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Stem cell-based immunomodulation after stroke: effects on brain repair processes.

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Introduction

Stroke is a major cause of death and disability worldwide. The inflammatory response is pivotal to the pathophysiology of ischemic stroke. It begins in the vasculature directly after arterial occlusion, continues in the brain, and systemically throughout all disease stages. Immune responses are tightly regulated and have both beneficial and detrimental properties after stroke; inflammation can result in considerable brain damage and/or inhibition of brain repair, including neurogenesis.[1] Variability in different inflammatory processes render the immune response a strong determinant of brain restoration and patient survival following stroke.[2] Directed modulation of the immune response could therefore be designed as a potential therapeutic approach to induce stroke recovery.

Modulation can be achieved with stem cell (SCs) therapy, and is now a widely investigated approach with multiple clinical trials for different diseases, including stroke (www.clinicaltrials.gov).[3, 4] Certain types of SCs are pluri- or multipotent and have the potential to create many neural cells, which may be important after stroke-based neuronal loss. Exogenous SC transplantations, primarily with neural stem/precursor cells (NSPCs) and mesenchymal-derived stem cells (MSCs), have been examined using different administration routes in various stroke animal models; increased functional recovery was often observed.[5–8] Besides NSPCs and MSCs, mixed adult stem cell populations from bone marrow or umbilical cord blood have been examined, showing improved outcomes as well.[9–11] However, the therapeutic time window of these mixed cell populations appears to be narrower, restricting their use to the acute and subacute stages after stroke as compared to the NSPCs and MSCs, which can be used in chronic stroke as well.[12] Therefore, the focus of this Topical Review lies on NSPC and MSC therapy in stroke. Despite many studies, their exact mechanisms behind the brain restoring effects are not completely understood. It is

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thought that SC-based approaches can induce post-stroke recovery via mechanisms such as neuronal replacement, promotion of angiogenesis, induction of brain plasticity, reduction of cell death or immunomodulation.[13] This Topical Review is the first to link SC-induced immunomodulation to different pro-regenerative processes; understanding these interactions is essential to develop successful stroke therapies.

Stem cell-based modulation of inflammation in stroke

The brain cytokine environment

The effects of cytokines released from resident and infiltrating leukocytes in stroke pathology are numerous; they include additional leukocyte recruitment to the site of injury, leukocyte activation and apoptosis induction.[14] SC transplantation has been demonstrated to modulate this cytokine environment, both at the injury site and in the periphery. For example, early SC administration after stroke (within 48 hours) decreases pro-inflammatory cytokine brain levels and increases anti-inflammatory cytokine levels.[15, 16] Liu *et al.* showed that cortical MSC administration following distal middle cerebral artery occlusion (dMCAO) decreased the infarct area and improved neurological function, likely through upregulated gene and protein expression of the anti-inflammatory cytokine IL-10, and a decrease in the pro-inflammatory cytokine TNF.[16] Decreased pro-inflammatory gene expression, including TNF, IL-1β and IFN-γ, was also observed after intravenous NSPCs transplantation.[17] These data show the anti-inflammatory effects of SCs, as confirmed by microarray analysis on mouse brain after intra-hippocampal MSC administration one day post-stroke (Table 1).[18]

Microglia/macrophage polarization

It is currently thought that pro-inflammatory M1 microglia/macrophages can exacerbate brain injury, whereas anti-inflammatory M2 microglia/macrophages are neuroprotective.[19] This dual role makes them an exciting target to enhance post-stroke brain recovery by shifting their balance from the detrimental M1 to the beneficial M2 phenotype. Accumulating evidence indicates that SCs can alter the polarization status of microglia/macrophages.[20, 21] *In vitro*, primary microglia/macrophages co-cultured with MSCs increased mRNA and protein expression of M2 markers such as Arg1, CD206, IL-10 and decreased expression of M1 markers such as IL-12 and TNF. This occurred both in a direct cell-contact and an indirect transwell environment, indicating the presence of paracrine factors.[22–25]

Ohtaki *et al.* first described M2 microglia induction by MSC administration in a transient MCAO model.[18] After MSC transplantation into the dentate gyrus at one day post-stroke, M2 protein expression of YM-1, IGF-1 and Galectin-3 was increased which correlated with improved neurological recovery. A later study suggested that MSC transplantation into the lateral ventricle of stroked rats decreased infarct volume and increased functional recovery by increasing IL-10 and decreasing TNF expression in the brain.[16] These M2-polarizing actions have been confirmed in several experimental stroke studies, which were all associated with improved neurological function.[26, 27] Thus, the observed beneficial actions of SCs are likely partly due to skewing microglia/macrophages toward a neuroprotective and neuroregenerative phenotype (Table 1).

