Mechanisms of Allogeneic Mesenchymal Stem Cell Improvement of Endothelial Function in Patients with Heart Failure and Frailty

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MECHANISMS OF ALLOGENEIC MESENCHYMAL STEM CELL IMPROVEMENT OF ENDOTHELIAL FUNCTION IN PATIENTS WITH HEART FAILURE AND FRAILTY

By

Courtney Heather Premer

A DISSERTATION

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MECHANISMS OF ALLOGENEIC MESENCHYMAL STEM CELL
IMPROVEMENT OF ENDOTHELIAL FUNCTION IN PATIENTS WITH HEART
FAILURE AND FRAILTY

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Cardiovascular disease (CVD) is a leading cause of morbidity and mortality in the United States and worldwide. Furthermore, CVD is heavily linked to frailty, a comorbidity afflicting elderly patients. Endothelial dysfunction—characterized by diminished endothelial progenitor cell (EPC) function and flow-mediated vasodilation (FMD)—is central to the pathophysiology of heart failure (HF) and frailty. Current therapies are unable to reverse or stop the progression of these diseases, lending way to the emergence of regenerative medicine approaches and the use of stem cells, most notably mesenchymal stem cells (MSCs). MSCs are pro-angiogenic, immunomodulatory, antifibrotic, and also stimulate endogenous endothelial cell proliferation and function, thus having the potential to restore endothelial dysfunction. Recent clinical trials in HF patients with ischemic and non-ischemic cardiomyopathy illustrate that MSC therapy improves cardiac function. However, the specific mechanisms underlying this therapeutic effect remain controversial.

In this study, we tested the hypothesis that allogeneic MSCs preferentially improve endothelial function by increasing EPC function and restoring FMD via a
mechanism involving the suppression of pathologic vascular endothelial growth factor (VEGF), stromal derived factor-1 alpha (SDF-1α), and tumor necrosis factor alpha (TNFα). Accordingly, EPC-colony forming units (EPC-CFUs) and FMD were measured in patients with dilated cardiomyopathy (DCM), ischemic cardiomyopathy (ICM), and frailty at baseline and three months post either allogeneic or autologous MSC therapy. The mechanism was studied in patients with DCM. More specifically, the vasculogenic potential of allogeneic versus autologous MSCs was measured in vitro. Additionally, patient serum VEGF and TNFα were measured at baseline and three months post MSC treatment, as well as MSC secretion of SDF-1α and TNFα.

Our results revealed exciting and important implications for the future design of stem cell trials. We found that patients with DCM, ICM, and frailty have endothelial dysfunction at baseline, evident by reduced EPC-CFUs and FMD. Allogeneic, but not autologous, MSCs were able to improve this dysfunction three months post treatment in patients with DCM and ICM. Mechanistically, we found human umbilical vein endothelial cells (HUVECs) with impaired vasculogenesis due to pharmacologic nitric oxide (NO) synthase inhibition, were rescued by allogeneic MSC-conditioned medium. Furthermore, circulating VEGF and TNFα were profoundly elevated in DCM patients and only allogeneic MSCs were able to restore these levels towards normal. Additionally, autologous MSCs secreted significantly higher levels of SDF-1α than allogeneic MSCs. There were strong correlations between EPC-CFUs and FMD, EPC-CFUs and VEGF, EPC-CFUs and SDF-1α, EPC-CFUs and TNFα, VEGF and TNFα, and VEGF and SDF-1α.
Ultimately, these findings reveal a novel mechanism by which allogeneic MSCs secrete normal levels of SDF-1α, which results in normal levels of VEGF signaling, an increase in EPC bioactivity, an improvement in FMD and NO bioavailability, and a reduction in the pro-inflammatory signaling of TNFα, resulting in a significant improvement in endothelial function. These findings have significant clinical and biological implications for the use of MSCs in HF and other disorders associated with endothelial dysfunction.
DEDICATION

My thesis is dedicated to my supportive and loving family: my parents, Ilene and Howard Premer, brother, Blake Premer, grandparents, Rita and Howard Ullman and Marlene and Arnie Premer, and Jorge Barragan.
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Chapter 1 Introduction

1.1 Cardiovascular disease and heart failure

Cardiovascular disease (CVD) is the leading cause of death in the United States, with approximately one million men and women dying from CV complications annually\(^1\). Post myocardial infarction (MI), the heart undergoes remodeling which hampers left ventricular (LV) function\(^2\). Despite significant therapeutic advancements over the past decade to slow progression, there are no therapies that repair cardiac structural damage or restore function.

1.1.1 Dilated cardiomyopathy

Notably, dilated cardiomyopathy (DCM) is the most prevalent type of non-ischemic cardiomyopathy\(^3\). DCM occurs when the left ventricle is enlarged resulting in the heart’s diminished ability to pump blood properly, ultimately leading to systolic dysfunction with normal LV wall thickness. Furthermore, cardiac remodeling after MI can play a significant role in accelerating LV dysfunction and can lead to progressive heart failure (HF). Emerging studies suggest there is a significant inflammatory component in the pathogenesis of DCM \(^4\) \(^5\), with endothelial dysfunction playing a vital role in the progression of HF in these patients.

Currently, there is no cure for DCM. Pharmacological interventions such as ACE-1 inhibitors, β-blockers, and mineralcorticoid antagonists are the standard of treatment for patients and often progression of the disease necessitates heart transplant \(^3\). Thus, it is imperative to explore novel treatments
for DCM, and stem cell therapy provides promising results as a new wave of treatment for DCM.

1.1.2 Ischemic cardiomyopathy

Ischemic cardiomyopathy (ICM) is the most prevalent cause of HF in the Western World. It is characterized by a lack of oxygen supply that causes considerably depressed LV function generally due to coronary artery disease (CAD). This dysfunction causes a reduction in blood supply to the body and in the infarcted myocardium, which is ultimately the main cause of morbidity and mortality. In particular, this ischemia results in cardiomyocyte apoptosis and necrosis during which the heart undergoes progressive remodeling and hypertrophy resulting in this diminished LV function.

Current therapies include pharmacologic intervention, coronary artery bypass grafting (CABG), and coronary artery stents, however similar to strategies for DCM, these therapies also fail to treat the pathophysiological changes following an ischemic injury. Thus, interventions that can regenerate damaged myocytes and myocardium is essential to treating ICM, again making stem cell therapy an attractive candidate.

1.2 Aging and frailty and their link to cardiovascular disease

Frailty is a multifaceted disorder that is heavily linked to CVD. Characterized by high levels of inflammatory markers, chronic fatigue, and poor gait speed, frailty and CVD share similar risk factors. Accumulating research shows that resident heart and bone marrow-derived stem or progenitor cells are
involved in maintaining CV homeostasis and may be activated to proliferate and
give rise to new cells in response to injury\textsuperscript{11, 12}. The age-related decrease in CV
tissue repair capacity may be attributed to impairment in differentiation and/or
functional capacity of these stem cells\textsuperscript{13}. Moreover, current research illustrates
that frailty clinically manifests as CVD. Ultimately, both diseases are burdened by
chronic inflammation\textsuperscript{14}, and currently there are no therapies to address this
pathologic inflammatory state.

Cell-based therapy has demonstrated sustainable improvements in heart
failure—most notably in patients with ICM\textsuperscript{15-18}—and therefore holds great
promise as a novel approach to treating frailty. It has been observed that the
heart loses its regenerative potential with aging, therefore restoring its
regenerative potential is a critical target for treating frailty.

1.3 Endothelial dysfunction

1.3.1 Endothelial dysfunction in heart failure and frailty

Endothelial dysfunction plays a key role in HF and frailty
pathophysiology\textsuperscript{19, 20, 21}(Figure1.1). Endothelial dysfunction occurs when the
vascular endothelium is unable to properly dilate and constrict in response to
signaling molecules or shear stress. More specifically, the vascular endothelium
controls the balance between nitric oxide (NO), reactive oxygen species (ROS),
vasomotor tone, and inflammation, and it is a crucial regulator of peripheral blood
flow\textsuperscript{19, 22}. Lack of NO bioavailability—which can most notably be caused by
diminished endothelial nitric oxide synthase (eNOS), impaired endothelial
progenitor cells (EPCs), alterations in cell signaling, and accelerated NO
degradation by ROS—modulates endothelial dysfunction\textsuperscript{23}. Ultimately, these impairments lead to pathological changes including modified anticoagulant and anti-inflammatory properties of the endothelium and restricted vascular remodeling, which are detrimental to patients already with HF and frailty and reciprocally can cause HF and frailty.

Specifically in HF patients, left ventricular function is impaired, which results in a reduction in blood flow and reduced stroke volume, and therefore reduced shear stress on the luminal surface of the endothelium\textsuperscript{24}. This reduction results in a lower production of endothelium-derived NO and consequently hindered NO-dependent dilation. Moreover, endothelial dysfunction contributes to the progression of reduced coronary and systemic perfusion.

Literature clearly demonstrates the link between frailty and CVD, and studies are beginning to emerge demonstrating the relationship between endothelial dysfunction, frailty, and CVD\textsuperscript{21}. In aging, there is an increase in oxidative stress, inflammation, and asymmetric dimethylarginine (ADMA)\textsuperscript{25}. This pro-inflammatory profile switch is one of the crucial factors mediating endothelial dysfunction in aging and frailty. Therapies for ameliorating endothelial dysfunction include exercise training, L-Arginine administration, ACE inhibitors, and antioxidant supplements, however none of these treatments cure endothelial dysfunction\textsuperscript{26}. 

Endothelial dysfunction and endothelial progenitor cells (EPCs) are mononuclear cells that predominantly reside in the bone marrow, and they promote proper endothelial function via endothelial regeneration and angiogenesis. Specifically, they play a pivotal part in maintaining vascular homeostasis as well as in mediating vascular repair in damaged endothelium by stimulating the release of NO from the endothelium. EPCs are paramount to preserving endothelium integrity and function. Notably, EPC function is critical to maintaining proper endothelial function, because pathologically elevated endothelial dysfunction results in cardiovascular disease (CVD) and frailty, and reciprocally CVD and frailty can cause endothelial dysfunction. Abbreviations include nitric oxide (NO), reactive oxygen species (ROS), endothelial progenitor cell (EPC), and vascular endothelial growth factor (VEGF).
mature endothelium has a limited regenerative capacity and therefore relies on 
EPCs for vascular repair\textsuperscript{29}. EPCs have the ability to form colony units \textit{in vitro}, 
which serves as a measure of endothelial function\textsuperscript{30, 31 32}.

