Evaluation of an Enhanced (Sialyl Lewis-X) Collagen Matrix for Neovascularization and Myogenesis in a Mouse Model of Myocardial Infarction

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Abstract

In cardiovascular disease the repair response is insufficient to restore blood flow, leading to the death of muscle and loss of tissue function. Therefore, strategies to augment the endogenous cell response and its effects may help improve tissue recovery and function. In this study we explored the use of tissue-engineered collagen matrices for augmenting endogenous regenerative processes after myocardial infarction. Treatment with the sLe^X-collagen matrix reduced inflammation and apoptosis and had a positive regenerative effect on the infarcted mouse heart, through improved vascular density and possibly enhanced cardiomyogenesis.

Additionally, we investigated the effects of cryopreservation on generating circulating angiogenic cells (CACs) from peripheral blood mononuclear cells (PBMCs), as a potential source of stem cells that could be used in combination with our collagen scaffold. Our findings show that despite PBMCs experiencing phenotypic changes after cryopreservation, they may still be used to generate the same therapeutic CACs as freshly procured PBMCs.

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List of Abbreviations

7-AAD	7-Aminoactinomycin D
ADSC	Adipose derived stem cell
APC	Allophycocyanin
APS	Ammonium persulfate
BM	Bone marrow
BMC	Bone marrow cell
BrdU	Bromodeoxyuridine
CAC	Circulating angiogenic cell
CAD	Coronary artery disease
CD	Cluster of differentiation
CIHR	Canadian Institutes of Health Research
CPC	Cardiac progenitor cells
CSC	Cardiac stem cell
CS-C	Chondroitin sulfate-C
CTGF	Connective tissue growth factor
CVD	Cardiovascular disease
DAPI	4',6-diamidino-2-phenylindole
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine–labeled
DMSO	Dimethyl sulfoxide
EBM	Endothelial basal medium
EC	Endothelial cell
ECD	Electron coupled dye
ECM	Extracellular matrix
EDC	N-ethyl-N-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EF	Ejection fraction
EPC	Endothelial progenitor cell
ESC	Embryonic stem cell
FAC	Fractional area change
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor-2
FITC	Fluorescein isothiocyanate
FOV	Field of view
GM-CSF	Granulocyte macrophage colony-stimulating factor
HGF	Hepatocyte growth factor
HPC	Hematopoietic progenitor cell
HPS	Hematoxylin phloxine saffron
HRP	Horse radish peroxidase
HSC	Hematopoietic stem cell
HSFO	Heart and Stroke Foundation of Ontario
IGF-1	Insulin-like growth factor 1
IgG	Immunoglobulin G
IMDM	Isocove's modified Dulbecco's medium

KDR	Kinase insert domain receptor
LAD	Left anterior descending artery
LDL	Low density lipoprotein
Lin	Lineage
LV	Left ventricle
LVEF	Left ventricle ejection fraction
MAC-1	Macrophage-1 antigen
MCP-1	Monocyte chemoattractant protein-1
MES	2-(N-morpholino) ethanesulfonic acid
mg	Milligrams
MHC	Myosin heavy chain
MI	Myocardial infarction
MMP	Matrix metalloproteinase
MNC	Mononuclear cell
MSC	Mesenchymal stem cell
NHS	N-hydroxysuccinimide
NHS	Normal horse serum
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PE	Phycoerythrin
PE-Cy5	Phycoerythrin-Cy5
PFA	Paraformaldehyde
rpm	Revolutions per minute
SDF-1	Stromal cell derived factor-1
SDS	Sodium dodecylsulfate
sLeX	Sialyl Lewis^{X}
SMA	Smooth muscle actin
SMC	Smooth muscle cell
TBS	Tris buffered saline
TEMED	Tetramethyethylenediamine
UCB	Umbilical cord blood
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
WHO	World Health Organization
PAGE	Polyacrylamide gel electrophoresis

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Statement of Contribution

Dr. Erik Suuronen came up with the concept and the design of the study. Ali Ahmadi conducted all the LAD ligation surgeries and aided with the treatment injections. Suzanne Crowe ran the flow cytometry and conducted the active caspase 3 staining. The RGN pathology lab was responsible for HPS staining.

For the cryopreservation experiments Drew Kuraitis and Dr. Erik Suuronen came up with the concept of the study. Jenelle Marier and Kim McEwan helped with the isolation of the PBMCs and the experiments. Ross Davies assisted by providing his statistical expertise.

INTRODUCTION

Introduction

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1. General Introduction

As cardiovascular disease (CVD) is the number one cause of death worldwide (WHO 2011), there has been much focus placed on eradicating this ailment and alleviating the high cost of health care associated with it. The World Health Organization (WHO) estimates that over 17 million people die from CVD, a disease affecting the heart and the vascular system, worldwide each year (WHO 2011). One major type of CVD is coronary artery disease (CAD). The symptoms of CAD manifest as narrowing and hardening, due to plaque build-up, of the coronary arteries that supply blood and essential nutrients to the heart tissue. This reduction in blood flow may lead to a myocardial infarction (MI) and ultimately heart failure and even death. Current therapies for patients ailing from this disease, such as bypass grafting and percutaneous coronary intervention, have been successful at extending and improving the quality of life of numerous afflicted individuals; however, not all patients are amenable to these treatments, and CAD still remains an incurable disease due to the large loss of cardiomyocytes leading to a decrease in contractility and severe functional deficits. In fact, it has been shown that irreversible cardiomyocyte loss begins to occur approximately 15 to 20 minutes after a coronary artery occlusion (Heyndrickx et al. 1978). Compensatory mechanisms such as cardiomyocyte hypertrophy and fibrotic scar formation, seen in both infarcted and non-infarcted myocardium, that assist the heart in maintaining cardiac output vital for sustaining other organs, place an extra burden on the already weakened myocardium, further progressing this disease (Jawad et al. 2008).

1.1 Stem Cell Therapy

With the discovery of adult stem and progenitor cells, regenerative therapies offer a promising approach to treat CAD by using these cells to rebuild new vasculature in the myocardium as well as to replace the dying cardiomyocytes. Stem cells are precursor cells, found in numerous organisms that can both self-renew and differentiate into a diverse number of specialized cell types that make up the biological body (Krause 2002). Meanwhile, progenitor cells are more lineage specific than stem cells, and are already committed to a certain cell line but are still immature and are not terminally differentiated. Another difference between stem and progenitor cells is that the stem cells can replicate indefinitely (Suda et al. 1987), while progenitor cells are more limited in their expansion potential (Bull et al. 2005; Xu et al. 2007). However, pluripotent stem cells that can proliferate indefinitely and differentiate into numerous lineages are rare in the adult. More common are the lineage-restricted stem and progenitor cells that are named after their tissue of origin or cell lineage such as adipose-derived stem cells (ADSC) or hematopoietic stem cells (HSC). In the adult, the stem and progenitor cells' function is to repair and replenish injured or worn out tissue.

Stem cells for therapy can be derived from an inner cell mass of a blastocyst, which are referred to as embryonic stem cells (ESCs); they can be induced from somatic cells, which are known as induced pluripotent stem cells (Park et al. 2008); or they can be extracted from either a donor or a patient's own tissues. Other than the ethical controversy that surrounds the use of ESCs, the benefit to using autologous cells instead of donor cells or ESCs is immuno-compatibility (Barrilleaux et al. 2006). Since the stem cells are derived from the patient's own tissue, there is no need to search for a compatible donor match or for the patient to take immuno-suppressant drugs to prevent cell rejection.

Also, using adult stem cells, which tend to be lineage-specific, instead of embryonic stem cells, which are more pluripotent, reduces the risk of malignancies and cells differentiating into tissue other than the one desired.

The use of adult stem and progenitor cells for repair of diseased or damaged tissue has already been applied to cases such as bone marrow cell (BMC) transplants for leukemia patients (Gahrton et al. 2000), neovascularization of ischemic muscles (Suuronen et al. 2006), tissue grafts (Bhattacharya et al. 2000; Stosich et al. 2007) and even for MI (Wollert et al. 2004; Dill et al. 2009). The goals in the use of stem cell therapy after a MI or cardiac ischemia are two-fold. Firstly, it is hoped that stem cell therapy will replace the damaged and dead cardiomyocytes, thereby, restoring some of the heart function and contractility that is lost after an infarct. Secondly, it is desired that the stem cell therapy also restores and repairs the vascular network to prevent further cardiomyocyte loss and to help sustain the newly formed cardiomyocytes. Both processes are required as generation of new cardiomyocytes without the supporting vasculature to provide blood flow and nutrients would result in apoptosis of the cells. Also, while restoring just the blood flow may prevent further cardiomyocyte loss, it will not replace the cardiomyocytes that have already died. Therefore, both these processes are desired for optimal repair of the heart post-MI.

1.1.1 Types of Stem Cells for Myocardial Repair

The exciting prospect of restoring the myocardium after an infarct has garnered much focus on the use of stem cell therapy and the identification of the ideal cell source for such treatments (see Table 1). In theory, the ideal stem cell should be able to: 1) undergo or augment myogenesis and neovasculogenesis; 2) effectively engraft and integrate into the infarcted myocardium so that it is synchronous with other beating cells or connected to functional blood vessels; 3) restore cardiac function; and 4) perform all of these functions with limited negative side effects such as differentiating into non-tissue specific lineages (Gallegos et al. 2008).

Several adult stem cell types have been identified both in the bone marrow (BM) and in the heart that have shown promising results in restoring heart function. The BM is one of the largest stores of adult stem and progenitor cells (Perin et al. 2006) and comprises of a mixture of cells including: HSCs, hematopoietic progenitor cells (HPCs), endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs). These cells can be easily mobilized into the circulation using factors such as granulocyte macrophage colony-stimulating factor (GM-CSF), statins and chemokines secreted during hypoxia or injury (Deng et al. 2006). They can also be gathered by bone marrow harvest or through collection and separation of peripheral blood mononuclear cells (PBMCs). The ease of collection and the relatively large numbers of BMCs makes them a great candidate for myocardial regeneration. The resident cardiac stem cells (CSC) and cardiac progenitor cells (CPCs) found in the heart are harder to extract and are present in lower numbers compared to BMCs. Thus far, both stem cells from the BM and the heart have shown promising results in restoring heart function after a MI in humans and animal models; however, the ideal cell type with the most therapeutic potential has yet to be elucidated.

Table 1. Summary of some of the current stem cells used for repair of the myocardium. Adapted from: Li SC et al. Cell Bio International 2009; 255-267.

Stem Cell Type	Abb.	Tissue of Origin	Differentiation	Mechanism of repair
Endothelial Progenitor Cells	EPC	BM, other tissue	Endothelial cells	Neovascularization
Resident Cardiac Stem Cells	CSC	Myocardium	Cardiomyocytes, Endothelial cells, Smooth muscle cells	Cardiomyogenesis, neovascularization
Mesenchymal Stem Cells	MSC	BM, other tissue	Cardiomyocytes, Endothelial cells	Cardiomyogenesis, Paracrine Factors
Skeletal Myoblasts	SM	Skeletal muscle	Cardiomyocytes, Muscle cells	Repopulate scar tissue
Adipose Derived Stem Cells	ADSC	Adipose tissue	Endothelial cells	Angiogenesis
Mononuclear Cells	MNC	Bone marrow	Endothelial cells, Cardiomyocytes, Smooth muscle cells	Neovascularization, Angiogenesis

1.1.1.1 Endothelial Progenitor Cells

Until the discovery of EPCs in 1997 (Asahara et al. 1997), vasculogenesis, *de novo* formation of blood vessels, was thought to be restricted to embryonic development. Prior to this, regeneration of the injured endothelium in the adult was attributed to migration and proliferation of neighbouring endothelial cells (EC), termed angiogenesis (Urbich et al. 2004). EPCs can give rise to endothelial cells (Prater et al. 2007), which line the lumen of the blood vessels. They have been found to home to the site of ischemia and injury and to incorporate into the new vasculature. Surprisingly, $CD34^+$ enriched cells were also reported to trans-differentiate into cardiomyocytes and smooth muscle cells (Imamura et al.; Badorff et al. 2003; Yeh et al. 2003; Imamura et al. 2010). Studies involving the use of EPCs for treatment after an MI have already been associated with greater preservation of left ventricular (LV) function, reduction of infarct size and inhibition of apoptosis in both mouse and rat animal models (Kocher et al. 2001; Iwasaki et al. 2006) as well as some clinical trials (Kang et al. 2004; Ince et al. 2005).

The description of EPCs still remains unclear today due to the lack of specific surface markers to identify this cell type (Urbich et al. 2004). This is because most of the cell surface markers used to select EPC populations such as CD34, vascular endothelial growth factor receptor 2 (VEGFR2) (Asahara et al. 1997) and CD133 (a marker of immature EPCs that disappears once they become more committed) (Peichev et al. 2000), are also found in HSCs and/or ECs (Kim et al. 2008). Another population of EPCs isolated from the PBMCs have been shown to express CD14, macrophage-1 antigen (MAC-1) and CD11c, indicating a monocyte or a macrophage origin (Perin et al. 2006). Once differentiated into ECs, the cells retain the cell surface markers CD34 and VEGFR2 and in addition express markers such as: vascular endothelial (VE)-cadherin, von

Willebrand factor (vWF) and CD31 (Hristov et al. 2003). Of late, a more stringent guideline has been proposed to describe EPCs. Simply culturing PBMCs for up to a week does not generate EPCs alone, but a more heterogeneous mixture of pro-angiogenic cell referred to as circulating angiogenic cells (CACs) (Yoder 2009).