Brain immune cell infiltration

The acute phase (first 48 hours) after stroke is characterized by cytokine and chemokine secretion, and blood-brain barrier disruption; this results in massive immune cell infiltration into the brain.[28] This first phase has devastating effects on stroke outcome.[29, 30] Acute and subacute (3–14 days) administration of both NSPCs and MSCs diminished Iba1$^+$ cells, which are either the resident microglia or infiltrated macrophages.[6, 17, 27, 31] Activated ED1$^+$ microglia/macrophage numbers also decreased in the striatum of NSPCs-transplanted ischemic rats.[7] In contrast, other studies showed increased numbers of microglia/macrophages in stroked animal models intracerebrally transplanted with NSPCs. They suggest this induces brain recovery by increased secretion of brain remodeling factors such as insulin growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF) and transforming growth factor-β1 (TGF-β1).[32, 33] These data suggest the beneficial effects of transplanted SCs are partly due to the inhibition of leukocyte infiltration (Table 1).

Stem cell-induced effects on the systemic immune response

Splenic contraction, a reduction in splenic cells and a corresponding increase in brain monocytes have been observed after transient MCAO,[34] indicating the importance of systemic immune responses. Systemically administered NSPCs in the acute phase post-stroke restored neurological function, and decreased brain edema and infarct volume. IL-1β, TNF, IL-23 and IL-17 expression levels in the ischemic hemisphere and blood were decreased, whereas TGF-β and IL-10 were increased in blood; an increase in blood T regulatory cells was also observed.[35] This suggests that peripheral immunomodulation can improve brain recovery post-stroke.

A spleen-dependent neuroprotective effect was observed after systemic administration of NSPCs in ischemic rats.[36] Intravenous injection of NSPCs 2 hours after stroke improved functional recovery, reduced infarct size and edema, and decreased brain inflammatory infiltration. Cytokine analysis demonstrated decreased pro-inflammatory expression of TNF, IL-6 and NF-κB in brain and spleen. Numerous NSPCs were also observed in the spleen; splenectomy eliminated the effects on brain edema and immune cell infiltration. A decrease in pro-inflammatory gene expression was confirmed in the MASTERS trial, a phase II randomized, double-blind, placebo-controlled trial evaluating SC treatment in acute strokes, at 7 days post-transplantation. Furthermore, a reduction in spleen size was prevented, indicating suppression of the peripheral immune response. [37, 38] These data suggest that solely modulating peripheral immunity could promote neurorestorative effects.

Chronic inflammation of the brain

The chronic stroke phase (>1 month) is characterized by persistent immune cell infiltration. B and T cells are present in the stroke core at 4–12 weeks post-stroke in different experimental models. In contrast to lymphocytic localization in the core, activated microglia/macrophages have been detected in the thalamus, striatum and internal capsule.[39] This immune cell infiltration has also been detected in postmortem human brain samples, even decades after stroke.[40] Despite this apparent chronic immune response, most SC transplantations have been performed during the earlier phases post-stroke. However, it was

shown that intravenous MSC administration in MCAO rats at 60 days post-stroke reduces brain and splenic inflammation.[41] Clinical studies with SC transplantation in the chronic stroke phase, like the Sanbio SB623 and PISCES trials, showed some promising neurological improvements, however its potential underlying immunomodulatory effects remain to be defined.[12] The Sanbio study demonstrated a transient FLAIR signal starting one week after MSC transplantation, which correlated with neurological recovery.[3] This signal may represent a beneficial inflammatory response, which could attenuate the chronic inflammatory response.

