BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 27(6), 522-529 (2019)

$S1P_1$ Regulates M1/M2 Polarization toward Brain Injury after Transient Focal Cerebral Ischemia

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Abstract

M1/M2 polarization of immune cells including microglia has been well characterized. It mediates detrimental or beneficial roles in neuroinflammatory disorders including cerebral ischemia. We have previously found that sphingosine 1-phospate receptor subtype 1 ($S1P_1$) in post-ischemic brain following transient middle cerebral artery occlusion (tMCAO) can trigger microglial activation, leading to brain damage. Although the link between $S1P_1$ and microglial activation as a pathogenesis in cerebral ischemia had been clearly demonstrated, whether the pathogenic role of $S1P_1$ is associated with its regulation of M1/M2 polarization remains unclear. Thus, this study aimed to determine whether $S1P_1$ was associated with regulation of M1/M2 polarization in post-ischemic brain. Suppressing $S1P_1$ activity with its functional antagonist, AUY954 (5 mg/kg, *p.o.*), attenuated mRNA upregulation of M1 polarization markers in post-ischemic brain at 1 day and 3 days after tMCAO challenge. Similarly, suppressing $S1P_1$ activity with AUY954 administration inhibited M1-polarizatioin-relevant NF-κB activation in post-ischemic brain. Particularly, NF-κB activation was observed in activated microglia of post-ischemic brain and markedly attenuated by AUY954, indicating that M1 polarization through $S1P_1$ in post-ischemic brain mainly occurred in activated microglia. Suppressing $S1P_1$ activity with AUY954 also increased mRNA expression levels of M2 polarization markers in post-ischemic brain, further indicating that $S1P_1$ could also influence M2 polarization in post-ischemic brain. Finally, suppressing $S1P_1$ activity decreased phosphorylation of M1-relevant ERK1/2, p38, and JNK MAPKs, but increased phosphorylation of M2-relevant Akt, all of which were downstream pathways following $S1P_1$ activation. Overall, these results revealed $S1P_1$-regulated M1/M2 polarization toward brain damage as a pathogenesis of cerebral ischemia.

Key Words: Transient middle cerebral artery occlusion (tMCAO), $S1P_1$, AUY954, M1/M2 polarization, Microglia

INTRODUCTION

Cerebral ischemia caused by blockage of blood supply to the brain can lead to irreversible neuronal damage. Mediators released by ischemia-induced damaging neurons such as proteases can trigger immune responses in the brain, leading to aggravation of neuroinflammatory cascades. Glial cells, particularly microglia, are considered as major cell types that participate in ischemia-induced neuroinflammatory events. Despite the fact that brain-resident microglia act as the first line of defense during neuroinflammatory conditions, their over-activation is neurotoxic (Block *et al*., 2007). Under normal condition, microglia exist in ramified morphology with small soma and highly branched processes. Following ischemic injury, microglial biology is altered as they undergo time- and region-dependent activation, morphological transformation into amoeboid cells (increased soma and reduced branched processes), and proliferation (Gaire *et al*., 2015; Sapkota *et al*., 2017), all of which can ultimately affect disease pathogenesis. Beside these features for activated microglia, another key feature following ischemic injury is their phenotypic shift commonly known as polarization (Hu *et al*., 2012; Xia *et al*., 2015). Depending on functional roles, microglial polarization is classified into two distinct phenotypes: pro-inflammatory M1 and anti-inflammatory M2 (Hu *et al*., 2012, 2015). M1 polarization is mainly involved in augmentation of inflammatory responses that trigger brain damage and worsen disease condition because M1-induced ischemic pathogenesis is mediated through the release of pro-inflammatory cytokines and chemokines. In sharp contrast, M2 polarization is involved in anti-inflammatory responses associated with damage repair and recovery after ischemic injury (Hu *et al*., 2012). Therefore, targeting M1/M2 polarization is considered an important therapeutic strategy in cerebral ischemia as it determines the fate

Open Access **https://doi.org/10.4062/biomolther.2019.005**

Received Jan 10, 2019 **Revised** Mar 16, 2019 **Accepted** Apr 9, 2019
Published Online Jun 11, 2019

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of secondary brain damage following ischemic injury.

