

Original Paper

Delivery of Placenta-Derived Mesenchymal Stem Cells Ameliorates Ischemia Induced Limb Injury by Immunomodulation

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Key Words

Mesenchymal stem cells • Peripheral artery disease • Immunomodulation • Ischemic Limb injury

Abstract

Background: Peripheral artery disease (PAD) is a major health burden in the world. Stem cell-based therapy has emerged as an attractive treatment option in regenerative medicine. In this study, we sought to test the hypothesis that stem cell-based therapy can ameliorate ischemia induced limb injury. **Methods:** We isolated mesenchymal stem cells derived from human placentas (PMSCs) and intramuscularly transplanted them into injured hind limbs. Treatment with PMSCs reduced acute muscle fibers apoptosis induced by ischemia. **Results:** PMSC treatment significantly enhanced regeneration of the injured hind limb by reducing fibrosis and enhancing running capacity when the animals were subjected to treadmill training. Mechanistically, injected PMSCs can modulate acute inflammatory responses by reducing neutrophil and macrophage infiltration following limb ischemia. ELISA assays further confirmed that PMSC treatment can also reduce pro-inflammatory cytokines, TNF- α and IL-6, and enhance anti-inflammatory cytokine, IL-10 at the injury sites. **Conclusion:** Taken together, our results demonstrated that PMSCs can be a potential effective therapy for treatment of PAD via immunomodulation.

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Introduction

Peripheral artery disease (PAD) is estimated to afflict 202 million people worldwide with major risk factors being age, smoking, and metabolic disorders such as diabetes. PAD has experienced a marked increase in prevalence throughout the first decade of the 21st century [1]. It is a disease process that has a predilection to the lower extremities, PAD is characterized by pathophysiologic arterial narrowing and consequent stenosis. Left untreated, clinical symptoms of end-organ, limb ischemia develop in susceptible patients. Revascularization via percutaneous angioplasty/stent placement or surgical bypass is essential to avoid amputation in critical cases, while catheter-based thrombolytic therapies remain of limited use.

As a better understanding of stem cell biology in both basic science and translational medicine continues to emerge for tissue renewal and repair, the use of stem cells as a potential therapy to prevent end-organ, muscle damage in PAD has been increasingly considered [2]. Among all types of stem cells, mesenchymal stem cells remain to be one of the most attractive and main sources for stem cell-based therapy due to their minimal tumorigenicity [3, 4] and immunogenicity [5], combined with their ease of accessibility [6]. Mesenchymal stem cells (MSCs) can be derived from various tissues and can similarly differentiate into numerous cell types. In addition, increasing evidence has shown that treatment with both native and differentiated MSCs has protective adaptations in paracrine signaling, neovascularization, and immunomodulation in some *n vivo* studies (summarized in the review article [7]). Given the low percentage of MSCs present in the bone marrow (0.001 – 0.01%), our group has worked primarily with MSCs derived from human placenta tissue (PMSCs)- a readily available medical waste product. We have previously shown that PMSC injection yields enhanced angiogenesis and expression of pro-angiogenic growth factors on enhancing excisional wound healing in diabetic Goto-Kakizaki rats [6]. However, our understandings of PMSCs on the local immunological response to muscle ischemia remain unknown [6]. In this study, utilizing an established mouse model of hind limb ischemia, we hypothesized that PMSC treatment would ameliorate the local inflammatory immunological response compared to PBS-treated controls. In our present study, we found that post-ischemia intramuscular PMSCs transplantation is associated with reduced neutrophil and macrophage counts, and an anti-inflammatory cytokine shift as compared to PBS control. The PMSCs-mediated inflammatory modulation led to decreased muscle cell apoptosis induced by ischemic injury and subsequently enhanced recovery of muscle function as demonstrated by running capacity on treadmill test. Taken together, our studies suggested that treatment with PMSCs can ameliorate ischemia-induced skeletal muscle injury by modulating the local inflammatory response.

Materials and Methods

Materials

Placenta mesenchymal stem cells (PMSCs) isolation and culture procedure was established in our previous publication [6]. Anti-Bcl-2, anti-myeloperoxidase and anti-F4/80 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Unless otherwise specified, all other reagents were purchased from Sigma-Aldrich.

