

Endothelial Progenitor Cell Transplantation Attenuated Synaptic Loss by Enhancing CR3 Dependent Microglial Phagocytosis in Mice

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Abstract

Background: Endothelial progenitor cell (EPC) transplantation has been shown to have therapeutic effects in cerebral ischemia. However, whether the therapeutic effect of EPCs is a result of the modulation of microglia activity remain elusive.

Methods: Adult male mice (n=184) underwent 90 minute-middle cerebral artery occlusion and EPCs were transplanted into the peri-infarct region immediately after the surgery. Microglia migration and phagocytosis were evaluated in the ischemic brain *in vivo* and underwent oxygen-glucose-deprivation culture condition *in vitro*. Complement receptor 3 was examined in ischemic brain and cultured primary microglia. Complement receptor 3 agonist leukadherin-1 was intraperitoneally injected to mice immediately after ischemia to imitate the EPC effect. Expression of synapse remodeling related synaptophysin and PSD-95 proteins was detected in the EPC and leukadherin-1 treated mice, separately.

Results: EPC transplantation increased the number of microglia in the peri-infarct region of the brain at 3 days after focal ischemia ($p<0.05$). The ability of phagocytizing apoptotic cells of microglia was higher in EPCs transplanted group at 3 days after ischemia compared to the controls ($p<0.05$). *In vitro* study showed that cultured microglia displayed a higher migration ($p<0.05$) and phagocytosis ability ($p<0.05$) under the stimulation of EPC conditioned medium or cultured EPCs compared to the controls. Complement receptor 3 expression in the ischemic mouse brain with EPC transplantation ($p<0.05$), and primary microglia treated by EPC conditioned medium or cultured EPCs was up-regulated ($p<0.05$). Leukadherin-1 reduced brain atrophy volume at 14 days ($p<0.05$) and ameliorated neurological deficiency during 14 days after cerebral ischemia ($p<0.05$). Both EPC transplantation and leukadherin-1 injection increased synaptophysin ($p<0.05$) and PSD-95 expressions ($p<0.05$) at 14 days after focal ischemia.

Conclusion: We concluded that EPC transplantation promoted regulating complement receptor 3 mediated microglial phagocytosis at acute phase, and subsequently benefited for attenuating synaptic loss at the recovery phase of ischemic stroke, which provided a novel therapeutic mechanism of EPC for cerebral ischemia.

Introduction

Ischemic stroke constitutes the majority of stroke, which is the leading cause of permanent disability and the second cause of death worldwide [1]. Currently, thrombolysis and endovascular thrombectomy are the only proved effective treatments. However, less than 15% of the patients are accessible to the treatments due to the limited therapeutic time window and tissue window [2]. Therefore, seeking other potential and alternative therapeutic methods is urgent and timely for the treatment of ischemic stroke.

Emerging pre-clinical and clinical studies recognized that stem cell therapy was a promising strategy that effectively ameliorated the neurological deficits and functionally enhanced recovery from ischemic stroke [3]. Endothelial progenitor cells (EPCs) hold particular promise for ischemic therapy due to its wide variety of sources, fewer ethical constraints and strong capacity for angiogenesis [4–6]. However, the exact therapeutic mechanism has not been understood yet, which limits its clinical translational application.

Numerous studies demonstrated that complement system was activated and exerted protective or detrimental effects during cerebral ischemia [7]. Complement 3 (C3) is the core of complement signal pathway and activates the downstream signal pathway mainly via binding with the receptor C3aR or complement receptor 3 (CR3) [7]. CR3 was exclusively expressed on resident microglia in the brain and mediated the phagocytosis function [8, 9]. C3/CR3 signal pathway was proved to be involved in promoting synapse elimination and phagocytizing debris, contributing to

nervous circuits development and functional recovery from neurological diseases [9–11]. However, emerging evidence implicated that C3/CR3 signaling was an etiologic factor in neurodegeneration [12]. Our previous study proved that EPC transplantation inhibited astrocyte releasing the complement component C3 and regulated C3/C3aR pathway on microglia, attenuating inflammatory response and neurological function deficit after ischemic stroke [13]. Whether EPC transplantation affecting CR3 to regulate microglia activity and exert the therapeutic function remains unexplored.

In this study, we transplanted EPCs into mice brains of transient middle cerebral artery occlusion (tMCAO) model, and explored the effect of EPCs on CR3 mediated microglial phagocytosis after stroke.

Materials And Methods

EPC isolation and identification

The procedure for EPC isolation was approved by Ethics Committee of Shanghai Jiao Tong University, Shanghai, China. The ethical standards were consistent with the Helsinki Declaration of 1975 that revised in 1983, confirmation that written informed consent was obtained from all donors. Human umbilical cord blood was obtained from the International Peace Maternity & Child Health Hospital of China (IPMCH, Shanghai, China). EPC purify was followed the procedure previously [14]. Briefly, monocytes were isolated and cultured in a 6-well plate coated with collagen I (Corning, Bedford, MA). EPCs within 5 to 8 passages were used in the following experiments. For immunofluorescent staining, EPCs were fixed with 4% paraformaldehyde, followed by 0.3% Triton-X 100 for 10 minutes and 10% donkey serum for 1 hour at room temperature. Then EPCs were incubated with primary antibodies of CD34 (1:50 dilution, BD Biosciences, Franklin Lakes, NJ) and KDR (1:50 dilution, R&D, Minneapolis, MN), CD133 (1:50 dilution, Abnova, Taipei, China) and KDR (1:50 dilution, R&D), and vWF (1:400 dilution, Abcam, Cambridge, MA) at 4°C overnight. After washing for three times, EPCs were incubated with the secondary antibodies: Alexa Fluor 594-conjugated donkey anti-mouse and Alexa Fluor 488-conjugated donkey anti-goat, or Alexa Fluor 488-conjugated donkey anti-rabbit (1:500 dilution, Invitrogen, Carlsbad, CA) for 1 hour at room temperature. Images were taken under a confocal microscope (Leica, Solms, Germany).

The preparation of EPC conditioned medium

To produce EPC conditioned medium (EPC-CM), EPCs were cultured in a 10 cm dish (Corning Incorporated, Corning, NY) at about a density of 1.5×10^6 cells. Fresh medium (EGM-2, EPC basic culture medium) was added in the cultured dish. After incubation of 24 hours for EPCs at 37°C. The medium, namely EPC-CM, was collected and centrifuged at 1000 rpm for 5 minutes, the supernatant was sterile filtered and frozen in -20°C until use. The EGM-2 was served as a control medium (EPC-free).

For EPC-O-CM, mixed medium (DMEM without glucose: EGM-2 without FBS=1:2) was used to culture EPCs in a hypoxic chamber, which was used for oxygen-glucose-deprivation (OGD) assay. After incubation of 24 hours at 37°C, the medium, namely EPC-O-CM was collected, centrifuged, sterile filtered and frozen in -20°C until use.