Stem cell-based immunomodulation of non-inflammatory repair processes after stroke

SCs also directly influence important brain repair processes, and multiple studies show that transplanted SCs induce angiogenesis.[6, 42–44] The expression and secretion of angiogenic factors such as VEGF, BDNF and fibroblast growth factor (FGF) are of key importance. SC transplantation also enhances brain plasticity by increasing axonal and dendritic sprouting.[33, 45, 46] Studies have shown that VEGF and thrombospondins 1 and 2 partially mediate these effects.[5, 47] Brain plasticity modulation also occurs at the synaptic level after transplantation, with changes in the number of excitatory and inhibitory synapses in different cortical layers of the brain.[48–50] Furthermore, SC transplantation increases the survival of endogenous glial and neuronal progenitors after ischemia.[17, 18, 51] Decreases in cell death are often accompanied by increased secretion of neurotrophic factors such as BDNF, FGF and VEGF,[13, 52] which generate survival signals in glia and neurons and can increase cellular resistance against oxidative stress.[51]

Numerous preclinical and a few clinical studies have shown beneficial SC-induced effects on angiogenesis, brain plasticity and brain cell death after stroke; these details are beyond the scope of this review, but are extensively reviewed elsewhere.[53–56] Despite the evidence demonstrating the beneficial effects of post-stroke SC transplantation on these processes, their exact mechanisms of action are unclear. It is most likely that stem cell-induced immunomodulation plays a central role.

Immunomodulation of angiogenesis

SC transplantation can polarize microglia/macrophages toward the anti-inflammatory and angiogenic M2 phenotype, and a classical factor secreted by M2 microglia/macrophages is VEGF.[57, 58] VEGF-dependent suppression of inflammation after intracerebral NSC transplantation was demonstrated in ischemic rats and associated with enhanced angiogenesis and functional recovery.[6] TGF-β, another prototypical M2 mediator induced after SC transplantation, also plays a prominent role in angiogenesis induction.[59] VEGF and TGF-β also interact to control angiogenesis, thereby strengthening each other's function.[60] Moreover, increased M2 polarization could result in less M1 microglia/macrophages and therefore less expression of their key cytokine IFN-γ; this cytokine has strong anti-angiogenic potential in diseases like cancer and atherosclerosis, and so could also be important in stroke.[61, 62]

The complement cascade is now recognized as more than a 'component' of the innate immune system; it is implicated in CNS development and regeneration, and is known to influence stroke.[63, 64] The effects of SC transplantation on the complement system are currently unknown; however the active complement factors C3a and C5a are associated with M2 microglia/macrophages and they can stimulate angiogenesis,[65, 66] making them interesting research targets. These data suggest SC-induced angiogenesis is mediated not only by expression of remodeling factors like VEGF, BDNF and FGF, but also induced by the anti-inflammatory effects of SCs (Figure 1a).

Immunomodulation of brain plasticity

Brain plasticity - in the form of neurogenesis, synaptic remodeling, axonal sprouting and dendritic branching - is essential for brain repair and functional recovery after stroke. Neuroinflammation, and activated M1 microglia in particular, can have detrimental effects on brain plasticity which can be reversed by administration of anti-inflammatory drugs.[67, 68] These effects are thought to be mediated by pro-inflammatory factors including TNF, IL-1β and NO.[68–70] TNF controls synaptic plasticity by regulating neuronal surface expression of excitatory AMPA and inhibitory $GABA_A$ receptors.[71, 72] IL-1β influences the surface expression of AMPA receptors in a similar fashion, albeit with lower efficacy. Post-stroke SC transplantation decreases microglia activation and secretion of pro-inflammatory cytokines, including TNF and IL-1β. Additionally, SC transplant after stroke enhances neurogenesis in the acute and subacute phase.[7, 31, 43] This suggests that decreased microglial activation can increase brain plasticity and improve functional recovery.

Cytokine-mediated interactions between microglia and astrocytes could also affect brain plasticity. After SC transplantation, increased IL-10 expression stimulates production of TGF-β by astrocytes, which decreases microglial activation and increases their phagocytotic capacity.[73] TGF-β secretion by astrocytes induces neuronal complement protein C1q expression, thereby targeting them for elimination by phagocytotic microglia.[73–75] Unwanted synapses are then pruned by microglia, crucial for brain remodeling. This demonstrates an indirect role for astrocytes in microglial-synapse elimination. Astrocytes can also monitor and modify synapse function directly, making their effects on brain plasticity context-specific.[74]

If the complement system is affected after SC transplantation, then microglia and astrocytes would likely respond to complement stimulation with the production of trophic molecules necessary for neuronal proliferation and renewal.[63] It is thus also possible that SC-induced complement activation induces brain plasticity (Figure 1b).

