



# M2 Phenotype Microglia-derived Cytokine Stimulates Proliferation and Neuronal Differentiation of Endogenous Stem Cells in Ischemic Brain

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Microglia play a key role in the immune response and inflammatory reaction that occurs in response to ischemic stroke. Activated microglia promote neuronal damage or protection in injured brain tissue. Extracellular signals polarize the microglia towards the M1/M2 phenotype. The M1/M2 phenotype microglia released pro- and anti-inflammatory cytokines which induce the activation of neural stem/progenitor cells (NSPCs). In this study, we investigated how the cytokines released by microglia affect the activation of NSPCs. First, we treated BV2 cells with a lipopolysaccharide (LPS; 20 ng/ml) for M1 phenotype microglia and interleukin-4 (IL-4; 20 ng/ml) for M2 phenotype microglia in BV2 cells. Mice were subjected to transient middle cerebral artery occlusion (tMCAO) for 1 h. *In vivo*, brain sections containing the subventricular zone (SVZ) were cultured in conditioned media of M1 and M2 phenotype-conditioned media for 3 d. We measured the expression of cytokines in the conditioned media by RT-PCR and ELISA. The M2 phenotype microglia-conditioned media led to the proliferation and neural differentiation of NSPCs in the ipsilateral SVZ after ischemic stroke. The RT-PCR and ELISA results showed that the expression of TGF- $\alpha$  mRNA was significantly higher in the M2 phenotype microglia-conditioned media. These data support that M2 phenotype microglia-derived TGF- $\alpha$  is one of the key factors to enhance proliferation and neural differentiation of NSPCs after ischemic stroke.

**Key words:** Ischemic stroke, TGF- $\alpha$ , microglia, neural stem/progenitor cells, proliferation, neuronal differentiation

## INTRODUCTION

Stroke is the dangerous disease accounting for the leading cause

of death or disorder worldwide. While stroke has a high mortality rate and causes serious suffer after recovery, there is no complete therapy. In ischemic stroke, inflammatory cells such as macrophage and monocyte move into the lesion area [1, 2]. Furthermore, microglia can quickly undergo morphologic transformation from a resting condition to an activating condition, where they become virtually indistinguishable from circulating macrophages [3, 4]. Activated microglia have been shown to release a variety of cytokines contributing to cell damage or cell repair leading to

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exacerbated brain injury [5]. In ischemic stroke, resting microglia can be polarized to the M1 phenotype, or alternatively to pro-phagocytic M2 phenotype through different signals [6-8]. The M1 phenotype promotes the transcriptional activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and generates high levels of pro-inflammatory cytokines and oxidative metabolites [9]. In contrast, the M2 phenotype is triggered by anti-inflammatory cytokines [10, 11], which are thought to inhibit inflammation and enhance tissue repair and wound healing [12].

In ischemic stroke, the progenitor cells in SVZ become into a new neural cell that could be one of the therapies to neurogenesis [13]. Following focal ischemia, enhanced neurogenesis including an increased proliferation of neural progenitors in the ipsilateral SVZ, migration of neuroblasts from the SVZ to the injured striatum, and functional differentiation and integration of newly generated cells in the damaged brain has been observed in many studies [14]. Activated microglia accumulate in postnatal SVZ and induce neurogenesis and oligodendrogenesis via released cytokines [15]. We and others have investigated how microglia affect the activation of progenitor cells in the SVZ after ischemic stroke. This is still unclear and controversial.

Therefore, the objective of this study is to explore further the effect of microglia-derived cytokine on the process of proliferation, neurogenesis and neural differentiation of NSPCs in SVZ after ischemic stroke.

## MATERIALS AND METHODS

### *BV2 cell culture*

Murine BV2 cell line, which was originally immortalized after infection with a v-raf/v-myc recombinant retrovirus [16], and maintained at 37°C at 5% CO<sub>2</sub> at Roswell Park Memorial Institute (RPMI)-1640 (Thermo Scientific) with 10% fetal bovine serum (FBS; Thermo Scientific) and 1% penicillin/streptomycin (Thermo Scientific). To polarize the M1 phenotype activated by LPS or M2 phenotype activated by IL-4, BV2 cells were treated with LPS (20 ng/ml, Sigma-Aldrich) and IL-4 (20 ng/ml, cell signaling), respectively. Cultured media containing the BV2 cells were transferred to serum-free RPMI-1640 media after 24 h stimulation and cultured further for 48 h. The serum-free RPMI-1640 conditioned media was collected with a 0.22  $\mu$ m filter and stored at -70°C until use [13]. To determine the role of TGF- $\alpha$  in M2 conditioned media, TGF- $\alpha$  antibody (0.2 ng/ml) was added to the M2 conditioned media for TGF- $\alpha$  neutralization. The control media used serum-free RPMI-1640 media.