Human umbilical vein endothelial cell (HUVEC) conditioned medium preparation

To produce HUVEC conditioned medium, namely HUVEC-CM and HUVEC-O-CM, HUVECs were cultured in a 10 cm dish (Corning Incorporated, Corning, NY) under normal and OGD conditions, separately. HUVEC basic culture medium for normal culture and mixed medium for OGD culture were added in culture dishes, separately. After incubation of

24 hours at 37°C, HUVEC-CM and HUVEC-O-CM were collected and centrifuged at 1000 rpm for 5 minutes, the supernatant was sterile filtered and frozen in -20°C until use.

Establishment of a mouse model of transient middle cerebral artery occlusion (tMCAO)

Animal studies were performed in accordance with ARRIVE guidelines. Procedure for using laboratory animals was approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University, Shanghai, China. The procedure of tMCAO was performed as described previously [5, 15]. Adult male ICR mice (n=184) weighing 25-30 grams were used in the study. Briefly, a 6-0 silicone-coated nylon suture was inserted into the external carotid artery, went along the internal carotid artery, and stopped at the origin of the middle cerebral artery. After 90 minutes, the suture was withdrawn. The success of occlusion and the reperfusion of cerebral blood flow was confirmed by a laser Doppler flowmetry (Moor Instruments, Axminster, Devon, UK). Mice with the cerebral blood flow in ipsilateral hemisphere not reduced to 80% of baseline were excluded from the study. Sham-operated animals underwent paralleled procedures except for the occlusion of the middle cerebral artery.

EPC transplantation

Mice were fixed on a stereotaxic frame (RWD life science, Shenzhen, China). EPC suspension with 3×10^5 cells was injected through the skull at a rate of 1000 nl/minute under the stereotactic brain inject system (RWD life science, China). The needle was inserted 2 mm lateral to the bregma and 2.5 mm under the dura. The needle was maintained for 10 minutes after the completion of injection in case of liquid leakage. After the needle withdrawal, the skull hole was sealed with bone wax and the skin incision was sutured.

Leukadherin-1 injection

CR3 agonist leukadherin-1 (LA1, Selleck, Shanghai, China) was dissolved in DMSO with a final working concentration of 10 mg/ml. The dissolved LA1 was packaged and stored in -20°C until use. LA1 was taken out of the freezer, thawed on ice, and was injected intraperitoneally into mice (1mg/kg) immediately after mice waking up from anesthesia of the tMCAO. LA1 injection continued once daily in the next three days after tMCAO. Mice underwent the identical procedure in the control mice were injected with the same volume of DMSO solution.

Brain atrophy volume measurement

Brains were collected and frozen immediately after euthanizing the mice. A series of 30 μ m coronal sections were cut from the anterior commissure to the hippocampus. A total of 20 coronal sections of each brain were mounted on the slides, stained with 0.1% cresyl violet, and used for brain atrophy volume measurement. The distance between 2 adjacent sections was 300 μ m. The brain area was measured using the ImageJ software (NIH, Bethesda, MD). The atrophy area was calculated by subtracting the area of normal brain in the ischemic hemisphere from the area of contralateral hemisphere. The total brain atrophy volume were further calculated according to the formula
$$\sum_1^n \left[(S_n + \sqrt{S_n * S_{n+1}} + S_{n+1}) * \frac{h}{3} \right]$$
, in which, h was the distance between 2 adjacent sections, and S_n and S_{n+1} were atrophy area of 2 adjacent sections, respectively.

Real-time PCR

Total RNA was extracted from ischemic brain using TRIzol reagent (Invitrogen) and transcribed to cDNA using ZymoScript II First Strand cDNA Synthesis Kit (Abconal, Shanghai, China) according to the manufacturer's

recommendations. The sequence of each primer used in the whole study was summarized in **Table 1**.

Table 1. Real-time PCR primers

Gene	Animal	Forward primers 5'3'	Reverse primers 5'3'	Amplicons size (bp)
CR3	mouse	ATGCTTACCTGGGTTATGCTTC	AGGCCCCAAAATAAGAGCCAA	177
CD68	mouse	CGGTGG AATACAATG TGTC	GCTGGAGAAAGAACT ATGCT	136
MerTK	mouse	AAGGACTGACGGTGTCCAAG	CCATCAAACCAGGGACCCA	127
MFG-E8	mouse	CAACCTAGCCTCCCGTTGT	CAGACGAGGCGGAAATCTGT	80
Synaptophysin	mouse	ACAGGCAGGTGAAGAGGACGAG	AGGGCAGAGAAAGGGTGGAGAAG	348
PSD-95	mouse	ACCGCTACCAAGATGAAGACAC	CCGTTACCTGCAACTCATATCCT	133
GAPDH	mouse	GGTTGTCTCCTGCGACTTCA	TGGTCCAGGGTTTCTTACTCC	183

Western blot analysis

Equal amounts of proteins were loaded onto a 10 % resolving gel for electrophoresis and were transferred onto polyvinylidene fluoride membranes (Immobilon-P, Billerica, MA). Then the membranes were blocked with 5% non-fat milk and incubated with primary antibodies CR3 (1:1000 dilution, Abcam), synaptophysin (1:1000 dilution, Abcam), PSD-95 (1:1000 dilution, Millipore, Billerica, MA) and β -actin (1:1000 dilution, Abcam) at 4 °C overnight. After three times washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit immunoglobulin G for 1 hour at room temperature. After three times washing, the membranes were reacted with an enhanced chemiluminescent substrate (Thermo Scientific, Waltham, UK). The results of chemiluminescence were semi-quantified using the ImageJ software (NIH).

Immunohistochemistry

Paraformaldehyde-fixed brain sections were washed with PBS for three times and then incubated with 0.3% Triton-X 100 for 10 minutes at room temperature. After blocking with 10% donkey serum for 1 hour at room temperature, the brain sections were incubated with primary antibodies at 4°C overnight. The primary antibodies included Iba1 (1:200 dilution, WAKO, Osaka, Japan), MAP2 (1:200 dilution, Millipore), CR3 (1:200 dilution, Abcam) and synaptophysin (1:200 dilution, Abcam). After three times washing, the brain sections were incubated with fluorescence-conjugated secondary antibodies for 1 hour at room temperature. Then the brain sections were incubated with 4', 6-diamidino-2-phenylindole (DAPI, Life Technologies, Mulgrave, VIC, Australia) for 5 minutes at room temperature. The brain sections were covered and sealed with mounting medium (Vector Labs, Burlingame, CA) for further study.

Cell counting

For analysis of Iba1 and TUNEL immunostaining, 4 fields were randomly selected in the peri-infarct regions at X20 or X40 objectives. Images were collected using a confocal microscope (Leica, Solms, Germany) under the same conditions. Four serial sections (1.10 mm to -0.70 mm from the bregma) were selected from each animal. The distance from each section was 300 μ m.

