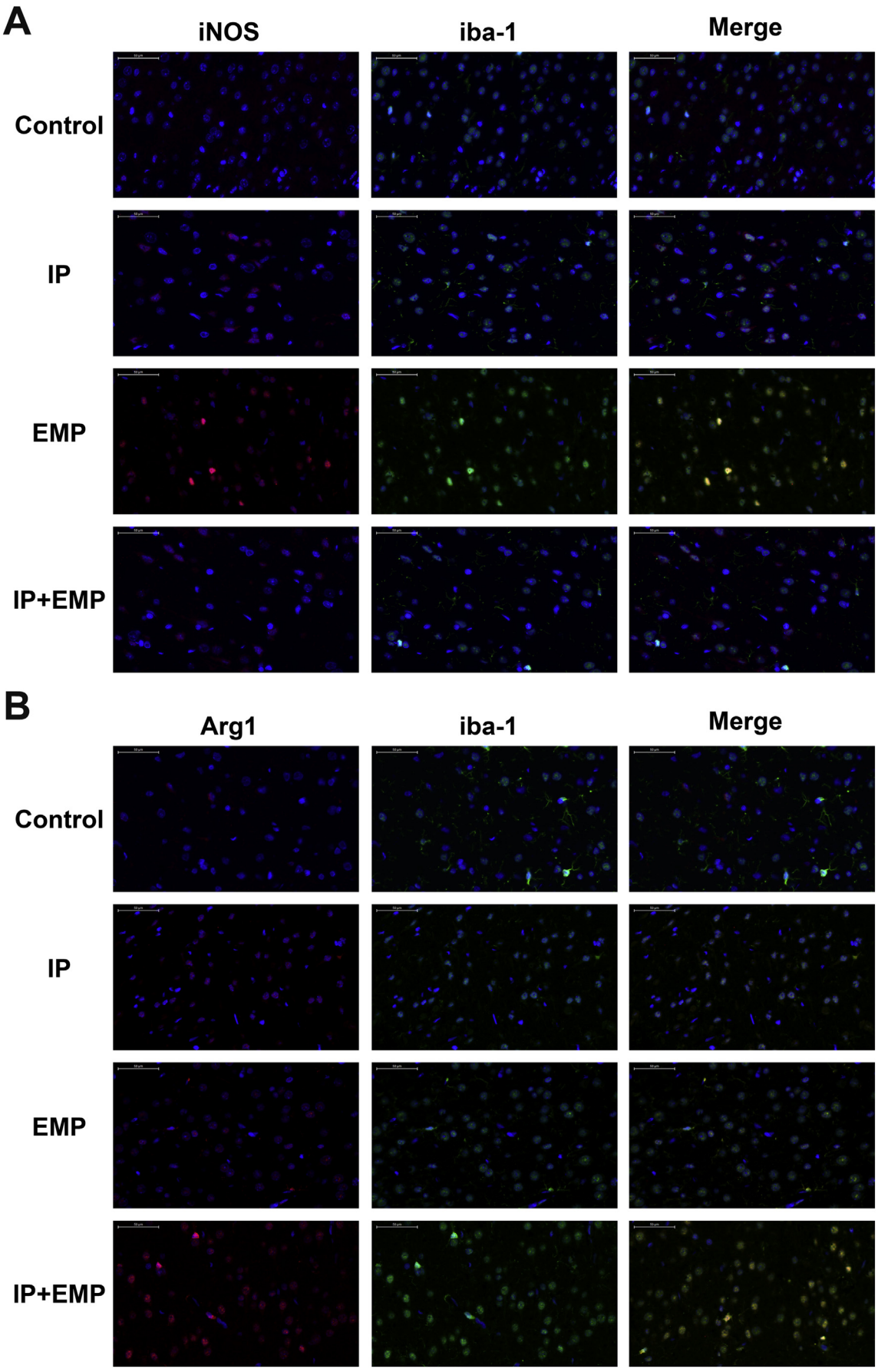

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Fig. 3. Switch in the pro-inflammatory/anti-inflammatory microglia activation phenotypes following EMP exposure with or without IP. (A) Co-staining of iNOS (red) and iba-1 (green) in rat brain sections following EMP. (B) Co-staining of Arg1 (red) and Iba-1 (green) in rat brain sections following EMP. Nuclei were labeled with blue fluorescence. Scale bars = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

