A Comparison of the Response of Intramuscularly Delivered Neonatal and Adult MSCs to Distant Source of Inflammation

by

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Doctor of Philosophy

Faculty of Dentistry/Institute of Biomaterials and Biomedical Engineering
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Abstract

Transplanted mesenchymal stromal cells (MSCs) have been shown to modulate host inflammatory responses in a wide range of immune disorders and tissue injuries. However, little is known of the relationship between MSC tissue source and their rate of response to an inflammatory trigger. We hypothesized that a neonatal source of MSCs, intramuscularly (IM)-delivered, will downregulate a distant source of inflammation more efficiently than an MSC population derived from an adult tissue source. The immunomodulatory response of MSCs was assessed systematically in-vitro, and comprehensively in-vivo from an anatomically distant location to the inflammatory trigger. The key anti-inflammatory tumor necrosis factor (TNF)-stimulated gene 6 (TSG-6)–only expressed upon stimulation–has been used as a surrogate to scrutinize the response time of human umbilical cord perivascular cells (HUCPVCs), and human and mouse bone marrow MSCs (BMMSCs). In-vitro, HUCPVCs, human and mouse BMMSCs were stimulated with recombinant TNF-α over various exposure times and concentrations. HUCPVCs showed higher sensitivity to TNF-α stimulation compared with hBMMSCs, but expression level varied across donors. Both human derived MSC sources showed higher response to TNF-α stimulation than mBMMSCs. Following the in-vitro studies, a unilateral hind paw
inflammation was created *in-vivo* with carrageenan in immunocompetent mice. The three sources of MSCs were IM-delivered in the contralateral quadriceps, at the first inflammatory peak. Post 24h, the TSG-6 level in the tissue at the site of cell injection was significantly higher in the HUCPVC compared to the hBMMSC group, and all measures of inflammation were downregulated more rapidly. Since HUCPVCs showed earlier response to inflammation compared with BMMSCs, we believe neonatal MSCs may be a stronger candidate to treat inflammatory diseases than adult BMMSCs.
Dedication

This thesis is dedicated to my lovely parents and beloved husband. You are the reason of what I have become today. Thank you for your unconditional love and support.
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First, I would like to thank my Ph.D. advisor, Prof. John E. Davies. His patience, immeasurable knowledge, and continuous encouragements were the main driving forces over the course of my Ph.D. studies at the University of Toronto. Without his valuable insights in every stage of my research, this thesis may never have been completed. It was an amazing experience to be a part of the Bone Interface Group in the Institute of Biomaterials and Biomedical Engineering and the Faculty of Dentistry.

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Chapter 1: Mesenchymal Stromal Cells- History, Mechanism of Immunomodulation, and Intramuscular Delivery Route

1.1 Mesenchymal Stromal Cells (MSCs)

1.1.1 Adult: Bone marrow-derived MSCs

1.1.2 Neonatal: Umbilical cord-derived MSCs

1.1.3 Immunosuppressive/Immunomodulatory Response of BMMSCs versus UC-MSCs

1.2 Activation Mechanisms of MSCs

1.3 Therapeutic response of MSCs to inflammation

1.3.1 Tumor necrosis factor-induced gene 6 protein

1.3.1.1 Mechanism of Activation and Action of TSG-6

1.3.1.2 TSG-6 Autocrine Effect and Modulation of Inflammatory Cells

1.3.2 Other soluble factors

1.4 Mechanism of carrageenan-induced inflammation

1.5 Choice of MSC Delivery Route

1.6 Rationale

1.7 Hypothesis

1.8 Specific aims

Chapter 2: Skeletal Muscle as a Delivery Route for Mesenchymal Stromal Cells
2.1 Clinical Safety of IM-MSC delivery .................................................................34
2.2 Pre-clinical studies: IM-Delivered MSCs to Treat Local Pathologies .............35
2.3 IM-delivered MSCs to treat distant and systemic conditions .........................37
2.4 Discussions: .................................................................................................38
2.4.1 Dwell-time of IM-delivered MSCs ..............................................................38
2.4.2 Cell Dose and Frequency of Injections .........................................................42
2.4.3 Differentiation of IM-delivered MSCs ..........................................................43
2.4.4 Biodistribution of MSCs post IM-delivery ..................................................44
2.4.5 Concluding Remarks ..................................................................................45

Chapter 3: Preliminary Assessment of Animal Models of Inflammation and Cell Tracking Methods .................................................................53
3.1 Animal models ...............................................................................................53
3.1.1 Osteoarthritis and Dunkin Hartley guinea pig model of osteoarthritis .........53
3.1.2 Carrageenan-induced Paw Model of Inflammation ......................................58
3.2 MSC labelling for in-vivo biodistribution studies .............................................59
3.2.1 Gold Quantum Dot Hybrids .........................................................................60
3.2.2 Far-red fluorescent protein (E2-Crimson) .....................................................64
3.2.3 Far-red lipophilic heptamethine carbocyanine (DiR) ......................................66
3.2.4 Comparison of fluorescence flux: E2-Crimson and DiR ...............................68
3.2.5 Gaussia Luciferase .......................................................................................69
3.3 Summary and conclusions ............................................................................73

Chapter 4: Effect of Tumor Necrosis Factor Alpha Dose and Exposure Time on Tumor Necrosis Factor Induced Gene-6 Activation by Neonatal and Adult Mesenchymal Stromal Cells ...............................................................75
4.1 Introduction .....................................................................................................75
4.2 Materials and Methods ...................................................................................78
4.2.1 HUCPVC culture and source: .................................................................78
Chapter 5: Human Umbilical Cord Perivascular Cells and Human Bone Marrow Mesenchymal Stromal Cells Transplanted Intramuscularly Respond to a Distant Source of Inflammation

5.1 Introduction

5.2 Materials and Methods

5.2.1 HUCPVCs culture and source

5.2.2 hBMMSCs culture and source
List of Tables

Table 2-1 Few examples of clinical studies of intramuscular-MSC therapy ........................................... 46
Table 2-2 Intramuscular-MSC therapies to treat local pathologies ............................................................. 47
Table 2-3 Intramuscular-MSC therapies to treat distant and systemic diseases ........................................... 50
Table 2-4 Intramuscular-MSC studies in healthy animals ............................................................................ 52

Table 3-1 Optical penetration depth for muscle tissue (postmortem) at different emission wavelengths. N=10; a Standard Error [291] ................................................................................................. 59
Table 3-2 Characteristics of Luciferases used for cell tracking ..................................................................... 70

Table 4-1 Statistical analysis of mRNA expression of pooled population of hBMMSCs (Fig.4-4) stimulated with (1 to 100ng/mL) of rhTNF-α. Two-way ANOVA-Multiple comparison Tukey statistical test was performed between the means of 3 replicates at significance level of 95%CI; data is expressed as means ± S.E.M. significant (p<0.01 to 0.05); very significant (p<0.001 to 0.01); and extremely significant (p<0.001); not significant (NS=p>0.05). ................................................................................................................................. 93

Table 4-2 Statistical analysis of mRNA expression of 5 donors of HUCPVCs (Fig.4-2) stimulated with (1 to 100ng/mL) of rhTNF-α. One-way ANOVA-Multiple comparison Tukey statistical test was performed between the means of 15 data points at significance level of 95%CI; data is expressed as means ± S.E.M. Significant (p<0.01 to 0.05); very significant (p<0.001 to 0.01); and extremely significant (p<0.001); not significant (NS=p>0.05); significant (p<0.01 to 0.05); very significant (p<0.001 to 0.01); and extremely significant (p<0.001); not significant (NS=p>0.05). ................................................................................................................................. 97

Table 5-1 Gene transcript, designed primer sequences (5’–3’), EMBL fragment size (amplicon length-bp) ................................................................................................................................. 112
List of Figures

Figure 1-1 Diagram depicting the origin of adult and neonatal-derived MSCs. Courtesy of John E. Davies. .......................................................... 5

Figure 1-2 Temporal decline in the colony forming unit-fibroblast (CFU-F) frequency relative to the number of mononuclear cells in the BM [51, 52]; Figure is adopted from Caplan et al., 2009, [51] and used with permission from John Wiley and Sons. ........................................... 7

Figure 1-3 Cross-section of human umbilical cord tissue. (A) Hematoxylin and Eosin staining of the cord illustrates the complete structure that consists of 2 arteries and 1 vein, perivascular regions (black arrows) from which HUCPVCs are isolated, Wharton’s Jelly matrix (intermediate and cord lining), and amniotic epithelium. Note the distinct separation of the perivascular cells (higher cellular and tissue density) and the intermediate Wharton’s Jelly matrix; (B) DAPI (4’,6-diamidino-2-phenylindole) nuclear staining partially illustrating the vein and cellular composition of the cord. The cellular density in the perivascular zone is higher than towards the amniotic epithelium. Note that there is a distinct separation between the tunica media of the vein and the perivascular cells. Images are adapted from Davies et al., 2017, [54] and used with permission from John Wiley and Sons. ............... 8

Figure 1-4 Representative flow cytometry histograms. Flow cytometry data for one cryopreserved cord sample at passage 2, one plated cord sample at passage 1, and one cryopreserved hBMSC sample at passage 2. The black histogram is the cell staining of the blank controls, the red histogram is the antibody-stained cells. Legend and Figure adopted from Matta et al., 2009 [61]. Abbreviation: hBMSCs, human bone marrow stromal cells. ................... 10

Figure 1-5 Profile of cell surface epitopes in HUCPVCs and BMSCs. (A) Abundance of cells positive for CD49e, CD90, CD146, CD31, CD117, Strol-1, and CD45, expressed as percentages, in HUCPVCs and bone marrow-derived BMCs; (B) Flow cytometric analysis showed that HUCPVCs express elevated levels of CD146. Abbreviations: BMSCs, bone marrow stromal cells; Ctrl., control; PE, phycoerythrin; R2, Region 2. Figure and partially legend is adopted from Baksh et al., 2007 [60]; used with permission from John Wiley and Sons........................... 10

Figure 1-6: Overview of TLR signaling pathways. Adopted from Kawai et al. 2010 [101]. Used with permission of Springer Nature Publishing Group................................. 14

Figure 1-7 MSC activation through TLR stimulation or directly by inflammatory mediators. Abbreviations: Lipopolysaccharide (LPS); double-stranded RNA (dsRNA); Complement 3 (C3); CD59 (a complement regulatory protein); Tolerogenic DC (Tol DC). Adopted from English et al. 2012 [111]. Used with permission from Springer Nature publishing group. .......................................................... 16

Figure 1-8: Summary of immune-suppressive effects of human mesenchymal stromal cells transplanted in mice. In different immune models, the major immunosuppressive factors secreted by hMSCs were observed to be tumor necrosis factor induced gene 6 (TSG-6), IL-1RA (IL-1 receptor antagonist), HO-1 (heme oxygenase 1), PGE2 (prostaglandin E2), TGF-β (transforming growth factor b), CCL2 (C-C motif ligand-2), IDO1, FasL (Fas
ligand), or PD-L1 (programmed death-ligand 1). Most of the factors primarily altered cells of the innate immune system, such as monocytes/macrophages, MDSCs (myeloid-derived suppressor cells), or antigen presenting dendritic cells. Subsequent waves of cytokines then suppressed inflammation and altered T cells to either increase or decrease the ratio of Th1/Th17:Th2 responses. Figure is adopted from a review article, Prockop et al., 2017, [128] and is used with permission from Elsevier. 20

Figure 1-9 Schematic of the potential mechanisms for the modulation of CXCL8 function by TSG and inhibition of neutrophil migration. Inhibition by TSG-6 of CXCL8/GAG interactions could antagonize (a) binding of CXCL8 to Heparan Sulfate Proteoglycans (HSPGs) on the luminal surface of the endothelium, thereby preventing the formation of haptotactic gradients and/or (b) binding of CXCL8 to GAGs on the ablumenal surface, thus impairing transcytosis of CXCL8. Very high local concentrations of TSG-6 might inhibit the CXCL8/CXCR2 interaction (c, d), thereby limiting the movement of neutrophils in response to a chemotactic gradient of CXCL8. Figure and legend adopted from Dyer et al. 2014[171]. Used with permission of The American Association of Immunologists, Inc. 24

Figure 1-10 Sulfated di-galactose structure of λ-carrageenan. The repeating disaccharide structural units demonstrate β-1➔4- and α-1➔3 galactosidic linkages connected residues. Sites of sulfation differ among the three major types of carrageenan (λ, κ, ι). Adopted from Bhattacharyya et al. 2010 [209]. Used with permission of Elsevier Publishing Group. 27

Figure 1-11 Schematic of inflammatory cascades involved in carrageenan-mediated activation. These particular pathways are shown in NCM460 cells (a normal human colon mucosal epithelial cell line). The pathways are believed to be by the 3 cascades (1) TLR-4, B-cell leukemia/lymphoma (BCL)10, IκB kinase (IKK)γ, and phosphor- IκBα-mediated activation of RelA; (2) TLR4, BCL10, phosphor-NF-κB-inducing kinase (NIK), IKKα-mediated activation of the non-canonical pathway leading to nuclear translocation of p52 and RelB; (3) ROS-mediated pathways requiring Hsp27, and IKKβ. Adopted from Bhattacharyya et al. 2011 [211]. 28

Figure 1-12 Simplified illustration of experimental setup, TNF-α mediated release of tumor necrosis factor-induced gene 6 proteins................................................................. 32

Figure 3-1 Cartilage fibrillation, loss of chondrocytes, and cellularity in OA (A &C) at 1.5 months; (B and D) 7 months; and (E) 10 months of age. Histopathological features seen include fibrillation and proteoglycan loss. In addition, chondrocyte cloning extended into the middle zone and the loss of Safranin O staining indicates changes in proteoglycan synthesis. (F) changes in the distal end of femur and proximal end of tibia, as well as joint spaces in the 33-35 months old Gp lateral-medialateral radiographs is indicative of the severity of OA when compared with the knee joints of 12-14th month old Gps. .......... 57

Figure 3-2 Biphasic pattern of carrageenan-induced edema in mice. An early inflammatory peak is shown to develop between 4-6h post-carrageenan injection. Adopted from Posadas et al., 2004 [186]....................................................................................... 58
Figure 3-3 Qualitative assessment of the degree of edema developed in carrageenan hind-paw model of inflammation in mice confirms reproducibility. Here we show 3 animals, but sample size analysis showed the minimum requirement of 5 mice/study group.

Figure 3-4 Gold-quantum dot hybrids (GQHs) assembly. (A) step by step assembly of GQHs involves gold nanoparticle (GNP) surface modification with alternating layers of PS-b-PAA and PAH (i-ii), then assembly of QDs around the coated layer (iii) with a further coating of PAH; (B) uniform coating of polymers around the GNP; (C) confirmation of assembly of QDs around the coated GNP; (D) confirmation of presence of QD by showing the lattice fringes (black arrow).

Figure 3-5 Gold-quantum dot hybrids (GQHs) endocytosis by HUCPVCs. (A) cross-section of a cell that has endocytosed multiple GQHs; (B) intracellular confirmation of a GQH in a vesicle; (C) confirmation of intact GQHs post endocytosis (black arrows point to the QDs attached around the polymer coated GNP.

Figure 3-6 Electroporated E2-Crimson HUCPVCs. (A) pCMV map of E2-crimson vector used for electroporation of HUCPVCs; (B) healthy morphology of electroporated HUCPVCs with Hoechst nucleus counterstain; (C) Efficiency of HUCPVCs post-electroporation, calculated from 5 areas of n=3 wells/condition, p≥0.05, C6P and C13P were electroporated with double the plasmid concentration; (D) fluorescence flux (radiant efficiency) generated by various cell densities detected with Xenogen-IVIS system; (E) In-vivo flux of fluorescence from IM-transplanted MSCs, as a measure of penetration depth of the fluorochrome that was not detected in dorsal position and dimly detected in ventral position.

Figure 3-7 DiR-labeled HUCPVCs. (A) healthy morphology of electroporated HUCPVCs; (B) Efficiency of HUCPVCs post-electroporation, measured by flow cytometry; (C) fluorescence flux (radiant efficiency) generated by various cell densities detected with Xenogen-IVIS system; (D) In-vivo fluorescence flux obtained from IM-transplanted MSCs, as a measure of penetration depth of the fluorochrome.

Figure 3-8 Measurement of fluorescent flux (Radiant Efficiency) (p/sec/cm²/sr)/(µW/cm²) of E2-Crimson and DiR labeled HUCPVCs at cell densities 1, 2.5, and 5 (×10⁵) in 2D culture plate measured with Xenogen-IVIS system.

Figure 3-9 Gaussia adino-transfected HUCPVCs. (A) Efficiency of HUCPVCs post modification, measured by flow cytometry; (B) Luminescence photon flux (radiance) generated by various cell densities detected with the Xenogen-IVIS system; (C) Linear correlation of the employed cell density and the generated photon flux; (D) In-vivo photon flux of luminescence from IM-transplanted Gluc-HUCPVCs.

Figure 3-10 Kinetics of Gaussia luciferase-coelenterazine. (A) IM-transplanted Gluc-HUCPVCs, IV-infused with 250 µg/100 µl sterile ddH₂O of coelenterazine and acquired open filter images every 1 min up to 15 min. The region of interest (ROI) used for quantification is encircled in red. (B) Quantification of the decay in flux at the encircled ROI over time.
Figure 4-1 mRNA Expression level of TSG-6 in unstimulated HUCPVCs and hBMMSCs. GeneChip® Human Gene 1.0 ST Array was used. Affymetrix data were normalized and summarized with multi-average (RMA) method implemented in the Affymetrix Expression Console. Data is expressed as means ± S.E.M, n=7-8 donors. ........................................ 84

Figure 4-2 Examples of the effect of TNF-α treatment on 3 reference genes used for normalization of RT-PCR data analysis. Cq values of the 3 most stable reference genes in response to TNF-α treatment of 1 to 100ng/mL across exposure time (A) 10 hr; and (B) 24 hr is represented as color Pink β2M (▲); Purple=RPL13A (■); Blue=GAPDH (●); Green=geometric mean of the 3 reference genes used to normalize target gene (▼). Data is expressed as means ± S.E.M, n=3. ................................................................................ 85

Figure 4-3 Growth profile (A) and doubling time (B) of a pooled population of HUCPVCs and hBMMSCs in culture; passage 2 to 3. The initial seeding density of both cell types was 1333 cells/cm² and daily yield of adhered and viable cells within 7 days of culture was measured and plotted. Data is expressed as means ± S.D. n=4. ....................................... 87

Figure 4-4 TSG-6 dose and response time of a pooled population of HUCPVCs stimulated with (1 to 100ng/mL) of rhTNF-α. mRNA fold change expression level of TSG-6 was determined by RT-PCR. Expression level was normalized to the geometric mean of β2M, RPL13A, and GAPDH and further normalized to the controls (untreated) of each timepoint. Two-way ANOVA-Multiple comparison Tukey statistical test was performed between the means of 3 replicates at significance level of 95%CI, data is expressed as means ± S.E.M. asterisk (*) represents p≤0.05, otherwise, no significant difference was noted p>0.05. .. 89

Figure 4-5 TSG-6 dose and response time of a pooled population of HUCPVCs stimulated with (1 to 100ng/mL) of rhTNF-α. Secreted protein concentration of TSG-6 in the supernatant of the same cultures in Fig.4-4, were quantified with ELISA and normalized to the total secreted protein/well. Two-way ANOVA-Multiple comparison Tukey statistical test was performed between the means of 3 replicates at significance level of 95%CI, data is expressed as means ± S.E.M. Asterisk (****) represents p≤0.0001, otherwise not significant (NS) p>0.05. ............................................................................................. 90

Figure 4-6 TSG-6 dose and response time of a pooled population of hBMMSCs stimulated with (1 to 100ng/mL) of rhTNF-α. mRNA fold change expression level of TSG-6 was determined by RT-PCR. Expression level was normalized to the geometric mean of β2M, RPL13A, and GAPDH and further normalized to the controls (untreated) of each timepoint. Two-way ANOVA-Multiple comparison Tukey statistical test was performed between the means of 3 replicates at significance level of 95%CI, data is expressed as means ± S.E.M. Statistical significance between groups is shown in Table 4-1. ........................................ 92

Figure 4-7 TSG-6 dose and response time of a pooled population of hBMMSCs stimulated with (1 to 100ng/mL) of rhTNF-α. Secreted protein concentration of TSG-6 in the supernatant of the same cultures in Fig. 4-6, were quantified with ELISA and normalized to the total secreted protein/well. Two-way ANOVA-Multiple comparison Tukey statistical test was performed between the means of 3 replicates at significance level of 95%CI, data is expressed as means ± S.E.M. Asterisks (*) represents p≤0.05, and (***) p≤0.01, otherwise not significant (NS) p>0.05. ............................................................................................. 94
Figure 4-8 TSG-6 dose and response time of pooled donors of mBM MSCs stimulated with (1 to 100ng/mL) of rmTNF-α. mRNA fold change expression level of TSG-6 is determined by RT-PCR. Expression level was normalized to the geometric mean of PPIA, YWHAZ, and GAPDH and further normalized to the controls (untreated) of each timepoint. Two-way ANOVA-Multiple comparison Tukey statistical test was performed between the means of 3 replicates at significance level of 95% CI. Data is expressed as means ± S.E.M. Asterisks (*) represents p≤0.05, (**) p≤0.01, (***) p≤0.001, otherwise not significant p>0.05.  

Figure 4-9 TSG-6 dose and response time of 5 HUCPVC donors stimulated with 1 to 100ng/mL of rhTNF-α. mRNA fold change expression level of TSG-6 was determined by RT-PCR. Expression level was normalized to the geometric mean of β2M, RPL13A, and GAPDH and further normalized to the controls (untreated) of each timepoint. One-way ANOVA-Multiple comparison Tukey statistical test was performed between the means of 15 data points ((n=3/donor) x 5 donors) at significance level of 95% CI; data is expressed as means ± S.E.M. Statistical significance between groups is shown in Table 4-2.  

Figure 5-1 Experimental design (A) time-line; (B) animal model of acute inflammation and the IM site of transplanted MSCs, dorsal thigh quadriceps muscle; (C) Method of paw circumference measurement. Hind paw circumference measurements were taken using a thread at 360° from digit 1 plantar to digit 1 dorsal, as illustrated with black ink.  

Figure 5-2 Hind paw circumference measurements (mm), 24 and 48 hours post induction of inflammation. Data for each animal is normalized to their baseline measurements of inflammation (4 hours post induction). Two-way ANOVA-Multiple comparison Tukey statistical test is performed between the means of measurements obtained from 5 mice/group and 4 measurements/animal/timepoint. Statistical analysis is presented at significance level of 95% CI, and data is expressed as means ± S.E.M. Asterisk (*) represents p≤0.05; (**) p≤0.01; (***) p≤0.010, and (****) represents p≤0.0001, otherwise no significant difference was noted p>0.05.  

Figure 5-3 Neutrophil abrogation in acute inflammation post MSC transplantation. (A) Giemsa staining of hind paw plantar 8-10µm cryo-embedded cross-sections. Nuclei (Blue), Neutrophilic cytoplasm (pink), black arrows show neutrophils, scale bar: 20µm; (B) MPO activity in the inflamed paw. Two-way ANOVA-multiple comparison Tukey statistical test is performed between the means of measurements obtained from 5 mice/group and 4 measurements/animal/timepoint. MPO measurements were obtained using an ELISA kit. Statistical analysis is presented at significance level of 95% CI, and data is expressed as means ± S.E.M. Asterisk (*) represents p≤0.05; (**) p≤0.01; (***) p≤0.001, and (****) represents p≤0.0001, otherwise no significant difference was noted p>0.05.  

Figure 5-4 Macrophage activity in acute inflammation post MSC transplantation in 8-10µm cryo-sections of the inflamed paw. Macrophages F4/80 positive (MCA497R and Alexa Fluor 647-red) and nucleic acid stain (Hoechst 33342-blue). Scale bars=250µm.  

Figure 5-2 Hind paw circumference measurements (mm), 24 and 48 hours post induction of inflammation. Data for each animal is normalized to their baseline measurements of inflammation (4 hours post induction). Two-way ANOVA-Multiple comparison Tukey statistical test is performed between the means of measurements obtained from 5 mice/group and 4 measurements/animal/timepoint. Statistical analysis is presented at significance level of 95% CI, and data is expressed as means ± S.E.M. Asterisk (*) represents p≤0.05; (**) p≤0.01; (***) p≤0.001, and (****) represents p≤0.0001, otherwise no significant difference was noted p>0.05. Green =Control (●); Gray=mBM MSCs (▲); Purple=hBM MSCs (■); Navy=HUCPVCs (●).  

Figure 5-3 Neutrophil abrogation in acute inflammation post MSC transplantation. (A) Giemsa staining of hind paw plantar 8-10µm cryo-embedded cross-sections. Nuclei (Blue), Neutrophilic cytoplasm (pink), black arrows show neutrophils, scale bar: 20µm; (B) MPO activity in the inflamed paw. Two-way ANOVA-multiple comparison Tukey statistical test is performed between the means of measurements obtained from 5 mice/group and 4 measurements/animal/timepoint. MPO measurements were obtained using an ELISA kit. Statistical analysis is presented at significance level of 95% CI, and data is expressed as means ± S.E.M. Asterisk (*) represents p≤0.05; (**) p≤0.01; (***) p≤0.001, and (****) represents p≤0.0001, otherwise no significant difference was noted p>0.05. Green =Control (●); Gray=mBM MSCs (▲); Purple=hBM MSCs (■); Navy=HUCPVCs (●).  

Figure 5-4 Macrophage activity in acute inflammation post MSC transplantation in 8-10µm cryo-sections of the inflamed paw. Macrophages F4/80 positive (MCA497R and Alexa Fluor 647-red) and nucleic acid stain (Hoechst 33342-blue). Scale bars=250µm.  

xvii
Figure 5-5 Serum level of tumor necrosis factor alpha (TNF-α) in acute inflammation, MSC treated, and healthy mice. Measurements are obtained using a Quantikine ELISA kit with assay range of 10.9-700 pg/mL, hence the values below the dash-line are all considered at the same level. One-way ANOVA-multiple comparison Tukey statistical test is performed between the means of measurements obtained from 3-4 mice/group. Statistical analysis is presented at significance level of 95% CI, and data is expressed as means ± S.E.M. Asterisk (**) represents p≤0.01 and (***), p≤0.001, otherwise no significant difference was noted p>0.05. Green =Control (●); Gray=mBMMSCs (▲); Purple=hBMMSCs (■); Navy=HUCPVCs (●). ..................................................................................................... 123

Figure 5-6 TSG-6 protein level in lysate of transplanted muscle (dorsal thigh quadriceps). (A) Chemiluminescent blots comparing mesenchymal stromal cell transplanted samples with controls; (B) Total protein loading per lane prior to primary antibody incubation using stain-free technology. TSG-6 protein has been normalized to the total protein of each sample; (C) Relative intensity of TSG-6 protein normalized to stain-free blots of total protein/column. One-way ANOVA-multiple comparison Tukey statistical test is performed between the means of measurements obtained from 5 mice/group. Statistical analysis is presented at significance level of 95% CI, and data is expressed as means ± S.E.M. Asterisk (**) represents p≤0.01, otherwise no significant (N.S.) difference was noted p>0.05. Green =Control (●); Purple=hBMMSCs (■); Navy=HUCPVCs (●). .... 124

Figure 5-7 Profile of cell surface epitopes of Unmodified and DiR-labeled HUCPVCs by flow cytometry-PI was added to exclude dead cells. (A) DiR-labeled HUCPVCs exhibited the same cell surface profile as unmodified cells with near 100% of cells positive for CD73, CD105, CD90 and MHC I (MSC markers) and negative for HLA-DR, CD45 and CD31. DiR effectively labeled 100% of viable HUCPVCs compared to control; (B) Abundance of cell surface receptors as measured by MFI. The MFI of DiR was a direct measure of the cytoplasmic membrane it labels. Abbreviations: HUCPVCs, human umbilical cord perivascular cells; PI, Propidium iodide; MFI, mean fluorescence intensity. ................ 125

Figure 5-8 Longitudinal study of 1.3x10^6 DiR labeled HUCPVCs, xenotransplanted intramuscularly in CD1 mice for 33 days. The longitudinal image is a representative image of n=3 mice. The fluorescence signal post day 7 is a false positive signal transmitted by the engulfed HUCPVCs possibly in the cytoplasm of phagocytes............................................. 126

Figure 5-9 Profile of cell surface epitopes of Unmodified and GLuc-Transfected HUCPVCs (GLuc-HUCPVCs) by flow cytometry-PI was added to exclude dead cells. (A) GLuc-HUCPVCs had the same cell surface profile as unmodified cells in both positive and negative markers. The only noticeable difference was the lower % of cells positive for CD10, CD146, and MHC-I in the modified cohort and higher in CD142; (B) Abundance of cell surface receptors as measure by MFI. Abbreviations: HUCPVCs, human umbilical cord perivascular cells; PI, Propidium iodide; GLuc, Gaussia Luciferase; MFI, mean fluorescence intensity............................................................. 127

Figure 5-10 Longitudinal study of 1.3x10^6 GLuc expressing HUCPVCs, xenotransplanted intramuscularly in CD1 mice. Mice were intravenously injected with Coelenterazine (250ug/100ul sterile ddH2O per 23g mouse) and in-vivo GLuc activity was measured at different time points (24, 48, 72, 96, and 120 hours post HUCPVC transplantation). Data
acquisition was obtained 2 minutes post substrate administration (exposure time=9s), longitudinal images are representative images of the behavior of cells in n=5 mice; (A) Reconstructed IVIS 3D images; (B) 2D images (open filter); (C) Longitudinal quantification of luminescence flux (p/sec/cm$^2$/sr) from the represented mouse in (b), region of interest (ROI) is circled with red. (B) GLuc reporter activity in the blood at different time points, n=5. Data represented as relative light unit (RLU) and normalized to controls at each time point. One-way ANOVA-multiple comparison Tukey statistical test is performed between the means of measurements obtained from 5 mice. Statistical analysis is presented at significance level of 95% CI, and data is expressed as means ± S.D. Asterisk (*) represents p≤0.05; (**) p≤0.01; otherwise no significant (N.S.) difference was noted p>0.05.

Figure 5-11 Fate of GLuc-HUCPVCs in the muscle tissue 5 days post transplantation. (A) high and (B) low magnification of GLuc-HUCPVCs in 5-7µm cryo-sections of the muscle tissue 120 h post transplantation. GLuc positive (E8023S and Alex Fluor 647-red) and nucleic acid stain (Hoechst 33342-blue). Scale bars: 125 µm.