Statistical analysis

Statistical analysis was performed using SPSS18.0 software (SPSS Inc., Chicago, IL). For parametric analysis, multiple comparisons were evaluated by one-way ANOVA followed by Bonferroni (homogeneity of variance) or the Tamhane test (heterogeneity of variance). For comparisons between two groups, an unpaired Student *t*-test was used. For nonparametric analysis, the Kruskal–Wallis test and Mann–Whitney U test were applied. All data were represented as mean±SD. *p*<0.05 was considered statistically significant.

Results

EPC isolation, identification, and transplantation

Monocytes were isolated from human umbilical cord blood cells and seeded on a 6-well plate with a density of 1×10^7 /well. At 10 days, the cobblestone-like EPCs appeared and remained cobblestone-like shape and held high proliferation ability at passage 6. Immunofluorescent staining showed that EPCs expressed both CD34 (red) and KDR (green), which were considered characteristic markers of EPCs [16, 17]. In addition, EPCs also expressed endothelial cell marker vWF (green, **Fig. 1A**). EPCs did not express the other stem cell marker CD133 (red, **Fig. 1A**). We injected GFP transfected EPCs into the peri-infarct region of the striatum in the ipsilateral hemisphere of the brain. The result showed that the intensity of GFP fluorescence was still strong in injected cells, suggesting that the grafted cells were still alive at 14 days after tMCAO (**Fig. 1B**).

EPC transplantation increased the number of M2 microglia in ischemic brain

To evaluate the effects of EPC transplantation on microglia, we first examined the number of microglia in mice following tMCAO. The results showed that the number of microglia was increased in EPCs treated group compared to the PBS treated group at 3 (**Fig. 2A**, *p*<0.05) and 14 days after tMCAO (**Fig. 2B**, *p*<0.05). Our previous study proved that EPC transplantation enhanced M2 phenotype of microglia related genes but reduced M1 phenotype related genes in the mouse brain [13]. We speculated that the increased microglia in the EPCs treated mice was M2 microglia.

EPC transplantation enhanced microglial phagocytosis via CR3

To clarify what function the increased microglia exerted in the EPCs treated mice, we then evaluated the ability of microglial phagocytosis after tMCAO. We found that there were less TUNEL⁺ cells but more TUNEL⁺/Iba1⁺ cells at 3 days after tMCAO in the EPCs treated mice (**Fig. 3A**, *p*<0.05). The results suggested that EPC transplantation promoted microglia to phagocytize more apoptotic cells at acute stage of tMCAO. To further determine which receptor mediating microglial phagocytosis, we detected MerTK, MFG-E8, CD68 and CR3 during tMCAO. The result showed that MerTK and MFG-E8 expressions were not changed but CD68 and CR3 expressions at mRNA level were increased at 3 days after tMCAO. EPC transplantation did not affect MerTK and MFG-E8 but further increased CD68 and CR3 mRNA expressions (**Fig. 3B**, *p*<0.05). Western blotting confirmed that EPC transplantation enhanced CR3 at 3 days after tMCAO (**Fig. 3C**, *p*<0.05). CR3 was a specific marker and receptor mediating microglial phagocytosis in the brain [7]. Iba1, CR3 and MAP2 colocalization in 3-dimensional space showed that Iba1⁺ microglia engulfed debris of MAP2 labeled neurons through CR3 (**Fig. 3D**). These results suggested that EPC transplantation enhanced microglial phagocytosis via up-regulating CR3.

EPC conditioned medium promoted microglial migration and phagocytosis *in vitro*

To further prove that EPCs could modulate microglia activity, we evaluated microglial migration and phagocytosis after EPC conditioned medium treatment *in vitro*. Transwell assay showed that EPC-CM and EPCs increased the

number of migrated microglia under both normal and OGD culture condition (**Figs. 4C,4D**, $p<0.05$). These results suggested that EPCs promoted microglial migration. Next, we detected microglial phagocytosis using fluorescent beads treated with EPC-CM and EPC-O-CM under LPS stimulation (**Fig. 4E**). The results showed that more beads were in the cell body of EPC-O-CM treated microglia (**Figs. 4F,4G**, $p<0.05$). Real-time PCR showed that EPC-O-CM treated microglia expressed the highest CR3 at mRNA level compared to the other groups (**Fig. 4H**, $p<0.05$), confirming that EPCs promoted microglial migration and enhanced microglial phagocytosis via up-regulating the receptor CR3.

EPC transplantation reduced synaptic loss in the brain after ischemic stroke

Microglia help synaptic pruning via synapse elimination during the development of nervous system [10]. To determine whether EPCs enhanced the ability of microglial phagocytosis to affect synapse remodeling, we detected the synapse remodeling related genes synaptophysin and PSD-95. Real-time PCR result showed that synaptophysin expression decreased (**Fig. 5A**, $p<0.05$), which was not affected by EPC transplantation at 3 days after tMCAO (**Fig. 5A**, $p>0.05$). Synaptophysin expression also had a decrease tendency at 14 days after tMCAO, while EPC transplantation reversed the tendency and increased synaptophysin expression (**Fig. 5A**, $p<0.01$). EPC transplantation did not affect PSD-95 expression at mRNA level at 3 and 14 days after tMCAO (**Fig. 5B**, $p>0.05$). Western blotting showed that synaptophysin and PSD-95 expression reduced at 3 and 14 days after tMCAO (**Figs. 5C,5D**, $p<0.05$), while EPC transplantation reversed this decrease (**Figs. 5C,5D**, $p<0.05$). Immunostaining confirmed that synaptophysin was reduced at 14 days after tMCAO, and EPC transplantation increased synaptophysin expression to the basic level close to that in the sham group (**Fig. 5E**, $p<0.05$). These results indicated that EPC transplantation reduced synaptic loss at the recovery phase of tMCAO.

CR3 agonist LA1 reduced neurological deficit and synaptic loss in ischemic mice

To clarify whether EPC transplantation improved neurological function and reduced synaptic loss via CR3 activating microglia, we intraperitoneally injected LA1 immediately after and following 3 days of tMCAO. The brain atrophy volume was smaller in the LA1 treated group compared to the DMSO control group at 14 days after tMCAO (**Figs. 6C,6D**, $p<0.01$). To evaluate neurological deficiency after tMCAO, we assessed motor, balance, and reflex functions in mice at 1, 3, 7, and 14 days using modified neurological severity scores (mNSS). mNSS was lower in whole examination period in the LA1 treated group compared to the control group (**Fig. 6E**, $p<0.05$). These results indicated that the LA1 injection attenuated ischemic brain injury and neurological deficiency. We further found that the synaptophysin and PSD-95 expressions after tMCAO decreased while EPC treatment reversed the synaptophysin and PSD-95 down-regulation at 14 days after tMCAO (**Fig. 6F**, $p<0.05$). Synaptophysin and PSD-95 expressions were also increased in the LA1 injected mice at 14 days after tMCAO (**Fig. 6F**, $p<0.01$). There was no significant difference of synaptophysin and PSD-95 expressions between the EPC treated and the LA1 treated mice. Immunostaining confirmed that the LA1 injection increased synaptophysin expression to the basic level close to that in the DMSO control group after 14 days of tMCAO (**Figs. 6G,6H**, $p<0.05$). These results demonstrated that LA1 injection had the similar effect on attenuating brain atrophy volume, neurological deficiency and synaptic loss after tMCAO compared to the EPC transplantation.

