Chapter 2
Interdisciplinary Advances Towards Understanding and Enhancing the Therapeutic Potential of Stem Cell-Based Therapies for Ischaemic Stroke

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Abstract Worldwide, stroke is the second single most common cause of death and is a major cause of permanent disability. Moreover, the highest incidence of these pathologies is observed in the elderly, increasing the socioeconomic burden in an aging population. Current available therapies lead to insufficient functional improvement or are not applicable to all patients. This stresses the urgent need for alternative strategies in treating stroke patients, for example cell-based therapies. These cells showed great preclinical potential although the underlying therapeutic mechanisms, preferential route of administration and most suitable stem cell-subtype are unknown. Mechanisms of action include neuroprotection, cell replacement, neurogenesis, immunomodulation and the promotion of both neuroplasticity and angiogenesis in damaged central nervous system regions. Moreover, stem cells have been genetically engineered to enhance their beneficial effects after transplantation. Additionally, noninvasive imaging can be used to provide detailed spatial and functional information on the donor cell fate and the response of the host microenvironment. This chapter provides an overview of recent advances in (bio-)medical research using or manipulating stem cell-based therapies for ischaemic stroke with a focus on their neuroprotective, neuroregenerative and immunomodulatory properties. Additionally, the use of noninvasive imaging to allow temporospatial evaluation of stem cell fate following transplantation in animal stroke models will be discussed.

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Abbreviation

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
Ang-1	Angiopoietin-1
ASC	Adipose-derived stem cell
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BLI	Bioluminescence imaging
BM-MNC	Bone marrow-derived mononuclear cells
BMMSC	Bone marrow-derived MSC
CCR2	C-C chemokine receptor type 2
CT	Computed tomography
CXCR4	C-X-C chemokine receptor type 4
DAMPs	Danger associated molecular pattern molecules
DPSC	Dental pulp stem cell
EC	Endothelial cells
ECM	Extracellular matrix
EGF	Epidermal growth factor
ESC	Embryonic stem cell
EVs	Extracellular vesicles
FGF	Fibroblast growth factor
FLI	Fluorescence imaging
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibrillary acid protein
hESC	Human embryonic stem cell
HGF	Hepatocyte growth factor
ICAM-1	Intercellular Adhesion Molecule 1
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-gamma
IGF-1	Insulin-like growth factor 1
IL	Interleukin
iPSC	Induced pluripotent stem cell
MCAO	Middle cerebral artery occlusion
MCP-1	Monocyte chemotactic protein 1
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MMP	Matrix metalloproteinase

MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cell
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
NK cells	Natural killer cells
NMDA	N-methyl-D-aspartic acid
NO	Nitric Oxide
NSC	Neural stem cell
OGD	Oxygen-glucose deprivation
PDGF-BB	Platelet-derived growth factor BB
PET	Positron emission tomography
PGE2	Prostaglandin E2
ROS	Reactive oxygen species
SDF-1α	stromal cell-derived factor 1 α
SGZ	Subgranular zone
SPECT	Single-photon emission computed tomography
SPIO	Superparamagnetic iron oxide
STAT3	Signal transducer and activator of transcription 3
SVZ	Subventricular zone
TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumour necrosis factor alfa
Treg	Regulatory T cell
VEGF	Vascular endothelial growth factor

1 Introduction

The pathophysiology of stroke is defined as a neurologic dysfunction of vascular origin with the rapid occurrence of symptoms and signs corresponding to the involvement of focal areas in the brain [1]. Two different types of stroke can occur: ischaemic stroke (80–85%) and haemorrhagic stroke (15–20%). Ischaemic stroke is most frequently caused by thromboembolisms while haemorrhagic stroke most often results from vessel wall pathology associated with hypertension and microaneurysms [2]. This chapter will only focus on ischaemic stroke as the main pathology.

Worldwide, stroke is the second most common cause of death and is a major cause of permanent disability [3, 4]. Moreover, the highest incidence of these pathologies is observed in the elderly, increasing the socioeconomic burden in an ageing population [4]. In ischaemic stroke, the blood supply to certain areas of the brain is compromised which triggers a cascade of deleterious events ultimately leading to neuronal cell death [5]. This in turn triggers the acute immune response which can have a persistent and detrimental effect on stroke outcome [6]. The resulting severe neurological

dysfunction is clinically translated into symptoms such as paralysis, sensory disturbances, aphasia, urinary incontinence and cognitive impairment. Limited stroke-induced endogenous neurogenesis can be observed in patients but adequate functional recovery is not achieved [7]. Recombinant tissue plasminogen activator is the only FDA-approved pharmacological treatment for stroke but comes with many restrictions. Administration should be started within a time window of 4.5 h post-ischaemia, limiting its use to merely 2–4% of the patients and leading to an insufficient functional improvement [8]. These indications highlight the urgent need for alternative strategies in treating stroke patients.