Clinical studies have demonstrated that circulating EPC levels serve as a 
predictor of CV events, frailty, and endothelial dysfunction\textsuperscript{30, 33 30, 34}. Low 
numbers of EPC-colony forming units (EPC-CFUs) have been linked to high 
Framingham risk scores for adverse CV health outcomes\textsuperscript{30}. Of note, there is 
evidence that NO-deficient environments stimulate mesenchymal stem cell 
(MSC) involvement in angiogenesis\textsuperscript{35}. We recently published that patients with 
HF had severely reduced EPC-CFUs and impaired endothelial function, and that 
allogeneic MSC therapy restored this deficit\textsuperscript{36}. Additionally, it has been recently 
reported that maintenance of vascular homeostasis by EPCs is attenuated with 
age due to functional deficits rather than depletion of progenitor cells\textsuperscript{13}.

\textit{1.3.3 Endothelial dysfunction and flow-mediated vasodilation}

Similar to measuring EPC-CFUs, flow-mediated vasodilation (FMD) 
percent is also used to assess endothelial dysfunction. FMD is the response of 
the endothelium to shear stress that results in vasodilation—predominantly NO 
mediated—and thus is a direct measurement of conduit artery function, or in 
other words, endothelial dysfunction. Measuring FMD\% via ultrasound is non-
invasive, making it an attractive method of assessing endothelial dysfunction 37. 
Specifically, FMD\% calculates the change in the arterial lumen diameter as a 
proportion of the initial diameter 38. Importantly, FMD has been shown to predict
long-term CV events\textsuperscript{39, 40}. Additionally, brachial reactivity has been shown to decline with age \textsuperscript{41 42}.

1.4 Signaling in heart failure, frailty, and endothelial dysfunction

1.4.1 Nitric Oxide

Nitric oxide (NO) bioavailability is a key player in the progression of heart failure and frailty \textsuperscript{43}. Notably, it affects myocardial function, systemic and pulmonary hemodynamics, and coronary and renal circulation\textsuperscript{19}. In heart failure, the release of NO via coronary circulation is impaired, and this impairment leads to an increased oxygen demand accompanied by a diminished peak oxygen consumption. Furthermore, NO is implicated in inflammation, cellular proliferation, and cytokine expression \textsuperscript{44}. Thus, it directly effects angiogenesis and is thought to modulate VEGF.

There is a critical balance between which NO is physiologic or pathologic. Under normal conditions in endothelial cells, NO synthase converts L-citrulline to L-arginine which enables NO to bind to soluble guanylyl cyclase and thereby activate cGMP as well as s-nitrosylate effector molecules \textsuperscript{44}. With regards to the heart, this signaling cascade enhances myocyte relaxation and diastolic function. Under pathologic conditions, overproduction of NO bioavailability is reduced and supplemented by the formation of ROS and cell damage. Most notably, peroxynitrites oxidize proteins involved in contractility and ion channels and also inhibit mitochondrial energy production\textsuperscript{44}. Ultimately, NO dysregulation resulting in endothelial dysfunction creates unfavorable changes in the nitro-redox balance.
and therefore has substantial negative effects in patients with heart disease and frailty.

1.4.2 Vascular endothelial growth factor (VEGF)

The vascular endothelial growth factor (VEGF) family is comprised of six ligands—VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and PIGF—that exert their function through two tyrosine kinase receptors, FLT-1 and KDR/Flk-1. VEGF signaling plays a fundamental role in both physiological and pathological vasculogenesis and angiogenesis, specifically responsible for stimulating endothelial cell survival, proliferation, and migration as well as NO release\(^45\).

VEGF is implicated in many different signaling pathways. It has been shown to activate the anti-apoptotic Akt/PBK signaling pathway which induces endothelial cell survival\(^46\). Additionally, VEGF can activate the MAP kinases ERK1/2 and JNK, which causes the proliferation of endothelial cells, and FAK which promotes migration and is thought to be the initiating step in angiogenesis\(^47\). VEGF can stimulate endothelial cells to produce NO, which in turn promotes vasculogenesis, angiogenesis, and vessel dilatation\(^45\). Furthermore, there is a reciprocal regulation in which VEGF synthesis can stimulate eNOS production of NO.

Ultimately, VEGF is a master regulator of vascular function. Aberrant signaling is implicated in chronic inflammation, ischemia, stroke, ischemic heart disease, diabetic retinopathy, and aging\(^48,49\), making it an attractive target for improving endothelial dysfunction, heart failure, and aging.
1.4.3 Stromal-derived cell factor 1 (SDF-1)

There is a dynamic interplay between stromal derived factor-1 (SDF-1) and VEGF. Particularly, it is has been shown that under ischemia, SDF-1 upregulates VEGF and in turn VEGF upregulates SDF-1\textsuperscript{50-52}. Furthermore, SDF-1 is the predominant chemokine involved in mobilizing EPCs from bone marrow\textsuperscript{31}, making it a crucial part of the mechanism involving EPCs and improved endothelial function.

SDF-1 is regulated by its G protein-coupled receptor, CXCR4, and it is ubiquitously expressed in many cell types ranging from endothelial cells to dendritic cells\textsuperscript{53}. SDF-1 was initially isolated as a T lymphocyte chemoattractant, and is thus thought to play a role in immunosurveillance \textsuperscript{54}. There are two known splice variants of SDF-1, SDF-1\textsubscript{α} and SDF-1\textsubscript{β}\textsuperscript{53}. Notably, SDF-1\textsubscript{α} is required for stem cell homing to bone marrow and accordingly plays a critical role in the mobilization of EPCs, MSCs, and CD117-positive stem cells\textsuperscript{55}. There is a short-lived upregulation of SDF-1\textsubscript{α} and subsequent recruitment of progenitor cells immediately after an ischemic injury—and notably a recruitment of EPCs that partake in neovascularization\textsuperscript{56}—however, this effect dissipates quickly\textsuperscript{57}.

It has been shown that SDF-1\textsubscript{α} can be utilized therapeutically to induce stem cell migration to infarcted myocardium in patients with ischemic cardiomyopathy and initiate tissue regeneration\textsuperscript{55}. However, like VEGF, there is a fine balance in which too much SDF-1\textsubscript{α} causes monocyte migration and inflammation\textsuperscript{55, 56}. Furthermore, in aging, wound healing is significantly impaired due to reduced levels of SDF-1\textsubscript{α} accompanied by diminished
neovascularization\textsuperscript{58}. In addition, SDF-1 has been shown to inhibit the apoptosis of T-cells, which is thought to contribute to the pathologic inflammation evident in aging and frailty\textsuperscript{59}. Lastly, it was demonstrated that rat MSCs overexpressing SDF-1\textsubscript{α} stimulate angiogenesis and ameliorate heart function \textsuperscript{60}, highlighting the need to elucidate the role of MSCs in enhancing SDF-1 signaling and thereby improving endothelial function in patients with heart failure and frailty.

\textit{1.4.4 Tumor necrosis factor alpha (TNF\textalpha)}

Tumor necrosis factor alpha (TNF\textalpha) is another cytokine that is heavily implicated in inflammation and plays a major role in heart failure, frailty, and endothelial dysfunction \textsuperscript{61,62,63}. TNF\textalpha is produced by macrophages, lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblast, and neurons after trauma and infection. Its primary role is to regulate immune cells and pro-inflammatory cytokine production, and accordingly causes apoptosis, cell proliferation, and both physiologic and pathologic inflammation via activation of the NF-kB, MAPK, and caspase pathways \textsuperscript{64}. Additionally, TNF\textalpha is produced by alloreactive T-cells, which is indicative of an immune rejection of foreign cells, and thus a cytokine of interest regarding allogeneic stem cell treatment\textsuperscript{65}.

Dysregulation of TNF\textalpha causes detrimental inflammatory reactions, tissue injury, and shock \textsuperscript{66}. Numerous studies have shown that TNF\textalpha is severely elevated in patients with heart failure \textsuperscript{67,68,69,63}, and it not only contributes to, but also causes, LV dysfunction\textsuperscript{70}. TNF\textalpha can initiate the downregulation of sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase via IL-1beta, which results in prolongation
of the Ca2+ transient. Furthermore, persistent TNFα signaling can uncouple the beta-adrenergic receptors from adenylyl cyclase. These effects are damaging to the heart’s calcium homeostasis and contractility, thus necessitating a therapy that can target abnormal TNFα signaling.

Increasing evidence demonstrates that aging and frailty is heavily associated with the upregulation of TNFα. Vascular dysfunction and inflammation are prevalent in frailty, and TNFα has been shown to worsen oxidative stress, vascular inflammation, endothelial apoptosis, and endothelial dysfunction. Unfortunately, there is no cure for halting the detrimental effects of TNFα signaling, making MSCs an appealing therapy due to their immunomodulatory and anti-inflammatory properties.

1.5 Mesenchymal Stem cells (MSCs)

1.5.1 Mesenchymal stem cell properties

Mesenchymal stem cells are a heterogeneous, multipotent stromal cell population that can be isolated from a variety of sources including bone marrow, cord blood, adipose tissue, dental pulp, muscle, and recently the heart. MSCs are capable of self-renewal and differentiation—specifically showing the capacity to differentiate into adipocytes, osteocytes, chondrocytes, and myocytes. Although human MSCs lack unique cell surface markers, they can be identified by the absence of hematopoietic markers CD34 and CD45 and the expression of CD105, CD73, CD90, and CD271. MSCs are further identified by their plastic-adherence in culture, fibroblast-like morphology, and ability to form colonies in vitro.
1.5.2 Mesenchymal stem cells and the immune system

The immunological properties of MSCs makes them a distinct stem cell type. In the human body, foreign molecules are recognized by a set of cell surface proteins, major histocompatibility complex (MHC), which bind to pathogenic or foreign fragments and display the epitope on the cells surface as a marker for T-cells’ recognition. This enables the immune system to discern whether the antigen is self or non-self, ultimately preventing the immune system from targeting its own cells. Furthermore, MHC markers are critical for assessing compatibility of donors for recipient cells and transplants. There are two main classes of MHC markers, MHC class I and MHC class II. MHC class I markers are displayed on all nucleated cells and present epitopes to killer T cells—ligand of the CD8 receptor—thereby mediating cellular immunity. On the other hand, MHC class II markers are expressed on antigen-presenting cells such as macrophages, B cells, and dendritic cells—with the main ligand being CD4 receptors from T-helper cells—therefore acting as the main response to allore cognition resulting in rejection of transplant cells. Specifically, human MSCs express low to moderate levels of histocompatibility complex class I (MHCI), and do not express major histocompatibility complex class II (MHCII) or costimulatory molecules B7 and CD40 ligand. Furthermore, they express low-intermediate levels of HLA class I antigens and low to no levels of HLA class II antigens. Therefore, MSCs do not elicit alloreactivity, and are thereby protected from innate immune system cell lysis during transplantation, and additionally they are able to escape recognition by alloreactive T cells. This is a critical characteristic
of MSCs, because traditional bone marrow derived stem cells contain lymphocytes, and notably T cells, which contribute to graft-versus-host disease (GVHD) during which the transplanted cells attack the recipient’s cells. Additionally, MSCs possess the ability to modulate the immune system via suppressing the formation of cytotoxic T cells, neutrophils, dendritic cells, natural killer cells, mast cells, and macrophages\textsuperscript{76}.