Discussion

In this study, we aimed to clarify whether EPC transplantation affected CR3 and regulated microglia activity to reduce ischemic brain injury. We demonstrated that EPC transplantation increased microglial migration and phagocytosis in ischemic brain and in cultured microglia. EPC transplantation up-regulated CR3 expression, which was associated

with enhanced phagocytosis of microglia at the acute stage and reduced synaptic loss at the recovery stage of tMCAO. Furthermore, we demonstrated that CR3 agonist LA1 mimicked EPC therapeutic effects on diminishing synaptic loss, contributing to alleviating brain injury and ameliorating neurological function after tMCAO.

Our previous study found that EPC transplantation reduced inflammatory response and ameliorated neurological deficit after MCAO [13]. The effect was partially related to inhibit C3/C3aR signal pathway between astrocytes and microglia, and increase M2 microglia related genes expression after EPC transplantation [13]. One of the primary functions of microglia was to migrate to the damaged tissue and phagocytize the debris to limit inflammatory response [18]. In the present study, we explored whether EPC transplantation affected microglial migration and phagocytosis to exert its beneficial function. The results demonstrated that EPC transplantation enhanced microglial migration and phagocytizing apoptotic cells at the acute stage of tMCAO, contributing to decreased synaptic loss at the recovery phase of tMCAO.

C3 is the core component of complement activation pathways and mainly exerts its functions via binding with C3aR and CR3, which are two major receptors of C3 [7]. Our previous data indicated that EPC transplantation reduced C3 but not affected C3aR expression after tMCAO [13]. We tried to clarify whether EPC transplantation affected the other crucial receptor of C3, CR3, to promote tissue recovery. We found that CR3 was up-regulated in the brain and EPC transplantation further increased CR3 expression at 3 days after tMCAO, associated with enhanced phagocytosis of microglia in ischemic brain. *In vitro* study showed that cultured BV2 and primary microglia had a higher migration and phagocytosis, and increased expression of CR3 after EPC-CM or EPCs treatment during hypoxia. These results further confirmed that EPCs could modulate microglia activity via up-regulating CR3.

Numerous studies proved that C3/CR3 signaling mediated microglial clearance of apoptotic neurons, degenerated synapses and extracellular debris in a variety of neurodegeneration diseases, exerting protective and beneficial effects [19–23]. In ischemic stroke, the role of C3/CR3 signaling was controversial. In a mouse model of embolic stroke, C3 inhibition suppressed complement-dependent synaptic uptake by microglia, contributing to reduced cognitive decline [24]. However, in a permanent MCAO model in rats, inhibition of CD11b, a subunit of CR3 (CD11b/CD18), with anti-CD11b mAb weakened microglial phagocytosis of myelin debris, resulting in aggravated ischemic brain injury [25]. *In vitro* study indicated that CR3 activation on microglia was involved in regulating synaptic activity and plasticity under hypoxia and LPS stimulation condition [26]. Our study supported the notion that CR3 in microglia was up-regulated under hypoxia and mediated microglial phagocytosis of apoptotic cells at acute stage of MCAO. Currently, there is no available specific inhibitor for CR3. We used CR3 agonist LA1 to clarify whether CR3 activation elicited beneficial effects via affecting microglial phagocytosis and synapse remodeling after tMCAO. Synaptophysin and PSD-95 were primary proteins reflecting synapse remodeling [27–29]. LA1 injection within 3 days after tMCAO reversed synaptophysin and PSD-95 decrease during 14 days after cerebral ischemia. The reduced synaptic loss was associated with ischemia induced brain atrophy and neurological function deficiency at 14 days after ischemic stroke. Our study proved that LA1 injection elicited the similar protective effects on reducing synaptic loss as EPC transplantation in stroke mice. In contrast to our expectation, there was no impact on microglial phagocytosis in ischemic mice at 3 days after LA1 injection (data was not shown).

LA1 is a small molecule allosteric activator of CR3 [30]. LA1 activated the subunit CD11b of CR3 by binding to a site distal from the site of ligand binding [31]. LA1 mainly promoted neutrophils adhesion to inflamed endothelium and restricted further tissue infiltration after injury, exerting anti-inflammatory function [32]. LA1 treatment could modulate inflammatory response and decrease tissue injury via modulating macrophage polarization [33]. Both residential microglia and tissue macrophages originated from myeloid cells and shared common characteristics [18]. We speculated that LA1 treatment at the acute stage of ischemic stroke possibly resolved acute stroke induced

inflammatory response, contributing to reducing synaptic loss and ischemic brain injury. Whether LA1 treatment affected microglia polarization in ischemic stroke needs to be studied in the future.

We used LA1 to mimic the effect of EPC transplantation on CR3 activation. We did not find changes of microglial phagocytosis in LA1 injected mice at 3 days after tMCAO but observed increased synaptophysin and PSD-95 expression at 14 days after tMCAO, suggesting that LA1 was an inappropriate agent to study the direct effect of EPC transplantation on CR3 activation. Some studies used the CD11b antibody to study CR3 function during pathological conditions [30, 34]; However, blocking CD11b with the CD11b antibody had no role in altering the chemotaxis and cytotoxicity of cells expressing CR3, while CD11b activation mimicked CR3 activation induced by pathophysiological conditions [35]. Therefore, using CD11b antibody to block and study the role of CR3 in ischemic stroke is also inappropriate. Much work is needed to develop a CR3 specific and functional agonist to investigate the precise mechanism of EPC transplantation on regulating CR3 and clarify the role of CR3 during cerebral ischemia.

In the brain, CR3 is exclusively expressed on residential microglia, mediating the clearance of debris for tissue remodeling and recovery after ischemic brain injury [7, 18]. Although the role of CR3 on microglial phagocytosis of injured neurons or damaged tissue or degenerated myelin were widely studied in many neurodegenerative diseases, its role on microglia in ischemic stroke remains elusive [11, 23, 36]. Currently, we found that EPC transplantation enhanced microglial phagocytosis of apoptotic neurons at 3 days after ischemic stroke and up-regulated synaptic related proteins of synaptophysin and PSD-95 expression at 14 days after tMCAO. To investigate which receptor mediated microglial phagocytosis, we further detected expressions of microglial phagocytosis related genes including MerTK, MFG-E8, CD68 and CR3. Only CR3 expression was up-regulated after EPC transplantation at 3 days after tMCAO. Iba1, CR3 and MAP2 colocalization in 3D space displayed that CR3 was involved in phagocytizing the debris of damaged neurons. Many circulating cells expressing CR3 including neutrophils, monocytes, eosinophils, natural killer cells, B and T lymphocytes could be recruited in the brain after cerebral ischemia as a result of the damage of blood-brain-barrier [37]. Consequently, it is difficult to identify which type of cells was the dominant type with up-regulated expression of CR3 and mediated phagocytosis after cerebral ischemia. Studies have demonstrated that the initial phagocytic response after focal brain ischemia mainly performed by residential microglia, while the majority of blood-borne macrophages were recruited secondarily to participate in the removal of necrotic tissue [38]. In addition, we transplanted EPCs via direct brain injection, causing a more direct impact on resident brain cells including microglia compared to circulating cells. Therefore, we considered that EPC transplantation mainly up-regulated CR3 expression on microglia and activated CR3 mediated phagocytosis of apoptotic cells at the acute stage of ischemic stroke, making a less harmful environment for synaptic remodeling and functional recovery after ischemic injury.

