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EDITORIAL

Progenitor cells as remote "bioreactors": Neuroprotection *via* modulation of the systemic inflammatory response

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Abstract

Acute central nervous system (CNS) injuries such as spinal cord injury, traumatic brain injury, autoimmune encephalomyelitis, and ischemic stroke are associated with significant morbidity, mortality, and health care costs worldwide. Preliminary research has shown potential neuroprotection associated with adult tissue derived stem/progenitor cell based therapies. While initial research indicated that engraftment and transdifferentiation into neural cells could explain the observed benefit, the exact mechanism remains controversial. A second hypothesis details localized stem/progenitor cell engraftment with alteration of the loco-regional milieu; however, the limited rate of cell engraftment makes this theory less likely. There is a growing amount of preclinical data supporting the idea that, after intravenous injection, stem/progenitor cells interact with immunologic cells located in organ systems distant to the CNS, thereby altering the systemic immunologic/inflammatory response. Such distant cell "bioreactors" could modulate the observed post-injury pro-inflammatory environment and lead to neuroprotection. In this review, we discuss the current literature detailing the above mechanisms of action for adult stem/progenitor cell based therapies in the CNS.

Key words: Traumatic brain injury; Cerebral stroke; Spinal cord injury; Stem cells; Bone marrow; Inflammation

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INTRODUCTION

Acute central nervous system (CNS) injuries such as spinal cord injury (SCI), traumatic brain injury (TBI), and ischemic stroke are associated with significant worldwide morbidity and mortality. Up to 5 million people are burdened by the morbidity associated with TBI annually with approximately 40% of patients reporting unmet needs 1 year after injury[1]. In addition, an initial cerebrovascular accident is associated with a lifelong loss of 9.5

quality adjusted life years[2]. Overall, the combined economic impact of SCI, TBI, and ischemic stroke surpasses several billion dollars annually in the United States[3,4].

Early preclinical research has shown potential benefit from adult tissue progenitor cell therapy for acute and chronic CNS injury. Adult tissue stem/progenitor cells are maintained in select microenvironments or niches throughout the body. Within the niche, stem cell proliferation, depletion, and involvement in resident tissue regeneration and repair is tightly regulated[5]. Stem/progenitor cells are prime candidates for novel therapies due to their observed capacity for self renewal and ability to differentiate down multiple cell lines[6].

Preliminary *in vivo* and *in vitro* research has shown potential benefit associated with stem/progenitor cell therapy after TBI[7], ischemic stroke[8], and SCI[9]. While initial research indicated that engraftment and transdifferentiation into neural cells could explain the observed benefit[10], the exact mechanism remains controversial. A second hypothesis details localized stem/progenitor cell engraftment with alteration of the loco-regional milieu; however, the limited rate of cell engraftment makes this theory less likely. There is a growing amount of preclinical data supporting the idea that, after intravenous injection, stem/progenitor cells interact with immunologic cells located in organ systems distant to the CNS thereby altering the systemic immunologic/inflammatory response. Such distant cell "bioreactors" could modulate the observed post-injury pro-inflammatory environment and lead to neuroprotection.

ENGRAFTMENT AND TRANSDIFFERENTIATION

Early preclinical research hypothesized that transplanted bone marrow-derived mesenchymal stromal cells (MSCs) could migrate and engraft at the site of injury and adopt neuronal cell markers indicating their differentiation into neurons [neuronal nuclei (NeuN)] and astrocytes [glial fibrillary acidic protein (GFAP)][11]. Additional work completed by Hayase *et al*[12] showed induction of neurospheres from MSCs *in vitro*. The neurospheres were then implanted into rodent cerebral cortex after focal ischemic injury and remained engrafted at the injury site for up to 100 d. The engrafted progenitor cells displayed neural markers with a concordant improvement in animal behavioral recovery[12]. Using a rodent spinal cord injury model, the Ha laboratory implanted human umbilical cord blood mononuclear cells (HUCBCs) into the injury region and found engrafted HUCBCs up to 8 wk after injury. HUCBCs were found to express the neural markers GFAP and microtubule-associated protein 2 (MAP2). Functional improvement via locomotor testing was observed in the animals for up to 8 wk[13].

Such preliminary work investigating the intravenous infusion of MSCs has been promising. However, much debate remains about the frequency and clinical significance of progenitor cell "transdifferentiation" and the validity of neural marker expression with most investigators believing this to be erroneous[14-16]. Coyne *et al*[17] showed that MSCs labeled with BrdU transferred their label to replicating neurons and gave the erroneous impression that MSCs were expressing these proteins when double labels were used. In addition, hematopoietic stem cells (HSCs) implanted into a spinal cord injury site[18] and murine striatum[16] failed to transdifferentiate into neurons and actually showed differentiation into macrophages and microglia. Furthermore, Hunt *et al*[19] observed the failure of transdifferentiation with collagen deposition and axonal injury after the implantation of MSCs into demyelinated spinal cord.