1.5.3 Mesenchymal stem cells and cell therapy

Recent clinical trials show the therapeutic potential of using bone marrow derived MSCs for patients with heart failure\textsuperscript{16, 79}. Specifically, MSCs have been shown to engraft into host tissue, have antifibrotic effects, as well as participate in immunomodulation, neovascularization, niche formation, and activate endogenous cells (Figure 1.2)\textsuperscript{74, 76 80}. Clinically, MSC trials for CVD have shown improvements in LV ejection fraction, end diastolic volume, stroke volume, myocardial mass, Minnesota Living with Heart Failure Questionnaire, and 6 minute walk test\textsuperscript{16, 17, 81 82}. Furthermore, research shows that MSCs are able to differentiate and mature into endothelial cells. Along these lines, we published that MSCs stimulate the bioactivity of EPCs in the failing heart in patients with depressed endothelial function and HF\textsuperscript{36}. Additionally, we found that allogeneic MSCs originating from young, healthy donors led to significantly greater improvements in endothelial function than autologous MSCS, which originated from older patients with comorbidities.
While many clinical trials have studied the effect of MSC therapy in patients with CVD, no studies have looked at MSC therapy in a frail population. Efimenko et al. demonstrated that adipose-derived MSCs from aged patients with coronary artery disease have impaired angiogenic potential, which parallels our finding that allogeneic MSCs have a greater therapeutic potential regarding improving endothelial function. Therefore, we are currently investigating the effect of allogeneic mesenchymal stem cell—derived from young, healthy donors—therapy in patients with frailty. 

**Figure 1.2** Therapeutic potential of mesenchymal stem cells (MSCs).
Chapter 2 Methods

2.1 Study Design

All patients were enrolled of one of three clinical trials: POSEIDON-DCM (NCT01392625), TRIDENT (NCT02013674), or CRATUS (NCT02065245). In both POSEIDON-DCM and TRIDENT, patients were randomized to receive MSCs by transendocardial delivery. In CRATUS, MSCs were delivered intravenously. Autologous MSCs were derived from the patient's bone marrow.

Figure 2.1 Endothelial function study design. Patients were recruited from three clinical trials and received treatment according to the figure schematic. Endothelial progenitor cell-colony forming units (EPC-CFUs), flow-mediated vasodilation (FMD), and serum was obtained at baseline and 3 months post injection to assess treatment outcome.
(iliac crest aspiration) 4-6 weeks before cardiac catheterization, while allogeneic MSCs were manufactured by the University of Miami Cell Manufacturing Program\textsuperscript{84}. Healthy subjects (n=10) were enrolled ranging in ages from 22-58 years and both genders. All subjects provided written informed consent.

### 2.2.1 Mesenchymal stem cell characterization

#### 2.2.1 Processing protocol

Both autologous and allogeneic MSCs were manufactured by the Foundation for Accreditation of Cellular Therapy (FACT)-accredited Good Manufacturing Practice (GMP) Cell Production Facility at the Interdisciplinary Stem Cell Institute, University of Miami Miller School of Medicine, as previously described\textsuperscript{79, 84}. Briefly, cells were plated and grown on plastic culture flasks and cultured in MEM alpha (Gibco) with 20% fetal bovine serum. Confluent cells were passed using 0.05% Trypsin (Gibco). Cells were released for patient administration after meeting the following criteria: negative for mycoplasma via polymerase chain reaction, ≥70% cell viability, growth assay via colony forming units-fibroblasts assay, positive for CD105 (>80%) and negative for CD45 by flow cytometry, and no growth of bacteria.

#### 2.2.2 Flow cytometry characterization

MSCs were removed from culture flasks by applying 0.05% Trypsin for 5 minutes. Cell pellets were resuspended with 1mL FACs buffer (1% BSA+5%FBS+PBS), counted, and divided into 1 million cells per FACs tube. Antibody cocktails conjugated with secondary antibodies (CD45, CD34, and CD105) were added to the cells and incubated on ice for one hour in the absence
of light. Subsequently, cells were washed twice with PBS and resuspended in 400µL of PBS for FACs analysis (Figure 2.2).

**Figure 2.2** Representative flow cytometry characterization of the autologous and allogeneic MSCs before injection. (A) Autologous MSCs are 97.6% CD105+. (B) Autologous MSCs are 97.5% negative for CD45. (C) Allogeneic MSCs are 96.2% CD105+ and 99.9% negative for CD45.

### 2.3 Endothelial progenitor cell-colony forming units (EPC-CFUs)

Peripheral blood samples were obtained from patients at baseline and three months after MSC injection. Mononuclear cells were isolated from blood samples using a Ficoll-Paque density gradient followed by subsequent washes. Red blood cells were lysed utilizing ACK lysis buffer. Five million cells were seeded on 6-well fibronectin-coated dishes (BD biosciences) in CFU-Hill medium (stem cell technologies, cat#05900)\(^{30, 85}\). 48 hours later, the non-adherent cells—purified endothelial progenitor cells—were collected and one million cells were seeded on 24-well fibronectin-coated dishes. On day five, EPC-CFUs were counted in six wells and the average was obtained. Colonies were counted as an EPC-CFU if they had a circular center of cells with characteristic endothelial cells uniformly projecting around the center (Figure 2.3). Conversely, colonies that failed to have
uniform projections around the center or were highly disorganized were not counted as EPC-CFUs (Figure 2.3).

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**Figure 2.3** Method used for identifying endothelial progenitor cell-colony forming units (EPC-CFUs). Top row illustrates positive EPC-CFUs, while bottom row illustrates clusters and disorganized colonies that were not considered EPC-CFUs.

2.4 Flow-mediated vasodilation (FMD)

Brachial artery diameter measurements and FMD% were performed in the morning, after an overnight fast. The subjects’ right arm was immobilized in an extended position, and the brachial artery was scanned via ultrasound 5-10cm above the antecubital fossa\textsuperscript{30, 37}. A brachial cuff was then inflated to a supra-systolic pressure (40 to 50mmHg above systolic pressure) for five minutes.
Subsequently, the cuff was deflated and the brachial artery diameter was recorded for three minutes.

2.5 Immunofluorescence

EPC-CFUs were directly fixed on fibronectin-coated dishes using 4% PFA. Cells were blocked in 10% normal donkey serum/0.3% Triton X-100/PBS for 1 hour and then incubated in anti-CD31 and anti-VEGFR overnight at 4º (DAKO #235218, Cell Signaling #55B1R). Next, cells were incubated in Alexa Flour 564 anti-mouse and Alexa Flour 488 anti-rabbit for 45 minutes at room temperature. Lastly, wells were cover slipped with Vectashield plus DAPI. Images were obtained using immunofluorescent microscopy.

2.6 Enzyme-linked immunosorbent assay (ELISA)

2.6.1 Vascular Endothelial Growth Factor (VEGF)

Vascular endothelium growth factor (VEGF) levels were measured in patient serum at baseline and 3 months after MSC treatment according to kit protocol (Life technologies #KHG0111). Serum was diluted 1:2 with standard diluent buffer.

2.6.2. Tumor necrosis factor alpha (TNFα)

Tumor necrosis factor alpha (TNFα) levels were measured in patient serum at baseline and 3 months post MSC treatment according to kit protocol (Life technologies #KHC3011). Serum was not diluted to increase sensitivity of TNFα detection. Additionally, TNFα was measured from MSC conditioned medium. Briefly, MSCs were cultured until 70% confluency and serum was removed from culture media. 24 hours after starvation, medium was collected, and spun in the
centrifuge at 500g to ensure no cells were collected. Conditioned medium was stored in -80° until use.

2.6.3 Stromal cell-derived factor 1 alpha (SDF-1α)

Stromal cell-derived factor 1 (SDF-1α) levels were measured from MSC conditioned medium according to kit protocol (Abcam #ab100637). Conditioned medium was collected as described above. Lastly, conditioned medium was not diluted to enhance assay sensitivity.

2.7 Matrigel assay

Human umbilical vein endothelial cells (HUVECs) were grown to passage seven in EGM-2 medium (LONZA). Autologous (n=7) and allogeneic (n=5) donor MSCs were grown to 70% confluence and the conditioned medium was collected. Briefly, MSCs were starved in MEM alpha for 24 hours at 5%, the supernatant was collected, centrifuged at 2000g for ten minutes, and stored at -20° until use. 50,000 HUVECs were plated on Matrigel (BD Biosciences) in 24-well plates and pre-treated with 15μM L-NAME (Cayman Chemical #80210) dissolved in alpha-MEM (GIBCO) for 45 minutes. 80% of either MSC conditioned medium (MSC-CM) or plain MEM alpha was added to respective treatment wells, and L-NAME was kept in the medium. After six hours, six pictures per well were taken and Image J was used to analyze tube length, networks, and vascular index (tube length x tube number).

2.8 Statistical analysis

To assess the difference between autologous and allogeneic groups, an unpaired, two-tailed T-test was used. To measure the difference before and after
treatment in each group, both a paired, two-tailed T-test and a one-way ANOVA was utilized. Correlations were measured using Pearson correlation, assuming a Gaussian distribution. Data are presented as mean and standard deviation of the mean. Both D'Agostino-Pearson omnibus normality test and Shapiro-Wilk normality tests were run to measure within-group variability on all data (only significant differences were reported as D'Agostino-Pearson).
Chapter 3. Patients with dilated cardiomyopathy have impaired endothelial function at baseline that is restored after allogeneic, but not autologous, MSC therapy

3.1 Endothelial function at baseline

3.1.1 EPC-CFU numbers are reduced and have an unhealthy morphology

Patients were assessed for colony formation before and three months after MSC treatment. At baseline, patients with dilated cardiomyopathy (n=23) had markedly reduced numbers of EPC-CFUs compared to healthy controls (n=10) (4±3 vs. 25±16, respectively, P<0.001; Figure 3.1A). Furthermore, colonies were disorganized in appearance, often clustering as balls of progenitor cells unable to properly form vascular networks (Figure 3.1B&C). Colonies were

![Figure 3.1](image)

Figure 3.1. Patients with dilated cardiomyopathy (DCM) endothelial progenitor cell colony formation at baseline. (A) Endothelial progenitor cell-colony forming units (EPC-CFUs) in patients with DCM (n=23) versus healthy controls (n=10) (P<0.0001). (B) Cluster images of impaired EPC-CFUs from DCM patients at baseline (20x).
stained for endothelial markers to illustrate they are positive for endothelial progenitor cell markers (Figure 3.2).

