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**Immunomodulation: the next target of mesenchymal stem cell-derived exosomes in the context of ischemic stroke**

Shan XQ *et al.* MSC-Exos on IS

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## **Abstract**

Ischemic stroke (IS) is the most prevalent form of brain disease, characterized by high morbidity, disability, and mortality. However, there is still a lack of ideal prevention and treatment measures in clinical practice. Notably, the transplantation therapy of mesenchymal stem cells (MSCs) has been a hot research topic in stroke. Nevertheless, there are risks associated with this cell therapy, including tumor formation, coagulation dysfunction, and vascular occlusion. Also, a growing number of studies suggest that the therapeutic effect after transplantation of MSCs is mainly attributed to MSC-derived exosomes (MSC-Exos). And this cell-free mediated therapy appears to circumvent many risks and difficulties when compared to cell therapy, and it may be the most promising new strategy for treating stroke as stem cell replacement therapy. Studies suggest that suppressing inflammation *via* modulation of the immune response is an additional treatment option for IS. Intriguingly, MSC-Exos mediates the inflammatory immune response following IS by modulating the central nervous system, the peripheral immune system, and immunomodulatory molecules, thereby promoting neurofunctional recovery after stroke. Thus, this paper reviews the role, potential mechanisms, and therapeutic potential of MSC-Exos in post-IS inflammation in order to identify new research targets.

**Key Words:** Mesenchymal stem cells; Exosomes; Ischemic stroke; Immunomodulation; Inflammation; Exosome therapy

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**Core Tip:** Mesenchymal stem cell-derived exosomes (MSC-Exos) are an emerging strategy for treating ischemic stroke (IS) and have demonstrated certain achievements in animal studies. Here, we review and discuss the mechanisms of MSC-Exos in treating

IS through immunomodulation, the current responses to the clinical limitations of MSC-Exos therapy, and the issues that need to be addressed in future MSC-Exos research.

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## INTRODUCTION

Stroke is one of the leading causes of death and permanent disability on a global scale; ischemic stroke (IS) accounts for approximately 80% of stroke cases<sup>[1]</sup>. Currently, the mainstay of acute treatment for IS is limited to reperfusion by intravenous recombinant tissue fibrinolytic activator (tPA, thrombolysis) and rapid recanalization utilizing devices (thrombectomy)<sup>[2]</sup>. In clinical practice, however, the thrombolytic treatment conditions are strictly limited to presentation within 4.5 h of symptom onset<sup>[3]</sup>. Although the therapeutic window for thrombectomy has been extended to 24 h, there may be a risk of cerebral hemorrhage, occlusion after revascularization, and over-perfusion brain injury<sup>[4,5]</sup>. In recent years, other treatments that researchers have actively explored have also been prevented from being implemented on a large scale in clinical practice due to a variety of disadvantages. For instance, hypothermia treatment may reduce body metabolism while affecting neuronal death mechanisms, resulting in increased immunosuppression and susceptibility to infectious complications<sup>[6]</sup>. Prophylactic antibiotic treatment can decrease the incidence of infectious complications. However, antibiotic therapy is targeted, and broad-spectrum antibiotics can affect the body's normal flora if they are misused, which may increase organismal resistance<sup>[7]</sup>. By 2050, there will be more than 200 million stroke survivors and almost 300 million disability-adjusted life-years, 25 million new strokes, and 13 million deaths from stroke annually<sup>[1]</sup>. Therefore, there is a pressing need to discover effective treatments for IS that can be administered on a large clinical scale.

In acute stroke management, time is brain. The focus of stroke research should be on extending the time window for treatment. Examples include early measurement of immune biomarkers<sup>[8]</sup>, improved efficiency of pre-hospital emergency transport<sup>[9]</sup>, improved levels of care<sup>[10]</sup>, and stem cell transplantation therapy<sup>[11]</sup>. Among these, stem cell transplantation therapy, which can extend the treatment window for IS to seven

days, has become a hot research topic<sup>[11]</sup>. This also offers promising treatment options for patients outside the golden treatment period. MSCs are among the most hopeful candidates for stem cell therapy compared to other types due to their comprehensive source, ease of culture, pluripotent differentiation, immune tolerance, high survival rate, and strong paracrine effects<sup>[11-13]</sup>. It has previously been proved that nutrient factors and extracellular vesicles secreted in situ by stem cells after transplantation enter the damaged brain and exert immunomodulatory, neuroprotective, angiogenic, and neural restructuring effects<sup>[13,14]</sup>. This phenomenon is known as the paracrine response (also called the “bystander” effect) and is the main mechanism by which stem cell’s function. In comparison, exosomes are key effectors in the paracrine response of stem cells<sup>[14]</sup>. Mesenchymal stem cell-derived exosomes (MSC-Exos) therapy applied to stroke is superior to cell therapy in biodistribution, stability, safety, and development potential while ensuring therapeutic efficacy as an alternative therapy to stem cells.

In addition to the problem of a narrowing treatment window, the poor prognosis of ischemic stroke is another pressing issue. Immunosuppression is the important cause of IS patients' poor prognosis and increased susceptibility. The inflammatory response underlies ischemic tissue damage. MSC-Exos, a highly promising treatment modality for brain injury, can effectively reduce neuroinflammatory reactions by modulating the immune system to promote recovery<sup>[15,16]</sup>. This paper reviews and discusses the immunomodulatory effects of MSC-Exos at the cellular and molecular levels following IS, as well as its application in therapy, in order to serve as a reference for future research and treatment.