### *Experimental animals*

Adult male ICR mice (8 weeks old, Coatech) were used in this study and kept under a 12 h light/dark cycle with freely accessible food and water. All animal experiments were approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University Health System (2011-0212) and performed in accordance with the National Institute of Health guidelines for the Care and Use of Laboratory Animals.

### *Transient middle cerebral artery occlusion*

Three days prior to tMCAO, all experimental animals were labeled with 5-bromo-2'-deoxyuridine (BrdU; 50 mg/kg, daily intraperitoneal (IP) injection, Sigma-Aldrich). Before the surgery, animals were anesthetized with Zoletil50<sup>®</sup> (30 mg/kg) and Rompun (10 mg/kg). Body temperature was maintained 37.5°C with a heating pad. Ischemic stroke was induced using an intraluminal suture as described earlier [17]. In brief, an uncoated 15 mm segment of 6-0 nylon mono filament suture with the tip rounded by a flame was inserted into the internal carotid artery via common carotid artery until the suture occluded the middle cerebral artery. After 60 min occlusion, the suture was removed and surgical incisions were closed. Animals were kept on the heating pad before awakening. Food and water were freely accessible in their cages.

### *Immunocytochemistry*

The characteristics of the BV2 cells after LPS or IL-4 induced stimulation were confirmed by immunocytochemistry. Samples were fixed with 4% paraformaldehyde, permeabilized with PBS in 0.025% Triton X-100 and blocked with goat serum at room temperature (RT). Primary antibodies as anti-rabbit CD68 (1:200, Santa Cruz) and anti-goat CD206 (1:200, Santa Cruz) were incubated for 3 h at RT. Samples were washed with PBS. The samples were incubated with Alexa-488 donkey anti-rabbit and Alexa-594 donkey anti-goat (1:200, Santa Cruz) for 1 h at RT. The samples were washed with PBS and counterstained with 1  $\mu$ g/ml 4',6-diamidino-2-phenylindole (DAPI; 1:500, Invitrogen) for 1 min at RT.

### *Organotypic cultures of nervous tissue*

Organotypic culture was prepared according to a previously described method [18]. After 6 h reperfusion, Mice were decapitated after 6 h reperfusion. The extracted brain samples were embedded in 5% low melting agarose gel and hardened at RT. The embedded brains were serially cut in the coronal plane into 200  $\mu$ m thick sections using a vibratome. The sectioned brains were transiently stored in artificial cerebrospinal fluid (ACSF; 119 mM

NaCl, 26.2 mM NaHCO<sub>3</sub>, 2.5 mM KCl, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 1.3 mM MgCl<sub>2</sub>, 10 mM glucose, and 2.5 mM CaCl<sub>2</sub>). ACSF was sterilized with a 0.22 µm filter apparatus and oxygenated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> for 10~15 min before use. The sliced brains with SVZ region were placed on a cell culture insert with 0.4 µm pore size (FALCON) in 6-well plates. The cell culture inserts were filled with 2 ml conditioned media (M0, M1, and M2 phenotypes). The sliced brain samples were incubated in 5% CO<sub>2</sub> incubator at 37°C for 72 h.

### Immunohistochemistry

All samples were fixed with 4% paraformaldehyde for 1 h and then washed with PBS with 1% Tween-20. For BrdU staining, the sliced brains were rinsed in PBS, denatured in 4 N HCl at RT for 30 min, and neutralized in 0.1 M boric acid for 10 min. Samples were rinsed and blocked with PBS include 10% FBS and 1% Tween 20. Primary antibodies of anti-rabbit Ki67 (1:500, Abcam), anti-mouse BrdU (1:200, Thermo Scientific), anti-rabbit DCX (1:200, Abcam), anti-rabbit NeuN (1:200, Abcam), and anti-rabbit Olig2 (1:200, Abcam) were added to the samples and incubated for 48 h at 4°C. Samples were washed with PBS with 1% Tween 2, followed by incubation with Alexa-594 donkey anti-rabbit and Alexa-488 donkey anti-mouse (1:200, Santa Cruz) at RT for 1 h. Samples were washed with PBS and stained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI; 1:500, Invitrogen) for 3 min at RT. The stained brain samples were captured using LSM 700 confocal microscope (Carl Zeiss).