Additional *in vitro* research has been carried out to investigate the capacity for stem/progenitor cell transdifferentiation in to neurons. Barnabe *et al*[20] have shown that MSCs could be chemically induced to produce the neuronal proteins NF-200, S100, β-tubulin Ⅲ, NSE and MAP-2; however, the cells had an apoptotic rate greater than 50%. *In vitro* electrophysiological recordings did not show neuronal properties as no sodium / potassium gradients or action potentials were observed[20]. Further research has shown that bone marrow derived multipotent adult progenitor cells (MAPCs) express the neural proteins β Ⅲ tubulin and NF200 at baseline. Culture of MAPCs in neural differentiation media failed to upregulate protein expression, indicating that the appearance of neural transdifferentiation based upon neural antigen expression can be misleading[21]. While preliminary work pointed towards transdifferentiation as a potential mechanism for cognitive improvement, a large body of preclinical research now indicates that this is an unlikely pathway towards functional benefit.

MODULATION OF THE LOCO-REGIONAL INFLAMMATORY RESPONSE

The observed functional benefit observed with intravenous stem/progenitor cell therapy could be secondary to localized engraftment and interaction with resident microglia leading to modulation of the loco-regional milieu. Work completed in the Cox laboratory measured the concentration of the pro-inflammatory cytokines interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor (TNF)-α, found in cortical tissue after TBI in a rodent model. A multiplex cytokine assay showed an increase in all of the measured pro-inflammatory cytokines measured (IL-1α, IL-1β, IL-6, and TNF-α) in the direct injury and penumbral areas of the injured brain as shown in Figure 1[22]. These results detail the post injury pro-inflammatory response and identify a potential target for novel therapies.

Early *in vitro* work investigated the co-culture of human immunologic cells with MSCs and showed an increase in production of the anti-inflammatory cytokines IL-4 and IL-10 in accordance with a decrease in production of the pro inflammatory cytokine interferon γ (IFN-γ).