Patient selection and tissue processing

The study was approved by the regulations of the Independent Ethics Committee of the Shanghai Ninth People's Hospital affiliated with Shanghai Jiao Tong University School of Medicine. Fresh placentas from normal, full term (38 to 40 weeks gestation) for healthy consent given donors were collected. The tissue processing has been previously described [6]. In brief, the umbilical cord blood was drained, then the placentas were dissected. All tissues were tested to make sure they were free of HIV, toxoplasmosis, cytomegalovirus and rubella virus infections. To maintain cell viability, all tissues were processed within 3 hours, after being evaluated by a certified pathologist.

Isolating, culturing and phenotyping Placenta-derived mesenchymal stem cells (PMSCs)

As described in our previous study [6], the processed pieces of tissue were washed four times in phosphate-buffered saline (PBS) and minced. Then they were digested in PBS solution containing 0.1% collagenase (Sigma-Aldrich, St. Louis, MO) and 10% FBS (Invitrogen, Grand Island, NY) at 37°C for 1 h. The digested tissue was filtered twice through a cell strainer (BD Biosciences, San Jose, CA) to eliminate undigested fragments. The cells were collected by centrifugation at 500 × g for 10 minutes. The remaining red blood cells were lysed with red blood cell lysis buffer for 5 minutes at 37°C, then the mixture was centrifuged at 300 × g for 5 minutes. The cell pellets were resuspended in DMEM medium supplemented with 1g/l of glucose, 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 1% nonessential amino acids. Cells were cultured at 37°C under a 5% CO₂ atmosphere for 4 days before the culture medium was first changed and a 70–80% cellular confluence was reached. The fibroblast-like phenotype was presented after 7 days in culture. After seeding the primary cells, the proliferation rate increased progressively, and cell morphology was kept for at least 8 passages [6]. PMSCs from passage 3 were used for phenotypic marker identification by flow cytometry. The cells were trypsinized (0.25% trypsin with 0.1% ethylenediaminetetraacetic acid (EDTA) in Hank's balanced salt solution (HBSS) and suspended in cold staining buffer. Approximately 5 × 10⁵ cells were incubated with fluorescence-conjugated antibodies for 30 min. The antibodies used were CD29 (APC-conjugated), CD13 (FITC-conjugated), CD73 (PE-conjugated), CD105 (PE-conjugated), CD49b (APC-conjugated), HLA-DR (FITC-conjugated), CD45 (PE-conjugated), and CD34 (FITC-conjugated). All of the antibodies were purchased from Becton, Dickinson and Company (BD Pharmingen, San Diego, CA). At least 15,000 events were counted by flow cytometry (FACScan, BD Biosciences), and analyzed with BD FACSDiva software (version 5.0, BD Biosciences).

Hind limb ischemia model

Adult male C57BL/6J mice (10–12 weeks, 20–25 g), provided by the Animal Facility, Shanghai Jiao tong University School of Medicine, were housed in the laboratory animal room and maintained at 25 ± 1 °C with 65 ± 5% humidity on a 12-h light/ dark cycle (lights on from 07:30 to 19:30) for at least 1 week before the start of the experiments. Animals were given food and water *ad libitum*. All experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experiment of Shanghai Jiao Tong University. Animals were maintained and handled in accordance with the guidelines published by the Shanghai Experimental Animal Center of the Chinese Academy of Sciences.

The immunomodulation of PMSCs was investigated in a murine model of hind limb ischemia. Mice were randomly grouped into 3 groups: 1) a surgery group with PMSCs treatment; 2) a surgery group with PBS treatment; 3) a sham group as a control. Mice were anesthetized with intraperitoneal injection of 50 mg/kg sodium pentobarbital. For the surgery groups, the left femoral artery, great saphenous artery, iliac circumflex artery/vein, and muscular branch were doubly ligated with 7-0 Prolene suture (Ethicon, Somerville, NJ) to induce left hind limb ischemia, then the mice were allowed to recover. For the sham group, all of the procedures were performed same as surgery groups except ligation.

PMSCs transplantation, histological and biomedical analysis

One day after surgery, the PMSCs (5 × 10⁵ cells in 50 µl PBS) were intramuscularly injected into the ischemic leg at four different sites [8]. The control group was injected with same amount of PBS. The animals were sacrificed at different time points. Part of the tibialis anterior (TA) muscle was harvested and cryopreserved in optimal cutting temperature (OCT) media compound (Bio-Optica). Frozen tissue sections were used for different pathological measurements, such as Hematoxylin and Eosin (H&E) staining for histological evaluation, Masson's trichrome staining for fibrosis and muscle regeneration, TUNEL staining for apoptotic cell quantification. For immune cell infiltration detection, the tissue slides were stained with myeloperoxidase (MPO) antibody for neutrophil staining and were stained with F4/80 antibody for mature macrophage. The immune cell infiltration was reviewed and quantified by qualified pathologist blinded with the treatments. The other part of TA muscle was lysed for ELISA analysis of inflammatory factors.