Conclusions

We provided a novel mechanism of EPC transplantation during MCAO. EPC transplantation enhanced microglial phagocytosis by regulating CR3 expression at the acute stage of tMCAO, contributing to attenuated synaptic loss at the recovery stage of tMCAO. Our results indicated that increasing microglial phagocytosis by regulating CR3 activity provides a novel therapeutic strategy for cerebral ischemia.

Abbreviations

C3: complement 3; CM: conditioned medium; CR3: complement receptor 3; EPCs: endothelial progenitor cells; LA1: Leukadherin-1; mNSS: modified neurological severity scores; OGD: oxygen-glucose-deprivation

Declarations

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Contributions

MYM prepared the figures and wrote the manuscript; JL performed the animal model and contributed to the analysis of data; WLP and LYQ did the neurological function assessment. LYF helped transplant EPCs into mice and collect animal samples; WYT helped analyze the results; ZZJ helped design and instruct the experiment; JD and YGY conceived the study and revised the manuscript.

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Ethics declarations

Ethics approval and consent to participate

All animal experimental protocols were approved by the Loma Linda University Animal Care and Use Committees (IACUCs).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

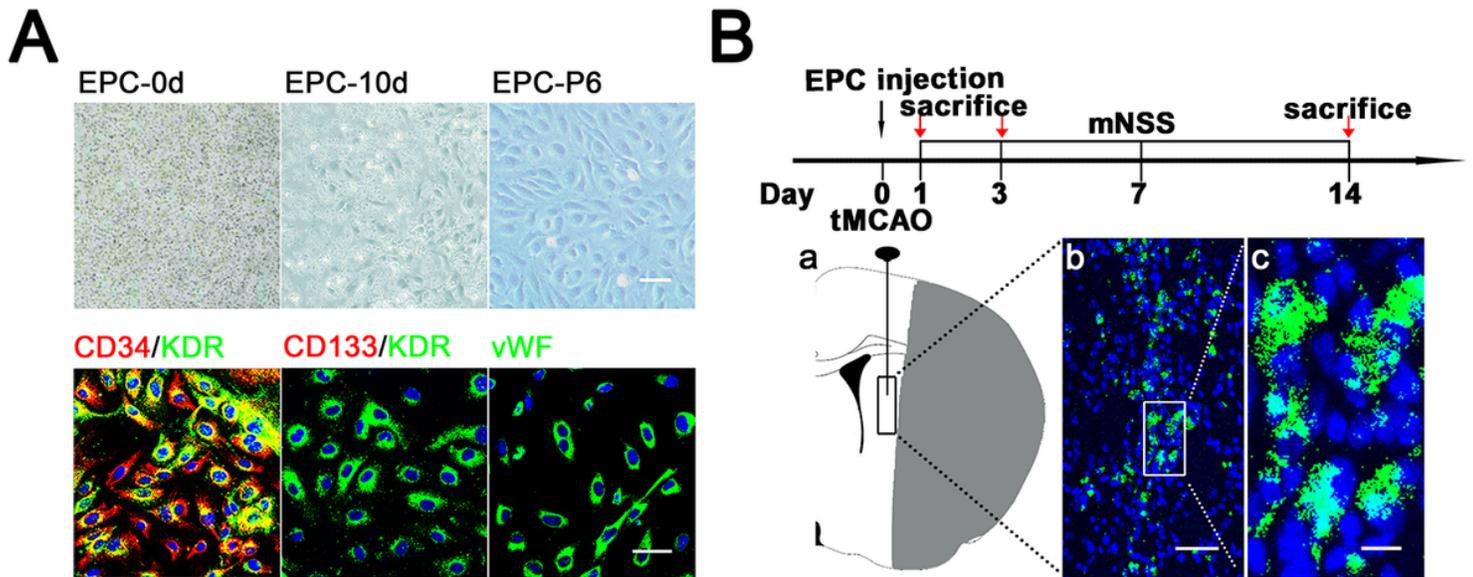


Figure 1

EPC isolation, identification, and transplantation (A) Monocytes were cultured on the 6-well plate after isolation; EPCs with cobblestone shape appeared at 10 days after isolation; EPCs maintained cobblestone shape at passage six. Scale bar=100 μ m; Representative immunofluorescence images of EPCs labeled by CD34 (red) and KDR (green), CD133 (red) and KDR (green), and vWF (green), respectively. Scale bar=50 μ m. (B) Flow chart showed the experimental design. Mice underwent tMCAO and were randomly assigned to different groups. EPCs were injected into the peri-infarct region of striatum within 120 minutes after tMCAO. The schematic diagram of ischemic brain showed the location of injected EPCs (a); Representative images of injected EPCs labeled by GFP in the peri-infarct region of striatum at 14 days after tMCAO (b). Scale bar=50 μ m; Magnification of the cells showed that the fluorescence intensity of GFP was strong and the nuclear (blue) of GFP labeled EPCs were clear (c). Scale bar=10 μ m.