### **IMMUNE RESPONSE AFTER IS**

IS is caused by thrombosis or embolism, which could interrupt blood flow to the brain. After acute ischemic events, blood stagnation and altered hemodynamics restrict the availability of oxygen and glucose (oxygen-glucose deprivation, OGD). Then brain cell metabolism shifts from the oxidative phosphorylation to high levels of glycolysis, producing excess lactic acid<sup>[17,18]</sup>. Excessive accumulation of lactic acid is able to trigger

tissue acidosis, edema, blood-brain barrier (BBB) dysfunction, and extensive necrosis<sup>[18]</sup>. Firstly, once the Na<sup>+</sup>/K<sup>+</sup> ATPase pump is affected, there will be an inward flow of Na<sup>+</sup> and an outward flow of K<sup>+</sup>, which depolarizes the neuronal plasma membrane and promotes the release of excitatory neurotransmitters (including glutamate)<sup>[18-20]</sup>. Excess glutamate activates the N-methyl-D-aspartate receptor and the  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor, thereby leading to cytotoxicity and cell death<sup>[19,21,22]</sup>. Next, an extracellular Ca<sup>2+</sup> inward flow occurs after affecting the Ca<sup>2+</sup> pump, which causes a dramatic rise in intracellular Ca<sup>2+</sup>. Ca<sup>2+</sup> overload activates calcium-dependent proteases, lipases, DNAses, kinase phosphatases, endonucleases, and other death signals, inducing ischemic core cell death<sup>[20,23]</sup>. Additionally, the Ca<sup>2+</sup> influx activates nitric oxide synthase (iNOS), which subsequently generates oxygen radicals and peroxynitrite (ONOO<sup>-</sup>), causing oxidative stress in neural tissue<sup>[24]</sup>. Meanwhile, the depletion of ATP production and overproduction of reactive oxygen species (ROS) leads to mitochondrial dysfunction, further exacerbating oxidative stress<sup>[22,25]</sup>. In summary, OGD results in subsequent energy disturbances, lactic acidosis, cellular excitotoxicity, and oxidative stress, ultimately leading to brain cell damage or death. This is the initial step of ischemia-induced damage, which triggers the subsequent cascade responses. Injured/dying cells emit “danger signals” and thus activate the immune system (**Figure 1**).

Once the immune system is activated, immune cells enter the brain parenchyma sequentially. Microglia (MG), as the resident macrophages of the central nervous system (CNS), are the first to detect ischemia and rapidly activate in response<sup>[26,27]</sup>. MG recognizes “danger signals” (danger-associated molecular patterns, DAMPs) released by dying and dead cells, primarily *via* the expressions of Toll-like receptors (TLR) and scavenger receptors. Then, the TLRs and scavenger receptors are activated, triggering a series of inflammatory events<sup>[28-30]</sup>. MG has been classified into two polarized phenotypes, including classical activation (pro-inflammatory, M1) and alternative activation (anti-inflammatory, M2). Anti-inflammatory cytokines [such as interleukin (IL)-4, IL-13, IL-10, and transforming growth factor (TGF)- $\beta$ ] activate the M2 phenotype.

4 The M2 cells promote translocation of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) and proliferation-activated receptor gamma, and promote the secretion of anti-inflammatory IL-10, IL-4, TGF- $\beta$  cytokines and 14 growth factors (such as brain-derived neurotrophic factor and vascular endothelial growth factor) to suppress inflammation and enhance tissue repair<sup>[31,32]</sup>. In contrast, lipopolysaccharide (LPS) and interferon gamma (IFN- $\gamma$ ) activate the M1 phenotype. The M1 cells promote the transcriptional activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), a member of the signal transducer and activator of the transcription family (STAT), and promote the 3 production of pro-inflammatory mediators like IL-12, tumor necrosis factor (TNF)- $\alpha$ , IL-6, IL-1 $\beta$  and NO, leading to the secondary brain damage<sup>[31,33,34]</sup>. Meanwhile, the chemokines and cytokines released by M1-MG and adhesion molecules highly expressed on endothelial cells can recruit peripheral blood leukocytes (including neutrophils, monocytes, and lymphocytes) to infiltrate the brain parenchyma, thereby mediating the adaptive immune response<sup>[28,30,31]</sup>. In the acute phase of brain injury, the M1 phenotype appears to predominate, whereas MG favors the M2 phenotype in the later stages. In addition, neurons can control MG activation by releasing “on” and “off” signals. MG is able to quickly recognize the “eat me” (CX3CL1) or “don't eat me” (e.g., CD47-SIRP $\alpha$  and CD200-CD200R) signal on a neuron and engulf the live ischemic neurons<sup>[35]</sup>. In the same way like MG, macrophages can be polarized into two phenotypes, M1 and M2. The two are often described as MG/macrophages, because their roles in stroke are mostly similar<sup>[36]</sup>. However, in contrast, the main inflammatory factors produced by both are skewed. MGs secrete relatively high levels of ROS and TNF- $\alpha$ , while macrophages produce relatively high levels of IL-1 $\beta$ <sup>[37]</sup>.

Astrocytes (Ast) are among the first brain cells to be activated after an ischemic event. Ast undergoes a dramatic transformation called “reactive astrogliosis” after ischemic injury, forming glial scarring<sup>[38]</sup>. Similar to MG, the harmful or beneficial effects of reactive Ast depend on the different phenotypes of Ast (neuronal toxicity phenotype A1 and neuroprotective phenotype A2)<sup>[38,39]</sup>. In addition to the activation of Ast by DAMPs, there is growing evidence regarding the importance of MG-Ast crosstalk for activating



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Ast. MG activation, followed by the release of IL-1 $\alpha$ , TNF- $\alpha$  and complement component subunit 1q (C1q), induces the activation of A1-type reactive Ast<sup>[38,40]</sup>. A1-Ast secrete pro-inflammatory mediators, like IL-6, TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IFN- $\gamma$ , NO, matrix metalloproteinases (MMP), superoxide and ONOO-, inducing neuron and oligodendrocyte death<sup>[38,41]</sup>. MG also induces the A2 phenotype of Ast and attenuates the inflammatory response. Li *et al* 19 have reported that Zinc finger E-box binding homeobox 1 (ZEB1) was highly expressed in MG of the ischemic hemisphere after experimentally induced strokes<sup>[42]</sup>. ZEB1 overexpression mediates the MG response primarily through a TGF- $\beta$ 1-dependent pathway and subsequently reduces CXCL1 production in Ast, thereby reducing neutrophil infiltration in the brain parenchyma. Likewise, Ast also can regulate the phenotype and function of MG through crosstalk between Ast and MG<sup>[43]</sup>. Thus, when the brain is disturbed, MG and Ast seem to respond as a unit.