Figure 5-12 Accidental rupture of a major vessel during IM-transplantation of MSCs. Image is acquired 24 h post incidence. MSCs have traveled systemically, some of which are detected at the ear tag injury site.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>ADMSCs</td>
<td>Adipose-derived MSCs</td>
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<tr>
<td>ARCs</td>
<td>Adventitial reticular cells</td>
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<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<tr>
<td>BM</td>
<td>Bone marrow</td>
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<tr>
<td>BMMSCs</td>
<td>Bone marrow-derived mesenchymal stromal cells</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<tr>
<td>C4S</td>
<td>Chondroitin-4-sulphate</td>
</tr>
<tr>
<td>CCL2</td>
<td>Chemokine (C-C motif) ligand 2</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFU-F</td>
<td>Colony-forming unit-fibroblast</td>
</tr>
<tr>
<td>CLI</td>
<td>Critical limb ischemia</td>
</tr>
<tr>
<td>CM-DiI</td>
<td>Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate</td>
</tr>
<tr>
<td>CXCL8</td>
<td>Chemokine (C-X-C motif)/ligand 8</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DHGp</td>
<td>Dunkin Hartley guinea pigs</td>
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<tr>
<td>DiR</td>
<td>DiIC₁₈(7) 1,1'-Dioc-tadecyl-3,3',3'-Tetramethylindotricarbocyanine Iodide</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas ligand</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GLuc</td>
<td>Gaussia Luciferase</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
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<tr>
<td>GQHs</td>
<td>Gold-QD hybrids</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronan</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Human leukocyte antigen–D Related surface antigens</td>
</tr>
<tr>
<td>HO-1</td>
<td>Hemo oxygenase 1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>---------</td>
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<tr>
<td>HSC</td>
<td>Hematopoietic stem cells</td>
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<tr>
<td>hsTNFR</td>
<td>Human soluble tumor necrosis factor receptor II</td>
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<tr>
<td>HUCPVCs</td>
<td>Human umbilical cord perivascular cells</td>
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<tr>
<td>hBMMSCs</td>
<td>Human bone marrow-derived mesenchymal stromal cells</td>
</tr>
<tr>
<td>IA</td>
<td>Intra-arterial</td>
</tr>
<tr>
<td>IC</td>
<td>Intracardiac</td>
</tr>
<tr>
<td>IDO1</td>
<td>Indoleamine-pyrrole 2,3-dioxygenase 1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IL-1RA</td>
<td>Interleukin 1 receptor antagonist</td>
</tr>
<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>iPSCs</td>
<td>Induced pluripotent stem cells</td>
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<tr>
<td>ISCT</td>
<td>International society of cellular therapy</td>
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<tr>
<td>IV</td>
<td>Intravenously</td>
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<tr>
<td>mBMMSCs</td>
<td>Mouse bone marrow-derived mesenchymal stromal cells</td>
</tr>
<tr>
<td>MDSCs</td>
<td>Myeloid-derived suppressor cells</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MLR</td>
<td>Mixed Lymphocyte Reaction</td>
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<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal stromal cell</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid-differentiation primary response protein 88</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NG2</td>
<td>Neuron-glial antigen 2</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>PAH</td>
<td>Poly(allylamine)</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PD-L1</td>
<td>Programmed death-ligand 1</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
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</table>
PHA Phytohemagglutinin
PLX-PAD Placental derived MSCs
pBMMSCs Porcine bone marrow-derived mesenchymal stromal cells
PS-b-PAA Poly(styrene)-block-poly(acrylic acid)
QDs Quantum dots
RANKL Receptor activator of nuclear factor kappa-B ligand
rBMMSCs Rat bone marrow-derived mesenchymal stromal cells
ROS Reactive oxygen species
sBMMSCs Sheep bone marrow-derived mesenchymal stromal cells
TGF Transforming growth factor
Th cell T-helper cell
TNF Tumor necrosis factor
TLR Toll-like receptor
TSG-6 TNF-stimulated gene 6
VEGF Vascular endothelial growth factor
WJ Wharton’s Jelly
α-SMA Alpha-smooth muscle actin
Preface

Mesenchymal stromal cells (MSCs), isolated from various sources, have shown to exhibit remarkable immunomodulatory potency in-vivo. Recently, focus is directed towards using MSCs from the neonatal sources due to their superiority in (1) colony-forming-unit fibroblast frequency, (2) growth rate, (3) low immunogenicity, and (4) immunosuppressant/immunomodulatory potency. However, little is known of the relationship between MSCs tissue source and their rate of response to an inflammatory trigger. This thesis is focused on assessment of the immunomodulatory efficiency of a neonatal source of MSCs (human umbilical cord perivascular cells) compared with an adult source (human and mouse bone marrow MSCs). Concluding results presented in this thesis demonstrate that human umbilical cord perivascular cells response to inflammation is more prompt compared with bone marrow MSCs, and therefore neonatal MSCs may be a stronger candidate to treat inflammatory diseases than adult bone marrow MSCs.

This thesis is divided in five chapters. Chapter 1 presents an overview of the immunoregulatory activities of MSCs. Chapter 2 includes a concise review of the intramuscular route of delivery of MSCs, dwell-time, fate and biodistribution of MSCs. In Chapter 3, the preliminary studies conducted to assess different animal models and cell tracking methods has been discussed in detail. Data presented in Chapter 4, systematically scrutinizes the differences in the response time of neonatally-derived MSCs and adult-derived MSCs when stimulated with a key pro-inflammatory factor (tumor necrosis factor-alpha), in-vitro. In Chapter 5, functional assessment of the response of the neonatal and adult populations of MSCs conducted in-vivo is presented. To better study the differences in the potency of the two populations of MSCs in response to inflammatory mediators, MSCs were delivered in the muscle site anatomically away
from the site of inflammatory trigger. Chapter 6 discusses overall discussion of the importance of our findings, limitations and strengths of our studies and techniques, concluding remarks, and future work.

This research thesis resulted in two published papers in peer-reviewed scientific journals and one mini-review article that is included in this thesis but not yet submitted. All manuscripts were drafted by Shiva Hamidian Jahromi accompanied with critical comments and revisions provided by Prof. John E. Davies. Shiva Hamidian Jahromi contributed to research papers by defining the objectives, designing the experiments, conducting the experiments, acquiring data, quantification, image analysis, data analysis, statistical analysis, and interpretation of the results, as well as preparation of the manuscripts. Contributions of other co-authors are provided below:


   **Contributions:** Li Y. assisted experimentally in RNA isolation and purification, and RT-PCR plate preparations. Davies JE supervised and funded the studies, in addition critically reviewed and revised the manuscript.


   **Contributions:** Estrada C. transfected HUCPVCs with Gaussia luciferase, Li Y. assisted in cell preparation, blood isolation, tissue collection, genomic DNA, RNA, and protein isolation,
and droplet digital PCR training. Cheng E. characterized surface markers of MSCs prior and post labeling using flow cytometer. Davies JE supervised and funded the studies, in addition critically reviewed and revised the manuscript.

Conference and Meeting Proceedings


Chapter 1: Mesenchymal Stromal Cells-History, Mechanism of Immunomodulation, and Intramuscular Delivery Route

1.1 Mesenchymal Stromal Cells (MSCs)

Alexander Friedenstein was the first to identify a group of fibroblast-like, colony-forming cells in the bone marrow (BM) of guinea pigs and mice [1]. Arnold Caplan in 1991 popularized them as “mesenchymal stem cells”, due to their capability to differentiate into cells of the mesodermal lineage [2]. However, since the word ‘stem’ implies a long-term self-renewing cell capable of multi-lineage differentiation in-vivo, Horwitz et al. in 2005 suggested to use the term multipotent “mesenchymal stromal cells” for MSCs derived from supportive stromal tissues [3].

The remarkable use of stem cell-based therapy within the biomedical disciplines called for standardization of minimum criteria for defining in-vitro characteristics of MSCs to provide an opportunity for better comparison of the study outcomes. According to the international society of cellular therapy (ISCT) in 2006, the following criteria define human MSCs: plastic-adherence in culture; expression of cluster of differentiation (CD)105, CD90, and CD73, and lack expression of CD45, CD34, CD14, or CD11ß, CD79α, CD19 and human leukocyte antigen–D Related (HLA-DR) surface antigens; and multipotent differentiation potential to osteoblasts, adipocytes, and chondroblasts in-vitro [4]. Nevertheless, it is worth noting that demonstration of in-vitro differentiation of MSCs does not guarantee in-vivo differentiation of the culture expanded MSCs, and vice-versa. The in-vivo physiological environment and trophic cues are far more complex than the limited in-vitro induced culture environment. More recently, Caplan and Correa in 2011 proposed an alternative term “medicinal signaling cells”, owing to their putative capacity to
immunomodulate/immunosuppress a host immune response as well as consequent commercialization of MSCs or their derivatives as drugs for a wide array of human diseases [5]. Although MSCs are currently known as therapeutic drugs, little is known about their developmental origin, phenotypic characteristics post-transplantation (in-vivo), and contribution to organogenesis.

Stromal cells share many characteristics with fibroblasts [6] including adherence to tissue culture plastic, fibroblastic morphology, and production of extracellular matrix (ECM). The distinct characteristics that discriminates this subpopulation (i.e., stromal cells) from other fibroblasts are their plasticity to undergo multilineage differentiation and lack of expression of fibroblast-specific protein-1 (FSP-1) [7]. Moreover, this subpopulation is shown to undergo clonogenic self-renewal referred to as colony-forming unit-fibroblastic (CFU-F) cells [8]. Each CFU-F is expected to give rise to a discrete colony composed of at least 50 cells by 14 days of culture. The frequency of the CFU-F is considered indicative of the frequency of the progenitor cells. Nevertheless, the single colonies derived from single MSC precursors, specifically from bone marrow-derived MSC (BMMSCs) population, have shown to exhibit heterogeneity [9, 10]. Cells from a colony when re-plated at clonal densities, form a new colony with cells that vary in size and differentiation potential [10]. Tremain et al. 2001, performed an extensive gene expression study on a single cell-derived colony of undifferentiated human BMMSCs (hBMMSCs) and reported expression of 2,353 unique transcripts that linked to various MSC lineages reflecting the developmental potential of the cells within a colony [9]. This pervasive heterogeneity has convoluted characterization of MSCs. A comprehensive review on MSCs [11] and a concise review on intra-colony heterogeneity ofMSCs is available elsewhere [12].
MSCs and pericytes located in the perivascular compartment of vessels share a few functional characteristics. This has shaped an inaccurate belief that all MSCs from any tissue are pericytes or MSCs are precursors of pericytes [13-15] or pericytes are precursors of MSCs [16]. Hence, a population of MSCs/pericytes expressing the pericyte markers are frequently considered a homogenous population of stem cells. Pericytes are closely associated with the walls of all blood vessels surrounding the endothelial cells [17, 18]. Pericytes are contractile cells that are shown to have critical immunoregulatory functions such as maintaining endothelial cell junctions extravasation during inflammation [19]. Pericytes have specialized counterparts such as adventitial reticular cells (ARCs) of the sinusoids outer lining in BM stroma [20, 21]. The density of pericytes are reported to vary around the vessels, depending on the function of the vessel. Pericytes are shown to be scattered around capillaries and abundant around the small venules and arterioles. Also, pericyte density varies in different organs, and different anatomical locations [22]. These specific cells (i.e., pericytes) are identified as CD146$^+$ [15, 23]. In addition, they can also express platelet-derived growth factor receptor $\beta$ (PDGFR$\beta$)$^+$ [24] in capillaries, arterioles, and venules; alpha-smooth muscle actin ($\alpha$-SMA)$^+$ [25] in arterioles and venules; neuron-glial antigen 2 (NG2)$^+$ [26] in capillaries and arterioles [25]. Interestingly, a fraction of the endothelial cells found in the circulation, in models of vascular disease [27], fibrosis [28], tumors [29] and inflammation [30] have been shown to express CD31$^+$ and CD146$^+$ that perhaps take part in neovascularization of the injured/inflamed tissues. Despite the wealth of literature that reported pericytes as stem cells, Guimaraes-Comboa et al. in 2017 through lineage tracing demonstrated that endogenous pericytes in brain, heart, skeletal muscle, and fat do not contribute to cell lineages [31]. Hence, it is suggested that the plasticity of the pericytes expanded in-vitro and transplanted in-vivo is perhaps different than the functionality of the endogenous pericytes [31].
There are various ways to classify MSCs: differentiation potential, origin, and developmental stage from which they are obtained (Fig. 1-1). **Totipotent** cells are found in the early developmental stage (fertilized oocyte and the few subsequent cell divisions). These cells can differentiate into embryonic (inner cell mass) and extra-embryonic cells (trophoblast) from which embryo, placenta, and chorion are formed [32]. **Pluripotent** stem cells from the inner mass are a more committed derivative of totipotent cells, they can give rise to all cell types of the three germ layers (i.e., ectoderm, endoderm, and mesoderm). “Pluripotent” stem cells from the trophoblast give rise to the extra-embryonic cells that form chorion and placenta which differentiate to multiple cell types, including cystotrophoblasts, syncytiotrophoblasts, villous core stroma cells, and pericyte/endothelial cells that participate in placental vasculogenesis. Embryonic stem cells as well as induced pluripotent stem cells (iPSCs) that are reprogramed somatic cells are considered pluripotent [33, 34]. Unlike most somatic stem cells [35], embryonic stem cells [36] have indefinite replicative lifespan in culture, within an undifferentiated state—does not reach senescence. Embryonic stem cells can differentiate into any somatic cells and reach senescence. A comprehensive review on the lifespan and senescence signaling in embryonic stem cells is reported elsewhere [37].

**Multipotent** stromal cells are found in most developed tissues with limited differentiation capacities. **Multipotent** MSCs are either derived from adult tissue sources or fetal and neonatal. MSCs derived from BM, peripheral blood, adipose tissue, dental pulp, tendon, skin, and muscle are categorized as adult MSC sources that are derived from mesoderm. The use of adult stem cells to regenerate damaged tissue evades the ethical issues associated with the use of those from an embryonic source or the emerging safety concerns of using iPSCs. MSCs derived from Wharton’s Jelly, amniotic fluid, amnion membrane, placenta, chorionic villi, and cord blood are all derived from fetal and neonatal tissues. MSCs derived from the neonatal tissues are shown to have
advantages over MSCs derived from adult tissues [38, 39]. Neonatal-derived MSCs, mainly Wharton’s Jelly (WJ) from the umbilical cord connective tissue have shown increased proliferation activity [40], low immunogenicity [41], increased immunosuppression [39, 41], and enhanced immunomodulatory potency [42-44] compared to adult tissue-derived MSCs. Detailed comparison of neonatal (e.g., Human umbilical cord perivascular cells-HUCPVCs) and adult-derived MSCs (e.g., BMMSCs) are provided in chapter 4.

**Figure 1-1** Diagram depicting the origin of adult and neonatal-derived MSCs. Courtesy of John E. Davies.
1.1.1 Adult: Bone marrow-derived MSCs

Stroma of BM is composed of a network of heterogenous cells, including fibroblasts, stromal progenitors, endothelial progenitor cells, adipocytes, and reticular cells. The predominant blood vessels in BM are large vessels that are called sinusoids covered with endothelial cells with outer surface covered with adventitial reticular cells (ARC), without an interface of basement membrane that in many tissues, defines the location of pericytes [20, 21]. Hematopoietic stem cells (HSCs) commonly isolated from BM give rise to all mature blood cell types of hematolymphatic system (e.g., monocyte/macrophages, neutrophils, eosinophils, basophils, erythrocytes, mast cells, dendritic cells, platelets, and T and B lymphocytes). BMMSCs are the non-hematopoietic fraction that are adherent population of MSCs, suggested to specifically originate from the perivascular compartment within BM [45, 46]. The MSC fraction, is shown to support BM-HSCs through regulation and development of the hematopoietic microenvironment, shown \textit{in-vitro} [47] and \textit{in-vivo} in myeloablative therapy [48]. To isolate BMMSCs, BM aspirates that consist of a highly heterogeneous population are plated in culture, then upon expansion, through subsequent passages, the hematopoietic cells (CD45+) are eliminated. The adhered cells are still heterogeneous and consist of a few stem cells among a majority of the mononuclear cells (e.g., osteogenic cells, fibroblasts, adipocytes, and smooth muscle cells), many of which have limited number of divisions and are eliminated through sequential passages. Efforts towards decreasing the heterogeneity of the cell population beyond donor variability and careful cell isolation and culture methods have been made by limiting the cultures to clonogenic cells. Clonogenic cells have improved the antigenic homogeneity, but even the single colonies, as previously discussed, have shown notable heterogeneity in size, differentiation potential, and antigen profile [10, 49, 50]. Although a stem cell-specific marker is not yet identified, Sacchetti \textit{et al.} have employed CD146+ selectivity as a marker of cells from the perivascular region to obtain an improved homogeneous population of
MSCs [15]. Despite the heterogeneity of BMMSCs, they have been extensively used for variety of therapeutic approaches, including tissue regeneration and immunomodulation. Apart from the invasiveness of the procedure of BM isolation and the ethical and regulatory concerns, there are other critical limitations associated with using BMMSCs: (i) human BM is shown to have low frequency of CFU-F (1:1×10^4 to 1:1×10^5 BM mononuclear cells), and (ii) a temporal decline in the number of MSCs throughout the process of aging (Fig.1-2) [51].

![Human MSCs Decline With Age](image)

**Figure 1-2** Temporal decline in the colony forming unit-fibroblast (CFU-F) frequency relative to the number of mononuclear cells in the BM [51, 52]; Figure is adopted from Caplan et al., 2009, [51] and used with permission from John Wiley and Sons.

These limitations, have shifted the focus of research to a simple tissue (i.e., less heterogenous) with rich CFU-F frequency of MSCs from a discarded neonatal source —WJ-derived MSCs from umbilical cord. WJ-MSCs are currently the focus of 108 clinical trials registered at ClinTrials.gov.
1.1.2 Neonatal: Umbilical cord-derived MSCs

Human umbilical cord tissue is comprised of 2 arteries, 1 vein, amniotic epithelium, vascular endothelium, and WJ (Fig. 1-3) (originated from extraembryonic mesoblast) that provides vascular support and is comprised of mesenchymal cells (progenitors and myofibroblasts) that elaborate ECM (collagen, glycoprotein, hyaluronan, sulfated glycosaminoglycan (GAG), and plasma proteins) [53].

Figure 1-3 Cross-section of human umbilical cord tissue. (A) Hematoxylin and Eosin staining of the cord illustrates the complete structure that consists of 2 arteries and 1 vein, perivascular regions (black arrows) from which HUCPVCs are isolated, Wharton’s Jelly matrix (intermediate and cord lining), and amniotic epithelium. Note the distinct separation of the perivascular cells (higher cellular and tissue density) and the intermediate Wharton’s Jelly matrix; (B) DAPI (4’,6-diamidino-2-phenylindole) nuclear staining partially illustrating the vein and cellular composition of the cord. The cellular density in the perivascular zone is higher than towards the amniotic epithelium. Note that there is a distinct separation between the tunica media of the vein and the perivascular cells. Images are adapted from Davies et al., 2017, [54] and used with permission from John Wiley and Sons.

Human umbilical cord vessels lack the tunica adventitia layer that is commonly found around the vessels as fibrous connective tissue and micro-vessels that nourish the intima and media. Tunica adventitia of arteries and veins is shown to contain MSC progenitors that anatomically and phenotypically are different from pericytes (expressed CD34 and lacked expression of CD146) [55]. Instead, the dense perivascular regions (Fig.1-3) in the umbilical cord that is avascular and aneural are shown not only to compensate for adventitial functional support
but also to specifically contain a rich population of progenitors that express CD146 and not CD34. Schugar et al., 2009, have shown that this population is exclusively expressed in the perivascular region (62%), the vessel walls (100%), and diffused throughout the WJ towards the epithelium layer [56]. Davies et al. 2017, suggests the perivascular region to contain more than 45% of the total WJ cells [57]. Interestingly, Davies et al. have shown strong and contained CD146+ expression in the smooth muscle vessel wall during the first trimester, whereas at term, the CD146+ cells are found in perivascular region, in the smooth muscle vessel wall, and in the vascular endothelium [57]. Considering this finding with evidence of the presence of platelet-derived growth factor (PDGF)-AB in the amniotic fluid [58], Davies et al., suggested that a number of CD146+ cells might have migrated out from the vasculature throughout gestation. The migrated cells are platelet derived growth factor-receptor beta positive (PDGFRβ+). These migrated cells also potentially proliferate to increase the volume of WJ [57]. Other common markers of MSCs are also expressed by the umbilical cord perivascular population that are CD90, CD105, CD44, and CD73 [56, 59], a fraction of these cells might be the ones that differentiate to myofibroblasts [57]. Despite the evidence on the anatomical regions of WJ that contain the majority of CD146+ cells, various reports have isolated MSCs from either the whole cord or other distinct areas and this defines the heterogeneity of the isolated population used. In this thesis, we have used a more homogeneous population of MSCs, that are well-characterized and distinctly isolated from the perivascular region of umbilical cord (HUCPVCs). Phenotypically, HUCPVCs are shown to express CD146, CD73, CD90, CD105, PDGFβ, major histocompatibility complex (MHC)-I and lack expression of CD40, CD45, CD31, CD34, CD80, CD86, and MHC-II [60, 61] (Fig. 1-4 and Fig. 1-5). As shown by Baksh et al., 2007 (Fig. 1-5), 52% of HUCPVCs and 15% of BMMSCs express CD146+ [60]. This significant difference is perhaps related to the reported CFU-F of HUCPVCs compared with BMMSCS; HUCPVCs: 1:300 [62] and BMMSCs: 1: 100000 [51]. This
may also infer a greater homogeneity of the HUCPVC population compared with the BMMSCs [60, 63].

**Figure 1-4** Representative flow cytometry histograms. Flow cytometry data for one cryopreserved cord sample at passage 2, one plated cord sample at passage 1, and one cryopreserved hBMSC sample at passage 2. The black histogram is the cell staining of the blank controls, the red histogram is the antibody-stained cells. Legend and Figure adopted from Matta et al., 2009 [61]. Abbreviation: hBMSCs, human bone marrow stromal cells.

**Figure 1-5** Profile of cell surface epitopes in HUCPVCs and BMSCs. (A) Abundance of cells positive for CD49e, CD90, CD146, CD31, CD117, Strol-1, and CD45, expressed as percentages, in HUCPVCs and bone marrow-derived BMCs; (B) Flow cytometric analysis showed that HUCPVCs express elevated levels of CD146. Abbreviations: BMSCs, bone marrow stromal cells; Ctrl., control; PE, phycoerythrin; R2, Region 2. Figure and partially legend is adopted from Baksh et al., 2007 [60]; used with permission from John Wiley and Sons.
1.1.3 **Immunosuppressive/Immunomodulatory Response of BMMSCs versus UC-MSCs**

MSCs from different sources, have all been shown to possess various functional phenotypes depending on the tissue source [64-72]. Both hBMMSCs and UC-MSCs have been employed in cell therapy, and UC-MSCs have been proposed as an alternative to MSCs derived from adult tissue sources. Indeed, the ideal MSC tissue source may depend upon the targeted therapy. Thus, some MSCs may be better than others for specific indications. For example, hBMMSCs produce higher levels of Vascular Endothelial Growth Factor (VEGF) and stem cell-derived factor-1 (SDF-1) than UC-MSCs and may be more suitable to support neovascularization or homing to the BM—by binding to the CXCR4 in the BM that is the ligand for SDF-1 [73].

Neonatal MSCs have shown low immunogenicity [41], increased immunosuppression [39, 41] and cause an increase in alternatively activated M2-type macrophages [42] compared to BMMSCs, although this may not be reflected in standard mixed lymphocyte reactions (MLR), where both a similar [74] and improved [75] functional phenotype has been reported. Prasanna et al. 2010, compared the potency of hBMMSCs and WJ-MSCs both unprimed, and TNF-α or IFN-γ primed, using an MLR assay *in-vitro*, and reported WJ-MSCs as a more potent population compared with hBMMSCs [75]. Several studies have highlighted the impact of BMMSC donor age on the plasticity and differentiation capacity of the MSCs [76-78].

Liu et al. (2016) showed that umbilical cord MSCs reduced the activation of pro-inflammatory signaling [44], and Raicevic et al. (2011) concluded that WJ-MSCs were more effective than either adipose tissue or BM-derived MSCs when an immunosuppressive action is required in the presence of an inflammatory stimulant [79]. Furthermore, Holley et al., using quantitative proteomics, reported small numbers of proteins that were either BMMSCs or
HUCPVC specific, although the functional relevance of these differences was not explored [80]. Please see Chapter 4 and Chapter 5 for further comparison of these MSC sources.

Understanding the differences between the potency of MSC populations from different sources in different inflammatory milieus enables more effective use of different MSC populations in a specific and tailored manner. In this thesis, we have particularly focused on the gold standard BMMSCs and recently popularized UC-MSCs.

**Part of the text in this subsection have previously been published as included in Chapters 4 and 5 of this thesis [81, 82].

1.2 Activation Mechanisms of MSCs

Inflammation, is the first response elicited by the innate immune cells towards a damage to tissue by either physical injury, pathogens, or chemicals [83]. The innate immune system is composed of mast cells, eosinophils, basophils, neutrophils, macrophages, dendritic cells (DC), and natural killer cells (NK). The first leukocytes to infiltrate the site of injury are the neutrophils that mark the start of an inflammatory response towards wound healing. Neutrophils are effector phagocytotic cells that destroy pathogenic microorganisms at the site of injury by means of phagocytosis, degranulation, release of reactive oxygen species (ROS), and neutrophil extracellular traps (NETs) [84, 85]. Consecutively, neutrophils recruit macrophages to the site of injury. Acute and chronic inflammation differ in the duration and pathology. Acute inflammation has a short timing during which, neutrophils and macrophages can successfully destroy the invaded foreign body. However, the release of cytokines, proteases, and other factors of cytoplasmic granules can also further damage the tissue. Excess infiltration and activation of neutrophils and macrophages at the site of injury causes tissue damage, leading to chronic inflammation [86]. Also, neutrophil mediated tissue damage during transmigration is reported in various disease pathologies
such as in pulmonary diseases [87, 88], autoimmune diseases [89], and ischemia reperfusion injury [90]. Therefore, it is crucial that the activity of neutrophils is finely controlled. The adaptive immune system, mainly CD4\(^+\) and CD8\(^+\) T-cells, and B-cells, is subsequently activated by the innate immune system [91].

The innate immune system relies on recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) which leads to activation of pro-inflammatory signaling pathways. PRRs include toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and retinoic acid-inducible gene I (RIG-I) like receptors. TLRs are type I membrane proteins expressed by both immune and non-immune cells [92]. TLRs activate the intracellular signaling pathways leading to induction of pro-inflammatory cytokines such as type I interferons (IFNs) and upregulation of costimulatory molecules that activate the adaptive immune system [92]. TLRs (1, 2, 4, 5, 6, and 10) are localized on the cell surface that recognize microbial membrane components (lipids, lipoproteins, and proteins) and TLRs (3, 7, 8, and 9) are expressed on the intracellular compartments (endoplasmic reticulum, endosomes, and lysosomes) and recognize viral nucleic acids [93, 94].

Intracellularly, post ligand recognition, myeloid-differentiation primary response protein 88 (MyD88) that is shared between all TLRs except TLR3 [95] activates mitogen-activated protein (MAP)-kinases (MAPKs) and nuclear translocation of the transcription factor-κB (NF-κB) (NF-κB:MyD88 dependent pathway) [96, 97], Fig. 1-6. Toll/IL-1R (TIR)-domain-containing adaptor protein (TIRAP) is another adaptor protein required for MyD88-dependent signaling utilized by TLR2 and TLR4 [95, 98]. An alternative pathway is triggered by TIRA-inducing INF-β (TRIF) that is employed through TLR3 and TLR4, (NF-κB:MyD88 independent pathway) that leads to the activation of NF-κB, MAPKs and transcription factors interferon-regulatory factors (IRFs),
responsible for induction of type I IFNs, specially IFN-β [99, 100], Fig. 1-6. TRIF-related adaptor molecule (TRAM) is another adaptor protein that is required for TRIF-dependent signaling through TLR4, not TLR3 [95, 98].

**Figure 1-6:** Overview of TLR signaling pathways. Adopted from Kawai et al. 2010 [101]. Used with permission of Springer Nature Publishing Group.

MSCs are either activated through their TLR receptors [102] or directly by the designated receptors for mediators such as tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and interleukin 1 beta (IL-1β) and thereby MSCs exert their immunomodulatory actions [75, 103] (Fig. 1-7). MSCs are also activated by other inflammatory mediators from the TNF family such as a proliferation inducing ligand (APRIL), and B-Cell activating factor (BAFF) proteins that are overexpressed in inflammatory diseases, mainly in cancer or chronic inflammatory conditions.
It is shown that inflammation modifies the pattern and function of TLRs expressed by MSCs. Waterman et al. 2010, introduced a new paradigm for the immunomodulatory mechanism of action of MSCs. Pro-inflammatory MSCs (MSC1) induced through TLR-4 produces pro-inflammatory mediators such as (macrophage inflammatory protein (MIP)-1α, MIP-1β, Regulated on Activation Normal T-cell Expressed and Secreted (RANTES), CXCL9, and CXCL10) that can lead to T-lymphocyte activation. Anti-inflammatory (MSC2) phenotype is induced by TLR3 that produces immunosuppressive factors (Indoleamine-pyrrole 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), Nitric Oxide (NO), Transforming Growth Factor (TGF)-β, Hepatocyte Growth Factor (HGF), and hemo oxygenase (HO)) that can lead to T-lymphocyte inhibition. Raicevic et al. has shown that the pattern of expression of TLRs on MSCs are source dependent. BMMSCs and AD-MSCs share similar pattern of TLR expressions (1, 2, 3, 4, 5, 6, and 9, and absence of mRNA for 7, 8, and 10). On the other hand, WJ-MSCs do not express TLR4; Cord Blood (CB)-MSCs, express low levels of TLRs 1, 3, 5, 9, and high levels of 4, and 6, with functional proteins TLR4, and TLR5. Human amnion MSCs express TLR1-10 at various levels. Liotta et al. 2008, reported that ligation of TLR3 and TLR4 in hBMMSCs inhibited the ability of these cells to inhibit T-cell proliferation. Raicevic et al. 2011, reported that ligation of TLR3 and TLR4 of BMMSCs, WJ-MSCs, and AD-MSCs differentially affected the 3 cell sources resulting in reduced ability of immunosuppressive potency of BMMSCs and not AD-MSCs or WJ-MSCs.
Figure 1-7 MSC activation through TLR stimulation or directly by inflammatory mediators. Abbreviations: Lipopolysaccharide (LPS); double-stranded RNA (dsRNA); Complement 3 (C3); CD59 (a complement regulatory protein); Tolerogenic DC (Tol DC). Adopted from English et al. 2012 [111]. Used with permission from Springer Nature publishing group.

1.3 Therapeutic response of MSCs to inflammation

MSCs modulate both innate and adaptive immune responses. The underlying mechanism of action by which MSCs exert their therapeutic effect is mainly through (i) paracrine activity and (ii) homing/migration to the site of injury to modulate the microenvironment via cell-cell interaction.

The classical view of the mechanism of immunomodulatory action of exogenously transplanted MSCs was through migration to the site of injury, engraftment and differentiation into tissue-specific cells, and perhaps to fusion with the host tissue. MSCs are indeed shown to migrate towards the injury site [13]. Rojas et al. in an in-vitro assay demonstrated that murine MSCs
preferentially migrated towards the bleomycin-injured mouse lung cells and not the healthy lung cells [112]. The preferential migration of MSCs is stimulated by the secreted cytokines, chemokines and growth factors such as PDGF-AB, and insulin-like growth factor 1 (IGF-1) [113]. Furthermore, chemokine and cytokine driven migration of MSCs is mediated by the pro-inflammatory factors \textit{i.e.} TNF-\alpha [113]. Also, transmigration of the circulating MSCs to the site of injury is mediated through the upregulated endothelial surface molecules (vascular cell adhesion molecule 1 (VCAM) and E-selectin) during injury that are the ligands for MSC surface molecules integrin \(\alpha 4/\beta 1\) and CD44 respectively [114]. Although MSCs preferentially migrate to the site of injury, they lack engraftment at the site of injury. MSCs exert their immunomodulatory effects by cell-cell contact through Jagged1-Notch1 interactions [110], and release of soluble factors that modulate the microenvironment of a pro-inflammatory milieu towards an anti-inflammatory and a reparative state. It is also shown that exogenously transplanted MSCs trigger endogenous MSCs and transfer their mitochondria and microRNAs to the impaired endogenous cells. MSCs are shown to exert anti-apoptotic effects mediated through secretion and stimulation of factors such as Vascular Endothelial Growth Factor (VEGF) [115, 116], HGF [115, 116], Insulin-like Growth Factor (IGF)-1 [115], Stranniocalcin-I [117], TGF-\(\beta\) [116], basic fibroblast growth factor (bFGF) [116], granulocyte-macrophage colony-stimulating factor (GM-CSF) [13, 116]. MSCs also exhibit an anti-fibrotic effect by augmentation of HGF [118]. In addition, MSCs are shown to enhance angiogenesis when employed at ischemic or injured sites. Kinnaird \textit{et al.} transplanted MSCs locally, in a murine model of hind limb ischemia and reported detection of bFGF, and VEGF around the transplanted site \textit{in-situ} [71]. Furthermore, MSCs support recruitment of endogenous progenitor cells as well as their growth and differentiation through secretion of G-CSF, M-CSF, leukemia inhibitory factor (LIF), and IL-6 [47, 119]. Most importantly, MSCs immunomodulate
through inhibition of T-cells [120-122], B-cells [123], suppression of NK cells [124, 125], and modulation of the secretory profile of macrophages [126], and DC [127].