EPCs are also thought to induce neovascularization through the secretion of cytokines and growth factors, which act in a paracrine fashion to induce angiogenesis by migration of ECs and EPCs from previously existing vessels and other host tissues. Several research groups have shown EPCs to secrete pro-angiogenic factors such as hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), stromal cell-derived factor-1 (SDF-1) and vascular endothelial growth factor (VEGF) (Majka et al. 2001; Urbich et al. 2005). This paracrine effect has been confirmed in experiments in which EPC-cultured medium was used, resulting in pro-angiogenic effects similar to the effects obtained with the administration of the EPCs themselves (Urbich et al. 2005).

The positive roles of EPCs and the more heterogeneous population of CACs in the improvement of heart function post-MI have already been established in animals, and in some clinical trials. The ease of procurement of these cells from peripheral blood and the BM, as well as their ability to home to ischemic tissue and to augment neovascularization for improved myocardial blood flow make them an advantageous cell type for restoring cardiac function.

1.1.1.2 Resident Cardiac Stem Cells

Traditionally it has been understood that the heart was a terminally differentiated organ with no capability for repair or replacement of worn-out myocytes. Resident CSCs and CPCs have only recently been discovered in the human endomyocardial areas (Beltrami et al. 2003; Ballard et al. 2008), leading to excitement over the prospect of expanding these cells and replacing dead or damaged heart tissue after a MI. These cells were identified as Lin$^-$ c-kit$^+$ cells that can give rise to: cardiomyocytes, smooth muscle cells (SMC) that encircle some blood vessels and ECs, which line blood vessels, when injected into rat MI hearts *in vivo*. While smaller than the mature cardiomyocytes, the incorporated CPCs still expressed cardiac myosin heavy chain (MHC), α-cardiac actinin and connexin 43 (Beltrami et al. 2003) showing their differentiation into new cardiomyocytes. These c-kit$^+$ CPCs were found to express, to some degree, early cardiac transcription markers such as GATA4 and Nkx2.5 (He et al. 2011), associated with early cardiac development (Durocher et al. 1997). More recently, resident CPCs were expanded to also express cell surface markers MDR1, Sca-1 (in mice) (Linke et al. 2005) and Isl-1 (Laugwitz et al. 2005) in variable amalgamation.

When injected into the rat MI hearts, these c-kit$^+$ CPCs limited the infarct size, decreased LV dilation and remodelling (Dawn et al. 2005), as well as differentiated into EC and SMA cells, which incorporated into large functional arteries that increased blood flow to the infarcted myocardium, and further enhanced cardiac function (Tillmanns et al. 2008). Expansion of these cells *in vitro* showed their capability to organize into clusters called cardiospheres, which predominantly expressed cardiac markers and some endothelial markers such as VEGFR2 and CD31 (Messina et al. 2004).

Given that c-kit+ CPCs are very rare in the heart, ~ 1 in 30000 myocytes are CPCs in rat heart (Beltrami et al. 2003) and 1.8%±1.7% in unsorted human myocytes (C. Bearzi et al. 2005), proliferation of these cells *in vivo* or their expansion prior to treatment *in vitro* are important aspects that need to be further studied in order to generate enough cells needed after a considerable cardiomyocyte loss post-MI. The fact that the CPCs can differentiate into both cardiac and vascular lineages make them a powerful potential candidate as a stem cell source for use in heart regeneration.

1.1.1.3 Mesenchymal Stem Cells

MSCs are a rare (1 in 10000 cells in the BM are of MSC phenotype (Chamberlain et al. 2007)), primarily BM-derived population that are precursors of non-hematopoietic, mesenchymal cells such as osteocytes, chondryocytes, and adipocytes (Minguell et al. 2001). The lack of a specific surface markers for MSC identification makes it hard to isolate and recognize this population of cells, but a combination of markers such as CD73, CD90 and CD105 has been used (Dominici et al. 2006). MSCs also do not express hematopoietic markers CD45, CD34, CD14 or CD11 (Chamberlain et al. 2007). Both *in vitro* and *in vivo* studies have demonstrated the cells' capacity for cardiomyogenic differentiation using human and animal MSCs (Shake et al. 2002; Toma et al. 2002; Xu et al. 2004). Additionally, some studies have shown that MSCs can be induced to generate ECs. When MSCs were cultured in the presence of VEGF they expressed endothelial-specific markers such as vWF and augmented their expression of VEGFR2 as well as being able to help form capillary-like structures in an angiogenesis assay (Oswald et al. 2004).

Treatment of MI hearts with MSCs has shown increased heart contractility, reduced wall thinning and overall improved heart function (Shake et al. 2002; Yoon et al. 2005). Paracrine effects of MSCs have also been noted. MSCs can release numerous cytokines vital to tissue repair or angiogenesis some of which are VEGF, FGF-2 and SDF-1 (Kinnaird et al. 2004; Kinnaird et al. 2004; Zhang et al. 2007). Similar to EPC conditioned media, conditioned media of MSCs showed beneficial properties such as reduced cell death and infarct size in MI rats (Gnecchi et al. 2006).

The advantages of MSCs as a potential stem cell source in myocardial regeneration are that they are easily acquired via BM, they can be expanded *in vitro*, they may differentiate into cardiomyocytes and they can augment neovascularization. MSCs also have low immunogenicity, allowing for minimal inflammatory response after transplantation (Kim et al. 2009).

1.1.1.4 Other sources of stem cells for repair

Other sources of adult stem cells have been investigated for cardiac repair such as skeletal myoblasts (SMs), adipose derived stem cells (ADSCs) and unsorted BM and PB derived mononuclear cells (MNCs). SMs are myogenic committed lineage cells that have a high proliferative potential. Clinical studies report having injected these cells into MI hearts, with an improvement in regional left ventricular function and increased wall thickening (Herreros et al. 2003; Menasche et al. 2003). However, marked increases in arrhythmia-related events and death following SM transplantation was observed, which was theorized to be due to lack of intercellular communication between the transplanted SM cells and the host cardiomyocytes (Stagg et al. 2006). ADSCs are another source of mesenchymal and endothelial cells (Colazzo et al. 2010; Zhang et al. 2011) that have been shown to have beneficial angiogenic and LV function effects (Mazo et al. 2008). Unsorted MNCs, derived from either BM or PB that contain MSCs and EPCs, also improved heart function and perfusion post-MI (Li et al. 2007; Tatsumi et al. 2007; Yao et al. 2008; Lipiec et al. 2009) .

1.1.2 Mechanisms in Cell Therapy for Myocardial Repair

The cardiac regeneration field is still relatively new and requires further study and comprehension. In order to better understand and optimize stem cell therapy, the underlying mechanisms of myocardial repair need to be explored. Currently, there are several theories as to the mechanism(s) responsible for the observed beneficial effect of stem cell therapy. It is still unclear as to which mechanism is more valuable over another, but it does seem that a multi-faceted approach may be the most optimal. Figure 1.1 summarizes some of the potential benefits of stem cell therapy after a MI.

1.2.1.1 Neovascularization

Neovascularization can occur via two different mechanisms: angiogenesis or vasculogenesis. Angiogenesis is the growth of new capillaries from pre-existing blood vessels. This usually occurs by the sprouting of new vessels, through proliferation or migration of ECs from the vicinal environment. On the other hand, vasculogenesis is the main mechanism of vascular network formation in embryogenesis, and until the discovery of angiogenic progenitor cells in the adult it was thought to only occur in the developmental stage of life. It involves the growth of new vessels *de novo* from precursor cells (Vailhe et al. 2001). As previously mentioned, EPCs and CACs have been implicated in neovascularization in the adult (Asahara et al. 1999; Hattori et al. 2001; Rookmaaker et al. 2005). Upon an infarct they home to the site of ischemia and can incorporate into the tissue to make new vasculature or they can release paracrine factors for augmentation and reformation of existing vasculature. Since the primary cause of an infarct is stenosis of a coronary artery, in order to return the blood flow to the ischemic tissue the new vasculature has to be connected to the pre-existing vessels through

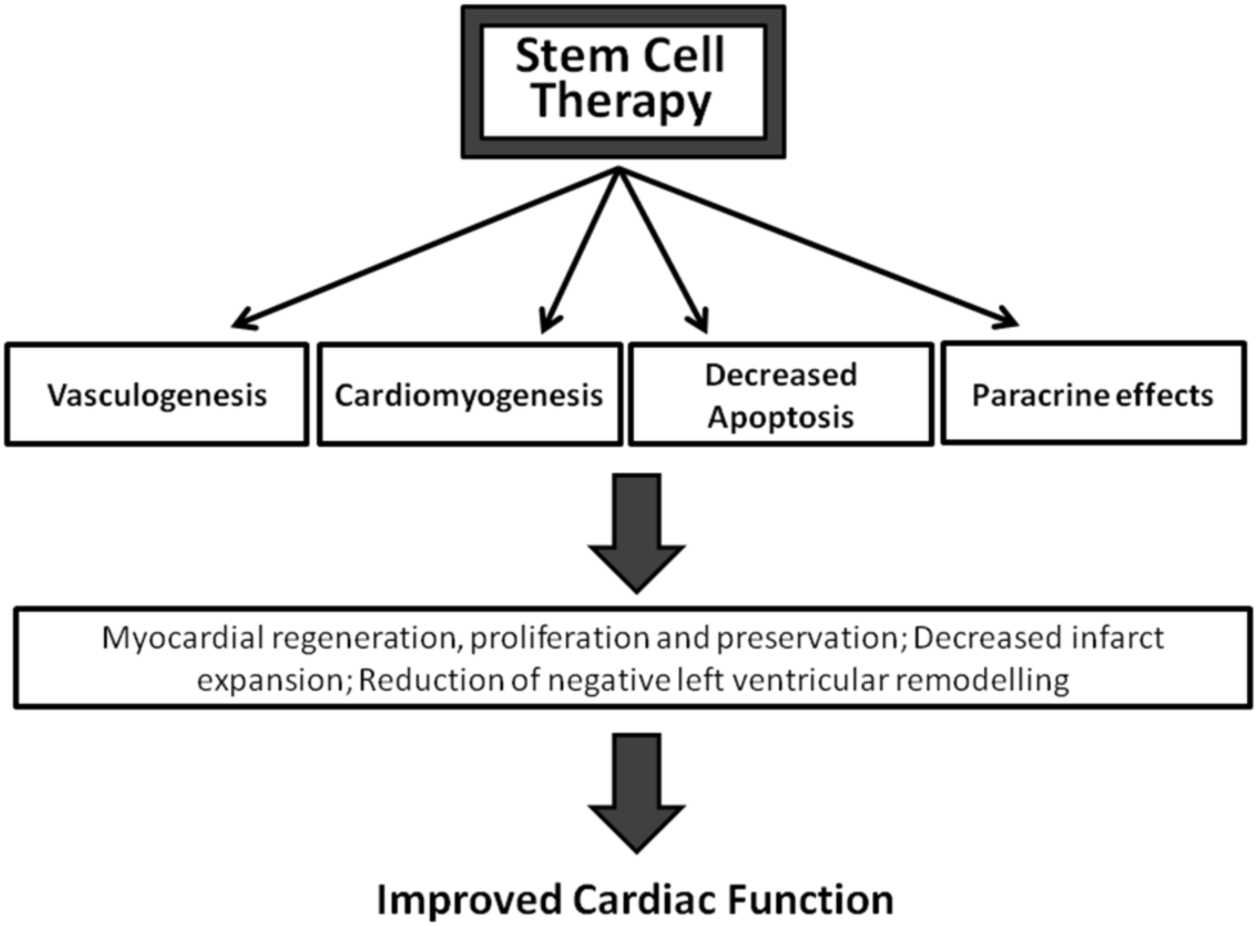

Figure 1.1. Potential beneficial mechanisms of stem cell therapy after a myocardial infarction. Vasculogenesis, decreased apoptosis of native cardiomyocytes, and paracrine effects may limit infarct expansion and preserve the myocardium. Proliferation and cardiomyogenesis can lead to myocardial regeneration. Combined together this can lead to diminishing of the infarct and reduced left ventricular remodelling, leading to improvements in cardiac function. Adapted from: Forrester J S et al. Circulation 2003;108:1139-1145.

angiogenesis. Additionally, remodelling of the impaired vessel and vicinal collateral vessels can occur to increase the luminal diameter and enhance the blood flow to the ischemic tissue (Semenza 2007).

1.1.2.2 Cardiomyogenesis

With the large loss of cardiomyocytes that occurs following an infarct, regeneration of the dead and damaged cells is the ultimate goal of cardiac repair. The fact that cardiomyocytes can be renewed has already been established, but controversy yet remains on the fraction of the cells in the heart that renew, with laboratories reporting ranges from 0-20% of cells per year (Kajstura et al. 2010). Stem cell sources such as MSCs and CPCs have been shown to have the capability to differentiate into cardiomyocytes *in vitro* and *in vivo* (Toma et al. 2002; Beltrami et al. 2003; Messina et al. 2004; Xu et al. 2004). So far the use of adult progenitor and stem cells for cardiac repair has shown limited cardiomyogenesis and thus inadequate functional improvement (Lee et al. 2010). However, this therapeutic strategy is still in its infancy, with knowledge and mechanisms yet to be elucidated for the proper stem cell source, differentiation, induction and expansion of these cells.

1.1.2.3 Paracrine Effects

Upon the onset of ischemia paracrine factors including cytokines and growth factors are secreted by the injured cells, newly recruited stem cells and the surrounding cells. These factors act to enhance the mobilization and homing of stem cells, which may originate from the circulation, BM or tissue itself, and their subsequent engraftment at the site of injury (Burchfield et al. 2008). Additionally, these factors can mediate cell apoptosis, proliferation and differentiation, cardiomyocyte protection, neovascularization and cell/matrix remodelling (Gnecchi et al. 2008), all of which can contribute to cardiac repair. A study conducted by Kinnaird et al (2004) using MSCs showed that their expression of pro-angiogenic factors (including VEGF, bFGF and monocyte chemoattractant protein-1 (MCP-1)) increased vascular density upon injection through paracrine effects without the direct incorporation of cells into the blood vessel structures (Kinnaird et al. 2004). In fact, conditioned media from MSC or CAC culture alone can improve vascular density and tissue repair upon injection after acute ischemia (Urbich et al. 2005; Chen et al. 2008). Other studies have shown that paracrine effects, which augment neovascularization and tissue repair, can be further sustained by the host tissue long after the transplanted cells disappear (Cho et al. 2007).