### Reverse transcription polymerase chain reaction

Total RNA was extracted from BV2 cells by TRIzol (Invitrogen) following the instructions protocol and the mRNA was quantified by spectrophotometric analysis. Total RNA (2 µg) from each sample was transcribed into cDNA and synthesized PCR amplicon using SuperScript III One-Step RT-PCR System with platinum<sup>®</sup> Taq DNA Polymerase (Invitrogen) according to the manufacturer's instructions; The following primers were used: TGF-α primer pairs forward: TACCGCTGGGTATCCTGTTA and reverse: TTCTCATGTCTGCAGACGAG, IL-10 primer pairs forward: CCAAGCCTTATCGGAAATGA and reverse: TTTTCACAGGGGAGAATCG and GAPDH primer pairs forward: TGGCACAGTCAAGGCTGAGA and reverse: CTTCTGAGTGGCAGTGAATGG. The thermal cycling conditions were set to 95°C for 10 min, followed by 95°C for 15 sec, 60°C for 30 sec, and 68°C for 1 min, repeated 40 times. The mRNA expression was normalized to the levels of GAPDH.

### ELISA assay

To determine the soluble TGF-α concentration in the different

phenotypes of supernatant in BV2 cells, the conditioned media were measured by the mouse TGF-α ELISA Kit (E-EL-M1190, Elabscience) according to the manufacturer's instructions. All steps were performed at 37°C. In a 96-well plate which is coated with the capture antibody, 100 ul of the standard solution, blank and samples were loaded for 90 min incubation. Absorbance was read at 450 nm using a microplate reader.

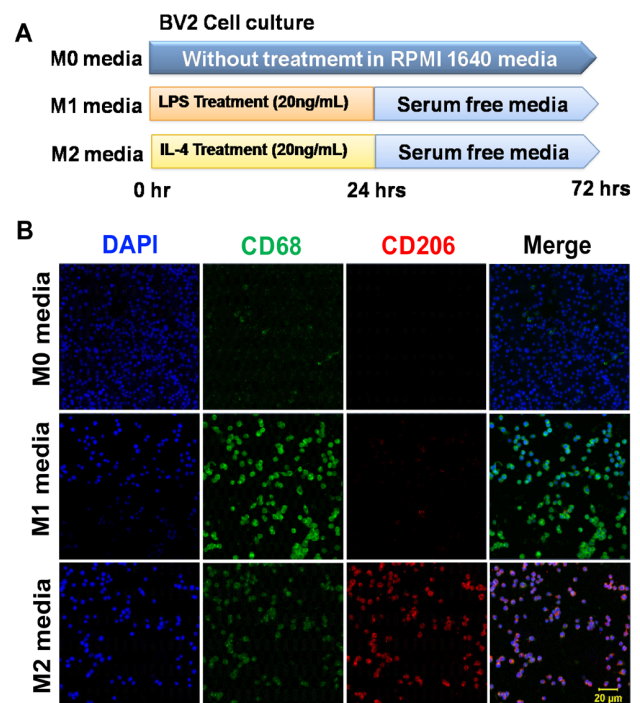
### Statistical analysis

Statistical comparisons were made between multiple groups using analysis of variance (ANOVA) followed by Tukey's Honestly significant difference (HSD) test. The level of significance was set at p≤0.05. Data were expressed as the mean±standard deviation (SD).

## RESULTS

### LPS or IL-4 induce M1/M2 type polarization of BV2 microglia cell

To confirm the polarization of microglia, BV2 microglia were



**Fig. 1.** Polarization of BV2 microglial cells using conditioned media. (A) Schematic time course of the *in vitro* experiments. (B) Immunohistochemistry of CD68 (M1 phenotype microglia; green) and CD206 (M2 phenotype microglia; red). In BV2 microglial cell, the expression of CD68 significantly increased in M1 conditioned media compared with M2 conditioned media. CD206 is expressed only in M2 conditioned media (Scale bar=20 µm, n=5/group).

treated with the LPS (M1 phenotype) or IL-4 (M2 phenotype) (Fig. 1A). After 72 h incubation, these supernatants were harvested in the M1 conditioned media and M2 conditioned media, respectively, from the BV2 cell culture. Serum free RPMI 1640 media were labeled as M0 conditioned media (M0 CM). We performed immunocytochemistry against CD 68 (M1 phenotype) and CD 206 (M2 phenotype) [19] in BV2 microglia cells polarized by LPS or IL-4 for 72 h (Fig. 1B). Overexpression of CD68 was observed in BV2 cells induced by M1 conditioned media, however, CD68 expression decreased in M2 conditioned media. CD206 expression was observed only in BV2 microglia cells induced by M2 conditioned media.