Different from other immune cells, the number of lymphocytes infiltrating into the stroke brain is relatively small<sup>[27]</sup>. T lymphocytes can enter the brain hours after a stroke and are preferentially accumulated at the edge of the lesion<sup>[44]</sup>. The T cells infiltrating into the ischemic tissue mainly comprise 18 CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs), CD4<sup>+</sup> T helper cells (Ths), and regulatory T cells (Tregs)<sup>[45]</sup>. Infiltrating MG/macrophages may stimulate the differentiation of activated CD4<sup>+</sup> T cells into Th1 or Th2 cells<sup>[46]</sup>. Th1 cells are able to secrete pro-inflammatory factors like IFN- $\gamma$ , IL-2, and IL-12 to exacerbate 8 inflammation. In contrast, Th2 cells produce anti-inflammatory factors, such as IL-4, IL-5, IL-10, and IL-13, to suppress inflammation<sup>[47]</sup>. CTLs directly or indirectly kill neurons and aggravate brain damage through cell interactions and the release of perforin after antigen-dependent activation<sup>[48]</sup>. Tregs exert their protective effects mostly by inhibiting IL-1 $\beta$  and TNF- $\alpha$  through the expression of IL-10<sup>[47,49]</sup>. The role of B lymphocytes in the immunology of stroke is not clear yet. Whereas, some studies have observed the local production of corresponding antibodies in the cerebrospinal fluid of stroke patients, indicating that B lymphocytes are indeed present in the ischemic brain and they may be involved in post-ischemic immunological events<sup>[50]</sup>.



Neutrophils are the initial blood-derived immune cells to cross the BBB and invade ischemic tissues, and they can be detected as soon as 1h after the event<sup>[51]</sup>. Neutrophils are activated and recruited to the injured brain parenchyma by inflammatory factors produced from some activated glial cells and dying neurons, and adhesion molecules expressed by endothelial cells (e.g., intercellular adhesion molecule 1, P-selectin and E-selectin)<sup>[27,52,53]</sup>. Traditionally, the neutrophil aggregation has been considered detrimental to stroke. After infiltration into ischemic tissues, activated neutrophils produce inflammatory factors, such as MMP, iNOS, and ROS, and form neutrophil extracellular traps (NETs) to increase BBB permeability and exacerbate inflammation<sup>[54-56]</sup>. In addition, the accumulation of neutrophils can further block local blood flow, resulting in “no reflux” of the microcirculation<sup>[57]</sup>. Neutrophils also exhibit two kinds of phenotypes, comprising N1 (pro-inflammatory) and N2 (anti-inflammatory) phenotypes. Neutrophil's different phenotypes may shape other cellular effector functions and be cleared by phagocytosis of MG/macrophages<sup>[58,59]</sup>.

In conclusion, the post-stroke ischemic environment induces immune cells to polarize into different phenotypes or type, acting either protectively or destructively. Hence, it is probably a promising mean to affect the immune cell heterogeneity and improve the post-stroke inflammatory environment.

### **EXOSOMES AS A REPLACEMENT THERAPY FOR MESENCHYMAL STEM CELLS ON IS**

All above, it is clear that the immune responses following IS can influence the development of ischemic brain injury. Anti-inflammatory and immunomodulatory therapies have shown beneficial effects on several experimental stroke models<sup>[60]</sup>. Among them, MSCs transplantation is one of the most important therapeutic tools involved in regulating immunity and repairing ischemic tissues in clinical practice<sup>[61]</sup>. Initially, researchers have assumed that the primary mechanism of MSCs transplantation mainly involved in MSC's ability to differentiate into parenchymal cells to repair and replace injured tissues. However, many preclinical studies suggested that

most MSCs were confined to the liver, spleen, and lungs, and only a few MSCs could reach the injury site, surviving and differentiating into neurons<sup>[62,63]</sup>. Interestingly, despite most transplanted MSCs stagnate in the organ, this does not prevent the therapeutic effect of MSCs transplantation. Thus, the distal therapeutic effect after transplantation of MSCs may be primarily attributable to the paracrine mechanism of MSCs<sup>[63]</sup>. MSC-Exos mainly mediate the paracrine secretion of MSCs. Exosomes are extracellular vesicles (EVs) with a single membrane structure of 30-150 nm in diameter, carrying proteins, lipids, nucleic acids (DNA, mRNA, miRNA, lncRNA, circRNA), and other substances<sup>[64]</sup>. When exosomes are circulating, the contents encapsulated within them can be delivered to target cells, mediating intercellular communication and regulating the function of the target cells<sup>[64,65]</sup>. This is essentially the role of the miRNAs contained by exosomes. MiRNAs are endogenous hairpin-loop structured non-coding RNAs, primarily binding to mRNA in specific ways to influence post-transcriptional events and regulate cellular behavior<sup>[66]</sup>.

Furthermore, there are multiple advantages of transplanting exosomes rather than the entire “factory” (cell) into the body: 1) In terms of biodistribution, as nano-scale cellular secretions that could escape the phagocytosis of macrophages and readily cross the BBB to reach the brain parenchyma, they are considered to be natural therapeutic agents and innate drug delivery system for brain diseases<sup>[67]</sup>. 2) In terms of stability, exosomes have a stable bimolecular phospholipid structure that prevents the contents’ biological activity from being broken down by extracellular hydrolytic enzymes<sup>[64]</sup>. 3) In terms of safety, compared to MSCs transplantation therapy, the cell-free therapy can avoid cell-mediated adverse effects, such as tumor formation, coagulation dysfunction, and infarction due to vascular occlusion<sup>[16]</sup>. 4) In terms of development potential, exosomes can be enriched in large quantities within the culture medium (mass production) and easily retouched/retrofitted (controllable). Moreover, some studies comparing the therapeutic effects of MSC-Exos with MSCs in stroke rat models suggest that MSC-Exos treatment is indeed superior to treatment with MSCs themselves<sup>[68,69]</sup>. For above reasons, we believe that MSC-Exos is a crucial effector of MSCs to exert their

immunomodulatory effects. Together with its unique advantages, MSC-Exos is expected to be a replacement therapy for MSCs in the treatment of stroke.