Western Blot Analysis

Seventy-two hours after ischemia injury, the TA muscle was isolated and lysed by RIPA buffer (25mM Tris-HCl (pH 7.6), 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (Roche, Applied Science). For each sample, 25 µg of total protein was loaded

and separated on 8% SDS polyacrylamide gels. Then proteins were transferred to polyvinylidene difluoride membrane and blotted with primary antibody and horseradish peroxidase conjugated secondary antibody. Peroxidase activity was developed with ECL kits (Pierce). Anti-Bcl-2 and anti-GAPDH antibodies were used in the experiments.

Treadmill Exercise Training

A subgroup of mice (10 mice per group) were exercised on a rodent treadmill (Columbus Instruments, Columbus, Ohio). The treadmill exercise training was performed at 0, 7 and 14 days after surgery to evaluate the *in vivo* function of injured muscle. The mice were initially trained on the treadmill at a speed of 5 m/min for 3 minutes and the speed was then increased to 8.5 m/min. The training was performed until the mice were unable to keep pace (greater than 3 consecutive seconds on the shock grid without attempting to reengage the treadmill). The running time for each mouse was recorded as a parameter for *in vivo* muscle function. Most of the uninjured mice can run a long time at the speed of 8.5 m/min, so when reach 4 hours of running, the treadmill test was stopped.

Results

Isolation and characterization of PMSCs

Most of the PMSCs strongly expressed CD29, CD13, CD73, CD105, and CD49b and were negative for HLA-DR, CD45, and CD34. The immunophenotype of PMSCs remained unchanged for more than eight cell passages (as described in our previously published paper [6]). Thus, in present study, we used primary cultured PMSCs with less than eight passages.

PMSCs treatment decreases apoptotic cells after ligation

It has been demonstrated that apoptosis of skeletal muscle fibers is one of main consequence of ischemic injury. Thus, we perform multiple experiments to test the potential effects of PMSCs in ischemia induced skeletal muscle apoptosis. First, as demonstrated by TUNEL assay, the frequency of apoptotic cells in the ischemic hind limb was decreased in mice treated with PMSCs (66 ± 11 cells/mm², $p < 0.05$) compared with PBS treatment (152 ± 26 cells/mm²) (Fig.1A and 1B). Second, in order to further determine the molecule that mediated ischemia induced apoptosis, we tested expression of Bcl-2, a well known anti-apoptotic protein. As shown in Fig. 1C, PMSCs treatment greatly enhanced expression of

Fig. 1. Injection of PMSCs reduces apoptotic cells after ischemia injury to mouse hind limb. A. Representative TUNEL staining pictures of muscle shows PMSCs treatment reduced apoptotic muscle fibers (nucleus was stained in brown color) as compared to PBS treatment. The tibialis anterior (TA) muscle was harvested 72 hrs after ischemic injury. Scale bar: 50 μ m. B. Quantification of positive cells stained by TUNEL technique from ischemic muscle tissue is similarly shown for sham, PBS-treated, and PMSC-treated groups. Cell counts are expressed in number of cells/mm² of tissue and are provided as mean \pm SEM; n = 6 per group; * $p < 0.05$, and ** $p < 0.01$ (versus PBS-treated group). C. western blot analysis shows that PMSCs treatment upregulated anti-apoptotic protein, Bcl-2, expression in skeletal muscle.