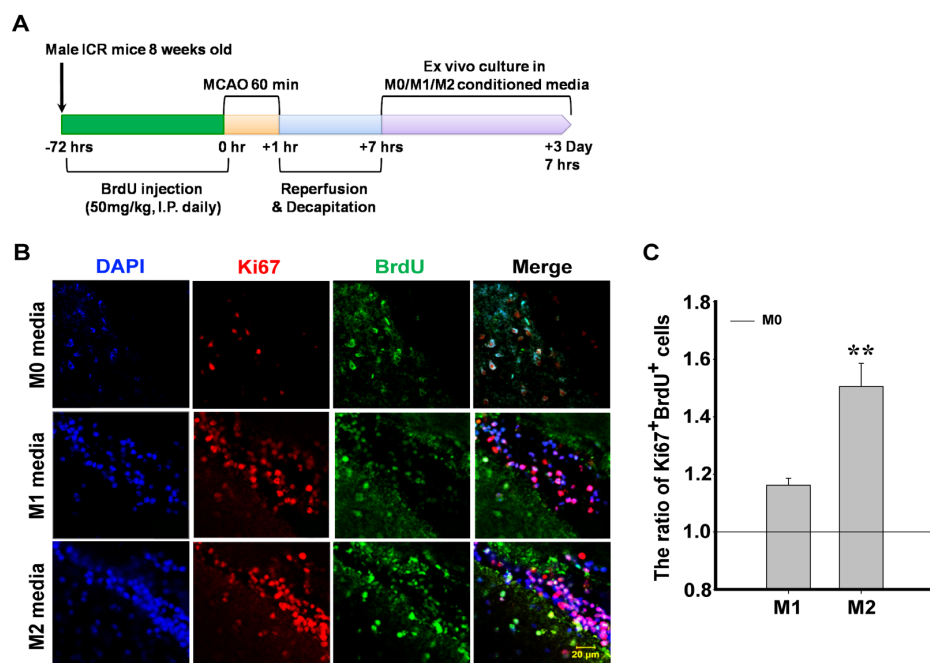
### ***M2 conditioned media stimulates the cellular proliferation in SVZ of the organotypic brain after ischemic stroke***

To investigate whether M0, M1, and M2 conditioned media affect cellular proliferation in SVZ of the organotypic brain after ischemic stroke, *ex vivo* organotypic culture was performed in 3 different conditioned media for 3 d (Fig. 2A). Ki67 expression increased significantly during the G1-, S-, M- and G2-phase of the cell cycle. BrdU incorporate into the newly synthesized DNA strands of S-phase cells and is useful for estimating the fraction of cells in S-phase. We counted the number of Ki67<sup>+</sup> and BrdU<sup>+</sup>-double labeled cells to confirm the cellular proliferation in SVZ for 7 d after ischemic stroke. Immunohistochemistry showed that Ki67<sup>+</sup> and BrdU<sup>+</sup>-double labeled cells were significantly increased in M2 conditioned media compared with M0 and M1 conditioned

media (Fig. 2B and 2C). These results suggest that M2 conditioned media strongly promoted the cellular proliferation in SVZ after ischemic stroke.