### **MSC-EXOS MODULATES IMMUNE RESPONSE AFTER IS**

Recently, numerous studies have shown that MSC-Exos can promote recovery after stroke, *via* modulating the innate and adaptive immune responses activated after IS<sup>[70-72]</sup>. Firstly, MSC-Exos is able to regulate cell differentiation, activation, proliferation, and intercellular communication by delivering functional molecules to cells involved in immunity, for example, MG, Ast, macrophages, neutrophils, lymphocytes, dendritic cells (DCs), etc (Table 1). There are three primary forms of action: 1) through the signaling molecules on its surface as ligands binding to specific receptors on the target cell, the intracellular signaling pathways are regulated; 2) *via* fusing with the corresponding target cell membrane and releasing the contents into the recipient cell; 3) by entering the target cell in the form of endocytosis and bringing the active factors into the cell<sup>[73]</sup>. Secondly, MSC-Exos can also mediate the immune response by down-regulating pro-inflammatory factors and/or up-regulating anti-inflammatory factors (Table 2).

### **MSC-Exos regulate the immune response through cells**

#### **MSC-Exos and CNS**

**Microglia:** MG is firstly activated after IS, as an immune sentinel of the CNS, exerting neuroprotective or neurotoxic effects<sup>[27,74]</sup>. A therapeutic strategy balancing the two polarization states of MG may become a future adjunctive stroke therapy. One study used protein blotting to analyze TLR-2, TLR-4 and TLR-6 Levels in MG of ischemia/reperfusion (I/R) mice and found that the TLR/NE- $\kappa$ B pathway was activated in MG after an ischemic event, leading to the secretion of pro-inflammatory factors (IL-1 $\beta$ , TNF- $\alpha$ , IL-6, etc.) and that this signaling pathway was important in promoting M1 transformation and exacerbating the inflammatory response<sup>[75]</sup>. TLRs are pattern recognition receptors widely expressed on the surface of immune cells and play

a key role in the immune response. NF- $\kappa$ B is a key regulator of the immune response and is intricately involved in MG/macrophage M1 and M2 phenotypic signaling<sup>[31,76,77]</sup>. Various miRNAs encapsulated in exosomes can regulate the expression of TLRs on MG surface, which act on NF- $\kappa$ B to influence MG polarization<sup>[29,30,78]</sup>. It has been reported that Cholesterol 25-hydroxylase (CH25H) is significantly increased during inflammation and contributes to the immune response by recruiting Iba-1-positive MG and activating TLR-3<sup>[79]</sup>. Meanwhile, an experiment used microarray to analyze the expression differences of miRNAs in ischemic brain tissue after exosome treatment, and found that miR-26b-5p expression increased significantly after exosome treatment and could target CH25H in MG to inactivate the TLR pathway to inhibit M1 polarization<sup>[75]</sup>. Besides, miR-542-3p prevents the expression of pro-inflammatory factors and the production of ROS by post-ischemic activated glial cells through inhibiting TLR<sup>[71]</sup>. miR-202-3p<sup>[80]</sup>, miR-182-5p<sup>[81]</sup>, miR-181c<sup>[82]</sup>, and miR-1906<sup>[83]</sup> also play a role in inhibiting M1 polarization through downregulation of TLR expression. Also, in a further explanation of the potential mechanism of miRNA-mediated TLR/NF- $\kappa$ B pathway, Liu *et al* have documented that miR-216a-5p activates the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling cascade through inhibition of TLR4/NF- $\kappa$ B, enabling the M1 to M2 phenotypic shuttle<sup>[84]</sup>. Some data also suggest that miR-145-5p downregulates inflammatory responses by inhibiting the Interleukin-1 receptor-associated kinase 1 (IRAK1)/TNF receptor-associated factor 6 (TRAF6) signaling pathway to reduce and increase the amount of M1-MG and M2-MG, respectively<sup>[85]</sup>. In contrast, overexpression of IRAK1 and TRAF6 is involved in the activation of TLR/NF- $\kappa$ B pathway and promotes the release of proinflammatory factors<sup>[78,85,86]</sup>. Hence, miR-145-5p may indirectly affect the TLR/NF- $\kappa$ B pathway by inhibiting the IRAK1/TRAF6 pathway. Exploring the crosstalk between TLR/NF- $\kappa$ B and other pathways may better interfere with MG polarization. As described above, exosome miRNAs acting on the TLR/NF- $\kappa$ B pathway and its upstream/downstream signaling pathways could influence MG phenotype as well as the expression of pro/anti-inflammatory factors to improve inflammation.

In the regulation of MG polarization, the TLR/NF- $\kappa$ B pathway has been studied the most. However, in stroke, MG polarization is complex and actually regulated by multiple factors. Thus, other pathways affecting the activation state of MG are discussed below. <sup>13</sup> miR-223 is one of the most abundant miRNAs in MSCs and their exosomes. In vivo and *in vitro* experiments have revealed that miR-223-3p down-regulates the transcription and expression of Cysteinyl leukotriene receptor 2 (CysLT2R) to induce a conversion from deleterious M1 to beneficial M2 phenotype<sup>[87]</sup>. CysLTs secreted by dying/dead cells are potent medium for inflammation. They are activated in various cell types during brain injury, further exacerbating the development of inflammation<sup>[88]</sup>. Zhao *et al* conducted an in-depth study involving the miR-223-3p inhibiting CysLT2R expression *in vivo* and *in vitro*. They found that miR-223-3p reversed M1 polarization by apparently downregulating the expression of ERK1/2 downstream of CysLT2R, which led to a decrease in <sup>5</sup> the secretion of pro-inflammatory factors and an increase in the secretion of anti-inflammatory and neurotrophic factors, thereby slowing down inflammatory damage<sup>[89]</sup>. In addition, miR-223-3p also effectively inhibits N-methyl-leukotriene C4/Leukotriene D4 to promote M1 to switch to M2<sup>[87,90]</sup>. Through targeting the autophagy-associated proteins Beclin-1 and Atg5, miR-30d-5p greatly inhibited autophagy-mediated polarization of MG towards M1 and reduced OGD-induced inflammatory responses <sup>[91]</sup>. Notably, autophagy may exert both beneficial and detrimental effects under IS conditions, depending on the degree of autophagy<sup>[92,93]</sup>. A moderate increase in MG autophagic activity can reduce MG activation and promote MG polarization towards the M2 phenotype, exerting a neuroprotective effect. Instead, excessive autophagy exacerbates cerebral ischemic injury. It has been demonstrated that regulation of autophagic flux and exosome biogenesis in MG plays a vital role in neuronal survival under conditions of cerebral ischemia<sup>[94]</sup>. Interestingly, the similar property was also reported in Ast<sup>[95,96]</sup>. As such, balancing the autophagic flux of immune cells after stroke may be a promising target for treating stroke. Cheng *et al* have <sup>1</sup> demonstrated that miR-26a-5p was downregulated and CDK6 was upregulated in