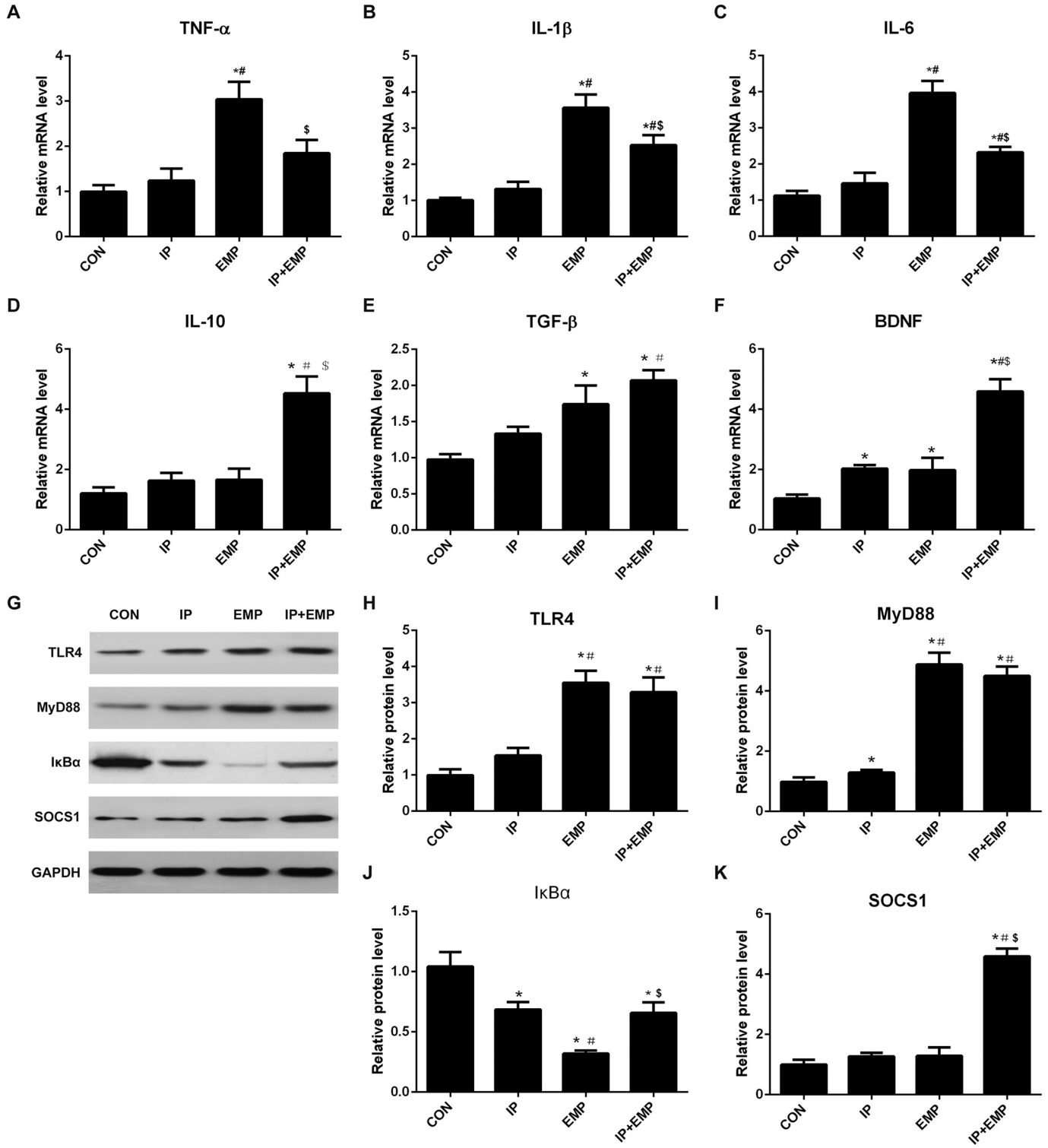

Fig. 4. Altered expression levels of various signaling molecules or cytokines/chemokines after EMP exposure with or without IP. (A–F) Relative mRNA levels of TNF-α, IL-1β, IL-6, IL-10, TGF-β, and BDNF, respectively, in different treatment groups (n = 6). (G-K) Relative protein levels of TLR4, MyD88, IκB-α, and SOCS1in different treatment groups (n = 6) examined by Western blot assay. The relative protein level was normalized to the intensity of respective GAPDH and expressed as fold of control (control = 1). *$p < 0.05$ vs. Con, #$p < 0.05$ vs. IP, \$$p < 0.05$ vs. EMP.