Immunomodulation of brain cell death

Acute and subacute SC transplantation decreases brain cell death and increases functional recovery, which is associated with decreased secretion of inflammatory mediators such as TNF, IL-1β, IL-6 and IFN-γ.[17, 18, 76] These cytokines are known apoptosis inducers, acting through the caspase cascade and expression of cell death receptors in several disease conditions including stroke.[77, 78] Accordingly, blocking TNF or IFN-γ prevents secondary infarct growth after stroke.[79] Expression of IL-10 and TGF-β is upregulated after SC

transplantation, which reduces microglial and astrocytic activation. Reduced cellular activation decreases reactive oxygen species levels, which are known to induce cell death.[80] Therefore, a direct link between cytokine levels and brain cell death may exist.

Excitotoxicity is a form of neurotoxicity and a major contributor of post-stroke neuronal injury; this phenomenon could also explain the decreased brain cell death observed after early post-stroke SC transplantation.[81] As described, TNF and IL-1β regulate synaptic plasticity by stimulating excitatory neurotransmission; when deregulated, neurotoxicity can result.[82] As SC transplantation reduces TNF and IL-1β levels, this could reduce excitotoxicity and thereby reduce brain cell death (Figure 1c).

Stem cells as a therapeutic strategy for stroke: future directions

Ischemic stroke is complex and affects a variety of brain regions, involving multiple interactions with the vasculature and immune system. Altering central or peripheral immune responses often improves functional recovery following stroke (Table 1), and there is strong evidence that SCs could be used as a clinically relevant therapy to target multiple pathways.

As described in this Topical Review, both the tissue specific NSPCs and the non-tissue specific MSCs can have advantageous effects with regard to immunomodulation of pro-regenerative processes. For both SC types, these immunomodulatory effects are mainly due to their bystander effect; the secretion of important proteins such as cytokines and trophic factors. Their mechanism of action seems to differ slightly when transplanted intravascularly, as MSCs primarily seem to control the immune response in the periphery, while NSPCs are prone to specifically home to the lesion site to exert their immunomodulatory effects there. [83] For both NSPCs and MSCs, true tissue restoration by integrating into the brain and differentiating into correctly functioning cells, such as neurons and glia, or endothelial cells is believed to have only a minor contribution to functional recovery. [84, 85] Indeed, evidence for this is scarce, although it has been shown that NSPCs can integrate into the brain and acquire neuronal characteristics, such as expression of synaptic proteins, synapse formation and appropriate electrophysiological aspects. [84] Whether this then is regulated via their immunomodulatory properties remains to be determined. Overall, a better understanding of the similarities between NSPCs and MSCs in the immunomodulation of pro-regenerative processes is needed, as this might reveal the essential immunomodulators for stroke recovery. In contrast, a better understanding of the differences between them will show SC specific actions in stroke recovery, which could help determine which particular SC type is needed for an optimal therapeutic effect, for example according to time post-stroke.

In addition, fundamental questions remain regarding the optimal route and dosage of SC transplantation, and why few (or no) transplanted cells engraft in the brain.[53] The survival rates of transplanted cells can vary,[86] which may indicate that secreted factors from transiently surviving or dying SCs have immunomodulatory roles. Clinical studies would benefit greatly from non-invasive imaging techniques to track the transplanted SCs longitudinally and repeatedly. [87] In this way, one can monitor their survival, migration, proliferation and the immune reactions they elicit. This all might help understand how their

pro-regenerative effects are generated. Additionally, *in vivo* brain imaging using MRI or PET would be useful to monitor the immunomodulatory response of the brain to the SCs. Altogether, this would be of great value to determine a patient-specific therapeutic approach**,** for example based on their lesion size and location. Despite the enormous potential of SC tracking and *in vivo* brain imaging, several problems exist regarding these technologies. It is essential to understand whether the tracking agents affect cellular functions and viability before this can be applied in the clinic. [88] Regarding *in vivo* brain imaging, each imaging technique has its own advantages and disadvantages, for example concerning spatial resolution and the use of contrast agents. Ideally, one should combine multiple imaging techniques to make this a non-invasive, safe and efficient way to serve as a qualitative and quantitative technique. [89]

We also consider combinatorial approaches to be of importance in future clinical studies. This rapidly emerging treatment option encompasses for example co-treatment with growth factors, or transplantation of genetically modified SCs. [90, 91] This approach will enable an improved understanding about the immunomodulation of pro-regenerative processes suggested in this Topical Review. Its effectiveness indeed was shown by a recent study in which MSCs were genetically modified to secrete abundant IL-10, which improved its therapeutic effects as compared to treatment with MSCs alone. [92] Another promising combination therapy is a tissue engineering approach using biomaterials. Biomaterials can serve as protective scaffold to ensure better survival of the graft and can among others enhance cellular infiltration into the lesion to stimulate regeneration. [93, 94] Interestingly, they can also be used for targeted delivery and sustained release of growth factors or cytokines, thereby serving as a promising tool to assess the benefits of immunomodulation on pro-regenerative processes post-stroke. However, before use in the clinic, one should be confident these combination therapies do not affect the SCs properties or induce adverse effects such as an inflammatory response.