Signaling through sphingosine 1-phospate (S1P) receptors has gained an increasing attention in cerebral ischemia because of neuroprotective effects of FTY720, a non-selective S1P receptor modulator that binds four of five S1P receptors except $S1P_2$, in several rodent ischemic models (Czech *et al*., 2009; Kraft *et al*., 2013; Nazari *et al*., 2016). FTY720 is also under clinical trials for acute stroke (Fu *et al*., 2014; Zhu *et al*., 2015), clearly indicating a crucial role of receptor-mediated S1P signaling in cerebral ischemia. In addition, exogenously administered S1P can trigger ischemic pathogenesis (Moon *et al*., 2015), further reaffirming the involvement of S1P receptors in ischemic pathogenesis. In line with this notion, we and others have recently reported that at least three S1P receptor subtypes ($S1P_1$, $S1P_2$, and $S1P_3$) exert pathogenic roles in a mouse model of focal cerebral ischemia (Kim *et al*., 2015; Gaire *et al*., 2018a, 2018b). Pathogenic mechanisms of $S1P_1$ and $S1P_3$ are related to microglial activation (Gaire *et al*., 2018a, 2018b) while $S1P_2$ is mainly involved in ischemia-induced vascular dysfunction (Kim *et al*., 2015). Both $S1P_1$ and $S1P_3$ can influence microglial activation, morphological transformation toward amoeboid shape, and proliferation in ischemic penumbra, all of which are linked to brain damage in post-ischemic brain. Interestingly, M1 polarization, but not M2 polarization, is triggered by $S1P_3$ in post-ischemic brain along with increased pro-inflammatory productivity, which occurs mainly in activated microglia (Gaire *et al*., 2018b). However, whether other S1P receptor subtypes besides $S1P_3$ can influence M1/M2 polarization in the brain after ischemic insult remains unclear.

$S1P_1$ is the most abundant subtype of S1P receptors in the brain (Moon *et al*., 2015). It plays a critical role as a regulator for microglial activation in injured areas following ischemic challenge (Gaire *et al*., 2018a). Although the link between $S1P_1$ and microglial activation as a pathogenesis in cerebral ischemia has been clearly demonstrated, whether the pathogenic role of $S1P_1$ in cerebral ischemia is associated with its regulation of M1/M2 polarization in post-ischemic brain remains unclear. Thus, the objective of the present study was to determine whether $S1P_1$ was associated with regulation of M1/M2 polarization in post-ischemic brain. We determined $S1P_1$-regulated M1/M2 polarization by measuring mRNA expression levels of M1 or M2 markers in post-ischemic brain after transient middle cerebral artery occlusion (tMCAO) challenge. In addition, we determined roles of $S1P_1$ on M1-relevant NF-κB activation in post-ischemic brain, especially in activated microglia. Finally, we analyzed activation of $S1P_1$-dependent signaling pathways, including PI3K and MAPKs known to be closely associated with M1/M2 polarization.

MATERIALS AND METHODS

Animals and induction of transient focal cerebral ischemia

Male ICR mice (6 weeks old, 34 ± 2 g) were bought from Orient Co., Ltd. (Gyeonggi, Korea) and acclimatized for a week under controlled laboratory conditions of temperature (22 ± 2°C), relative humidity (60 ± 10%), and a 12-h light/dark cycle (light on from 07:00 to19:00). Mice were provided free access to food and water. Institutional animal care and use guidelines of Gachon University approved animal protocols (LCDI-2014-0079 and LCDI-2015-0048) for animal handling and surgical procedures.

Transient focal cerebral ischemia was induced by tMCAO in mice as described previously (Gaire *et al*., 2015, 2018a). Briefly, under anesthesia, right common carotid artery was carefully separated from vagus nerve and silicon coated 5-0 monofilament was inserted into internal carotid artery from the carotid bifurcation to occlude the middle cerebral artery (MCA). At 90 min after occlusion, the monofilament was withdrawn to allow reperfusion. Mouse body temperature was maintained at 37 ± 0.5°C throughout the operation period. Identical operation procedures were performed for sham-operated mice except for the occlusion of MCA.

Drug administration and brain sampling

AUY954, a functional antagonist of $S1P_1$, was dissolved in 10% Tween-80 (vehicle), and orally administered to mice (5 mg/kg) immediately after reperfusion. Brain sampling for histological and biochemical analysis was performed as described previously (Gaire *et al*., 2018a, 2018b). Briefly, mice brains were transcardially washed with ice-cold phosphate-buffered saline (PBS) at 1 or 3 days following tMCAO challenge. Ipsilateral brain hemispheres were harvested for RNA and protein expression analysis. For histological analysis, brain samples were perfused with 4% paraformaldehyde following transcardial wash with ice-cold PBS. Obtained brains were cryoprotected in 30% sucrose, frozen in tissue-tek optimum cutting temperature (OCT) solution, and sectioned (20 μm in thickness) using a cryostat microtome (J4800AMNZ, Thermo, Dreieich, Germany).