Herein, we have focused on the MSC trophic factor-mediated action on the acute phase of inflammation. The potential immunomodulatory effects of human MSCs transplanted in immunocompetent mice is comprehensively reviewed by Prockop et al. 2017 (Fig. 1-8) [128]. As illustrated in Fig. 1-8, hBMMSCs have been shown to modulate/suppress the immune response through the release of a number of trophic factors, including TNF-stimulated gene 6 (TSG-6), interleukin 1 receptor antagonist (IL-1RA), PGE2 [129], TGF-β, chemokine (C-C motif) ligand 2 (CCL2) [130], IDO1, Fas ligand (FasL) [131], programmed death-ligand 1 (PD-L1) [132], and HO-1. However, the response of MSCs is shown to vary with the nature of the stimulant in a disease setting. For example, in an autoimmune disease, such as rheumatoid arthritis or multiple sclerosis, MSCs suppress T-helper cells (Th)1/Th17 [133], and in a model of allergic asthma MSCs suppress Th2/Th17 inflammation [134]. Moreover, in peritonitis, MSCs suppress NF-κB signaling in macrophages through the release of TSG-6 [135]. Similarly, in many other studies, TSG-6 along with other modulators secreted by MSCs increased the number of anti-inflammatory macrophages and regulatory T-cells to modulate the microenvironment of inflammation [128]. MSCs are shown to modulate the generic pathway of innate inflammation through two negative feedback loops: (i) activation of MSCs by the proinflammatory mediators to upregulate expression of COX2 as well as other components of arachidonic acid pathway, thereby MSCs increase secretion of PGE2 which drives the shift of resident macrophages from inflammatory (M1) phenotype to anti-inflammatory (M2) leading to secretion of IL-10 and IL-1RA; (ii) activated MSCs increase expression of TSG-6 that interacts with CD44 on resident macrophages (directly or in a complex
with HA) to dissociate CD44 from TLR2 to limit TLR2 from activation of NFκB. In this way, MSCs control secretion of TNF-α and other proinflammatory mediators [136].

To give a few more examples, in a model of bleomycin-induced lung injury (sterile inflammation), Ortiz et al. 2007, demonstrated that mouse BMMSCs (mBMMSCs), transplanted intravenously (IV), decreased the anti-inflammatory response mainly by the release of IL-1RA. It was also observed that MSCs were only effective when administered at the same time as bleomycin, during the acute phase of inflammation, and not at a later timepoint [137]. In a zymosan mouse model of peritonitis, Choi et al. 2011, demonstrated that hBMMSCs abrogated neutrophil infiltration and inhibited activation of (TLR)2/NF-κB signaling of macrophages to pro-inflammatory phenotype (M1). The mechanism of action by which TSG-6 interacted with macrophages is shown to be via the CD44 receptor. MSCs that were pre-activated by TNF-α and MSCs that were unstimulated were transplanted intraperitoneally, 15 min post zymosan induction, and both showed significant abrogation of inflammatory cues [135]. The mechanism of action was suggested to be through upregulation of IL-10 by anti-inflammatory macrophages (M2) and therefore downregulation of TNF-α and IL-1α. Both studies highlighted the anti-inflammatory properties of naïve mouse and human MSCs, respectively, and suggested that transplanted MSCs are activated in-vivo by the inflammatory cues [138]. In-vitro cultured hBMMSCs were shown to secrete low levels of IL-1β (≤200pg/mL), and IL-6 [139]. The observed continuous secretion of pro-inflammatory cytokines through cell passages suggested that ex-vivo expanded MSCs might show a pro-inflammatory phenotype which can be further enhanced via stimulation [139]. It should also be noted that, as discussed previously, in-vitro polarization of MSCs via stimulation towards pro- or anti-inflammatory phenotypes depends on the activation of specific TLRs [106] that their expression is highly MSC source dependent [79, 108, 109].
**Figure 1-8:** Summary of immune-suppressive effects of human mesenchymal stromal cells transplanted in mice. In different immune models, the major immunosuppressive factors secreted by hMSCs were observed to be tumor necrosis factor induced gene 6 (TSG-6), IL-1RA (IL-1 receptor antagonist), HO-1 (heme oxygenase 1), PGE2 (prostaglandin E2), TGF-β (transforming growth factor b), CCL2 (C-C motif ligand-2), IDO1, FasL (Fas ligand), or PD-L1 (programmed death-ligand 1). Most of the factors primarily altered cells of the innate immune system, such as monocytes/macrophages, MDSCs (myeloid-derived suppressor cells), or antigen presenting dendritic cells. Subsequent waves of cytokines then suppressed inflammation and altered T cells to either increase or decrease the ratio of Th1/Th17:Th2 responses. Figure is adopted from a review article, Prockop et al., 2017, [128] and is used with permission from Elsevier.

### 1.3.1 Tumor necrosis factor-induced gene 6 protein

Tumor necrosis factor-induced gene 6 (TNFIP6 or TSG-6) maps to human chromosome 2q23.3 and is a 35 kDa secreted multifunctional anti-inflammatory glycoprotein, originally identified as a cDNA derived from human fibroblasts treated with TNF-α [140, 141]. TNFIP6 is composed of a connecting link module and complement subcomponents C1r/C1s-Uegf-BMP-1 (CUB) module [141-143]. Link modules are found in proteins that interact with glycosaminoglycans (GAGs) [144] and hyaluronan (HA), that is a major constituent of ECM.
TSG-6 is involved in extracellular matrix remodeling by interacting with GAGs, HA, chondroitin-4-sulphate (C4S) [145], aggrecan [146], and serine protease inhibitor inter-α-inhibitor [144, 147-149].

1.3.1.1 Mechanism of Activation and Action of TSG-6

TSG-6 is expressed by numerous cell types (i.e., skin fibroblasts, monocytes, synovial cells, smooth muscle cells, and MSCs) in inflammatory conditions, and the expression level is shown to vary with different cell types [142]. Constitutive upregulation of TSG-6 in healthy adult tissues is negligible but it is highly expressed in inflammatory conditions in response to proinflammatory mediators, predominantly TNF-α, and IL-1, and LPS [148, 150]. An exception is shown in rabbit vascular smooth muscle cells for which the primary stimulators were TGF-β, FGF, epidermal growth factor (EGF), and IL-1 rather than TNF-α [142, 151, 152]. This is suggestive of differential upregulation pathways [153] for TSG-6 with various kinetics in different cell types [142, 154]. TSG-6 gene expression is tightly regulated and complex [150]. TNF-α and IL-1 enhance transcription of TSG-6 gene through the promoter regions -165 to -58 containing the transcription factors activator protein 1 (AP-1) and nuclear factor IL-6 (NF-IL6) sites [155]. Interferon responsive element (IRF-E) overlaps with the AP-1 site of the TSG-6 promoter (-119 to -126), 3 base pairs apart from the NF-IL6 site (-106 to -115) [140]. NF-IL6 is a family of (c/EBP) that is synthesized upon stimulation by IL-1, IL-6, TNF-α and LPS [156]. Activation of TSG-6 gene is shown to require two adjacent binding sites for NF-IL6 [150]. Activation by TNF-α requires both the AP-1 site as well as the NF-IL6 sites. However, activation of TSG-6 by IL-1 requires the NF-IL6 site, although activation can be enhanced through the AP-1 site [150]. Indeed, TNF-α and IL-1 do not function through identical pathways. Two binding sites of NF-IL6 function as activator or inhibitor of TSG-6 transcription, dependent on the ratio
of the activator to the inhibitor. However, TNF-α or IL-1 stimulation can lead to an increase in the level of activator NF-IL6 [157].

Human TSG-6 cDNA is shown to have 94% homology with amino acid sequences of mice and rabbits [148]. Wisniewski et al. have reported a correlation between the level of TSG-6 in the synovial fluid of OA patients and the progression of the disease [158]. Strong association was found between high levels of TSG-6 and rapid progression of OA [158]. Hence, Wisniewski et al. have suggested TSG-6, as a biomarker of OA [158]. TSG-6 has been identified as one of the most upregulated genes in hBMMSCs, and plays a critical role in the immunomodulation and down-regulation of inflammation [159]. Knockdown or silencing of TSG-6 has been shown to result in the loss of therapeutic activity of both BMMSCs [159] and UC-MSCs [44]. Intravenous (IV)-administration of a single dose of hBMMSCs or two doses of TSG-6 recombinant protein in a mouse model of traumatic brain injury is reported to decrease neutrophil extravasation, decrease expression of metalloproteinase (MMP) 9, and reduce blood brain barrier leakage and the size of the lesion [160]. Numerous reports have shown TSG-6 to be important in inhibition of inflammation and joint destruction in autoimmune disease [161], acceleration of wound closure and reduction of fibrosis through altering TGF-β1 and TGF-β3 synthesis from a scar promoting (high) to an anti-fibrogenic (low) ratio [162], and inhibition of MMP mediated degradation of the cartilage matrix [163]. Thus, TSG-6 has been suggested as a biomarker of potency of MSCs [164].

### 1.3.1.2 TSG-6 Autocrine Effect and Modulation of Inflammatory Cells

As mentioned above, TSG-6 protein is a member of HA-binding family, including lymphocyte adhesion receptor CD44 family of proteins [165, 166]. In an inflammatory milieu, the proinflammatory cytokines increase HA expression on the vascular endothelial cells [167], and it is shown that integration between HA and CD44 controls leukocyte rolling [168]. TSG-6
protein is also shown to abrogate neutrophil migration and infiltration [169] through interference with the chemokine/GAGs and chemokine (C-X-C motif)/ligand 8 (CXCL8) interactions [170, 171], Fig. 1-9. Also, TNFIP6-deficient transgenic mice, showed enhanced neutrophil extravasation in proteoglycan-induced arthritis [172]. Furthermore, TSG-6 is shown to interact with macrophages through the CD44 receptor to decrease translocation of NF-κB [135]. Furthermore, TSG-6 shifts the phenotype of macrophages from M1 to M2, secondary to suppression of TLR4/NF-κB signaling and STAT1 and STAT3 activation [173].

TSG-6 activation of NF-κB in hBMMSCs has been correlated with increased expression of TSG-6. In contrast, inhibition of NF-κB signaling has been shown to result in decreased expression of TSG-6 and, interestingly, an increase in the osteogenic differentiation of hBMMSCs [164]. Tsukahara et al., have shown that TSG-6 downregulates osteoblastic differentiation induced by bone morphogenic protein (BMP)-2 [174] through direct binding to receptor activator of NF-κB ligand (RANKL) and BMPs (except BMP-3) via Link and CUB domains [175]. Hence, TSG-6 secretion by MSCs has potent autocrine effects [174, 175]. TSG-6 is also shown to inhibit osteoclast activity, RANKL-mediated bone erosion in synergy with osteoprotegerin (OPG) [176], therefore can be important in treatment of osteoporosis.

TSG-6 is also shown to modulate plasmacytoid dendritic cells (pDCs) by downregulation of TNF-α and IFNs, mediated through CD44, and thereby IRF-7 (a key transcriptional factor involved in regulating IFN-α gene in lymphoid cells) phosphorylation [177, 178]. TSG-6 is also shown to delay the onset of autoimmune diabetes by suppressing the activation of T-cells as well as the antigen-presenting cells—suppression of Th1 development and enhancing tolerogenicity [179].
In this thesis, we have chosen TNF-α as a direct activation method for upregulation of the TSG-6 gene. TNF-α is a key pro-inflammatory mediator in inflammation, and specifically in neutrophil migration, and allodynic responses in carrageenan-induced inflammatory process [180]. Rocha et al., 2006, have discussed the significance of TNF-α in a mouse model of inflammation induced by carrageenan in the hind paw. The level of neutrophil activity in the paw is decreased by ~50% when TNF-α is blocked [180]. We have chosen TSG-6 as it has been shown among the anti-inflammatory secretome of MSCs, to be one of the primary mediators, hence a surrogate for...
the anti-inflammatory function of MSCs. Besides TNF-α and IL-1, Other pro-inflammatory mediators are also shown to have significant indirect and secondary effect on TSG-6 upregulation.

### 1.3.2 Other soluble factors

Prostaglandins are lipid mediators that are synthesized from arachidonic acid via the action of cyclooxygenase (COX) enzyme in response to injury, or stimuli [181]. The pathway of PGE2 synthesis involves COX-1 and COX-2 production of prostaglandin H2 from arachidonic acid and further conversion into prostaglandins. MSCs constitutively express COX2 that is necessary for production of PGE2. Furthermore, pro-inflammatory stimuli such as TNF-α and IFN-γ enhances PGE2 synthesis by MSCs [182]. PGE2 secreted by MSCs is shown to mediate modulation/suppression of T-cells, NK cells, DC, macrophages [127, 183], and mast cells through a COX-2 dependent mechanism [184]. MSC-mediated immunosuppression by PGE2 is exerted in combination with other immunosuppressive molecules. For example PGE2 MSC-mediated immunosuppression in humans acts with IDO to alter T-cell proliferation and inhibit NK cell proliferation and cytotoxicity and cytokine production [183, 185]. PGE2 has a major role in edema formation. It has been demonstrated that edema formation and the inflammatory response following carrageenan injection into mice paw is associated with local production of PGE2 (mediated by COX-1 and COX-2) and NO derived from eNOS and iNOS [186].

IDO is a intracellular monomeric enzyme that controls the pathway of Kynurenine (KP) that degrades tryptophan (an essential amino acid required for T-cell proliferation) [187]. IDO expression is not constitutive, but pro-inflammatory cytokines such as INF-γ [188], TNF-α and IL-1 induce expression of IDO [189] that functions locally. MSC-derived IDO mediates shift of macrophage phenotype to M2 anti-inflammatory [190], promotes Th1-Th2 switch [191], induces
tolerogenic DCs and Treg, and also influences T-cell differentiation [192] and T and NK cell proliferation [111, 125, 182].

NO is catalyzed by nitric oxide synthase (NOS) and expressed by 3 genes, iNOS (inducible mainly in macrophages); nNOS, in neurons; and eNOS, in endothelial cells [193]. NO, apart from having a direct effect on macrophage function but also at high concentrations inhibits T-cell receptor signaling, and induced proliferation [194-196]. NO is highly unstable, hence only mediates the effects locally on proximal immune cells [197, 198]. Inflammatory cytokines such as INF-γ, TNF-α and IL-1 were shown to induce NO synthesis in mice MSCs [193].

Other cytokines, chemokines, and growth factors: MSCs deficient in CCL2 chemokine is shown to exhibit lower capacity in inhibition of IL-17 secretion by activated T-cells [199]. CCL-2 is also shown to suppress the production of immunoglobulin by plasma cells [200]. Other immunoregulatory factors that are produced by MSCs or other progenitor cells upon inflammatory stimulation are inhibitory surface protein PD-L1 or B7-H1 [201], HO-1, IL-6, galactins, LIF, TGF-β and regulatory T-cells, and many more reviewed in the following references [13, 111, 202-204].

1.4 Mechanism of carrageenan-induced inflammation
Carrageenan-induced edema in hind paw was first introduced by Winter et al. 1962, for the purpose of testing the effect of anti-inflammatory drugs on the induced inflammation and edema [205]. The lambda form of carrageenan (Fig. 1-10) does not gel at room temperature which facilitates injection. Following subcutaneous injection of λ-carrageenan, various pro-inflammatory factors are released i.e. bradykinin, histamine, tachykinins, complement and reactive oxygen that cause acute inflammation with cardinal signs of edema, hyperalgiesia, and erythema [206]. The initial phase is the release of histamine, bradykinin, 5-hydroxytryptamine (5-HT) and the second phase of inflammation when swelling starts is attributed to induction of COX-2 and local
production of PGE2 [207]. Local neutrophil infiltration further produces pro-inflammatory cytokines, mainly TNF-α [180] and also oxygen derived free radicals [186, 208].

Figure 1-10 Sulfated di-galactose structure of λ-carrageenan. The repeating disaccharide structural units demonstrate β-1\(\rightarrow\)4- and α-1\(\rightarrow\)3 galactosidic linkages connected residues. Sites of sulfation differ among the three major types of carrageenan (λ, κ, ι). Adopted from Bhattacharyya et al. 2010 [209]. Used with permission of Elsevier Publishing Group.

Structurally, carrageenan is a sulfated polysaccharide (Fig. 1-10) that resembles the naturally occurring sulfated GAGs, mainly chondroitin sulfate, dermatan sulfate and keratan sulfate. However, instead of having a β-galactosidic bond, carrageenan has a α-galactosidic bond which is recognized as immune epitope if used in humans, since humans lack the enzyme α-1\(\rightarrow\)3-galactosyltransferase [209]. Interestingly, it also shares similar structure with LPS with regards to α-Gal (1\(\rightarrow\)3)Gal epitope. Similar to LPS, the carrageenan-induced inflammatory cascade is shown to involve TLR4-MyD88 mediated NFκB activation leading to IL8 production (Fig. 1-11) [209, 210].
Figure 1-11 Schematic of inflammatory cascades involved in carrageenan-mediated activation. These particular pathways are shown in NCM460 cells (a normal human colon mucosal epithelial cell line). The pathways are believed to be by the 3 cascades (1) TLR-4, B-cell leukemia/lymphoma (BCL)10, IκB kinase (IKK)γ, and phosphor- IκBα-mediated activation of RelA; (2) TLR4, BCL10, phosphor-NF-κB-inducing kinase (NIK), IKKα-mediated activation of the non-canonical pathway leading to nuclear translocation of p52 and RelB; (3) ROS-mediated pathways requiring Hsp27, and IKKβ. Adopted from Bhattacharyya et al. 2011 [211].

1.5 Choice of MSC Delivery Route

The therapeutic capacity of exogenously transplanted MSCs to treat immune disorders and tissue injuries has been demonstrated in a wide range of animal models of human diseases [159, 212, 213] and clinical trials [214, 215] that have mainly delivered MSCs by intravenous (IV) injection, which is the most common route to effectuate systemic therapy. It has frequently been shown that small numbers of IV delivered MSCs, which are rapidly entrapped in the capillary beds of lungs, [159] lodge in other organs, mainly the liver and spleen [216-219]. Nevertheless, during entrapment in the lungs, MSCs have been shown to alter the tissue microenvironment of a remote
injured/inflamed site through their secreted soluble factors [159]. Thus, it is clear that the physical presence of the delivered MSCs at the target site is not essential for therapeutic immune modulation.

Other routes, intramuscular (IM), intraperitoneal (IP) and subcutaneous (SC) have been explored and recently compared by Braid et al. (2018) [220] which reported the dwell-time of hMSCs when delivered IM (5 months), IP or SC (3-4 weeks), and IV (3 days) in healthy athymic mice [220]. While several authors have reported that the number of IM injected cells decrease over time, there is little evidence of distribution to other organs. Creane et al. [221] detected hBMMSCs 3 months after injection in the thigh and calf musculature of BALB/c Nude mice, but no MSCs were detected in the brain, heart, lungs, kidneys, spleen or liver. IM delivery of MSC to the hamstrings, has been shown to increase cardiac function [222, 223], predominantly through the release of VEGF [224], although other trophic factors may also play an important role [225]. Therefore, IM delivery of MSCs may also represent a valuable alternative to treat systemic conditions where the long dwell-time of secretorily active cells would provide an advantage over the rapid disappearance of cells from the lungs following IV delivery.

We have chosen the IM-delivery route in this thesis to ensure that the majority of the delivered MSCs reside locally while we assess the anti-inflammatory response of the different populations to a distant source of inflammation through their trophic factors. In addition, we aimed to assess the potential of using the IM-MSC delivery route to treat a distant inflamed site as a principal proof-of-concept study with the prospective of using such a delivery method to simultaneously treat multiple sites.

**Part of the text in this subsection have previously been published as included in Chapters 4 and 5 of this thesis [81, 82].
1.6 Rationale

IM delivery of MSCs has recently been promoted as an alternative to IV delivery. The majority (~95%) of IV-delivered MSCs are immediately entrapped in the lungs and only very few cells reach a site of injury/inflammation [159]. However, the entrapped MSCs have been shown to have an immunomodulatory effect on a distant source of injury, through the release of trophic factors [159]. Thus, the therapeutic potency of MSCs is a function of a plethora of trophic factors rather than a minimal engraftment at the site of injury.

In many disease pathologies, a sustained modulation of inflammation is necessary to obtain an optimal therapeutic effect, which cannot be achieved through the transient residence of MSCs infused through the IV route, in particular, when multiple doses of MSCs are necessary during the course of therapy. An extended in-vivo dwell-time for MSCs, can be achieved through IM-delivery [220], which has mainly been employed to treat local injuries of skeletal or myocardial diseases. Nevertheless, majority of such studies have raised the concern that injecting exogenous MSCs into an already inflamed muscle with myogenic cues and lymphocyte infiltrates, could affect the putative potency of transplanted MSCs [226, 227]. To the best of our knowledge, the only anti-inflammatory study of IM-delivered MSCs—as a prophylactic to abrogate the inflammatory effects of a rheumatoid arthritis model—was only successful with gene-modified MSCs [228]. However, it is generally agreed that MSCs are constitutively anti-inflammatory [229] [138]. Among the plethora of anti-inflammatory cytokines released by MSCs, TSG-6 is of particular interest, since there is a direct correlation between the levels of the inflammatory marker TNF-α and the secretion of TSG-6 [148, 159, 164]. In summary, and based on the available literature, it can be stated that:

1) MSCs are constitutively anti-inflammatory, although MSCs from different tissue sources have been shown to possess varying anti-inflammatory potencies.
2) Neonatal MSCs have been shown to be more effective anti-inflammatory mediators than MSCs derived from adult tissues.

3) IM-delivery of MSCs provides extended dwell-time and secretory activity of the cells.

4) Even with IV delivery of MSCs, and the transient dwell-time when they are trapped in the lungs, they have been shown to have anti-inflammatory effects on a distant organ—the heart—through the secretion of TSG-6.

5) Unmodified MSCs have been shown to have no anti-inflammatory effect on systemic inflammation if delivered prior to the onset of inflammation.

Considering the above-mentioned observations with the anti-inflammatory phenotype of MSCs, the question would be: Could the IM-delivery of unmodified MSCs be employed to combat an established inflammation at a distant anatomical site?

1.7 Hypothesis

A neonatal source of MSC, transplanted intramuscularly will downregulate a distant source of inflammation more efficiently than an MSC population derived from an adult tissue source.
1.8 Specific aims

**AIM I:** Study the effects of TNF-α dose and exposure time on TSG-6 activation by neonatal and adult mesenchymal stromal cells (Fig. 1-12).

**Questions:**

- What is the functional response, in particular, TSG-6 of neonatal HUCPVCs to different doses of an inflammatory stimulant compared to BMMSCs?
- What is the effective priming condition of neonatal HUCPVCs to achieve the maximum upregulation of anti-inflammatory TSG-6 in comparison with adult hBMMSCs for downstream *in-vivo* applications?

![Figure 1-12](image.png)

*Figure 1-12* Simplified illustration of experimental setup, TNF-α mediated release of tumor necrosis factor-induced gene 6 proteins.

**AIM II:** Study the *in-vivo* functional response of unmodified neonatal and adult mesenchymal stromal cells transplanted intramuscularly to a distant source of inflammation.

**Questions:**

- Can unmodified MSCs, transplanted intramuscularly, modulate and downregulate a distant source of inflammation?
- What is the biodistribution of MSCs transplanted intramuscularly?
- What is the fate of IM-xenotransplanted HUCPVCs in an immunocompetent mouse?
Chapter 2: Skeletal Muscle as a Delivery Route for Mesenchymal Stromal Cells

The following chapter will be submitted for publication.

Mesenchymal stromal cells [3], have demonstrated extensive capacity to limit injury and promote regeneration through signaling and secretion of trophic factors [230]. Indeed, MSCs provide a putative treatment for immune-related, infectious, and degenerative diseases, without a requirement for engraftment [231]. Despite these therapeutic effects of MSCs, one challenge is the short dwell-time of the delivered cells in-vivo [232]. However, in a recent study, Braid et al. [220] reported the dwell-time of human MSCs (hMSCs) delivered intravenously (IV), subcutaneously (SC), interperitoneally (IP), and intramuscularly (IM) in athymic healthy mice as 3 days (IV), 3-4 weeks (SC and IP), and 5 months (IM) respectively. Thus, skeletal muscle may provide an advantage for increasing the dwell-time of delivered MSCs.

To date, skeletal muscle has principally been employed as a delivery route for local treatment of myopathic, neurodegenerative, and vascular related diseases. However, recent studies have emphasized the opportunity afforded by IM-delivery to effect systemic changes. The three main advantages of skeletal muscle MSC delivery are: (i) Extended dwell-time provided by dense muscle fibers that retain the MSCs in-situ; (ii) High vascular density that provides a conduit for systemic release of MSC trophic factors and (iii) An abundance of tissue that provides for multiple injection sites. Although the IM-delivery of MSCs has been shown to be clinically safe [233-239], it is important to critically evaluate the fate of MSCs post-delivery in the skeletal muscle.

We discuss herein the evidence for engraftment and differentiation of MSCs delivered into skeletal muscle, their secreted factors both local and systemic, their dwell-time and their biodistribution.
2.1 Clinical Safety of IM-MSC delivery

Clinical trials that have adopted IM delivery of bone marrow-derived cells include both bone marrow-mononuclear cells (BM-MNC) and MSCs [240]. Clinical IM-MSC delivery has targeted both the promotion of angiogenesis in patients with peripheral artery disease (PAD) and thromboangiitis obliterans (TAO)/Buerger disease, and amelioration of motor neuron loss in amyotrophic lateral sclerosis (ALS) patients. Previously, the chosen route of MSC delivery for PAD and TAO was either IV or intra-arterial (IA) in anticipation that the cells would reach ischemic sites. However, IV-delivered MSCs are entrapped in the capillary beds of lungs with minimal engraftment to ischemic sites [241]. Clinical studies employing IM-MSC delivery have validated the safety of this approach (see Table 2-1). Gupta et al., IM delivered allogeneic BMMSCs in the ischemic limb of patients and reported improvement in clinical scores, without edema [233]. Furthermore, due to the low frequency of MSCs in BM aspirates, Bura et al. [234], assessed the safety and efficacy of IM-delivery of adipose-derived MSCs (AD-MSCs) in PAD patients with clinical limb ischemia and reported no sign of edema or necrosis at the site of injury. Clinical signs such as leg pain, ulcer size, and pain-free walking were all reported to be significantly improved—potentially due to revascularization.

The IM delivery of MSCs has, more recently, been pursued as an alternative to intrathecal (IT) and/or IV transplantation in ALS patients. Petrou et al. 2016 [239] reported no significant complications, and only slight edema, associated with injection, at 24 sites (1x10^6/site) in the biceps and triceps, of BMMSCS—induced in culture to express neurotrophic factors (NTF)—to promote regeneration and neuroprotection. Due to the nature of ALS, direct intrathecal delivery of MSC-NTFs together with peripheral IM administration of MSC-NTF was considered to enhance the efficacy of MSC-therapy compared with the IM-MSC delivery alone [239]. However, the
systemic effects of the release of NTF were not assessed. While all clinical studies have confirmed the safety of IM-MSC delivery, the only clinical study that has assessed a systemic effect of IM-delivered MSCs was conducted in critical limb ischemia (CLI) patients receiving allogeneic placenta-derived MSCs (PLX-PAD) where modulation of dendritic/natural killer cell interactions was observed [242].

2.2 Pre-clinical studies: IM-Delivered MSCs to Treat Local Pathologies

MSCs have been IM delivered for local treatment or to locally treat complications associated with systemic diseases (see Table 2-2). These studies have focused predominantly on the local angiogenic and neuro-supportive effects of MSCs but, although systemic disease models have been employed, the systemic sequelae of the secreted trophic factors have not been assessed. 

Diabetic polyneuropathy (DPN), similar to PAD, is a complication associated with diabetes. Shibata et al., 2008 [243] delivered rat BMMSCs (rBMMSCs), IM, in streptozotocin (STZ)-induced diabetic Sprague-Dawley (SD) rats. Four weeks post-delivery, the cells were observed in the gaps between the muscle fibers. In addition, a significant increase in the levels of bFGF and VEGF were observed in the treated muscle. In a similar model in balbC mice, Kim et al. 2011, delivered mBMMSCs along the sciatic nerve and reported improvement in motor nerve conduction as early as 2 weeks, while no further improvement was observed after 4 weeks. [244]. On the other hand, Han et al. [245] delivered allogeneic rBMMSCs in the thigh muscle of DNP-STZ induced Wistar rats near the sciatic nerve, and reported engraftment along the vasa nervosa after 4 and 8 weeks. Additionally, upregulation of angiogenic and neurotrophic genes, myelin protein, and nerve growth factor receptor gene in the transplanted muscle were all observed.
Following their initial use of neural progenitor cells [246], Suzuki et al. 2007 pursued delivery of glial cell derived growth factor (GDNF) transfected MSCs into various muscle groups [247]. In a SD-SOD1<sup>G93A</sup> rat model of ALS—that develops neurodegeneration of spinal motor neurons and progressive motor deficits—<sup>GDNF</sup>hBMMSCs were delivered together with daily cyclosporine (CsA). First, to ameliorate hBMMSCs survival, a focal muscle injury was induced with injection of bupivacaine hydrochloride prior to cell delivery. MSC delivery into the muscle led to significant reduction in the number of denervated endplates, and abrogation of motor neuron loss. IM-transplanted MSCs were detected after 8 weeks in the muscle at the site of injection [247].

In other IM-MSC studies, human cells were xenotransplanted in animal models of CLI for pre-clinical and translational assessment of the human MSC functionality in ischemia. Prather et al. 2009 [248], IM-transplanted luciferase expressing PLX-PAD, 5h post arterial ligation. Cells were delivered locally at the site of injury in both immunocompetent (balbC) and immunocompromised (NOD/SCID) mice. Loss of luminescence signal in the immunocompetent balbC mice was observed after 4 days, whereas in NOD/SCID mice, cells were still detected for 3 weeks. Similarly, Francki et al., IM-delivered placenta-derived adherent cells (PDACs), luciferase-transduced, in the ischemia-induced hind limb muscle of BalbC mice 24h after injury, and reported significant improvement in the blood flow and vascular density by 35-49 days. Furthermore, at 49 days, the injured muscle showed a reduction of inflammatory infiltrate and improvement in the structure of the regenerated muscle fibers [249]. Beegle et al., in a similar CLI model, IM-transplanted hBMMSCs over-expressing VEGF in the hamstrings of immunocompromised NOD/SCID-IL2Rγ-/- (NSG) mice. Significant loss of MSCs was reported within the first 28 days; a small number of cells were detected after 4.5 months, but no cells were detected after 6 months [250]. Although all of these studies reported local upregulation of angiogenic growth factors, they showed variations in the dwell-time of MSCs.
2.3 IM-delivered MSCs to treat distant and systemic conditions

MSCs are shown to secrete a plethora of immunomodulatory factors in response to inflammatory stimuli [202] and also to stimulate endogenous cell regeneration [251, 252]. IM delivery of MSCs has demonstrated a potential to treat distant or systemic conditions where the long dwell-time of secretorily-active cells would provide an advantage over the rapid disappearance of cells from the lungs following IV delivery. The systemic release of the IM-delivered MSC secretome was first demonstrated by Bartholomew et al., 2001. Baboon MSCs, genetically modified to express human erythropoietin (EPO), were IM-delivered in NOD/SCID mice and released hEPO for up to 1 month [253].

Shabbir et al. 2009, IM-delivered porcine BMMSCs (pBMMSCs), 2 injections 2 weeks apart, into the hamstrings of cardiomyopathic TO2 hamsters. Significant ventricular function improvement (i.e., attenuated chamber dilation) and increased systolic wall thickening were reported 3 weeks after a second IM-delivery of MSCs. MSCs were also shown to reduce apoptosis and myocardial tissue injury, as well as decreased myocardial-pathological fibrosis by ~50%. The systemic increase in the level of HGF, LIF, and GM-CSF were suggested to be the mediators of myocardial repair which was concomitant with upregulation of HGF, IGF-II, and VEGF in the myocardium [223]. Similarly, Zisa et al. 2009, IM-delivered hBMMSCs in the hamstrings of TO2 hamsters and reported improved left ventricle ejection fraction (LVEF) by 30%, 4 weeks post MSC therapy [254]: VEGF was considered to be the main factor that improved cardiac repair. Similarly, Mao et al. 2017, IM-delivered human umbilical cord Wharton’s jelly MSCs (hWJMSCs) into both forelimb and hindlimbs of doxorubicin-induced SD rats (a model of dilated cardiomyopathy), 2 injections 2 weeks apart. Improved cardiac function with increased systemic levels of HGF, IGF-1, LIF, GM-CSF, and VEGF and cardiac tissue expression level of HGF, VEGF, and IGF-1 was
observed 2 weeks after the second MSC injection [225]. Furthermore, Liu et al. 2013 using human soluble tumor necrosis factor receptor (hsTNFR) transduced hBMMSCs demonstrated a prophylactic reduction in joint inflammation in an antibody-induced/LPS-challenged murine rheumatoid arthritis (RA) model, although the naïve hBMMSCs showed no effect [228]. In another study, Braid et al. 2016 showed that a depot of IM-delivered human umbilical cord perivascular cells (HUCPVCs), genetically modified to secrete an anti-viral monoclonal antibody, provided systemic protection against exposure to Venezuelan equine encephalitis virus (VEEV), with secretorily-active MSCs detectable for 109 days [255]. The engineered HUCPVCs were IM-delivered in the thigh muscle of balbCnu/nu mice 24h or 10 days prior to intranasal inoculation with VEEV. No significant difference was observed between 24h or 10 days prophylactic protection. We have also delivered hBMMSCs, mBMMSCs, or HUCPVCs, IM in the hind limb of immunocompetent CD1 mice, and reported systemic downregulation of TNF-α and abrogation of neutrophil infiltration at an anatomically distant (contralateral) site of inflammatory injury [81].