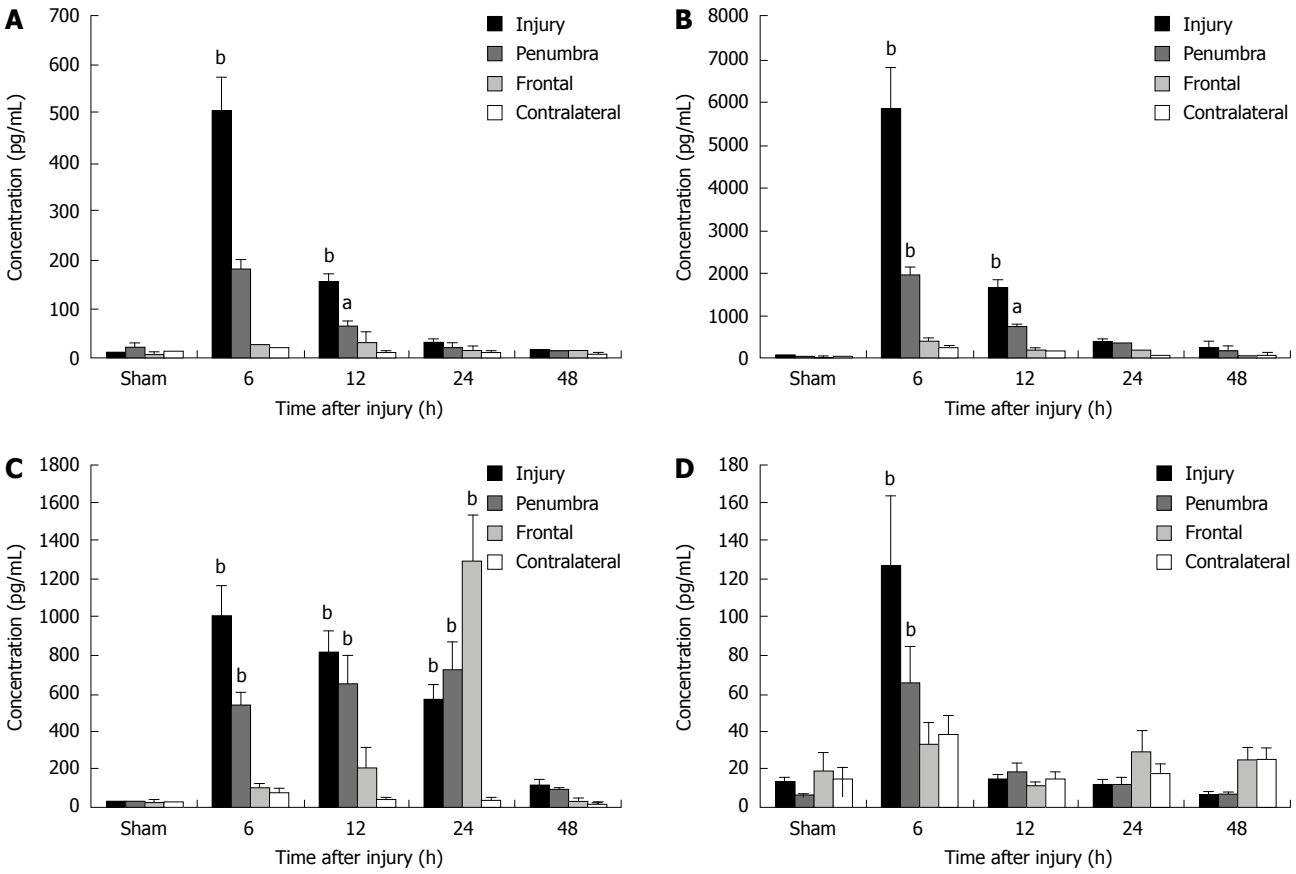


Figure 1 Elevated intracerebral cytokines identified in specific areas and at specific time points relative to the traumatic brain injury. The proinflammatory cytokines interleukin (IL)-1α (A), IL-1β (B), IL-6 (C), and tumor necrosis factor-α (D) were significantly elevated 6 h after CCI in the injury and penumbral regions when compared with sham animals ($^bP < 0.01$ for all). IL-1α, IL-1β, and IL-6 remained elevated through 12, 12 and 24 h, respectively ($^bP < 0.01$ or $^aP < 0.05$). In the frontal area, IL-6 was significantly increased at 24 h (33- to 50-fold; $P < 0.01$; Dunnett's test), but not at 6 or 12 h after traumatic brain injury. Reproduced with permission[22].

Additionally, an increase in T regulatory cell (a known mediator if the anti-inflammatory response) differentiation was observed[23]. Walker *et al*[24] found an increase in the cytokine interleukin 6 (IL-6) in rodent brain tissue supernatant after the direct intrathecal implantation of MSCs using a TBI model. To explore the potential mechanisms of action, a series of *in vitro* MSC and neuronal stem cell (NSC) co-culture experiments was devised. Direct contact co-culture led to activation of the NSC NFκB pathway with a concordant decrease in NSC apoptosis which was not replicated in transwell (non contact) cultures, indicating the need for direct MSC/NSC contact for effect[24]. Additional work investigating the direct intracerebral implantation of MSCs using a stroke model found increased intracerebral IL-10 with a corresponding decrease in TNF-α production. The observed modulation of the loco-regional milieu led to functional improvement[25]. Pluchino *et al*[26] have shown that the intravenous delivery of neurosphere-derived stem/progenitor cells in a chronic CNS inflammatory model leads to the engraftment of cells in perivascular niches. Upon engraftment, the neurosphere-derived cells induce apoptosis in circulating blood born T cells thereby decreasing the amount of inflammatory neuronal injury[26]. Such preliminary work has shown a potential mechanism to explain the observed benefit; however, the majority of studies are based upon the direct intracerebral or intrathecal implantation of progenitor cells.

A potential barrier to the direct implantation of stem/progenitor cells is related to the size or the multifocality of the lesion. A significant injury cavity can occur after TBI, SCI, or ischemic stroke that could potentially require multiple stereotactic injections (needle tracts) which could exacerbate the inflammatory response to injury. More commonly, there are multiple foci of diffuse injury that would make stereotactic implantation impractical. In order to circumvent the need for multiple injections, alternate delivery methods such as intravenous injection need to be considered.

The intravenous delivery of stem/progenitor cells is attractive subject to the potential for widespread distribution and the lack of invasiveness from the procedure. Biodistribution studies completed by Fischer *et al*[27] have shown that the vast majority of injected progenitor cells remain sequestered in the lungs, as illustrated in Figure 2, and have described this as a significant pulmonary first pass effect[27]. These findings have been replicated by many investigators with Harting *et al*[28] showing only 0.001% of intravenously transplanted cells engrafted in the brain parenchyma with significant sequestration of