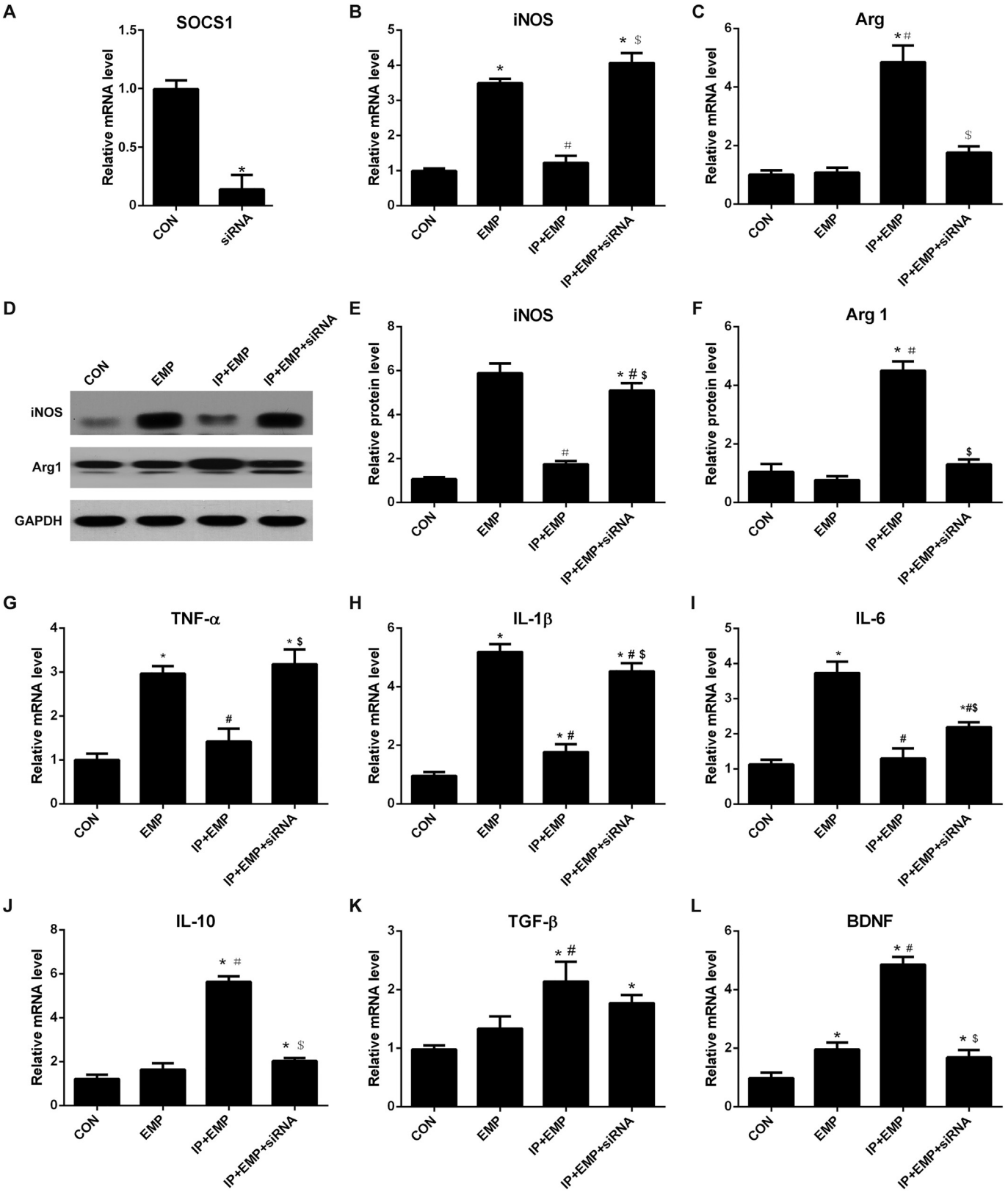

Fig. 5. SOCS1 siRNA blocked the protective effects of IP in EMP exposure and IP-induced switch of pro-inflammatory/anti-inflammatory microglia phenotypes. (A) SOCS1 siRNA knockdown efficiency. (B and C) Relative transcript levels of iNOS (pro-inflammatory microglia marker) and Arg1 (anti-inflammatory microglia marker) revealed by qPCR (n = 6). (D–F) Relative protein levels of iNOS and Arg1(n = 6). (G–L) Relative mRNA levels of TNF-α, IL-1β, IL-6, IL-10, TGF-β, and BDNF, respectively (n = 6). *$p < 0.05$ vs. Con group, #$p < 0.05$ vs. EMP group, \$$p < 0.05$ vs. IP + EMP group.

microglia, including IL-10, TGF-β, and BDNF, were unaltered or only moderately upregulated compared to the control or IP group following EMP, but were markedly increased in the IP + EMP group ($p < 0.05$, Fig. 4D–F).

Toll-like Receptor (TLR) 4/MyD88/IκB-α pathway plays a key role in microglia-mediated inflammation, we thus assessed the activities of this pathway. Results from western blot assay revealed that the protein levels of TLR4 and MyD88 were higher in the EMP group compared to the CON group (Fig. 4G–I) while the IκB-α level was greatly downregulated after EMP exposure (Fig. 4J). IP did not alter the expression levels of TLR4 and MyD88 in EMP treated animals but significantly increased the protein level of IκB-α ($p < 0.05$, Fig. 4G–J). Finally, the expression levels of the suppressor of cytokine signaling 1 (SOCS1) was evaluated. SOCS1 was not affected by EMP but greatly upregulated in the IP + EMP group ($p < 0.05$, Fig. 4K).

3.4. SOCS1 siRNA attenuated the protective effect of IP in EMP exposure