These studies provide evidence that factors released from MSCs are the primary therapeutic mediators independent of their engraftment and differentiation at the site of injury. Nevertheless, three important factors that affect the efficacy of IM-MSC delivery for a systemic effect are the dwell-time of the cells, the cell dose and frequency of injection.

2.4 Discussions:
2.4.1 Dwell-time of IM-delivered MSCs

The extended dwell-time of transplanted MSCs in the skeletal muscle (compared to other routes of administration) enables putative extended therapeutic effects. Nevertheless, the reported dwell-time of MSCs delivered to the skeletal muscle varies from 72h to 8 months. Two key factors profoundly affect these dwell-time variations: (1) immune-rejection and (2) the methods used for
MSC detection. Although autologous MSCs are often used in clinical trials, they can show disease [256] or age-related [257] impairments. Therefore, allotransplantation provides an advantage since MSCs exhibit low immunogenicity, and are expected to evade the immune system. MSCs in-vitro exhibit low expression of MHC-I, and co-stimulatory molecules CD40, B7-1 (CD80) and B7-2 (CD86)—which are involved in T-cell co-stimulation or co-activation—and lack expression of MHC-II [258]. However, it is not clear whether MSCs maintain their low immunogenicity post transplantation [259], especially in an inflamed site. Hemeda et al. demonstrated that MSCs exposed to IFN-γ increased MHC class I expression and also triggered the expression of MHC-II cell surface markers [260]. Ishikane et al. 2008, showed a significantly lower number of T-lymphocytes in rBMMSC-transplanted healthy muscle compared to ischemia-induced MSC-transplanted muscle [261]. Even with autologous transplantation, in-vitro cell culture expansion conditions may cause phenotypic changes that facilitate innate recognition of the cells when transplanted, [262] resulting in physiological clearance. The only pre-clinical IM-autologous MSC transplantation study reported herein, records a dwell-time of 6 weeks at the transplanted site, [263] which is significantly less than the dwell-time of MSCs in immunocompromised animals. Importantly, even immunocompromised animal models differ in their reaction to xenotransplanted MSCs. Athymic-nude rodents do not produce mature T-cells and have high activity of macrophages, natural killer (NK) and dendritic cells (DC) [264, 265]. In contrast, SCID mice have impaired production of mature T-cells, and severely reduced macrophages NK and DC activity. These factors all affect the dwell-time of exogenously transplanted MSCs [266-268].

The majority of the pre-clinical studies are conducted in small animals and MSCs are often allotransplanted. Such studies have shown 17 days to 4 weeks of in-situ dwell-time [71, 261, 269-271], but the length of the study also affects the reported dwell-time. A somewhat extended dwell-time, ranging from less than 4 to more than 8 weeks, is reported when MSCs are allo-IM-
transplanted in non-injured muscles in models of systemic conditions such as STZ-induced DPN [243-245]. MSCs IM-delivered in immunosuppressed (CsA)-rats exhibited a dwell-time of 8 weeks when transplanted in a knock out ALS model [247]. It is important to note that CsA blocks recipient T-lymphocyte reactions, [272] and compromises granulocyte migration during acute inflammation. When hMSCs are transplanted in immunocompetent animals, a short dwell-time of 4-8 days is reported by Prather et al. [248], Francki et al. [249], and Hamidian Jahromi et al. [81]. Exceptions are the studies by Mao et al. [225] and Shabbir et al. [223] have reported therapeutic effects for 4 weeks that may infer survival of hWJ-MSC or pBMMSCs in immunocompetent SD-rats and TO2 hamsters respectively, although not assessed. On the contrary, some of the studies that have IM-transplanted MSCs in genetically immunocompromised animal models have reported significant dwell-times of 3-24 weeks in injured muscle [248, 250, 273], ~4-16 weeks in intact skeletal muscle of animals with systemic disease [228, 255], and ~4-32 weeks in intact healthy animals [220, 253, 274-276]. One factor that was similar in all reports was the fast decay in cell density over the first 14 days with further decline up to 28 days. For example, Liu et al. [271], transplanted mouse AD-MSCs into the hindlimb adductor muscle of ischemic C57BL/6 mice 24 hours post injury; gradual loss of the IM-transplanted MSCs was reported over 28-days. Ishikane et al. IM-delivered rBMMSCs or rat fetal membrane MSCs (rFM-MSCs) in a CLI model in MHC mismatched rats [261]. Loss of MSC engraftment was observed 3 weeks post IM-MSC delivery with a small quantity of cells still present at the site of injury. The fraction of cells remaining in the muscle for a longer period has been reported to be 10% of the transplanted cells after 8 months [274].

Suzuki et al., have reported a short MSC dwell-time when transplanted into intact muscle. A focal injury in the skeletal muscle, prior to transplantation, extended the MSCs dwell-time [247]. The short dwell-time of MSCs in intact muscle does not corroborate the findings of Shibata et al.,
2008 [243], Kim et al.[244], and Han et al. 2016 [245] where cells were injected in the intact muscle of DPN-STZ induced animal models and other studies that have injected MSCs in healthy mice [220, 253, 263, 274-276]. Interestingly, Laurila et al. reported detection of MSCs near the needle injury site [277] and Braid et al. 2018 [220] reported accumulation of MSCs around the site of needle injury which indeed was more pronounced when the density of IM-delivered MSCs declined over time. Although the discussed work does not support the notion of extended dwell-time of MSCs in an injured site, it is understood that needle injury itself is a small focal injury created in every IM-delivery model.

It is also worth noting that the different studies listed in Table 2-2 to Table 2-4 have used various methods with different sensitivity ranges to locate the IM delivered cells and/or to assess differentiation of the IM-delivered MSCs. Regarding the biodistribution studies, using in-vivo luminescence or fluorescence systems are limited to detection of higher cell numbers only. Histological detection methods are limited by the ability to find sections that provide an indication of the biodistribution of the cells. Genomic DNA assessment is the most sensitive method, but it is important to sample from a true pooled population of cells to be able to draw conclusions on the presence of the cells in an organ. Therefore, the best practice is to use multiple methods collectively to drive a conclusion regarding the biodistribution or the dwell-time of the delivered MSCs. Likewise, assessment of the phenotype of MSCs post IM-delivery, across different studies using various methods is compared in Tables 2-2 to 2-4. Detection of endothelial cells as an indication of angiogenesis was assessed using Lectin where no differentiation was reported in any of the studies using rBMMSCs [245, 261]. On the other hand, Al-khaldi et al. 2003, employed endothelial factor (F VIII) as a marker to assess differentiation of MSCs to endothelial cells and reported differentiation of the delivered rBMMSCs that were also LacZ+ [269]. Similarly, Iwase et al. 2005 reported detection and differentiation of the transplanted rBMMSCs using Von
Willebrand Factor (VWF), an endothelial marker [270]. These differences can be minimized by using various methods of detection and different markers prior to drawing definitive conclusions.

2.4.2 Cell Dose and Frequency of Injections

To date, MSC dosing, both in clinical trials and animal studies has been chosen rather arbitrarily. For IV infusion in humans, 1-2 x 10⁶ cells/Kg body weight is commonly employed. As expected for local delivery, lower cell numbers are reported; examples of which are from 1 x 10⁶-10⁸ for injection into OA knee joints [278], and 6 or 18 x 10⁶ cells delivered into the intervertebral disc for the treatment of lower back pain caused by degenerative disc disease [279]. Interestingly, the latter clinical study showed no therapeutic advantage of using the higher dose, although the clinical study was based, in part, on a sheep study employing both a low 0.5 x 10⁶, and high 4 x 10⁶ ovine BMMSCs in which the higher dose was more effective [280]. In an ex-vivo pig lung dose escalation study using HUCPVCs, Mordant et al. [281] found a medium dose (5 x 10⁷ cells) to be more effective than either a lower or higher dose.

For IM delivery of MSCs, little information is currently available and is contradictory. For example, although Petrou et al. [239] undertook a dose escalation study in patients with ALS (see above), no differential effects of the 3 dosing cohorts of combined IT and IM-delivered autologous BMMSCs were reported. In pre-clinical studies, Suzuki et al. [247] delivered 0.12x10⁶ gene-modified human neonatal BMMSCs (see above) either unilaterally or bilaterally into three muscle groups (tibialis anterior, triceps brachii and dorsal trunk musculature) of rats at 24h, 1 week and 2 weeks after local muscle injury. While the number of surviving cells was reported to increase with multiple injections, no other differences were attributed to the multiple dosing. On the contrary, Kang et al. [282], delivered high and low doses of hBMMSCs in ischemic limbs of Balb/c mice and reported no dose-effect relationship but enhanced results were obtained with higher frequency
of MSC injection. Similarly Mao et al. injected hUCMSCs twice into both fore- and hind-limb musculature of DCM rats (see above), 2 weeks apart, but reported no differences in outcome with low and high dose (0.25 or 1 x 10^6 cells) although the second treatment did result in significant increase in left ventricular ejection fraction [225]. On the other hand, Shabbir et al. reported that the highest injection dose employed, of 0.25, 1 and 4x10^6 pBMMSMCS into bilateral hamstrings, resulted in the most effective cardiac function improvement in the recipient hamsters [223].

As all MSCs populations are heterogeneous, but to varying extents, the therapeutically optimum cell dose for a particular delivery route can be expected to vary with MSC tissue source and the therapeutic target condition in addition to variations in the dosing regimen which, for IM administration, can include the number of IM sites chosen, their anatomical location and frequency (for multiple deliveries). Furthermore, gene-modified cells could be expected to be employed at different dosing regimens than unmodified populations. Several authors have shown that neonatal MSCs are more potent than those derived from adult tissues including higher MSC frequency, growth rate, life span, and superior immunomodulatory properties [38-41, 79, 82, 283, 284].

2.4.3 Differentiation of IM-delivered MSCs

Environmental cues can drive the phenotype of transplanted MSCs. IM-MSC delivery has also been employed to treat other local pathologies in local muscle injuries. De Bari et al., 2003 [273], assessed myogenic differentiation of human synovial membrane (hSM)-MSCs-LacZ⁺, delivered either IV or IM, to treat Latoxan-induced muscle injury in NMRI nu⁻/⁻ mice. After 4 weeks, cells expressing human myosin heavy chain type IIx/d (MyHC-IIx/d)—a terminal differentiation marker—were found in both injured and non-injured tibialis anterior (TA) muscles. In addition, human β2-microglobulin (β2M) was detected between the basal lamina and muscle fibers at the injured site, but without fusion with the latter. Similar results were obtained when
hSM-MSCs were IM-transplanted in the TA muscle of Dystrophin-deficient mdx mice (C57BL/10ScSn DMD<sup>mdx/J</sup>) immunosuppressed with Tacrolimus (FK506). After 4 weeks, human dystrophin and MyHC-IIx/d were detected in the injected muscle implicating differentiation and contribution of hSM-MSCs to regeneration of myofibers but without fusion [273]. Similar results were demonstrated by Suzuki et al. as the hBMMSCs transplanted in focally-induced skeletal muscle expressed β-actin and hMyHC-IIx/d suggesting myogenic differentiation [247]. Furthermore, 3 weeks post IM-transplantation of rBMMSCs, Iwase et al. reported detection of double positive PKH26/von Willebrand (vWF) cells [270]. Similarly, in a CLI Lewis rat model, Al-Khaldi et al. demonstrated that rBMMSCs transplanted in the ischemic limb of rats express factor VIII, α-SMA actin and desmin, markers of endothelial, smooth muscle and skeletal muscle cells respectively and concluded that the transplanted cells spontaneously regenerated the various components of muscular tissues [269]. Ishikane et al. assessed fusion of MSCs with blood vessel endothelial cells after 1 week of MSC transplantation in the ischemic limb and did not observe GFP+/Lectin double positive cells [261] which was similar to the reported results of Han et al. observed after 4 and 8 weeks [245]. Studies that did not use specific markers reported that MSCs reside in the gaps between the fibers without differentiation [71, 243]. The collective opinion is that myogenic environmental cues affect the phenotype of exogenously transplanted MSCs, and that this may happen earlier in an injured site.

2.4.4 Biodistribution of MSCs post IM-delivery

The biodistribution of MSCs is important for both safety and survival of MSCs. It is important to assess whether MSCs distribute to unwanted organs post-delivery, cause microembolism, or disappear which shortens the duration of therapeutic effect. Although it has been shown by many that MSCs can migrate towards the site of injury, this was not demonstrated with the IM-delivery route, except if the injury site was local [245]. MSCs transplanted in the skeletal
muscle are shown to reside locally and secrete trophic factors that enter systemic circulation. Upon loss of the IM-delivered MSCs from skeletal muscle, either a small (1.5%) portion was found in the liver [274], or none was observed in any organs other than the muscle site where transplanted [81, 220, 248-250, 275, 277]. Furthermore, it has been shown that if the needle accidently punctures a major blood vessel, then the IM-delivered MSCs rapidly enter the circulation and transfer into distal organs. This could cause a problem more specifically in small animals i.e., mice that exhibit small size muscles.

2.4.5 Concluding Remarks

The studies reviewed collectively support the notion of broadening the applicability of IM-delivery route from a local therapy to the treatment of systemic disease. Multiple studies have shown that IM-delivered MSCs safely reside in-situ for an extended dwell-time and are secretorily active. Current assessment of the fate of MSCs post IM-delivery is largely limited to conditions where MSCs are transplanted in an injured site consisting of a significant amount of inflammation. This is a concern, since local injury environmental cues are shown to both impair MSC viability and functionality while driving phenotypic change and lineage differentiation.

This raises many questions, of which the following are key examples: What degree of inflammation primes MSCs without affecting their viability and engraftment? What is the degree of inflammation in which MSCs can survive and still exert an immunomodulatory response? and What is the timeframe for a change in MSC phenotype? Answers to these questions are vital in determining the dose of a particular MSC population, and the frequency of their IM-delivery to optimize therapeutic performance.
Table 2-1 Few examples of clinical studies of intramuscular-MSC therapy

<table>
<thead>
<tr>
<th>Disease</th>
<th>MSC Source</th>
<th>Delivery Site</th>
<th>Cell Dose</th>
<th>Single/Multiple</th>
<th>Outcome</th>
<th>Complications</th>
<th>Follow-up Period</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLI</td>
<td>Allogenic BMMSCS</td>
<td>Gastrocnemius</td>
<td>2×10^6 cells/kg</td>
<td>40-60 sites at proximity</td>
<td>Improvement in clinical scores (rest pain scores and ankle pressure)</td>
<td>Safety without occurrence of edema at the site of injury</td>
<td>24 w</td>
<td>[233]</td>
</tr>
<tr>
<td>PAD + CLI</td>
<td>Autologous ADMSCs</td>
<td>Internal and External gastrocnemius and anterior compartment of the ischemic leg</td>
<td>1×10^8</td>
<td>15 sites in each muscle</td>
<td>Clinical signs such as leg pain, ulcer size, and pain-free walking was reported as significantly improved.</td>
<td>No sign of edema, or necrosis at the site of injury</td>
<td>24 w</td>
<td>[234]</td>
</tr>
<tr>
<td>ALS</td>
<td>Autologous BMMSC-NTF treated</td>
<td>Biceps and Triceps</td>
<td>24 × 10^6</td>
<td>24 sites at proximity</td>
<td>Improvement in the CAMP amplitude</td>
<td>Slight edema at the site of transplantation. No infection. No tumor formation</td>
<td>24 w</td>
<td>[239]</td>
</tr>
</tbody>
</table>
**Table 2-2** Intramuscular-MSC therapies to treat local pathologies

<table>
<thead>
<tr>
<th>Disease Model</th>
<th>Species</th>
<th>L.S.</th>
<th>MSC Source</th>
<th>Delivery Site</th>
<th>Cell Dose*</th>
<th>Single/ Multiple</th>
<th>Engraftment &amp; Differentiation</th>
<th>Systemic Effect</th>
<th>Bio- distribution</th>
<th>Method of assessment of biodistribution/ engraftment/ differentiation</th>
<th>Study Length</th>
<th>IM-dwell time</th>
<th>Comments &amp; General outcome</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPN-STZ induced</td>
<td>SD-Rat</td>
<td>N/</td>
<td>rBMMSCs</td>
<td>Thigh and Soleus</td>
<td>1</td>
<td>Single, unilateral</td>
<td>Engraftment in the gaps between fibers, no differentiation</td>
<td>Not assessed</td>
<td>Transplanted muscle</td>
<td>Muscle histology, PKH26&lt;sup&gt;+&lt;/sup&gt;</td>
<td>4 w</td>
<td>4 w</td>
<td>↑bFGF, ↑VEGF, Amelioration of diabetes complications</td>
<td>[243]</td>
</tr>
<tr>
<td>DPN-STZ induced</td>
<td>balbC Mice</td>
<td>N/A</td>
<td>mBMMSCs</td>
<td>Along the sciatic nerve</td>
<td>1</td>
<td>Single, 4 sites, bilateral</td>
<td>Engraftment, did not assessed differentiation</td>
<td>Not assessed</td>
<td>Transplanted muscle</td>
<td>Functional loss of improvement</td>
<td>4 w</td>
<td>&lt;4 w</td>
<td>↑NGF, ↑NT-3, Improvement in sciatic motor nerve conduction</td>
<td>[244]</td>
</tr>
<tr>
<td>DPN-STZ induced</td>
<td>Wistar-Rats</td>
<td>N/A</td>
<td>rBMMSCs</td>
<td>Thigh near sciatic nerve</td>
<td>5</td>
<td>Single, bilateral</td>
<td>Engraftment to sciatic nerve, no differentiation, assessed with Lectin after 4-8 w</td>
<td>Not assessed</td>
<td>Transplanted muscle, along vasa nervosa</td>
<td>Muscle histology, Dil-MSCs</td>
<td>8 w</td>
<td>&gt;8 w</td>
<td>Upregulation of factors involved in angiogenesis, neural function, and myelination</td>
<td>[245]</td>
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<tr>
<td>ALS + local IM injury (Cardiotoxin-induced)</td>
<td>SD- SOD&lt;sub&gt;G93A&lt;/sub&gt; Rats</td>
<td>Cyclo</td>
<td>hBMMSC-GDNF</td>
<td>Tibialis anterior, forelimb triceps brachii, long muscles of the dorsal trunk muscle</td>
<td>0.12</td>
<td>Multiple:3 injections 1 w apart, bilateral</td>
<td>Engraftment and differentiation. Expression of human myosin heavy chain IIx/d(MyHC-IIx/d) gene in rat muscle</td>
<td>Not assessed</td>
<td>Transplanted sites</td>
<td>Histology and RT-PCR (hcDNA)</td>
<td>8 w</td>
<td>8 w</td>
<td>Amelioration of motor neuron loss locally and within the spinal cord which connected to the limb muscles with transplants</td>
<td>[247]</td>
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<tr>
<td>Limb Ischemia</td>
<td>MHC mismatched ACI Rats</td>
<td>N/A</td>
<td>rBMMSCs or rFM</td>
<td>Ischemic thigh muscle</td>
<td>5</td>
<td>Single, 5 sites, unilateral</td>
<td>Engraftment but not differentiation (assessed with Lectin after 1 week)</td>
<td>Not assessed</td>
<td>Transplanted site</td>
<td>Histology, GFP+ MSCs</td>
<td>3 w</td>
<td>3 w</td>
<td>Improvement of blood perfusion and capillary density</td>
<td>[261]</td>
</tr>
<tr>
<td>Limb Ischemia</td>
<td>Cell Type</td>
<td>N/A</td>
<td>mADMSCs</td>
<td>Ischemic Muscle</td>
<td>N/A</td>
<td>N/A</td>
<td>Transplanted Site</td>
<td>Vital Signs</td>
<td>Histology</td>
<td>Improvement</td>
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<td></td>
</tr>
<tr>
<td>C57BL/6 mice</td>
<td>N/A</td>
<td>mADMSCs</td>
<td>Ischemic adductor muscle</td>
<td>N/A</td>
<td>1</td>
<td>Single, unilateral</td>
<td>Engraftment, did not assess differentiation</td>
<td>Not assessed</td>
<td>Transplanted site</td>
<td>Bioluminescence imaging</td>
<td>4 w</td>
<td>4 w</td>
<td>Improvement of vascular density and perfusion rate</td>
<td></td>
</tr>
<tr>
<td>balbC mice</td>
<td>N/A</td>
<td>hPLX-PAD</td>
<td>Thigh musculatures</td>
<td>N/A</td>
<td>1</td>
<td>Single, unilateral</td>
<td>Engraftment, did not assess differentiation</td>
<td>Not assessed</td>
<td>Transplanted site</td>
<td>Bioluminescence imaging</td>
<td>3 w</td>
<td>4 d</td>
<td>Improvement of vascular density and blood flow, reduction of endothelial damage</td>
<td></td>
</tr>
<tr>
<td>NOD/SCID mice</td>
<td>N/A</td>
<td>hPDAC</td>
<td>proximal and distant to the ischemic muscle</td>
<td>N/A</td>
<td>1</td>
<td>Single, 2 sites, unilateral</td>
<td>Engraftment, did not assess differentiation</td>
<td>Not assessed</td>
<td>Transplanted site</td>
<td>Bioluminescence imaging</td>
<td>49 d</td>
<td>&lt;8</td>
<td>Improvement of vascular density and blood flow</td>
<td></td>
</tr>
<tr>
<td>NOD/SCID-IL2Rγ−/− (NSG) mice</td>
<td>N/A</td>
<td>hBM MSCs-VEGF</td>
<td>Ischemic hamstring</td>
<td>N/A</td>
<td>0.5</td>
<td>Single, 2 sites, unilateral</td>
<td>Engraftment, did not assess differentiation</td>
<td>Not assessed</td>
<td>Transplanted site</td>
<td>Bioluminescence imaging and PCR (hgDNA)</td>
<td>24 w</td>
<td>4 w</td>
<td>Improvement of vascular density and blood flow</td>
<td></td>
</tr>
<tr>
<td>Lewis Rats</td>
<td>N/A</td>
<td>rBM MSCs</td>
<td>Anteromedial ischemic muscle</td>
<td>N/A</td>
<td>5</td>
<td>Single, unilateral</td>
<td>Engraftment and differentiation to endothelial cells that expressed (F VIII), also differentiation to skeletal muscle fibers (expression of desmin) and adipocytes</td>
<td>Not assessed</td>
<td>Transplanted site</td>
<td>Histology LacZ+ MSCs, X-gal identification</td>
<td>3 w</td>
<td>3 w</td>
<td>Improvement of vascular and arteriolar density, regeneration</td>
<td></td>
</tr>
<tr>
<td>BalbC</td>
<td>N/A</td>
<td>mBM MSCs</td>
<td>Adductor muscle proximal to the site of injury</td>
<td>N/A</td>
<td>1</td>
<td>Single, 6 sites, unilateral</td>
<td>Engraftment. B-gal+ cells found distributed between muscle fibers without incorporation into the vessels, therefore no</td>
<td>Not assessed</td>
<td>Transplanted site</td>
<td>Histology (GFP+, β-gal+)</td>
<td>17 d</td>
<td>17 d</td>
<td>Improvement in perfusion of ischemic tissue and collateral modeling. Attenuation of muscle atrophy and fibrosis</td>
<td></td>
</tr>
<tr>
<td>Condition</td>
<td>Species</td>
<td>Source</td>
<td>Cells Type</td>
<td>Engraftment</td>
<td>Location</td>
<td>Method</td>
<td>Time 1</td>
<td>Time 2</td>
<td>Result</td>
<td></td>
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</tr>
<tr>
<td>Limb Ischemia</td>
<td>Lewis Rats</td>
<td>N/A</td>
<td>rBMMSCs</td>
<td>Single, unilateral</td>
<td>Transplanted site</td>
<td>Histology PKH 26</td>
<td>3 w</td>
<td>3 w</td>
<td>Improvement of vascular density and blood flow</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Ischemic thigh muscle</td>
<td></td>
<td>Engraftment, and some transplanted MSC were positive for VWF, an endothelial marker</td>
<td>Not assessed</td>
<td></td>
<td></td>
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<tr>
<td>Muscle Dystrophy-CTX (Latoxan)-induced</td>
<td>NMRI nu-/- mice</td>
<td>N/A</td>
<td>hSM-MSCs</td>
<td>Single, unilateral</td>
<td>Transplanted site</td>
<td>In situ hybridization for human Alu repeats and PCR (hDNA)</td>
<td>24 w</td>
<td>4 w, Small quantity 4 w</td>
<td>Transplanted MSCs differentiated and contributed to myofibrils and satellite cells but did not fuse with murine cells</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Injured muscle</td>
<td></td>
<td>Engraftment and detection of human β2M and human dystrophin and MyHC-IIx/d in injured muscle</td>
<td>Not assessed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease Model</td>
<td>Species model</td>
<td>L.S.*</td>
<td>MSC Source</td>
<td>Delivery Site</td>
<td>Cell Dose**</td>
<td>Single/ Multiple</td>
<td>Engraftment &amp; Differentiation</td>
<td>Systemic Effect</td>
<td>Bio-distribution</td>
<td>Method of assessment of biodistribution / engraftment/ differentiation</td>
<td>Study Length</td>
<td>IM-dwell time</td>
<td>Comments &amp; General outcome</td>
<td>Ref.</td>
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</tr>
<tr>
<td>Dilated cardiomyopathy</td>
<td>TO2 hamster</td>
<td>N/A</td>
<td>pBMMSCs</td>
<td>Intact hamstrings</td>
<td>0.25, 1, 4</td>
<td>Multiple, 2 injections, 2 w apart, bilateral</td>
<td>Not assessed at the IM transplantation site</td>
<td>↑ LIF and GM-CSF, ↓ cTnl</td>
<td>Not assessed</td>
<td>Functional effect</td>
<td>4 w</td>
<td>4 w</td>
<td>Mobilization of progenitor cells to the heart. Significant cardiac output improvement and higher MSC dose resulted in higher functional improvement.</td>
<td>[223]</td>
</tr>
<tr>
<td>Dilated cardiomyopathy</td>
<td>Bio-TO2 hamster</td>
<td>N/A</td>
<td>hBMMSCs, pBMMSCs</td>
<td>Intact hamstrings</td>
<td>2-4</td>
<td>Single, bilateral</td>
<td>Not assessed at the IM transplantation site</td>
<td>VEGF</td>
<td>Not assessed</td>
<td>Functional effect</td>
<td>4 w</td>
<td>4 w</td>
<td>Improved cardiac output. hBMMSCs express less VEGF compared with pBMMSCs</td>
<td>[224]</td>
</tr>
<tr>
<td>Dilated cardiomyopathy- doxorubicin-induced</td>
<td>SD-rats</td>
<td>N/A</td>
<td>hUC-WJMSCs</td>
<td>fore- and hind-limb skeletal muscles</td>
<td>0.25, 1</td>
<td>Multiple, 2 sites, 2 injections, and 2 weeks apart, bilateral</td>
<td>Not assessed at the IM transplantation site</td>
<td>↓ BNP and cTnl, ↑ LIF, HGF, GM-CSF, and VEGF</td>
<td>Not assessed</td>
<td>Functional effect</td>
<td>4 w</td>
<td>4 w</td>
<td>Improved cardiac output, MSC-dose independent</td>
<td>[225]</td>
</tr>
<tr>
<td>RA-antibody/LPS induced</td>
<td>BalbC/SC ID mice</td>
<td>N/A</td>
<td>hBMMSCs, hBMMSCS- hsTNFR</td>
<td>Thigh quadriceps</td>
<td>2</td>
<td>Single, bilateral</td>
<td>Not assessed at the IM transplantation site</td>
<td>hsTNFR</td>
<td>Not assessed</td>
<td>Functional effect</td>
<td>27 d</td>
<td>27 d</td>
<td>Prophylactic concept, only hsTNFR transduced hBMMSCs protected joints from inflammation, 20 d of hsTNFR detection in circulation</td>
<td>[228]</td>
</tr>
<tr>
<td>Focal paw inflammation, induced by γ-carrageenan</td>
<td>CD1 mice</td>
<td>N/A</td>
<td>HUCPVCs, hBMMSCs, mBMMSCs</td>
<td>Contralateral to the injured limb, quadriceps muscle</td>
<td>1.3</td>
<td>Single, unilateral</td>
<td>Engraftment, did not assess differentiation</td>
<td>Reduced TNF-α, increased TSG-6 (indirect measurement)</td>
<td>Transplanted site, no distribution to organs</td>
<td>Histology, RT-PCR (hgDNA),</td>
<td>48 h</td>
<td>48 h</td>
<td>Reduction of inflammation in the paw, reduction of neutrophil infiltration, HUCPVCs and hBMMSCs performed better than mBMMSCs, HUCPVCs showed earliest response</td>
<td>[81]</td>
</tr>
<tr>
<td>Study Description</td>
<td>Animal Model</td>
<td>Protocol</td>
<td>Treatment</td>
<td>Time Points</td>
<td>Outcome Measures</td>
<td>Time</td>
<td>Therapeutic Theme</td>
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<tr>
<td>Systemic virus infection-induced with VEEV</td>
<td>balbCnu/nu</td>
<td>N/A</td>
<td>Thigh skeletal muscle</td>
<td>2.5</td>
<td>Single, bilateral</td>
<td>123 d</td>
<td>Prophylactic concept, protection against exposure to a VEEV</td>
<td></td>
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<tr>
<td></td>
<td>HUCPVC5-anti-VEEV</td>
<td></td>
<td></td>
<td></td>
<td>Engraftment, did not assess differentiation</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Anti-VEEV IgG</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Transplanted site</td>
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<td></td>
<td></td>
<td></td>
<td>Secreted IgG, bioluminescence imaging</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>109 d</td>
<td>Bioluminescence, Systemic IgG secretion</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Disease Model</td>
<td>Species model</td>
<td>L.S.*</td>
<td>MSC Source</td>
<td>Delivery Site</td>
<td>Cell Dose **</td>
<td>Single/ Multiple</td>
<td>Engraftment &amp; Differentiation</td>
<td>Systemic Effect</td>
<td>Bio-distribution</td>
<td>Method of assessment of biodistribution/ engraftment/ differentiation</td>
<td>Study Length</td>
<td>IM-dwell time</td>
<td>Comments &amp; General outcome</td>
<td>Ref.</td>
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</tr>
<tr>
<td>Healthy</td>
<td>balbCnu/nu mice</td>
<td>N/A</td>
<td>HUCPVCs, hBMMSCs</td>
<td>Thigh skeletal muscle</td>
<td>1 Single, unilateral</td>
<td>Engraftment, did not assess differentiation</td>
<td>Not assessed</td>
<td>Transplanted site</td>
<td>Histology, bioluminescence</td>
<td>5 m</td>
<td>5 m</td>
<td>IM: 5 m dwell-time and no distribution to organs.</td>
<td>[220]</td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>NOD/SCI D mice</td>
<td>N/A</td>
<td>hBMMSCs-hEPO</td>
<td>Quadriceps muscle</td>
<td>1 Single, bilateral</td>
<td>Engraftment, did not assess differentiation</td>
<td>Increased human EPO and hematocrit level</td>
<td>Not assessed</td>
<td>Secreted hEPO</td>
<td>4 w 4 w</td>
<td>Non-human primate MSCs can also be engineered to deliver biological products.</td>
<td>[253]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>balbCnu/nu mice</td>
<td>N/A</td>
<td>hADMSCs</td>
<td>Thigh muscles</td>
<td>1 Single, bilateral</td>
<td>Engraftment, did not assess differentiation</td>
<td>Not assessed</td>
<td>Transplanted site and liver</td>
<td>Histology, bioluminescence imaging, genomic hybridization (hgDNA)</td>
<td>8 m</td>
<td>8 m</td>
<td>Majority of transplanted cells disappeared within 1 w, 10% remained in the muscle through 8 m</td>
<td>[274]</td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>nu/nu Foxn1 mice</td>
<td>N/A</td>
<td>hBMMSCs</td>
<td>Hind limb skeletal muscles</td>
<td>1.5 Single, bilateral</td>
<td>Engraftment, did not assess differentiation</td>
<td>Not assessed</td>
<td>Transplanted site</td>
<td>MRI</td>
<td>26 d 26 d</td>
<td>Significant reduction in signal between 3 to 12 d</td>
<td>[275]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>Merino-cross sheep</td>
<td>N/A</td>
<td>sBMMSCs</td>
<td>Gastrocnemius muscle</td>
<td>2 Single, unilateral</td>
<td>Engraftment, did not assess differentiation</td>
<td>Not assessed</td>
<td>Transplanted site</td>
<td>Histology</td>
<td>6 w 6 w</td>
<td>CM-DiI labelled sBMMSC into skeletal muscle showed dye retention for 6 w</td>
<td>[263]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>NOD/SCI D mice</td>
<td>N/A</td>
<td>PLX-PAD</td>
<td>Thigh muscle</td>
<td>1 Single, 2 sites, unilateral</td>
<td>Engraftment, did not assess differentiation</td>
<td>Not assessed</td>
<td>Transplanted site</td>
<td>Histology, RT-qPCR (hgDNA)</td>
<td>3 m</td>
<td>3 m</td>
<td>80% of cells lost within 1 m. Neither single nor multiple caused toxicity</td>
<td>[276]</td>
<td></td>
</tr>
</tbody>
</table>

* L.S. (Immuno-suppressant); ** cell dose (x10⁶); r (rat); m (mouse); h (human); p (porcine); s (sheep); b (baboon); d (day); w (week); m (month); LIF (Leukemia inhibitory factor); GM-CSF (Granulocyte-macrophage colony-stimulating factor); cTn1 (cardiac troponin 1); HGF (Hepatocyte growth factor); VEGF (Vascular endothelial growth factor); bFGF (basic Fibroblast growth factor); NGF (nerve growth factor); NT-3 (Neurotrophin-3); GFP (Green fluorescent protein); gDNA (genomic DNA); cDNA (complementary DNA); RT-qPCR (Reverse transcription polymerase chain reaction)
Chapter 3: Preliminary Assessment of Animal Models of Inflammation and Cell Tracking Methods

3.1 Animal models

First, we assessed the feasibility of undertaking our fundamental studies on two animal models of inflammation: (i) a spontaneous model of OA with low-grade chronic inflammation, and (ii) a carrageenan hind paw model of acute inflammation.