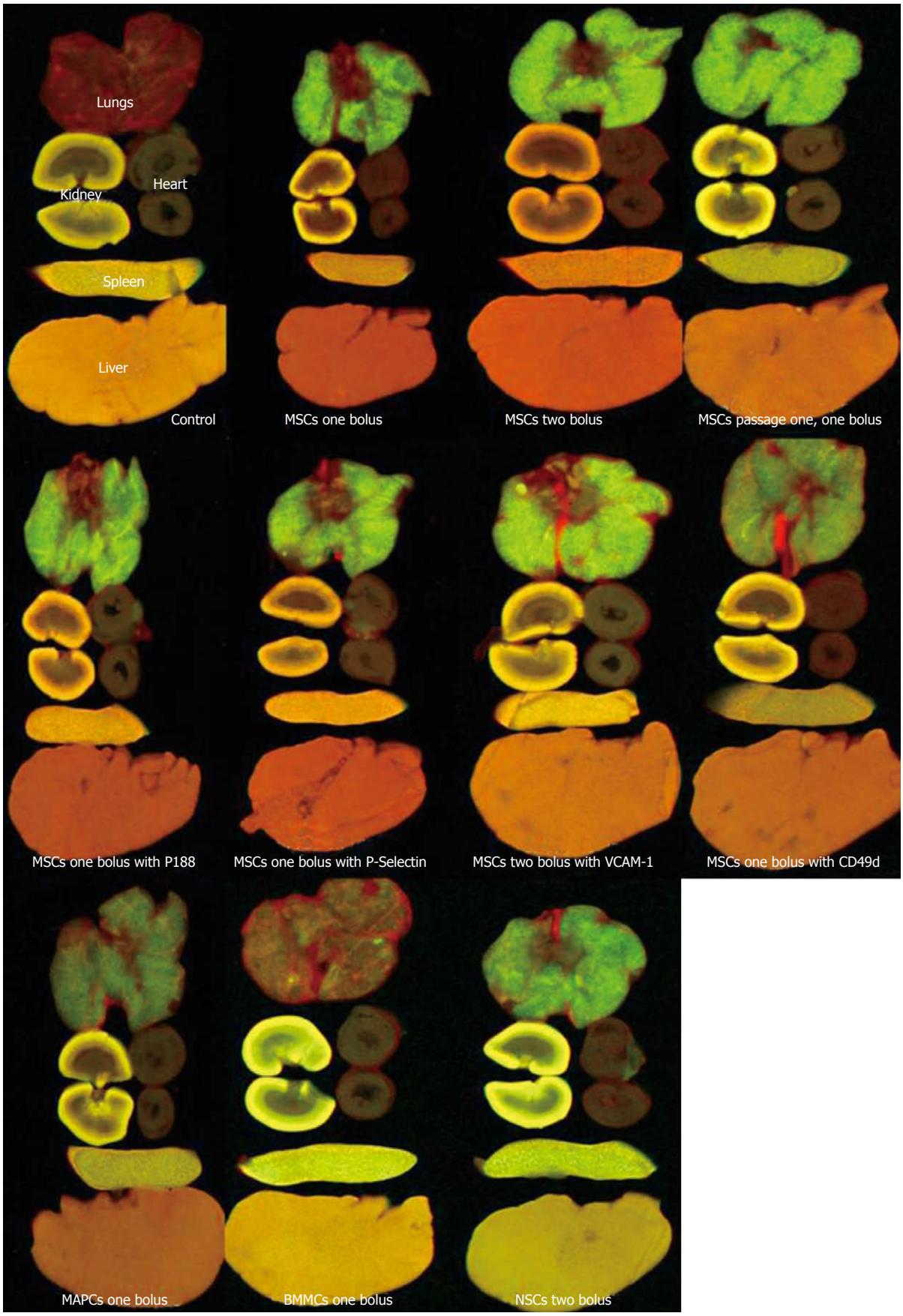


Figure 2 Fluorescent imaging of QDOT (green) labeled mesenchymal stromal cells, neuronal stem cells, multipotent adult progenitor cells, and bone marrow mononuclear cells after intravenous injection. Less than 1% of mesenchymal stromal cells (MSCs) bypassed the lungs into the arterial circulation (as shown by high levels of green fluorescence). A two fold increase in pulmonary bypass was observed with neuronal stem cells (NSCs) and multipotent adult progenitor cells (MAPCs) with a 50 fold increase observed with bone marrow mononuclear cells (BMMCs). Reproduced with permission[48].

MSCs in the lung up to 3 d after injection. Additionally, Lee *et al*[29] found significant pulmonary sequestration with only 0.001% of intravenously injected MSCs in any distant organ system.

The observed significant pulmonary first pass effect greatly decreases the number of stem/progenitor cells that reach the systemic circulation, thereby limiting the quantity that could interact with the injury area and engraft. It is possible that only a few MSCs are needed to activate resident microglia leading to modulation of the loco regional inflammatory/immunologic response. However, emerging data indicates that the hypothesis of stem/progenitor cells acting as local "bio-reactors" seems more unlikely and requires further investigation.

MODULATION OF THE SYSTEMIC INFLAMMATORY RESPONSE

The intravenous delivery of stem/progenitor cells remains the ideal delivery vehicle due to the potential for widespread distribution and simplicity although cell delivery is limited by a significant pulmonary first pass effect. Despite the limited number of cells reaching the systemic circulation, multiple investigators have reported neuroprotection with intravenous therapy[30,31]. Such results indicate that it may not be necessary for a large number of cells to reach the injury zone to produce effect. It is also possible that the stem/progenitor cells are interacting with immunologic cells in remote organ systems and acting as distant "bioreactors" which alter the systemic inflammatory/immunologic response and lead to the observed benefit. The possible locations of remote progenitor/immunologic cell interactions include the lung, spleen, liver, lymph nodes, and kidney.