**Figure 3.2** Endothelial progenitor cell-colony forming units are positive for the endothelial markers CD31 and vascular endothelial growth factor receptor (VEGFR) (20x).

3.1.2 **FMD% is decreased in all patients**

Similarly, FMD% was assessed in patients at baseline and three months post MSC treatment. FMD% was also diminished in patients with DCM (n=23) at baseline compared to healthy controls (n=10) (6.1±3.5 vs. 8.9±2.2, P=0.03; Figure 3.3A).
3.2 Patient’s results three months post MSC therapy

3.2.1 Patients who received allogeneic MSC therapy have an improvement in EPC-CFU numbers, EPC-CFU morphology, and FMD%

Patients who received allogeneic MSC therapy (n=11) had dramatic improvements in endothelial function three months post treatment. Specifically, EPC-CFUs significantly increased post treatment (3±2 vs. 13±4, P<0.0001; Figure 3.4A). Furthermore, colonies were organized and healthy in appearance, with sparse clusters present (Figure 3.4 B&C). Correspondingly, FMD% also increased three months post allogeneic MSC treatment (5.5±3.8 vs. 8.7±5.0, P<0.0001; Figure 3.5). Together, these findings suggest that transendocardial
allogeneic MSC therapy stimulates EPC bioactivity and vessel dilation response in DCM patients.

**Figure 3.4** Endothelial colonies in patients with dilated cardiomyopathy (DCM) who received allogeneic mesenchymal stem cells (MSCs). (A) Endothelial progenitor cell-colony forming units at baseline and 3 months post treatment allogeneic MSC treatment (p<0.0001). (B) Representative images of colonies 3 months post treatment (20x).
3.2.2 Patients who received autologous MSC therapy have no improvement post injection

On the other hand, patients who received autologous MSCs (n=12) had no improvement in endothelial function from baseline to three months post treatment. Particularly, EPC-CFUs did not improve post injection (5±3 vs. 6±5, P=NS; Figure 3.6A), and there was a mixture of colonies and clusters with notable cellular disorganization in all patient samples (Figure 3.6B). Accordingly, FMD% did not increase from baseline to three months post autologous MSCs (6.6±3.2 vs. 7.5±2.8, P=NS; Figure 3.7).
Figure 3.6 Colonies in patients who received autologous mesenchymal stem cells (MSCs) (A) Endothelial progenitor cell (EPC) colonies from baseline to 3 months post injection after autologous MSC injection (n=12) (P=0.2). (B) Representative images of EPC colonies after autologous MSCs (20x).

Figure 3.7 Flow-mediated vasodilation (FMD) in patients with dilated cardiomyopathy who received autologous mesenchymal stem cells (n=12) (P=0.7).
3.3 Comparison of allogeneic MSC therapy versus autologous MSC therapy

3.3.1 There is a significant difference between EPC-CFUs and FMD% comparing allogeneic versus autologous MSC therapy

We compared allogeneic MSC treatment versus autologous MSC treatment in DCM patients and found there was a significant difference in endothelial functional outcome depending on the cells received. Patients who received allogeneic MSCs had a significantly greater positive change in EPC-CFUs from baseline to three months post injection compared to patients who received autologous MSCs (Δ10±3 vs. Δ0±3, respectively, P=0.0001; Figure 3.8A). Likewise, patients who received allogeneic MSCs had a greater positive improvement in FMD% from baseline to three months post injection compared to patients who received autologous MSCs (Δ3.2±2.3 vs. Δ0.3±2.8, respectively, P=0.005; Figure 3.8B). These results highlight the allogeneic MSC advantage.
3.3.2 There is a strong correlation between EPC-CFUs and FMD%

We defined endothelial function as the number of EPC-CFUs and the FMD%, and thus we looked at the correlation between these two parameters. There was a strong correlation between the change in EPC-CFUs and the change in FMD% from baseline to three months post injection in all patients (N=23, R=0.5, P=0.03; Figure 3.9). Additionally, comparing the baseline and three months post injection results in all patients and controls markedly demonstrated that patients who received allogeneic MSCs improved towards normal—evident by the overlapping error bars in the three month post injection allogeneic group and the healthy controls—while patients who received autologous MSCs did not (Figure 3.10).

**Figure 3.8** Comparison of allogeneic mesenchymal stem cell (MSC) therapy versus autologous MSC therapy. (A) Change in endothelial progenitor cell-colony forming units from baseline to 3 months post treatment comparing patients who received allogeneic MSCs (n=11) to patients who received autologous MSCs (N=12)(P=0.0001). (B) Change in flow-mediated vasodilation (FMD %) from baseline to 3 months post injection comparing patients who received allogeneic MSCs to patients who received autologous MSCs (P=0.005).
Figure 3.9 Correlation between the change in flow-mediated vasodilation percent (FMD%) and the change in endothelial progenitor cell (EPC) colonies in all patients receiving allogeneic (n=11) and autologous (n=12) MSCs (R=0.5, P=0.03).

Figure 3.10 Flow-mediated vasodilation (FMD%) versus number of endothelial progenitor cell (EPC) colonies at baseline and three months post injection in controls (n=10), patients receiving allogeneic mesenchymal stem cells (MSCs)(n=11), and patients receiving autologous MSCs (n=12).
Chapter 4. Patients with ischemic cardiomyopathy have diminished endothelial function at baseline and this is improved after allogeneic MSC therapy

4.1 Endothelial function at baseline

4.1.1 EPC-CFU numbers are reduced

Similar to our findings in patients with DCM, patients with ICM (n=16) also had impaired EPC-CFU numbers at baseline (4±3 vs. 25±16, P<0.001; Figure 4.1A). Moreover, colonies were disorganized and incomplete, exemplified by scattered tube formation and clusters that failed to form into functional colonies (Figure 4.1B).

![Figure 4.1](image_url)

**Figure 4.1** Endothelial progenitor cell-colony forming units (EPC-CFUs) from patients with idiopathic cardiomyopathy (ICM) at baseline. (A) Number of colonies at baseline comparing patients with ICM (n=16) to healthy controls (n=10) (P<0.0001). (B) Representative images of EPC colonies before allogeneic MSC treatment (20x).
4.1.2 FMD is diminished

FMD% was also assessed at baseline in patients with ICM. Patients had severely impaired FMD% compared to healthy controls (4.9±2.1 vs. 8.9±2.2, P=0.0002; Figure 4.2A).

![Figure 4.2](image)

**Figure 4.2** Flow mediated vasodilation (FMD%) at baseline in patients with idiopathic cardiomyopathy (ICM). (A) Patients with ICM (n=16) versus healthy controls (n=10)(P=0.0002). (B) Representative baseline and (C) peak FMD ultrasound trace. (D) Representative reactive hyperemia graph used to score FMD.

4.2 Patient results three months post allogeneic therapy

4.2.1 Patients have improved EPC-CFUs and FMD% post MSC therapy

All patients with ICM were treated with allogeneic MSCs. Similar to patients with DCM who received allogeneic MSCs, ICM patient’s had a significant improvement in EPC-CFUs three months post treatment (Δ5±10, P=0.003; Figure 4.3A). Morphology and organization also significantly improved post
treatment. (Figure 4.B). Comparably, FMD% significantly improved three months post treatment in all patients with ICM ($\Delta 2.2 \pm 2.5$, $P<0.0001$; Figure 4.4).

**Figure 4.3** Endothelial progenitor cell (EPC) colony forming units in patients with idiopathic cardiomyopathy (ICM). (A) Before and 3 months after allogeneic MSC treatment in patients with ICM ($n=16$) ($P=0.003$). (B) Representative images of EPC-CFUs 3 months after allogeneic MSC treatment (20x).

**Figure 4.4** Flow mediated vasodilation (FMD) in patients with idiopathic cardiomyopathy (ICM) and baseline and 3 months post allogeneic mesenchymal stem cell therapy. ($n=16$) ($P<0.0001$).
4.2.2 EPC-CFUs and FMD% correlate

Again, the correlation between each endothelial function parameter was assessed. There was a significant correlation between the change in FMD% and the change in EPC colonies from baseline to three months post allogeneic MSC therapy ($R=0.8$, $P<0.0001$; Figure 4.5).

![Figure 4.5](image)

**Figure 4.5** Correlation between the change in flow mediated vasodilation percent (FMD%) to the change in endothelial progenitor cell colonies from baseline to 3 months post allogeneic mesenchymal stem cell treatment ($R=0.8$, $P<0.0001$).
Chapter 5. Systemic allogeneic MSCs may improve endothelial function in patients with frailty

5.1 Endothelial function at baseline

We first assessed endothelial function at baseline in patients with frailty. Compared to heart failure patients (N=45), frail patients (N=28) had similar numbers of EPC-CFUs at baseline (4±3 versus 6±6, P=NS; Figure 5.1). Similarly, patients with heart failure and frailty had no difference in baseline FMD% (5.6±3.0 vs. 5.2±2.6, P=NS; Figure 5.2). Therefore, we concluded that patients with frailty have impaired endothelial function at baseline that is comparable to patients with HF.

Figure 5.1 Number of endothelial progenitor cell colonies at baseline comparing patients with heart failure (N=45) versus patients with frailty (N=28)(P=0.2)
5.2 Endothelial function three months post MSCs or placebo

5.2.1 EPC-CFUs three months post treatment

All frail patients were injected intravenously with either 100 million allogeneic MSCs (N=10), 200 million allogeneic MSCs (10=9), or a placebo control (N=9). There was a positive change in EPC-CFUs from baseline to three months post treatment in patients who received 100 million allogeneic MSCs (6±4 to 11±6 colonies, P=0.01) and in the placebo group (6±4 to 10±4, P=0.04), and there was no difference between groups (Figure 5.3A). Furthermore, the greatest trend in improvement was evident in the 100 million MSC group. Patients who received 200 million MSCs had a trending increase in EPC-CFUs,
although not statistically significant (3±3 to 7±5 colonies, P=NS), suggesting that
dose may play a role in efficacy. Only three patients had a frail score above 5,
and all three received MSCs and had an improvement in EPC-CFUs post MSC
infusions. There were no patients in the placebo group with a score above 5 to
compare with these patients.