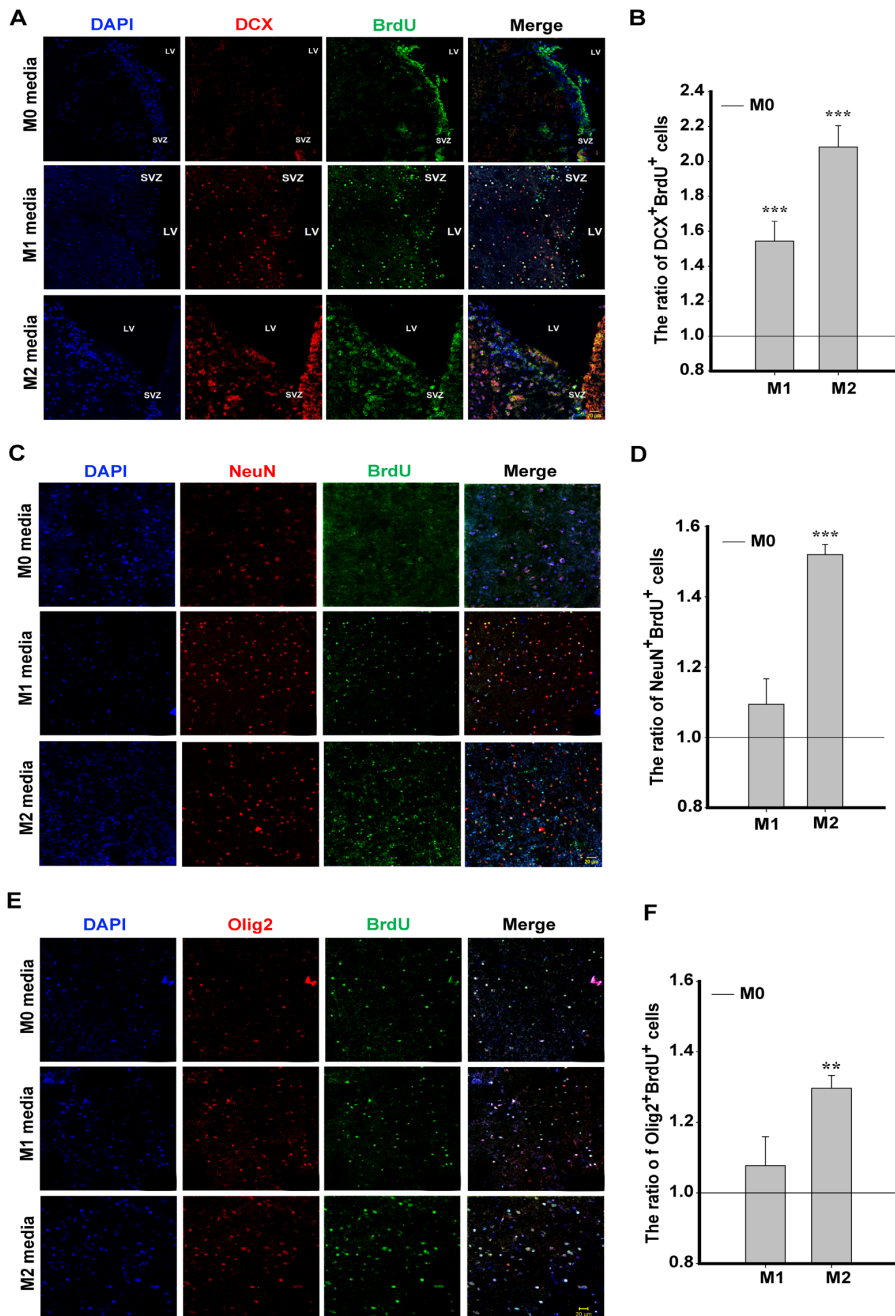
### ***M2 conditioned media stimulates the neurogenesis, oligodendrogenesis and neural differentiation of SVZ-NSPCs in the organotypic brain after ischemic stroke***

To determine the characteristics of proliferating in SVZ of the organotypic brain after ischemic stroke, immunohistochemistry was performed using doublecortin (DCX), NeuN, Olig2, and BrdU. DCX, which is a microtubule-associated protein essential for the migration of neurons in the human brain, is highly expressed in neuronal progenitor cells or migrating neuroblasts. The results showed that M2 conditioned media significantly increased DCX<sup>+</sup> and BrdU<sup>+</sup> cells in SVZ compared with M0 and M1 conditioned media after ischemic stroke. The quantification graph shows significantly increased cell number in M2 conditioned media (Fig. 3A and 3B). Next, to determine the neural differentiation of SVZ-NSPCs after ischemic stroke, immunohistochemistry was performed using NeuN, Olig2, and BrdU were performed. NeuN reacts with most neuronal cells in the nervous system. The results showed that M2 conditioned media significantly increased NeuN<sup>+</sup> and BrdU<sup>+</sup> cells in SVZ compared with M1 conditioned media after ischemic stroke. The quantification graph shows significantly increased cell number in the M2 conditioned media (Fig. 3C and 3D). Olig2 is specifically expressed in nervous tissue as a gene regulator of oligodendrogenesis. The result showed



**Fig. 2.** Proliferation SVZ-NSPCs in M2 conditioned media. (A) Schematic time course of the *in vivo* and *ex vivo* experiments. (B) Immunohistochemistry of Ki67 and BrdU in M0, M1, and M2 conditioned media shows the proliferation of SVZ-NSPCs in THE organotypic brain. (C) Quantification graph of the ratio of Ki67<sup>+</sup> and BrdU<sup>+</sup> cells of SVZ-NSPCs in organotypic brain (Scale bar=20  $\mu$ m, \*\* $p$ <0.05 vs. M0 conditioned media,  $n$ =5/group).