MSCs-derived exosomes of middle cerebral artery occlusion (MCAO) and OGD model<sup>[97]</sup>. They then hypothesized that CDK6 might be a direct target of miR-26a-5p and further confirmed the correlation between exosome miR-26a-5p and CDK6 using a luciferase reporter gene assay. The data showed that miR-26a-5p inhibited MG apoptosis and attenuated I/R injury in mice by mediating CDK6 downregulation<sup>[97]</sup>. Besides, miR-424 can also reduce ischemic brain injury by targeting key activators of the G1/S transition in MG (including CDK6, CDC25A, and CCND1) to inhibit BV-2 microglia activation<sup>[98]</sup>. CDK6 seems to be a good target of miRNAs in neuroprotection. As well as alleviating inflammation, MSC-Exos can also alleviate neuronal death by regulating MG polarization to downregulate inflammatory mediators relating to pyroptosis<sup>[99]</sup>. In vitro data suggest that the non-coding RNA H19 carried by MSC-Exos could attenuate M1 polarization and inflammatory responses by sponging miR-29b-3p and further inhibit neuronal apoptosis<sup>[100]</sup>. miR-29b-3p may prevent ischemic-hypoxic brain injury by activating the PI3K/Akt pathway *via* downregulating the protein phosphatase and tensin homolog (PTEN)<sup>[101]</sup>. Most studies have reported routes associated with miRNAs affecting M1 polarization, while studies acting on pathways associated with M2 polarization are still lacking and deserve further exploration.

**Astrocytes:** Ast, the most abundant brain cells in the CNS, plays an essential role in neuroinflammation and neuroregeneration<sup>[40,41]</sup>. Following ischemic injury, Ast is activated by DAMPs and/or MGs and undergoes a transformation known as “reactive astrogliosis”<sup>[38]</sup>. Features include hypertrophy of the shape and overexpression of glial fibrillary acidic protein (GFAP)<sup>[102]</sup>. The activated Ast phenotype matches MG and is divided into pro-inflammatory A1 and anti-inflammatory A2. Notably, recent studies have shown that the inflammatory response mediated by Ast appears to last longer and induces more damage than MG<sup>[103]</sup>. This possibility further underlines the importance of targeted inhibition of Ast activation or induction of Ast phenotypic transformation in the treatment of IS. It has been demonstrated that Nrf2-related pathways are involved

in the inflammatory response of Ast<sup>[104-106]</sup>. In one study, immunofluorescence experiments were performed after *in vivo* and *in vitro* administration of MSC-Exo, respectively, and protein blots showed that MSC-Exo reduced the expression of GFAP (Ast marker), C3 (A1 marker) and ki67 (cell proliferation marker) in LPS-stimulated cultured primary hippocampal Ast<sup>[104]</sup>. Meanwhile, the data show that MSC-Exo could reverse hippocampal Ast oxidation (e.g., upregulation and nuclear translocation of Nrf2) and inflammation phenotypes (e.g., NF-κB activation and translocation)<sup>[104]</sup>. These results suggest that MSC-Exo can inhibit inflammation-induced Ast activation by modulating the Nrf2-NF-κB signaling pathway. Nrf2 is a regulator of redox homeostasis and a target for the induction of inflammatory responses. In brain diseases with simultaneous inflammation and oxidative stress (e.g., IS), the interaction between Nrf2 and NF-κB signaling pathway is the fundamental mechanism regulating these responses<sup>[107]</sup>. miR-146a-5p, one of the most abundant cargo miRNAs in human umbilical cord-derived MSC-Exos, dramatically decreased the expression of A1 markers [C3 and lipid chain lipoprotein-2 (LCN2)) by inhibiting the NF-κB signaling cascade, thereby reversing the neurotoxic phenotype of Ast<sup>[108]</sup>. Among them, LCN2 has been identified as a potent mediator of astrocyte neurotoxicity<sup>[109]</sup>. LCN2 secreted by reactive Ast can accelerate or propagate neuronal cell death and promote the activation of resting Ast and MG<sup>[109]</sup>. Moreover, a recent study identified high LCN2 expression in a mouse transient middle cerebral artery occlusion model and detected that IS patients with higher plasma LCN2 Levels were more likely to develop a post-stroke infection<sup>[110]</sup>. Overexpression of miR-138-5p negatively regulates the LCN2 expression in Ast, thereby inhibiting inflammation and reducing ischemic nerve injury<sup>[111]</sup>.

More importantly, the increase of reactive Ast results in further glial scarring. In the acute phase of IS, these physical barriers can limit the inflammation spread and infarct area to maintain CNS homeostasis. However, in the recovery phase of IS, their presence may impede the circulation and neurological tissue regeneration, affecting functional recovery in late stroke <sup>[41]</sup>. It has been demonstrated that in several previous cerebral ischemia and hypoxia models, transplantation of MSCs markedly reduced reactive Ast