3.1.1 Osteoarthritis and Dunkin Hartley guinea pig model of osteoarthritis

Osteoarthritis (OA) is a chronic, degenerative disease that leads to deterioration of the articular cartilage and breakdown of the subchondral bone. Articular cartilage is a specialized tissue that distributes load across the joint and provides a low-friction articulating surface for the synovial joints. Its mechanical properties are determined by its matrix and fluid composition that is kept at homeostasis by the chondrocytes. However, in the case of traumatic injury, since cartilage matrix is devoid of blood vessels, lymphatics, and nerves, chondrocytes have limited capacity to intrinsically repair the matrix. The proinflammatory environment and catabolic signaling imbalance in an OA affected joint, further stimulate the chondrocytes to secrete matrix degrading enzymes that results in progressive tissue loss. The number of endogenous MSCs in OA affected joints, have shown to be increased with progression of the disease [285]. Once those MSCs from patients with end-stage OA have been isolated and cultured in-vitro, they have shown reduced proliferation and differentiation abilities [285]. Also, the synovial fluid extracted from OA joints in late stages, is shown to inhibit the chondrogenic differentiation ability of MSCs [286]. This suggests loss of capacity of the cartilage chondrocytes and endogenous MSCs, within the joint.
tissue, to maintain/regain homeostasis in such a catabolic and inflammatory condition. Stem cell-based therapies by taking advantage of the immunomodulatory function of mesenchymal stem cells (MSCs), have empowered the prospect of using exogenously transplanted MSCs for the repair of cartilage and/or slowing the progression of OA.

The OA animal models available resemble either the secondary (induced) or the primary (spontaneous) type of OA. However, it is important to take into consideration that animal models do not fully resemble the mechanical and biochemical pathology of human disease. Specifically, in OA that the load bearing joints are more affected, most animals are quadrupeds and some of the load is distributed on their front legs. Besides the spontaneous animal models of OA, the other models are either genetically modified, or OA-induced (surgically/ enzymatically/chemically induced) [287]. The spontaneous models of OA are mice (DBA/1, STR/ORT, C57BL/6j, C57), guinea pig (Dunkin-Hartley), Macaque (Rhesus, Cynomolgus), and horse [288]. Use of non-human primates is limited due to the number of primates needed for statistically meaningful studies and also consistently of reproducing lesion severity in the animals under study [287]. Within the small order animals that develop spontaneous OA, we chose Dunkin Hartley Guinea Pigs.

Dunkin Hartley guinea pigs (DHGp) represent a spontaneous OA model with biomechanical and biochemical onset at about 3-4 months of age with low grade inflammation at early time points that slightly increases at the late stages of cartilage degradation around 12-18 months [289]. Lesions appear on the medial side of the knee joint, bilaterally, due to the varus alignment of its knee anatomy. The mal-alignment increases load on the medial compartment that is similar to the primary osteoarthritic human joints [290]. This model represents a naturally obese OA model. We aimed to investigations whether IM-delivered MSCs can shift the intra-articular inflammatory state in mid-late OA towards anti-inflammatory/reparative state that can delay
further degeneration of the knee joint. Our initial assessment of the pathology of the knee joints are shown in Fig. 3-1. To confirm the development and progression of OA in the DHGps, and to assess the degree of variation between the lesions developed in each Gp as an indication of the variation in the degree of inflammation, the right knee joint of Gps aged 1.5 to 10-month-old were isolated for histological assessments. Gp knee joints were fixed, decalcified, dehydrated, and embedded in paraffin. The right knee joints of Gps were then serial sectioned in transverse plane to investigate any signs of cartilage fibrillation in the medial and lateral compartment of the knee joint. Sections (5μm) were stained with Safranin O and counter stained with Fast green for evaluation of OA signs and proteoglycan (PG) loss at the sites of cartilage fibrillation. Fig. 3-1A and C illustrate that we observed a smooth surface of intact cartilage from 1.5-month Gp knee joint with no signs of OA. Furthermore, progression of OA over time was seen (Fig. 3-2 B, D, and E) with extensive fibrillation and PG loss. In addition, chondrocyte cloning extended into the middle zone and (loss of Safranin O staining indicates changes in the PG synthesis). The radiographs in Fig. 3-1F shows further development and progression of OA that is also evident in the lateral-mediolateral radiographs up to 35 months. The variation in the severity of the developed OA between same age/weight Gps, resulting in a variation in the degree of inflammation in their corresponding joints created initial concerns about the reproducibility of OA in such animal model. In addition, the relatively old age of the Gps at the desired level of OA also led to other physiological complications (e.g., kidney stone, sudden heart attack, and liver disease), of which a few Gps died during our preliminary studies. The mentioned complications, disqualified this animal model for our fundamental studies that required an animal model with a controlled and reproducible degree of inflammation. However, it should be noted that DHGp model of spontaneous OA is the closest model to the spontaneous OA (in the small order animals) that develops in humans and maybe suitable for studies of multiple injury sites (joints). Although we
subsequently switched to the carrageenan model of inflammation, we radiographically assessed the time-course and the degree of degeneration of OA in DHGPs joints up to 35 months (Fig. 3-1F and Appendix I-Fig. A1-1). Our assessment of humroradial, glenohumeral, and femoroacetabular joints, as well as lumbar facet joints of forelimb hind paw (Appendix I-Fig. A1-1) indicated that the knee joints are the only two joints that show subchondral degeneration and severe signs of OA in DHGPs.
Figure 3-1 Cartilage fibrillation, loss of chondrocytes, and cellularity in OA (A & C) at 1.5 months; (B and D) 7 months; and (E) 10 months of age. Histopathological features seen include fibrillation and proteoglycan loss. In addition, chondrocyte cloning extended into the middle zone and the loss of Safranin O staining indicates changes in proteoglycan synthesis. (F) Changes in the distal end of femur and proximal end of tibia, as well as joint spaces in the 33-35 months old Gp lateral-mediolateral radiographs is indicative of the severity of OA when compared with the knee joints of 12-14th month old Gps.

The carrageenan-induced hind-paw model is a well-characterized and reproducible model of inflammation. Carrageenan (lambda) is a polysaccharide that does not gel strongly at room temperature and is routinely used to induce an acute inflammatory response (please see Chapter
Induction of inflammation by carrageenan is characterized by edema, hyperalgesia, and erythema. Fig. 3-2 [186] demonstrates a biphasic response with a primary peak developed between 4-6h and a secondary peak at around 48-72h [206].

3.1.2 Carrageenan-induced Paw Model of Inflammation

In our preliminary studies, we assessed the reproducibility of induction of inflammation in the sub-plantar area of immunocompetent CD1 mice hind paw using 1% carrageenan (Fig. 3-3) and optimized our method of induction to attain maximum reproducibility, for 48h IM-transplantation studies.

**Figure 3-2** Biphasic pattern of carrageenan-induced edema in mice. An early inflammatory peak is shown to develop between 4-6h post-carrageenan injection. Adopted from Posadas et al., 2004 [186].
Figure 3-3 Qualitative assessment of the degree of edema developed in carrageenan hind-paw model of inflammation in mice confirms reproducibility. Here we show 3 animals, but sample size analysis showed the minimum requirement of 5 mice/study group.

3.2 MSC labelling for \textit{in-vivo} biodistribution studies

To investigate the dwell-time and biodistribution of MSCs post-IM transplantation, it was important to use a labeling technique with a high tissue penetration depth that is typically achieved by near infrared (NIR) emission wavelengths. In addition, the cell-tracking technique was required to have high quantum yield, low cytotoxicity, high photostability, high efficiency, and low exocytosis. However, it is impossible to find a single cell-tracking probe that simultaneously exhibits all the afore-mentioned characteristics. In an effort to find the most optimum labeling technique, we assessed four labeling methods: (\textit{i}) colloidal semiconductor nanocrystals known as quantum dots (QDs), (\textit{ii}) far-red fluorescent protein (E2-Crimson), (\textit{iii}) far-red lipophilic heptamethine carbocyanine (DiR), and (\textit{iv}) reporter-gene, a bright luciferase (Gaussia). The optical penetration depth through a muscle tissue for different wavelengths is shown in table 3-1 [291].

\begin{table}
\centering
\caption{Optical penetration depth for muscle tissue (postmortem) at different emission wavelengths. N=10; *Standard Error [291]}
\begin{tabular}{ll}
\hline
(\text{nm}) & (\text{mm}) \\
\hline
\(\lambda_{\text{em}}= 632.8\) & 1.47\(\pm\)0.1\
\hline
\(\lambda_{\text{em}}= 675\) & 1.63\(\pm\)0.1\
\hline
\(\lambda_{\text{em}}= 780\) & 3.46\(\pm\)0.23\
\hline
\(\lambda_{\text{em}}= 835\) & 3.72\(\pm\)0.29\
\hline
\end{tabular}
\end{table}
3.2.1 Gold Quantum Dot Hybrids

Quantum dots are known for their narrow emission band-gap (*i.e.*, full-width at half maximum <30 nm), high quantum yield, photostability, and resistance to chemical and metabolic degradation. Major limitations of using QDs for cell tracking include the size of QDs, cytotoxicity, and the blinking effect. To further evaluate the feasibility of using a QD-based labeling technique in our studies, we utilized a previously developed method of gold-QD hybrids (GQHs) [292], and prepared GQH samples for our preliminary cell labeling studies. Following the reported method by Song *et al.* and with his help in the laboratory of Professor Warren Chan, we prepared two batches of GQHs using two different sizes of gold nanoparticles (GNPs), 60 nm and 80 nm (Cytodiagnostics, Cat. CG-60 and CG-80, respectively), as cores of the GQHs to increase brightness of QDs and suppress the unwanted blinking effect. Using a layer-by-layer polymer deposition technique, we coated alternating layers of poly(styrene)-block-poly(acrylic acid) (PS-b-PAA) (negatively charged) (Polymer source Inc., Cat. P4873A-SAA) or Poly(allylamine) (PAH) (positively charged) (Polysciences Inc., Cat. 24826) with the aim of increasing the thickness of polymer layers roughly to 11 nm, reported to be the optimized polymeric shell thickness around the GNPs. We then assembled core/shell CdSe/ZnS QDs (diameter=6.5 nm; $\lambda_{\text{em}}$=665nm) (Cytodiagnostics, Cat. Fn-665) around the GQHs with a final coat of PAH to confer a final positive hybrid charge (Fig. 3-4A). CdSe cores were coated with a ZnS shell to increase the quantum yield of the bare QDs and reduce cytotoxicity. Uniform coating of the polymers around the GNPs were confirmed with transmission electron microscopy (TEM), shown in Fig. 3-4B. Successful assembly of the QDs around the GNP-polymer structure was also confirmed with TEM (Fig. 3-4C). To further confirm the presence of QDs, Fig. 3-4D shows the lattice fringes of one single QD embedded in the polymer coating. Various concentrations of GQHs were incubated with HUCPVCs for 48h in a chemically defined media (Lonza, Cat. 00192125) without the presence of
Fetal Bovine Serum (FBS). Positively charged GQHs were endocyto sed by the cells (Fig. 3-5A). We enzymatically detached HUCPVCs 48h post-incubation with GQHs for further assessments. The detached cells were pelleted and fixed, dehydrated, stained, resin embedded, and sectioned using ultramicrotome for intracellular visualization (TEM) of the endocytosed GQHs.

Figure 3-4 Gold-quantum dot hybrids (GQHs) assembly. (A) step by step assembly of GQHs involves gold nanoparticle (GNP) surface modification with alternating layers of PS-b-PAA and PAH (i-ii), then assembly of QDs around the coated layer (iii) with a further coating of PAH; (B) uniform coating of polymers around the GNP; (C) confirmation of assembly of QDs around the coated GNP; (D) confirmation of presence of QD by showing the lattice fringes (black arrow).
Higher magnification images confirmed the stability of the GQHs post 48h (Fig. 3-5B and C). Although, some of the GQHs were successfully endocytosed by the HUCPVCs, but after 24h of culture period, significant agglomeration and/aggregation of GQHs was observed in the culture media. Although effect of proteins in the culture media on the agglomeration/aggregation of nanoparticles have been reported in the literature [293, 294], but none of these studies were conducted with MSCs in serum-free media, as we have and yet we obtained similar results.

Therefore, various factors have made it challenging to achieve high efficiency labeling of MSCs with this method beyond 24h of culture time. These factors include the batch-to-batch size variation of the commercially available QDs and GNPs, possible cytotoxicity of QDs, the critical size-dependency of endocytosis-exocytosis, and aggregation of GQHs that affects significantly the cells uptake.
Figure 3-5 Gold-quantum dot hybrids (GQHs) endocytosis by HUCPVCs. (A) cross-section of a cell that has endocytosed multiple GQHs; (B) intracellular confirmation of a GQH in a vesicle; (C) confirmation of intact GQHs post endocytosis (black arrows point to the QDs attached around the polymer coated GNP.)
3.2.2 Far-red fluorescent protein (E2-Crimson)

E2-Crimson with an emission wavelength, $\lambda_{em}=646$ nm, and an excitation wavelength, $\lambda_{ex}=611$ nm, is a NIR fluorescent protein. E2-Crimson is derived from tetrameric red fluorescent protein Discosoma red (DsRed)-Express2 [295, 296]. E2-Crimson is highly photostable and has reduced cytotoxicity and phototoxicity which makes it a suitable choice for in-vivo cell tracking. In order to avoid genetic manipulation, HUCPVCs were electroporated to insert E2-Crimson vectors (Fig. 3-6A). 21 conditions were tested for optimization of the electroporation. We tested two different conditions of HUCPVCs; one batch was recovered post-cryopreservation for a period of 48h in culture prior to electroporation and another batch was directly thawed from cryopreserved vials, washed, and electroporated with pCMV-E2 vectors at 48μg DNA plasmid/1x10^5cell density (recommended for cell electroporation in suspension for seeding in 24-well plate) using a Neon transfection system kit (ThermoFisher Scientific, Cat. MPK1025). Both HUCPVC groups were re-seeded post-electroporation for recovery and imaging. The batch of HUCPVCs that was not recovered prior to electroporation resulted in significant reduction of viability and impaired adherence to culture plastic (~15%) post-electroporation. Through our screening and optimization studies, the best parameters for optimum electroporation of HUCPVCs with E2-Crimson were found to be 2 pulses (C13) of 1100 volts with 20 ms pulse width, which resulted in ~68% efficiency (Fig. 3-6B). Doubling the amount of the plasmid to 96μg (C13P) resulted in reduced mean percentage efficiency (~52%) that was not statistically significant compared to C13 (Fig. 3-6C). The second-best choice was 1 pulse (C16) of 1100 volts with 30ms pulse width, which resulted in ~58% efficiency. Doubling the amount of the plasmid to 96μg (C6P) also resulted in reduced mean percentage efficiency (~48%) that was not statistically significant compared to C6 (Fig. 3-6C). Statistical analysis of groups was performed by means of One-Way ANOVA followed by post hoc Tukey statistical test between the means of replicates at significance level of 95% CI. To assess
the fluorescence signal generated from E2-Crimson-HUCPVCs with Xenogen-IVIS system, we plated the cells at 1, 2.5, and 5 ($\times 10^5$) in a 24 well-plate and changed the medium to water prior to imaging. As shown in Fig. 3-6D, the obtained radiant efficiencies were 2.36, 3.93, and 5.49 ($\times 10^9$) (p/sec/cm$^2$/sr)/(µW/cm$^2$), respectively (Fig. 3-6D). Although, a strong signal was obtained from the E2-Crimson-labelled HUCPVCs in the culture dish, but IM-delivered E2-Crimson-HUCPVCs ($1\times 10^6$) in the dorsal thigh musculature of hind limb were not detectable with epi-illumination in dorsal position (Fig. 3-6E) and weakly detectable in the ventral position of the animals.

The emission wavelength of E2-Crimson is in the far-red range and therefore it was expected to show moderate penetration depth in the 5-8mm thickness of mouse hind limb muscle tissues. But, the signal obtained from $1\times 10^6$ FACs sorted HUCPVCs was not strong enough for our biodistribution studies that maybe, in part, due to the low penetration depth. Therefore, in the next step, we assessed the efficiency and penetration depth of DiR (membrane dye) that is considered to be the closest to IR range among other lipophilic tracers.
Figure 3-6 Electroporated E2-Crimson HUCPVCs. (A) pCMV map of E2-crimson vector used for electroporation of HUCPVCs; (B) healthy morphology of electroporated HUCPVCs with Hoechst nucleus counterstain; (C) Efficiency of HUCPVCs post-electroporation, calculated from 5 areas of n=3 wells/condition, p≥0.05, C6P and C13P were electroporated with double the plasmid concentration; (D) fluorescence flux (radiant efficiency) generated by various cell densities detected with Xenogen-IVIS system; (E) *In-vivo* flux of fluorescence from IM-transplanted MSCs, as a measure of penetration depth of the fluorochrome that was not detected in dorsal position and dimly detected in ventral position.

3.2.3 Far-red lipophilic heptamethine carbocyanine (DiR)

DiIC_{18}(7) 1,1'-Dioctadecyl-3,3',3'-Tetramethylindotricarbocyanine Iodide (DiR), \( \lambda_{ex}=750 \) and \( \lambda_{em}=780 \), is the farthest red fluorescent dye among the family of lipophilic tracers.
DiR, once in contact with a cell membrane, diffuses laterally within the cytoplasmic lipid bilayer and intracellular plasma membrane. DiR has two 18-carbon long hydrophobic chains that intercalate in the plasma membrane and makes it stable. DiR is reported to be photostable, with no cytotoxicity or dye transfer to the neighboring cells, and is commonly used in short-term in-vivo biodistribution studies [297-300]. For the preliminary DiR studies, seeded HUCPVCs were trypsinized after 48h recovery and washed with 1x phosphate buffered saline (PBS). The washed HUCPVCs were then incubated in fresh media containing 3.5μg/mL DiR (Invitrogen, Cat.D12731) for 19 min at 37°C. HUCPVCs were then washed twice with PBS and re-seeded for recovery and imaging. DiR labeling of HUCPVCs resulted in normal morphology (Fig. 3-7A), high percentage of viability (~94%), and high labeling efficiency (99.5%), measured by flow cytometry (Fig. 3-7B). To assess the fluorescence signal generated from DiR-HUCPVCs with the Xenogen-IVIS system, the cells were seeded at 1, 2.5, and 5 (×10⁵) in a 24 well plate, and the media was changed to water prior to imaging. As shown in Fig. 3-7C, the obtained fluorescence fluxes (radiant efficiency) were 2.99, 8.31, and 14.3 (×10⁹) (p/sec/cm²/sr)/(µW/cm²), respectively (Fig. 3-7C). To ensure maximum cell detection in the muscle, we IM-transplanted 1×10⁶ DiR labeled HUCPVCs in mouse hind limb followed by epi-illumination in the dorsal (Fig. 3-7D) and ventral positions. DiR-HUCPVCs were clearly visualized in both dorsal and ventral positions.
3.2.4 Comparison of fluorescence flux: E2-Crimson and DiR

We computed the fluorescence flux (radiant efficiencies) obtained from different cell densities (in a 2D culture dish) of E2-Crimson and DiR-labeled HUCPVCs (Fig. 3-8). As the results suggest, DiR-HUCPVCs resulted in a higher radiant efficiency compared to E2-Crimson-HUCPVCs. Hence, DiR labeling method was chosen for our 48h in-vivo studies. We further assessed the MSC markers and phenotype changes post-DiR labeling (refer to Chapter 5 for results). Despite the above-mentioned advantages of DiR labeling, they have two limitations: (i) dilution post-proliferation, and (ii) active fluorescence in the lipid bilayer remnants of degenerated cells. We did not expect MSCs to proliferate within our 48h study, but we did expect host immune response to the xenotransplanted MSCs in the immunocompetent animals.
3.2.5 Gaussia Luciferase

In order to assess the fate of the xeno-transplanted MSCs in immunocompetent mice, and to evaluate the systemic secretion activity of the IM-transplanted MSCs, a labeling probe dependent on the cell-metabolism was required to allow detection of viable, secretorily active cells. Various reporters are used for *in-vivo* tracking/biodistribution of cells (Table 2-2). However, Gaussia luciferase (GLuc) is the only reporter with detectable secreted protein. Gaussia luciferase is an ATP-independent luciferase [301-306] derived from marine copepod that extracellularly secretes small size protein (19.9 kDa).
### Table 3-2 Characteristics of Luciferases used for cell tracking

<table>
<thead>
<tr>
<th>Luciferase</th>
<th>Species</th>
<th>MW (kDa)</th>
<th>λ&lt;sub&gt;em&lt;/sub&gt; (nm)</th>
<th>Secreted</th>
<th>Kinetics</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gaussia</strong></td>
<td>Coepod/Gaussia princeps, Metridia pacifica and Metridia longa</td>
<td>19.9</td>
<td>480-600</td>
<td>Yes</td>
<td>Flash</td>
<td>[305-308]</td>
</tr>
<tr>
<td><strong>Firefly</strong></td>
<td>Photoni firefly/Photinus pyralis and Vargula hilgendorfii</td>
<td>62</td>
<td>550-570</td>
<td>No</td>
<td>Glow</td>
<td>[309, 310]</td>
</tr>
<tr>
<td><strong>Renilla</strong></td>
<td>Sea pansy/Renilla reniformis</td>
<td>34</td>
<td>482</td>
<td>No</td>
<td>Flash</td>
<td>[311, 312]</td>
</tr>
<tr>
<td><strong>Bacterial</strong></td>
<td>Photobacterium/Vibrio fischeri</td>
<td>~76</td>
<td>490</td>
<td>No</td>
<td>Glow</td>
<td>[313]</td>
</tr>
</tbody>
</table>

Gaussia luciferase is a suitable reporter for assessment of secretorily active MSCs in-vivo. Hence, we adeno-transfected HUCPVCs to express Gaussia luciferase. A pre-made GLuc (GLuc) recombinant adenovirus was purchased from Vigene Biosciences (Rockville, MD, USA). Please refer to Appendix I, Fig.A1-2 for the vector map. Dr. Catalina Estrada has performed the adeno-transduction of MSCs. HUCPVCs were seeded at a cell density of 2.3×10⁴ cells/cm² and 24h later were exposed to the virus at a multiplicity of infection (MOI) of 100 in 100μl/cm² of culture medium. After 24h of incubation at 37°C and 5% CO₂, cells were washed with PBS three times and fresh growth medium was added. After 3 days, engineered HUCPVCs were harvested. The efficiency of transfection was assessed using intracellular staining of GLuc (Fig. 3-9A). Briefly, HUCPVCs were fixed with 4% paraformaldehyde, washed, and permeabilized with 100% methanol for 30 min at -20°C. Cells were then incubated with 2μL GLuc (rabbit IgG) antibody (New England BioLabs, Cat.E8023S) in antibody diluent (PBS + 0.5% BSA +0.5% Tween 20) for 30 min at 4°C in dark. Cells were then washed and incubated with 0.5 μL goat anti-rabbit IgG Alexa Fluor 546 secondary antibody (Life Technologies, Cat. A-11010) in antibody diluent for 30 min at 4 °C in the dark. Cells were then washed and resuspended in flow buffer for analysis by
flow cytometry. We further assessed the MSC markers and phenotype changes post-modifying the HUCPVCs (see Chapter 5 for results). The morphology of HUCPVCs were normal post-transfection, and the efficiency of transfection was measured to be 85.4% (Fig. 3-9A). To assess the luminescence flux generated from GLuc-HUCPVCs with Xenogen-IVIS system, we pipetted the cells at $1 \times 10^6$, 8.75, 7.5, 6.25, 5, 3.75, 2.5, and 1.25 ($1 \times 10^5$) cell densities in a black clear bottom tissue culture treated 24-well plate (Visiplate) (PerkinElmer. Cat.1450-605). Coelenterazine (Nanolight, Cat. 3031) (300 µg/300 µl sterile ddH2O/well) was added using a multichannel pipette immediately prior to open filter imaging using the Xenogen IVIS system (Fig. 3-9B). The photon flux of GLuc (Fig. 3-9C) is linearly correlated with cell density and it is reported to linearly correlate with the concentration of secreted protein in blood [307]. Although bioluminescence is known to have a low tissue penetration due to the scattering of the emitted photons within tissues, the quantum yield (i.e., high flux intensity) of GLuc has overcome this limitation [314]. $1 \times 10^6$ GLuc-HUCPVCs IM-transplanted in mice (Fig. 3-9D) clearly shows the flux intensity and detection level of Gaussia Luciferase. One of the limitations of GLuc is the flash kinetics and its fast signal decay due to the inactivation (i.e., degradation and auto-oxidation) of substrate coelenterazine [315], as shown in Fig. 3-10. This disadvantage also limited transformation of in-vitro photon flux data of cell densities to our in-vivo 3D images acquired for biodistribution and xenoreactivity studies. The algorithms in the Living Image software® of the IVIS system by PerkinElmer assumes consistent light output which is not applicable with Flash kinetics of Gaussia luciferase.

Coelenterazine binds to serum proteins and thereby has a fast systemic clearance rate [316]. Our kinetic studies (Fig. 3-10) suggested that all animals should be imaged within 1 min post-IV administration of coelenterazine, to produce comparable longitudinal data. To perform the kinetic
study, we IM-transplanted $1 \times 10^6$ GLuc-HUCPVCs, intramuscularly, IV-transplanted 250 μg/100 μl sterile ddH$_2$O of coelenterazine and acquired open filter images every 1 min up to 15 min. Fig. 3-10A illustrates 5 selected images and the encircled region of interest (ROI) used for quantification. Note the increase in the exposure time and decrease in signal to noise ratio over time. As can be seen in Fig. 3-10A, it is easy to make an erroneous conclusion on the biodistribution of cells with the noise generated as a result of higher exposure time. Hence, pre-assessment of the decay kinetic, consistent imaging time post-perfusion of substrate, and constant exposure time among all animals are critical requirements for longitudinal studies. Fig. 3-10B illustrates the computed decay of luminescence flux over time.

**Figure 3-9** Gaussia adino-transfected HUCPVCs. (A) Efficiency of HUCPVCs post modification, measured by flow cytometry; (B) Luminescence photon flux (radiance) generated by various cell densities detected with the Xenogen-IVIS system; (C) Linear correlation of the employed cell density and the generated photon flux; (D) *In-vivo* photon flux of luminescence from IM-transplanted Gluc-HUCPVCs.
Figure 3-10 Kinetics of Gaussia luciferase-coelenterazine. (A) IM-transplanted Gluc-HUCPVCs, IV-infused with 250 μg/100 μl sterile ddH₂O of coelenterazine and acquired open filter images every 1 min up to 15 min. The region of interest (ROI) used for quantification is encircled in red. (B) Quantification of the decay in flux at the encircled ROI over time.

3.3 Summary and conclusions

Our preliminary assessment of a suitable model of inflammation to fundamentally evaluate the potency of IM-transplanted MSCs to downregulate a distant source of inflammation required a consistent level of inflammation. Therefore, we chose the well-characterized carrageenan hind paw model of inflammation. This model allowed us to unilaterally induce inflammation in an easily accessible and measurable site (hind paw). We transplanted MSCs contralaterally in the dorsal thigh quadriceps muscle and performed our analysis. Our preliminary assessment of a suitable MSC tracking method for assessment of the biodistribution of IM-transplanted MSCs required a method with intracellularly retained (non-transferable to other cells), high efficiency, high signal penetration depth, and low cytotoxicity. Therefore, we chose DiR as our labeling method for the...
48h study. To assess the fate of xenotransplanted human MSCs in mice, we genetically modified MSCs (adeno-transfected) with a high flux intensity reporter (Gaussia Luciferase) that extracellularly released detectable proteins.
Chapter 4: Effect of Tumor Necrosis Factor Alpha Dose and Exposure Time on Tumor Necrosis Factor Induced Gene-6 Activation by Neonatal and Adult Mesenchymal Stromal Cells

The following chapter is published in the Journal of Stem Cells and Development. The reference numbers have been adjusted for this thesis and supplementary information is included.

Hamidian Jahromi S, Li Y, Davies JE (2018) Effect of Tumor Necrosis Factor Alpha Dose and Exposure Time on Tumor Necrosis Factor-Induced Gene-6 Activation by Neonatal and Adult Mesenchymal Stromal Cells, Stem Cells Dev. 27(1):44-54

4.1 Introduction

Mesenchymal stromal cells can be derived from various adult tissue sources such as BM, peripheral blood, adipose tissue, dental pulp, tendon, skin, and muscle, or fetal and neonatal tissue sources such as amniotic fluid, amnion membrane, placenta, chorionic villi, umbilical cord blood and tissue. The MSCs from these various sources, have all been shown to possess the minimum criteria defined for MSCs [4] while exhibiting various functional phenotypes depending on the tissue source [64-72].

Of the neonatal tissues, umbilical cord connective tissue—“Wharton’s Jelly” is the most frequently employed source of MSCs and is the focus of 108 clinical trials registered at ClinTrials.gov, of which about 34 are currently active [57]. Thus, a growing body of reports compare the functional potency of WJ-MSCs with the more traditional adult derived BM tissue source [39, 41, 75].

Indeed, MSCs from both placenta [38] and umbilical cord [40] exhibit increased
proliferative activity and CFU-F frequency [62] when compared to MSCs derived from adult tissue sources. Specifically, Sarugaser et al. [62] reported a (CFU-F) frequency of 1:300 in HUCPVCs, whereas that in the whole umbilical cord tissue is 1:1609 [317] both of which are significantly higher than that of adult BMMSCs (1:1x10^5) [51]. This may suggest a greater homogeneity of the HUCPVC population compared with the BMMSCs [60, 63]. Neonatal MSCs have also shown low immunogenicity [41], increased immunosuppression [39, 41] and cause an increase in alternatively activated M2-type macrophages [42] compared to BMMSCs, although this may not be reflected in standard mixed lymphocyte reactions where both a similar [74] and improved [75] functional phenotype has been reported. Holley et al., using quantitative proteomics, reported small numbers of proteins that were either BMMSCs or HUCPVC specific, although the functional relevance of these differences was not explored [80].