Stem cell therapy is a promising approach to minimize neurological damage and enhance functional recovery after stroke. Preclinical studies in animal stroke models using for instance neural stem cells (NSC) [9], mesenchymal stem cells (MSC), induced pluripotent stem cell (iPSC)-derived cells delivered encouraging results (See Table I and Table II in [10]). However, the optimal stem cell-source, mechanisms of action, cell fate and optimal treatment protocol remain to be elucidated. Mechanisms of action include neuroprotection, cell replacement, immunomodulation and the promotion of both neuroplasticity and angiogenesis in damaged central nervous system regions [10]. Moreover, stem cells that were genetically engineered to overproduce growth factors such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), Noggin and angiopoietin-1 (Ang-1) have been previously shown to enhance post-stroke recovery [11–13]. Additionally, noninvasive imaging can be used to provide detailed spatial and functional information on the donor cell fate and the response of the host microenvironment following cell-transplantation into animal stroke models [10].

This chapter aims to provide an up to date overview of current interdisciplinary advances in preclinical stroke research, focussing on neuroregeneration, neuroprotection and immunomodulation supported by noninvasive imaging opportunities.

2 Therapeutic Approaches and Evaluation of the Post-stroke Microenvironment

The multiple mechanisms that have been proposed for stem cell-mediated therapies include brain protection, cell replacement, immunomodulation and promoting both brain plasticity and angiogenesis in damaged brain regions (Fig. 2.1) [10]. Interestingly, these mechanisms are mainly thought to be mediated by the paracrine effect of the transplanted cells on endogenous stem cells and on the host microenvironment instead of directly replacing the lost cells, although encouraging results have also been achieved with cell-replacement studies. Therefore, the engrafted cells can be seen as a vehicle for persistent growth factor delivery at the stroke lesion which can also respond dynamically to changes in the local microenvironment. In addition, the transplanted cells directly or indirectly influence extracellular matrix (ECM) remodelling and glial scar formation.

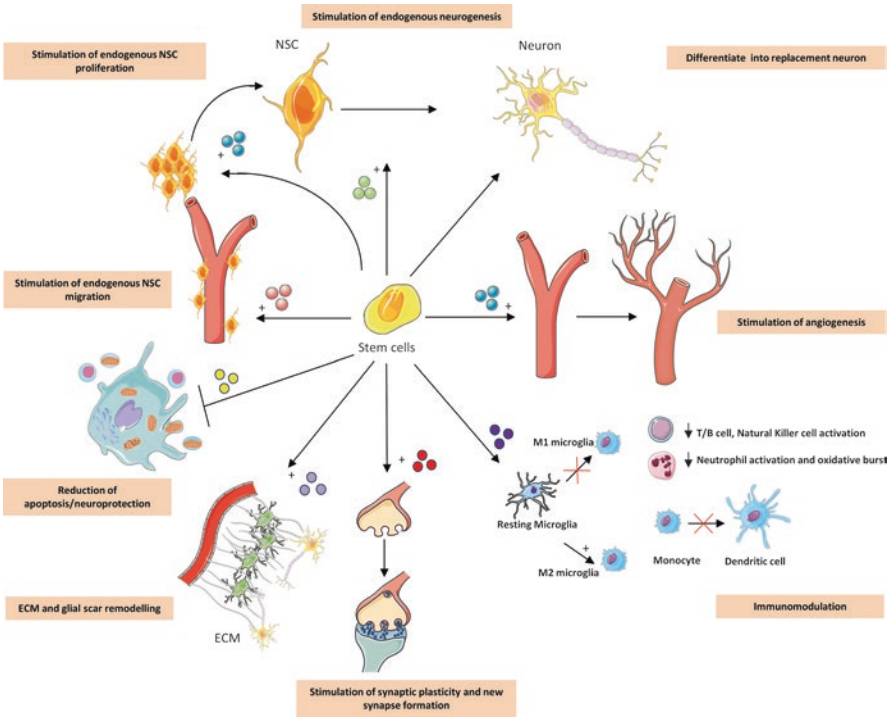

Fig. 2.1 Mechanisms of action of cell-based therapies in ischaemic stroke. The poststroke microenvironment can be influenced by exogenously delivered stem cells by multiple mechanisms to trigger tissue repair. Stem cells contribute to poststroke recovery by stimulating endogenous NSC migration toward the stroke lesion, where proliferation and differentiation toward replacement neurons can be triggered. Additionally, transplanted stem cells are thought to be able to replace the lost neurons themselves. Moreover, the formation and attraction of novel blood vessels toward the ischaemic lesion and the stimulation of synaptogenesis and synaptoplasticity contribute to brain repair. In addition to directly stimulating the formation of new brain tissue, the degradation of resident cells is inhibited by neuroprotective mechanisms and transplanted cells can influence the extent of glial scar formation. Immunomodulatory effects are also observed and include the inhibition of neutrophil activation and migration, effector T-cell and B-cell inhibition, reducing the activation and attraction of peripheral dendritic cells, and stimulating the M2 microglial phenotype. These effects are predominantly caused by the soluble factors released by the stem cells, but also cell–cell interactions appear to play a role. Image was adapted from [10] with permission

2.1 The Neuroprotective Effect of Stem Cell-Based Therapies

The ideal therapeutic approach for stroke would be to prevent neuronal cell death induced by the ischaemic insult, thereby minimizing neurological damage and stroke severity. Any strategy that aims to inhibit or antagonize the pathophysiological cascade of biochemical events resulting in irreversible cell damage and neuronal cell death is considered a neuroprotective approach [14].