NF-κB/Iba1 immunohistochemical analysis

Role of $S1P_1$ signaling in ischemia-induced NF-κB expression in activated microglia was determined through NF-κB/Iba1 double immunolabeling at 3 days after tMCAO challenge as described previously (Gaire *et al*., 2018b). Briefly, brain sections were exposed to antigen retrieval by heating them in 1X Tris-EDTA at 100°C for 30 min followed by blocking with 1% fetal bovine serum (FBS). These sections were labelled with a primary antibody against NF-κB (1:100; Santa-Cruz Biotechnology Inc., CA, USA) overnight at 4°C followed by incubation with a secondary antibody at room temperature for 2 h. These sections were incubated with avidin-biotin complex (1:100; Vector labs, Burlingame, CA, USA) kit. NF-κB signals were then visualized using 3, 3'-diaminobenzidine tetrahydrochloride hydrate (DAB, Sigma-Aldrich, St. Louis, MO, USA) substrate (0.02% in 0.05 M Tris solution containing 0.01% hydrogen peroxide). After visualizing NF-κB signals, sections were washed several times, blocked with 1% FBS, and incubated overnight with Iba1 primary antibody (1:500; Wako Chemicals, Richmond, VA, USA). Sections were then labelled with Cy3 conjugated secondary antibody (1:1000; Jackson ImmunoResearch, West Grove, PA, USA), mounted with vectashield (Vector labs, Burlingame, CA, USA), and photographed. For quantification, three 20X images were obtained from a single mouse brain and immunopositive cells were manually counted in whole areas of each image. The average number of immunopositive cells was obtained from these three images and converted to the number of cells/mm^2 area for a single mouse. Then mean value of each experimental group was obtained. Representative images were prepared using Adobe Photoshop 7.0 (Adobe, San Jose, CA, USA).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from ipsilateral brain hemisphere at 1 and 3 days after tMCAO challenge using TRI reagent (Sigma-Aldrich). For qRT-PCR, RNA (1 μg) was reverse-transcribed in a reaction mixture containing 3 mM $MgCl_2$, 1 U RNase inhibitor, 0.5 mM dNTP, 1x RT buffer, 500 ng of random primers, and 10 U reverse transcriptase (Agilent, Santa Clara, CA, USA). The synthesized cDNA was used as a template for qRT-PCR using StepOnePlus™ qRT-PCR system (Applied Biosystems, Foster City, CA, USA) and gene-specific primers (Supplementary Table 1).

Western blot analysis

For Western blot analysis, protein samples were obtained from the ipsilateral brain hemisphere at 1 day after tMCAO challenge. Ipsilateral brain hemisphere was triturated with neuronal protein extraction reagent containing phosphatase and protease inhibitors to extract proteins. Proteins (30 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk and incubated with primary antibodies against rabbit p-Akt, Akt, p-ERK1/2, ERK1/2, p-p38, p38, p-JNK, JNK (1:1000; cell signaling, Danvers, MA, USA), or mouse GAPDH (1:5000; Santa-Cruz Biotechnology Inc.) overnight at 4°C followed by incubation with respective secondary antibodies (1:10000; Jackson ImmunoResearch) at room temperature for 2 h. Signals were visualized with enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific, Rockford, IL, USA). Band intensity of each protein blot was analyzed using ImageQuant™ TL software (GE Healthcare Bio-Science, Uppsala, Sweden), normalized with GAPDH, and expressed as fold change compared to sham-operated group.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). All statistical tests were performed using Graph Pad Prism 5 (Graph Pad Software Inc., La Jolla, CA, USA). Differences among groups were analyzed by a one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons. Statistical significance was set at $p<0.05$.

RESULTS

Suppressing $S1P_1$ activity attenuates tMCAO-induced M1 polarization

Transient ischemic challenge results in classical polarization normally known as M1 phenotype. In case of ischemic resolution or after the proper therapeutic intervention, immune cells become alternatively activated, which is termed as M2 phenotype (Hu *et al*., 2012, 2015; Walker and Lue, 2015). To determine whether $S1P_1$ activity in the ischemic brain was linked to M1 polarization, expression levels of surface and soluble markers of M1 cells were determined. At one day after tMCAO, mRNA expression levels of surface markers of M1 polarized cells including CD11b, CD16, CD32, and CD86 were significantly increased in vehicle-administered mice whereas AUY954-administration markedly attenuated expression levels of these markers (Fig. 1A-1D). We also determined expression levels of these M1 surface markers at 3 days following ischemic insult. Expression levels of these surface markers were not attenuated in AUY954-administered mice. Unexpectedly, a few of them (CD16 and CD32) were elevated (Fig. 1E-1H).