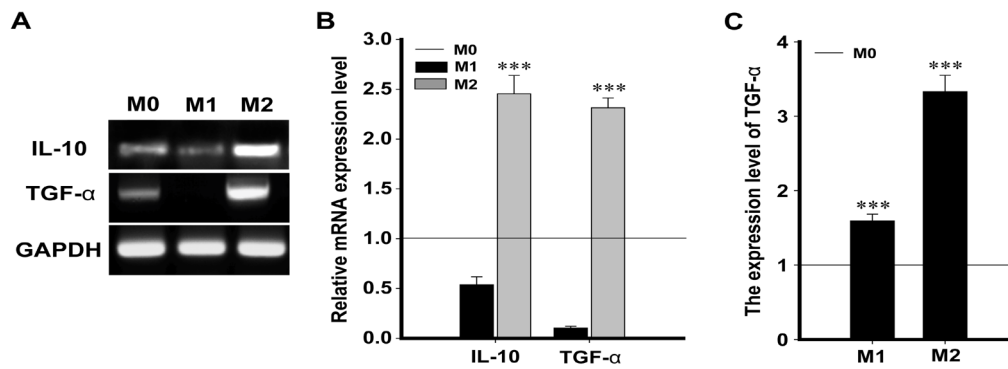


**Fig. 3.** Differentiation of SVZ-NSPCs in M2 conditioned media. M0, M1, or M2 conditioned media induced the differentiation of SVZ-NSPCs in the organotypic brain. (A and B) Immunohistochemistry and quantification graph of the ratio of DCX<sup>+</sup> and BrdU<sup>+</sup> cells of SVZ-NSPCs in the organotypic brain. (C and D) Immunohistochemistry and quantification graph of the ratio of NeuN<sup>+</sup> and BrdU<sup>+</sup> cells of SVZ-NSPCs in the organotypic brain. (E and F) Immunohistochemistry and quantification graph of the ratio of Olig2<sup>+</sup> and BrdU<sup>+</sup> cells of SVZ-NSPCs in the organotypic brain (Scale bar=20 um; \*\*p<0.05, \*\*\*p<0.01 vs. M0 conditioned media; n=5/group).

that M2 conditioned media was significantly increased Olig2<sup>+</sup> and BrdU<sup>+</sup> cells in SVZ compared with M0 and M1 conditioned media after ischemic stroke. The quantification graph shows significantly increased cell number in M2 conditioned media (Fig. 3E and 3F). Together, these results ascertain that M2 conditioned media promotes the neurogenesis, oligodendrocyte and neural differentiation of SVZ-NSPCs after ischemic stroke.

**TGF-α is one of the major cytokines in M2 conditioned media**

Using RT-PCR and ELISA, we subsequently identified a key cytokine in M2 conditioned media, which promoted the proliferation, neurogenesis and neural differentiation of SVZ-NSPCs after ischemic stroke. The expression of IL-10 and TGF-α mRNA was significantly increased in M2 conditioned media compared with M0 and M1 conditioned media (Fig. 4A and 4B). ELISA assay was performed to quantitate TGF-α in the M2 conditioned media. The data showed that the level of TGF-α was



**Fig. 4.** TGF- $\alpha$  is one of the major cytokines in M2 conditioned media. (A) RT-PCR of IL-10 and TGF- $\alpha$  in M0, M1, or M2 conditioned media. IL-10 and TGF- $\alpha$  expression is significantly increased at the mRNA level in M2 conditioned media. (B) The graph depicts the relative mRNA expression of IL-10 and TGF- $\alpha$  in M0, M1, and M2 conditioned media. The expression of these genes is significantly increased in M2 conditioned media. (C) The expression level of TGF- $\alpha$  in M0, M1, and M2 conditioned media using ELISA assay. The quantity of TGF- $\alpha$  is significantly increased in M2 conditioned media compared with M1 (\*\*\*) $p$ <0.01 vs. M0 conditioned media,  $n$ =5/group).

significantly increased in M2 conditioned media compared with M0 and M1 conditioned media (Fig. 4C). The results indicate that TGF- $\alpha$  is one of the major cytokines in M2 conditioned media and may be affected in the proliferation, neurogenesis, and neural differentiation of SVZ-NSPCs after ischemic stroke.