2.1.1 The Complexity of the Ischaemic Cascade

Neuronal cells located in the ischaemic core die within minutes after stroke onset, whereas peripheral cells residing in the penumbra provided with limited collateral blood flow become dysfunctional but do not undergo acute cell death. However, if left untreated, the neuronal cells in the penumbra are likely to progress into delayed neuronal cell death hours to days after the ischaemic insult [15]. Therefore, a restricted time window exists wherein reversibly damaged neuronal cells can be salvaged from cell death in order to limit infarct size and improve functional outcome after stroke.

In the ischaemic core and penumbra, a series of neurochemical events occur, described as the ischaemic cascade [16]. The brain depends on oxygen and glucose to assure normal neuronal cell function and maintain ionic homeostasis. Impairment of cerebral blood flow causes disturbances in these vital energetic processes [5]. Ischaemia leads to dysfunction of ATP-dependent ion pumps, including the Na^+/K^+ pump, which results in alterations in the membrane potential and depolarisation of neurons and glial cells [17]. Subsequently, voltage-dependent Ca^{2+} channels are activated and excitatory neurotransmitters, including glutamate, are released into the extracellular space. This accumulation of glutamate in the extracellular space leads to the stimulation of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA)-type glutamate receptors on adjacent neurons. Consequently, these neurons become depolarized, which results in additional Ca^{2+} influx and glutamate release, causing an exacerbation of the initial ischaemic insult. Due to the ionic imbalance, intracellular water accumulation occurs, which is responsible for the formation of cytotoxic oedema [5, 16]. Furthermore, the increase in intracellular Ca^{2+} leads to the activation of Ca^{2+}-dependent enzymes, including proteases, endonucleases, phospholipases and cyclooxygenases. These enzymes cause extensive cell damage and are partially responsible for the generation of reactive oxygen species (ROS). ROS are important mediators of cellular damage by inducing DNA damage, lipid peroxidation and protein denaturation, which ultimately results in mitochondrial failure and membrane disruption [5, 16, 17]. The outcome of these detrimental molecular events is cell death via necrosis or apoptosis, depending on the degree of ischaemic injury [17, 18].

2.1.2 Stem Cell-Mediated Neuroprotection

Numerous neuroprotective agents which target different components of the pathophysiological cascade have been investigated in the context of stroke (reviewed by Moretti et al. [19]). These molecular targets comprise various mechanisms of calcium influx, glutamate excitotoxicity, ROS scavenging, NO metabolism and apoptosis. Despite promising preclinical results, no neuroprotective agent has passed clinical trials in stroke patients. Alternatively, approaches like therapeutic hypothermia and decompressive craniectomy have been explored to minimize neurological damage. Both these approaches seem to result in mild improvements in

neurological scores in stroke patients. However, these patients still have a poor functional outcome after 6 months [20, 21].

Stem cell-based therapy is considered a promising treatment strategy to minimize neurological damage and enhance functional recovery following stroke. By secreting neurotrophic and anti-apoptotic factors, stem cells can provide support to reversibly damaged neurons present in the penumbra. In this way, the stem cells can exert neuroprotective effects and rescue neurons which otherwise would progress into delayed cell death, thereby preventing additional neuronal damage [22, 23]. It has been demonstrated that MSC, including bone-marrow-derived MSC (BMMSC) and dental pulp stem cells (DPSC), secrete a plethora of paracrine factors comprising BDNF, NGF, neurotrophin-3 (NT-3) and glial cell-derived neurotrophic factor (GDNF), which are considered hallmark neurotrophic and neuroregulatory factors [24–27].

Neuroprotective effects mediated by stem cells have been observed in numerous *in vitro* studies performed by independent research groups. It has been shown that MSC protect neuroblastoma cells against hypoxia and glutamate excitotoxicity [28–30]. Furthermore, MSC are able to rescue primary cortical neurons which are exposed to oxygen-glucose deprivation and trophic factor withdrawal [25, 31]. Additionally, MSC can also prevent cell death in ischaemic human astrocytes [32]. Extensive evidence of neuroprotective effects mediated by MSC has also been provided in *in vivo* stroke models. Injection of the conditioned medium of stem cells as well as the stem cells as such have proven to exert neuroprotective effects in experimental stroke models [28, 33–37]. The paracrine factors secreted by the stem cells are believed to be responsible for this neuroprotective effect, since no or very limited integration of the stem cells in the lesion site is observed. These studies comprised the use of MSC derived from different sources, including adipose-derived stem cells, umbilical tissue-derived stem cells, BMMSC and DPSC. Furthermore, different routes of administration, cell dosages and time points of transplantation have been used in these studies.