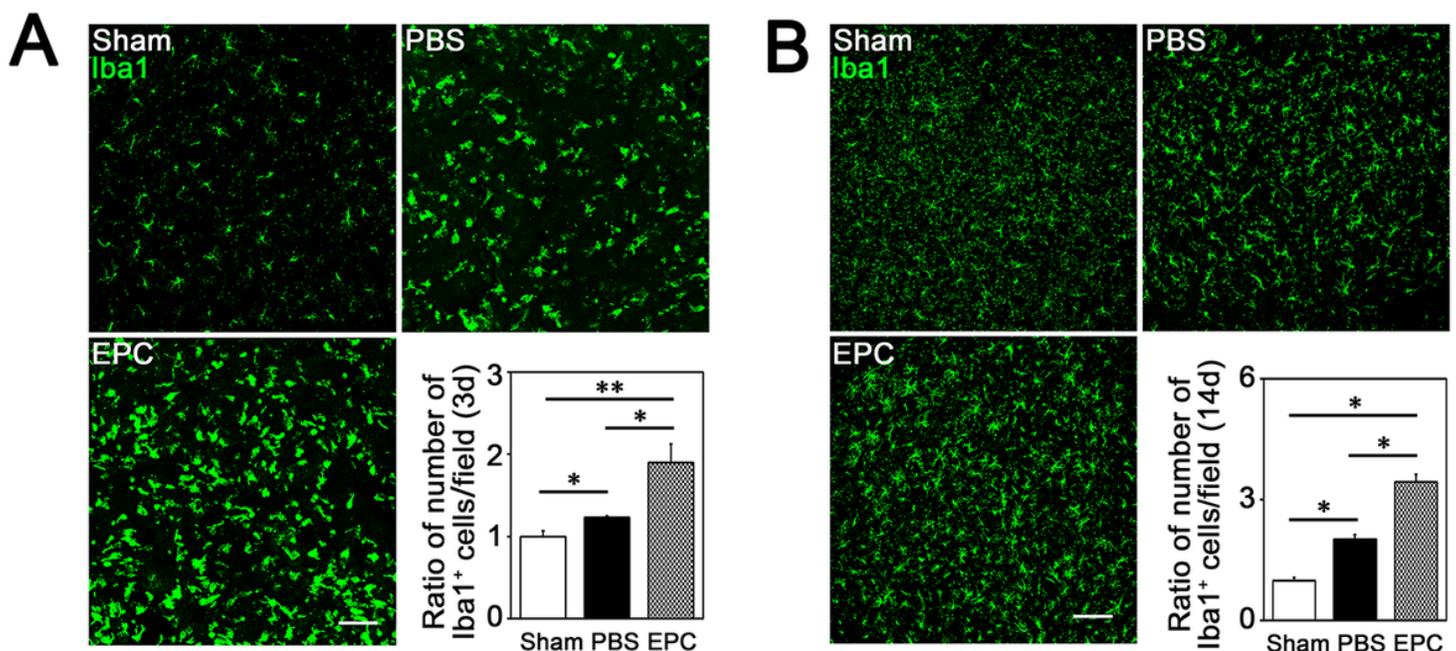


Figure 2

EPC transplantation enhanced the number of microglia after tMCAO (A-B) Immunofluorescence images of Iba1+ microglia (green) in the peri-infarct region of ischemic brains in Sham, PBS and EPC groups at 3 and 14 days after tMCAO, respectively. The bar graphs showed the ratios of number of microglia among groups. The data in Sham group was normalized to 1. Data were mean±SD. n=3-4 per group. *, p<0.05, **, p<0.01.

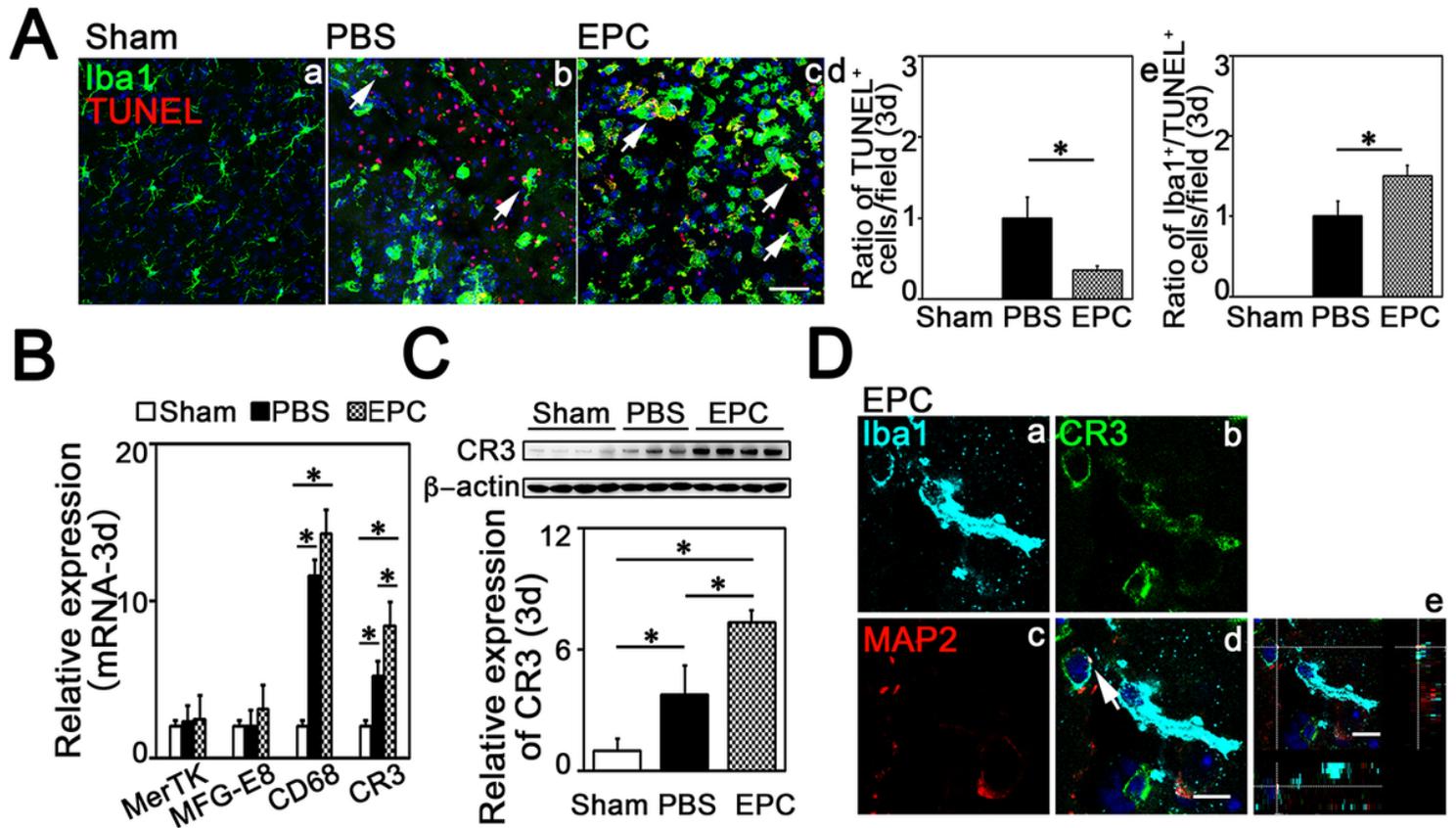


Figure 3

EPC transplantation promoted microglial phagocytosis via enhancing CR3 (A) Immunofluorescence images of Iba1+ microglia (green) and TUNEL labeled apoptotic cells (red) among groups at 3 days after tMCAO. Scale bar=50 μ m. Bar graphs showed the ratio of TUNEL+ cells (d) and Iba1+/TUNEL+ cells (e) among groups at 3 days after tMCAO, respectively. n=3-4 per group. *, p<0.05. (B) Quantifications of microglial phagocytosis related genes (MerTK, MFG-E8, CD68 and CR3) expression at 3 days after tMCAO. (C) Detection of CR3 expression using Western blot in the brain at 3 days after tMCAO. Bar graph showed the quantification of CR3 expression. Data were mean±SD. n=3-4 per group. *, p<0.05. (D) Immunostaining of Iba1 (light blue, a), CR3 (green, b), and MAP2 (red, c) labeled cells and merge images (d) of the cells in the mouse brain at 3 days after tMCAO. Scale bar=10 μ m. Confocal image of Iba1, CR3 and MAP2 colocalization in three-dimensional space (e). Scale bar=10 μ m.