Pulmonary immunologic cells

Secondary to the significant pulmonary first pass effect, the majority of stem/progenitor cells are sequestered within the lung after intravenous injection, indicating a high probability of interaction between the transplanted cells and resident pulmonary immunologic cells[27]. Mei *et al*[32] found a reduction in lipopolysaccharide (LPS)-induced pulmonary inflammation after the intravenous injection of MSCs in a murine acute lung injury model. A further reduction in alveolar inflammation and permeability was observed when the MSCs were transfected with vasculoprotective gene angiopoietin 1 (ANGPT1) prior to injection. A reduction in neutrophils as well as the pro inflammatory cytokines IFN-γ, TNF-α, IL-6 and IL-1β was found with both treatment groups[32].

A recent study completed in the Mezey laboratory investigated the effect of intravenous MSC therapy on systemic inflammation due to sepsis using a murine cecal ligation and puncture (CLP) model. This seminal study characterized the cellular interactions between MSCs and lung-derived monocytes/macrophages. MSC treatment improved survival, organ function and reduced pro-inflammatory cytokines (TNF-α and IL-6) in the serum, as shown in Figure 3. The injected MSCs were found to directly interact with pulmonary macrophages resulting in an increase in serum levels of IL-10, which is produced by monocytes and macrophages[33] and associated with a reduction in the migration of neutrophils[34] and decreased oxidative damage[35]. The role of IL-10 production in the observed improvement in mortality and end organ function was confirmed via a series of experiments using IL-10 knockout mice and IL-10 receptor antibodies as shown in Figure 4. Furthermore, a series of *in vitro* and *in vivo* experiments was completed showing that MSC derived prostaglandin E_2 production stimulated resident macrophages to produce IL-10 *via* activation through EP2 and EP4 receptors[36].

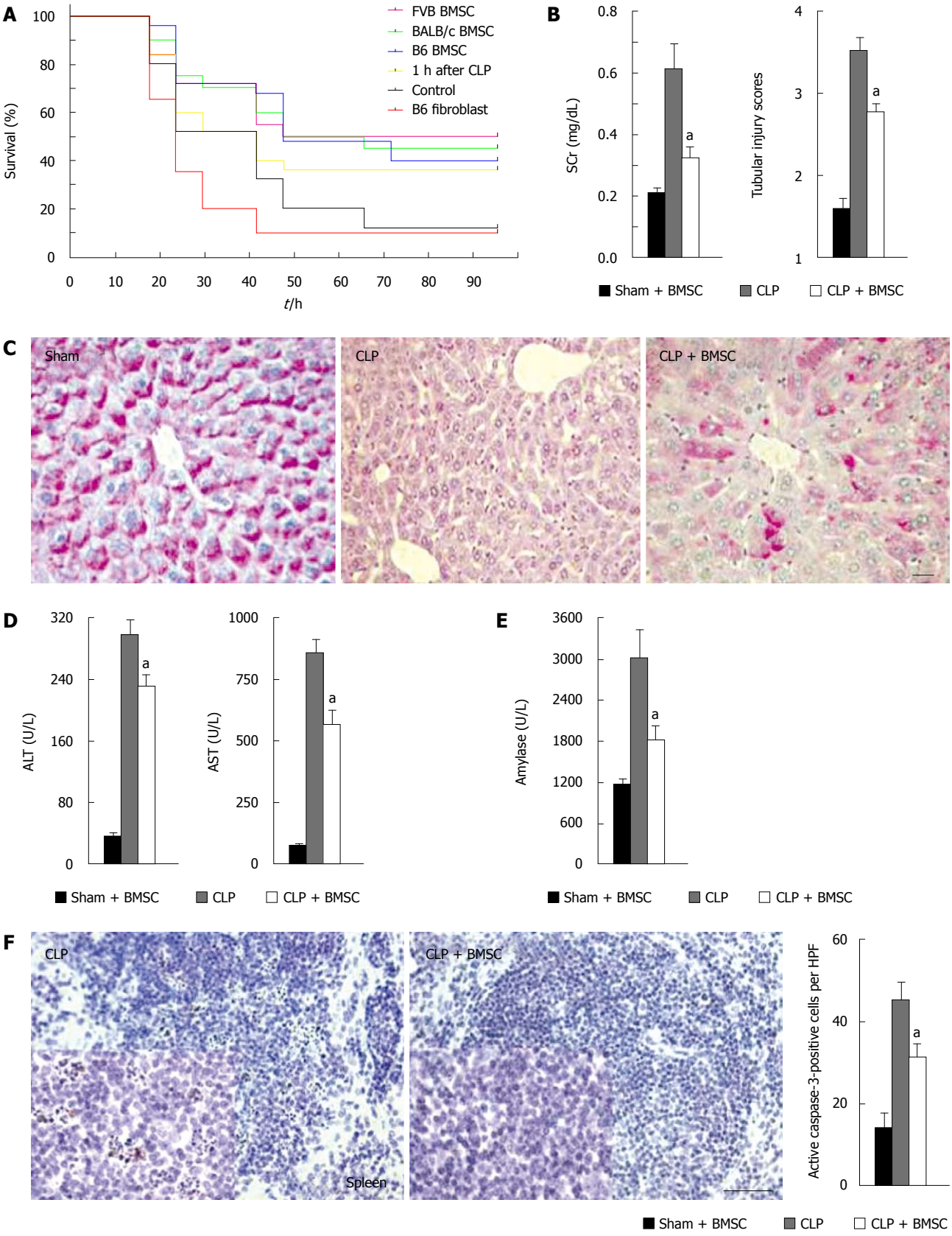
These preliminary studies have shown the potential importance of interactions between transplanted stem/progenitor cells and pulmonary macrophages. The observed interaction appears to modulate both the local and systemic inflammatory response increasing anti-inflammatory cytokine production which could lead to enhanced neuroprotection.