2.2 Regeneration of Endogenous Tissue by Stem Cells After Ischaemic Stroke

In the 1990–2000s, MSC therapy gained a lot of attention to be used as therapy after stroke because of their assumed ability to transdifferentiate into neuronal cells, endothelial cells and glial cells. Numerous *in vitro* experiments demonstrating this so-called transdifferentiation potential sparked hope to use these stem cells for neuronal tissue replacement. However, these properties are merely induced under certain artificial cell culture conditions, not representing their endogenous properties. Another disadvantage is that this processes turned out to be time-consuming and very inefficient. Despite of this, transplantation of undifferentiated MSC has been explored with the rationale that they would locally replace the neural tissue. Intracranial transplantation of MSC showed that the cells migrated towards the

infarct region, survived in the host brain and stimulated functional recovery [38]. However as already mentioned above, numerous studies indicated that only a small percentage of the transplanted MSC survived and locally differentiated preferentially towards astroglial cells instead of the desired neurons [10, 34, 39]. It is generally accepted that functional replacement of the lost neurons is not the main mode of action of MSC.

Embryonic stem cells (ESC) and iPSC have the tremendous potential to differentiate in all possible cells from the nervous system including glial cells and neurons. However, achieving mature neurons from these pluripotent stem cells is even *in vitro* very time-consuming, costly and inefficient. Also their possible tumorigenicity when used in undifferentiated state is a major disadvantage [40, 41]. Therefore, iPSC are irreversibly pre-differentiated *in vitro* in order to minimize tumour formation and improve the functional outcome as only the undifferentiated iPSC form teratomas. The true potential of iPSC and ESC in stroke research is to apply them *in vitro* as a patient-in-a-dish model, trying to understand disease mechanisms and for drug discovery [42]. Especially the novel method culturing cerebral organoids resembling the 3D brain structure is a big step forward closing the gap between 2D cell cultures and animal models [43, 44].

When it comes to the use of stem cells for neuronal tissue regeneration as well as for neuroprotection, the current focus lays on the angiogenic and neurotrophic factors, cytokines and chemokines that are able to enhance the endogenous repair mechanisms after cerebral ischaemia, i.e. vascular remodelling, activation of endogenous neuroregeneration and remodelling of the extracellular matrix.

2.2.1 Vascular Remodelling After Stroke

Cerebral ischaemia leads to increased vascular remodelling in both the acute and chronic phase [45]. During acute blood flow obstruction, arteriogenesis also referred to as collateralisation can occur. This development of a functional blood flow from pre-existing arterial anastomoses is induced by mechanical forces such as shear stress and is independent of a hypoxia state [46]. By contrast, angiogenesis, the development of new capillaries sprouting from existing small blood vessels, is a key endogenous process induced by chronic hypoxia. The angiogenic process is a complex cascade of events, involving breakdown of the extracellular matrix, activation of endothelial cells (EC), followed by the proliferation, migration of EC. In a final step, pericytes are recruited towards the formed tubular network of EC resulting in mature blood vessels. A multitude of angiogenic factors and signalling molecules such as vascular endothelial cell growth factor (VEGF), fibroblast growth factor (FGF), angiopoietin-1 (Ang-1), Platelet-derived growth factor BB (PDGF-BB), nitric oxide (NO) have to co-operate in concert with spatiotemporal precision [47, 48]. Although distinct triggering mechanisms induce either collateralisation or angiogenesis, similar growth factors, cytokines and signalling molecules are shared by both modes of vascular remodelling [45]. A lot of angiogenic therapies applying recombinant proteins gained disappointing results in clinical trials [49, 50]. This can

be partly explained by the fact that the most studied angiogenic factor VEGF, is linked to the generation of immature and unstable vessels leading to oedema and vessel regression over time, aggravating stroke progression. In addition, the majority of angiogenic therapy regimens only involved administration of a single angiogenic protein. In that respect, MSC which have been showed to secrete numerous cytokines and angiogenic factors and as a surplus can act as a pericyte, can create the right angiogenic microenvironment [51]. Furthermore, numerous animal stroke studies support the pro-angiogenic properties of MSC obtained from different tissues and that the reported improved recovery is associated with increased blood vessel density. For example, increased VEGF and VEGFR2 expression was observed after intravenous injection of human BMMSC in a rat model of ischaemic stroke [52]. Another research group reported elevation of Ang-1 and Ang-2 mRNA levels in BMMSC treated rats [53]. Wakabayashi et al. showed that intravenous injection human MSC in a rat middle cerebral artery occlusion (MCAO) model induced functional improvement and reduced infarct volume by producing angiogenic factors. Moreover, MSC locally secreted IGF-1 in the ischaemic core and interestingly, this IGF-1 production was only detected *in vivo*, suggesting its specific induction by the ischaemic environment. In addition, the transplanted MSC affected the host cells, as endogenous VEGF, EGF, and bFGF levels were significantly elevated in stem cell-treated rats 7 days after injury [54]. Not only the stem cells themselves have a significant therapeutic potential in ischaemic stroke but also their extracts were shown to have a therapeutic effect. Intraperitoneal injection of a cell-free extract derived from MSC was shown to dramatically decrease the ischaemic volume and improve motor function after stroke [55].