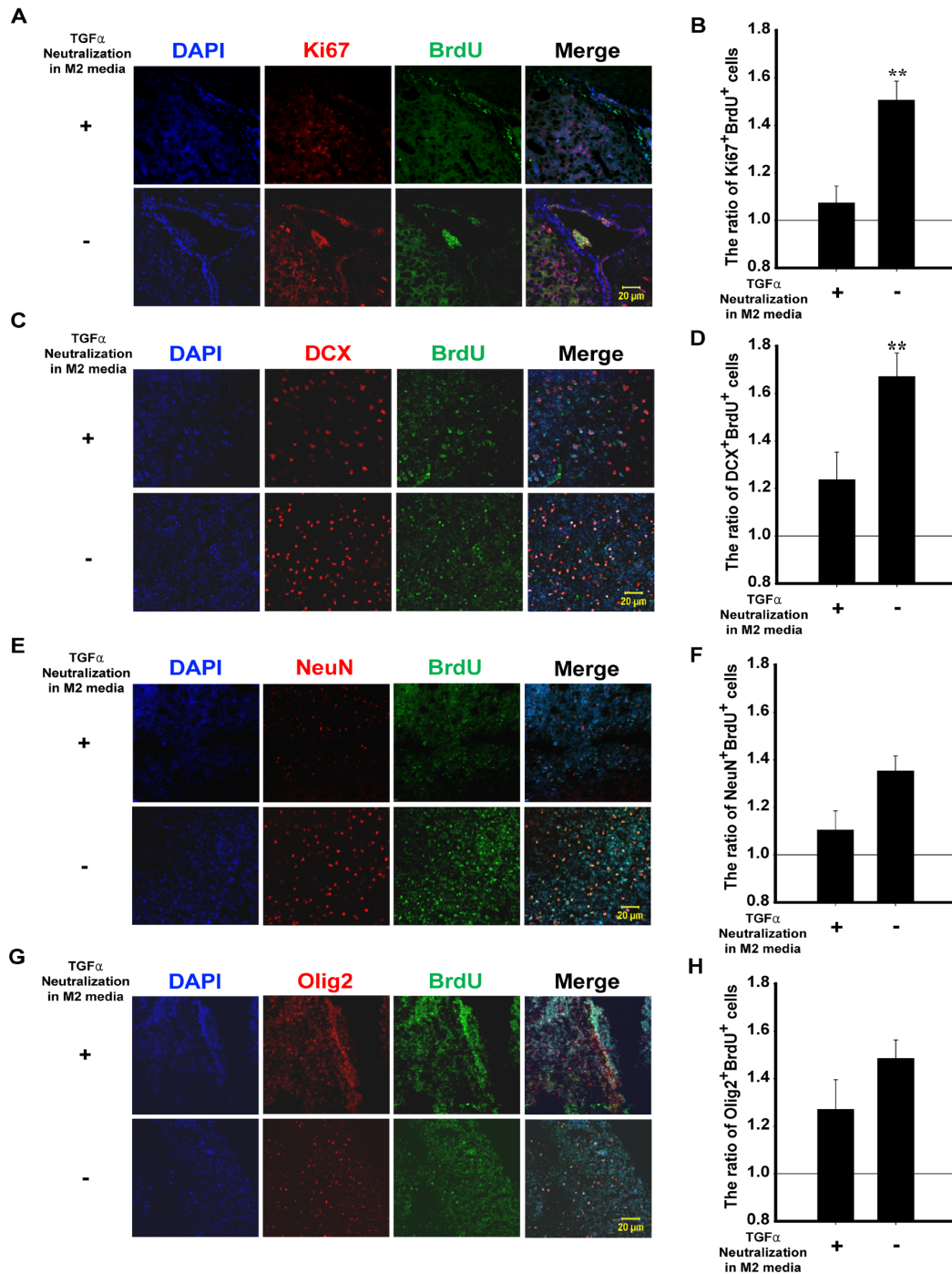
#### ***TGF- $\alpha$ plays key role in the proliferation and neurogenesis of SVZ-NSPCs after ischemic stroke***

To investigate the role of TGF- $\alpha$  in the proliferation, neurogenesis, and neural differentiation of SVZ-NSPCs, we treated the SVZ-NSPCs with or without neutralizing antibody against TGF- $\alpha$  in M2 conditioned media. Immunohistochemistry analyses revealed that Ki67<sup>+</sup> and BrdU<sup>+</sup> of SVZ-NSPCs was significantly increased (1.6 fold) in no neutralized TGF- $\alpha$  in M2 conditioned media (Fig. 5A and 5B). Next, we assessed the neural progenitor cells or migrating neuroblasts of SVZ-NSPCs after ischemic stroke using immunohistochemistry of DCX and BrdU. This data showed that DCX<sup>+</sup> and BrdU<sup>+</sup> of SVZ-NSPCs was significantly increased (1.8 fold) in non-neutralizing TGF- $\alpha$  in M2 conditioned media (Fig. 5C and 5D). To determine the neuronal differentiation of SVZ-NSPCs after ischemic stroke, immunohistochemistry was performed using NeuN and BrdU. This data showed that the number of NeuN<sup>+</sup> and BrdU<sup>+</sup> of SVZ-NSPCs did not significantly change, but not change with or without neutralizing TGF- $\alpha$  in M2 conditioned media (Fig. 5E and 5F). To determine the oligodendrogenesis of SVZ-NSPCs after ischemic stroke, immunohistochemistry was conducted using Olig2 and BrdU. This data showed that the number of Olig2<sup>+</sup> and BrdU<sup>+</sup> cells of SVZ-NSPCs also slightly increased, but not changed with or without neutralizing TGF- $\alpha$  in M2 conditioned media (Fig. 5G and 5H). Therefore, these results suggest that TGF- $\alpha$  might play a

key role in the proliferation and neurogenesis of SVZ-NSPCs after ischemic stroke, but not in neural differentiation.