1.1.3 Stem Cell Therapy Limitations

As promising as stem cell therapy seems to be there are still numerous unanswered questions and limitations preventing the most favourable outcome for regenerating the myocardium post-MI. Aside from establishing the optimal cell source and its mechanism(s) of action, it is still unclear: what the optimal time for cell delivery is; how to limit the non-specific tissue cell delivery; how to prevent cell death of transplanted stem cells; and how to improve cell mobilization and engraftment.

The optimal time for treatment is a very important aspect of stem cell therapy. An essential balance has to be reached between waiting for the acute inflammatory process to abate and providing treatment before scar formation and hypertrophy set in (ter Horst 2010). Right after an acute MI, the inflammatory and apoptotic environment makes it an unfavourable setting for cell engraftment and survival (Wang et al. 2006). This inflammatory phase occurs during the first 72 hours post-MI with increases in proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (Il-1β), which recruit leukocytes to clear away the debris and dead cells (Frangogiannis 2006). This increase in inflammatory cytokines has been observed to continue up to a week after a MI (Ono et al. 1998). Injecting cells 24h post-MI was found to have no beneficial effect while treatment 4-7 days after an infarct showed positive outcomes of stem cell therapy (Zhang et al. 2009). BM stem cells are mobilized into the circulation after an infarct due to the cytokines released by the injured cells. One study of hindlimb ischemia saw the EPC population peak one week after injury (Takahashi et al. 1999), while another found that EPC and MSC population fluctuated from day 3 to day 28 post-MI (Wang et al. 2006). Other limits of stem cell therapy are poor cell engraftment and survival along with non-specific cell delivery. Due to these factors only 1-10% of the transplanted cells remain in the myocardium (Aicher et al. 2003; Retuerto et al. 2004).

1.2 Tissue Engineering

One strategy for overcoming some of the cell therapy limitations is via the use of tissue engineering to construct a scaffold upon which the cells can adhere to, proliferate and grow. Tissue engineering is a field that aims to replace, repair, maintain or improve human tissue functions through use of a combination of cells, engineering, biomaterials, and suitable biochemical factors (Barnes et al. 2008). There are several desirable traits that scaffolds should have in order to qualify them as a candidate for tissue engineering. One of the main properties is biocompatibility. Upon placement, the scaffold should not trigger a foreign body response or other immunological systems in order to minimize adverse tissue reactions to the implanted material (Williams 2008). The scaffold should also be biodegradable and is ideally able to be broken down by the body at the rate that it is being replaced by newly regenerated tissue. Furthermore, the by-products of the degraded scaffold should also not produce any toxic effects in the body. Another important aspect to be considered in tissue engineering is the mechanical properties of the scaffold. The scaffold should be mechanically similar in tensile strength and stiffness to the tissue it is replacing in order to be able to bear the load and stress that is placed on that tissue during function (Lee et al. 2008). Finally, the biomaterial scaffold should provide surface area for migration, adhesion and differentiation of cells to support the growth of new tissue (Zippel et al. 2010).

In general, polymeric materials have received much focus due to their ability to mimic the characteristics and structure of the natural extracellular matrix (ECM) environment for specific tissue types (Lee et al. 2008). The ECM is a gel-like fluid that interconnects cells in the tissue and their cytoskeletal elements. The ECM also provides structural support, acts as a compression buffer against the stress placed on the tissue, and provides cells with a medium in which to migrate (Fisher et al. 2007). Interactions between the ECM and the cells are very important, as cells are very sensitive to their environment and the extrinsic signals from the ECM can regulate cell fate and behaviour (Keely et al. 1995; Stevens et al. 2005). In turn, cells can also respond to their microenvironment, and aid in the synthesis of new ECM elements or the degradation of the ECM through matrix metalloproteinases (MMPs) (Haas et al. 1998; Wang et al. 1998; Chen et al. 2004). One way that that ECM-cell interaction occurs is through receptor-mediated signalling, while another way is through binding and sequestering of growth and differentiation factors, thereby changing the cytokines’ concentrations in various areas and influencing cell proliferation and phenotype (Rosso et al. 2004). The ECM-cell interactions are especially important when it comes to stem cells, where a controlled environment is needed to direct the proliferation and differentiation of the cells as required for the damaged tissue. In one study, chondrogenic or osteogenic differentiation of MSCs was dependant on the type of exogenous ECM they were cultured in (Hwang et al. 2011); while another study showed that MSC's morphology was influenced by their adhesion to the surrounding ECM via integrin receptors (McBeath et al. 2004).

One type of polymeric materials is referred to as natural - meaning the materials are derived from living creatures. Natural materials are generally composed of polymers such as collagen, chitosan, alginate, fibrin...etc. These polymers are usually well-suited for tissue engineering as they are naturally found in the tissue, do not elicit a major immunological response and are inherently broken down in the organism, usually by enzymes (Yannis 2004).

1.2.2 Collagen Matrices

Of the ECM-type materials used for scaffold generation, collagen is perhaps the most frequent. It compromises a large portion of the body's ECM, and accounts for up to 30% of total protein in the human body (Fuhrmann et al. 2010). Collagen fibers weave together to form a network which plays an important role in preserving the ECM structure. It is also highly dynamic and undergoes remodelling as needed for physiological function (Cen et al. 2008). An advantage in the use of collagen for a scaffold material is its ability to be crosslinked, with glutaraldehyde or other chemicals, allowing for fine-tuning of mechanical and physical properties to better suit the target tissue. Also, since collagen is a part of ECM and basement membranes of many cells, the cells have the innate ability to adhere and interact with it through their integrin surface receptors (Schoichet 2010).

1.2.2.1 Collagen matricies for augmented endogenous repair

In addition to using collagen matrices for improving the efficacy of cell therapy, such biomaterials may also serve to ameliorate the endogenous repair response. While the body does have an ability to repair itself after an injury such as a myocardial infarct, the process is often insufficient in restoring the lost tissue and heart function. Collagen based matrices can augment the endogenous responses through the recruitment of progenitor cells and their cytokine production (Suuronen et al. 2009; Kuraitis et al. 2011). This approach to treating disease is desirable as it uses the host's autologous cells and enhances the innate healing processes for improvement of heart function. Tissue engineered matrices can also be used in combination with stem cell therapy to further enhance regeneration of tissue by augmenting the infarcted environment and providing transplanted cells a less hostile, physiologically pertinent milieu to engraft and differentiate within.

1.2.2.2 Collagen Matrices for neovascularization

Collagen is one of the major components of the blood vessel wall. In normal vessel structures the basement membrane of the ECM is in contact with ECs and is imperative to vessel stability and function. It has been shown that in the initial stages of angiogenesis the basement membrane is broken down by proteinases such as MMPs. This change in the microenvironment causes rapid EC growth and migration in the interstitial ECM abundant in collagen (Davis et al. 2005). In addition, EC-ECM interactions, in particular EC-collagen interactions, influence neovascularization through the control of EC proliferation, migration, morphology and survival (Davis et al. 2005). One study has shown that the blocking of ECs' integrin receptors involved in collagen binding reduced

VEGF-mediated angiogenesis by 90% *in vitro* (Senger et al. 1997). Additionally, *in vivo* neovascularization was inhibited in a developing chick animal model upon interference of collagen assembly, cross-linking and/or deposition (Ingber et al. 1988). It has also been observed that ECs cultured on collagen type I undergo morphological changes similar to pre-capillary cord formation that occurs in embryonic and adult neovascularization and subsequently, go on to form tube-like structures indicative of a capillary network (Delvos et al. 1982; Montesano et al. 1983).

The use of a collagen scaffold for neovascularization has already been investigated in animal models of ischemia and MI. Our lab has shown that treatment with collagen-based matrices in the mouse hindlimb ischemia model improved arteriole density and perfusion compared to the untreated control (Suuronen et al. 2009). Other studies have additionally seen improvement in neovascularization and in turn, cardiac function, in animal models of MI with the use of collagen scaffolds (Huang et al. 2005; Xiang et al. 2006; Miyagi et al. 2011).

1.2.2.3 Collagen Matrices for cardiac regeneration

Collagens type I, III, IV, V, VI and VIII have all been identified to be present in the myocardium. Of particular importance are collagens I and III, which contribute to cardiomyocyte support; collagen I (~85% of collagen in the heart) through its strength and collagen III (~11% of collagen in the heart) through its elasticity (Marijianowski et al. 1995; Jugdutt 2003). The collagen network in the myocardium is essential for proper and normal heart function. In diastole (heart relaxation), the collagen fibrils uncoil and bear most of the pressure-volume force preventing cardiomyocytes from stretching, while in systole (heart contraction) the collagen fibrils keep the cardiomyocytes aligned

(Fomovsky et al. 2010). Post-MI, when a large loss of cells is observed, collagen is produced and deposited in the infarcted ventricle (Cleutjens et al. 1995) in an effort to reduce the stress on the remaining cardiomyocytes. One previous study observed that rat MI hearts injected with collagen matrices showed a thickened infarct wall and improved LV stroke volume and ejection fraction (Dai et al. 2005).

1.3 Cryopreservation of PBMCs and CACs

Another potential method for improving stem cell therapy is through the cryopreservation of stem cells. Cryopreservation offers a means to maintain cells as they are generated, until they are required for therapy. More importantly, cryopreservation allows a patient to store his or her own autologous cells until needed, thereby avoiding the risks associated with the graft-versus-host disease (Stockschlader et al. 1995). This has value for cardiovascular disease and neovascularization given that EPCs and CACs are not available off-the-shelf and their frequency in circulating PBMCs is rather low, at about 0.0001% to 0.01% (Khan et al. 2005) and 2% of total PBMCs (Dimmeler et al. 2001), respectively. Furthermore, diabetes and cardiovascular disease deplete EPC numbers and degenerate their function (Tepper et al. 2002; Chen et al. 2004), making it difficult to obtain therapeutically-relevant and potent cells for application in therapy.

Cryopreservation preserves cells by dramatically reducing biological metabolism at low temperatures (between -136°C and -196°C). At this temperature, even the biochemical pathways leading to cell apoptosis are suspended. Although this process preserves cells it may also cause damage to certain cell types as well as potentially change their function (Motta et al. 2010; Haack-Sorensen et al. 2011). Cryoprotectants are used to decrease the damage to the cells that is incurred through the freezing steps.

During the freezing phase, the fluid in the ECM and the cell begins to turn to ice. As the fluid in the ECM begins to freeze the remaining fluid surrounding the cells becomes more concentrated leading to lethal concentrations of salts which causes osmotic stress on the cells and their dehydration (Mazur 2004). Furthermore, intracellular ice crystals can form with the remaining fluid in the cell. These events can lead to changes in structure and function of the cell membrane, disruption of cellular organelles and gas bubble formation in the cell (Fuller 2004; Mazur 2004). Cryoprotectants are defined as an additive to the cell prior to the freezing step that can yield a higher post-thaw survival (Fuller 2004). They work by dissolving in an aqueous solution and simply increasing the solute concentration in the cell, which leads to a lower melting point of water. This attenuates the high salt concentrations that form upon the start of freezing and decreases the dehydration of the cells (Wowk 2007).

Cryopreservation has been applied for some time in the medical field, ranging from freezing blood and bone marrow cells for transplantation, to embryo preservation for *in vitro* fertilization and long term gamete storage for cancer patients. Transplants of frozen BMCs and PBMCs have been successful in restoring precursor cells for patients whose own BMCs were damaged by disease, chemotherapy or radiation (Gallardo et al. 2009; Bertz et al. 2011). However, it has been noted that cryopreservation can affect the phenotype and function of the frozen cells (Lanza et al. 1999; Costantini et al. 2003; Owen et al. 2007). One study demonstrated that cryopreservation of T-cell subsets caused an increase in the expression of CXCR4 and CD69, while expression of L-selectin (CD62L) was decreased (Faint et al. 2011). The consequence of cryopreservation on CACs and their generation from PBMCs, yet remains to be thoroughly investigated.

1.4 Research Plan

1.4.1 Rationale

As collagen is one of the most abundant and important ECM components in both the heart and the blood vessels and its interactions with vicinal cells is crucial in numerous processes, it was rationalized that it would have beneficial properties in restoring vascular density and cardiac function after an infarct. Furthermore, addition of the oligosaccharide sialyl Lewis^X (sLe^X), a ligand of the adhesion receptor L-selectin, to promote CAC and EC homing and engraftment (Biancone et al. 2004), was contemplated to additionally enhance the recruitment and attachment of the stem cells imperative for neovascularization and cardiac regeneration.

Previous work in our lab has used a sLe^X-collagen matrix in the rat and mouse model of hindlimb ischemia. In the rat hindlimb ischemia model, the sLe^X-collagen matrix was found to up-regulate paracrine factors, increase the number of CACs in the circulation and restore vascularization compared to the collagen-only matrix or the PBS control (Suuronen et al. 2009). In addition, the sLe^X-collagen matrix was further found to augment the myogenic response, with significantly more regenerating myocytes and myogenic cytokines expressed in the ischemic mouse hindlimb tissue treated with the sLe^X-collagen matrix, as well as an observed decrease in apoptosis (Kuraitis et al, unpublished data). With the beneficial effects of the sLe^X-collagen matrix seen in the ischemic hindlimb tissue, the next step was to investigate the potentially beneficial properties of this matrix in the infarcted mouse heart.