Thus, of the major paracrine signaling functions of MSCs, their anti-inflammatory properties have attracted considerable attention [229]. Placental and umbilical cord MSCs have been shown to have profound anti-inflammatory and wound healing effects in both cutaneous healing [43, 44, 318, 319] and acute liver failure [320] respectively. In particular, Liu et al. (2016) showed that umbilical cord MSCs reduced the activation of pro-inflammatory signaling [44], and Raicevic et al. (2011) concluded that WJ-MSCs were more effective than either adipose tissue or BM-derived MSCs when an immunosuppressive action is required in the presence of an inflammatory stimulant [79].

Tumor necrosis factor (TNF)-stimulated gene 6 protein (TSG-6) is a key anti-inflammatory mediator whose gene, TNFA1P6, is highly expressed in inflammation. It has been identified as one of the most upregulated genes in hBMMSCs and plays a critical role in the immunomodulation and down-regulation of inflammation [159]. Knockdown, or silencing, of TSG-6 has been shown to result in the loss of therapeutic activity of both BMMSCs [159], and umbilical cord MSCs [44].
TSG-6 is shown to interact with macrophages through the CD44 receptor to decrease translocation of NF-κB [135]. Increased activation of NF-κB in hBMMSCs has been correlated with increased expression of TSG-6 while inhibition of NF-κB signaling has been shown to result in decreased expression of TSG-6 and, interestingly, an increase in the osteogenic differentiation of hBMMSCs [164]. TSG-6 has been also shown by Tsukahara et al., 2006 to downregulate osteoblastic differentiation induced by BMP-2 [174] by direct binding to RANKL and BMPs (except BMP-3) via Link (a HA domain) and CUB domain [175]. Hence, TSG-6 secretion by MSCs has potent autocrine effects [135, 174, 175].

Furthermore, TSG-6 protein is also shown to abrogate neutrophil migration and infiltration [169] through interference with chemokine/ GAGs CXCL8 interactions [170]. In addition, a number of studies have shown TSG-6 to be important in inhibition of inflammation and joint destruction in autoimmune disease [161], acceleration of wound closure and reduction of fibrosis through altering TGF-β1 and TGF-β3 synthesis from a scar promoting (high) to an anti-fibrogenic (low) ratio [162], and inhibition of metalloproteinase mediated degradation of the cartilage matrix [163]. Hence, TSG-6 expression has been suggested as a biomarker of potency of MSCs [164].

Therefore, while MSCs have an anti-inflammatory phenotype, and TSG-6 has been shown to be an important mediator of this putative therapeutic outcome, little is known of the comparative sensitivity of MSC populations to a temporal exposure to a specific inflammatory stimulant. To address this issue, and using TSG-6 expression as a surrogate for an anti-inflammatory phenotype, we compared HUCPVCs with adult BMMSCs by addressing the following questions: (1) What is the TSG-6 gene expression response to different doses of TNF-α stimulation? and (2) Is there an optimum priming condition for HUCPVCs to achieve the maximum upregulation of TSG-6 compared to hBMMSCs? Also, in preparation for an in-vivo xenotransplantation study in a mouse
model of inflammation, we included cohorts of mBMMSCs to compare with both human cell types. We hypothesized that TNF-α activated neonatal HUCPVCs would express an enhanced level of TSG-6 activation compared with adult BMMSCs.

4.2 Materials and Methods

4.2.1 HUCPVC culture and source:

HUCPVCs were isolated, from the perivasular region of human umbilical cord. Briefly, the amniotic membrane was removed, and the umbilical vessels were separated. The perivasular Wharton’s Jelly was dissected from the tunica media of the individual vessels, diced, and used as a tissue source for explant culture. HUCPVCs were isolated and cultured using a xeno-free process. HUCPVCs were isolated and provided by Tissue Regeneration Therapeutics Inc., Toronto, Canada. Human umbilical cords were collected under a protocol approved by the Health Sciences Research Ethics Board of the University of Toronto and the Research Ethics Board of Mount Sinai Hospital, Toronto (#s 28546 and 13-0066-E respectively). Passage 1 cells were seeded at 1333 cells/cm², incubated at 37°C in a 5% CO₂ atmosphere in a humidified incubator and expanded in Lonza TheraPEAK™ MSCGM-CD™ (Lonza, Cat. 00192125) medium, which was changed every 3 days. The cells were enzymatically dissociated from the culture dish at approximately 70% confluency using TrypLE Select CTS (Invitrogen, Cat. A12859-01). HUCPVCs derived from 5 donors were either individually expanded to passage 3 and/or grown to passage 3 as a pooled population.

4.2.2 hBMMSCs culture and source:

hBMMSCs, isolated from human BM aspirates, were obtained as frozen vials at passage 1 from the Center for the Preparation and Distribution of Adult Stem Cells [321]. Two frozen vials with approximately 1 million cells in each from 2 different donors were thawed at 37°C in a water bath followed by re-suspension in complete culture medium MSCGM Bullet Kit (Lonza cat. PT-
3238 and PT-4105). Cells were pooled and seeded at 1333 cells/cm², incubated at 37°C in a 5% CO₂ atmosphere in a humidified incubator. The medium was changed every 3 days followed by enzymatic dissociation of cell from the culture dish at 70% confluency using TrypLE Select CTS (Invitrogen, Cat. A12859-01). Cells were then re-seeded for growth up to passage 3.

4.2.3 mBMMSCs culture and source:

mBMMSCs, isolated from mouse BM aspirates, 15 donors, were obtained as frozen vials at passage 1 were kindly donated by Professor Donald Phinney at Scripps Research Institute. Frozen vials were thawed at 37°C in water bath followed by re-suspension in complete culture medium consisting of α-MEM (GIBCO, Cat. 32561-037), 10% fetal bovine serum-FBS (Atlanta Biologicals, Optima, Cat. S12450, Lot. M13174), and 1% Penicillin/streptomycin (Gibco, Cat. 15140122). mBMMSCs were seeded at 5000 cells/cm², incubated at 37°C, under hypoxic conditions (5% O₂, 5% CO₂ atmosphere) in a humidified incubator. The medium was changed every 4 days followed by enzymatic dissociation of cells from the culture dish at 70% confluency using 0.25% trypsin-EDTA (1x) (Sigma, Cat. T3924). Cells were then re-seeded for growth up to passage 3.

4.2.4 Cell growth rate measurements:

HUCPVCs, hBMMSCs and mBMMSCs at passage 2 were cultured as described above. The medium was changed every 3 days. Cells in culture were washed with 1xPBS, and enzymatically detached from the dish using TrypLE Select Enzyme (ThermoFisher Scientific, A1217701), followed by centrifugation. Viable cells were then counted using a hemocytometer. The exclusion cell viability test used a 1:1 ratio of trypan blue 0.4% (ThermoFisher Scientific, Cat. 15250061). Individual plates were set up for each time point up to 7 days, n=4/time point. The doubling time (DT) was calculated based on the following formula [322]:

---

79
\[ DT = T \frac{Ln2}{Ln\left(\frac{X_e}{X_b}\right)} \]

Where: \( T \): Culture time in hours; \( X_e \): Cell number at harvest; \( X_b \): Cell number at seeding; \( Ln \): natural logarithm.

4.2.5 Baseline measurement of HUCPVCs and hBMMSCs:

We assessed the comparative baseline expression of 47322 genes in HUCPVCs and hBMMSCs, from which we present the TNAIP6 (TSG-6) expression level herein (Fig. 4-1). The microarray experiments were performed using the Affymetrix GeneChip Human Gene 1.0 ST oligonucleotide arrays of cDNA on HUCPVCs and hBMMSCs both cultured under the same conditions, in α-MEM (GIBCO, Cat. 32561-037) supplemented with 10% FBS (Gibco/Invitrogen: Cat #12483 / Lot #1404231) at passage 3. Measurements and analysis was performed by Center for Applied Genomics-The Hospital for Sick Children (Toronto, ON).

4.2.6 TNF-α treatment in cultures:

Cells were harvested at passage 3, plated at 12,000 cells/cm² in 6-well plates in complete media. After 18-24 hours, when cells had adhered to the culture plates, the medium was changed to that supplemented with recombinant human/mouse tumor necrosis factor alpha—rh/rmTNF-α (rhTNF-α, R&D systems, Cat. 210-TA-020/CF; rmTNF-α aa 80-235, R&D systems, Cat. 410-MT-050/CF) (n=3). Through our preliminary studies, we found 1ng/mL to be the minimum concentration of TNF-α for reproducible cell activation. Thus, for this study, the TNF-α concentrations for MSC activation were 1, 10, 50, and 100ng/mL. Cells were exposed to TNF-α for 0.5, 1, 10, 24, and 48 hours. Experiments were conducted under one-time induction of TNF-α at a defined concentration; TNF-α was not continuously added.
4.2.7 Total RNA and protein sample collection:

At each time point, supernatant was collected followed by immediate addition of 1x protease inhibitor cocktail without EDTA (Bioshop, Cat. PIC002.1) that contains 2mM AEBSF HCL, 130µM Bestatin, 14µM E-64, 1µM Leupeptin, and 0.3µM Aprotinin. Cells were briefly washed with phosphate buffered saline (PBS 1x, Gibco, Cat. 10010023) followed by immediate lysis and collection of total RNA using Arum™ Total RNA Mini Kit (Bio-Rad, Cat. 7326820), followed the company’s recommended procedure. Samples were stored at -80°C for further analysis.

4.2.8 RNA purification and reverse transcription:

RNA was immediately purified using Arum™ Total RNA Mini Kit (Bio-Rad, Cat. 7326820). Purity and quantity of RNA was measured through absorbance using spectrophotometric quantification (NanoDrop™ 1000, Thermo Fisher Scientific). The A260/A280 ratio for all RNA samples was 1.9-2. Genomic DNA elimination, and reverse transcription (RT) was performed using iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad, Cat. 1725035) according to the manufacturer’s instructions. Approximate amount of 300ng of RNA from each sample was reverse transcribed to cDNA (Bio-Rad C1000™ Thermocycler) under the following synthesis reaction protocol: priming (5mins at 25°C), reverse transcription (20mins at 46°C) RT inactivation (1min at 95°C) followed by incubation at 4°C.

4.2.9 Reference gene selection:

A reference gene panel consisting of 30 reference genes (Bio-Rad, H384, Cat. 10025899; M384, 10029516) was used to identify the most stable reference genes with regards to TNF-α treatment. cDNA samples of cells exposed to different concentrations and exposure time of TNF-α was tested on the panel. Three reference genes were chosen based on average expression stability (M-value) and their coefficient of variation on the normalized relative quantities (CV). The
reference genes used for quantification of human samples were human ribosomal protein L13a (RPL13A), human beta-2-Microglobulin (β2M), and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH)—mean M value (0.0795), mean CV (0.0325) for HUCPVC samples; mean M value (0.0798) and mean CV (0.1881) for hBMMSCs samples. The most stable reference genes chosen for mouse were mouse tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ), mouse peptidylpropyl isomerase A (PPIA), and mouse GAPDH—mean M value (0.3519), mean CV (0.1442). All primers were validated by Bio-rad with the following unique assay IDs: human RPL13A (qHsaCED0045063), human β2M (qHsaCID0015347), human GAPDH (qHsaCED0038674), mouse Ywhaz (qMmuCED0027504), mouse PPIA (qMmuCED0041303), mouse GAPDH (qMmuCED0027497). Fig. 4-2 illustrates the quantification cycle (Cq) value, as proposed by Bustin et al. (2009) [323], of the selected reference genes across concentrations and exposure times. The starting material and loading concentration of the cDNA was maintained at the same quantity. Thus, the values of Cq represents the variation in the expression level of the genes when cells were treated with TNF-α.

4.2.10 Real-time polymerase chain reaction (RT-PCR) and quantification:

RT-qPCR was carried out on the synthesized cDNA using SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad, Cat. 1725272) according to the manufacturer’s instructions (10µl reaction volume) at an annealing temperature of 65°C. Each sample was run in triplicate and a Bio-Rad CFX384 Touch™ System was used for fluorescence measurement. Validated Bio-Rad TSG-6 primers had the following unique assay IDs: human TSG-6 (qHsaCID0008311), and mouse TSG-6 (qMmuCID0010069). Quantification was done by normalizing the expression levels of TSG-6 to the geometric mean of the three reference genes mentioned previously, using the Pfaffl method. PCR efficiency, linearity (R²), slope, and y-intercepts, respectively, according to the MIQE’s guidelines for each cell type and primer calibration are listed: HUCPVCs-TSG-6
(101.1%, 0.99, -3.298, 44.961); HUCPVCs-RPL13A (95.4%, 0.99, -3.436, 44.621); HUCPVCs-
β2M (102.8%, 0.99, -3.255, 41.03); HUCPVCs-GAPDH (97.7%, 0.99, -3.379, 41.264);
hBMMSCs-TSG-6 (104.7%, 0.99, -3.214, 43.578); hBMMSCs-RPL13A (104.3%, 0.99, -3.222,
4.139); hBMMSCs-β2M (103.3%, 0.99, -3.246, 37.588); hBMMSCs-GAPDH (104.1%, 0.97, -
3.228, 38.192); mBMMSCs-TSG-6 (92.4%, 0.99, -3.519, 45.855); mBMMSCs-PPIA (99.3%,
0.96, -3.339, 38.805); mBMMSCs-YWHAZ (100.9%, 0.99, -3.302, 41.727).

4.2.11 Enzyme-linked immunosorbent assay (ELISA) and quantification:

Culture supernatant samples supplemented with protease inhibitor stored at -80°C were
tested for TSG-6 levels in the human samples using a validated sandwich-based ELISA kit
(RayBiotech, Cat. ELH-TSG6), as employed by others. Prior to measurement, protein samples
were centrifuged at 1000xg for 5mins at 4°C to remove cellular debris. A standard curve was
generated using the recombinant TSG-6 protein in the kit and all Samples were tested in triplicate
and measurements followed the company’s recommended procedure. Sample absorbance was
measured at 450nm using a plate reader (Molecular Devices, SpectraMax i3x Multi-Mode
Detection Platform). Total protein of the same samples was measured using Pierce BCA protein
assay kit (Thermo Fisher scientific, Cat. 23225) and measurements were then normalized to the
total protein.

4.2.12 Statistical Analysis:

Statistical analysis of groups/time points was performed by means of factorial ANOVA-
multiple comparison followed by post hoc Tukey statistical test between the means of replicates
at significance level of 95% CI. Each figure legend indicates details of the statistical analysis
performed. Statistical analysis was performed using GraphPad Prism 6.01 (GraphPad Software
Inc., La Jolla, CA, USA).
4.3 Results

4.3.1 Baseline of TSG-6 in HUCPVCs and hBMMSCs:

mRNA expression level of TNAIP6 (TSG-6) in unstimulated HUCPVCs with hBMMSCs grown under the same culture condition demonstrates 2.14-fold higher in HUCPVCs compared with hBMMSCs (Fig. 4-1).

**Figure 4-1** mRNA Expression level of TSG-6 in unstimulated HUCPVCs and hBMMSCs. GeneChip® Human Gene 1.0 ST Array was used. Affymetrix data were normalized and summarized with multi-average (RMA) method implemented in the Affymetrix Expression Console. Data is expressed as means ± S.E.M, n=7-8 donors.

4.3.2 Effect of TNF-α on reference genes:

Assessment of the effect of TNF-α stimulation on reference genes (Fig. 4-2A and B) 10 and 24 hr post TNF-α exposure (respectively), demonstrates that all reference genes are affected
by TNF-α treatment. We have demonstrated this change by representing the Cq values with different stimulant concentration and exposure time. We have further represented the geometric mean to elaborate on the importance of using the geometric mean of at least 3 most stable (out of a pool of 30) reference genes to diminish the variation [323, 324].

Figure 4-2 Examples of the effect of TNF-α treatment on 3 reference genes used for normalization of RT-PCR data analysis. Cq values of the 3 most stable reference genes in response to TNF-α treatment of 1 to 100ng/mL across exposure time (A) 10 hr; and (B) 24 hr is represented as color Pink β2M (▲); Purple=RPL13A (■); Blue=GAPDH (●); Green=geometric mean of the 3 reference genes used to normalize target gene (▼). Data is expressed as means ± S.E.M, n=3.
4.3.3 Growth rate of HUCPVCs and adult BMMSCs:

HUCPVCs when compared with hBMMSCs, exhibited faster growth rates of about 43% and 63%, post seeding, on day 4 and 7 respectively (Fig. 4-3A). The short population doubling time of HUCPVCs, Fig. 4-3B, further highlights their growth rate from day 2 of seeding compared to hBMMSCs. Cultures of mBMMSCs represented a morphologically heterogenous population with slow growth rate (not shown).
Figure 4-3 Growth profile (A) and doubling time (B) of a pooled population of HUCPVCs and hBMMSCs in culture; passage 2 to 3. The initial seeding density of both cell types was 1333 cells/cm² and daily yield of adhered and viable cells within 7 days of culture was measured and plotted. Data is expressed as means ± S.D. n=4.

4.3.4 Response time of activated HUCPVCs:

The TSG-6 mRNA expression profile of pooled HUCPVCs in response to TNF-α stimulation was assessed at various stimulant concentrations and exposure times. As shown in Fig. 4-4, upregulation of TSG-6 across all stimulant concentrations was evident in HUCPVCs
compared with unstimulated HUCPVCs. Stimulation of HUCPVCs with 1ng/mL of TNF-α resulted in the highest upregulation of TSG-6 expression in the first 30 minutes (\( \bar{X} = 11.36 \) fold increase), while no significant differences were observed with higher stimulation concentrations. Further stimulation of HUCPVCs for a longer period of 1 and 10 hours, neither significantly reduced the expression level of TSG-6, nor represented a significantly different pattern with increased concentration of stimulant. On the contrary, 24 or 48h stimulation resulted in a gradual downregulation of TSG-6 expression at all concentrations. The concentration of TSG-6 protein released in the supernatant of the same cell population (Fig. 4-5) further confirmed the response level (i.e., fold change) of HUCPVCs to TNF-α stimulant. TSG-6 increased by \(~2\) fold across all stimulant concentrations compared with controls. However, no significant differences were observed across different concentrations or exposure times.
Figure 4-4 TSG-6 dose and response time of a pooled population of HUCPVCs stimulated with (1 to 100ng/mL) of rhTNF-α. mRNA fold change expression level of TSG-6 was determined by RT-PCR. Expression level was normalized to the geometric mean of β2M, RPL13A, and GAPDH and further normalized to the controls (untreated) of each timepoint. Two-way ANOVA-Multiple comparison Tukey statistical test was performed between the means of 3 replicates at significance level of 95%CI, data is expressed as means ± S.E.M. asterisk (*) represents p≤0.05, otherwise, no significant difference was noted p>0.05.
Figure 4-5 TSG-6 dose and response time of a pooled population of HUCPVCs stimulated with (1 to 100ng/mL) of rhTNF-α. Secreted protein concentration of TSG-6 in the supernatant of the same cultures in Fig.4-4, were quantified with ELISA and normalized to the total secreted protein/well. Two-way ANOVA-Multiple comparison Tukey statistical test was performed between the means of 3 replicates at significance level of 95%CI, data is expressed as means ± S.E.M. Asterisk (****) represents p≤0.0001, otherwise not significant (NS) p>0.05.
4.3.5 Response time of activated adult BMMSCs:

To test the difference in TSG-6 expression pattern of HUCPVCs with an adult population of MSCs, we chose hBMMSCs to represent the most commonly employed mesenchymal cells in both research and clinical therapies. The TSG-6 mRNA expression profile of pooled hBMMSCs in response to TNF-α stimulation was assessed at various stimulant concentrations and exposure times, and is shown in Fig. 4-6, and statistical analysis is presented in Table 4-1.

Stimulation of hBMMSCs with 50ng/mL of TNF-α after 60 minutes resulted in the highest upregulation of TSG-6 expression ($\bar{X} = 9.49$ fold increase) compared to either lower or higher TNF-α concentrations within the same exposure time. Stimulation of hBMMSCs for a longer exposure time of 10 hours showed dose dependency from 1ng/mL to 50ng/mL with an increase in the TSG-6 expression level from $\bar{X} = 1.07$ to a 5.42 fold increase. On the contrary, as hBMMSCs were stimulated for a prolonged period of 24 or 48 hours, the level of TSG-6 expression approached, and was not significantly different from, the level of untreated hBMMSCs (controls). The concentration of TSG-6 protein released in the supernatant of the same cell population (Fig. 4-7) was close to that of controls across all concentrations and incubation times. Interestingly, the level of TSG-6 protein detected in culture medium of control BMMSCs was higher at 60 minutes than at 24 or 48 hours. Statistical analysis showed a difference in the protein concentration detected in the 50ng/mL at 60 mins exposure time compared to controls at 48 hours, but not at 60 mins.

The response pattern of TSG-6 upregulation in the pooled population of mBMMSCs when stimulated with TNF-α is shown in Fig. 4-8. Statistical analysis suggests the highest stimulation to be achieved with TNF-α of 1ng/mL at 48 hours of exposure time ($\bar{X} = 2.1$ fold increase). As stimulation of mBMMSCs with TNF-α did not result in an enhanced upregulation of TSG-6 expression, we did not measure the protein released in the supernatant of the cultures.
Figure 4-6 TSG-6 dose and response time of a pooled population of hBM-MSCs stimulated with (1 to 100ng/mL) of rhTNF-α. mRNA fold change expression level of TSG-6 was determined by RT-PCR. Expression level was normalized to the geometric mean of β2M, RPL13A, and GAPDH and further normalized to the controls (untreated) of each timepoint. Two-way ANOVA-Multiple comparison Tukey statistical test was performed between the means of 3 replicates at significance level of 95% CI, data is expressed as means ± S.E.M. Statistical significance between groups is shown in Table 4-1.
Table 4-1 Statistical analysis of mRNA expression of pooled population of hBMSCs (Fig.4-4) stimulated with (1 to 100ng/mL) of rhTNF-α. Two-way ANOVA-Multiple comparison Tukey statistical test was performed between the means of 3 replicates at significance level of 95%CI, data is expressed as means ± S.E.M. significant (p<0.01 to 0.05); very significant (p<0.001 to 0.01); and extremely significant (p<0.001); not significant (NS=p>0.05).

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Figure 4-7 TSG-6 dose and response time of a pooled population of hBMMSCs stimulated with (1 to 100ng/mL) of rhTNF-α. Secreted protein concentration of TSG-6 in the supernatant of the same cultures in Fig. 4-6, were quantified with ELISA and normalized to the total secreted protein/well. Two-way ANOVA-Multiple comparison Tukey statistical test was performed between the means of 3 replicates at significance level of 95%CI, data is expressed as means ± S.E.M. Asterisks (*) represents p≤0.05, and (**) p≤0.01, otherwise not significant (NS) p>0.05.
4.3.6 Donor variation in HUCPVCs in response to TNF-α stimulation:

TSG-6 expression in hBM-MSCs have previously been shown to be donor dependent [164]. Thus, we assessed TSG-6 donor variation in HUCPVCs in response to various concentrations and exposure times of TNF-α stimulation (Fig. 4-9). Data from 5 independently cultured and stimulated HUCPVC populations plotted against stimulant concentration and exposure time are shown in Fig. 4-9. As can be seen in Fig. 4-9, TSG-6 expression is highly donor dependent in HUCPVCs. Across all donors, the highest expression was achieved with 1ng/mL of stimulation.
with TNF-α within a short exposure period of 30-60 minutes. Higher concentrations and prolonged exposure times, inversely affected the expression level of TSG-6 in HUCPVCs. Statistical analysis is presented in Table 4-2.

**Figure 4-9** TSG-6 dose and response time of 5 HUCPVC donors stimulated with 1 to 100ng/mL of rhTNF-α. mRNA fold change expression level of TSG-6 was determined by RT-PCR. Expression level was normalized to the geometric mean of B2M, RPL13A, and GAPDH and further normalized to the controls (untreated) of each timepoint. One-way ANOVA-Multiple comparison Tukey statistical test was performed between the means of 15 data points ((n=3/donor) x 5 donors) at significance level of 95%CI, data is expressed as means ± S.E.M. Statistical significance between groups is shown in Table 4-2. Red =Donor 1 (●); Green=Donor 2 (■); Navy blue=Donor 3 (♦); Aqua=Donor 4 (▲); Olive=Donor 5 (▼).
Table 4-2 Statistical analysis of mRNA expression of 5 donors of HUCPVCs (Fig.4-2) stimulated with (1 to 100ng/mL) of rhTNF-α. One-way ANOVA-Multiple comparison Tukey statistical test was performed between the means of 15 data points at significance level of 95%CI, data is expressed as means ± S.E.M. Significant (p<0.01 to 0.05); very significant (p<0.001 to 0.01); and extremely significant (p<0.001); not significant (NS=p>0.05); significant (p<0.01 to 0.05); very significant (p<0.001 to 0.01); and extremely significant (p<0.001); not significant (NS=p>0.05).
4.4 Discussion

Both hBMMSCs and UCMSCs have been employed in cell therapy, and UCMSCs have been proposed as an alternative to MSCs derived from adult tissue sources. In this work, we studied the anti-inflammatory potency of HUCPVCs with respect to hBMMSCs in response to varying doses of TNF-α and monitored the pattern of upregulation of TSG-6.

Elevated serum and tissue levels of TNF-α, produced in inflammatory and infectious conditions, directly correlate with the severity of the inflammation or infection [325]. It has been shown that TNF-α induces an inflammatory response through the NF-κB signaling pathway [326]. Thus, TSG-6 that is shown to inhibit the NF-κB pathway is important in the regulation of TNF-α transcription. MSCs in contact with inflammatory stimulants switch to an activated state, upregulate anti-inflammatory associated genes and release anti-inflammatory mediators to immunosuppress/immunomodulate the inflammatory cascade. TSG-6 is identified as the most important mediator released by MSCs that shifts the pro-inflammatory milieu to an anti-inflammatory state [327]. Although in-vitro assays cannot recapitulate the complex and dynamic physiological network, they can provide valuable insights into the cellular response to a distinct key mediator. The circulating concentration level of TNF-α in minor and advanced inflammation cases in humans ranges from 0.02-6ng/mL [328-334]. Interestingly, we found that in contrast to hBMMSCs, HUCPVCs demonstrated an early response (0.5 hr) to 1ng/ml of TNF-α, irrespective of the activation dose (Figs. 4-4 and 4-6). The prompt response of HUCPVCs corroborates the reported response of WJ-MSCs where both unprimed, and TNF-α or IFN-γ primed, WJ-MSCs demonstrated an earlier and higher, extent of immunosuppression compared with hBMMSCs in an MLR assay [75]. The early response of WJ-MSCs was also reported to lead to early activation
of the co-stimulatory molecule CTLA4 on peripheral blood mononuclear cells (PBMCs), which was not evident with hBMMSCs [75]. This prompt response of HUCPVCs may be associated with the age of UCMSCs compared with MSCs isolated from adult tissue sources. Several studies have highlighted the impact of BMMSCs donor age on the plasticity and differentiation capacity of the MSCs [76-78], which would corroborate our results. The concentration of TSG-6 protein in the supernatant of HUCPVCs compared with hBMMSCs also reflected the low response of hBMMSCs to TNF-α activation compared to controls (Figs. 4-5 and 4-7). Additionally, when we compared the baseline gene expression of unstimulated HUCPVCs with hBMMSCs, the TNAIP6 (TSG-6) expression level was 2.14-fold higher in the former (Fig. 4-1).

The effect of TNF-α stimulation on the therapeutic effects of MSCs, from several tissue sources, have previously been studied as a function of dose and exposure time. It has been demonstrated that pre-treatment of MSCs, prior to transplantation, can significantly enhance their therapeutic activity. Leij et al. (2017) studied the effect of IFN-γ/TNF-α stimulation (1, 20, and 50ng/mL) of hBMMSCs at 24 hours and reported enhanced expression of migration and adhesion receptors of MSCs [335]. Ziaei et al. (2014) studied the effect of TNF-α stimulation on hBMMSCs for 2, 10, 24 and 48 hours and observed greater CXCR4 expression with 10ng/mL compared to 1ng/mL of TNF-α stimulation after 24 compared to 2 hours exposure time [336]. Interestingly, in the case of hBMMSCs, our results for TSG-6 were higher at 1 hour than later time points, and response to 10ng/mL was greater than 1ng/mL. This was not the case for HUCPVCs. Migration activity and CXCR4 upregulation has also been demonstrated in TNF-α stimulated (100ng/mL) human adipose derived MSCs (hAD-MSCs) [337]. Lee et al., (2010) studied the effect of TNF-α stimulation (0.1-10ng/mL) on hAD-MSCs and observed an enhanced secretion of IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), and CXCL6 at an optimal concentration of 10ng/mL at 48 hours of exposure time [338]. Liu et al. (2016) also studied the effect of TNF-α
(20ng/mL) stimulation on hADMSCs for 24 hours and observed the upregulation of MCP-1 at 24h exposure time [339]. In an in-vivo study, Kwon et al. (2013) studied the effect of pre-treatment of hADMSCs with TNF-α (10ng/mL), intramuscularly injected into athymic nude mice and observed an enhanced homing of endothelial progenitor cells and angiogenesis [340]. Furthermore, Liu et al. (2016) has reported upregulation of TSG-6 in human umbilical cord MSCs stimulated with a single dose of 10ng/mL of TNF-α [44].

The afore-mentioned in-vitro and in-vivo studies confirm that the pre-treatment of adult tissue derived MSCs with TNF-α enhances both the migratory and immunomodulatory capacity of the cells and is dose-dependent.

In this study, we observed the differential activation patterns of neonatal and adult tissue sourced MSCs (Figs. 4-4 and 4-6). Importantly, as shown in Fig. 4-4, HUCPVCs upregulated TSG-6 independent of the stimulation dose, whereas adult hBMMSCs, corroborating previous reports and shown in Fig. 4-6, demonstrated TNF-α dose dependency. Additionally, prolonged exposure of MSCs to TNF-α did not lead to an increase in the expression level of TSG-6 in either HUCPVCs, or hBMMSCs, independent of stimulation dose. This finding suggests that HUCPVCs and hBMMSCs could be activated at a shorter timescale than previously reported for pre-treatment of MSCs. The observed downregulation in TSG-6 expression with prolonged culture time could be attributed to the short half-life of TNF-α. It has been reported that intravenously infused TNF-α has a short plasma half-life of 0.5 h in rats [341]. This supports our in-vitro findings that suggest the effective response of MSCs to TNF-α to be within the first 0.5-1h.

Lee et al., reported fold changes up to 80-120 for TSG-6 expression level of hBMMSCs when exposed to a single dose of 10ng/mL of TNF-α for 48h [159]. The difference in the observed fold changes of hBMMSCs in our study compared to Lee et al., could be associated with the
difference in the stimulation methods. Lee et al., serum starved hBMMSCs during TNF-α stimulation, and Wang et al., 2012 have shown that serum starvation increases induction of TSG-6 in MSCs [342]. Clearly, it was not necessary to serum-starve the HUCPVCs, as they were grown entirely in serum-free conditions; however, we did not serum starve either the human or murine BMMSCs to avoid sudden changes in the biological milieu of these cells. Another difference is the reference gene normalization method employed. To reduce the effect of reference gene variation, we followed MIQE guidelines [323] and normalized our TSG-6 expression data to the geometric mean [324] of 3 of the most stable (out of 30 tested) reference genes (Fig. 4-2) to achieve a more reliable baseline for normalization of qPCR datasets. We believe this approach is important because reference gene expression is not protected from environmental changes in the cellular milieu. Yet, GAPDH is commonly, and singularly, used for normalization of data involving tissues or cells treated with TNF-α and a number of such gene expression analyses have been done on TSG-6 in an inflammatory context [159, 343, 344].