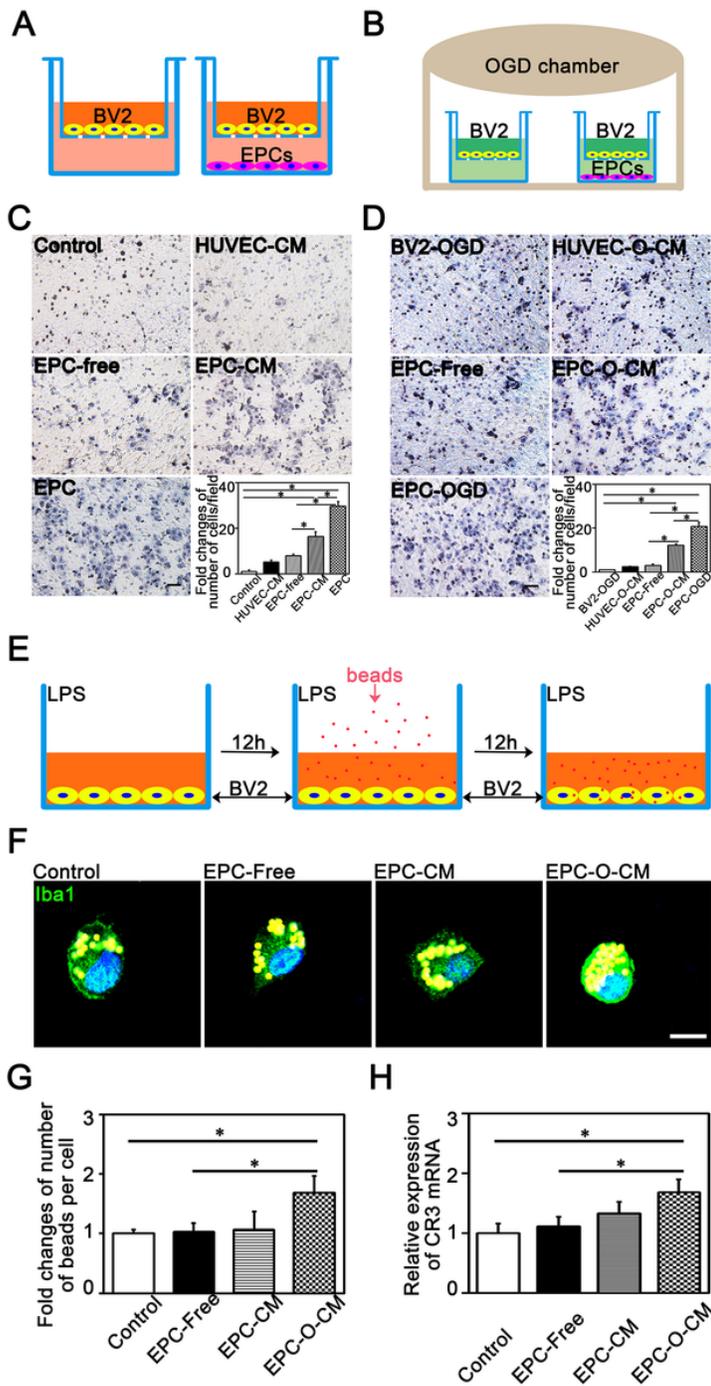


Figure 4

EPC-CM or EPCs promoted microglial migration and phagocytosis (A-B) Diagram of transwell assay: BV2 cells were cultured on the transwell with basic culture medium under normal culture condition or with glucose free DMEM OGD medium in a hypoxic chamber. (C-D) Migrated BV2 cells and quantifications of the number of cells per field among groups. Scale bar=50 μ m. Control group was normalized to 1. Data were mean \pm SD. n=3 per group. *, p<0.01. (E) Diagram of phagocytosis assay. (F) Images of phagocytized beads (yellow particles) per microglia (green, Iba1) among groups under LPS stimulation. Scale bar=10 μ m. (G-H) Quantifications of the number of beads per microglia and the CR3 mRNA of microglia among groups under LPS stimulation. Control group was normalized to 1. n=3 per group. *, p<0.05.