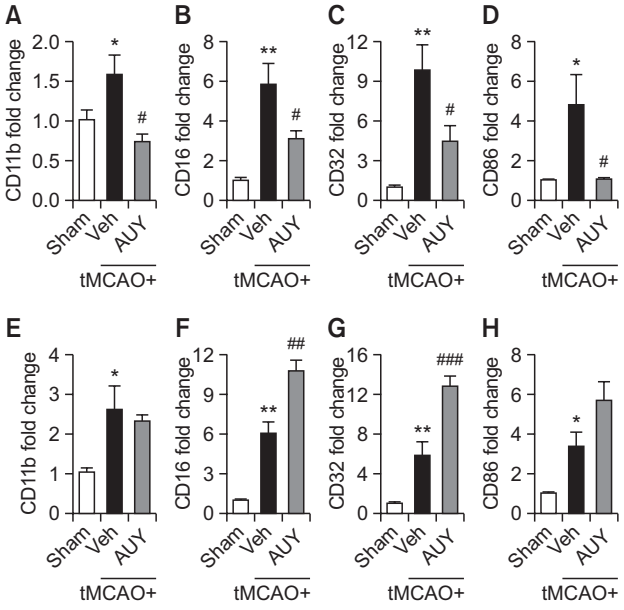

Fig. 1. Effects of AUY954 on mRNA expression levels of M1 surface markers in post-ischemic brain. Mice were challenged with tMCAO. AUY954 (AUY, 5 mg/kg, p.o.) was then administered immediately after reperfusion. Expression levels of CD11b, CD16, CD32, and CD86 mRNAs were assessed in the brain through qRT-PCR analysis at 1 day (A-D) and 3 days (E-H) after tMCAO challenge. n=4-5 mice per group. $^{*}p<0.05$ and $^{**}p<0.01$ versus sham group. $^{\#}p<0.05$, $^{\#\#}p<0.01$, and $^{\#\#\#}p<0.001$ versus vehicle-administered tMCAO mice (tMCAO+veh).

Following indecisive results of $S1P_1$ activity on the expression of M1 surface markers between acute phase (1 day) and chronic phase (3 days) of ischemia, we next determined the role of $S1P_1$ signaling in functionally important soluble markers of M1 polarized cells. Soluble markers of M1 polarized cells such as proinflammatory cytokines are inflammatory mediators. These soluble markers are considered to play functionally more important role of M1 polarized cells because they are mainly involved in the augmentation of inflammatory cascades following ischemic insult (Patel *et al*., 2013; Doll *et al*., 2014; Xiong *et al*., 2016). After receiving AUY954, mice showed significant attenuation in mRNA expression levels of TNF-α, IL-6, and IL-1β at 1 day after tMCAO challenge, confirming that suppression of $S1P_1$ in the ischemic brain reduced inflammatory response of polarized immune cells through inhibition of functionally important M1-polarized markers (Fig. 2A-2C). Importantly, these effects of $S1P_1$ suppression in attenuation of expression of M1 soluble markers in the ischemic brain lasted up to the chronic stage of ischemic challenge as a single administration of AUY954 at the time of reperfusion attenuated expression levels of TNF-α, IL-6, and IL-1β at 3 days following ischemic challenge (Fig. 2D-2F). These data on M1 soluble markers clearly indicated that despite having no effects on M1 surface markers, $S1P_1$ suppression remarkably attenuated M1 polarization even at 3 days after ischemic challenge. Collectively, these data demonstrated that suppression of $S1P_1$ activity inhibited M1 polarization in post-ischemic brain.

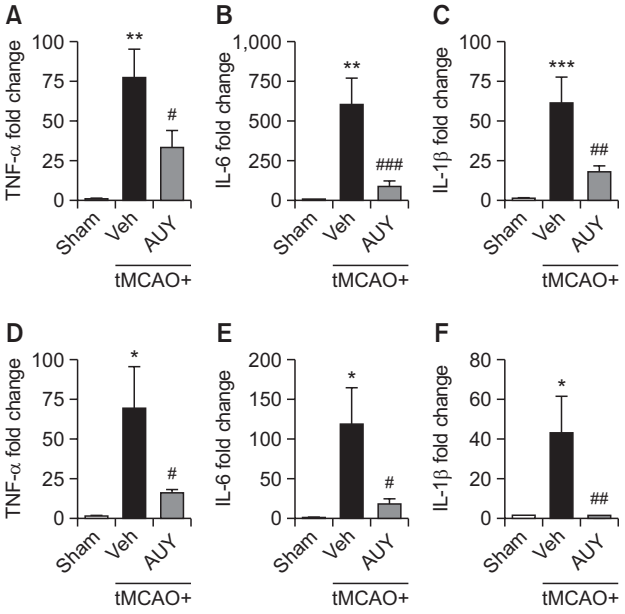

Fig. 2. Effects of AUY954 on mRNA expression levels of M1 soluble markers in post-ischemic brain. Mice were challenged with tMCAO. AUY954 (AUY, 5 mg/kg, p.o.) was then administered immediately after reperfusion. Expression levels of TNF-α, IL-6, and IL-1β mRNAs were assessed in the brain through qRT-PCR analysis at 1 day (A-C) and 3 days (D-F) after tMCAO challenge. n=4-5 mice per group. *p<0.05, **p<0.01 and ***p<0.001 versus sham group. $^{\#}p$<0.05, $^{\#\#}p$<0.01, and $^{\#\#\#}p$<0.001 versus vehicle-administered tMCAO mice (tMCAO+veh).