In addition to HUCPVCs and hBMMSCs, we also explored the anti-inflammatory potency of mBMMSCs in a similar set of experiments. Stimulation of mouse BMMSCs with 10ng/mL of TNF-α has previously shown to have minimal effect on upregulation of TSG-6 [159]. Indeed, Lee et al., 2009 reported that the response of hMSCs to TNF-α far exceeded that of human fibroblasts, but that murine MSCs showed an order of magnitude lesser response. We wanted to investigate if lower or higher concentrations of TNF-α would increase the induction of TSG-6 in mBMMSCs. Our results corroborated the earlier report [159]—both human MSC populations exhibited enhanced TSG-6 upregulation upon stimulation with TNF-α compared with mBMMSCs. mBMMSCs demonstrated a low-grade response to TNF-α exposure, with maximum upregulation of TSG-6 obtained after 48 hours of exposure. Similarly to HUCPVCs, the TSG-6 expression level of mBMMSCs was independent of the stimulant concentration.
MSCs derived from different donors are shown to have different growth rate and potency. It has previously been reported that the expression level of TSG-6 by hBMMSCs is highly donor-dependent [164] and we confirmed similar variation from HUCPVC donors (Fig. 4-9). However, our results also demonstrate an enhanced and prompt response of HUCPVCs to TNF-α stimulation compared to hBMMSCs. The question remains whether these in-vitro results would correlate with an observable in-vivo functional response. This we have done, and which we shall report elsewhere. If so, this would suggest that the functional potency of an MSC potentially could be screened, in-vitro, with a rapid low dose assay.

4.5 Conclusions

HUCPVCs showed higher sensitivity to TNF-α stimulation compared with hBMMSCs, but expression level varied across donors. Thus, MSC response to TNF-α stimulation is MSC population and source dependent, but neonatal MSCs may be a stronger candidate population than those derived from adult BM to treat inflammatory diseases.
Chapter 5: Human Umbilical Cord Perivascular Cells and Human Bone Marrow Mesenchymal Stromal Cells Transplanted Intramuscularly Respond to a Distant Source of Inflammation

The following chapter is published in the Journal of Stem Cells and Development. The reference numbers have been adjusted for this thesis and supplementary information is included.


5.1 Introduction

The potential therapeutic capacity of exogenously transplanted MSCs to treat immune disorders and tissue injuries has been extensively demonstrated in a wide range of animal models of human diseases [159, 212, 213] and clinical trials [214, 215]. These studies delivered MSCs by IV injection, which is the most common route to effectuate systemic therapy, although other routes, IM, IP and SC have been explored and recently compared by Braid et al. (2018) [220].

One important advantage of IM delivery of MSCs is their demonstrated increase in dwell-time in-situ. This was first shown by Bartholomew et al. 2001, [253] who delivered baboon MSCs, engineered to express human EPO, IM to NOD/SCID mice, and reported serum EPO levels, above those of controls, up to 1 month. An 8 month dwell-time was reported by Vilalta et al. [274] for human adipose derived MSCs transplanted in the thigh muscle of BALB/c homozygous nude nu/nu mice, although the majority of cells disappeared from the muscle within the first week.
Braid, et al. 2016, also showed that a depot of IM delivered HUCPVCs, genetically modified to secrete an anti-viral monoclonal antibody, provided protection against exposure to Venezuelan equine encephalitis virus, with secretorily active MSCs detectable for approximately 4 months [255]. More recently, Braid et al. 2018, compared the dwell-time of hMSCs when delivered IM (5 months), IP or SC (3-4 weeks), and IV (3 days) in healthy athymic mice [220].

While several authors have reported that the number of IM injected cells decreases with time, there is little evidence of distribution to other organs. Ramot et al. reported that placental-derived human MSCs remained at the thigh musculature injection site in NOD/SCID mice for 3 months [276]. Similar results were reported by Creane et al. [221] who detected hBMMSCs 3 months after injection in the thigh and calf musculature of BALB/c Nude mice, but no migration of MSCs to brain, heart, lungs, kidneys, spleen or liver. On the contrary, it has been frequently shown that small numbers of IV delivered MSCs, which are rapidly entrapped in the capillary beds of lungs, [159] lodge in other organs, mainly the liver and spleen [216-219]. Nevertheless, during entrapment in the lungs, MSCs have been shown to alter the tissue microenvironment of a remote injured/inflamed site through their secreted soluble factors [159]. Thus, it is clear that the physical presence of the delivered MSCs at the target site is not essential for therapeutic immune modulation. Indeed, it is now generally accepted that trophic factors released by MSCs are responsible for the majority of their therapeutic effects. Thus, IM delivery of MSC to the hamstrings, has been shown to increase cardiac function [222, 223], predominantly through the release of VEGF [224], although other trophic factors may also play an important role [225].

Nevertheless, IM delivery of MSCs, has mainly been employed for treatment of focused pathologies including myocardial ischemia [345, 346], muscular dystrophy [222], and peripheral nerve lesion-associated muscular atrophy [347]. The output measures in these studies focused on local effects in the affected muscle, cardiac muscle or limb, and the systemic sequelae of the IM-
MSC administrations were not explored [346, 347]. But, IM delivery of MSCs may also represent a valuable alternative to treat systemic conditions where the long dwell-time of secretorily active cells would provide an advantage over the rapid disappearance of cells from the lungs following IV delivery. Indeed, Consentius et al. showed that mis-matched placental-derived MSCs, while administered locally into CLI patients, also inhibited immune responses by modulating dendritic/natural killer cell interactions [242]. Furthermore, Liu et al., demonstrated a prophylactic reduction in joint inflammation in an antibody-induced/LPS-challenged murine rheumatoid arthritis model, when gene-modified hBMMSCs were delivered IM (bilateral hind limbs) [228]. But, they also showed that un-transduced MSCs were not effective in abrogating inflammation, which would apparently contradict the findings of Shabbir et al. [222] who reported reduced circulating plasma myeloperoxidase (MPO) levels following IM-hBMMSC administration.

The lack of uniformity between reported MSC source, delivery route, and animal model complicates comparison of available pre-clinical data, and has hindered development and adoption of IM MSC delivery in clinical studies. To begin to address this issue we compared the efficacy of three MSC sources—hBMMSCs; HUCPVCs and mBMMSCs—in an established murine hind paw carrageenan-induced inflammation model [206] in immune competent mice. To avoid the local effects of delivering cells into the ipsilateral limb, in each case the cells were delivered, post inflammation induction, into the contralateral quadriceps. Then we monitored paw circumference, myeloperoxidase levels, and ingress of both neutrophils and macrophages into the paw tissue. As TNF-α is a potent inflammatory mediator, we measured circulating TNF-α levels and the level of TSG-6 at the IM-injection site, and tracked biodistribution and dwell-time of the delivered cells. We hypothesized (a) that IM transplanted MSCs could effectively downregulate such a remote source of acute inflammation and (b) that the efficacy of the MSC population, in abrogating the inflammation, would depend upon the tissue source of the cells.
5.2 Materials and Methods

5.2.1 HUCPVCs culture and source

HUCPVCs were isolated, from the perivascular region of human umbilical cord. Briefly, the amniotic membrane was removed, and the umbilical vessels were separated. The perivascular Wharton’s Jelly was dissected from the tunica media of the individual vessels, diced, and used as a tissue source for explant culture. HUCPVCs were isolated and cultured using a xeno-free process. HUCPVCs were isolated and provided by Tissue Regeneration Therapeutics Inc., Toronto, Canada. Human umbilical cords were collected under a protocol approved by the Health Sciences Research Ethics Board of the University of Toronto and the Research Ethics Board of Mount Sinai Hospital, Toronto (#s 28546 and 13-0066-E respectively). Passage 1 cells were seeded at 1333 cells/cm², incubated at 37°C in a 5% CO₂ atmosphere in a humidified incubator and expanded in Lonza TheraPEAK™ MSCGM-CD™ (Lonza, Cat. 00192125) medium, which was changed every 3 days. The cells were enzymatically dissociated from the culture dish at approximately 70% confluence using TrypLE Select CTS (Invitrogen, Cat. A12859-01). HUCPVCs derived from 5 donors were pooled and expanded to passage 3 (P3).

5.2.2 hBMMSCs culture and source

hBMMSCs, isolated from human BM aspirates, were obtained as frozen vials at passage 1 from the Center for the Preparation and Distribution of Adult Stem Cells (http://medicine.tamhsc.edu/irm/msc-distribution.html). Two frozen vials with approximately 1 million hBMMSCs/vial from different donors were thawed at 37°C in water bath followed by re-suspension in complete culture medium MSCGM Bullet Kit (Lonza cat. PT-3238 and PT-4105). Cells were pooled and seeded at 1333 cells/cm², incubated at 37°C in a 5% CO₂ atmosphere in a humidified incubator. The medium was changed every 3 days followed by enzymatic dissociation of cell from the culture dish at 70% confluence using TrypLE Select CTS (Invitrogen, Cat.
A12859-01). Cells were then re-seeded for growth up to passage 3.

5.2.3 mBMMSCs culture and source

mBMMSCs, isolated from mouse BM aspirates, were obtained as frozen vials at passage 1 (donated by the Phinney Group at Scripps Research Institute). Frozen vials were thawed at 37°C in water bath followed by re-suspension in complete culture medium consisting of α-MEM (GIBCO, Cat. 32561-037), 10% fetal bovine serum (Atlanta Biologicals, Optima, Cat. S12450, Lot. M13174), and 1% Penicillin/streptomycin (Gibco, Cat. 15140122). mBMMSCs were seeded at 5000 cells/cm², incubated at 37°C, under hypoxic conditions (5% O₂, 5% CO₂ atmosphere) in a humidified incubator. The medium was changed every 4 days followed by enzymatic dissociation of cells from the culture dish at 70% confluency using 0.25% trypsin-EDTA (1x) (Sigma, Cat. T3924). Cells were then re-seeded for growth up to passage 3.

5.2.4 TNF-α treatment in cultures

Only mBMMSCs were primed (pre-treated) prior to IM-delivery. mBMMSCs were harvested at passage 3, plated at 12,000 cells/cm² in T175 flasks in complete media. After 18-24 h, when cells had adhered to the culture plates, the medium was changed to that supplemented with recombinant mouse tumor necrosis factor alpha—rmTNF-α (80-235, R&D systems, Cat. 410-MT-050/CF). The priming condition was 1ng/mL of rmTNF-α and 1 h incubation time.

5.2.5 MSC Fluorescence labeling

Post trypsinization, cells were washed 1x with phosphate buffered saline (PBS), (Gibco, Cat. 10010023), and then incubated in fresh media containing 3.5ug/mL 1,1’ dioctadecyltetramethyl indotricarbocyanine iodide (DiR) (Invitrogen, Cat.D12731) for 19 minutes at 37°C. The labeled MSCs were then centrifuged for 5 min at 1000 rpm at 4°C, and washed twice with PBS. A sample of labeled MSCs were checked for viability, fluorescence, and MSC surface markers post-labelling with a flow cytometer.
5.2.6 Gaussia Luciferase transfection of HUCPVCs

A pre-made GLuc recombinant adenovirus was purchased from Vigene Biosciences (Rockville, MD, USA). GLuc is a secreted luciferase protein that is used to monitor biological processes in-vivo [301, 302, 305, 306]. HUCPVCs were seeded at a cell density of 23000 cells/cm² and 24 h later were exposed to the virus at a MOI of 100 in 100μl/ cm² of culture medium. After 24 h of incubation at 37°C, 5% CO₂, cells were washed with PBS 3 times and fresh growth medium was added. Engineered HUCPVCs were harvested 3 days later for experimental use.

5.2.7 Flow cytometry on MSC surface markers post labeling

Briefly, 1x10⁵ of freshly harvested HUCPVCs, unlabeled or labeled with DiR, were washed in PBS containing 1% BSA and 2mM EDTA (flow buffer) and incubated for 30 min at 4°C in the same buffer containing the following conjugated anti-human antibodies (at 1:5–1:20 dilutions): HLA-DR-FITC (eBioscience, Cat. 11-9952-42), CD31-APC (eBioscience, Cat. 17-0319-42), CD45-FITC (eBioscience, Cat. 11-9459-42), CD105-PE (BD Bioscience, Cat. 560839), MHC I-APC (BD Biosciences, Cat. 555555). The cell suspensions were washed with the flow buffer and then resuspended in the flow buffer. Immediately before analysis on Cytomix FC 500 flow cytometer (Beckman Coulter), cells were stained with Propidium Iodide (PI) to exclude dead cells. Next, 5000 live or PI-negative events were collected. Labeling efficiency was assessed by measuring DiR fluorescence in FL5, while surface marker detection via antibodies was measured in FL1 for FITC and FL2 for PE. Flow cytometry data were analyzed using Kaluza Software (Beckman Coulter) (Fig. 5-7). The efficiency of DiR-HUPVCs was measured as 99.54%.

In the case of GLuc transfected HUCPVCs, flow cytometry was used first to determine the efficiency of transfection by intracellular detection of the GLuc protein. Surface marker profile was then assessed following the same procedure described above. Control and GLuc-transfected
cells were washed in the flow buffer and fixed in 2% paraformaldehyde solution. Cells were then washed twice with the flow buffer and permeabilized in pre-chilled (-20°C) 100% methanol at -20°C. This was followed by washing twice with the detergent solution (PBS+0.5% BSA+0.5% Tween 20) and resuspension in the detergent solution. A rabbit anti-human primary antibody against GLuc protein (New England Biolabs, Cat. E8023S) was added at 1:50 dilution and incubated for 30 min at 4°C. Cells were again washed twice and resuspended in the detergent solution for incubation with a secondary goat anti-rabbit antibody conjugated to Alexa Fluor 546 (Life Technologies, Cat. A-11010). Finally, cells were washed twice with the detergent solution and resuspended in the flow buffer for flow cytometric analysis. Gating of HUCPVCs was based on FS and SS characteristics and 5000 of the gated events were counted. Transfection efficiency was determined by measuring the fluorescence signal of Alexa Fluor 546 in the FL2 channel. The efficiency of GLuc-HUCPVCs was measured as 85.4%. For phenotyping, control and GLuc-transfected HUCPVCs were incubated with the following conjugated anti-human antibodies (at 1:5 – 1:50 dilutions): HLA-DR-FITC (eBioscience, Cat. 11-9952-42), CD45-FITC (eBioscience, Cat. 11-9459-42), CD10-FITC (eBioscience, Cat. 11-0106-42), CD142-APC (eBioscience, Cat. 17-1429-42), CD31-APC (eBioscience, Cat. 17-0319-42), CD34-APC (eBioscience, Cat. 17-0349-42), CD90-FITC (BD Bioscience, Cat. 561969), CD73-PE (BD Bioscience, Cat. 550257), CD105-PE (BD Bioscience, Cat. 560839), CD166-PE (BD Bioscience, Cat. 559263), CD146-PE (BD Bioscience, Cat. 550315), CD140b-PE (BD Bioscience, Cat. 558821) and MHC I-APC (BD Biosciences, Cat. 555555). PI was added prior to analysis and 5000 live events were collected (Fig. 5-9).

5.2.8 Animal studies

The animal experiments were performed in accordance with Canadian Council on Animal Care and the University of Toronto Animal Care Committee under protocol number 20011728.
Eight weeks old adult CD1 female mice (Charles River Laboratories, Gatineau, QC, Canada) were acclimatized for 1 week prior to animal studies. Mice were then randomized into groups. Mice were fed with alfalfa-free pellets (Harlan Teklad, Cat. TD97184) during acclimatization and throughout the study period. Lack of chlorophyll in Alfalfa-free pellets reduces auto-fluorescence in the stomach and intestines and hence improves fluorescent optical imaging.

5.2.9 Carrageenan Induction in mice hind paw

We used a well-developed model of inflammation (carrageenan-induced hind paw), and induced 9-weeks old CD1 mice with 1% w/v carrageenan (unilaterally). Mice were anesthetized with Isoflurane in nitrous oxide and oxygen, (900 ml total flow rate; 3-4% induction and 2% maintenance). 25 µl of 1% w/v λ-Carrageenan (Sigma, Cat. 22049) solution in 0.9% saline was injected (needle gauge 30) subcutaneously into the plantar region of the right hind paw.

5.2.10 MSC-delivery and measurement of inflammation

At the first peak of inflammation, 4h post-induction, mice were anaesthetized to have control over the site of injection. Next, different sources of MSCs (i.e., mBMMSCs, hBMMSCs, HUCPVCs) at a density of 1.3x10^6 cells resuspended in 0.9% saline were delivered contralaterally in the dorsal thigh musculature, n=6-7/group. Note: Due to insufficient total cell number at P3, we were only able to deliver mBMMSCs for the designated 48h time point. The circumference of the inflamed paw was measured using a cotton thread prior and post MSC delivered at 4, 24, and 48 h post-induction. Paw measurements were taken at 360° from digit 1 plantar to digit 1 dorsal, as illustrated with black ink in Fig. 5-1C.

5.2.11 Assessment of Bio-distribution of IM transplanted MSCs

I. Real-time longitudinal assessment (in-vivo imaging)

To assess the bio-distribution of MSCs, mice were imaged prior and post MSC delivery, at 24, 48h, and up to 33 days. Mice were imaged under anesthesia. The fluorescent flux emitted by
the DiR-labeled HUCPVCs were quantified using an IVIS® imaging system. Images were acquired using 2D epi-illumination to identify the region of interest where fluorescent signal was detected. Next, each mouse was imaged with 3D FLIT (with an exposure time of 200 ms) which includes a Computed Tomography (CT) dataset of the mice’s surface tomography and multiple fluorescent trans-illumination images to allow 3D reconstruction (excitation/emission at 745/840nm). For bioluminescence, we aimed to correlate the in-vivo cell number to the luminescence flux, for which \(1 \times 10^6, 8.75, 7.5, 6.25, 5, 3.75, 2.5, \text{and } 1.25 \times \text{10}^5\) cell densities of HUCPVCs were suspended in PBS in black clear bottom tissue culture treated 24-well plate (Visiplate) (PerkinElmer. Cat.1450-605). Coelenterazine (Nanolight, Cat. 3031) (300ug/300ul sterile ddH2O/well) was added using a multichannel pipette immediately prior to open filter imaging using Xenogen IVIS system (Fig. 3-9B, C). Then we assessed the kinetics of Gaussia-coelenterazine for which we IM-delivered \(1 \times 10^6\) GLuc-HUCPVCs, intramuscularly, followed by IV perfusion of 250ug/100ul coelenterazine in sterile ddH2O and acquired open filter images every 1 minute up to 15 minutes (Fig. 3-10A) on auto exposure. The quantification of the region of interest (ROI) encircled in red, is computed in Fig. 3-10B. For the primary study, mice were intravenously injected with Coelenterazine (250ug/100ul sterile ddH2O per 23g mouse) and in-vivo GLuc activity was measured at different time points (24, 48, 72, 96, and 120h post HUCPVC delivery). Data acquisition was obtained 1-minute post substrate administration (exposure time=9s. Fluorescent and luminescence 3D reconstruction was performed using the Living Image software. Anatomical co-registration was performed using the Xenogen digital mouse atlas included in the IVIS software.

II. Polymerase chain reaction (PCR) and droplet digital PCR (ddPCR):

Custom primers were designed against mouse SRY, murine actin, human FARS2, and human/mouse GAPDH using the National Center for Biotechnology Information (NCBI) website
To assure primer specificity, we blasted against human/mouse genome (NCBI-BLAST). RT-qPCR was the carried out on the synthesized cDNA using SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad, Cat. 1725272) according to the manufacturer’s instructions (10µl reaction volume) at an annealing temperature of 65°C. Each sample was run in triplicates and Bio-Rad CFX384 Touch™ System was used for fluorescence measurement. Murine Actin, and h/m GAPDH was used for normalization of expression level of mouse SRY and human FAR2. For more accuracy, we assessed the copy number of human/male mouse genomic DNA using ddPCR technique on organs and the induced paw. PCR reaction containing QX200 ddPCR EvaGreen Supermix (BioRad, Cat., 186-4034), with various concentrations of DNA– as low as 50ng of and as high as 1µg (using HindIII restriction enzyme; New England Biolab, Cat. R0104T). BioRad Automated Droplet Generator was used following manufacturer recommendations followed by amplification in a thermal cycler for ddPCR. Using QX200 droplet reader and QuantaSoft software, the positive and negative droplets for target DNA was quantified. Please see table 5-1 for the primer design details.

Table 5-1 Gene transcript, designed primer sequences (5’–3’), EMBL fragment size (amplicon length-bp)

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Sequence</th>
<th>Description, EMBL accession number</th>
<th>Amplicon length(bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FARS2</td>
<td>F: 5’-TGCCCATTAGCAGCTGGTT-3’&lt;br&gt;R: 5’-GTTGATCCTGCCCTACACCA-3’</td>
<td>Homo sapiens chromosome 6, GRCh38.p7, Primary Assembly, NC_000006.12</td>
<td>193</td>
</tr>
<tr>
<td>h/m GAPDH</td>
<td>F: 5’-CAGCCAGCCACCCACCTCCACCTT-3’&lt;br&gt;R: 5’-CATTGCCACCCACCCTGTTGCT-3’</td>
<td>Homo sapiens chromosome 12, GRCh38.p7, Primary Assembly, glyceraldehyde-3-phosphate dehydrogenase, isoform 1, NC_000012.12</td>
<td>228</td>
</tr>
<tr>
<td>Mouse SRY</td>
<td>F: 5’-GTCAAGCGGCCCATGATGAT-3’&lt;br&gt;R: 5’-AGTTGCTATTTCTCTCCTG-3’</td>
<td>Mus musculus sex determining region of Chr Y (Sry), mRNA, NM_011564.1</td>
<td>202</td>
</tr>
<tr>
<td>Murine Actin</td>
<td>F: 5’-GATGCAAGGTGGCTAAGTGGA-3’&lt;br&gt;R: 5’-CACTCGGGCAGGTGAAACT-3’</td>
<td>Mus musculus strain C57Bl/6j chromosome 5, GRCh38.p4 C57Bl/6j actin, cytoplasmic 1, NC_000071.6</td>
<td>121</td>
</tr>
</tbody>
</table>
5.2.12 Blood and tissue collection

Terminal blood collection was performed through intracardiac (IC) puncture while mice were under deep anesthesia. Terminal blood samples were collected at 4, 24, and 48h post-induction of inflammation. The induced paw and the injected muscle, as well as organs (kidneys, lungs, liver, heart, and spleen) were immediately excised post-sacrifice, frozen in liquid nitrogen, and further cryogenically grinded into fine powder with mortar and pestle. Fine and well homogenized powders were immediately stored at -80°C for further analysis.

5.2.13 Protein isolation and purification

Approximately 40mg frozen tissue powder from both muscle and paw were weighed followed by an immediate addition of 1ml mammalian cell lysis buffer supplemented with Benzonase Nuclease and Protease Inhibitor solution provided in the protein extraction kit, Qproteome Mammalian Protein Prep Kit (QIAGEN, Cat. 37901). Tissue dissociation was performed by means of ceramic beads (Omni International, Cat. 19-646) and Mini-BeadBeater (MIDSCI, Cat. 607), 2x45 seconds. Samples were then centrifuged at 1000xg for 5 min at 4°C to remove tissue debris and the supernatant was aliquoted and stored at -80°C for further use.

5.2.14 DNA isolation and purification

Approximately 100 mg frozen tissue powder from paw, kidneys, lungs, liver, heart, and spleen were weighed followed by an immediate addition of 720µl Purelink Genomic Digestion Buffer supplemented with 80µl Proteinase K provided in DNA purification Kit (Invitrogen, Cat. K182001). Tissue dissociation was performed by means of ceramic beads and Mini-BeadBeater, 2x30 seconds. Samples were then incubated on a heat block overnight at 55°C to obtain maximum lysis (recommended by the kit). Samples where then centrifuged at 14,000xg for 3 min at room temperature to remove debris. DNA lysis was further purified using the aforementioned kit and the recommended protocol. DNA samples were then aliquoted and stored at -80°C for further use.
**5.2.15 Enzyme-linked immunosorbent assay (ELISA) and quantification**

Blood serum supplemented with protease inhibitor cocktail (BioShop, Cat. PIC001.1) that contains 2mM AEBSF HCL, 130µM Bestatin, 14µM E-64, 1µM Leupeptin, and 0.3µM Aprotinin. Samples stored at -80°C were then tested for TNF-α levels using TNF-α Quantikine ELISA Kit (R&D systems, Cat. MTA00B). Prior to measurements, protein samples were centrifuged at 1000xg for 5min at 4°C to remove cellular debris. A standard curve was generated using the recombinant TNF-α protein in the kit and all samples were tested in triplicate and measurements followed the recommended procedure. Sample absorbance was measured at 450nm using a plate reader (Molecular Devices, SpectraMax i3x Multi-Mode Detection Platform).

Paw lysates that were extracted from tissue powders (Qproteome Mammalian Protein Preparation Kit) contained protease inhibitor and were stored at -80°C. MPO activity in the paw lysates was measured using MPO ELISA kit (Hycult biotech, Cat. HK210). Prior to measurement, protein samples were centrifuged at 1000xg for 5min at 4°C to remove cellular debris. A standard curve was generated using the protein standard provided in the kit. Sample absorbance was measured at 450nm. Total protein of the same samples (i.e., same aliquots), were measured using Pierce BCA protein assay kit (Thermo Fisher scientific, Cat. 23225) and normalized to the total measured protein. All samples were tested in triplicate and measurements followed the recommended dilution and procedures.

**5.2.16 Western Immunoblotting**

Protein lysates from muscle tissue were quantified using Pierce Bovine Serum Albumin Standard (ThermoFisher Scientific, Cat. 23208). Total protein concentration of 5µg was chosen to run on 4-15% Mini-PROTEAN® TGX stain-Free™ Protein Gels (BioRad, Cat. 4568083). Protein samples, including the positive control recombinant TSG-6 protein, prior to running on gel, was added to 4x Lammeli sample buffer (BioRad, Cat. 1610747) that contained 2-Mercaptoethanol
(BioRad, Cat. 1610710), with ratio 3 parts sample with 1-part Lammeli. Samples were then incubated on a heat block at 95°C for 5 min, followed by 5 min on an ice bath. Next, the denatured protein samples were centrifuged at 14xg for 2 min at 4°C and proceeded with gel electrophoresis at 165V for 35-40 min. 10μl All Blue Pre-stained protein ladder (BioRad, Cat. 1610373) was loaded on the first column followed by the positive control standard recombinant TSG-6 protein (BioRad, 2326-TS) and protein samples in the consecutive columns. The Running buffer used was 1x Tris/Glycin/SDS (BioRad, Cat. 1610732) that contained 25mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3. The gels were then activated for 1 min using ChemiDoc™ MP Imaging System (Biorad). Gels were then transferred to LF PVDF blots following the manual for Trans-Blot Turbo™ RTA Mini LF PVDF Transfer Kit (BioRad, Cat. 1704274) using Trans-Blot® Turbo™ Transfer System (BioRad) at 25 V, 1.3 A, for 7 min. The blots were then rehydrated in 100% methanol followed by transfer to 1x Tris-buffered saline, 0.1% Tween 20 (TBST). Protein blots were then imaged in the ChemiDoc™ MP Imaging System (Biorad) for chemiluminescent detection of total protein used for data normalization and loading control. The blots were then blocked with 3% Bovine Serum Albumin (BSA) at room temperature for 90 min. Primary antibody to human/mouse TSG-6 (R&D Systems, Cat. 259820) was diluted 1:1000 in 1% BSA and incubated for 60 min at room temperature with mild agitation, followed by 4x15 min washes with TBST. The blots were then incubated in horseradish Peroxidase-linked secondary antibody, goat anti-mouse (Jackson ImmunoResearch Laboratory, Cat. 115-035-146), 1:2000 dilution in 1% BSA, for 30 min followed by 4x15 min washes with TBST. Bands were detected using Clarity Max Western ECL Substrate (BioRad, Cat. 1705062) according to manufacturer’s protocol. Quantification was performed digitally using Image Lab™ Software (BioRad). Band signals were normalized to the digitally quantified total protein (BioRad stain-free system), (Fig. 5-6B). Note:
Protein levels of TSG-6 were only measured in mice treated with human MSCs since the mice treated with mBMMSCs showed little improvement in downregulation of inflammation.

5.2.17 GLuc measurement in blood circulation

To assess the secretory activity of IM-xenotransplanted HUCPVCs, we collected about 20µl blood samples from tail vein of mice in EDTA-treated cuvettes at 24, 48, 72, 96, and 120 h post HUCPVC delivery. Bioluminescence was tested using BioLlix GLucassay kit (New England Biolabs, Cat. E3300L). 5µl whole blood x 3 replicates of each sample was tested in white luminescence Greiner 96 flat bottom, using BMG PHERA star injector-equipped luminometer with the following setting: 2-10 seconds of signal integration, and 50µl of injection volume, gain 4000, 400rpm double orbital shaking for 30 seconds to mix the whole blood with the substrate before reading. Data was normalized to controls.

5.2.18 Giemsa and Immunofluorescence labeling

To assess the activity of neutrophils and macrophages in the inflamed hind paw of mice prior and post MSC delivery, we used Giemsa staining to identify neutrophils and anti-F4/80 to immunofluorescently (IF) label macrophages in cryo-sections. Post sacrifice, mice paw were fixed in 4% PFA in PBS for 24h at 4°C. Samples were then washed, and decalcified using Immunocal (StatLab, Cat. 1414-32) and decalcification was confirmed by MicroCT scout-view. Samples were then embedded in optimal cutting temperature (OCT) compound. Transverse plantar cross-sections were consistently obtained from the same area of the hind paw. Sections are taken from the soft tissue layers before reaching the bones. When choosing the sections, to be consistent, the difference in the thickness of the inflamed paw in the control group in comparison to the paws from MSC-treated group with minimal edema was taken into consideration. 8-10µm cross-sections of the plantar hind paw was stained either with Giemsa or IF using anti-F4/80 (ABD-BioRad, Cat. MCA497R) 1:300 for 1h at room temperature; and goat anti-rat cross adsorbed Alexa Fluor 647
(Invitrogen, Cat. A21247) 1:400 for 30 minutes at room temperature. Antigen retrieval was performed on the cryo-sections used for IF staining, using 20ug/ml Proteinase K (ThermoFisher, Cat. EO0491) in TE buffer (50mM Tris and 1mM EDTA at pH 8) for 15 mins at room temperature, followed by permeabilization with 0.3% Triton in PBS and blocking using 10% goat serum for 2.5h at room temperature in humidified chamber. Nucleic acid staining was performed using Hoechst 1:1000 for 5 mins at room temperature.

To investigate the fate of GLuc-HUCPVCs in the muscle tissue, the injected muscle tissues were cryo-sectioned (5-7µm) and fluorescently labeled using anti-Gluc (E8023S) 1:400 for 1h at room temperature and goat anti-rabbit-cross adsorbed Alexa Fluor 568 (Invitrogen, Cat. A11011) 1:600 for 30 mins at room temperature. Antigen retrieval, permeabilization, blocking, and nucleic acid staining protocol was the same as previously mentioned.

**5.2.19 Statistical Analysis**

Statistical analysis of groups/time points was performed by means of one-way or two-way ANOVA-multiple comparison Tukey Statistical test between the means of replicates at significance level of 95% CI. Each figure legend indicates details of the statistical analysis performed. Statistical analysis was performed using GraphPad Prism 6.01 (GraphPad Software Inc., La Jolla, CA, USA).

**5.3 Results**

**5.3.1 Reduced edema in the mouse paw**

As shown in the timeline in Fig. 5-1A, MSCs were delivered IM in the contralateral quadriceps muscle (Fig. 5-1B) at 4h post-induction of inflammation. This time was chosen because acute inflammation developed by 1% carrageenan has been shown to elicit a biphasic response with an initial peak at 4h with a second peak of inflammation starting from 6h and rising to 72h before resolving around 96h [186]. Changes in the paw circumference post treatment were
measured as shown in Fig. 5-1C.

Figure 5-1 Experimental design (A) time-line; (B) animal model of acute inflammation and the IM site of transplanted MSCs, dorsal thigh quadriceps muscle; (C) Method of paw circumference measurement. Hind paw circumference measurements were taken using a thread at 360° from digit 1 plantar to digit 1 dorsal, as illustrated with black ink.

The change in the paw circumference (Fig. 5-2), as a metric of the amount of edema, substantially increased throughout the 48h study in the untreated, compared to normal values. On the contrary, edema in both the hBM MSC and HUCPVC groups reduced over the period of 48h. The greatest effect was seen with the HUCPVC group at 24h with a statistically significant difference compared to controls at the same time point. The hBM MSC group only achieved this level of edema reduction by 48h. Edema reduction further decreased in the HUVPVC group from 24 to 48h where the significance of the difference between controls increased to P<0.001 due, in part, to the continuing increase in circumference of the control group paws. The mBM MSC group showed little difference but not significant (P=0.8694) from the controls at 24h although this was somewhat increased by 48h (P<0.05).
5.3.2 Neutrophil and macrophage activity abrogation in the mouse paw

To assess the effect of IM transplanted MSCs on immune cell extravasation to the carrageenan-induced paw, we first assessed the degree of neutrophil infiltration into the tissue surrounding the vasculature in cryo-sections prepared from a representative animal from each group (Fig. 5-3A). At 48h, the number of neutrophils in the paws of untreated mice was qualitatively higher (~60%) than the MSC-treated ones. Moreover, the HUCPVC group showed a lower number of neutrophils compared with hBMMSC group at both 24h and 48h.