2.2.2 Endogenous Regeneration After Stroke

For a long time, cells of the adult central nervous system were considered to be incapable of regeneration. However, it was demonstrated that human adult NSC reside in the dentate gyrus of the hippocampus and in the subventricular zone (SVZ) [56, 57]. Under normal physiologic conditions, adult NSC predominantly produce neurons, interneurons of the olfactory bulb for SVZ-derived cells, and dentate granule cell neurons for SGZ-derived cells. Ischaemic stroke enhances proliferation of the SVZ cell population and these cells migrate towards the lesion and differentiate into mature striatal neurons and replace damaged neurons. SDF-1α/CXCR4 and MCP-1/CCR2 receptor signalling has been shown to regulate the directed migration of these NSC to the injured area [58, 59]. NSC themselves have been shown to produce matrix metalloproteinase (MMP)-3 and MMP-9 in response to these extrinsic signals. Blocking the expression of MMP-3 or MMP-9 in NSC interferes with both their differentiation and migration [60], suggesting a prominent role of these MMPs in the endogenous NSC response. The leading fraction of the migrating NSC is closely associated with blood vessels, suggesting that this interaction provides a scaffold to direct the NSC towards the damaged brain region [61]. However, the amount of endogenous cells generated is considered to be too low to have a significant impact

on functional recovery after stroke. Nevertheless, various preclinical trials have been performed to enhance neurogenesis after stroke using EGF, VEGF, erythropoietin and statins [62, 63]. These investigations have also formed the basis to investigate the ability of cell transplantation to activate NSC and to mediate their differentiation towards neurons.

Neurotrophins/growth factors found in the MSC secretome include GDNF, NT-3, NGF and BDNF [10, 24, 30, 64]. To our knowledge, the effect of MSC on NSC migration and/or differentiation has not yet been tested *in vitro*. CM of DPSC, BMMSC and Wharton Jelly MSC have shown to enhance neuronal maturation of a pre-differentiated neuroblastoma cell line SH-SY5Y cells [65, 66]. In addition, the MSC secretome has been demonstrated to enhance neurite outgrowth in various types of primary neurons including, dopaminergic, primary cortical neurons and neurons derived from dorsal root and the retinal ganglia [10, 24, 30, 64]. In contrast to the overwhelming preclinical evidence on the induction of angiogenesis by MSC transplantation, only few reports on activation of endogenous NSC proliferation, migration and maturation are available. Munoz et al. showed that hMSC injected stereotactically induced migration of BrdUrd-labeled endogenous cells throughout the dorsal hippocampus, which were doublecortin-positive, and expressed markers for astrocytes as well as for neural or oligodendrocyte progenitors 7 days after treatment. In addition, 30 days after implantation, the newly generated NSC expressed markers for more mature neurons and astrocytes [67]. Another study in a rat stroke model, demonstrated that human MSC transplanted intracranially induce proliferation of endogenous NSC and subsequent migration as shown by double staining of BrdU and doublecortin at 1 and 2 weeks after MCAO induction [63]. Recent work in the setting of traumatic brain injury (TBI) showed that transplanted exogenous MSC are able to guide the migration of endogenous cells from the neurogenic site to the area of injury in the cortex via the formation of a 'biobridge' between the neurogenic and ischaemic site. This biobridge, visualized immunohistochemically and laser captured, corresponded to an area between the neurogenic SVZ and the injured cortex and consists of an altered endogenous expression of MMPs and ECM [68]. Despite the fact that MSC transplantations have been shown to induce both proliferation and differentiation of SVZ-derived NSC, neuronal differentiation rates were very low. As a consequence, there is a controversy on the fact whether or not MSC-induced enhancement of endogenous neurogenesis significantly contributes to an enhanced post stroke recovery [69, 70].

2.2.3 ECM and Scar Tissue Remodelling

After ischaemia, gliosis also referred to as scar formation is strongly induced at the infarct boundary. Damaged neurons initially interact with the adjacent astrocytes, which become activated and show increased expression GFAP, musashi-1 and secrete inflammatory cytokines [71, 72]. These triggered astrocytes in turn rapidly surround the infarct with fibrils [73]. The possible role of this demarcation consisting of activated astrocytes and ECM, but also microglia and oligodendrocytes is to separate the necrotic tissue

from viable brain and avoid further spreading of damage. Furthermore, this seal has also shown to play a role in maintenance of ion and fluid balance, preventing further inflammation, free radical scavenging and increasing tropic and metabolic support of the nerve tissue and for blood vessel ingrowth. On the other hand, it has a devastating effect on functional recovery as it impedes axonal regeneration [71, 72]. The ECM compound represents a physical barrier for new regenerating axons to cross. In addition, the reactive astrocytes secrete growth-inhibitory molecules such as Nogo [74]. According to this rationale, therapies that are able to reduce gliosis would thus be beneficial and enhance stroke recovery [72]. Several studies indicate that MSC secrete MMPs that cleave the ECM and would play a role in scar tissue destruction. BMMSC were able to produce active MMP-2, MMP-3 and also membrane-bound MT1-MMP [75, 76]. MSC are also able to activate exogenous proMMP-2 and proMMP-13. Interestingly, a recent study showed that the majority of the MMP activity is associated with the MSC cell surface while they secrete high levels of TIMPs, which strongly inhibits soluble MMPs. Since they bind and activate MMPs at their surfaces, the net result is a very controlled pericellular localization of MMP activities by MSC [77]. However, in the context of stroke, the contribution of this MMP production and the beneficial effects of MSC treatment remains to be elucidated. Only a few reports are available that studied the effect of stem cells on scar tissue formation. MSC treatment reduced the thickness of the scar wall and reduced the number of microglia/macrophages within the scar wall 4 months after surgery in a rat MCAO stroke model [71]. The same research group reported that long-term follow up (more than 1 year) of BMMSC injected in the carotid artery 1 day after MCAO significantly reduced thickness of the lesion scar wall and the number of Nogo-positive cells [78]. The exact molecular mechanisms behind this reduction of the scar wall thickness remain to be elucidated.