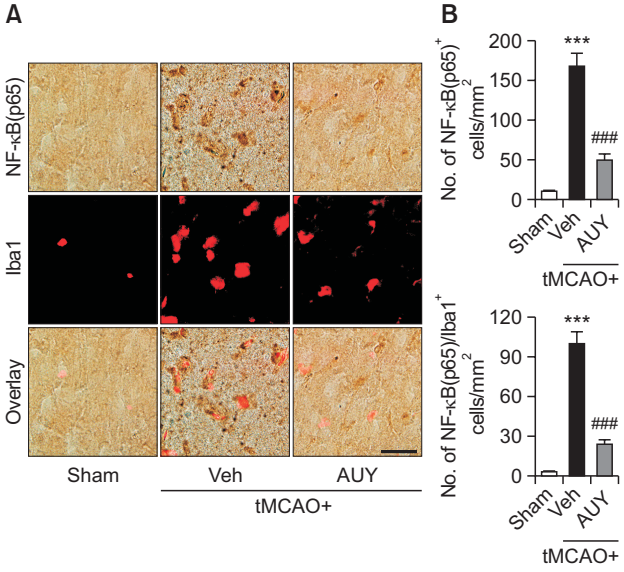

Fig. 3. Effects of AUY954 on microglial NF-κB expression in post-ischemic brain. Mice were challenged with tMCAO. AUY954 (AUY, 5 mg/kg, p.o.) was then administered immediately after reperfusion. Expression of NF-κB(p65) and microglial NF-κB(p65) was determined through immunohistochemical analysis at 3 days after tMCAO challenge. (A) Representative photographs and (B) quantification of the number of NF-κB(p65)- and NF-κB(p65)/Iba1-immunopositive cells in the ischemic core region. Scale bar=50 μm. n=4-5 mice per group. ***p<0.001 versus sham group. $^{\#\#\#}p$<0.001 versus vehicle-administered tMCAO mice (tMCAO+veh).

$S1P_1$-mediated M1 polarization following tMCAO challenge mainly occurs in activated microglia

NF-κB is an important signaling molecule for M1 polarization in neuroinflammatory diseases, including cerebral ischemia (Xia *et al*., 2015; Zhou *et al*., 2018). In order to reaffirm the role of $S1P_1$ in M1 polarization, we determined NF-κB expression in post-ischemic brain at 3 days after tMCAO challenge by immunohistochemical analysis. We found that suppressing $S1P_1$ activity dramatically attenuated the number of NF-κB(p65)-immunopositive cells in post-ischemic brain at 3 days after ischemic challenge (Fig. 3). These results further demonstrated that suppressing $S1P_1$ activity could attenuate M1 polarization in post-ischemic brain even at 3 days after ischemic challenge. Next, we determined whether M1 polarization could occur in activated microglia of post-ischemic brain. In previous studies, we have observed activated microglia with amoeboid morphology in the ischemic core region at 3 days following tMCAO challenge (Gaire *et al*., 2015; Sapkota *et al*., 2017; Gaire *et al*., 2018a). These amoeboid microglia as main source of proinflammatory mediators are believed to be polarized toward M1 phenotype (Tam and Ma, 2014). In addition, activated microglia can release proinflammatory mediators through transcriptional activation of NF-κB. Therefore, NF-κB activation is considered a major signaling pathway also for microglial M1 polarization following neuroinflammatory disorders, particularly cerebral ischemia (Xia *et al*., 2015). Consequently, we tempted to determine whether suppression of $S1P_1$ activity in the ischemic brain affected NF-κB signaling in activated microglia. The effect of AUY954 on tMCAO-induced NF-κB expression in the activated microglia was determined by double immunolabeling with NF-κB and Iba1 in post-ischemic brain at 3 days following ischemic challenge. AUY954 administration dramatically decreased NF-κB expression in the activated microglia (Fig. 3) as evidenced by attenuated NF-κB(p65)/Iba1-double immunopositive cells in the ischemic core region. These results further confirmed that suppression of $S1P_1$ activity could lead to attenuation of M1 polarization through inhibition of NF-κB-mediated inflammatory signaling in microglia. These *in vivo* effects of $S1P_1$ activity on M1 microglial polarization were further supported by our previous study in which suppression of $S1P_1$ activity in LPS-stimulated mouse primary microglia resulted in attenuated mRNA expression of soluble markers (TNF-α, IL-6, and IL-1β) of M1 polarization (Gaire *et al*., 2018a). Our previous study results and current findings indicate that the pathogenic role of $S1P_1$ activity in post-ischemic brain is associated with M1 polarization of activated microglia.

Suppressing $S1P_1$ activity augments tMCAO-induced M2 polarization

After confirming the association between $S1P_1$ activity and ischemia-induced M1 microglial polarization in which attenuation of $S1P_1$ activity in ischemic brain reduced the skewing of microglia toward M1 phenotype, we next determined whether $S1P_1$ activity was also associated with M2 polarization in post-ischemic brain. First, mRNA expression levels of CD206, CCL-22, Arg1, IL-10, TGF-β1, and Ym1 as markers of M2 polarized cells were determined in post-ischemic brain at 1 and 3 days after ischemic challenge. As previously suggested (Hu *et al*., 2012; Zhang *et al*., 2018), most M2 markers were upregulated