EPC-CFUs were also examined for morphology. At baseline, EPCs from
frail patients had disorganized and incomplete colony formation (Figure 5.3B),
resulting in clusters that failed to form functional colonies. Three months after
treatment, patient colonies were organized and healthy in appearance (Figure
5.3B)

Figure 5.3 The change in endothelial progenitor cell colonies from baseline to
3 months post injection. (A) The change in colonies comparing frail patients
who received placebo (N=9)(within group P=0.04), 100 million allogeneic
MSCs (N=10)(within group P=0.01), or 200 million allogeneic MSCs (N=9)
(P=NS, ANOVA). (B) Representative images of the change in colony
morphology from baseline to 3 months post (20x).
5.2.2 FMD% three months post treatment

FMD% was measured three months post treatment. FMD% improved in all three groups three months after treatment (Δ1.9±1.4 placebo, P=0.005 vs. Δ2.3±3.2 low dose MSCs, P=0.04 vs. Δ1.2±1% high dose MSCs, P=NS), and there was no difference between groups (Figure 5.4). As evident with the change in EPC-CFUs, also with the FMD%, the greatest trend in improvement was seen in the 100 million MSC group, followed by placebo group, and lastly the 200 million MSC group. Additionally, the three patients with frail scores above 5 had an improvement in FMD% three months after allogeneic MSCs.

Figure 5.4 The change in flow-mediated vasodilation (FMD%) from baseline to 3 months post injection comparing frail patients who received placebo (N=9)(within group P=0.005), 100 million allogeneic MSCs (N=10)(within group P=0.04), or 200 million allogeneic MSCs (N=9)(within group P=0.009).
5.2.3 There is a significant correlation between EPC-CFUs and FMD% from baseline to 3 months post all treatments

We next assessed whether there was a correlation between the change in EPC-CFUs and the change in FMD% from baseline to three months post all treatments. Similar to our findings in patients with heart failure, there was also a significant correlation between these parameters regardless of treatment (R=0.6, P=0.0006; Figure 5.5), suggesting these improvements were not random.

Figure 5.5 Correlation between the change in endothelial progenitor cell colonies and the change in flow-mediated vasodilation (FMD) percent from baseline to 3 months post treatment (N=28)(R=0.6, P=0.0006).
Chapter 6. Allogeneic MSC therapy improves endothelial function in a paracrine mechanism involving an improvement of NO bioavailability, and modulation of VEGF, SDF-1α and TNFα

6.1 Allogeneic MSCs preferentially stimulate vasculogenesis in endothelial cells with impaired nitric oxide production compared to autologous MSCs

FMD is directly related to the endothelium’s response to nitric oxide (NO). Therefore, in order to start testing the mechanism of MSC improvement of endothelial function, we looked at whether MSC secretion could rescue the vasculogenic potential of endothelial cells with impaired NO synthesis. Accordingly, human umbilical vein endothelial cells (HUVECs), pre-treated with L-NAME to block endogenous NO synthesis, were subsequently treated with either autologous or allogeneic MSC-conditioned media (CM). Vasculogenesis was examined in Matrigel assays. HUVECs treated with L-NAME exhibited severely impaired vasculogenesis (Figure 6.1B), evident by their decreased tube length (40.3±0.8 vs. 25.7±3.4, P<0.01; Figure 6.2A) severely impaired formation of networks (18.6±8.2 vs. 1±0, P<0.01; Figure 6.2B) and their depressed vascular index (305·2±196·8 vs. 1170·9±352·6, P<0.01; Figure 6.2C). Allogeneic MSC-CM was significantly favored for rescuing tube length and vascular networks over autologous MSC-CM, and only the addition of allogeneic MSC-CM prevented the L-NAME-induced impairment in the total vascular index (Figure 6.2). Specifically, both allogeneic and autologous MSC-CM restored tube length (25.73.4 L-Name alone vs. 47.5±3.8 L-NAME+ Allogeneic MSC-CM vs. 40.2±2.9 L-NAME+ Autologous MSC-CM, P<0.01; Figure 6.2A). However, there was a significant difference comparing the restorative effect of allogeneic-CM versus autologous-CM, with allogeneic MSC-CM having a greater effect in restoring tube
length (P<0.05). Vascular networks were also restored by both allogeneic and autologous MSC-CM (1±0 L-NAME alone vs. 22.1±3.1 L-NAME+ Allogeneic MSC-CM vs. 15.3±5.2 L-NAME+ Autologous MSC-CM; P<0.01 and P<0.05, respectively; Figure 6.2B), with allogeneic MSC-CM being more effective (P<0.01) than autologous (P<0.05) MSC-CM. Most strikingly, only allogeneic MSC-CM restored the total vascular index (305.2±196.8 L-NAME alone vs. 1113±296·2 L-NAME+ Allogeneic MSC-CM, P<0.01; Figure 6.2C), highlighting

**Figure 6.1** The vasculogenic potential of human umbilical vascular endothelial cells (HUVECs) exposed to allogeneic and autologous mesenchymal stem cell (MSC) secretion. (A) Normal HUVECs tube formation after 6 hours (20x). (B) HUVECs treated with the nitric oxide synthase inhibitor, L-NG-Nitroarginine methyl ester (L-NAME). (C) HUVECs treated with L-NAME for 40 minutes and then subjected to allogeneic MSC conditioned medium (CM). (D) HUVECs treated with L-NAME for 40 minutes and then subjected to autologous MSC-CM.
the preferential ability of allogeneic MSCs in restoring the vascular potential of endothelial cells.

Figure 6.2 Quantification of human umbilical vascular endothelial cells (HUVECs) treated with L-N\textsuperscript{G}\textsubscript{-Nitroarginine methyl ester (L-NAME) followed by either allogeneic or autologous mesenchymal stem cell conditioned medium (MSC-CM). (A) Tube length in HUVECs (n=3) treated with L-NAME (n=3), L-NAME with allogeneic MSC-CM (n=5) or autologous (n=7) MSC-CM added 40 minutes after treatment. (B) Vascular networks in HUVECs treated with L-NAME, L-NAME with allogeneic MSC-CM, or L-NAME with autologous MSC-CM. (C) Vascular index in all treatment and control groups. ANOVA results reported as *P<0.05 and **P<0.01.
6.2 VEGF plays a role in the mechanism of MSC improvement of endothelial function

6.2.1 VEGF is elevated in all patients at baseline and only allogeneic MSCs restore these levels towards normal

VEGF signaling is closely linked to angiogenesis and vasculogenesis, and thus succeeding our vasoculogenesis findings, we investigated the role of VEGF. At baseline, patients with DCM (n=21) had profoundly elevated levels of VEGF compared to controls (n=11) (581.2±812.2 vs. 2.0±5.9 pg/mL, P=0.04; Figure 6.3A). Only DCM patients who received allogeneic MSCs (n=10) had reduced levels of VEGF three months post treatment (Δ-267.1±252.1, P=0.01; Figure 6.3B). On the other hand, DCM patients who received autologous MSCs (n=10) had a further increase in VEGF (Δ511±702.3 pg/mL, P=0.04; Figure 6.3B). Correspondingly, there was a significant difference comparing autologous versus allogeneic MSC therapy (P=0.005).

Figure 6.3 Vascular endothelial growth factor (VEGF) in patients with dilated cardiomyopathy (DCM) and healthy controls. (A) VEGF in patients with DCM (n=21) versus controls (n=10) at baseline (P=0.04) (B) The change in VEGF from baseline to 3 months post either allogeneic (n=10, P=0.01) or autologous MSCs (n=11, P=0.04), comparing the different treatments (P=0.005).
6.2.2 VEGF correlates with EPC-CFUs

We next assessed the correlation between VEGF and endothelial function. There was a significant correlation between the change in VEGF and the change in EPC-CFUs from baseline to three months post treatment (R=-0.7, P=0.001; Figure 6.4). Notably, high levels of VEGF correlated with low levels of EPCs, evidenced by the autologous group. Conversely, lower levels of VEGF correlated with high levels of EPC-CFUs, illustrated by the allogeneic group. Taken together, these data demonstrate that allogeneic MSCs stimulate EPC mobilization and suppress compensatory elevations in circulating VEGF concentrations.

Figure 6.4 Correlation between change in VEGF and change in endothelial progenitor cell (EPC) colonies from baseline to 3 months post allogeneic or autologous mesenchymal stem cells (MSCs) (n=21)(R=-0.7, P=0.001)
6.3 MSC secretion of SDF-1α plays a role in the mechanism

6.3.1 Autologous MSCs secrete higher levels of SDF-1α than allogeneic

SDF-1α is part of the VEGF signaling pathway and is heavily implicated in endothelial function\textsuperscript{86}. Accordingly, we assessed allogeneic (n=4) versus autologous (n=7) SDF-1α MSC secretion. Remarkably, autologous MSCs secreted significantly higher levels of SDF-1α compared to allogeneic MSCs (79.3±16.7 vs. 14.2±9.4 pg/mL, P=0.0001; Figure 6.5). These results paralleled VEGF secretion, in which patients who received autologous MSCs had increased levels of VEGF post treatment, suggesting that both VEGF and SDF-1α are involved in a compensatory mechanism.

\textbf{Figure 6.5} Allogeneic (n=4) and autologous (n=7) mesenchymal stem cell (MSC) stromal cell-derived factor 1 alpha (SDF-1α) secretion (P=0.0001).
6.3.2 SDF-1α strongly correlates to VEGF and EPC-CFUs

We subsequently tested if SDF-1α levels correlated with the change in serum VEGF levels and the change in EPC levels. There was a striking correlation between all parameters. Specifically, there was a positive correlation between SDF-1α and the change in VEGF (N=12, R=0.9, P=0.0002; Figure 6.6A), in which patients who received allogeneic MSCs secreted low levels of SDF-1α which translated in a reduction in elevated VEGF post treatment, and vice versa. On the other hand, there was a negative correlation between SDF-1α and the change in EPC colonies (N=12, R=-0.9, P<0.0001; Figure 6.6B). Notably, patients who received allogeneic MSCs which secreted low levels of SDF1α, had an increase in EPC colonies post treatment.