We believe that SC-induced immunomodulation can be one of the central players in post-stroke recovery, via direct anti-inflammatory effects of the transplanted cells, or via its stimulating effects on angiogenesis and brain plasticity. Therefore, managing post-stroke inflammation through SCs administration is a worthwhile focus for future studies. Given the promising results obtained from pre-clinical and clinical research to date, there is significant belief that a better mechanistic understanding of the complex interactions required to develop successful immunomodulatory SC therapies for stroke is within reach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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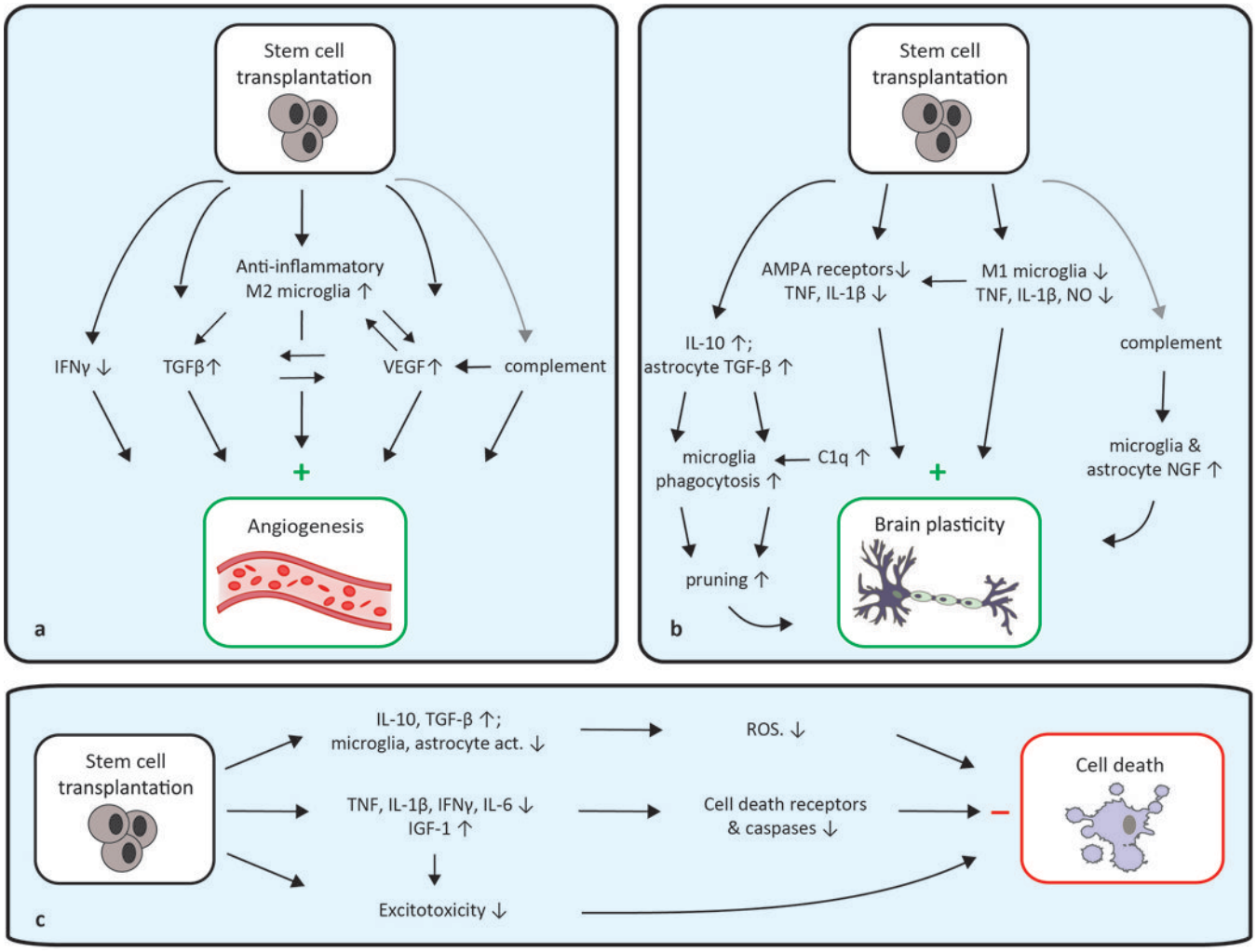