In order to explore the mechanisms underlying the switch between pro-inflammatory/anti-inflammatory microglia phenotype after EMP exposure induced by IP, we used N9 microglial cells to examine the role of SOCS1 in this process. We first confirmed the efficiency of SOCS1 siRNA knockdown (Fig. 5A). Next, consistent with previous findings, EMP induced an elevation of iNOS, at both mRNA and protein levels (Fig. 5B, D and E). IP nearly completely prevented the EMP-induced upregulation of iNOS, and this effect was blocked by SOCS1 siRNA (Fig. 5B, D and E). There were no changes in the expression level of Arg1 after EMP, but a dramatic increase of Arg1 expression in the IP + EMP group (Fig. 5C, D and F). Again, the increase of Arg1 in the IP + EMP group was completely abolished with the use of SOCS1 siRNA, suggesting a critical role of SOCS1 in the IP-regulated polarization of microglial cells.

We further assessed the expression of pro- or anti-inflammatory cytokines or chemokines in cultured N9 microglial cells following EMP with or without IP in the presence of SOCS1 siRNA. Similar to in vivo results, in EMP-exposed N9 cells the transcript levels of pro-inflammatory mediators including TNF-α, IL-1β, and IL-6 were increased. IP prevented such EMP-induced increase in a SOCS1-dependent manner (Fig. 5G–I). As for anti-inflammatory mediators including IL-10, TGF-β, and BDNF, EMP alone did not statistically change or only moderately increased their expression. However, in IP + EMP group, the mRNA levels of IL-10, TGF-β, and BDNF were substantially higher than those in the control group, and the difference was again greatly attenuated by SOCS1 siRNA (Fig. 5J–L).