1.4.2 Aims and Objectives

The primary goal of my research was to investigate a collagen-based matrix containing sLe^X (sLe^X-collagen matrix) and its effect on restoring cardiac function in a mouse MI model. Specifically, for aim #1 the objectives were to assess:

1. The recruitment of the endogenous stem and progenitor cells in MI mice injected intramyocardially with either: phosphate buffered saline (PBS), collagen or the sLe^X-collagen matrix;
2. The effect of the treatments on vascularity and function of the infarcted heart for restoration and improvement of the myocardium;
3. The effect of treatment on cardiomyogenesis

A second project was conducted to look at the cryopreservation effects on the PBMCs and CACs. In particular, for aim #2, the intent was to investigate the human PBMCs' ability to be stored via cryopreservation processes, and if the freezing process affected the generation of CACs. The objectives were to assess:

1. The changes, if any, in the phenotype and function of cryopreserved PBMCs.
2. If viable CACs can be generated from cultured cryopreserved PBMCs.
3. The changes, if any, in the phenotype and function of CACs that were generated from cryopreserved PBMCs.

1.4.3 Hypotheses

For aim #1, it was hypothesized that the sLe^X-collagen matrix would augment cell mobilization from the BM and enhance vascularization and regeneration in the mouse heart tissue post-MI, thereby improving the heart's function and contractility more effectively than the PBS and collagen treatments. Furthermore, it was theorized that the collagen-only treatment would also have beneficial effects on restoring some cardiac function.

For aim #2, it was hypothesized that cryopreservation of PBMCs would not affect their ability to generate functional CACs and, additionally, that the PBMCs and CACs would not be functionally and phenotypically altered.

MATERIALS & METHODS

2. Materials and Methods

2.1 METHODS PART A: sLe^X-collagen matrix in MI mouse model

2.1.1 Matrix Preparation

Preparation of sLe^X-collagen matrix has previously been described (Suuronen et al. 2009). Briefly, 1mM sLe^X (Cedarlane Laboratories, Hornby, Canada) was added to 0.1M 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 5.0) containing 1:1 (molar equivalent) cross-linking mixture of N-ethyl-N-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide (EDC/NHS; 13mM). The mixture was kept on ice during the process with further addition of 0.375% rat tail collagen type I (w/v, BD Biosciences, Mississauga, Canada) with 100µL of 40% chrondroitin sulfate-C (CS-C; w/v) (Wako Chemicals, Osaka, Japan). The final pH was adjusted to 7.2 – 7.4 using NaOH. Collagen-only matrices were prepared in the same manner but without the addition of sLe^X. The collagen-based matrices are liquid at the time of injection but upon exposure to physiological temperatures (~37°C), they solidify into a gel.

2.1.2 Animal Model and Surgical Procedure

All procedures were performed with the approval of the University of Ottawa Animal Care Committee. Female C57BL/6J mice (6-7 weeks old; Charles River, Sherbrooke, Canada) underwent a permanent left anterior descending (LAD) coronary ligation to induce MI. An hour before surgery the mice were intramuscularly injected with 0.1mL of buprenorphine for pain reduction. Prior to the start of the surgery, the mice were sedated by 2%-3% isoflurane administered nasally with 2% O_2 air supply. The hair on the chest was depilated using a hair removal cream and 100-150µL of 0.9% saline solution (Baxter Corporation, Toronto, Canada) was injected subcutaneously, for hydration, using a 1mL syringe (BD Biosciences) and 23G1 needle (BD Biosciences). The mice then underwent oral intubation using a 20GA (1.1x25mm) catherer (BD Biosciences), and were connected to an air pump set at 200uL of air and 150 strokes /minute while still under isoflurane. An incision in the chest was made and the ribs were retracted to obtain a view of the heart. Using a 6.0 silk suture (Syneture, Mansfield, USA), the LAD was ligated with one stitch, and additional stitch(es) were done if the blanching of the heart, indicating the size of the affected area, was not large enough. The ribs and the skin were sutured with #5 synthetic thread (Syneture, Mansfield, USA). The mice were allowed to recover in a cage with 2% circulated O_2 and were treated with 100µL of buprenorphine, twice a day for an additional 3 days.

One week or two weeks post-surgery the mice were randomly assigned to receive treatment with 3-4 injections (50uL total) of either: i) PBS, ii) collagen-only matrix, or iii) sLe^X-collagen matrix, using a 27G ¼ needle (BD Biosciences). The injections were guided by echocardiogram, while the mice were sedated with 2% isoflurane anaesthetic, applied as described above. The mice were then observed for 4 weeks post-treatment, after which they were subsequently sacrificed. The heart tissue was harvested, aspirated by a saline solution, frozen in O.C.T. compound (Sakura Finetek, Torrence, USA), and stored in -80°C until required for sectioning and immunohistochemistry. For Western blots and cytokine arrays, the infarcted tissue was dissected and snap-frozen in liquid nitrogen after which it was transferred to -80°C freezer for storage. Figure 2.1 summarizes the animal method and the procedure timeline.

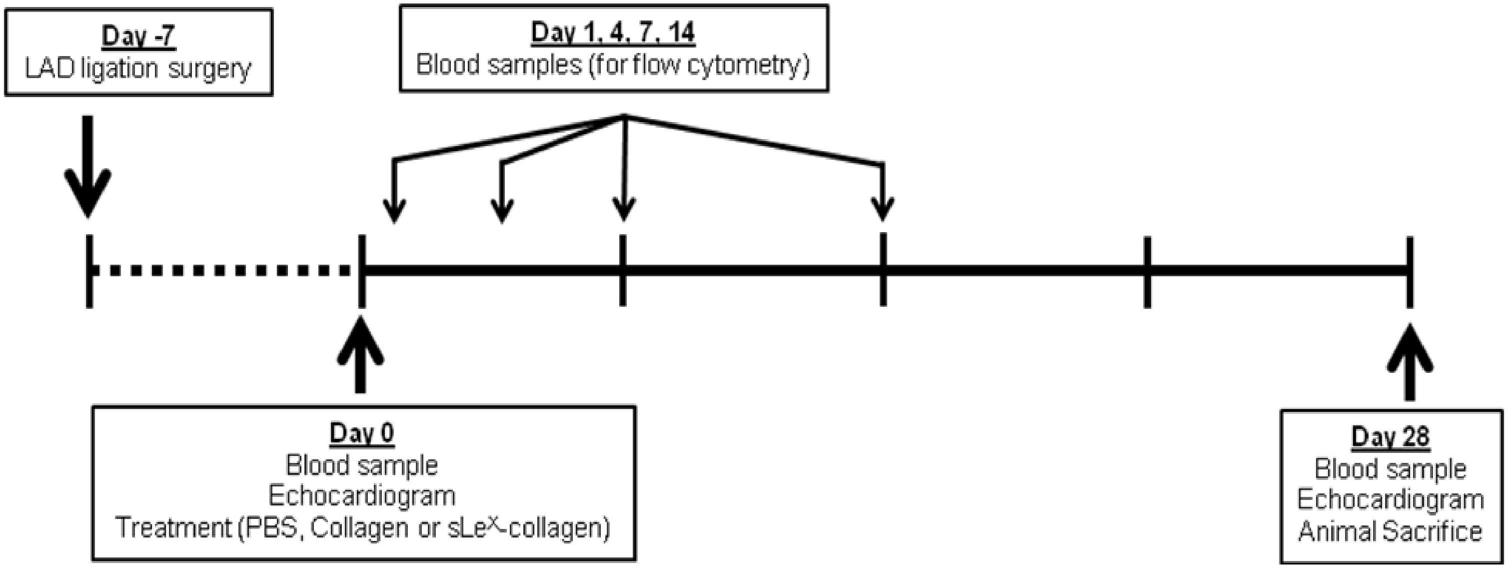

Figure 2.1. Timeline of the methods and procedures for the 1 week mouse MI model. A week after the LAD ligation surgery the mice were treated with either: PBS, collagen or sLe^X-collagen matrix. Blood samples were taken at various time points over a span of 4 weeks. At 4 weeks post-treatment, cardiac parameters were measured via echocardiogram and the heart tissue was harvested for additional experiments. In total, the mice were observed for a period of 5 weeks.

2.1.3 Echocardiography

Echocardiography was performed just prior to treatment (baseline), and again 4 weeks post-treatment using a 270Hz probe and the Vevo 770 V3.0 system (VisualSonics, Toronto, Canada). Left ventricular ejection fraction (LVEF), fractional area change (FAC) and LV mass parameters were measured using the provided Vevo program. Mice with an LVEF at baseline falling in the range of 25-45% were selected for inclusion in the study.

2.1.4 Flow Cytometry

Blood was procured for flow cytometry using a 23 ½ gauge needle. Between 50-80uL of blood was sampled by pricking the vein of the left or right leg of the mice and collected in blood cuvettes (Sarstedt, Germany) containing ethylenediaminetetraacetic acid (EDTA) to prevent blood coagulation. The blood was layered on Histopaque 1077 (Sigma-Aldrich, Oakville, Canada) and separated via density-gradient centrifugation by spinning the sample for 30 minutes at 2160 rotations per minute (rpm). The buffy coat containing the PBMCs was isolated and stained for 30 minutes in 100µL of PBS containing the following antibodies: i) 5µL of CD34-phycoerythrin (PE)-Cy5 (BioLegend, San Diego, USA), ii) 3µL of Flk-1(VEGFR2)-Pacific Blue (BD Biosciences), iii) 5µL of CD45-Fluorescein isothiocyanate (FITC) (R&D systems, Minneapolis, USA), iv) 2µL of c-kit-R-PE (Southern Biotech, Birmingham, USA), and v) 3µL of CXCR4-allophycocyanine (APC) (BD Pharmingen, San Diego, USA). Samples were also stained with the same quantity of the appropriate IgG isotype-matched controls. The amount of positive cells were analyzed and quantified with a BD FACSAria cell sorter (BD Biosciences).

2.1.5 Histology and Immunohistochemistry

Frozen heart tissue was cut into 10µm sections, which were placed on Superfrost microscope slides (VWR, Mississauga, Canada) and stored in -80°C until needed. Sections of 10µm were prepared to ensure that the thin, infarcted wall would not shatter or break during the sectioning process. Each mouse heart sample was used to generate 5-7 sections obtained at different levels of the heart. To stain tissue for various markers, the tissue was first fixed by placing it in 4% paraformaldehyde (PFA) or acetone for 10-15 minutes. The tissue was then washed with either 1x PBS or Tris buffered saline (TBS), three times for five minutes each.

Hematoxylin phloxine saffron (HPS) staining was conducted by placing the hematoxylin solution on the tissue for 7 minutes, following a wash in running tap water for 2 minutes. Next, the stain was differentiated in 0.2% acid alcohol by dipping them 30 times in the solution, and then washed for 2 minutes in running tap water. The tissues were dipped to blue the colour in lithium carbonate and washed again, this time for 5 minutes in warm running water. Phloxine stain was applied for 1 minute, followed by a wash and rehydration in absolute alcohol. The tissues were next stained with saffron for 2 minutes followed by a wash in alcohol and then a 1 minute rinse in the toluene. The infarct thickness was calculated using the Image J program: the average of three random measures of infarct thickness were divided by the average of three thickness measurements of the interior LV wall.

For immunostaining, the tissue was blocked for an hour, in 10% (v/v) of fetal bovine serum (FBS) or normal horse serum (NHS) diluted with PBS. Following the blocking step, the tissue was incubated with one or more of the following : i) 1:200

dilution of smooth muscle actin (SMA) (abcam, Cambridge, USA); ii) 1:50 dilution of CD31 (abcam); iii) 1:100 dilution of connexin 43 (abcam); iv) 1:50 dilution of nkx2.5 (Santa Cruz Technology, Santa Cruz, USA); v) 1:500 active caspase 3 (abcam); vi) 1:50 dilution of CXCR4 (abcam); vii) 1:800 dilution of CD68 (abcam) or viii) 1:50 dilution of c-kit (Santa Cruz). The slides were incubated with the primary antibody overnight at 4°C. The following day the tissue was washed using 1x PBS or 1xTBS containing 0.1% Tween and then incubated for an hour at room temperature with an appropriate secondary antibody either conjugated with horse radish peroxidase (HRP) (Cell Signaling Technology, Danvers, USA), alexa 488 (Invitrogen, Burlington, Canada), alexa 594 (Invitrogen) or FITC (Santa Cruz Technology). Subsequently, the tissue was once again washed three times for five minutes with either 1x PBS or 1xTBS containing 0.1% Tween, then mounted with 4',6-diamidino-2-phenylindole (DAPI) mounting media (Vector Laboratories, Burlington, Canada). Fluorescent-conjugated antibodies were viewed using an Olympus B×60 fluorescent microscope (Olympus Canada Inc., Markham, Canada). Four to five pictures of the infarcted area were taken per heart section and the number of positive cells was counted per field of view (FOV). For connexin 43 staining, the number of green stained pixels was quantified using Adobe Photoshop and calculated as a percentage over the total pixel count of the tissue.

In the case of the HRP-linked secondary antibodies, further steps needed to be taken prior to mounting of the slides. After the washing of the secondary HRP antibody, the slides were incubated with ImmPACT DAB peroxidase substrate (Vector Laboratories) for 10-15 minutes, which stains the positive tissue brown. The slides were washed two times for 5 minutes with distilled water and further stained with hematoxylin solution (Sigma-Aldrich) for 1 minute, which stains the nuclei blue for visualization of the cells. The slides were once again washed two times for 5 minutes with distilled water and then mounted as described above. Negative controls for each antibody were performed following the procedure described above except with omission of the primary antibody step. Negative controls were analyzed qualitatively for non-specific staining.