and further eliminated glial scarring around the lesion, promoting neuronal regeneration and relieving inflammation<sup>[112,113]</sup>. Recently, the *in vitro* studies reported that MSCs improved brain function after transplantation mainly by reducing the number of hypertrophic Ast and GFAP overexpression through inhibition of p38 MAPK, JNK, and its downstream targets p53 and STAT1 activation by paracrine factors<sup>[114]</sup>. In addition, miR-124 attenuated Ast proliferation and migration by blocking the STAT3 pathway, thereby reducing the width of glial scarring and improving neurological function<sup>[115]</sup>. Meanwhile, miR-124 may also involve in the reprogramming neuronal progenitor cells by Ast through decreasing Notch1 expression and increasing Sox2 expression<sup>[115]</sup>. As Ast and neurons originate from the same precursor cells, Ast can be reprogrammed into neurons under some specific conditions, which can be achieved, for instance, by adjusting the expression of certain specific transcription factors (including Notch1, NeuroD1, Mash1, Ascl1, etc.) *in vivo*<sup>[115-117]</sup>. If Ast transdifferentiated neurons homed to the ischemic lesion and replaced lost neurons, this would help limit glial scar formation and neural connectivity after injury, contributing to neural remodeling. This may be used as an alternative therapy for neuronal replenishment after stroke in the future. Furthermore, exosomes can also mediate communication between MSCs and Ast to enhance neurological recovery after stroke. MSCs communicate with Ast and neurons by releasing miR-133b-containing exosomes and transferring miR-133b into neurons and Ast to promote neuroprotection regeneration<sup>[118]</sup>. Hong *et al* further showed that miR-133b shared into Ast downregulated the expression of connective tissue growth factor, thereby reducing glial scar thickness in cerebral infarction<sup>[119]</sup>. All in all, Ast may be a potential target for the intervention in stroke. However, whether the responsiveness and function of Ast should be further reduced or enhanced may depend on the duration of the ischemic lesion, the location of the Ast, and the specific subtype of Ast.

### **MSC-Exos and peripheral immune system**

**Neutrophil:** Neutrophils are the first peripheral immune cells to infiltrate into the brain parenchyma crossing the damaged BBB. Neutrophil infiltration after IS is now believed to be detrimental to stroke<sup>[27,54,56]</sup>. MSC-Exos can mitigate the harmful effects of neutrophils in several ways. Firstly, MSC-Exos can reduce neutrophil infiltration and inhibit neutrophil respiratory burst, thereby decreasing the expression of inflammatory mediators, including IL-1b, IL-6, and TNF- $\alpha$ , as well as suppressing the production of ROS in neutrophils<sup>[120]</sup>. Also, further studies have revealed that MSC-Exos may prevent the subsequent recruitment of monocytes/macrophages and lymphocytes after reducing neutrophil infiltration at the cerebral ischemia site<sup>[121]</sup>. Secondly, MSC-Exos inhibits neutrophil apoptosis and enhances neutrophil phagocytosis, then contributing to the clearance of cellular debris and eliminating inflammation and infection. This may result from the presence of IL-6 in MSC-Exos and its transfer into the neutrophil cytoplasm, which subsequently exerts an autocrine effect on neutrophils, thereby prolonging the survival of these cells and maintaining their effective function and viability to further improve the inflammatory response<sup>[122,123]</sup>. Thirdly, MSC-Exos inhibits the formation of terminal complement complexes on neutrophils *via* CD59, thus attenuating neutrophil activation and inhibiting the release of NETs and IL-17 from neutrophils<sup>[124]</sup>. Besides, human umbilical cord blood-derived MSC-derived extracellular vesicles (exosomes) can also repair and enhance neutrophil mitochondrial function by transferring functional mitochondria, then reducing the formation of NETs<sup>[125]</sup>. Of greater importance, Soni *et al* further investigated the differences in the regulation of neutrophil function by exosomes from different sources of MSCs. The results suggested that bone marrow-derived MSCs (BMMSCs)-derived exosomes (B-Exos) were more effective in prolonging the neutrophil lifespan. In contrast, adipose-derived MSCs (ADMSCs)-derived exosomes (A-Exos) were more prominent in increasing the phagocytic capacity of neutrophils and inhibiting the formation of NETs<sup>[72]</sup>.

**Macrophage:** Activated macrophages are morphologically like MGs, which can be divided into neurotoxic M1 and neuroprotective M2 types<sup>[36]</sup>. Numerous experiments have demonstrated that MSC-Exos can effectively inhibit the effector function of M1 pro-inflammatory macrophages and/or promote the effector function of M2 anti-inflammatory macrophages, which contributes to alleviating the immune inflammatory response. It has been suggested that interferon regulatory factor (IRF) 5 could be reversibly induced by inflammatory stimuli in macrophages and IRF5 is associated with the plasticity of macrophage polarization (up- or down-regulation of M1 or M2 macrophage phenotypic marker expression)<sup>[126]</sup>. Overexpression of miR-22-3p promotes the polarization to macrophage M2, suppresses the inflammation, and attenuates I/R injury through downregulating IRF5, which is supported by increased expression of the M2 macrophage marker mannose receptor (CD206) and decreased expression of the M1 macrophage marker CD86<sup>[127]</sup>. B-Exos-derived miR-125a also exerts neuroprotective effects by targeting negative regulation of IRF5 to promote M2 phenotypic polarization<sup>[128]</sup>. Furthermore, A-Exos can promote M2 polarization by activating the M2 macrophage-specific transcription factors MafB and Stat6 to induce the expression of genes related to anti-inflammatory functions, supported by a mechanism that upregulates the expression of the M2 macrophage markers CD163, arginase-1 (Arg1) and CD206<sup>[129]</sup>. A-Exos also increased CD163, Arg1, CD206, TGF- $\beta$ 1, and IL-10 expression levels and decreased TNF- $\alpha$ , IL-6, and IL-8 expression levels by targeting the ROCK1 (Rho associated coiled-coil containing protein kinase 1)/PTEN pathway. The above results suggest that A-Exos may improve the inflammatory environment by promoting M2 polarization to increase the secretion of anti-inflammatory molecules and/or inhibiting M1 polarization from decreasing the secretion of pro-inflammatory factors<sup>[130]</sup>. And similar to MG, miR-21, miR-146a, and miR-301a can also regulate macrophage polarization by inhibiting the TLR/NF- $\kappa$ B pathway<sup>[131-133]</sup>.