Figure 6.6 Correlation between mesenchymal stem cell (MSC) stromal cell-derived factor 1 alpha (SDF-1α) secretion and the change in VEGF and colonies. (A) MSC SDF-1α secretion correlates to the change in VEGF from baseline to 3 months post injection (N=12, R=0.9, P=0.0002). (B) MSC SDF-1α secretion correlates with the change in endothelial progenitor cell colonies from baseline to 3 months post injection in patients receiving both autologous (N=7) and allogeneic (n=5) MSCs (R=-0.9, P<0.0001).
6.4 MSC TNFα suppression is involved in the mechanism mediating the improvement in endothelial function

6.4.1 TNFα is elevated in all patients and allogeneic MSCs preferentially reduce these levels

MSCs are known to function in the suppression of inflammation, which in turn improves endothelial function. Accordingly, we measured TNFα in DCM patients before and after allogeneic (n=8) and autologous (n=7) MSC therapy. At baseline, patients had elevated levels of serum TNFα (22±9.4 pg/mL). Three months post allogeneic MSCs, DCM patients had a reduction in serum TNFα (Δ-7.1±3.1 pg/mL, P=0.0005; Figure 6.7A). Conversely, three months post autologous MSCs, DCM patients had no improvement in circulating TNFα (Δ17.1±20.6 pg/mL, P=NS; Figure 6.7B). Interestingly, at 6 months post MSCs, both allogeneic and autologous MSCs reduced serum TNFα. More specifically, patients who received allogeneic MSCs had a reduction from 24.5±5.6 to 5.3±5.4 pg/mL 6 months post treatment (P<0.0001; Figure 6.7A), and similarly, patients who received autologous MSCs had reduction from 25.3±13.3 to 5.5±3.6 pg/mL 6 months post treatment (P=0.004; Figure 6.7B). Ultimately, both allogeneic and autologous MSCs reduced serum TNFα, with a strong preference towards allogeneic MSCs and their earlier effect at 3 months.
6.4.2 TNFα strongly correlated with VEGF and EPC-CFUs

To further delve into the mechanism, we subsequently looked at the interplay between TNFα, VEGF, and EPC colonies. There was a compelling correlation between the change in TNFα and the change in VEGF from baseline to three months post both allogeneic and autologous MSC therapy (N=15, R=0.9, P<0.0001; Figure 6.8A). In addition, the greater the reduction in VEGF the greater the reduction in TNFα. Similarly, there was a significant correlation between the change in TNFα and the change in EPC colonies from baseline to three months post both allogeneic and autologous MSC treatment (R=-0.6, P=0.01; Figure 6.8B). Notably, there was a reciprocal relationship in the change
in EPCs and the change in TNFα, highlighting that the greater the positive change in EPCs post treatment, the greater the reduction in TNFα.

6.4.3 MSC secretion is not responsible for elevated TNFα

After detecting elevated serum TNFα in patients that remained elevated after autologous MSC therapy, we looked at whether MSCs themselves were contributing to these levels. Both allogeneic (n=4) and autologous MSCs (n=7) secreted very low levels of TNFα (0.01±0.14 vs. 0.4±0.6 pg/mL; Figure 6.9). Furthermore, there was no difference comparing allogeneic versus autologous secretion (P=NS). Therefore, we concluded that autologous MSC secretion was not responsible for elevated TNFα in patients.

Figure 6.8 Correlations of tumor necrosis factor alpha (TNFα) in patients with dilated cardiomyopathy. (A) Correlation between the change in TNFα and the change in vascular endothelial growth factor (VEGF) from baseline to 3 months post both allogeneic and autologous MSCs (R=0.9, P<0.0001) (B) Correlation between the change in TNFα and the change in endothelial progenitor cell colonies from baseline to 3 months post both allogeneic and autologous treatment (N=15) (R=-0.6, P=0.01).
Figure 6.9 Allogeneic and autologous mesenchymal stem cell (MSC) tumor necrosis factor alpha (TNFα) secretion (P=0.6).
Chapter 7. Discussion

7.1 The role of endothelial dysfunction in patients with heart failure

There is an increasing awareness of the central role endothelial dysfunction plays in CVD. Here we demonstrated that patients with both DCM and ICM have impaired endothelial function as measured by EPC-CFUs, FMD%, and inflammatory cytokine signaling.

7.1.1 The role of endothelial progenitor cells

EPCs are integral to proper endothelial function and cardiac function due to their ability to incorporate into damaged endothelium and release signaling factors crucial to proper vasodilation and LV diastolic function. In this study, we showed that all HF patients had severely diminished numbers of EPC-CFUs at baseline, and that only after allogeneic MSCs, EPC-CFUs were dramatically improved. Multiple studies have shown that low numbers of EPC-CFUs strongly correlate with endothelial dysfunction and are associated with a high Framingham risk score for adverse CV health outcomes. Furthermore, circulating EPC levels predict CV events—specifically in patients with coronary artery disease, heart failure, atherosclerosis, and angina.

Ashara et al. first identified and characterized EPCs. Subsequently, Hill et al. forged the field with their landmark paper showing that bone marrow derived EPCs have a continuing role in endothelial repair, and depletion of these cells was paramount to CVD progression. Additionally, they were the first to describe that EPC-CFUs strongly correlate with endothelial function and can serve as biomarker for vascular function in patients with CVD. Since then,
numerous studies have been published validating that patients with CVD have low numbers of EPC-CFUs that correlate with endothelial dysfunction\textsuperscript{29,87}. Kissel \textit{et al.} showed the migratory capacity of EPCs, as well as illustrated that EPC-CFUs were profoundly reduced in patients with ischemic cardiomyopathy\textsuperscript{90}, while Valgimigli \textit{et al.} showed that EPC-CFUs diminish as heart failure progresses in patients with idiopathic dilated cardiomyopathy, thus paralleling our results.

There is an emerging interest in EPCs as a biomarker for CVD. It is well established that healthy subjects have high numbers of circulating EPCs as well as EPC-CFUs that are superior in angiogenic assays and resistant against oxidative stress\textsuperscript{91,92,93,94}. Similarly, we showed that healthy volunteers had high numbers of EPC-CFUs compared to DCM and ICM patients. However, there is a debate about whether measuring circulating EPCs via staining for CD34+/CD133+/VEGFR2+ or measuring EPC-CFUs is more appropriate for identifying endothelial dysfunction and cardiovascular progression risk. Thiess \textit{et al.} demonstrated that circulating EPC numbers were highly elevated in patients with DCM, however despite these high numbers, there was reduced homing to diseased myocardium and functional impairment, emphasizing the need to functionally measure EPCs over counting circulating numbers\textsuperscript{95}. Accordingly, EPC-CFUs have been shown to strongly correlate with functional properties and capacity, while flow cytometry via surface marker sorting has been more correlated with EPCs ability to proliferate. In this regard, George \textit{et al.} showed that EPC flow cytometry analysis based on CD34, CD45, CD133, and KDR was relevant for measuring circulating EPCs, however, there was no correlation
between the high numbers of circulating EPCs and EPC-CFUs\textsuperscript{96}. Additionally, they showed that only EPC-CFUs measured functional capacity. A high amount of circulating EPCs resulting in a low number of functional EPC-CFUs may occur because many circulating EPCs may not be mature enough to incorporate into damaged tissue\textsuperscript{97}. Additionally, EPCs are highly diverse, and thus not all circulating EPCs play a role in vascular homeostasis\textsuperscript{98}. Lastly, a significant proportion of FACs sorted EPCs have been shown to be apoptotic, thus their role in improving endothelial function is questionable\textsuperscript{99}. These discrepancies emphasize the need to further investigate which circulating EPCs are specifically contributing to endothelial function, and therefore incorporate into EPC-CFUs, in order to truly delineate the best biomarker for endothelial function in patients with CVD.

7.1.2 The role of flow mediated vasodilation

In the 1990s, FMD was established as a means to quantify endothelial dysfunction via measuring the ability of the brachial artery to vasodilate as a response to supra-systolic cuff stress\textsuperscript{37}. Since then, the role FMD and endothelial dysfunction has been heavily studied in patients with cardiovascular disease. A multitude of studies have shown that FMD inversely correlates with future cardiovascular events\textsuperscript{100-103}. Accordingly, Ras \textit{et al.} performed a meta-analysis of 23 studies with a total of 14,753 subjects and reaffirmed there is a significant inverse correlation between baseline FMD and future CV events\textsuperscript{104}.

In our study, we found that patients with both ischemic and non-ischemic cardiomyopathy had impaired FMD at baseline which correlated with low levels
of EPC-CFUs. Additionally, three months after allogeneic MSCs, but not autologous, FMD was significantly improved, and this again correlated with EPC-CFUs. While few studies focus on FMD in DCM, Sitges et al. measured FMD and TNFα in patients with DCM and had similar finding to us: patients with DCM have diminished FMD and high levels of TNFα. Vsllbracht-Israng examined the relationship between inflammatory cytokines and FMD in patients with DCM and found that these patients had endothelial dysfunction that correlated with elevated levels of sIL-12p70. With regards to ischemic cardiomyopathy and heart failure, many studies have demonstrated the relationship between FMD and endothelial dysfunction in this patient population. Notably, our study is the first to demonstrate that allogeneic MSC improves FMD while autologous MSCs do not.

Despite the widespread use of FMD, there are important limitations. FMD is time-dependent and there is no current consensus on when the maximal increase in diameter occurs. Some studies look at 60 seconds after release of the brachial cuff while others look at 45, and these time differences can result in misrepresentative conclusions. Additionally, results can significantly vary due to quality of ultrasound image, technician, and personal interpreting the images. Thus, FMD should be supplemented with additional testing to ascertain endothelial dysfunction. Accordingly, we correlated FMD with EPC-CFUs and inflammatory cytokines, ultimately demonstrating that patients with CVD have endothelial function measured by multiple parameters.
7.2 The role of endothelial dysfunction in aging and frailty

Aging and frailty are heavily associated with inflammation—a culprit of vascular dysfunction \(^{114}\,21\,115\). Accordingly, it has been shown that EPC-CFUs and FMD decreases with age \(^{41}\,116\), while inflammatory cytokines increase \(^{62}\). Frailty is a recently classified disease, and therefore studies involving “frail” patients are limited. The Toledo Study for healthy aging is one of the first studies to categorize elderly patients as frail, and then show there is a link between endothelial dysfunction and clinically termed frail patients \(^{21}\). It has also been demonstrated that endothelial dysfunction is substantial in aged patients both with CVD \(^{117}\) and without \(^{118}\), thereby demonstrating that endothelial dysfunction may be an underlying feature of frailty. Here we saw no differences between EPC-CFUs and FMD at baseline comparing heart failure patients to frail patients. Notably, Rodriguez-Mañas et al. described a potential mechanism of age-associated endothelial dysfunction, specifically showing there is increase in oxidative stress and a pro-inflammatory profile with aging\(^{118}\).