2.1.6 Bromodeoxyuridine

A subset of mice, which underwent the ligation and the injection procedures already described previously, were given 0.25mg/mL of bromodeoxyuridine (BrdU; Sigma-Aldrich), made fresh daily and provided in their drinking water for the duration of the 4 week post-treatment period, excluding weekends. Following the 4 weeks, the mice were sacrificed and their heart tissue was harvested, frozen in O.C.T. and stored in -80°C. The staining procedure was conducted as described above, except prior to the blocking step the slides were incubated with 1x sodium citrate buffer and heated up in a microwave for 5 minutes, followed by three washes of 5 minutes each with PBS. The primary BrdU antibody (BD Biosciences) concentration used was 1:500 diluted in 10% FBS.

2.1.7 Cytokine Array

The thin infarcted tissue was dissected from the frozen mouse hearts and the protein was extracted using lysis buffer accompanying the cytokine array kit with a dissolved protease inhibitor cocktail (Roche, Laval, Canada). The concentration of the protein in each sample was quantified using a Bradford Assay Kit (Thermo Scientific, Ottawa, Canada). Cytokine array of 80ug of heart tissue samples were conducted according to manufacturer’s protocol. Selected capture antibodies, spotted in duplicate on glass slides (RayBiotech, Norcross, USA) were purchased and incubated with provided antibodies and reagents. Briefly, the array was left to air dry for 2 hours before blocking the antibodies with the 1× blocking buffer (provided) for 30 minutes. The blocking buffer was decanted and 80ug of tissue lysate per array was incubated overnight at 4°C with gentle shaking. The following day, samples were decanted and the arrays were washed with the provided buffers. The arrays were next incubated with 70µL of biotin-conjugated antibodies for 2 hours at room temperature followed by further washing. Finally, the array slides were incubated with 70µL of fluorescent dye-conjugated streptavidin for 2 hours before washing and air drying. Membranes were then exposed and the light produced at each spot (proportional to the amount of cytokine bound) was quantified using AlphaEaseFC. For a complete list of the cytokines refer to the Appendix, Table 2.

2.1.8 Western Blots

Protein samples extracted for the cytokine array, as mentioned above, were also used to conduct Western blots. Protein (50µg per mouse sample) was run on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel made by mixing: 4.0mL of 30% acrylamide mix (Bio-Rad, Mississauga, Canada), 2.5mL of 1.5M Tris buffer pH~8.8, 0.1mL of 10% SDS (Sigma), 0.1mL of ammonium persulfate (APS; Sigma), 4uL of tetramethyethylenediamine (TEMED) in 3.3mL of water per gel. The running buffer contained 3g/L Trizma-base, 14.5g/L of glycine and 1g/L of SDS mixed in double distilled water. A rainbow ladder (BioRad) was used in order to identify the approximate weight of the targeted protein. The gel was run at ~100 volts, following which it was transferred onto a nitrocellulose-membrane (BioRad). The recipe for the transfer buffer is as follows: 25mM Trizma-base, 192mM glycine, 20% methanol (v/v) in double distilled water. The gel was transferred for 75 minutes at 130 volts. The blots were blocked in 5% skim milk diluted with 0.1% Tween-Tris buffered saline (TBST) for an hour and primed overnight at 4°C with the following antibodies: 1:1000 dilution of caspase 9 (abcam) and 1:1000 dilution of connective tissue growth factor (CTGF) (abcam). The primary antibodies were probed with 1:13000 dilutions of appropriate HRP-conjugated secondary antibodies (Cell Signaling Technology) for an hour and washed 4 times for 5 minutes in TBST. An ECL Western kit (Thermo Scientific) was used to visualize the bands by ECL machine FluorChem 9900 (Thermo Scientific).

The blots were stripped with a buffer composed of 2% SDS (w/v), 700uL of β-mercaptoethanol made up to 100mL with TBST. This was done by submerging the blots in the stripping buffer for 30 minutes at ~50°C. The blots were then washed 4 times for 5 minutes each with TBST and probed with 1:1000 dilution of α-tubulin (Cell Signaling Technology), as a loading control. A similar procedure as above was used to probe for the primary antibody and visualize the protein with the ECL kit. Image J program was utilized to quantify the protein bands. The quantity of each sample was divided by the quantity of α-tubulin measured for the same sample. This was done to standardize the amount of protein loaded onto the gel.

2.1.9 Statistical Analysis

Values are expressed as means ± standard mean error. Statistical analysis between groups was performed using a one-way Anova. For individual two-group comparisons, a student’s paired t-test was performed. *P*-values of ≤ 0.05 were considered statistically significant.

2.2 METHODS PART B: Cryopreservation of PBMCs

2.2.1 Cell Isolation

The study was approved by the Human Research Ethics Board of the University of Ottawa Heart Institute, and informed consent was obtained from all donors. Total PBMCs from 100µL of blood were isolated from fresh samples of seven healthy human donors by layering ~20mL of the blood on 20mL of Histopaque 1077 (Sigma-Aldrich) and using density-gradient centrifugation to separate the different portions of the blood. The layered blood was spun at 2160rpm for 30 minute in a centrifuge and the cells within the buffy coat were collected using a plastic pipette. The cells were washed two times with wash buffer (0.833mL of 6.5% EDTA, 0.5mL of FBS diluted to 50mL with PBS) and concentrated by spinning them down for 10 minutes at 1400rpm. Upon the last wash the cells were either analyzed (baseline PBMCs), cultured to generate CACs, or cryopreserved.

2.2.2 Cryopreservation

PBMCs retrieved from the buffy coat were reconstituted in a 1ml solution of Isocove's Modified Dulbecco's Medium (IMDM; Stem Cell Technologies, Vancouver, Canada) containing 5% dimethyl sulfoxide (DMSO; Sigma-Aldrich) and 6% donor cell-matched serum and placed into a cryovial (Corning Incorporated, Corning, USA). The DMSO was mixed with the IMDM prior to addition of blood serum and cells in order to prevent clump formation. The cells were cooled to -80°C over 3 hours before being transferred to liquid nitrogen for storage.

For rapid thawing and to prevent toxicity to cells, cryovial tubes were taken from the freezer and immediately placed in a 37°C water bath until the ice crystals disappeared, after which the cells were diluted 5× with IMDM and centrifuged at 1400 rpm and immediately re-suspended with fresh EBM.

2.2.3 CAC Culture

CACs were generated by seeding fresh PBMCs, or PBMCs thawed at days 1 or 28 post-freezing, on fibronectin (Sigma-Aldrich)-coated plates in Endothelial Basal Media (EBM-2; Clonetics, Guelph, Canada) supplemented with EGM-2-MV-SingleQuots (Clonetics) containing 5% fetal bovine serum, 50ng/mL human VEGF, 50ng/mL human insulin-like growth factor 1, 50ng/mL human epidermal growth factor and antibiotics for 4 days. After 4 days, the media was removed and the cells were lifted using gentle pipetting with PBS and prepared for subsequent assays.

2.2.4 Flow Cytometry Staining

Cells were counted using a Vi-Cell analyzer (Beckman Coulter, Mississauga, Canada). PBMCs or CACs (3×10^5) in 200µL of EBM were stained for 30 minutes at 4 ºC with antibodies against the following antigens: CD31-FITC (Beckman Coulter), CD34-PECy7 (BD Biosciences), KDR-PE (R&D Systems) and L-selectin-ECD (Beckman). Samples were also stained with appropriate IgG isotype-matched controls. Immediately prior to analysis, the viability stain 7-actinomycin D (7-AAD) (Invitrogen) was added to samples to make a final concentration of 8.3µg/mL. Characterization also included incubating 3×10^5 cells with 2 µg/mL of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine–labeled acetylated low density lipoprotein (DiI-LDL) (Invitrogen) for one hour at 37ºC after which the cells were pelleted, fixed for 10 minutes with fixation buffer (BD Biosciences) and further incubated for an hour at 37ºC with 10µg/mL of FITC labelled lectin from *Ulex europaeus* agglutinin-1 (Sigma-Aldrich).

Fluorescent cells were analyzed and quantified with a BD FACSAria cell sorter (BD Biosciences).

2.2.5 Functional Assays

Cells were suspended in 50µg/mL of DAPI (Sigma-Aldrich) in PBS for 30 minutes at 37ºC, after which the cells were pelleted and counted. *Migration.* DAPI stained PBMCs or CACs (2×10^4) were suspended in 100µL of VEGF-free EBM and placed in upper chamber of a 24-well Boyden chamber (Corning Inc.) with the lower chamber coated with fibronectin and containing 350µL of 0.5µg/mL VEGF (Cedarlane) in EBM. The cells were incubated for 24 hours in 5% CO_2 allowing them time to migrate, via chemotaxis towards VEGF, through the membrane separating the two chambers and into the lower chamber where they could adhere. Following the 24 hours, the Boyden chamber and the VEGF media were removed and the cells were fixed with 4% PFA and washed with PBS. *Adhesion.* DAPI-stained cells (2×10^4) were plated in 1mL of EBM on fibronectin coated 24-well plates and incubated for 1 hour in 5% CO_2 after which they were fixed with 4% PFA. The number of DAPI stained cells for both assays were counted in 5 random fields-of-view for each well at 20× magnification using Olympus B×60 fluorescent microscope (Olympus Canada Inc). Assays were performed in duplicate.

2.2.6 Statistical Analysis

Results obtained for each phenotype and each cell type were analyzed for differences between days using a One-Way Anova adjusted for donor. Sub-analyses were performed when a Bonferonni significant result was obtained using a paired t-test. Results were Bonferonni adjusted for the number of tests performed for each of viability, phenotype and function separately, to achieve an alpha of 0.05. *P*-values which are Bonferonni corrected are denoted p_B; *p*-values given for sub-analyses are not corrected. Statistical analyses were performed using R (R development core team, Vienna, Austria).

A summary of the cell populations, methods and time points are presented in Figure 2.2.

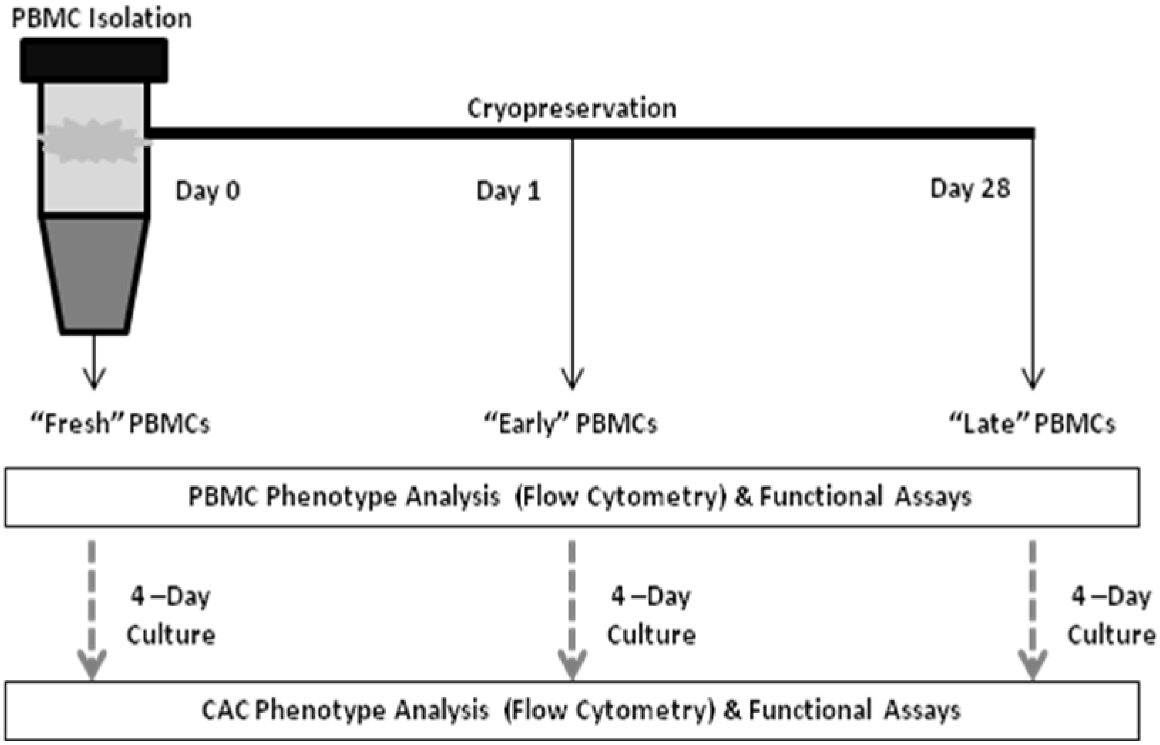

Figure 2.2. Summary of the cryopreservation methods. In brief, PBMCs were isolated and either analyzed (fresh), plated to generate CACs, or cryopreserved for 1 or 28 days after which the cryopreserved cells were thawed and analyzed or plated to generate CACs from cryopreserved PBMCs. Phenotype analysis of the cells was conducted by staining the cells for various surface markers and quantifying the number of positive cells by flow cytometry. Functional assays including: LDL-uptake, lectin binding, adhesion and migration assays were conducted on both the fresh and cryopreserved cells.

RESULTS

3. Results

3.1 RESULTS PART A: sLe^X-collagen matrix in MI mouse model

3.1.1 Two-week MI mouse model

As it was already well-established in our lab, we first chose to pursue our studies using a MI mouse model, with a treatment time point of 2 weeks post-surgery. The 2 week time point was initially chosen for comparison with another set of experimental animals that were originally scheduled for imaging studies by positron emission tomography (PET). The PET imaging protocol for cell tracking had required treatment delivery at the 2 week period post-MI induction to ensure that the inflammatory process had subsided, in order to reduce background for the specific imaging procedure to be implemented.