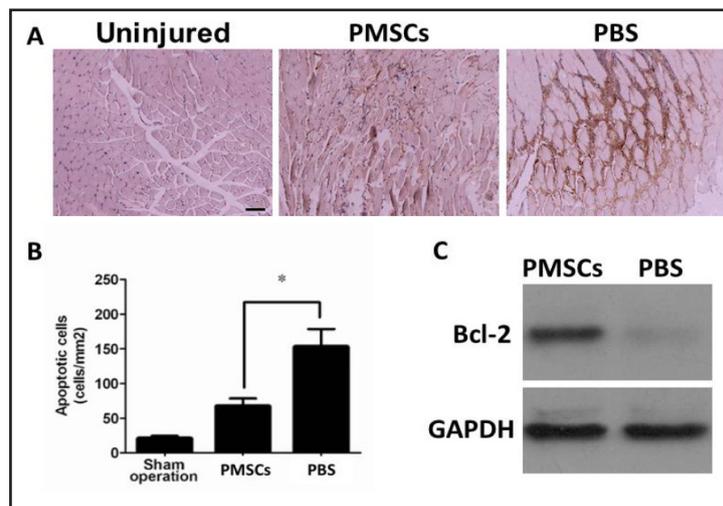


Fig. 2. PMSCs treatment improves pathologies of injured hind limbs induced by ischemia. A. H&E staining of injured B. Masson's trichrome staining shows less fibrosis in PMSCs treatment skeletal muscle tissues 2 weeks after injury. Scale bar: 50 μ m.

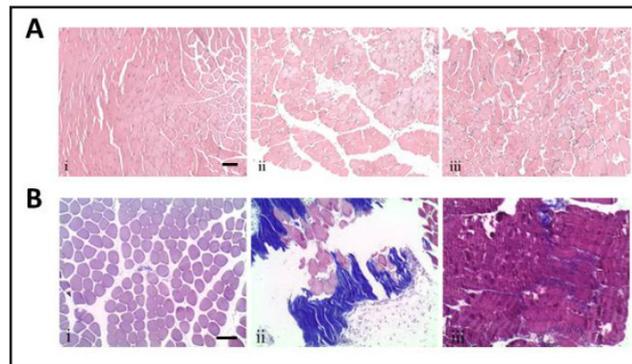
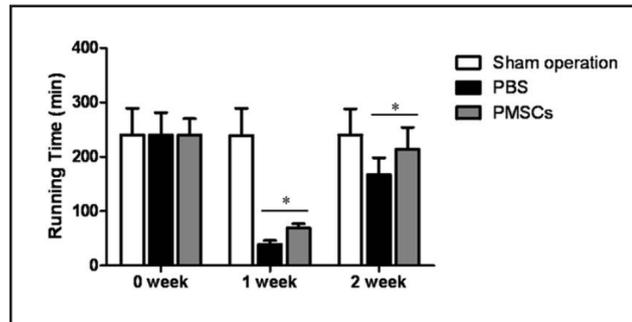


Fig. 3. PMSCs treatment improves muscle function after ischemic injury. Animals were subjected to treadmill running test at indicated time points. PMSCs treatments significantly improved running ability of the animals after ischemic injury as compared to PBS control. The data were presented as mean \pm SEM; n = 10 per group; * p<0.05.



Bcl-2, indicating the anti-apoptotic effects of PMSCs might be through activation of Bcl-2 pathway.

Histological assessment of PMSC transplantation

Histological examination of the muscle revealed extensive muscle degeneration and pronounced interstitial fibrosis in the mice in the PBS-treated ischemic hind limb group (Fig. 2A, ii) compared to sham operation. In contrast, remarkably reduced fibrosis and less muscle degeneration were observed in the PMSC-treated group (Fig. 2A, iii). On Masson's trichrome staining, compared with mice with untreated ischemic hind limbs, mice in the PMSC group again exhibited significantly less fibrosis (Fig. 2B, iii) and more muscle regeneration (Fig. 2B, ii).

PMSCs treatment enhances the recovery of muscle function after hind limb ischemia

To examine the functional recovery of injured hind limb, we tested the running capacity of the mice by our treadmill exercise protocol. As shown in Fig. 3, the treatment of PMSCs enhanced running capacity of injured mice as compared to PBS control, as evidenced by the total running time on treadmill.

PMSC treatment reduced inflammatory cell infiltration

One of the pathological responses to muscle ischemia is acute inflammation, which induces apoptosis of local tissue if left uncontrolled. To test the effect of PMSCs on inflammatory cell infiltration in the ischemic hind limb muscle, we evaluated MPO positive neutrophils and F4/80 positive mature macrophages by immunohistochemistry. As shown in Fig. 4, PMSC treatment reduced both neutrophil and macrophage infiltration in the ischemic muscle.

PMSCs regulated the local inflammatory response during post-ischemia recovery.