Results of our in vivo experiments suggested that TLR4/MyD88/IκB-α pathway was activated by EMP and likely modulated by IP. Therefore, we sought to examine the effect of SOCS1 siRNA on the expression of TLR4, MyD88, and IκB-α. The protein levels of TLR4 and MyD88 were significantly increased by EMP exposure and appeared insensitive to either IP or SOCS1 siRNA (Fig. 6A–C). However, EMP induced a drastic decrease of the IκB-α level, and this reduction was fully reversed by IP in a SOCS1-dependent fashion (Fig. 6A and D).

4. Discussion

In the current study using both in vivo and in vitro models, we demonstrated that microglial cells activated by EMP exposure were predominantly pro-inflammatory microglia, which then released pro-inflammatory IL-1β, IL-6 and TNF-α through activation of TLR4/MyD88/NFκB pathway, causing apoptosis of cortical neurons. Instead, IP prior to EMP exposure induced microglia activation that presented characteristics of mainly anti-inflammatory phenotype, with reduced production of IL-1β, IL-6 and TNF-α, but increased expression of anti-inflammatory IL-10, TGF-β and BDNF, which lead to attenuated apoptosis and improved neuroprotection. We further presented evidence that such neuroprotective property of IP involved upregulation of SOCS1, for SOCS1 siRNA nearly completely abolished the beneficial effects of IP in EMP-induced neuroinflammation. SOCS1 likely inhibited the activation and translocation of NFκB, thereby suppressed the production of pro-inflammatory cytokines, which lead to a change of cytokine levels in the microenvironment of microglial cells, facilitating a switch from pro-inflammatory to anti-inflammatory activation phenotype.

Inflammation in the CNS contributes to the pathogenesis of various brain diseases including Alzheimer's Diseases (AD), Parkinson's Diseases (PD), stroke, and EMP-induced neural damages [15,16]. For instance, during the progression of AD, the increased aggregation of Aβ induces a local enrichment of activated microglial cells, which can act as a "double-edged sword". On one hand, anti-inflammatory microglial cells facilitate the clearance of Aβ plaques through phagocytic activities, suppress inflammatory responses by releasing anti-inflammatory mediators such as IL-10 and BDNF, and thereby promote tissue repair and restoration of neurological functions. On the other hand, Aβ, as an endogenous abnormal proteolytic byproduct, can activate microglial cells through interactions with TLR4, subsequently eliciting massive production and secretion of pro-inflammatory mediators such as TNF-α, IL-6, and IL-1β, via recruitment of MyD88 and NFκB. When the homeostatic regulation of immune responses is disrupted and Aβ accumulation overwhelms Aβ clearance, the changes in the microglia microenvironment may promote a pro-inflammatory pro-inflammatory-skewed activation phenotype, and the excessive inflammation gradually results in the destabilization of synapses and neuronal degradation, contributing to the cognitive deficits of AD patients [17–19]. Likewise, in the development and progression of PD, microglia-mediated chronic neuroinflammation and release of inflammatory mediators play a prominent role in the progressive degeneration of dopaminergic neurons [20,21]. In a similar manner, we discovered in the current investigation that EMP exposure activated the TLR4/MyD88/NFκB pathway in microglial cells, which then presented properties of pro-inflammatory microglia and potentiated the inflammation, causing increased neuronal death. However, IP prior to EMP exposure effectively prevented the neural damages by inducing a shift in the microglia polarization, from neurotoxic pro-inflammatory phenotype to the neuroprotective anti-inflammatory phenotype. Consequently, the production of anti-inflammatory mediators was upregulated, suppressing the neuroinflammation induced by EMP.