Although several studies emphasize that reactive astrocytes after CNS injury induce glial scar formation, which inhibits axon regeneration and impedes functional recovery, others indicated a neuroprotective role of astrocytes in CNS injury [72]. In that respect, it is worth to study the impact of stem cells on astrocyte survival. Indeed, MSCs suppress astrocyte apoptosis induced by OGD *in vitro*, an effect that has been attributed through the MSC-induced activation of IL-6 signalling in injured astrocytes [32, 79]. In addition, Song et al. showed that both BMMSCs as wells as DPSCs attenuated OGD-induced GFAP, nestin, and musashi-1 expression and inhibited OGD-induced ROS and interleukin-1β production in activated astrocytes *in vitro* [32].

2.3 Immunomodulatory Properties of Candidate Stem Cell-Based Therapies for Ischaemic Stroke

Whereas the neuroregenerative and neuroprotective effect of transplanted stem cells on the stroke-affected microenvironment has been studied thoroughly as described in the previous sections, the effect of the transplanted cells on the immune system and the infiltrating immune cells remains to be fully characterized.

2.3.1 Introduction to Stroke Immunology

The immune system and inflammation play a key role in the pathophysiology of stroke and can greatly influence stroke outcome [80]. Moreover, as a response to the ischaemic insult, the brain exerts a suppressive effect on the systemic immune system which leads to systemic lymphocytopenia [81]. This makes patients more susceptible to infections and is a major cause of stroke-associated morbidity and mortality [82, 83].

The various elements of the immune system are involved in all stages of ischaemia-induced brain loss. Early vascular events after arterial occlusion initiate inflammation where hypoxia, the production of ROS and changes in blood flow trigger the coagulation cascade, blood platelets and complement [84–86]. These events are followed by the upregulation of adhesion molecules on the platelet—and EC surface such as P-selectin, E-selectin and intercellular adhesion molecule 1 (ICAM-1) [87]. Moreover, the production of pro-inflammatory signals/cytokines is increased as well as the production of the vasodilator nitric oxide (NO) [86, 87]. Ultimately, EC junctions are weakened which allows protein and cellular extravasation into the perivascular space where mast cells and macrophages are activated and secrete proteases and pro-inflammatory mediators leading to blood-brain barrier (BBB) damage and leukocyte infiltration [86, 88].

In the subsequent phase of ischaemic cell death, the dying neuronal cells send out danger signals that activate the immune system [89]. These so-called danger associated molecular pattern molecules (DAMPs) include extracellular ATP or other nucleotides [90], heat-shock proteins, ECM breakdown proteins [91] and the high mobility group box 1 protein (HMGB1) [92] which are released from dying brain tissue following stroke [89, 93]. These DAMPs activate ionotropic purine receptors and scavenger—or pattern recognition receptors on inflammatory cells, leading to the production of pro-inflammatory mediators by resident brain cells and infiltrating leukocytes (for in-depth DAMP signalling, see review Gelderblom et al. [93]).

Stroke-induced inflammation eventually diminishes and triggers several pathways needed for the repair and reorganisation of the injured brain. This switch from a tissue-damaging pro-inflammatory stroke microenvironment to an anti-inflammatory, repair-stimulating environment remains poorly understood, but is coordinated by an intertwined cascade of inter- and intracellular signalling [86]. In this transition, macrophages and microglia switch from a pro-inflammatory M1 phenotype to an M2 phenotype that stimulates repair processes and attenuates the inflammatory response [94, 95]. Dead cells and debris attract and activate infiltrating macrophages and microglia which subsequently phagocytize the lost tissue. Phagocytosis induces the production of cytokines such as transforming growth factor beta (TGF-β), IGF-1 and IL-10 which were shown to have a neuroprotective and/or an anti-inflammatory effect [96–98].

In addition to the innate immune system, the adaptive immune system was also shown to contribute to inflammation-induced neuronal damage. DAMPs from damaged cells can also function as antigens that are presented to cells of the adaptive immune system, leading to immunity against these antigens [99]. Although the

damage to the post-ischaemic brain does not appear to be caused by an autoimmune response, the observed injury also does not fit the profile of classical adaptive immunity due to the temporal profile of the cellular infiltrate [86]. Blocking postischaemic trafficking of T cells 24–48 h after ischaemia provides a neuroprotective effect [100], whereas the classical adaptive immune response takes up to 1 week to develop and damage the ischaemic tissue. Interestingly, B cells do not significantly contribute to brain injury [101] and T-cell mediated damage is associated with γδ T cells which release the pro-inflammatory cytokine interleukin-17 (IL-17) [102, 103] and blocking the IL-17 signalling axis was shown to decrease neutrophil infiltration and ameliorate stroke outcome [103]. The contribution of natural killer (NK) cells and NK T cells to stroke injury remains to be elucidated [100].