Figure 1. Stem cell-based immunomodulation of non-inflammatory repair processes after stroke.

a, SC-induced immunomodulation of angiogenesis is mediated by anti-inflammatory actions; they induce M2 microglia/macrophages with strong angiogenic potential via secretion of anti-inflammatory cytokines and growth factors, and by inhibition of pro-inflammatory cytokines. Increased VEGF expression and secretion acts either directly on endothelial cells for induction of angiogenesis, or indirectly through anti-inflammatory actions. **b**, SC-induced immunomodulation of brain plasticity is mediated by decreased M1 microglia activity, thereby decreasing inflammatory cytokine production and subsequently inducing brain plasticity. Decreased inflammatory expression results in less AMPA receptor surface expression, enhancing brain plasticity. Astrocyte-microglia interactions can increase synapse pruning, and complement activation may also affect plasticity. **c**, The anti-inflammatory actions of transplanted SCs decrease cell death receptor expression, inhibit the caspase cascade and decrease excitotoxicity, all decreasing brain cell death. Grey arrows indicate possible connections. ROS: reactive oxygen species; act.: activity.

Table 1.

Stem cell-induced immunomodulatory actions in *in vivo* ischemic stroke models

Model	Host	Cell type	Timing	Findings	Ref
Acute (>48 h) administration intravenous					
Transient MCAO	LE rat	MSCs c.m. allograft	0 dps	<7 dpt recovery ↑ Microglia/macrophages ↓ Trend toward decreased infarct size Neural progenitor cells ↑	31
Permanent MCAO	SD rat	MSCs allograft	30 min ps	1–14 dpt recovery ↑ Brain gene expression GFAP ↓, VEGF, SYP, Olig-2, NF ↑ 14 dpt apoptosis penumbra ↓	49
Transient MCAO	Wistar rat	MSCs xenograft	1 dps	$Iba1^+$ and $GFAP^+$ cells in core and penumbra ↓ Brain gene expression iNOS, MCP1, COX-2 ↓, IL-4 ↑	27
Permanent MCAO	SD rat	MSC-derived MVs allograft	2 dps	3–7 dpt recovery ↑ Striatal $GFAP^+$ cells ↓ Anti-inflammatory cytokines ↑ Infarct size ↓ Angiogenesis ↑ Neurogenesis ↑	43
Acute (>48 h) administration intracerebral					
Transient MCAO	C57Bl/6, C57Bl/6/SCID mice	MSCs in hippocampus xenograft	1 dps	1–4 dpt recovery ↑ Hippocampal M2 microglia & gene expression YM1, IGF-1, Gal-3 ↑ 1–4 dpt neuronal death hippocampus ↓	18
Permanent MCAO	SD rat	MSCs in lateral ventricle allograft	1 dps	14 dpt recovery ↑ Brain gene expression IL-10 ↑, TNF ↓ 1, 4 dpt infarct volume ↓	16
Transient MCAO	Nude rat	NSPCs in striatum xenograft	2 dps	6–12 wpt recovery ↑ Striatal inflammation ↓ Activated microglia ↓ Striatal neurogenesis ↑ SVZ proliferation ↑	7
Sub-acute (3–14 days) administration intravenous					
Transient MCAO	C57Bl/6 mice	NSPCs allograft	3 dps	> 18 dpt recovery ↑ Brain gene expression TNF, IL-1β, IL-6, IFN-γ ↓ Brain immune cell infiltrate ↓ Reactive astrocytes ↓ Neuronal death ↓	17
Sub-acute (3–14 days) administration intracerebral					
Permanent MCAO	Nude rat	NSPCs ipsilesional xenograft	7 dps	1–4 wpt recovery ↑ $Iba1^+$ cells ↓ VEGF neovascularization VEGF ↑ BBB integrity ↑	6

MCAO: middle cerebral artery occlusion; LE: Lewis Evans rat; SD: Sprague Dawley rat; dps: days post-stroke; ps: post-stroke; wpt: weeks post-transplantation; dpt: days post-transplantation; c.m.: conditioned-medium; MVs: microvesicles; SVZ: subventricular zone; SYP: synaptophysin; Olig-2: oligodendrocyte; NF: neurofilament; GFAP: glial fibrillary acidic protein.