3.1.1.1 Collagen based- sLe^X matrix improves heart function

The percent of ejection fraction (%EF), two weeks after LAD ligation, was on average 30-35% (Figure 8.1, Appendix). The change in %EF and LV mass between pre-treatment (baseline) and 4 weeks post treatment (d28) was calculated and graphed (Figure 3.1 A-B, n=5-7). Mice treated with sLe^X-collagen matrix showed an increased in %EF by 2.9±2.6%. This was significantly greater than the PBS-treated group, which showed a decline in %EF of -9±2.5% (p=0.009). The collagen-only treated mice showed a decline as well in %EF of -1.7%±1.5%, although it was less severe than the PBS-treated group (Figure 3.1A). The change in LV mass in milligrams (mg) was measured by echocardiogram and calculated as the difference between baseline (pre-treatment) and 4 weeks post-treatment. The change in LV mass was highest in sLe^X-collagen group (30.6±18.1mg; n=5), while a decline was observed in the collagen-treated group (-13.6±13.7mg, n=4). The PBS group showed an LV mass change of 11.5±7.1mg (n=7); however, no significant differences were found between the three treatments.

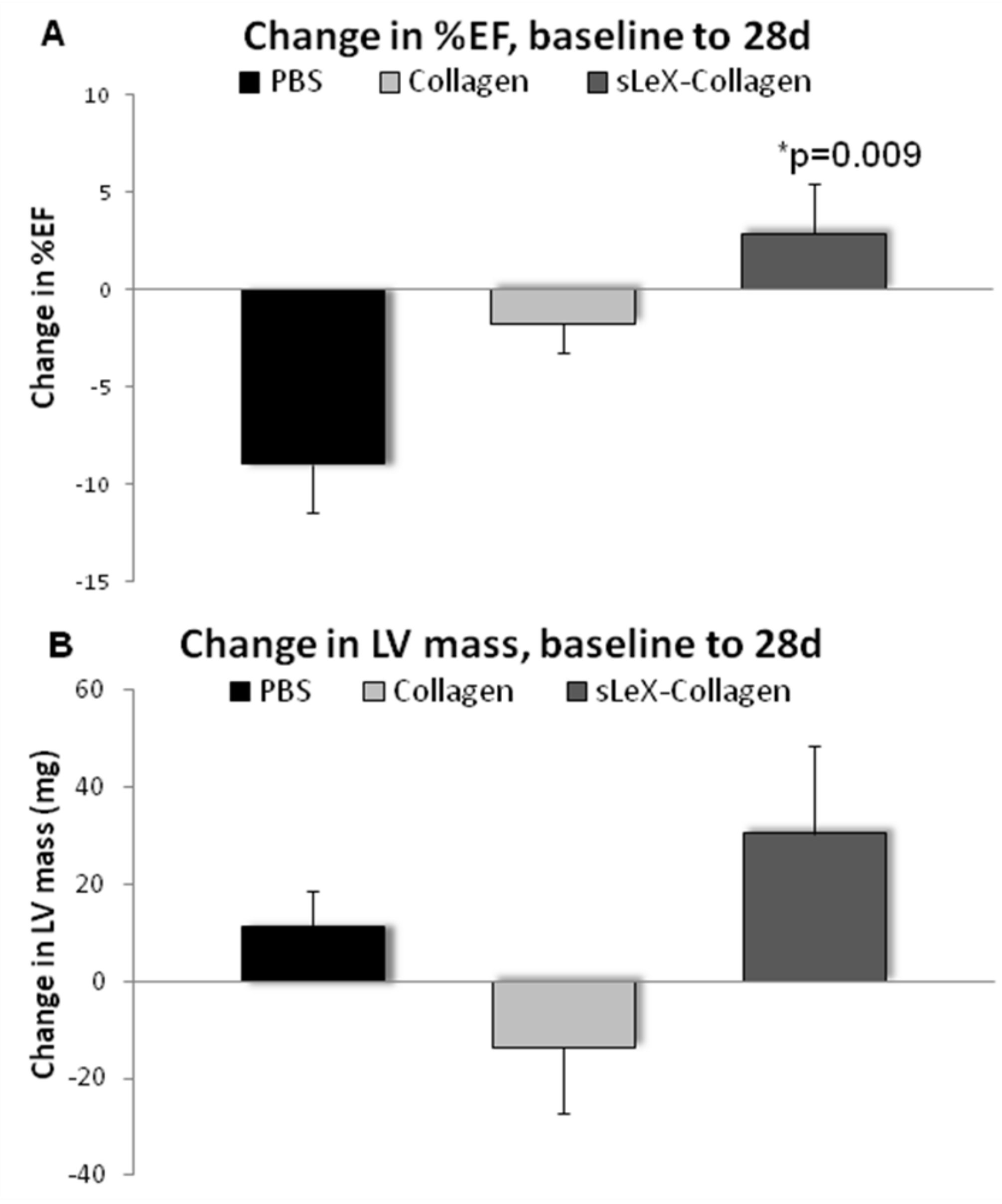

Figure 3.1. %EF and LV mass change in MI mice treated 2 weeks after infarct surgery. (**A**) %EF and (**B**) LV mass were measured using an echocardiogram, and the difference between the baseline (pre-treatment) and 4 weeks post-treatment (d28) were calculated and presented as an average change between the three treatments. (***** indicates statistical significance when compared to PBS, 4≤n≤7)

3.1.1.2 Cell mobilization into the circulation is unaffected

Blood samples were taken throughout the 4 weeks post-treatment (day 0, 1, 4, 7, 14 and 28) to analyze mobilization of progenitor and angiogenic cells in the circulation of the MI mice. There was no statistical difference or observable trends between the three treatments for the different cell surface markers thought to be expressed on angiogenic and progenitor cells. The graphs are shown in Figure 3.2 A-E and the values at each time point are represented as fold-change relative to pre-treatment (day 0) cell numbers.

3.1.1.3 Collagen based matrices improve wall thickness but not vasculature

Histology of the MI tissue sections 4 weeks post-treatment (6 weeks post-MI induction) was conducted using the HPS stain. The thickness of the infarct as a ratio to the interior wall thickness was measured using the program Image J. This measurement provides an indication of how well the infarct wall was preserved - a higher ratio indicates a thicker infarcted wall. MI mice treated with the sLe^X-collagen matrix (0.26±0.11; n=4) or with the collagen-only matrix (0.24±0.04; n=5) showed greater preservation of wall thickness ratio compared to the PBS-treated group (0.15±0.05; n=5) (Figure 3.3 A; *p*=0.08 and *p*=0.01, respectively). Frozen heart tissue sections of the MI mice were also stained for smooth muscle actin (SMA) and CD31, an endothelial marker found on the surface of the lumen in blood vessels, to observe the blood vessel density in the ischemic tissue. The number of arterioles, determined as SMA$^+$ smooth muscle cells surrounding CD31$^+$ endothelial cells, per field of view were counted. No significant difference was observed between the three treatments; however, a trend for greater vascular density was observed in the sLe^X-collagen group (4.2±2.3 arterioles/FOV), compared to collagen (3.9±2.0) and PBS (3.1±1.7) (Figure 3.3 B, n=5).

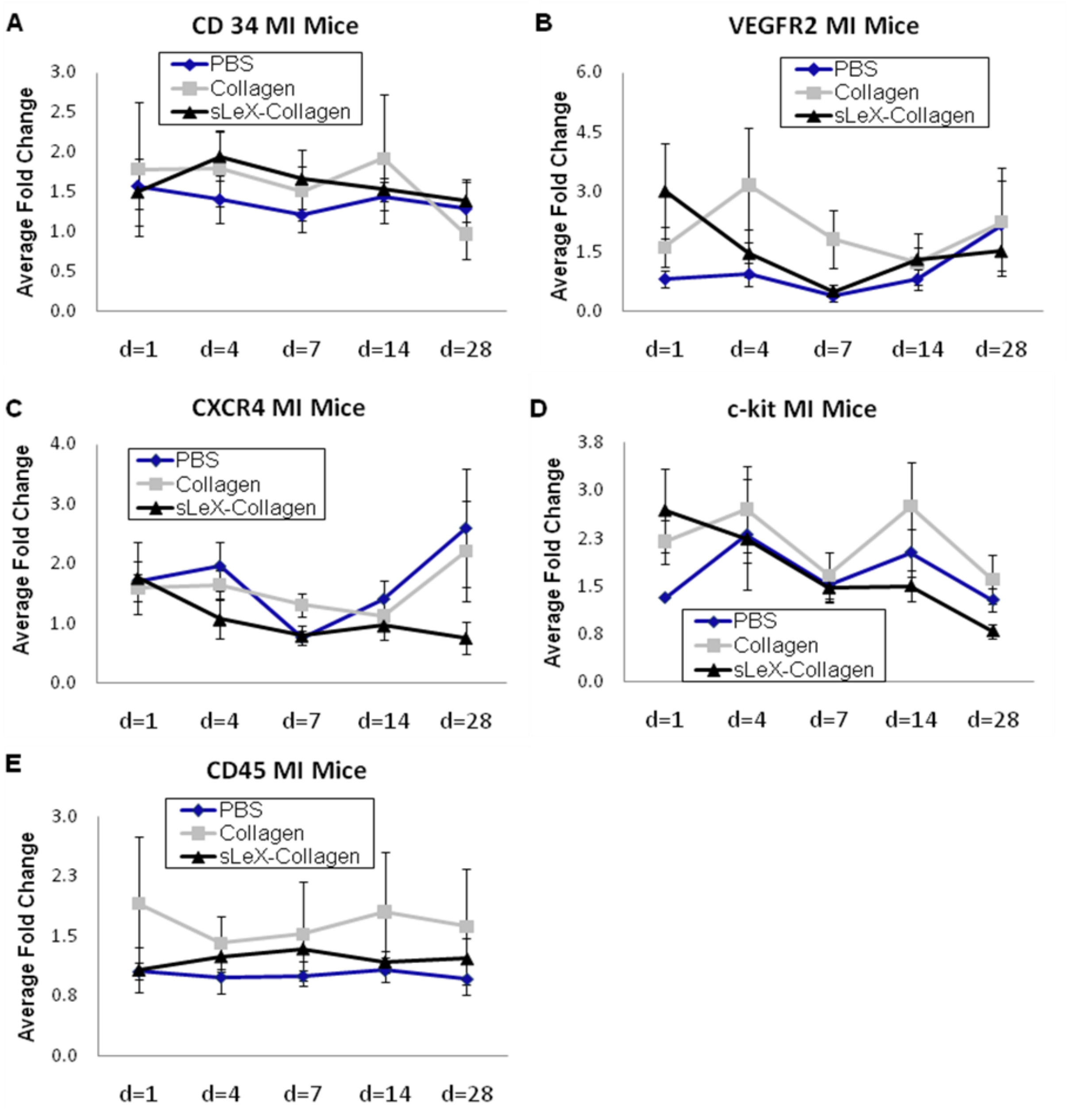

Figure 3.2. Mobilization of cells expressing A) CD34 and D) c-kit (progenitor markers), B) VEGFR2 and C) CXCR4 (angiogenic markers), and E) CD45 (leukocyte marker) quantified using flow cytometry (2 week mouse model). Blood samples (50-80uL) were taken from mice on days 0, 1, 4, 7, 14 and 28 and the PBMCs were isolated using gradient centrifugation. The cells were stained and quantified by flow cytometry and are represented as a fold change relative to pre-treatment levels ($4 \leq n \leq 7$).

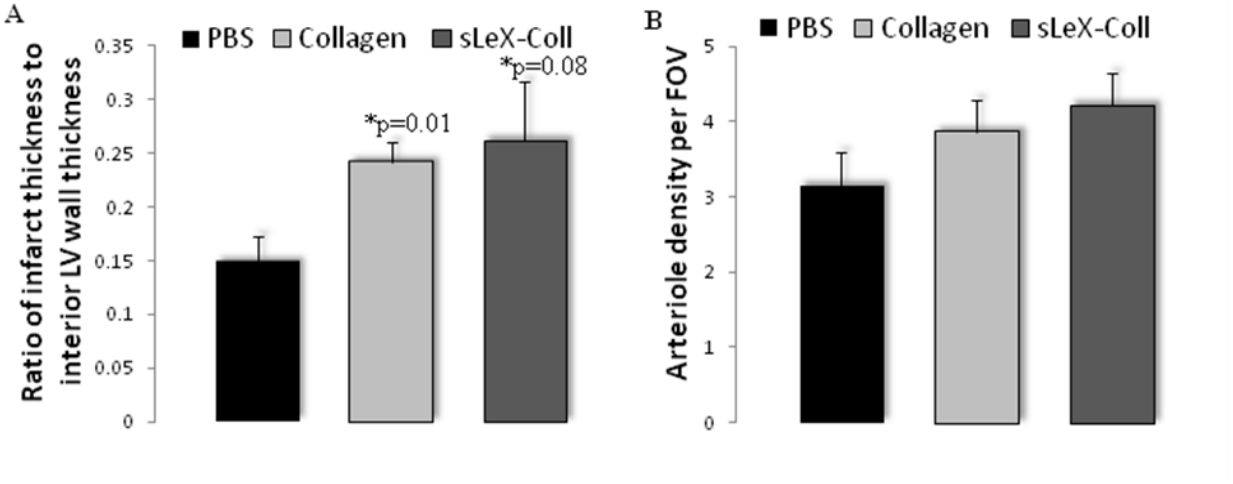

Figure 3.3. Histological assessment of the MI heart tissue. The tissue was stained with: (**A**) HPS, which allowed for the measurement of infarcted wall thickness; or (**B**) CD31 and SMA for arteriole density measurements. (* indicates statistical significance compared to PBS, n=5)

The data for the MI mice injected 2 weeks post-MI surgery with our matrices while showing some benefit when compared to PBS, was not as successful as we had hoped for. Although a change in the time of treatment delivery would preclude comparison with animals used in a related imaging study, it was determined that an earlier time of treatment (1 week) may be more favourable, since previous data has shown that mobilization of EPCs and other BMCs peaks in the first week after a MI (Takahashi et al. 1999). Furthermore, another study conducted in our lab where collagen matrices were injected with/without a mixture of CACs at one week post-MI surgery, demonstrated a much more beneficial effect (Ahmadi et al., unpublished data). Therefore, we decided to continue the experiments with the delivery of treatment at one week post-MI.