Interaction with splenocytes

Recent work completed in the Pennypacker laboratory has shown the release of immunologic T cells from the spleen into the systemic circulation with a concordant reduction in splenic mass after ischemic stroke in a rodent model. Adrenergic output appeared to mitigate this effect as treatment with the pan adrenergic blocker, carvediol, reversed the observed loss in splenic mass and reduced stroke cavity volume[37]. Vendrame *et al*[38] showed that the observed reduction in splenic mass associated with ischemic stroke was likely due to the release of cytotoxic CD8+ T cells which could contribute to the secondary injury seen after stroke. Injection of HUCBCs 24 h after ischemic stroke restored splenic mass, secondary to the retention of the splenocyte derived cytotoxic T cells. Results also showed a reduction in injury cavity volume as well as an increase in IL-10 and decreases in the pro inflammatory cytokines TNF-α and INF-γ[38].

Similar work carried out by Schwarting *et al*[39] using a rodent ischemic stroke model has shown increased levels of the pro-inflammatory cytokines TNF-α and IL-1β in the serum as well as chemokine receptor 2 and CX3CR1 within splenocytes. After intravenous injection, HSCs were found primarily in the spleen with levels of TNF-α, IL-1β, CX3CR1, and chemokine receptor 2 towards sham levels. A reduction in microglial activation and macrophage infiltration was also observed in the peri-injury parenchyma with a concordant decrease in injury cavity volume and neuronal cell apoptosis[39].

Lee *et al*[40] have investigated the role of NSC therapy for the treatment of intracerebral hemorrhage in a rodent model. The intravenous injection of human NSCs 2 h after injury was associated with improved functional outcomes and decreased cerebral edema as well as decreased intracerebral inflammatory infiltration and neuronal apoptosis. In addition, a reduction in the pro inflammatory cytokines TNF-α and IL-6 was measured in the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B
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Tubular injury scores
a
Sham + BMSC
CLP
CLP + BMSC
C
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CLP + BMSC
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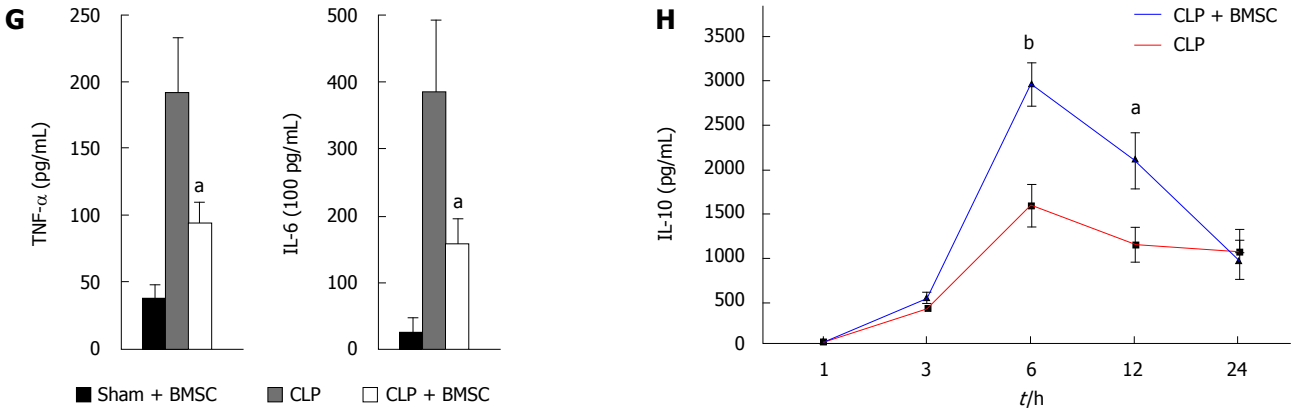