This brief introduction in post-ischaemic inflammation provides several targets for stem cell-directed therapies for immunomodulation. Starting at the early onset, for example ROS scavenging can reduce the initial ischaemia-reperfusion injury, whereas stem cell-mediated therapies can also influence stroke outcome by modulating other aspects of the inflammatory cascade, as will be discussed next.

2.3.2 Mechanisms of Stem Cell-Mediated Immunomodulation

Although stem cell survival can be influenced by the host immune system, the transplanted cells themselves are believed to possess immunomodulatory properties [104, 105]. When considering immunomodulation as a stem cell-based therapy for ischaemic stroke, the post-stroke systemic immunosuppression needs to be taken into account. Additional systemic immunosuppression by cell-based therapies could worsen stroke outcome. Fortunately, no adverse effects on systemic cytokine levels were observed following syngeneic BMMSC transplantation in a mouse model of ischaemic stroke [105]. Moreover, the majority of completed clinical pilot studies with autologous MSC showed no adverse effects and improved clinical outcome, although post-stroke immunosuppression was not investigated [106, 107].

When considering mechanisms of stem cell-mediated immunomodulation, several *in vitro* reports are available. BMMSC and their extracellular vesicles (EVs) were shown to suppress T-cell proliferation [108, 109], BMMSC and adipose-derived stem cells (ASC) suppressed lymphocyte proliferation and the mixed lymphocyte reaction (MLR) [110]. Similarly, DPSC possess immunomodulatory properties [111]. Demircan et al. showed that the suppressive actions on T cells were mediated via paracrine effects by means of a transwell and MLR assay [112]. Increased levels of hepatocyte growth factor (HGF), TGF-β, ICAM-1, IL-6, IL-10, VEGF and human leukocyte antigen-G were found in DPSC/T cell co-cultures [112], the latter factor was additionally shown to suppress T cell and NK cell function and induce regulatory T cell function when secreted by BMMSC [113]. Moreover, the expression of pro-inflammatory cytokines by T-cells such as interferon-gamma (IFN-γ), IL-2, IL-12, IL-17A and tumour necrosis factor alpha (TNF-α) were decreased in the transwell system whereas the expression of the anti-inflammatory cytokine inducible protein-10 was upregulated [112]. Interestingly,

the expression of the regulatory T cell (Treg) markers CD4, CD25 and Foxp3 was increased. T cell apoptosis was increased after 24 h incubation with DPSC [112]. A similar paracrine mediated immunosuppression was exerted by the secretome of porcine and human DPSC [114–116]. In addition to T cells, BMMSC were shown to inhibit NK cell activation by producing prostaglandin E2 (PGE2) and indoleamine 2,3-dioxygenase (IDO) [117]. The proliferation, activation, maturation and antigen presentation of dendritic cells was also inhibited by MSC subtypes [118–122] and macrophage/microglia polarization was shifted towards an M2 phenotype after exposure to MSC, their secretome or EVs [120–125]. This effect was presumably mediated by PGE2 [124] and by inhibiting the nuclear factor kappa B (NF-κB) pathway and stimulating the signal transducer and activator of transcription 3 (STAT3) pathway [126]. These reports include both paracrine-mediated immunomodulation [124–126] as direct co-cultures [120, 121, 123, 124, 126]. M2 polarized macrophages increased the production of the anti-inflammatory cytokines IL-10 and IL-6 and of arginase-1 while production of TNF-α was decreased [120, 123, 126]. Interestingly, pro-inflammatory cytokines were shown to stimulate MSC to secrete PGE2 and IDO and upregulate the expression of cell adhesion molecules, thereby stimulating the immunomodulatory capacity of MSC [127–129].

In addition to MSC, iPSC were also shown to modulate the immune system. Naïve major histocompatibility complex (MHC)-matched and/or -mismatched iPSC were reported to have superior immunomodulatory properties compared to MHC-matched and/or -mismatched MSC [130]. iPSC- or ESC-derived MSC acquired similar immunomodulatory properties as adult MSC and were able to inhibit lymphocyte proliferation and function [131–133] and NK cell function [132]. Soluble factors secreted by bone marrow-derived mononuclear cells (BM-MNC) were able to prevent macrophage-microglia induced neuronal cell death and ROS induced neurotoxicity [134]. Conversely, NSC were shown to upregulate arginase-1 production when exposed to inflammatory cytokines [135]. Whereas most *in vitro* proof for the immunomodulatory properties of stem cell-based therapies comes from MSC-centred research, proof for NSC-mediated immunomodulation mostly comes from *in vivo* data [136] and is mainly thought to be an effect of the transplanted cells on peripheral immunosuppression after intravenous administration [137, 138].