**Lymphocyte:** First of all, T lymphocytes are at the heart of the adaptive immune system. Despite some subtypes of T lymphocytes exert a neuroprotective role in the early post-

stroke phase, such as Tregs and Th2, on the whole, they have a negative impact on IS, as do neutrophils<sup>[45,134]</sup>. Accordingly, it appears to be a viable clinical treatment for stroke to modulate the differentiation, activation and function of various T lymphocyte subsets. In vitro studies indicated that A-Exos significantly inhibited the activation and proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and reduced IFN- $\gamma$  release, with directly immunosuppressive properties<sup>[135]</sup>. In vivo experiments showed that a dramatic reduction in the number of CTL was observed in a rat model of cerebral infarction injected intra-arterially with MSC-EV<sup>[136]</sup>. Another study has documented that MSC-Exos also promoted the Treg proliferation and induced the conversion of Th1 to Th2 by enhancing intracellular IL-10 and TGF- $\beta$  secretion, thereby boosting its immunosuppressive capacity<sup>[137]</sup>. Soni *et al* have further explored and found that MSC-Exos containing miR-146, miR-155, miR-21, and miR-29 may regulate the activation pathways of Th1 and Th2<sup>[72]</sup>. And they showed that different sources of MSC-Exos all inhibited the proliferation of T lymphocytes. But in comparison, Wharton Jelly-derived exosomes and B-Exos had a better inhibitory capacity than A-Exos <sup>[72]</sup>. Additionally, DCs activate T cells through delivering co-stimulatory molecules, such as CD80 and CD86, to naive T cells<sup>[138]</sup>. However, MSC-Exos can reduce T-lymphocyte activity, increase IL-10 and TGF- $\beta$  secretion, and reduce IL-6 secretion by inhibiting the maturation and differentiation of DCs<sup>[135]</sup>. Next, activation and isotype-transformed B-lymphocyte infiltration contribute to poor outcomes after IS. It has been previously reported that MSCs can reverse the unfavorable outcome by inhibiting B lymphocyte activation, proliferation, differentiation, and chemotactic response<sup>[139]</sup>. Recent studies have demonstrated that when co-cultured with lymphocytes derived from healthy human peripheral blood, B-Exos significantly inhibited lymphocyte proliferation and IgM production, particularly exhibiting effects on B lymphocyte-specific mRNA expression<sup>[70]</sup>.

### **MSC-Exos regulate the immune response through inflammatory mediators**

In addition to immune cells, changes in inflammatory mediators, such as cytokines and chemokines, are also observed in ischemic area. Among them, <sup>5</sup> TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 are the more typical pro-inflammatory factors, and their expression is significantly upregulated after ischemic events. MSC-Exos containing lncRNA ZFAS1<sup>[140]</sup>, lncRNAH19<sup>[100]</sup>, miR21-3p<sup>[141]</sup>, miR-146a-5p<sup>[85]</sup>, miR-138-5p<sup>[111]</sup>, and miR182-5p<sup>[81]</sup> was able to reduce immunosuppression by downregulating the secretion of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. Of these, lncRNA ZFAS1 and lncRNAH19 may be associated with the competitive binding of miR-15a-5p and miR-29b-3p<sup>[100,140]</sup>. MSC-Exos also down-regulate other pro-inflammatory factors such as IFN- $\gamma$ , iNOS, and IL-8<sup>[81,91,130,135]</sup>. Apart from down-regulating pro-inflammatory factors, some MSC-Exos <sup>1</sup> can up-regulate the expression of anti-inflammatory factors such as IL-10, IL-4, and TGF- $\beta$ 1<sup>[91,100,130,142,143]</sup>. Furthermore, some studies have shown that MSC-Exos could reduce the secretion of chemokines (e.g., C-C motif ligand 2, CCL-2) and cellular chemotactic proteins (e.g., monocyte chemotactic protein, MCP-1), thereby inhibiting the migration and aggregation of peripheral immune cells and alleviating the inflammatory response<sup>[71,75,81]</sup>. Inflammasomes are equally important inflammatory mediators in regulating the onset and progression of IS. NLRP3 inflammasome contains a caspase-1 precursor that cleaves to caspase-1 (C1) upon stroke stimulation. C1 not only is a critical executioner of pyroptosis (cleave full-length GSDMD to release GSDMD N-terminus) but also can convert <sup>17</sup> precursors of IL-1 $\beta$  and IL-18 into mature pro-inflammatory cytokines exacerbating inflammation <sup>[144]</sup>. Liu *et al* found that NLRP3 inflammasome was downregulated in MCAO mice after MSC-Exos treatment, thereby reducing inflammation and pyroptosis. They also observed that MSC-Exos contributed to MG polarization towards the M2 phenotype by inhibiting NLRP3<sup>[99]</sup>. On the one hand, it may be due to the high plasticity of the MG phenotype, which can dynamically switch according to brain environmental variables<sup>[74]</sup>. On the other hand, it may be related to the amount of autophagy<sup>[91,99,145]</sup>.

Collectively, MSC-Exos could improve the immune inflammatory response after IS *via* affecting the activation of MG/macrophages and Ast, reducing reactive astrocyte



hyperplasia, decreasing excessive infiltration of neutrophils, balancing the functional status of T cell subsets, suppressing the proliferation of B cells, inhibiting DC maturation, and regulating the secretion of inflammatory mediators. Therefore, MSC-Exos exhibits immunomodulatory effects and may help to reduce neurological damage and promote neurological repair after IS.

### **FROM BENCH TO BEDSIDE: RESPONSES TO THE LIMITATIONS OF MSC-EXOS THERAPY FOR IS**

To date, a growing number of studies have demonstrated the great potential of MSC-Exos in treating IS. However, lacking target ability of natural exosomes produced by MSCs has greatly limited their clinical application<sup>[146]</sup>. Regarding exosome producing, the cell membrane invaginates to form endosomes, which in turn form multivesicular bodies (MVBs), and the MVBs finally fuse with the plasma membrane to release luminal vesicles (called exosomes) into the extracellular matrix (ECM)<sup>[147]</sup>. At present, basing on the production process of exosome, two strategies are proposed to improve the targeting ability of exosomes, comprising “cell engineering” (pre-isolation) and “exosome engineering” (post-isolation)<sup>[147]</sup> (**figure 2**). For example, the cyclin (Arg-Gly-Asp-DTyr-Lys) peptide [c(RGDyK)] and the rabies virus glycoprotein (RVG) peptide have been used explicitly to target the brain. B-Exos loaded with cholesterol-modified miR-210 coupled to c(RGDyK) could bind to integrin  $\alpha v \beta 3$  on the BBB and deliver miR-210 to the site of cerebral infarction, thereby ameliorating post-stroke symptoms<sup>[148]</sup>. Additionally, c(RGDyK)-modified MSC-Exos loaded with curcumin (cRGD-Exo-cur) was used in a study, followed by tail vein injection to target the area of cerebral ischemic injury and enter neurons, MG and Ast, effectively inhibiting the inflammatory response and cellular apoptosis<sup>[146]</sup>. RVG fused with exosomes protein lysosome-associated membrane glycoprotein 2b (Lamp2b) could bind to acetylcholine receptors on the BBB and effectively deliver miR-124 to the infarct site, therefore promoting post-IS neurogenesis and reducing ischemic injury<sup>[149]</sup>. In another study, high-mobility group box 1 (HMGB1)-siRNA was loaded into RVG-modified exosomes (RVG-Exos) by