A great amount of effort has been focused on the regulation of microglial polarization as a potential means of intervention for promoting immune homeostasis, and a promising therapeutic strategy against neurological diseases that are associated with neuroinflammation. A prior study by *Yang* and colleagues showed that resveratrol promoted microglia polarization toward the anti-inflammatory phenotype and reduced the release of pro-inflammatory cytokines in lippopolysacharide (LPS)-induced neuroinflammation [22]. In a report by *Aryanpour* et al., progesterone gave rise to a switch from pro-inflammatory to anti-inflammatory microglia phenotype and inhibited the functions of NLRP3 inflammasome in a cuprizone-induced demyelination model [7]. However, contradictory results were generated by some clinical studies using non-steroidal anti-inflammatory drugs (NSAIDs), probably because the molecular targets of these drugs were not readily involved in the homeostatic regulation of immune responses [23]. The activation phenotypes of microglial cells are dictated by the microenvironment. The shift of pro-inflammatory/anti-inflammatory polarization is achieved by the interactions between microglia and local molecular mediators. For example, IL-4 and IL-10 have been shown to induce microglia polarization toward the anti-inflammatory phenotype, whereas activation of NFκB promoted a pro-inflammatory phenotype [24].

NFκB is the downstream effector of the TLR signaling pathway, a trimer constituted by p65, p50, and IκB at resting state. When activated, the p65/p50 dimers dissociate from IκB and translocate into the nucleus. The p65 subunit can function as a transcription factor and initiate