Studies that specifically focused on stem cell-based immunomodulation in stroke after transplantation *in vivo* are scarce. Similar to NSC, systemically injected BMMSC home towards the spleen, where TNF-α production is diminished. Moreover, the percentage of MHC-II-activated immune cells in the brain is reduced [139]. Intracranial ASC administration decreased the number of Iba-1$^+$ cells in the brain [140] and IL-10 production was increased after stem cell transplantation in a monkey stroke model [141]. Interestingly, intravenous delivered BMMSC-derived EVs had a similar functional outcome than BMMSC transplantation and attenuated postischaemic immunosuppression in the peripheral blood without altering the number of brain-infiltrating immune cells [142].

Although several encouraging results have been achieved and underlying mechanisms of stem cell-mediated immunomodulation were elucidated *in vitro*, the systemic and local effect of stem cells or stem cell-derived therapies such as EVs on

immunomodulation *in vivo* remains elusive. In accordance with previous sections, noninvasive imaging modalities can be applied to acquire insight in underlying immune mechanisms in ischaemic stroke.

3 Noninvasive Monitoring of Stem Cells in the Stroke Microenvironment

For many years, medical imaging focused on the anatomical changes taking place following ischaemic stroke. During the last decade, however, there has been a major shift in medical imaging towards the molecular processes underlying these anatomical changes. A large number of noninvasive imaging methods have now been developed to study molecular processes such as metabolic changes, gene expression and cell migration. Visualizing these processes can be of great benefit in the diagnosis and treatment follow-up in ischaemic stroke.

These noninvasive imaging methods comprise magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), single-photon emission computed tomography (SPECT) and optical methods such as bioluminescence imaging (BLI) and fluorescence imaging (FLI).

In order to noninvasively track stem cell therapy *in vivo*, cells need to be labelled with a specific imaging probe. This can be done by means of incubation of the cells *in vitro* with contrast agents or radioactive tracer molecules prior to injection, which is referred to as direct cell labelling. Contrast dilution and leakage of these agents from the cells hampers long-term imaging, which has led to the development of indirect cell labelling methods. Hence, so-called "imaging reporter genes" are introduced into the cells and their expression enables the accumulation of imaging probes on a cellular level. This enables repeated stem cell visualization *in vivo* over time within the same subject [143].

Imaging stem cells in the field of ischaemic stroke research is mainly focused on determining the optimal injection route, cell dosing, engraftment, survival and effect on the lesion volume. ESC-derived NSC were imaged with BLI and MRI after labelling with superparamagnetic iron oxide (SPIO) particles, and migration as well as differentiation of the NSC towards neural lineages was confirmed [144]. MRI has also been used in a clinical setting of stem cell transplantation. For example, Zhu et al. have tracked autologous SPIO-labelled NSC transplanted in a patient with brain trauma. Cells migrated towards the lesion site but the signal disappeared 7 weeks after the transplantation [145].

ESC-derived NSC were genetically engineered to express the herpes simplex virus type 1 thymidine kinase (HSV1-tk) reporter gene for PET and labelled with SPIO for MRI. 3 months after stroke, PET and MRI showed a decrease infarct size and functional engraftment of the transplanted cells [146].

Intra-arterial injection of stem cells for the treatment of ischaemic stroke seems to be a favourable injection route. MRI tracking of ASC in a rat MCAO model has

shown that the neuroprotective effect might be due to the secretion of trophic factors. Intra-arterially transplanted cells actively migrated towards the lesion sites, but only a low number of cells survived 8 weeks post transplantation [147]. Human umbilical cord blood-derived stem cells were labelled for MRI and injected intra-arterially 60 min after stroke. Researchers found an improved cerebrovascular function, a reduced infarct size and improvement in behavioural deficits [148]. Furthermore, Grudzenski et al. have shown using MRI that not only the injection route, but also the number of cells injected is important in the treatment of ischaemic stroke [149].

As all imaging modalities have their specific strengths and weaknesses, modern molecular imaging often combines several modalities. Multimodal imaging of MSC transplanted in rats with ischaemic stroke has combined MRI together with SPECT and FLI, using one single tri-modal probe (^{125}I-fSiO4@SPIOs). MSC transplanted intracerebrally or intravenously both improved neurobehavioral outcomes of these stroke animals [150].

4 Conclusion

Multiple advances have been made to understand stem cell-mediated mechanisms of brain regeneration following ischaemic stroke. Stem cell-based therapies applied via various administration routes have shown great promise in *in vitro* and *in vivo* models of ischaemic stroke focussing on a plethora of regenerative mechanisms including neuroregeneration, ECM and vascular remodelling and/or angiogenesis, stimulating endogenous repair and immunomodulation. However, these studies were not able to pierce the veil and pinpoint precise mechanisms of action of the transplanted cells. Nonetheless, harmonized stroke- and stem cell research will continue to contribute to the discovery of new targets and modulable pathways potential therapeutic approaches could be directed at. Moreover, noninvasive imaging methods allow changes in host microenvironment caused by the transplanted cells or cell-derived therapies to be connected with functional improvement.

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