Figure 3 Effect of intravenous injection of BMSCs on the course of sepsis after cecal ligation and puncture. A: Survival curves of mice after cecal ligation and puncture (CLP) and a variety of treatments using BMSCs from C57/BL6, FVB/NJ and BALB/c mice, as well as C57/BL6-derived fibroblasts; B: BMSC treatment effects on kidney function, as reflected by serum concentration of creatinine (SCr). The number of mice in all measurements is as follows: sham, n = 5; CLP, n = 13; CLP + BMSC, n = 14. Tubular injury scores are shown at right; C: Intense PAS staining of hepatocytes is shown after sham operation and BMSC treatment. No staining can be seen in CLP. After treatment (CLP + BMSC), the red staining by PAS in hepatocytes indicates partial glycogen storage capacity. Scale bar, 20 μm; D: Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations in the liver after sham and BMSC, CLP or CLP and BMSC treatment; E: Serum amylase concentrations after sham and BMSC, CLP or CLP and BMSC treatment; F: DAB staining of caspase-3 cells in untreated spleen sections and BMSC-treated spleen sections. A quantitative comparison between the numbers of apoptotic splenic cells in treated versus untreated mice (right) shows a significant decrease with BMSC treatment. Scale bar, 100 μm; G: Serum tumor necrosis factor (TNF)-α and interleukin (IL)-6 concentrations after sham and BMSC, CLP or CLP and BMSC treatment; H: Serum IL-10 concentrations at 3, 6 and 12 h after CLP. n = 8-11 at each time point. Error bars represent means ± SE; $^aP < 0.05$; $^bP < 0.01$. Reproduced with permission[36].