at mRNA expression levels in post-ischemic brain at both time points. Mice that received AUY954 showed further significant increase in the expression of Arg1 and TGF-β1 at 1 day after ischemic challenge (Fig. 4A-4D), revealing that suppression of $S1P_1$ activity in the ischemic brain skewed polarized cells toward the anti-inflammatory M2 phenotype. The role of $S1P_1$ attenuation in augmented M2 polarization was also observed in post-ischemic brain of mouse at 3 days after ischemic challenge, in which AUY954 administration resulted in increased expression levels of Arg1, CD206, and TGF-β1 (Fig. 4E-4H). These data indicate that suppression of $S1P_1$ activity in the ischemic brain can enhance the anti-inflammatory M2 polarization, which could be a key mechanism for neuroprotective effects of $S1P_1$ suppression in post-ischemic brain (Gaire *et al*., 2018a). Collectively, these data suggest that $S1P_1$ activity in ischemic brain could regulate differentially polarized microglia toward their neurotoxic phenotype.

Suppressing $S1P_1$ activity reduces MAPK phosphorylation and increases Akt phosphorylation in post-ischemic brain

$S1P_1$ activation is linked to effector pathways such as MAPKs and PI3K/Akt (Choi and Chun, 2013). Interestingly, these pathways are also linked to M1/M2 polarization with contrasting roles (Jiang *et al*., 2001; Olson *et al*., 2007; Pan *et al*., 2013; Vergadi *et al*., 2017). MAPKs activation triggers M1 polarization whereas PI3K/Akt activation increases M2 polarization and attenuates M1 polarization. We determined whether activation of these effector pathways was involved in $S1P_1$-dependent M1/M2 polarization in post-ischemic brain through Western blot analysis. All three MAPKs, including ERK1/2, p38, and JNK, were activated in post-ischemic brain as evidenced by their increased phosphorylation which was significantly attenuated by AUY954 administration (Fig. 5). On the other hand, Akt phosphorylation was reduced in post-ischemic brain, which was reversed by AUY954 administration (Fig. 5). These data demonstrated that $S1P_1$ activation could influence MAPKs and PI3K/Akt in post-ischemic brain. Furthermore, these data indicate that both increased M1 polarization and decreased M2 polarization in post-ischemic brain upon $S1P_1$ activation may be regulated by MAPKs and PI3K/Akt pathways.

DISCUSSION

In this study, we identified a critical role of $S1P_1$ in M1 polarization, particularly microglial M1 polarization, in post-ischemic brain. Pharmacological suppression of $S1P_1$ activity attenuated mRNA upregulation of M1 polarization markers and microglial NF-κB expression in post-ischemic brain. Moreover, it attenuated activation of MAPKs known to be not only $S1P_1$-associated effector pathways, but also M1 polarization-relevant signaling pathways. In addition to its regulatory role in M1 polarization, $S1P_1$ also influenced M2 polarization in post-ischemic brain. Suppressing $S1P_1$ activity further increased mRNA expression levels of M2 markers and activated M2 polarization-relevant PI3K/Akt in post-ischemic brain. Therefore, the current study identified $S1P_1$ as a novel regulator for M1/M2 polarization in post-ischemic brain.

Receptor-mediated S1P signaling in regulation of immune

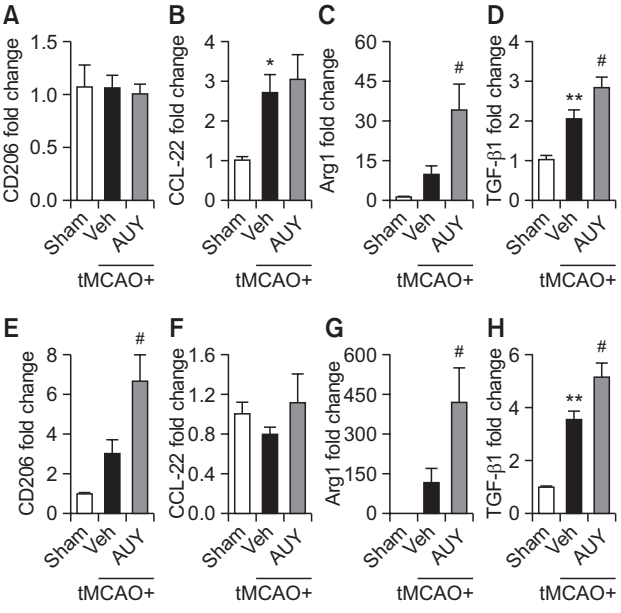

Fig. 4. Effects of AUY954 on mRNA expression levels of M2 markers in post-ischemic brain. Mice were challenged with tMCAO. AUY954 (AUY, 5 mg/kg, p.o.) was then administered immediately after reperfusion. Expression levels of CD206, CCL-22, Arg1, and TGF-β1 mRNAs were assessed in the brain through qRT-PCR analysis at 1 day (A-D) or 3 days (E-H) after tMCAO challenge. n=4-5 mice per group. **p*<0.05 and ***p*<0.01 versus sham group. #*p*<0.05 versus vehicle-administered tMCAO mice (tMCAO+veh).