3.1.2 *One-week MI mouse model*

3.1.2.1 Collagen based matrices improve heart function and wall thickness

Echocardiogram measurements of the mouse MI heart pre-treatment and 4 weeks post-treatment were conducted with the mice showing %EF of around 35% at 1-week post-MI (Figure 8.2 A, Appendix). This is reduced in comparison to the normal %EF of mouse hearts, which are recorded at 60-75%. A small improvement in the left ventricle ejection fraction (LVEF) of the sLe^X-collagen treated group (2.5±2%, n=18) was seen, whereas the collagen group remained unchanged (0.6±1.9%, n=20) and the PBS-treated mice showed a decline in LVEF (-4.1±1.1%, n=19) (Figure 3.4 A). The LVEF was greater in the sLe^X-collagen matrix and collagen-only treated mice, compared to the PBS group (p=0.008 and p=0.05, respectively). The percentage of fractional area change (FAC), another measurement of how well the heart contracts, followed the same trend between sLe^{X}-collagen (1.1±1.3%, n=18), collagen (0.6±1.3%, n=20) and PBS (-2.3±0.7%, n=18) treatment groups (Figure 3.4 B, *p*=0.03 and *p*=0.06 for sLe^{X}-collagen and collagen-only matrix compared to PBS, respectively).

LV mass was also measured by the echocardiogram (data shown in Appendix, Figure 8.2 B) and the endpoint measurements were compared to the body weight (Figure 8.2 C, Appendix) of the animals at the same time point. An increase in heart mass to body mass ratio has been found to correlate with cardiomyopathies (Zhang et al. 2001). The sLe^{X}-collagen matrix treated mice had a trend for a slightly lower ratio (6.8±0.5 mg/g; n=17) compared to PBS (7.2±0.6 mg/g; n=16) and collagen (7.1±0.5 mg/g; n=19) (Figure 3.4 C).

Histology of the MI tissue sections 4 weeks post-treatment (5 weeks post-MI induction) was conducted using the HPS stain. The thickness of the infarct (a ratio compared to the interior wall) was found to be non-significantly greater in the sLe^{X}-collagen matrix- and collagen matrix-treated groups (0.43±0.07 and 0.42±0.06, respectively; n=6) compared to PBS treatment (0.26±0.05, n=6; Figure 3.4 D; $p \leq 0.07$).

3.1.2.2 Mobilization of progenitor and angiogenic cells in the circulation

Blood samples were taken throughout the 4-week post-treatment period to analyze mobilization of progenitor and angiogenic cells in the circulation of the MI mice. Overall, a trend for increased numbers were observed in animals receiving either matrix treatment compared to the PBS group, although no significant differences were observed (Figure 3.5, n=12). The number of CD34^{+} cells in the circulation were higher at all time points in the collagen and sLe^{X}-collagen matrix treatment groups compared to PBS (Figure 3.5 A, n=12). VEGFR2^{+} cells in the circulation were higher in the collagen treated group during the first week post-treatment, after which it declined to levels similar to the PBS group (Figure 3.5 B, n=12). C-kit$^+$ cells also showed a slight increase in circulation compared to PBS treated groups (Figure 3.5 C, n=12). CD45$^+$ cells did not show many differences between the three treatments, except for the first day, when the sLe^X-collagen treated group showed a trend for higher CD45+ cell mobilization compared to PBS (Figure 3.5 D, n=12). CXCR4$^+$ cells were not significantly different between the three treatment groups, except for day 1, where PBS-treated mice had a higher number of these cells compared to sLe^X-collagen (Figure 3.5 E, n=12). CD45$^-$CD34$^+$VEGFR2$^+$ cells, a subset of circulating angiogenic cells thought to be involved in blood vessel regeneration(Case et al. 2007; Farace et al. 2007), showed a trend for a spike in numbers in the mice treated with sLe^X-collagen in the first week post-treatment (Figure 3.5 F, n=12).

3.1.2.3 Collagen matrices increase the blood vessel density network in MI mice

Frozen heart tissue sections of the MI mice were stained for smooth muscle actin (SMA) and CD31 to observe the blood vessel density in the ischemic tissue. The number of arterioles, SMA$^+$ smooth muscle cells surrounding CD31$^+$ endothelial cells, per FOV were significantly higher in both collagen and sLe^X-collagen treated matrices (7.9±0.6, 8.0±0.5, respectively, n=6) compared to PBS (5.5±0.5, n=6) (Figure 3.6 A; $p \leq 0.004$). Total CD31$^+$ vessel networks were also significantly higher in sLe^X-collagen groups (10±0.8, n=6) and collagen groups (9.8±0.8, n=6) compared to PBS (7.1±0.7, n=6) (Figure 3.6 B; $p \leq 0.03$).

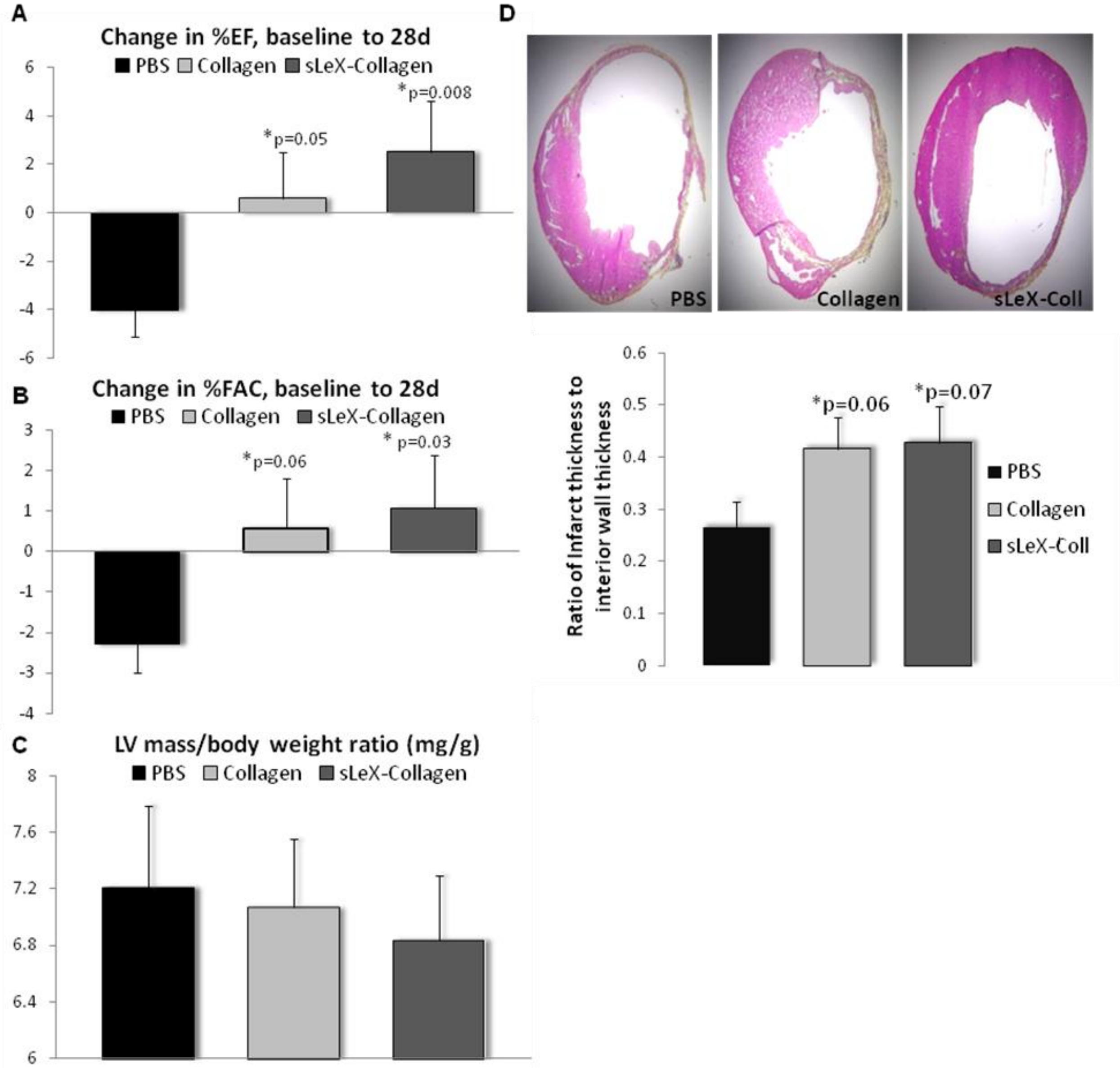

Figure 3.4. Treatment with collagen matrices improves heart function and wall thickness. The mouse heart function was measured pre-treatment (baseline at 1-week post-MI) and again 4 weeks later via echocardiogram. The changes in ejection fraction % (**A**) and fractional area change % (**B**) were calculated by subtracting the difference between the two time points. The ratio of LV thickness (as measured by echocardiogram)/body weight at 4 weeks is expressed in mg/g, ($18 \leq n \leq 20$) (**C**). Following 4 weeks, the hearts were sectioned and stained with HPS which stains cytoplasm and muscle pink, and collagen yellow. The infarct thickness is expressed as a ratio of posterior to interior wall thickness. Representative images of the HPS stained tissue are shown (**D**) (* indicates a statistical significance of $p \leq 0.1$ when compared to PBS; n=6)

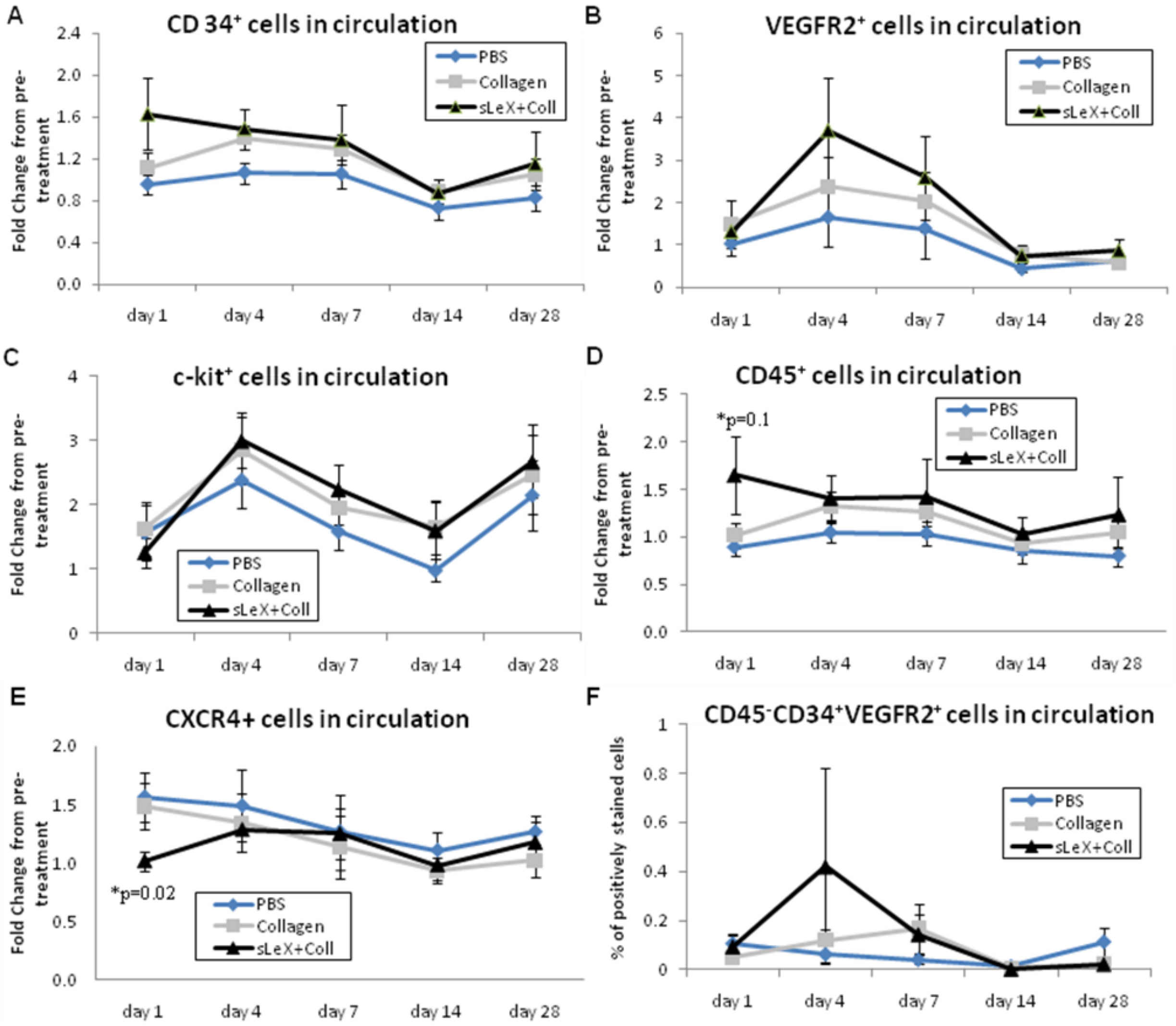

Figure 3.5. Mobilization of cells expressing A) CD34 and C) c-kit (progenitor markers), B) VEGFR2 and E) CXCR4 (angiogenic markers), and D) CD45 (leukocyte marker) quantified using flow cytometry (1 week mouse model). Blood samples were taken from mice on days 0, 1, 4, 7, 14 and 28 of the experiment and PBMCs were isolated using gradient centrifugation. The cells were stained and quantified by flow cytometry and are represented as a fold-change relative to pre-treatment levels (**A-E**). A subpopulation of circulating angiogenic cells CD45$^-$CD34$^+$VEGFR2$^+$ was also quantified and is represented as a percentage (**F**). (* indicates statistical significance of *p*≤0.1 when compared to PBS, n=12).