#### **DISCUSSION**

Neural stem cells (NSCs) are mainly located on the subgranular zone of the dentate gyrus of the hippocampus and the forebrain SVZ in the postnatal mammalian brain [20, 21]. Numerous studies demonstrated that activated microglia are important in neurogenesis in the adult SVZ; however, microglial contribution to neurogenesis is complex [22, 23]. Recently, the role of microglia in neurogenesis has been examined mainly in pathological conditions [24, 25]. Microglia, which is a kind of immune cell, perform main function of immune response in the brain [26-28]. Microglia have two efficiencies which are totally different effects. Once activated by extracellular signals, activated microglia have predominantly harmful effects in the acute stages of ischemic stroke, and that most beneficial effects appear in delayed stages [29, 30]. Microglia also play diverse roles in neurogenesis in both the embryonic and postnatal adult stages [31]. Recently, a study demonstrated that microglia related to neurogenesis of embryonic SVZ. During the late stages of cortical neurogenesis, microglia regulated the size of the neuronal precursor pool through the phagocytosis of Tbr21 and Pax61 cells [32]. Remarkably, most precursor cells, which were targeted by microglia, did not undergo cell death. Activated microglia accumulate in the SVZ during the early postnatal period and then disperse to white matter where they became more ramified. In *in vivo* and *in vitro* studies, activated microglia in the early postnatal SVZ also enhanced neurogenesis and oligodendrogenesis via the released cytokine [15]. The enhancement of neurogenesis was suppressed by a



**Fig. 5.** Effect of TGF- $\alpha$  on the proliferation and neurogenesis of SVZ-NSPCs after cerebral ischemia. (A and B) Immunohistochemistry and quantification graph of the ratio of Ki67<sup>+</sup> and BrdU<sup>+</sup> cells of SVZ-NSPCs in the organotypic brain. (C and D) Immunohistochemistry and quantification graph of the ratio of DCX<sup>+</sup> and BrdU<sup>+</sup> cells of SVZ-NSPCs in organotypic brain. (E and F) Immunohistochemistry and quantification graph of the ratio of NeuN<sup>+</sup> and BrdU<sup>+</sup> cells of SVZ-NSPCs in the organotypic brain. (G and H) Immunohistochemistry and quantification graph of the ratio of Olig2<sup>+</sup> and BrdU<sup>+</sup> cells of SVZ-NSPCs in the organotypic brain (Scale bar=20  $\mu$ m, \*\* $p$ <0.05 vs M0 conditioned media, n=5/group).

combination of function-blocking antibodies to cytokines, but not by any single antibody in the *in vitro* co-culture system of NSPCs and microglia [15]. This variability in the effects of microglia on

neurogenesis displayed the different polarizations of microglia and/or the precise status of NSCs/neuronal progenitor cells (NPCs) [33-35] and crosstalk between them [36]. Another study

showed that microglia-conditioned media rescue the *in vitro* formation of neuroblasts from NSPCs in SVZ [37]. To determine the role of activated microglia in functional neurogenesis, we collected M1 and M2 phenotype microglia-conditioned media and found that M2 phenotype microglia released TGF- $\alpha$ .

TGF- $\alpha$  is a member of the epidermal growth factor (EGF) family that activates the EGF-receptor (EGF-R) trans-membrane tyrosine kinase [38, 39]. This mechanism shows that the increase in intracellular calcium levels, glycolysis and expression of certain genes, such as EGF-R gene, ultimately lead to DNA synthesis and cell proliferation [40]. Both TGF- $\alpha$  and EGF-R are located in the SVZ where they regulate the activity of neural progenitor cells [41]. Previous reports have suggested that TGF- $\alpha$  also significantly amplify the proliferative responses of neural stem cells in the SVZ [42], but causes minimal migration in response [43]. In the ischemic stroke model, intraventricular TGF- $\alpha$  also reduced the infarct volumes. These neuroprotective effects are driven by anti-apoptotic and anti-inflammatory mechanisms [44]. Recently, study also showed that macrophage secrete TGF- $\alpha$  and even when the tissue was cultured in media including TGF- $\alpha$  from surrounding infarct lesion, this protective effect was maintained [42].

In the current study, we found that M2 phenotype microglia release TGF- $\alpha$ , which it may affect the neurogenesis of NSPCs in SVZ. This event was associated with the effect of TGF- $\alpha$  on the proliferation and migration of NSPCs. However, in the current study, we failed to see any changes of neuron and oligodendrocyte of SVZ-NSPCs with or without TGF- $\alpha$  in M2 conditioned media. We report for the first time that microglia-derived TGF- $\alpha$  plays a role in the proliferation and neurogenesis of NSPCs in the SVZ after ischemic stroke and is involved in neurological recovery. We also suggest that microglia-derived cytokines should be studied further in neurogenesis related brain injury.

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