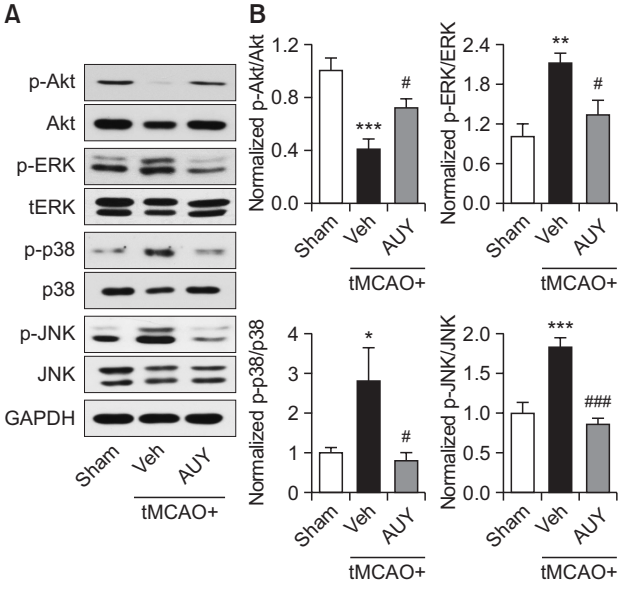

Fig. 5. Effects of AUY954 on phosphorylation of Akt and MAPKs in post-ischemic brain. Mice were challenged with tMCAO. AUY954 (AUY, 5 mg/kg, p.o.) was then administered immediately after reperfusion. (A) Expression levels of p-Akt, Akt, p-ERK1/2, ERK1/2, p-p38, p38, p-JNK, JNK, and GAPDH proteins were assessed in the brain by Western blot analysis at 1 day after tMCAO challenge. (B) Quantifications. n=4 mice per group. **p*<0.05, ***p*<0.01, and ****p*<0.001 versus sham group. #*p*<0.05 and ###*p*<0.001 versus vehicle-administered tMCAO mice (tMCAO+veh).

cell biology including their recruitment, migration, and proliferation has been well characterized (Cyster and Schwab, 2012; Aoki *et al*., 2016). This signaling also triggers proinflammatory responses through modulation of immune cells (Chi, 2011; Aoki *et al*., 2016; Tsai and Han, 2016). Proinflammatory role of S1P signaling has also been reported in the CNS (Moon *et al*., 2015; Qin *et al*., 2017; Rothhammer *et al*., 2017), particularly in activated microglia (Noda *et al*., 2013; Qin *et al*., 2017; Rothhammer *et al*., 2017). In LPS-stimulated microglia, FTY720, a non-selective S1P receptor modulator, attenuates microglial activation and suppresses production of proinflammatory cytokines (TNF-α, IL-6, and IL-1β) (Noda *et al*., 2013). In particular, these proinflammatory cytokines are featured soluble markers of M1 polarization (Mirendil *et al*., 2015; Lan *et al*., 2017), indicating that receptor-mediated S1P signaling is a possible regulator of M1 polarization. In fact, FTY720 administration can reduce ischemia-induced tissue damage in the white matter through inhibiting microglial M1 polarization (Qin *et al*., 2017). Moreover, exogenous S1P exposure can increase expression of representative soluble markers of M1 polarization including TNF-α, IL-1β, and iNOS in LPS-stimulated BV2 microglia (Nayak *et al*., 2010). It can also trigger TNF-α upregulation in post-ischemic brain (Moon *et al*., 2015). These independent studies indicate that S1P signaling may be a critical factor to regulate M1 polarization in neuroinflammatory diseases, including cerebral ischemia. Notably, our previous study has identified $S1P_3$ as the first S1P receptor subtype to have a regulatory role for M1 polarization in post-ischemic brain, showing that $S1P_3$ signaling can trigger microglial M1 polarization as a core pathogenesis of cerebral ischemia (Gaire *et al*., 2018b). Besides $S1P_3$, $S1P_1$ could also play critical roles in M1 microglial polarization in post-ischemic brain because $S1P_1$ activation can promote morphological changes of activated microglia from ramified to amoeboid cells in the ischemic core region (Gaire *et al*., 2018a). Amoeboid microglia in post-ischemic brain are believed to be polarized into M1 phenotypes because they can secret proinflammatory mediators as featured markers of M1 microglia (Tam and Ma, 2014). Indeed, the current study revealed that $S1P_1$ was an additional S1P receptor subtype to promote M1 polarization in the brain following ischemic insult because suppressing $S1P_1$ activity attenuated expression levels of both soluble and surface M1 markers in post-ischemic brain. Furthermore, in the current study, we found that this regulatory action of $S1P_1$ in M1 polarization occurred in activated microglia in post-ischemic brain because suppressing $S1P_1$ activity attenuated activation of a major M1 signaling, NF-κB expression (Byles *et al*., 2013; Xia *et al*., 2015; Gaire *et al*., 2018b), in activated microglia following ischemic insult. This $S1P_1$-dependent microglial M1 polarization is also supported by our previous *in vitro* study using mouse primary microglia (Gaire *et al*., 2018a), in which $S1P_1$ knockdown can attenuate mRNA expression levels of M1 soluble markers in cultured microglia stimulated with LPS, a well-known trigger of M1 polarization.