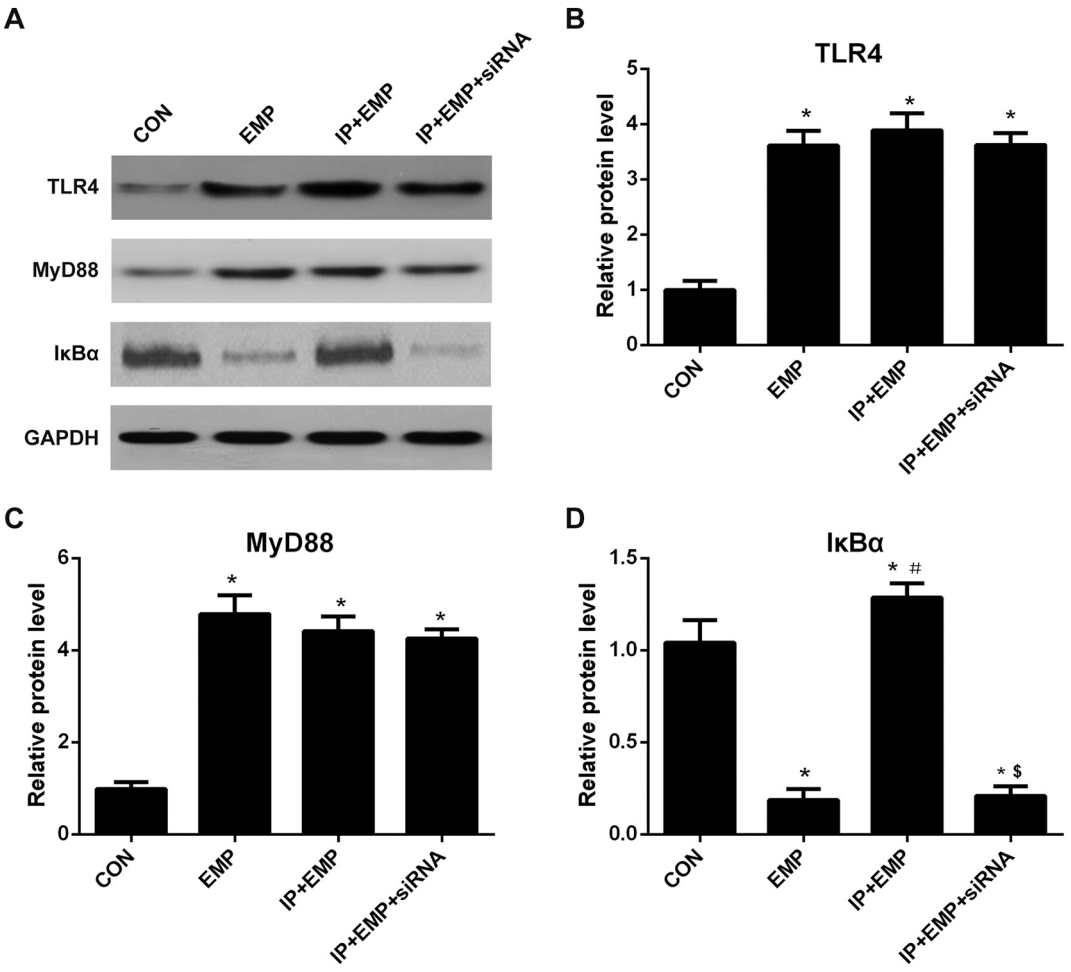

Fig. 6. Effects of SOCS1 siRNA on TLR4/MyD88/IκB-α pathway in EMP-exposed N9 microglial cells with or without IP. (A–D) Relative protein levels of TLR4, MyD88, and IκB-α revealed by Western blot assay (n = 6). The relative protein level was normalized to the intensity of respective GAPDH and expressed as fold of control (control = 1). $*p < 0.05$ vs. Con group, $\#p < 0.05$ vs. EMP group, $\$p < 0.05$ vs. IP + EMP group.

the expression of various target genes, including markers for pro-inflammatory microglia and pro-inflammatory cytokines such as IL-6 [25]; whereas the homodimers of p50 can increase the expression of markers for anti-inflammatory microglia, such as Arg1 and Ym1, inducing an anti-inflammatory phenotype [26]. SOCS family of proteins, consisting of SOCS1-7 and CIS, is a group of endogenous negative-feedback signaling molecules to properly terminate signal transduction of cytokines and maintain hemostasis. NFκB is a pivotal component in the feedback regulation of TLR4 pathway by SOCS1, but the particular mechanism by which SOCS1 interacts with NFκB remains controversial. It is believed by some researchers that SOCS1 limits NFκB activities by binding to p65 and inducing degradation of p65 via ubiquitination [27]. Yet others argue that SOCS1 exerts feedback regulation on TLR4/MyD88/NFκB pathway by reducing the cell's sensitivity to cytokines and generating inhibitory signals [28].

In our in vivo as well as in vitro experiments, we consistently observed upregulated expression of SOCS1 and IκB-α with IP prior to EMP. Further, after silencing of SOCS1 with the use of siRNA, the expression of IκB-α in the IP + EMP group was reduced, while the expression of iNOS, a marker for pro-inflammatory microglia, was elevated. Hence, we speculated that IP promoted the shift from pro-inflammatory to anti-inflammatory phenotype through upregulation of SOCS1, which in turn inhibited the translocation of NFκB and production of pro-inflammatory cytokines. Given these beneficial effects of IP against inflammation, further studies are warranted to verify our findings in other animal models, in order to not only confirm our discovery but also reveal any potential adverse effects. A comprehensive knowledge on potential effects, beneficial and detrimental, is critical in determining the future clinical value of IP.

Our data indicated that the polarization of microglia and the homeostasis of inflammatory responses are important pathological mechanisms in the EMP-induced neural injuries. However, it is noteworthy that discrepancies remain in the literature regarding the biological impacts of EMF or EMP in various formats. For instance, EMF equivalent to mobile phone usage was reported to protect against neural impairments and to enhance brain mitochondrial functions [29,30]. Similarly, chronic exposure to low frequency magnetic field was suggested to exert beneficial effects on injured spinal cord [31]. It was also demonstrated that EMP at the frequency of 75 Hz reduced hypoxia and inflammatory damages in glia and brain cells [32]. The apparent inconsistence between these reports and the findings from our study and others may be attributable to the different intensities, frequencies, as well as exposure durations of EMF/EMP that was examined. Further, EMP possesses several unique characteristics including extremely short rise time and high peak voltages, which may well contribute to the adverse effects we observed in the current study. Taken together, the use-dependent variable biological effects of EMP/EMF clearly imply the complexity of physiological processes activated by or involved in the EMP/EMF exposure. Further investigations are warranted to depict the mechanism underlying the effects of EMP/EMF as well as the protection by IP in the EMP-induced neural damage.

5. Conclusion

In the present study, we characterized the neuroprotective effect of IP in the EMP-induced neuroinflammation and neural damage, using both in vivo and in vitro models. We established that IP exerted its beneficial effect by inducing a switch of activated microglia from the pro-inflammatory to anti-inflammatory phenotype, and SOCS1 was required in such process.

Declaration conflict of interest

The authors declare that they have no conflict of interest.

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