In our study, we showed that patients with frailty have reduced numbers of EPC-CFUs and FMD, consistent with literature on elderly patients and the Toledo Study. However, a major limitation of our study is that frail patients with endothelial dysfunction improved three months post both treatment and placebo, with a preference towards patients receiving 100 million MSCs. Reversal of endothelial dysfunction in elderly patients has been shown when patients start exercising, change their diet, and take vitamin supplements\(^{119}\). Wray et al. revealed that antioxidant supplements composed of vitamin C, vitamin E, and \(\alpha-\)}
lipoic acid reversed endothelial dysfunction in elderly\textsuperscript{119}. Similarly, Chauhan \textit{et al.} showed that endothelial dysfunction was reversed by L-Arginine administration, suggesting NO bioavailability is a crucial component of endothelial dysfunction in an aged population\textsuperscript{120}. Exercise has been shown to have anti-inflammatory effects that combat oxidative damage. Accordingly, DeSouzza \textit{et al.} illustrated that regular exercise not only prevented age-related declines in endothelial dysfunction, but furthermore restored endothelial dysfunction in previously sedentary men\textsuperscript{121}. Lastly, Jablonski \textit{et al.} researched the effect of dietary sodium restriction on endothelial function and showed that reducing sodium intake in elderly patients reversed endothelial dysfunction\textsuperscript{122}.

Another limitation of our study was only three patients had a frailty score above 5, therefore majority of patients had mild frailty. Furthermore, endothelial function was a secondary endpoint, thus this trial was not powered for this outcome. However, notably, these three patients did have a significant improvement in EPC-CFUs and FMD\% post MSC infusions. Yet, more frail patients are necessary to study the effect of MSCs on endothelial function in this population.

Ultimately, we propose that a placebo effect may have occurred due to patients believing they received cells and therefore changing their diet and/or exercise routines. Notably, EPC-CFUs correlated with FMD, suggesting that these changes were not random. While MSCs had unique and specific outcomes in patients with heart failure, larger clinical trials need to be completed to determine whether they may have specific effects in different stages of frailty.
7.3 MSC therapy implications and the allogeneic advantage

MSCs are adult stem cells that are prototypically found in bone marrow and have the capacity to differentiate into multiple cell types\textsuperscript{76}. Importantly, they stimulate the proliferation and differentiation of endogenous precursor cells and play a crucial role in maintaining stem cell niches\textsuperscript{76}. In addition, MSCs secrete paracrine factors that participate in angiogenesis, cardiomyogenesis, neovascularization, stimulation of other endogenous stem cells, and regulation of the immune system\textsuperscript{35, 123}. While MSCs are known to stimulate cardiac precursor cells and cell cycle activity in the heart\textsuperscript{18}, their role in stimulating other endogenous precursor populations has heretofore been unknown. Here we report that MSCs stimulated endogenous EPC activation, increasing the number and quality of functional EPCs. These findings suggest that augmentation of functional EPCs may represent a novel mechanism of action by which MSCs exert favorable biological effects.

We found that allogeneic MSCs restored endothelial function in patients to a degree greatly exceeding that of autologous MSCs. One possible explanation for this may be the age of the cells. Recent studies highlight that MSC's therapeutic function declines as a result of aging\textsuperscript{124, 125}. Efimenko \textit{et al.} showed that adipose-derived MSCs from aged patients with coronary artery disease have impaired angiogenic potential\textsuperscript{124}. Similarly, Kasper \textit{et al.} demonstrated that MSC function is altered and diminished with age, specifically showing lower actin turnover and therefore decreased motility, decreased antioxidant power, decreased responsiveness to chemical and mechanical signaling, and increased
senescence\textsuperscript{126}. Stolzing \textit{et al.} also reported a decline in “fitness” as a result of aging, as evidenced by a decline in colony-forming unit-fibroblasts and increase in reactive oxygen species levels and oxidative stress\textsuperscript{127}. In our study, all allogeneic stem cell donors were healthy, young donors between the ages of 20 and 35. Patients receiving their own stem cells not only had underlying chronic diseases, but also were older (between the ages of 45 and 75). MSC aging may impair the survival, differentiation, and ability to recruit EPCs to areas of damage, ultimately reducing their therapeutic efficacy\textsuperscript{125}. Additionally, due to underlying patient comorbidities, the autologous MSC microenvironment may be negatively altered due to systematic inflammation. Consistent with this notion, Teraa \textit{et al.} showed that systemic inflammation affects the bone marrow microenvironment, disturbing EPC function\textsuperscript{128}. Although more studies are necessary to validate that the advantage evident here is due to the health and age of MSCs, this study supports the encouraging idea of using “off the shelf” allogeneic MSCs over autologous MSCs.

As use of allogeneic MSCs in human is a newly emerging field, it is important to highlight concerns regarding allogeneic MSCs before moving to an “off the shelf” model. Huang \textit{et al.} evaluated the ability of rat allogeneic MSCs to retain their immunoprivilege and functional efficacy and reported that allogeneic MSCs transitioned from an immunoprivileged to immunogenic state post differentiation, which hampered therapeutic efficacy\textsuperscript{129}. Albeit, no studies have been able to reproduce these results. For example, Liu \textit{et al.} had opposite findings, demonstrating that osteogenic cells differentiated from MSCs retained
their immunogenicity in vitro and in vivo \textsuperscript{130}. Furthermore, our lab has shown that MSCs delivered in a porcine ischemic cardiomyopathy model retain their efficacy and differentiation status three moths post treatment \textsuperscript{131}. Also, it has been reported that MSCs may not be immunoprivileged, but rather immune evading due to their ability to dramatically suppress the immune system, and more specifically T and B lymphocytes \textsuperscript{132}. Interestingly, a study by Crawford \textit{et al.} showed that MSCs produce a CD25+/CD4+ regulatory T cell population during differentiation, raising the idea that these cells can later differentiate and generate T cells that can subsequently stimulate an immune reaction\textsuperscript{133}. While this has not been studied in our clinical trials, Stanzani \textit{et al.} demonstrated that CD25 expression on donor T cells is linked with allogeneic rejection and ultimately graft-versus-host disease \textsuperscript{134}, thus highlighting the need to study the immune effects of allogeneic transplantation to see if donor MSCs later differentiate into CD25+/CD4+ cells. Notably, in all our clinical trials, allogeneic MSCs have proven to be both safe and effective. However, as studies are starting to test multiple injections of allogeneic MSCs, further research will need to address whether serial injections elicit an immune reaction. Eliopoulus \textit{et al.} observed a rejection of serial allogeneic MSC injections due to mismatched donors and recipients in mice, suggesting that MSCs are not inherently immunoprivileged. Ultimately, in our study, we found allogeneic MSC administration to be both safe and effective in improving endothelial function.
7.4 MSC mechanism in patients with DCM

While many clinical trials have demonstrated the powerful beneficial effects of MSCs in heart failure\textsuperscript{79, 81, 135, 136}, there is a lack of mechanistic insight in the literature. \textit{In vitro}, MSCs are known to secrete anti-inflammatory factors and cytokines—such as IL-2, TGF-β1, hepatocyte growth factor, NO, prostaglandin 2, and SDF-1—which can modulate the mobilization of EPCs from bone marrow\textsuperscript{76, 137}. Additionally, it has been shown that human MSCs stimulate vasculogenesis and combat inflammation\textsuperscript{76}, however no studies have addressed the mechanism of MSCs effect on endothelial function in a clinical model. In this study, we utilized MSCs from our clinical trials along with patient EPCs and serum, thereby offering novel insight into the mechanism of improvement of endothelial function via stimulating vasculogenesis and restoring VEGF, SDF-1α, and TNFα levels towards normal.

7.4.1 MSCs stimulation of vasculogenesis

We found that allogeneic MSCs stimulated vasculogenesis in endothelial cells that had impaired NO production to a much larger extent than autologous MSCs. Many studies have shown the ability of MSCs to enhance vascular tube formation of endothelial cells both in vitro and in vivo\textsuperscript{138, 139, 140}. Moreover, it is well established that NO dysregulation plays a major role in the pathophysiology of heart failure, and MSCs have been shown to participate in neovascularization by enhancing NO bioavailability\textsuperscript{43, 74, 76}. Kanki-Horimoto \textit{et al.} demonstrated that implantation of MSCs alone and MSCs overexpressing eNOS dramatically improved right ventricular impairments caused by pulmonary hypertension in
rats—with a preference towards eNOS MSCs—suggeting that eNOS plays a critical role in mediating the beneficial effects of MSCs 141. We similarly found that, in least in part, the beneficial efficacy of MSCs was due to their ability to override endothelial eNOS impairments and thereby promote vasculogenesis in vitro. More specifically, endothelial cells that were treated with L-NAME to block endogenous NO production had rescued tube length and tube network formation when subjected to both autologous and allogeneic MSC-CM. Notably, these defects were rescued to a much greater extent by allogeneic MSC-CM, and only allogeneic MSC-CM was able to restore the vascular index. Along these lines, Kwon et al. showed that MSCs secrete multiple paracrine factors that promote endothelial cell angiogenesis by activating EKR, FAK, and Akt-dependent eNOS pathways 142. Interesting mechanistic insight was shown by Sato et al. in which they demonstrated that MSCs suppressed T-cell production which enabled high amounts of NO production, and furthermore when MSCs were subjected to L-NAME, NO production was hampered and T-cell proliferation was restored 143. These results suggest that MSCs production of NO mediates T-cell suppression providing key insight into the mechanism of MSCs immunomodulation.

7.4.2 The role of VEGF

In this study, we found that patients with DCM had severely elevated levels of VEGF, and these levels were restored towards normal only after allogeneic MSCs. Elevated levels of circulating VEGF are linked to endothelial dysfunction and HF144, 145. In this regard, Eleuteri et al. demonstrated that elevated levels of VEGF correlated with HF disease progression146. Moreover,
Wei et al. investigated circulating EPCs and VEGF levels in patients with cerebral aneurysm and found that decreased levels of circulating EPCs and increased levels of plasma VEGF were associated with chronic inflammation in the vascular walls of cerebral arteries and the development of cerebrovascular abnormalities leading to aneurysm formation and rupture\textsuperscript{147}. Thus, endothelial dysfunction is a central feature of CVD, and may represent a powerful surrogate marker in the development of new treatments for CVD.

Over the last decade, there has been an emerging interest in the use of MSCs in CV disorders\textsuperscript{148, 149}. Clinical trials have demonstrated a major safety profile for MSC administration, and suggested efficacy in patients with HF\textsuperscript{79, 148}; however, underlying mechanism(s) of action continue to be vigorously debated. Our finding that allogeneic MSC injections in patients with both ischemic and non-ischemic HF results in an improvement in endothelial function, specifically by restoring EPC function and FMD and reducing VEGF, SDF-1\textsubscript{α}, and TNF\textsubscript{α} levels towards normal, offers a major new insight into the mechanisms of action of MSCs. In the study population, increased serum VEGF correlated with diminished EPC-CFUs, consistent with the idea that VEGF plays a compensatory role, a finding also reported in patients with cerebral aneurysm\textsuperscript{147}. This is also supported by the study of Vasa et al. which showed a diminished response of EPCs to VEGF in patients with CAD\textsuperscript{150}. Moreover, Alber et al. found that a key beneficial effect of atorvastatin therapy is reducing the levels of plasma VEGF in patients with CAD\textsuperscript{151}. This coincides with our study using MSCs, rather than a
pharmacological intervention, to decrease pathologic VEGF and increase endothelial function.