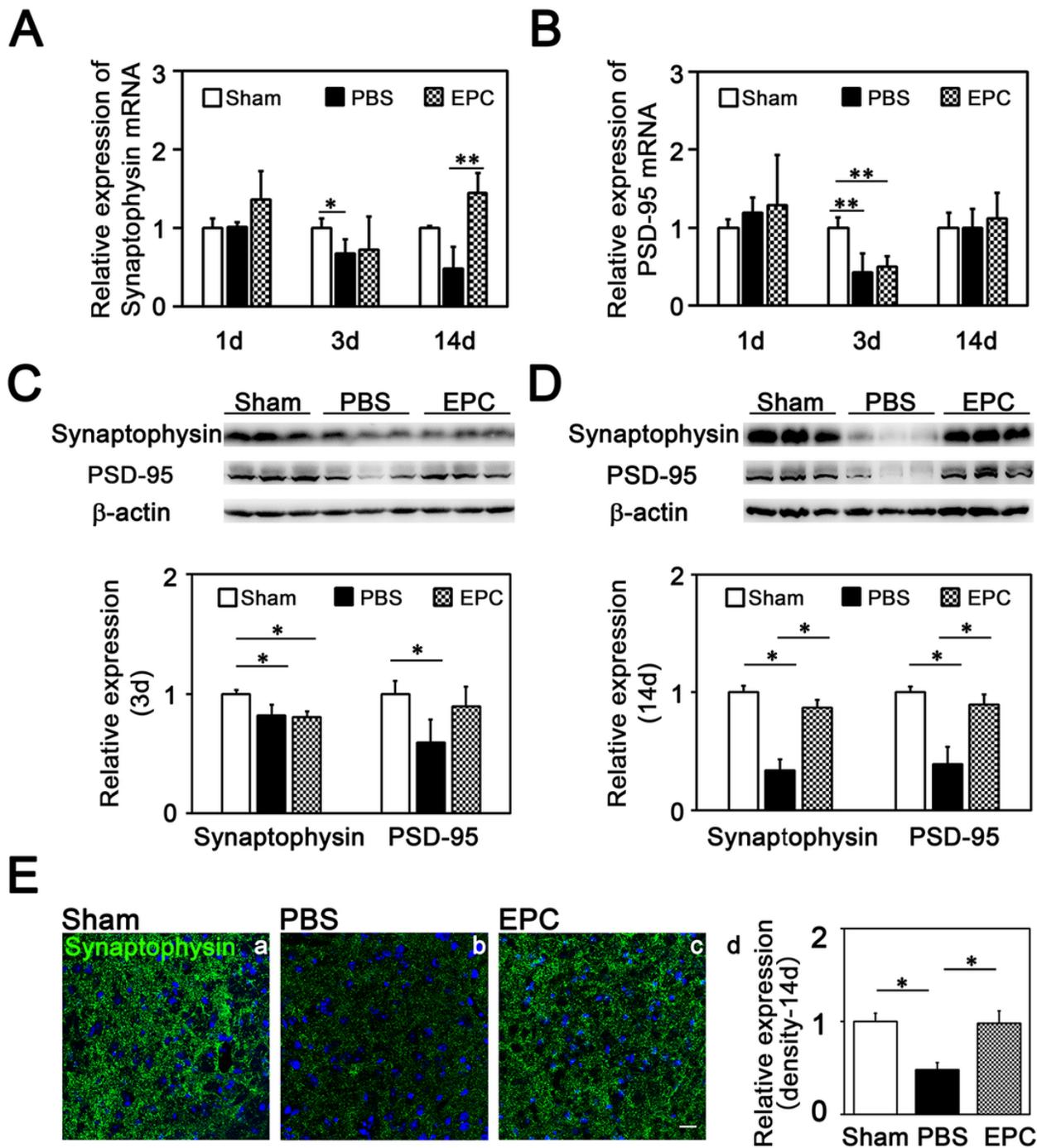


Figure 5

EPC transplantation reduced synaptic loss after tMCAO (A-B) Quantifications of synaptophysin and PSD-95 mRNA expressions in ischemic brains among groups at 1, 3 and 14 days after tMCAO, respectively. (C-D) Western blot showed the synaptophysin and PSD-95 expressions in the brain at 14 days after tMCAO. Bar graphs showed the quantifications of synaptophysin and PSD-95 expressions. (E) Representative images of synaptophysin immunostaining (green) among groups at 14 days after tMCAO. Scale bar=20 μ m. Bar graph showed the quantification of fluorescence intensity of synaptophysin. The data in Sham group was normalized to 1. Data were represented as mean \pm SD. n=3-4 per group. *, p<0.05, **, p<0.01.

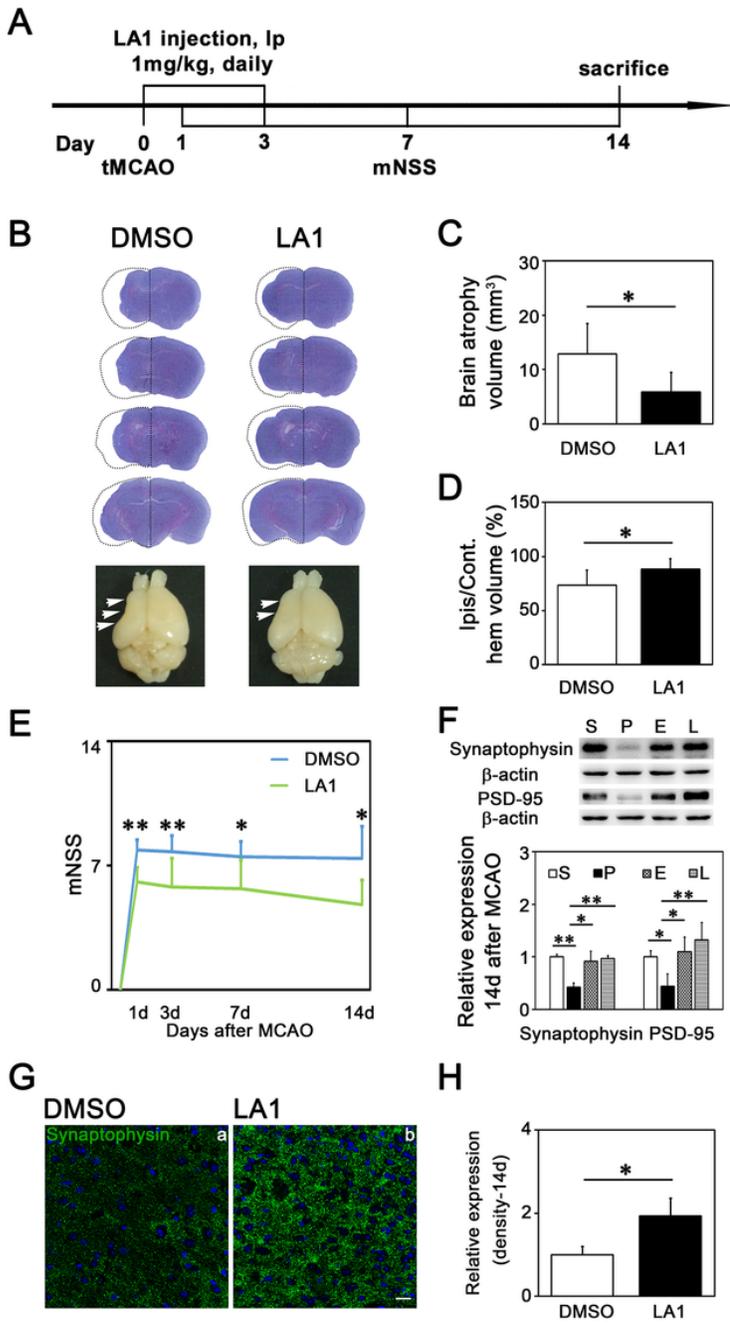


Figure 6

Injection of LA1 reduced brain atrophy, neurological function deficit and synaptic loss after tMCAO (A) The flow chart showed the experimental design. At 3 and 14 days, mice were sacrificed and brains were collected for mRNA, protein and immunostaining. (B) Images of cresyl violet staining and the atrophy was shown in the whole brain. Dashed line showed the original size of the ischemic brain side. (C) Quantification of the atrophy volume between DMSO and LA1 groups at 14 days after tMCAO. Data were mean±SD. n=6 per group. *, p<0.05. (D) Quantification of the ratio of ipsilateral and contralateral ischemic brain atrophy volume between DMSO and LA1 groups. Data were mean±SD. n=6 per group. *, p<0.05. (E) mNSS between DMSO and LA1 groups after tMCAO. Data were mean±SD. n=6-14 per group. *, p<0.05, **, p<0.01. (F) Bands of synaptophysin and PSD-95 in Western blot and its' quantifications. Sham group was normalized to 1. Data were mean±SD. n=4 per group. *, p<0.05, **, p<0.01. (G) Images of synaptophysin

immunostaining (green) between DMSO and LA1 groups at 14 days after tMCAO. Scale bar=20 μ m. (H)
Quantification of fluorescence intensity of synaptophysin. Data were mean \pm SD. n=4 per group. *, p<0.05.