To quantify these differences, we measured MPO in homogenized paw tissue lysates (5 mice/group). MPO is an enzyme released from the neutrophil azurophilic granules that is known
as a hallmark for the neutrophilic activity. As shown in Fig. 5-3B, neutrophilic activity in untreated mice paws was primarily increased to $\bar{X}=9.51 \times 10^4$ ng/mL at 24h post inflammation induction, following which it reduced by 33.54% to $\bar{X}=6.32 \times 10^4$ ng/mL at 48h. Although the same pattern was observed in the hBMMSC and HUCPVC groups, their MPO activity was significantly lower than untreated mice. hBMMSCs showed an initial increase in MPO activity of $\bar{X}=5.43 \times 10^4$ ng/mL at 24h that reduced by ~55% to $\bar{X}=2.44 \times 10^4$ ng/mL at 48h. On the other hand, HUCPVCs had attained this level at 24h ($\bar{X}=2.55 \times 10^4$ ng/mL), which further reduced by ~55.3% to $\bar{X}=1.14 \times 10^4$ ng/mL at 48h. While the difference was eradicated by 48h, when both cell populations were equally effective, a more rapid response of the HUCPVCs suggests that HUCPVCs abrogated neutrophil infiltration more effectively than hBMMSCs. No significant (MPO) difference was observed in the mBMMSCs-treated group at 48h compared to controls ($P=0.4286$). Macrophage numbers in the soft tissue of the carrageenan-induced paw were reduced in hMSC-treated groups compared to controls (Fig. 5-4).
Figure 5-3 Neutrophil abrogation in acute inflammation post MSC transplantation. (A) Giemsa staining of hind paw plantar 8-10µm cryo-embedded cross-sections. Nuclei (Blue), Neutrophilic cytoplasm (pink), black arrows show neutrophils, scale bar: 20µm; (B) MPO activity in the inflamed paw. Two-way ANOVA-multiple comparison Tukey statistical test is performed between the means of measurements obtained from 5 mice/group and 4 measurements/animal/timepoint. MPO measurements were obtained using an ELISA kit. Statistical analysis is presented at significance level of 95% CI, and data is expressed as means ± S.E.M. Asterisk (*) represents p≤0.05; (**) p≤0.01; (***) p≤0.001, and (****) represents p≤0.0001, otherwise no significant difference was noted p>0.05. Green =Control (●); Gray=mBMMSCs (▲); Purple=hBMMSCs (■); Navy=HUCPVCs (●).
Figure 5-4 Macrophage activity in acute inflammation post MSC transplantation in 8-10µm cryo-sections of the inflamed paw. Macrophages F4/80 positive (MCA497R and Alexa Fluor 647-red) and nucleic acid stain (Hoechst 33342-blue). Scale bars=250µm.

5.3.3 Reduced level of TNF-α in the blood serum:

To assess the quantity of circulating inflammatory mediators, we measured the amount of TNF-α, as a principal inflammatory moderator at the 48h timepoint. As shown in Fig. 5-5, the level of TNF-α in the control group ($\bar{x}$=15.83 pg/mL) was significantly higher (~23.46%) than the circulating level of TNF-α in mBMMSC treated group ($\bar{x}$=11.88pg/mL). However, the level of TNF-α in hBMMSC, HUCPVC, and healthy control groups were all lower than the detection limit of the utilized assay kit and thereby were all considered at the same level, and all significantly lower than the control and mBMMSC groups. It should also be noted that one animal showed significantly higher TNF-α serum measurement compared with the rest of the samples ($\bar{x}$=47pg/mL) which we considered an outlier and was not included in the statistical analysis, but it is illustrated in Fig. 5-5.
5.3.4 Increased level of TSG-6 in the MSC transplanted muscle

Next, we measured the level of TSG-6 [348]–a key anti-inflammatory protein released by activated MSCs in response to TNF-α stimulation–in muscle tissue lysate from the MSC transplant site. As shown in the chemiluminescent blots (Fig. 5-6A), the intensity of the bands increased in the MSC transplanted muscle tissues compared to controls. TSG-6 protein has been normalized to the total protein (Fig. 5-6B) of each sample. In Fig. 5-6C, TSG-6 in the HUCPVC-transplanted muscle tissues, normalized to stain-free blots of total protein/column, showed relative intensity unit of $\bar{X}=1.69 \times 10^7$ at 24h that is significantly higher (52.6%) compared to that from the hBMMSC
group $\bar{X}=8.01 \times 10^6$, which was not statistically raised above the control level. However, by 48h both the HUCPVC and hBMMSC groups had increased significantly, $\bar{X}=5.24 \times 10^7$ and $4.54 \times 10^7$ respectively, to reach approximately the same level.

Figure 5-6 TSG-6 protein level in lysate of transplanted muscle (dorsal thigh quadriceps). (A) Chemiluminescent blots comparing mesenchymal stromal cell transplanted samples with controls; (B) Total protein loading per lane prior to primary antibody incubation using stain-free technology. TSG-6 protein has been normalized to the total protein of each sample; (C) Relative intensity of TSG-6 protein normalized to stain-free blots of total protein/column. One-way ANOVA-multiple comparison Tukey statistical test is performed between the means of measurements obtained from 5 mice/group. Statistical analysis is presented at significance level of 95% CI, and data is expressed as means ± S.E.M. Asterisk (**) represents $p \leq 0.01$, otherwise no significant (N.S.) difference was noted $p>0.05$. Green =Control (●); Purple=hBMMSCs (■); Navy=HUCPVCs (●).
5.3.5 Biodistribution of IM transplanted MSCs

To assess MSC biodistribution, HUCPVCs were fluorescently labeled with the membrane dye DiR–prior IM-delivery. To evaluate the stability of the MSC phenotype post-labeling, a panel of surface markers were tested using flowcytometry (Fig. 5-7). DiR labeling of HUCPVCs did not result in any change in the expression levels of CD73\(^+,\) CD105\(^+,\) CD90\(^+,\) MHCI\(^+,\) HLA-DR\(^-\) surface receptors (Fig. 5-7).

**Figure 5-7** Profile of cell surface epitopes of Unmodified and DiR-labeled HUCPVCs by flow cytometry-PI was added to exclude dead cells. (A) DiR-labeled HUCPVCs exhibited the same cell surface profile as unmodified cells with near 100% of cells positive for CD73, CD105, CD90 and MHC I (MSC markers) and negative for HLA-DR, CD45 and CD31. DiR effectively labeled 100% of viable HUCPVCs compared to control; (B) Abundance of cell surface receptors as measured by MFI. The MFI of DiR was a direct measure of the cytoplasmic membrane it labels. Abbreviations: HUCPVCs, human umbilical cord perivascular cells; PI, Propidium iodide; MFI, mean fluorescence intensity.

DiR-labeled HUCPVCs (1.3x10\(^6\)) were then transplanted in the quadriceps muscle and followed by IVIS imaging (Fig. 5-8). Although the carrageenan-induced inflammation was resolved after 96h (Δ paw circumference = 0), the DiR fluorescence signal was still detectable for at least 33 days post-transplantation. Interestingly, no fluorescence signal was detected in other organs within the course of the study. We further investigated the presence of xenotransplanted
male MSCs (genomic DNA-sex determining region Y) in the female mice organs (lungs, heart, kidneys, spleen, liver, and inflamed paw). Using both RT-qPCR and droplet digital PCR (ddPCR), no cells were detected in lungs, heart, liver, kidneys, spleen or paws, except in one inflamed paw from the hBMMSC group.

![Figure 5-8](image-url) Longitudinal study of 1.3x10^6 DiR labeled HUCPVCs, xenotransplanted intramuscularly in CD1 mice for 33 days. The longitudinal image is a representative image of n=3 mice. The fluorescence signal post day 7 is a false positive signal transmitted by the engulfed HUCPVCs possibly in the cytoplasm of phagocytes.

**5.3.6 Temporal host immune response to xeno-IM transplanted MSCs**

To further verify the bioactivity of the IM-transplanted HUCPVCs in the CD1 immunocompetent mice, we adeno-transfected the HUCPVCs with Gaussia Luciferase. To evaluate the stability of the MSC phenotype post-transfection, a panel of surface markers were tested using flow cytometry (Fig. 5-9A). GLuc transfection of HUCPVCs resulted in a reduction in the expression level of CD10 by 44.11%, CD146 by 11.84%, MHCI^+ by 16.25%, and an increase in the expression level of CD142 surface receptors by 27.43%. Other markers, CD90^+, CD73^+, CD105^+, CD166^+, CD140b^+, HLA-DR^- did not significantly change post-transfection (Fig. 5-9).
As shown in Fig. 5-10A-C, 48 h post IM-transplantation of GLuc-HUCPVCs, the signal was reduced by 62.5 at 48h%; and 89.5% by 72h; 97.36% by 96h; and 99.3% by 120h. To assess the rate at which the secreted proteins in the muscle enter the blood circulation, we measured the secreted GLuc-protein in the whole blood (Fig. 5-10D). As shown in Fig. 5-10D, there is a strong correlation between the density of IM-residing HUCPVCs and the circulating GLuc level. Circulating GLuc-protein decreased by 44.5% 48h after transplantation; 77.2% after 72h; 95.7% after 96h; and 99.7% after 120h. We further investigated the fate of GLuc-HUCPVCs in the muscle tissue 5 days post transplantation. As the MSC-transplanted muscle cross-sections in Fig. 5-11 show, GLuc-HUCPVCs were detectable in the muscle after 5 days of xenotransplantation, although at low density which was below the detection limit of IVIS system. The low level of GLuc-protein detected in the blood at day 5 correlates with the low density of MSCs detected in the muscle. On the contrary, the membrane dye (DiR) (Fig. 5-8) survived beyond 33 days.
Figure 5-10 Longitudinal study of 1.3x10^6 GLuc expressing HUCPVCs, xenotransplanted intramuscularly in CD1 mice. Mice were intravenously injected with Coelenterazine (250ug/100ul sterile ddH2O per 23g mouse) and in-vivo GLuc activity was measured at different time points (24, 48, 72, 96, and 120 hours post HUCPVC transplantation). Data acquisition was obtained 2 minutes post substrate administration (exposure time=9s), longitudinal images are representative images of the behavior of cells in n=5 mice; (A) Reconstructed IVIS 3D images; (B) 2D images (open filter); (C) Longitudinal quantification of luminescence flux (p/sec/cm²/sr) from the represented mouse in (b), region of interest (ROI) is circled with red. (B) GLuc reporter activity in the blood at different time points, n=5. Data represented as relative light unit (RLU) and normalized to controls at each time point. One-way ANOVA-multiple comparison Tukey statistical test is performed between the means of measurements obtained from 5 mice. Statistical analysis is presented at significance level of 95% CI, and data is expressed as means ± S.D. Asterisk (*) represents p≤0.05; (**) p≤0.01; otherwise no significant (N.S.) difference was noted p>0.05.

Additionally, we should emphasize that, during IM delivery, care must be exercised to avoid puncture of major vessels. In one of our animals, we observed an immediate extravasation
of blood, upon “IM” delivery in the hind limb musculature, indicating that a major blood vessel was inadvertantly punctured. Although we excluded this mouse from our studies, we observed a systemic distribution of MSCs—including signal from the site of ear tag injury used to identify this mouse following MSC delivery (Fig. 5-12).

Figure 5-11 Fate of GLuc-HUCPVCs in the muscle tissue 5 days post transplantation. (A) high and (B) low magnification of GLuc-HUCPVCs in 5-7µm cryo-sections of the muscle tissue 120 h post transplantation. GLuc positive (E8023S and Alex Fluor 647-red) and nucleic acid stain (Hoechst 33342-blue). Scale bars: 125 µm.
5.4 Discussion

In this study, we demonstrated that unstimulated and non-engineered IM-delivered MSCs have the potency to diminish an existing acute source of inflammation in an anatomically distant location through systemic distribution of anti-inflammatory factors. We have further demonstrated that a neonatal source of MSCs (HUCPVCs) exhibits higher sensitivity to inflammatory stimuli, in vivo, than an adult source (BMMSCs), resulting in more effective downregulation of inflammation. These findings corroborate our in vitro results, which showed that HUCPVCs exhibited higher sensitivity, and a prompter response, to TNF-α stimulation compared to hBMMSCs [82].

Clinical studies have already confirmed the safety of IM-delivery of MSCs in patients with CLI [233, 236, 237], with no signs of tumorigenesis or necrosis at the IM site of MSC delivery. Despite this proven safety, MSC IM-delivery has been mainly employed to treat focal pathologies. However, even in such therapies, Laurila et al., 2009, reported a local increase in VEGF that was
of host (rat) rather than human origin [277]. This observation further emphasizes the paracrine effect of the exogenously transplanted cells rather than their engraftment and differentiation [277]. One of the main concerns in local IM-therapy is identifying the best time for delivery with respect to the stage of the inflammation, to avoid MSC survival and potency being impaired by harsh environmental tissue cues [226, 227]. These observations and concerns further condone transplanting MSCs away from the site of injury.

Some studies have employed the IM-delivery route to target cardiac myopathy. Shabbir et al. reported significant cardiac improvement in a hamster model of heart failure by hind limb IM-transplanted MSCs. Increased systemic levels of HGF, leukemia inhibitory factor (LIF), and GM-CSF were measured in the MSC-treated groups that resulted in myocardial repair [223]. Similarly, Mao et al., showed improved cardiac function following fore- and hind-limb IM delivery of MSCs in a rat model of dilated cardiomyopathy; with increased levels of HGF, insulin-like growth factor-1 (IGF-1), LIF, GM-CSF, and VEGF compared to controls [225]. Both studies reported plasma levels of cytokines and growth factors related to angiogenesis (IGF-1, VEGF), apoptosis (HGF, GM-CSF), or support of growth and differentiation of stem and progenitor cells (LIF) [349]. However, to our knowledge, Liu et al., is the only study which has evaluated the potency of IM-delivered MSCs to downregulate inflammation, in a mouse model of antibody mediated/LPS challenge arthritis. The MSCs were delivered prior to induction of inflammation and no immunomodulatory effect was observed for IM-transplanted MSCs that were not engineered to express human soluble tumor necrosis factor receptor II (hsTNFR) [228]. On the contrary, in our studies we have demonstrated that MSCs, IM-delivered post inflammation induction, significantly downregulated macrophage activity, systemic levels of TNF-α and neutrophil infiltration at the inflammatory site. As a counterpoint to Liu et al., our findings show the importance of the time-frame within which MSCs should be administered to attain their immunomodulatory action.
In this study, we delivered MSCs 4h after induction of inflammation to not only coincide with the peak inflammatory response [186] but also to assess the effect of MSCs on alleviation of such acute inflammation. PGE2, as a potentiator of acute inflammation, has been shown to cause vasodilation followed by the release of histamine and bradykinin from tissue resident sentinel leukocytes leading to formation of edema that is involved in the first phase of acute inflammation [350]. Following vasodilation, neutrophils are recruited to the inflamed tissue and, as the inflammation resolves, the cells die apoptotically and the remnants are then removed by macrophages and DC[85]. Our results demonstrate that HUCPVCs have abrogated neutrophil infiltration more effectively than adult sources of MSCs at earlier timepoints (confirmed by MPO levels and histology of the induced paw tissue). This could partly be attributed to the higher inherent expression level of TSG-6 in unstimulated HUCPVCs compared with hBMMSCs and their prompt response to low-grade inflammation that was observed in our in-vitro studies [82].

TSG-6 protein has been shown to abrogate neutrophil migration and infiltration [169] through interference with chemokine/ GAGs and chemokine CXCL8 interactions [170]. In addition, TSG-6 protein has been shown to interact with macrophages through CD44 receptor to decrease translocation of NF-κB [135] and inhibit inflammation. Our results provide additional insights to the previously demonstrated therapeutic advantages of neonatal MSCs [40, 79, 283, 284], including higher MSC frequency, growth rate and life span, and also superior immunomodulatory properties compared to adult tissue-derived MSCs [38, 39, 41, 317, 351].

It should be noted that we did not observe MSC migration to the site of inflammation that has been reported for IV-delivered MSCs. Vilalta et al., reported detection of 10% of MSCs at the IM delivery site and colonization of 1.5% MSCs in the liver over an 8-months study [274]. However, in our study, in agreement with other reported studies of IM-delivered MSCs [221, 255, 345], we
did not observe migration to, or colonization of, distant organs—delivered MSCs remained in the muscle mass (except in the one case when we were aware of vasculature puncture). Our assessments of the genomic DNA of inflamed paw tissue and organs (i.e., heart, lungs, liver, spleen, and kidneys), together with both fluorescence and luminescence imaging in two consecutive studies, confirmed that IM-delivered MSCs remained in the skeletal muscle.

Assessment of the therapeutic efficacy of human-derived MSCs xenotransplanted in immunocompetent mice, have mainly been conducted in short-term studies over a period of a few days due to the rapid host immune rejection. Nevertheless, Prockop et al., 2017 have recently strongly endorsed the validity of such models in pre-clinical studies [128]. However, the poor response of mBMMSCs compared to hMSCs in our experiments beg additional comment. First, as discussed by Prockop et al. [128], mouse MSCs are known to exhibit genomic instability, and grow slowly in culture. On the contrary, hMSCs can undergo 35-55 population doublings without undergoing spontaneous transformation [352] and are thus more stable. Second, mMSCs are known to effect immune suppression by different mechanisms to hMSCs [128, 189]. Both of these differences may have contributed to the greater efficacy of hMSCs in our assays. Indeed, in this study we have used TSG-6, which is a key anti-inflammatory protein secreted by MSCs and that has been proposed as a biomarker to predict the efficacy of MSCs [164], as a surrogate for the library of anti-inflammatory mediators secreted by MSCs. Our objective was not to prove the mechanism of TSG-6 action—which has been done so convincingly by others, or illustrate the plethora of anti-inflammatory agents released by MSCs, but to compare the potency of the MSCs harvested from different tissue sources in this experimental model. Nevertheless, we believe that future mapping of comprehensive cytokine responses in such models could inform the current debate on the therapeutic functional phenotypes of MSCs harvested from diverse tissue sources.
We showed that the delivered cells were only secretorily functional for up to 4 days and undetectable after 5 days. On the contrary, DiR-labeled human cell membrane fragments were still detectable at 33 days, potentially resulting from remnant phagocytosis by host macrophages, as has previously been described [353]. Thus, longer in-vivo survival of a xenogeneic stromal cell would require pharmacological immunosuppression.

Finally, current systemic MSC therapy is described as a “hit and run” phenomena [218], primarily owing to the short dwell-time of infused MSCs which are transiently effective. To extend the period over which sustained modulation of an inflammatory milieu is attained, multiple timed doses of MSCs have been administered [354-356]. An alternative would be to employ MSCs at a site that allows extended dwell-time of secretorily active MSCs for optimal therapeutic efficacy per dose. Although the experiments reported herein were, of necessity, of short duration in an immune-competent murine inflammation model, in a clinical setting the extended MSC dwell-time afforded by IM delivery could potentially be an advantage over the transitory efficacy of IV delivery. Indeed, human skeletal muscles account for about 40% of total adult body weight, and comprise large striated muscle fibers [357] that may provide suitable adherence sites for exogenously delivered MSCs. In addition, the high level of vascularization present in skeletal muscle, together with the contractile movement of muscles, provide an efficient putative route for the transport of the therapeutic agents into the systemic circulation [358].

5.5 Conclusions

The MSC response to TNF-α stimulation, in vivo, is MSC population and source dependent. Neonatal MSCs, delivered IM at a distant site, exhibited higher levels of secreted TSG-6 protein, and an earlier response, to a contra-lateral acute inflammation than MSCs derived from
adult BM. Thus, neonatal MSCs may represent a stronger candidate than those derived from adult BM to treat inflammatory diseases.
Chapter 6: General Discussion, Conclusions, and Future Work

6.1 General discussions and limitations

The hypothesis underlying the work presented herein was that a neonatal source of MSC, IM-delivered in the skeletal muscle will downregulate a distant source of inflammation more efficiently than an MSC population derived from an adult tissue source. The data presented in Chapter 4 demonstrates that HUCPVCs elicit a prompt response to TNF-α independent of the TNF-α dosing level compared with hBMMSCs. Furthermore, the functionality of HUCPVCs in-vivo, in response to immune and inflammatory responses also demonstrated the efficient response of HUCPVCs compared with hBMMSCs. The outcome of the presented studies favors the original hypothesis and corroborates the reported literature that also suggest superior immunosuppressive potency of neonatally-derived MSCs, in particular, the studies originating from umbilical cord tissue compared with adult tissue sources [39, 41, 75]. In the discussion section of Chapter 4 and 5, we have discussed each study independently. In this Chapter, we will assess the similarities and differences in the results observed across in-vitro and in-vivo studies.

6.1.1 Immomodulatory response of MSCs: In-vitro vs. In-vivo

In Chapter 4, we reported the anti-inflammatory response of HUCPVCs, hBMMSCs, and mBMMSCs to TNF-α stimulation, in-vitro. TNF-α was chosen as a key pro-inflammatory mediator that directly stimulates upregulation of TSG-6, a key anti-inflammatory mediator. There are certainly advantages and challenges to a 2D culture environment. It is advantageous that we can systematically assess the effect of a single mediator (TNF-α) on the expression pattern of TSG-6, but disadvantageous as the microenvironment, in-vitro, is far from the complex
microenvironment *in-vivo* where various other mediators would directly/indirectly influence the expression pattern of TSG-6.

In terms of overall response of MSCs, the results both *in-vitro* and *in-vivo* suggest that HUCPVCs have an earlier response to an inflammatory stimulant which, *in-vitro*, was found to be dose-independent. One major difference between the two studies is the response time. *In-vitro*, the maximum TSG-6 expression of hBMMSCs was observed in 60 min which for all concentrations declined at 24 and 48h after stimulation. However, *in-vivo*, the local level of TSG-6 protein was measured to be higher at 48h compared with 24h. Therefore, the *in-vitro* timeframe of the response of MSCs can only be used for *in-vitro* pre-activation (priming) purposes and does not represent the response time of MSCs *in-vivo*.

In another set of experiments in Chapter 4, we primed the MSC sources *in-vitro*, to investigate the response time of MSCs from different sources. We were mainly interested in the early time-points, while majority of MSC studies, routinely, incubate MSCs for 10-24h with TNF-α to activate and enhance anti-inflammatory cytokine production. Based on our findings, presented in Chapter 4, there is a need for reassessment of MSC activation protocols. Our results showed that TSG-6 expression level at 24 and 48h post-stimulation was downregulated almost in all cell sources and for all concentrations. In addition, TNF-α treatment can be performed by either single addition to the cultures, similar to our methods, or continuous multi-step delivery over the course of incubation. Two major factors that may potentially cause downregulation of the anti-inflammatory potency of MSCs post 24, and 48h, *in-vitro*, could be (*i*) degradation of the recombinant TNF-α (rTNF-α) in the supernatant over time; (*ii*) desensitization of the cells to soluble TNF-α stimulation.
Another interesting difference that we observed between the *in-vitro* and *in-vivo* results was the anti-inflammatory/immunomodulatory effect of hBMMSCs in response to the dose of stimulant. *In-vitro*, hBMMSCs were only significantly responsive when stimulated with higher concentration of TNF-α (50ng/mL) after 60 min of stimulation which is beyond the range of physiological level of circulating TNF-α in minor and advanced inflammation cases in humans (0.02-6ng/mL) [328-334]. On the contrary, HUCPVCs response to 1ng/mL further highlights their potential use in inflammatory conditions.

*In-vivo*, the response was more time-dependent since hBMMSCs were equally effective as HUCPVCs at 48h. Conclusively, both neonatal and adult-derived MSCs efficiently downregulated a distant source of inflammation but HUCPVCs showed a faster response. It is important to re-iterate that while each source of MSCs has distinct cell surface receptor number and pattern [359], they might also use different mechanisms of immunomodulation [75].

### 6.1.2 Importance of the method of analysis to reduce bias

**PCR data:** In Chapter 4, we extensively demonstrated and discussed the importance of assessment of the stability of reference genes used for normalization. We reported that TNF-α stimulation of MSCs also affects the reference genes and reiterated the importance of testing a panel of reference genes (we tested 30 genes) and choosing minimum of 3 most stable genes and normalizing data to the geometric mean of the 3 genes. This is important as the expression pattern and fold change data depends on this normalization step. MIQE’s guidelines provide details on the best PCR quantification practices [323].

**Western Blot data:** In Chapter 5 we have shown quantification of TSG-6 protein in the lysate of muscles. We used the semi-quantitative method of western blot. Western blot has for long been relying on the use of reference genes for quantification of proteins. To avoid reliance on
reference genes, the stain-free technology that we used provides the possibility of acquiring an image of total protein prior to specific antibody labeling. This method reduces the need of reliance on a reference gene quantification, as it allows quantification to the total protein loading. Data presented by many studies are only relying on the functionality of a reference gene that could have been adversely affected by the treatment, as we demonstrated in Chapter 4.

6.1.3 Potential Effect of Cell Culture on MSC Secretome

MSC isolation, preparation, and adaptation has indeed been an important factor that has caused variation in the results obtained from different laboratories. To minimize the impact of such factors, we have obtained all populations of cells from master bank distributors such as for hBMMSCs (Center for the Preparation and Distribution of Adult Stem Cells), mBMMSCs (Scripps Research Institute), and HUCPVCs (Tissue Regeneration Therapeutics) and we have followed the distributor’s recommended standard operating procedure (SOP) for culture expanding the MSCs. Furthermore, we have mentioned in Chapter 4, that Wang et al., 2012 have shown that serum starvation increases induction of TSG-6 in MSCs [342]. Clearly, it was not necessary to serum-starve the HUCPVCs, as they were grown entirely in serum-free conditions; however, we did not serum starve either the human or murine BMMSCs to avoid sudden changes in the biological milieu of these cells.

6.2 Overall Conclusions

From the studies presented in this thesis, it can be concluded that:

- HUCPVCs are more sensitive to TNF-α stimulation compared with hBMMSCs, but expression level varies across donors.
- Baseline expression level of TSG-6 in unstimulated HUCPVCs is 2.14-fold higher than unstimulated hBMMSCs.
- MSC response to TNF-α stimulation is MSC population and source dependent, but neonatal MSCs may be a stronger candidate than those derived from adult BM to treat inflammatory diseases.
- IM delivery of MSCs may be an option for the treatment of remote sites of inflammation, while MSCs have extended-dwell time in-situ in the skeletal muscle.

### 6.3 Future Prospective

From the studies performed, future studies that can be proposed are extended to the following questions:

- **What is the cause of the decline in the expression level of TSG-6 at 24 and 48h in HUCPVCs and hBMMSCs? Is it due to the degradation of TNF-α in culture or desensitization of MSCs to TNF-α stimulation?**

In inflammatory conditions and more persistently chronic diseases such as rheumatoid arthritis, persistent TNF signaling in various cell types lead to continuous cellular activation, inflammatory cues, migration of leukocytes, and tissue damage. Cytokines have short half-life and generate signals of short duration [360]. The duration of NF-κB activation is autoregulated through transcription of IκB-α (NF-κB inhibitor) [361]. Synthesized IκB-α retains NF-κB in the cytoplasm, but when expressed in the nucleus, it also inhibits interaction of NF-κB with DNA [362]. TNF signaling can be downregulated by (i) shedding of soluble receptors [363, 364]; (ii) ligand-induced downregulation of cell surface receptor by receptor-mediated endocytosis [365, 366]; (iii) interaction between TNF signaling and intracellular inhibitors [367, 368]. Human vascular endothelial cells exposed to TNF-α have shown rapid activation of NF-κB (≤20 min) which was persistent for (>20h) [369]. Although IκB-α degraded within 30 min, it reappeared by
4h and again degraded by 22h [369]. Conversely, Poppers et al. have shown that normal human fibroblasts persistently exposed to TNF-α demonstrated rapid degradation of IκB-α followed by incomplete reappearance. Suggesting that fibroblasts exposed to TNF-α, do not become completely desensitized to TNF-α signaling, only removal of TNF-α from the cultures resulted in complete IκB-α resynthesis [370]. The differences in the pattern of activation and desensitization of cells might be cell source dependent. We also observed differences in the activation pattern of HUCPVCs compared with hBMMSCs, and further reduction in the level of TSG-6 at 24 and 48h post exposure to TNF-α. It is important to first assess the possibility of degradation of TNF-α in the cultures over time by temporal monitoring of the level of TNF-α. Then to conduct desensitization assessment of the cells, re-stimulate both HUCPVCs and hBMMSCs after 24 and 48h and evaluate whether the cells are still responsive to TNF-α stimulation. To better understand the activation pattern of HUCPVCs and hBMMSCs independent of the expression level of TSG-6 by each cell population, it is important to directly measure NF-κB and IκB-α activities.

- **What is the phenotype of IM-delivered MSCs post-repair of needle injury?**

As discussed in Chapter 2, the phenotype of IM-delivered MSCs is not well understood. Since the IM-delivery route potentiates extended dwell-time for the exogenously transplanted MSCs, their extended therapeutic potency should also be assessed. This knowledge is necessary in understanding the fate of delivered MSCs and determining the required treatment frequency. It is well-known that needle creates a small injury at the muscle site where MSCs are delivered [220] [277] that may affect the activation and fate of the delivered MSCs. Therefore, it is necessary to assess the fate of IM-delivered MSCs in healthy mice by comparing MSCs pre- and post- needle-injury phenotypes. Transplanted muscles can be digested, and the collected cells can be sorted to separate the transplanted MSCs for multi-parametric phenotypic assessment.
• **Can IM-delivered MSCs be re-stimulated in-vivo? What is the optimal frequency of IM-delivery of MSCs?**

As discussed in Chapter 2, some clinical and pre-clinical studies have used multiple injection strategies to maximize the therapeutic effects of the IM-delivered MSCs. While the number of surviving cells was reported to increase with multiple injections, either no other differences were attributed to the multiple dosing [247] or an enhanced therapeutic effect was observed [282]. In our in-vitro studies, we have shown that HUCPVCs can be significantly stimulated with a low dose of TNF-α (1ng/mL). Once (A) the fundamental mechanisms contributing towards the downregulation of TSG-6 upon long-term exposure to TNF-α, and (B) the phenotype and effective period of immunomodulatory response of IM-delivered HUCPVCs are understood, then (C) it would be intriguing to assess the possibility of the re-activation of the originally IM-delivered MSCs in-vivo with a low dose of TNF-α (1ng/mL). TSG-6 measurements can be conducted to assess the activation state of the MSCs.

• **Assess the difference between HUCPVCs and hBMMSCs with regards to other anti-inflammatory/immunomodulatory factors.**

In Chapter 4, we have assessed the anti-inflammatory response of HUCPVCs compared with hBMMSCs through stimulation with TNF-α and measurement of TSG-6 gene expression. In Chapter 5, MSC functional response to a source of inflammation was assessed. The results indicated that the difference in the response of HUCPVCs and hBMMSCs lies in the sensitivity of the neonatal source of MSCs to inflammation and faster response time compared with hBMMSCs. We have mentioned two points in chapter 1: (i) TSG-6 gene expression can also be stimulated, indirectly, using other cytokines and growth factors (i.e. IL-6, IFN-γ, TGF-β, PGE2), although TNF-α and IL-1β are the main stimulants; (ii) Different populations of MSCs can have different therapeutic and immunoregulatory benefits. Therefore, it is important to assess the effect of various
other mediators and also to further assess the other therapeutic potencies of BMMSCs and HUCPVCs to enable choosing the right source of MSCs for the targeted therapy.


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322. Mediators Inflamm.


### Appendix I

**Humeroradial Joint**

**Glenohumeral Joint**

**Joints of Forelimb**

**Joints of Hind Paw**

**Lumbar Facet Joints**

**Femoroacetabular Joint**

**Fig. A1-1** Radiographs of a 35 months old Dunkin Heartly guinea pig. Assessment of humroradial, glenohumeral, joints of forelimb and hind paw, lumbar facet joints and femoroacetabular joints. As judged by the radiographs, there are no severe lesions of OA in the assessed joints except the knee joints shown in Fig. 3-1.
Figure A1-2 pEnter vector map in which Gluc has been cloned into the MCS section.