To explore the molecular mechanism of reduced inflammation in injured muscles, we next investigated the effect of PMSCs on the production of local inflammatory cytokines in ischemic tissue. ELISA analysis showed that PMSC treatment decreases the local levels of proinflammatory cytokines, TNF- α (Fig. 5A) and IL-6 (Fig. 5B), and increases anti-

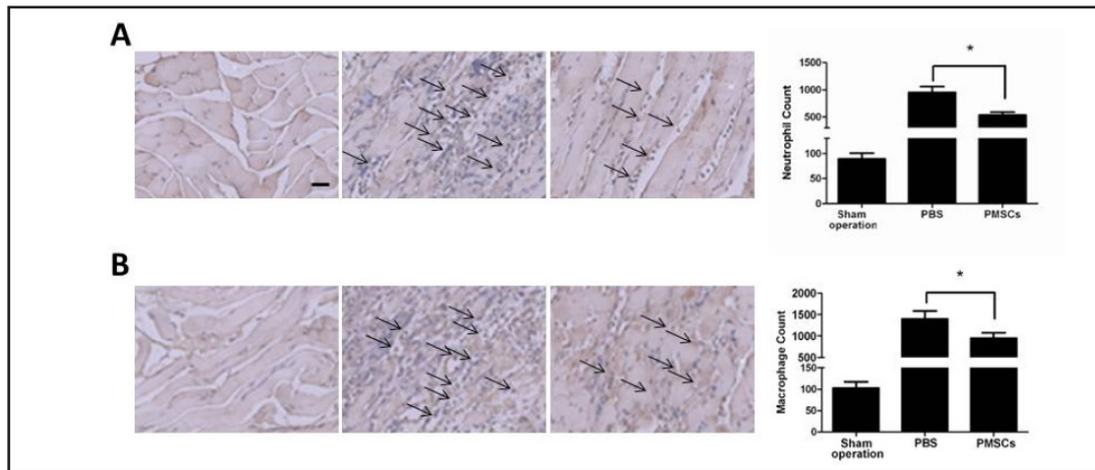


Fig. 4. PMSCs treatment inhibits acute inflammatory responses by reducing neutrophil and macrophage infiltration following ischemia. A. The number of MPO positive neutrophils (stained in brown color as pointed by arrows) remained significantly reduced at 72h post-ligation in the PMSC-treated group (532 ± 60 cells) compared to the PBS-treated group (953 ± 106 cells; $p < 0.05$). B. In addition, F4/80 stained mature macrophage infiltration (stained in brown color as pointed by arrows) in ischemic tissue was also significantly higher in the PBS-treated group (1399 ± 186 cells) than in the PMSC-treated group (946 ± 130 cells). Scale bar: $20 \mu\text{m}$.

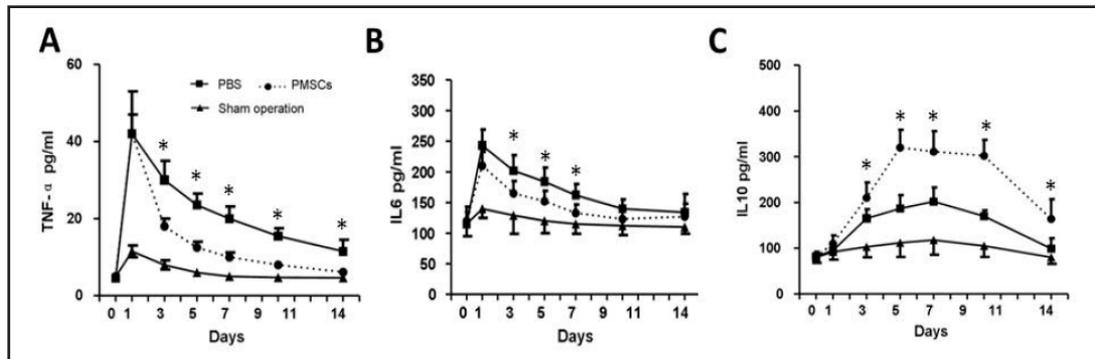


Fig. 5. PMSCs treatment regulates secretion of inflammatory factors. Inflammatory factors in blood circulation were determined at indicated time points. PMSCs treatment can effectively inhibit secretion of pro-inflammatory factors, TNF- α (A) and IL-6 (B) and promote secretion of anti-inflammatory factor, IL-10 (C). The data were presented as mean \pm SEM; * $p < 0.05$.

inflammatory cytokine IL-10 (Fig. 5C), which might explain why PMSCs treatment reduced inflammatory response after ischemic injury to skeletal muscles.