Alternatively activated M2 cells can produce a wide range of molecules that help neuronal repair in several neurodegenerative diseases. M2 microglia are involved in neuroprotection either directly by producing growth and neurotrophic factors (Butovsky *et al*., 2006; Thored *et al*., 2009) or indirectly by releasing anti-inflammatory cytokines (IL-4, IL-10) and other markers (Arg1, TGF-β1, Ym1/2) (Cherry *et al*., 2014). In ischemia-induced white matter injury, FTY720 administration can promote M2 microglial polarization (Qin *et al*., 2017), indicating that receptor-mediated S1P signaling might modulate skewing of activated microglia toward their M2 phenotype. In the current study, we found that suppressing $S1P_1$ activity increased expression levels of M2 markers such as Arg1 and TGF-β1 at both acute and chronic phases of cerebral ischemia. These data suggest that suppressing $S1P_1$ activity could skew activated microglia toward their anti-inflammatory phenotype in post-ischemic brain. Combined with the role of $S1P_1$ in promoting M1 polarization, our current study demonstrated that $S1P_1$ contributed to brain injury in cerebral ischemia by driving cell polarization toward inflammatory phenotypes likely by promoting detrimental M1 polarization and suppressing beneficial M2 polarization. This notion further indicates that $S1P_1$ antagonism could not only reduce brain damage, but also facilitate damage repair in the brain after ischemic challenge. Interestingly, the currently identified S1P receptor subtype, $S1P_1$, seems to influence M1/M2 polarization differently compared to the previously identified $S1P_3$ in post-ischemic brain (Gaire *et al*., 2018b). $S1P_3$ was linked to M1 polarization, but not M2 polarization (Gaire *et al*., 2018b), indicating that $S1P_3$ antagonism could reduce brain damage in cerebral ischemia rather than enhance brain damage repair.

$S1P_1$ is coupled with G_i protein, leading to activation of several effector pathways including PI3K/Akt and MAPKs (Choi and Chun, 2013). A growing body of evidence has suggested that these PI3K/Akt and MAPKs play critical roles in M1/M2 polarization (Jiang *et al*., 2001; Olson *et al*., 2007; Pan *et al*., 2013; Vergadi *et al*., 2017): MAPKs phosphorylation promotes M1 polarization whereas Akt phosphorylation not only promotes M2 polarization, but also suppresses M1 polarization. Persistent activation of MAPKs can trigger transcriptional activation of NF-κB signaling (Olson *et al*., 2007; Gu *et al*., 2013; Pan *et al*., 2013), leading to secretion of various proinflammatory mediators such as cytokines and chemokines (Gabriel *et al*., 1999; Mattson and Camandola, 2001; Harari and Liao, 2010). Additionally, Akt activation can suppress M1 polarization and augment M2 polarization through negative regulation of NF-κB signaling (Wang *et al*., 2016; Vergadi *et al*., 2017). In the current study, suppressing $S1P_1$ activity attenuated MAPKs phosphorylation and increased Akt phosphorylation in post-ischemic brain, demonstrating that $S1P_1$ might influence G_i-dependent effector pathways in the pathogenesis of cerebral ischemia. Moreover, given roles of these effector pathways in the regulation of M1/M2 polarization (Jiang *et al*., 2001; Olson *et al*., 2007; Pan *et al*., 2013; Vergadi *et al*., 2017), their altered activation states by suppressing $S1P_1$ activity again support the notion that $S1P_1$ in post-ischemic brain can regulate both M1 and M2 polarization by shifting detrimental phenotypes.

In summary, the current study identified $S1P_1$ as a novel regulator of M1/M2 polarization in cerebral ischemia, demonstrating its pathogenic role. In particular, functional roles of $S1P_1$ in both detrimental M1 polarization and beneficial M2 polarization were distinguishable from $S1P_3$ that was involved solely in M1 polarization. Our findings further suggest that $S1P_1$ antagonism in post-ischemic brain could attenuate brain damage and enhance brain repair by attenuating M1 polarization and promoting M2 polarization. Now, two S1P receptor subtypes, $S1P_1$ and $S1P_3$, have been identified with different roles linked to M1/M2 polarization in cerebral ischemia. Other S1P receptor subtypes might also have certain roles

for regulating M1/M2 polarization in post-ischemic brain such as $S1P_2$, a recently identified pathogenic factor for cerebral ischemia through vascular dysfunction (Kim *et al*., 2015) that can trigger NF-κB activation in endothelial cells (Zhang *et al*., 2013). Therefore, it is tempting to address roles of other S1P receptors in regulating M1/M2 polarization in the brain following ischemic challenge in the future.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by grants from the National Research Foundation (NRF) to JWC [NRF-2017R1A2B4002818 and NRF-2014M3A9B6069339].

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