electroporation and injected into an MCAO model *via* tail vein. The results showed that RVG-Exos loaded with HMGB1-siRNA was effective in reducing the level of brain apoptosis and infarct size and had the potential to target IS<sup>[150]</sup>.

Next, the “low yield bottleneck” of MSC-Exos is also one of the main causes limiting its clinical application. Some researchers have illustrated that the pretreatment of MSCs appears to increase the yield of MSC-Exos. For instance, the three-dimensional porous scaffold structure increases the surface area for cell-ECM interaction, compared to the traditional two-dimensional culture of BMSCs. In addition, the three-dimensional culture more closely resembled the natural ECM conditions, providing a better environment for cell attachment and growth, thus substantially increasing the yield of MSC-Exos<sup>[151]</sup>. Some studies have indicated that cultures using microcarriers and hollow fiber bioreactor can provide cells a larger attachment area and further enhance the secretion of MSC-Exos<sup>[152,153]</sup>. Moreover, a recent study found that pretreatment of MSCs with small molecule modulators (N-methyldopamine and norepinephrine) tripled the production of exosomes without altering the intrinsic regenerative effects of MSC-Exos and the level of total exosomes protein expression<sup>[154]</sup>.

To sum up, enhancing the targeting of exosomes by modifying them and improving the yield of exosomes by pretreating MSCs can both improve the therapeutic ability of exosomes. And further exploration of exosome improvement methods offers the possibility of transitioning from bench to bedside.

## **CONCLUSION**

IS is a severe cerebrovascular disease that adversely affects patient’s health and quality of life. A growing body of evidence suggests that the immune inflammatory response plays a critical role in pathogenesis of IS. It has emerged as a promising target for intervention in stroke therapy. After IS, the boundary between the CNS and peripheral immune system disappears due to the disruption of the BBB. Subsequently, the CNS and peripheral immune system can interact with each other, providing a unique opportunity to regulate the pathological process of IS and the repair process. At the



same time, immunomodulation is one of the main mechanisms by which MSC and MSC-Exos exert their therapeutic effects on IS. MSC-Exos is expected to be an alternative therapy to MSC in treating stroke due to its parental cell-like capabilities and specific advantages. MSC-Exos exerts immunomodulatory effects mainly by affecting the inflammatory phenotype of glial cells in the CNS, inhibiting peripheral immune cell activation, proliferation, differentiation, and hyperinfiltration, and regulating the secretion of immune-related molecules. Meanwhile, to complement or enhance the therapeutic suitability of exosomes, researchers are actively exploring novel methods to expand, modify or enhance their therapeutic capacity, such as modifying exosomes (to improve targeting) and pretreating MSC (to increase exosome yield).

Although the results of numerous preclinical studies have shown MSC-Exos to be one of the key breakthroughs in treating IS. However, the study of MSC-Exos in the treatment of IS is still in its infancy in clinical practice. Currently, there is only one study in the Clinical Trials Registry database to determine the effect of MSC-Exos administration on improving functional impairment after IS. This trial used the administration of miR-124-enriched isoform MSC-Exos to treat IS and entered into a phase I/II clinical trial ([NCT03384433](#))<sup>[155]</sup>. There are many challenges to overcome to transfer MSC-Exos therapy further into the clinic. 1) Optimal duration of treatment and effective dose. Numerous studies have shown that post-IS inflammatory cells play a dual role (beneficial and detrimental) and the inhibition of the same pathway at the wrong time may exacerbate ischemic damage<sup>[27,36,38,45]</sup>. Therefore, during developing new therapeutic strategies for IS, we need to pay more attention to the duration of treatment. Interestingly, there are also cases where the timing of transplantation based on previous cellular therapies may affect the therapeutic outcome<sup>[156]</sup>. Thus, we need to further investigate the optimal timing of treatment with exosomes that may be influenced by parental cells. Most preclinical trials have currently chosen to administer a single dose of MSC-Exos in the acute phase of the stroke while showing beneficial effects. As such, the next step should investigate the effects of delayed-time dosing compared to acute phase dosing, in order to determine the optimal timing of treatment.

However, determining the optimal timing of dosing may be difficult in practice, so we could further consider multiple repeat dosing. 2) A single research direction. Current experimental studies on the immunomodulatory aspects of MSC-Exos treatment with IS have focused on MG/macrophages, while other immune-related cells or factors remain poorly studied. Furthermore, the immune response following IS results from crosstalk within and between different cell types, which is complex and chronological. However, most experimental studies have usually explored a single mechanism of action mainly in a particular cell. There is no consensus on the exact molecular mechanism of MSC-Exos treatment of IS and further in-depth studies in multiple directions are urgently needed. 3) Lack of clinical trials. 4) Stroke models combined with relevant clinical conditions. Current studies targeting MSC-Exos for treating IS have almost always been conducted in healthy animals. Therefore, when using stroke models, it should be as close as possible to achieve the clinical situation, like hypertension, diabetes, heart disease, atherosclerosis, secondary infections, *etc.*, as these diseases may affect the formation, treatment, and prognosis of stroke. To summarize, there are still many animal experiments and clinical trials to be finished before the fact that MSC-Exos can be applied as a routine treatment for stroke. However, based on the available evidence, we believe that MSC-Exos therapy is an emerging therapeutic strategy based on cellular therapy with great potential for future use in IS treatment, particularly in immunomodulation.

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