Discussion

Our results show that intramuscular PMSC transplantation leads to a reduction in local inflammatory immunological responses in a mouse model of hind limb ischemia. Compared to PBS-treated controls, PMSC treatment was associated with much less fibrosis and degeneration on muscular histology. Similarly, TUNEL staining and western blot indicated decreased apoptosis in PMSC-treated muscles, and IHC displayed reduced infiltration of neutrophils and macrophages. Finally, ELISA analysis of PMSC-treated samples showed a local decrease in pro-inflammatory cytokines TNF- α and IL-6 and an increase in anti-inflammatory cytokine IL-10. Together, our studies provide novel evidence

of immunomodulation by PMSCs and hence advance their therapeutic potential in settings of PAD and other pathologies relating to end-organ ischemia.

One of the concerns for our protocol for xenograft model is the potential rejection after human PMSCs were implanted into immunocompetent mice. Interestingly, PMSCs have been applied to multiple species, including mice [9, 10], rats [11], rabbits [12] and dogs [13] in multiple disease settings, such as dermal wound, spinal cord injury and neurodegeneration diseases. Consistent with these studies, we did not observe obvious rejection responses after human PMSCs were implanted into mice. Although the molecular mechanisms for these observations are still largely unknown, our data did provide evidence to show the low immunogenicity of human PMSCs.

MSCs are being seen as increasingly more promising as a cell-based therapy for tissue renewal and repair. MSCs can be both derived from and differentiated into various different cell types. Beneficial effects of MSC-based treatments have been linked to *de novo* differentiation into local tissue, stimulation of local tissue, and protective pathways of paracrine signaling [14]. PMSCs specifically provide the advantages of being readily available without the need for additional invasive procedures. Clonally-expandable PMSCs share many markers with both human bone marrow-derived MSCs and embryonic stem cells, yet their cellular appearance is distinct from other progenitor populations. With special regards to hind limb ischemia as an experimental model of PAD, the efficacy of MSCs and other progenitor cells has been recognized in terms of angiogenesis and muscular regeneration [15]. The roles of immunomodulation and potential for PMSC-based therapies, however, have remained largely unexplored, hence our inspirations to pursue the current study.

While PMSCs' pro-angiogenic effects have previously been demonstrated, this is the first report of local immunomodulation related to a PMSC-based therapy for hind limb ischemia [6]. Of note, however, through either secretion of immunoreactive factors or direct cell-cell contact, MSCs have been shown to suppress immunological responses in several cell-based *in vivo* studies [16]. Specifically, MSCs can inhibit lymphocyte proliferation, prevent differentiation of antigen-presenting cells, and reduce levels of inflammatory mediators [16]. The placenta serves a critical role to protect the fetus during pregnancy, as such PMSCs have been proposed to promote immunosuppression via expression of indoleamine 2,3-dioxygenase (IDO), programmed death-1 ligand (PD-L1) and FasL molecules, IFN- γ signaling, and release of immunoreactive exosomes [17] [18-21]. *In vitro*, PMSCs have been linked to modified levels of immunostimulatory cytokines and inhibition of T cell survival, proliferation, and activation [18]. *In vivo*, PMSC treatment conferred decreased local immunoreactivity and improved functional outcomes in a rat stroke model and protected against bronchiolitis obliterans and reduced local neutrophil infiltration in a murine tracheal transplant model [22, 23]. Collectively, these studies indicate a significant role for PMSCs in immunomodulation, and our results contribute to the above-described understandings and advance the therapeutic potential of PMSCs for ischemic pathologies.

Despite our novel findings of PMSC-mediated immunomodulation, our studies have inherent limitations with regards to our model. For example, the treadmill test might not provide enough data to reveal the detailed regeneration processes with or without PMSCs treatment. *Ex vivo* or *in vivo* muscle contractility studies will be required for dissect the muscle recovery after PMSCs treatment. A laser Doppler measurement will be required for characterize vascular regeneration with PMSCs treatment. Hind limb ischemia is a well-established and widely used model of PAD, yet it does not fully represent the chronic pathophysiology of the disease process. We envision, however, that our experiments will serve as a baseline for further investigations into the therapeutic potential of PMSCs. Our use of PMSC treatment in mice encourages application to both transgenic and larger species for improved biological understandings and increased translational relevance respectively. Finally, cell-based studies will further elucidate the specific immunomodulatory and organ-protective mechanisms employed by PMSCs in post-ischemic pathologies.

In conclusion, we have demonstrated that intramuscular PMSC transplantation provides protective local immunomodulation in a mouse model of hind limb ischemia. Our results

expand the growing body of knowledge with regards to MSC-based therapies for PAD, and the specific immunological mechanisms and translational potential of post-ischemia PMSC treatment is currently under investigation.

Disclosure Statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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