7.4.3 The role of SDF-1α

In this study, we reported positive systemic effects including improvement in endothelial function and a decrease in elevated levels of VEGF, SDF-1α, and TNFα from local, cardiac transendocardial MSC injections. We have previously shown that MSCs engraftment after intramyocardial injection is approximately 10 to 20%, suggesting that these cells migrate and circulate systemically. MSCs have been shown to secrete paracrine factors that stimulate resident cells. Accordingly, we demonstrated that MSCs secretion of SDF-1α is crucial to the mechanism of MSCs, and furthermore there is a distinctive effect of secretion comparing allogeneic versus autologous MSCs.

It is widely accepted that SDF-1 plays an integral role in stem cell homing towards ischemic myocardium and additionally is a powerful recruiter of progenitor cells. While we are the first study to link SDF-1α as a critical part of the mechanism of MSC therapy in human, Abbott et al. demonstrated the necessity of SDF-1 in a mice model of MI. Notably, they demonstrated that SDF-1 was required for recruiting bone marrow-derived cells—primarily endothelial cells—to the injured heart, and furthermore SDF-1 effected VEGF expression. Askari et al. had similar findings in an ischemic cardiomyopathy rat model, demonstrating that SDF-1 induces stem cell homing to injured myocardium which results in significantly enhanced cardiac function. Human recombinant SDF-1 was utilized by Xinchun et al. to illustrate that SDF-1α activates the JNK3
pathway through an eNOS dependent mechanism which ultimately enhances endothelial cell migration and has significant implications on positively enhancing endothelial function\textsuperscript{155}. In accordance with these studies, our results indicate that MSC SDF-1\textsubscript{α} secretion levels correlate with the change in VEGF and the change in EPC-CFUs from baseline to post both allogeneic and autologous MSC therapy.

An interesting finding of ours revealed the differential secretion between allogenic and autologous MSCs. While many studies have highlighted the positive effects of SDF-1 and stem cell homing, we found an opposite effect from autologous derived MSCs. Shibata \textit{et al.} found that overexpression of SDF-1 induces gastric dysplasia and accelerates the development of inflammation during infection\textsuperscript{156}. Zgraggen \textit{et al.} showed that increased SDF-1 expression induces inflammatory angiogenesis and vascular remodeling that contributes to pathologic chronic inflammation\textsuperscript{157}. These studies highlight that increased SDF-1 has detrimental effects on inflammation, paralleling our results in which autologous MSCs secreted elevated levels of SDF-1\textsubscript{α} which resulted in elevated levels of VEGF and no improvements in endothelial function or TNF\textsubscript{α} levels.

\subsection*{7.4.4 The role of TNF\textsubscript{α}}

We found that all patients had elevated levels of serum TNF\textsubscript{α}, and that only allogeneic MSCs were able to reduce these levels at 3 months post injection. It is well established in the literature that patients with both DCM and ICM have high levels of TNF\textsubscript{α} associated with pathologic inflammation and vascular dysfunction\textsuperscript{63,72}. However, we are the first to show that MSCs isolated
from healthy donors reduce these detrimental levels, while MSCs isolated from patients had no effect on modulating TNFα levels. The implication of using allogeneic MSCs for combatting pathologic inflammation mediated by TNFα is an important finding. Elevated levels of TNFα increase with acute ischemia and accordingly progress the severity of the disease as well as can progress LV dysfunction 70 158. Additionally, TNFα is detrimentally augmented in patients post-MI and therefore puts patients at increased risk for recurrent coronary events 63. Tögel et al. demonstrated that MSC administration in patients with kidney disease protected patients against ischemic renal failure in a paracrine mechanism mediated by reducing levels of TNFα, IL-1β, IFN-γ 159. Remarkably, we found that 6 months post both allogeneic and autologous MSCs, serum TNFα was reduced in all patients, suggesting that autologous MSCs are able to modulate the immune system, but do so at a much slower rate compared to allogeneic MSCs. Along this line, SheveZla et al. demonstrated that MSCs derived from patients with hematological malignancies were 50% less effective in immunosuppressive activity compared to healthy donor MSCs160.

In our study, TNFα levels correlated with VEGF levels and EPC-CFUs. In line with these findings, Hong et al. identified that TNFα down-regulated EPC expression which resulted in decreased EPC-CFUs as well as diminished incorporation into HUVEC networks in children with vasculitis 161. Interestingly, in our study, there was no difference in the amount of TNFα secreted by allogeneic versus autologous MSCs themselves, suggesting that MSCs—regardless of being isolated from healthy subjects of HF patients—are not secreting TNFα
themselves, but rather mediating levels in patients. Given the correlation to EPC-CFUs and VEGF, one plausible explanation for this is that increased circulating patient TNFα activated allogeneic MSCs, but not autologous, to stimulate EPCs, ultimately improving endothelial function. Kwon et al. used an ischemic hindlimb model to illustrate a similar idea, specifically showing that MSCs pre-treated with TNFα promoted EPC homing and angiogenesis. More studies need to be performed to truly elucidate the exact link between MSCs, EPCs, and TNFα.

Ultimately, our findings establish a previously unappreciated therapeutic principle whereby allogeneic MSCs can be employed to stimulate EPC bioactivity, improve arterial physiologic vasodilatory responses, and decrease unfavorable cytokine mobilization in patients with CVD and other disorders associated with endothelial dysfunction.

7.4.5 TNFα, SDF-1α and their implications on the effect of MSCs on the immune system

Multiple studies have demonstrated that MSCs modulate the immune system via suppressing inflammatory cytokines as well as T and B cell proliferation, thus affecting both the innate and adaptive immune system. With regards to the adaptive immune system, MSCs can inhibit the activation and proliferation of effector T cells as well as induce the proliferation of T-cell inhibitory regulatory T cells. Additionally, MSCs have been shown to regulate B cell proliferation and function. Interestingly, both Ren et al. and Groh et al. showed that MSCs require activation by inflammatory cytokines such as IFN-Ɣ, IL-1, and TNF-α to elicit these immunomodulatory effects. In our study, we found that at three months post MSC treatment, only allogeneic MSCs
suppressed circulating TNFα, and that at six months post MSC treatment, both allogeneic and autologous MSCs suppressed elevated TNFα. Moreover, we recently published that at six months post treatment, both allogeneic and autologous MSCs—with a preference towards allogeneic MSCs—modified several immunologic markers, specifically lowering Temra T-cells and exhausted B-cells while increasing switch memory B-cells. Together, these data show that MSCs have the potential to restore immune competence in a microenvironment with elevated TNFα.

With regards to the innate immune system, MSCs have been shown to secrete SDF-1, which is a powerful chemoattractant for neutrophils, monocytes, basophils, NK cells, and endothelial progenitor cells. Following a cardiac injury, the immune system plays a vital role in both the acute inflammatory response as well as the regenerative response. This response is mediated by leukocytes, inflammatory cytokines, neutrophils, and macrophages, therefore making MSC therapy an attractive candidate for homing these crucial cells to injured areas. In our study, we found that both allogeneic and autologous MSCs secreted SDF-1α, however autologous MSCs secreted far higher levels which translated in poor EPC function and endothelial dysfunction. We proposed that these elevated levels secreted by autologous MSCs were compensatory for dysfunctional signaling, comparable to our VEGF findings. Although we are the first to propose this mechanism of action, Di et al. illustrated the importance of the health of donor MSCs. Specifically, they demonstrated that senescent human MSCs isolated from umbilical cord secreted elevated levels of IL-6,
thereby activating the STAT-3 pathway and ultimately promoting cancer activity. This fits in line with our findings that both elevated SDF-1α and VEGF translated in the inability to suppress elevated TNFα three months post autologous MSCs, ultimately promoting endothelial dysfunction. Together, our TNFα and SDF-1α findings demonstrate a potential mechanism of allogeneic immune modulation in patients with dilated cardiomyopathy.

7.5 Summary and working model

For the first time, we report a potential multilevel mechanism of the differential effect of allogeneic and autologous MSCs in patients with DCM (Figure 7). Specifically, we propose that allogeneic MSCs secrete normal levels of SDF-1α, which reduces elevated VEGF, reducing inflammation via TNFα, and ultimately improving endothelial function via increasing functional EPCs and FMD. On the other hand, autologous MSCs secrete elevated levels of SDF-1α which increases VEGF in a compensatory mechanism, and results in no improvement in TNFα or endothelial function.
7.6 Limitations and future directions

There are several limitations of our study. All ICM patients received allogeneic MSCs, therefore we were unable to study the received autologous MSCs had higher FMD% at baseline compared to patients who received allogeneic MSCs. Notably, however, all patients receiving autologous MSCs had lower FMD% post treatment, highlighting the allogeneic advantage. Furthermore, patients with ICM received different total number of cells (either 20 or 100 million cells). Regardless, all patients had an improvement in endothelial function and
there was no intergroup variability. In addition, there was variability within our control group for circulating VEGF levels. The majority of our controls had too low levels of circulating VEGF to detect, ultimately highlighting the elevated levels of VEGF evident in HF patients. Lastly, our results in patients with frailty were inconclusive, highlighting the need to study the effect in a larger population.

Despite these limitations, we are confident our results provide novel insights into the positive endothelial function effect of allogeneic MSCs in patients with HF.

Future directions are aimed towards unraveling the exact differences between allogeneic and autologous MSCs. As clinical trials aim towards improving the efficacy of treatment, it is important to discern what factors mediated the allogeneic advantage. Additionally, our study focused on the mechanism of endothelial improvements in patients with DCM. Thus, all mechanistic studies need to be further evaluated in patients with ICM and frailty.

In conclusion, this study demonstrates a potent and clinically relevant efficacy outcome of transendocardial therapy with MSCs in patients with advanced HF. In patients with DCM, allogeneic MSCs secreted normal levels of SDF-1α and restored flow mediated brachial artery dilatation, EPC bioactivity, VEGF, and TNFα levels towards normal. Additionally, we observed that patients with clinically designated frailty have impaired endothelial function that is comparable to patients with HF. As abnormalities in the vascular function of patients with CVD is shown to be highly predictive of adverse outcomes and disease progression, targeting endothelial function is a significant therapeutic strategy. Together, these findings offer a new mechanism of action underlying
potentially clinically relevant responses to the use of allogeneic MSCs in CVD and potentially frailty.
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