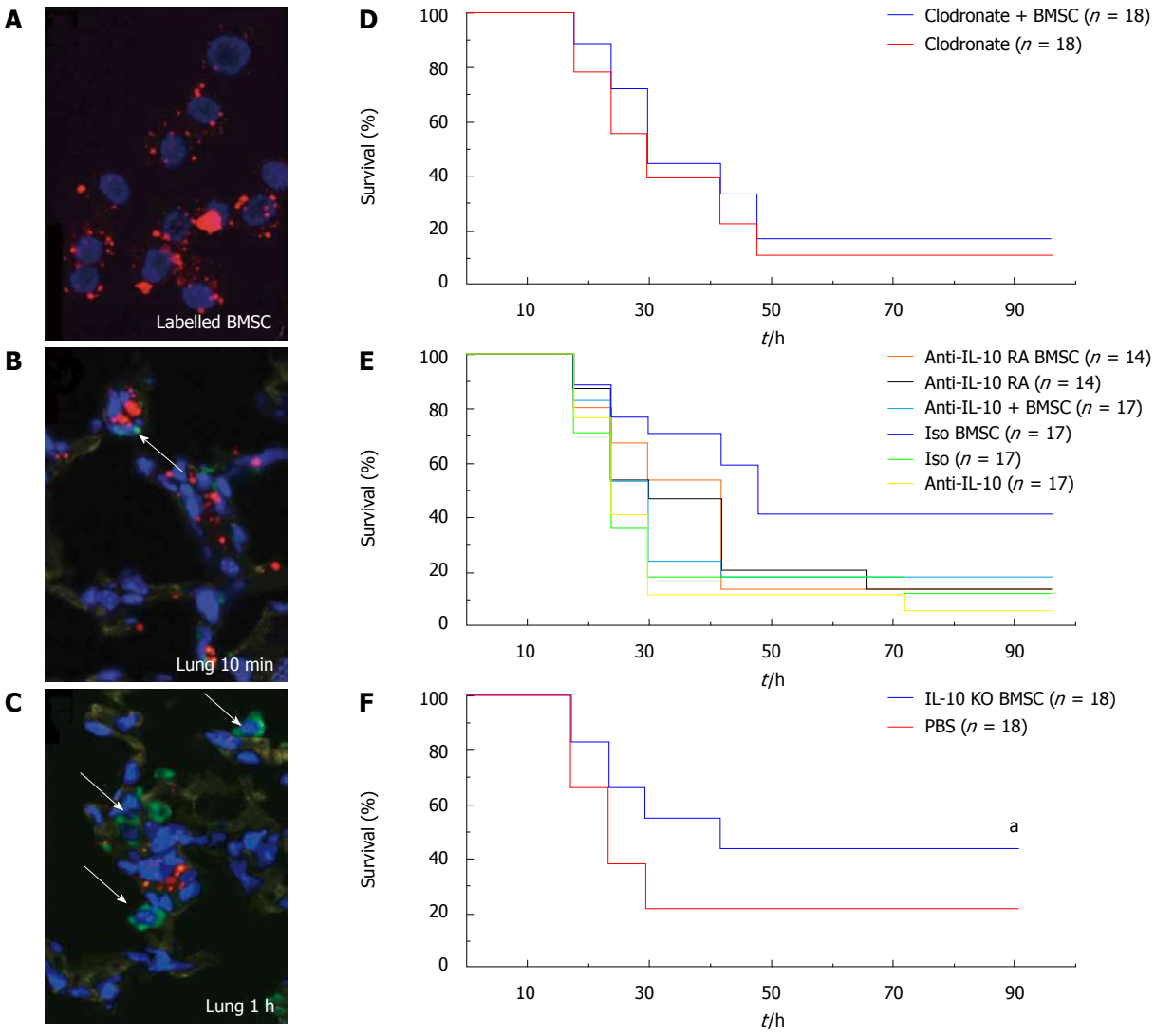

Figure 4 Fate of injected BMSCs and effect of BMSC treatment on survival of normal and immune cell-depleted mice. A-C Immunohistochemical staining

showing that BMSCs pre-labeled with Q-dot (red punctate staining; (A) travel to the lung (B) and take up residence in close proximity to macrophages (C); The latter cells were immunostained with an antibody to Iba1 (ionized calcium-binding adaptor molecule-1, a specific marker of the macrophage lineage47) and visualized with Alexa-Fluor-488 conjugated to a secondary antibody (green). Scale bar, 10 μm; (D-F) Summary of the effectiveness of BMSC treatment of mice genetically lacking or depleted of certain subsets of immune cells or soluble mediators. Survival curves show survival percentage of macrophage-depleted mice with or without BMSC treatment (D), survival percentage of BMSC-treated CLP mice and untreated mice after neutralizing IL-10 or blocking the IL-10 receptor (e) and survival percentage of after treatment with BMSCs derived from Il10-/- septic mice (F). $^{a}P < 0.05$. Reproduced with permission[36].

brain and spleen. Histology completed to track the NSCs showed very few to be engrafted in the cortical tissue; however, a higher number of NSCs were found in the marginal zone of the spleen. Further experiments completed with a non-specific cell line (fibroblasts) or with rats after splenectomy failed to show functional benefit or decreased edema, thereby confirming the need for the splenocyte/progenitor cell interaction to obtain the observed immunomodulation[40].

These data represent a growing field of research into the role of the spleen in post injury inflammation and the ways that progenitor cells may modulate that response. In stroke models, progenitor cell therapy has been shown to preserve splenic mass and modulate the inflammatory response. More studies are required to further investigate the mechanism of immunomodulation in order to optimize the timing and dosage for cell delivery.

Other distant organ systems

The Uccelli laboratory recently completed a series of *in vivo* and *in vitro* experiments to investigate the potential role of MSCs in a murine experimental autoimmune encephalomyelitis (EAE) model. Co-culture of MSCs and T cells inhibited T cell proliferation with a concordant decrease in TNF-α and IFNγ production. The intravenous injection of MSCs in the murine encephalomyelitis model showed MSC engraftment in the lymphoid tissue and a decrease in the autoimmune response secondary to T cell unresponsiveness[41]. Additional work carried out by Kassis *et al*[42] using a similar model showed engraftment in lymphoid tissue associated with a decrease in both mortality and CNS inflammation as well as protection of the resident axons.

Research completed by Refei *et al*[43] using a murine EAE model showed a decrease in spinal cord CD4+ T cell infiltration associated with the amelioration of symptoms after the intraperitoneal injection of MSCs. The observed benefit is secondary to the inhibition of CD4+ T cell activation via suppression of STAT3 phosphorylation by MSC-derived CCL2[43].

There is limited data on the interaction of implanted adult stem/progenitor cells with other organ systems in the setting of neurological injury. Distribution studies have demonstrated stem/progenitor cells to engraft in the liver and kidney as well as the lung and spleen[44-47]. At the time of this review, there is no published data on the liver and/or kidney acting as potential bioreactors to modulate the systemic inflammatory or immune response.

CONCLUSION

Preliminary research has shown the potential benefit of adult tissue stem/progenitor cell therapy for a wide array of acute and chronic CNS injuries. While initial work indicated that the transdifferentiation of stem/progenitor cells into new neurons could account for the observed neuroprotection, the frequency of CNS engraftment and clinical significance of transdifferentiation remains controversial. A growing amount of evidence supports the idea that injected stem/progenitor cells interact with distant organ systems and immunologic cells leading to modulation of the systemic inflammatory response. Multiple investigators have shown a decrease in the pro-inflammatory response to injury which could account for the observed neuroprotection. Such promising work should stimulate the design of additional pre-clinical experiments to further outline the therapeutic mechanism prior to implementation of clinical trials.

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