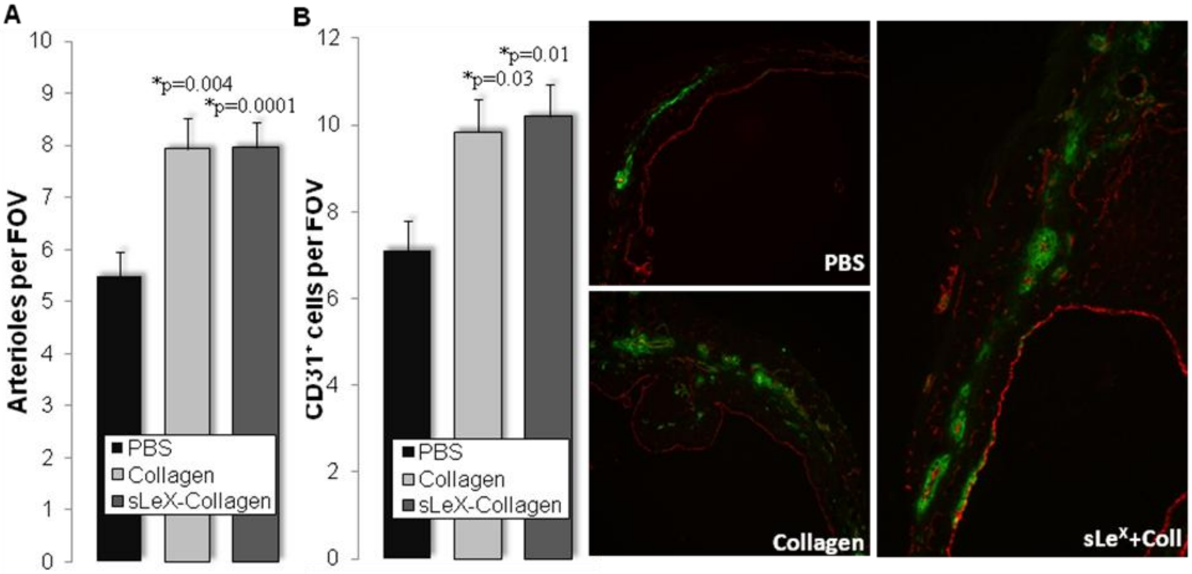

Figure 3.6. Collagen matrices increase vasculature *in vivo* in the mouse MI model. The number of (**A**) SMA$^+$ arterioles per FOV, and (**B**) CD31$^+$ vessels per FOV were determined from stained tissue sections of the infarcted heart for the 3 treatment groups. Representative pictures of slides stained for smooth muscle actin (green) and CD31 (red) are shown at a 20x magnification. (* indicates a statistical significance when compared to PBS, n=6).

3.1.2.4 sLe^X-collagen matrix stimulates heart regeneration in MI mice

Frozen heart tissue sections were stained for the gap junction protein connexin 43, thought to have a crucial role in the synchronized contraction of the heart (Oyamada et al. 1994). Mice treated with the sLe^X-collagen matrices exhibited significantly higher connexin 43 expression (2.4±0.2%; n=6), calculated as the percentage of positively stained area over the total tissue area, compared to both collagen and PBS (1.8±0.1% and 1.7±0.1% n=6, respectively) (Figure 3.7 A; p=0.0002 and p=0.0004, respectively). MI mice treated with the sLe^X-collagen matrix also had a higher number of cells per FOV that expressed nkx2.5 (5.2±0.4; n=6), an early cardiomyocyte differentiation marker (Lints et al. 1993), compared to PBS (2.5±0.7; n=6) (Figure 3.7 B; p=0.006). No significant differences were observed in the number of CXCR4$^+$ cells between PBS (44±5; n=6), collagen (49±2; n=6) and sLe^X-collagen (48±5; n=6) (Figure 3.7 C). The number of c-kit+ cells, a marker of resident cardiac progenitor cells (Beltrami et al. 2003), in the infarcted tissue was significantly higher in sLe^X-collagen matrix-treated groups (2.0±0.2; n=6) compared to PBS (1.5±0.1; n=6), while collagen treated groups had 1.9±0.3 cell per FOV (Figure 3.7 D, p=0.02). Of note, although no statistically significant difference was observed, there was a trend for a higher number of connexin 43$^+$, nkx2.5$^+$ and c-kit$^+$ cells with collagen-only treatment compared to the PBS-treated group (Figure 3.7 A, B, D).

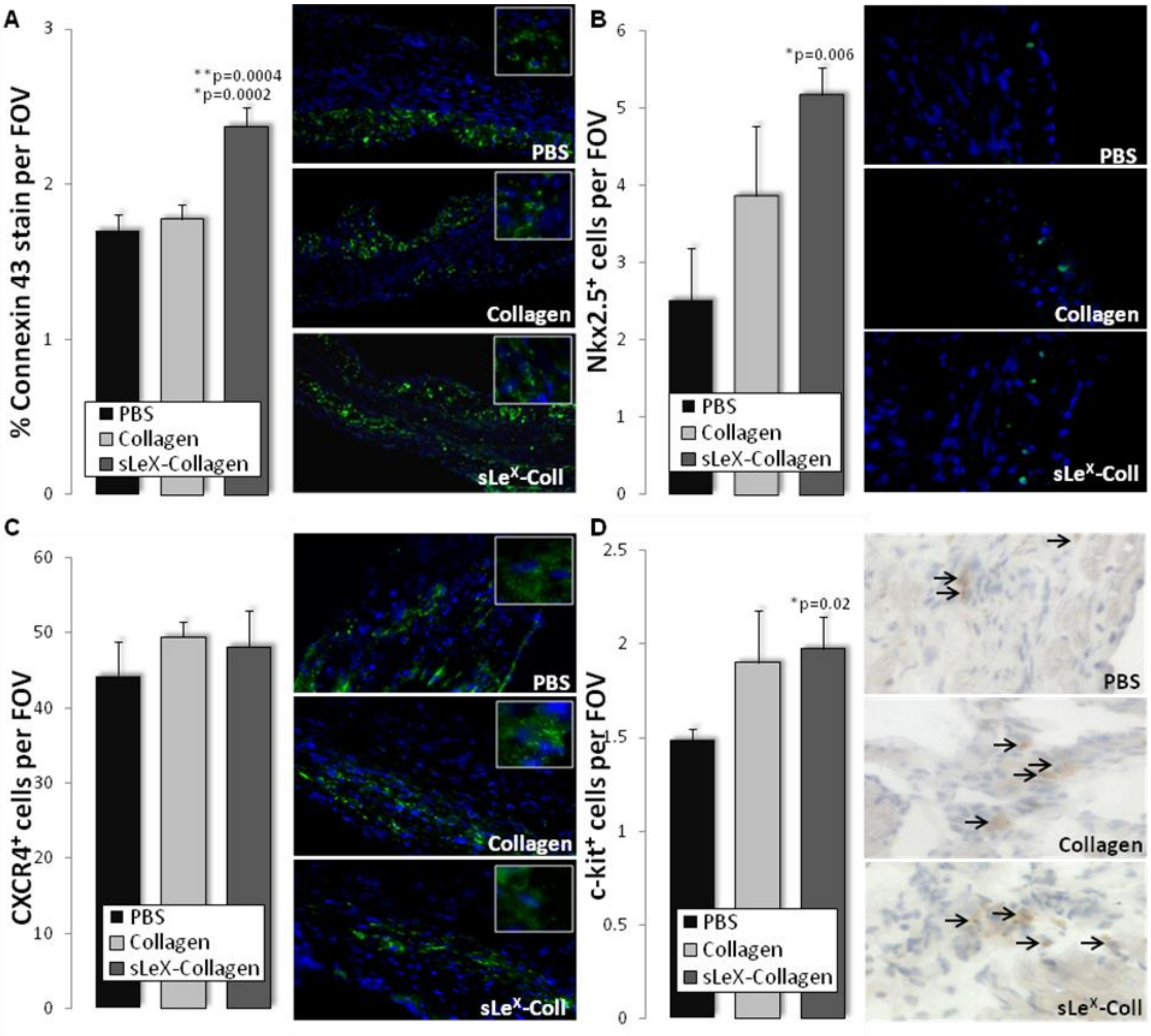

Figure 3.7. sLe^X-collagen matrix stimulates heart regeneration in MI mice. The infarcted heart tissue was sectioned and stained for DAPI (blue) and: (**A**) connexin 43 (green; 20x magnification); (**B**) Nkx2.5 (green; 60x magnification) and (**C**) CXCR4 (green; 40x magnification) and the number of positively stained cells per FOV was determined. C-kit$^+$ cells were stained with HRP (brown) and hematoxylin for nuclear visualization (blue) at 40x magnification; positive cells are indicated with arrows (**D**). (*indicates statistical significance when compared to PBS, ** indicates statistical significance when compared to collagen, n=6).

3.1.2.5 Collagen based matrices enhance the expression of regenerative cytokines

Protein extracted from infarcted hearts was used for cytokine arrays and Western blot analysis. E-cadherin, involved in cell-to-cell adhesion as well as cell migration, was higher in matrix treated groups (1.24±0.06 and 1.27±0.08 fold for sLe^X-collagen and collagen, respectively; n=6) compared to PBS (1.00±0.04; n=5), (Figure 3.8 A; p=0.01 and 0.02, respectively). Pro-MMP-9, a precursor form of MMP-9, a protein that breaks down extracellular matrix, was significantly increased in sLe^X-collagen treated mice (2.2±0.5 fold; n=7) compared to PBS (1.0±0.2; n=6). However, no significant differences were observed for another protein responsible for ECM remodelling, MMP-2. TWEAK (TNF related weak inducer of apoptosis) and its receptor TWEAK-R have been shown to positively regulate cardiomyocyte proliferation in development and adulthood. However, in adulthood the TWEAK-R is down-regulated making it harder for TWEAK to influence cell proliferation (Novoyatleva et al. 2010). TWEAK production was higher with both collagen (1.17±0.07; n=6) and sLe^X-collagen (1.10±0.03; n=7) treatments compared to PBS (1.00±0.03; p=0.04), while its receptor expression was higher only in the sLe^X-collagen-treated hearts (1.22±0.07; n=6) compared to PBS (1.00±0.04; n=6) and collagen (0.092±0.08 fold; n=6; Figure 3.8 A; p=0.02). L-selectin, the ligand of sLe^X, which was incorporated into our collagen matrix, and VEGFR2, an important receptor in cell mobilization, proliferation and vasculogenesis, did not significantly differ between the three groups (Figure 3.8 A).

Western blot analysis for connective tissue growth factor (CTGF), implicated in extracellular matrix remodelling, endothelial cell function and angiogenesis (Takahashi et al. 1999; Brigstock 2002), showed increased expression in the sLe^X-collagen treated hearts (2.1±0.9 fold) compared to the PBS group (1.0±0.5 fold; *p*=0.04), but it was not different from the collagen treated mice (1.4±0.4 fold; *p*=0.1; n=5; Figure 3.8 B).

3.1.2.6 sLe^X-collagen based matrix decreases apoptosis in MI mice

Western blots for apoptotic factor caspase 9 were conducted from infarct tissue protein samples. Although no significant difference was observed between the three treatments, the collagen based matrices (0.85±0.1, n=6 for both) showed a trend for a decrease in caspase 9 compared to PBS (1±0.2, n=6) (Figure 3.9 A). The number of cells per FOV expressing active caspase 3, which is activated by caspase 9 and plays a role in the execution phase of cell apoptosis, was found to be significantly decreased in the group treated with the sLe^X enhanced matrix (2.1±0.2, n=5) compared to PBS (3.1±0.3; n=6) (Figure 3.9 B; *p*=0.02).

3.1.2.7 Collagen matrices decrease inflammatory responses in MI mice

Arrays of MI heart tissue were conducted for various cytokines including ones implicated in inflammatory responses such as interferon gamma (IFN-γ), interleukin-1 alpha (IL-1α), tumor necrosis factor alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-1). IFN-γ was found to be significantly decreased in collagen (0.74±0.06; n=5) and sLe^X-collagen (0.78±0.08; n=7) treated groups compared to PBS (1.00±0.05; n=6) (Figure 3.10 A; *p*=0.01and *p*=0.05, respectively). Although no significant difference was found, a trend for decreased expression of inflammatory cytokines IL-1α and TNF-α was also observed in the matrix treated groups compared to PBS (Figure 3.10 A). MCP-1, a chemokine that recruits inflammatory cells such as T-cells and monocytes, was significantly lower in the collagen matrices (collagen: 0.62±0.6; n=5, sLe^X-collagen: 0.70±0.1; n=7) compared to PBS (1.00±0.08; Figure 3.10

A; *p*=0.005 and *p*=0.05, respectively). Furthermore, staining of the MI tissue sections for CD68, a macrophage marker, showed a significant decrease in the number of CD68$^+$ cells in collagen (18.7±1.4, n=6) and sLe^X-collagen (21.5±1.3, n=6) groups compared to the PBS (27.8±2.1, n=6) treated group (Figure 3.10 B; *p*=0.005 and *p*=0.03, respectively).

3.1.2.8 sLe^X-collagen based matrix enhances proliferation in infarcted tissue

A subset of mice, which underwent LAD ligation, was fed with BrdU infused water for 4 weeks after the injection of PBS, collagen or sLe^X-collagen. BrdU is an analogue of the DNA base thymidine, and as such incorporates into newly replicating DNA, which can be used for identifying proliferating cells. The number of BrdU positive cells per FOV, was significantly higher in sLe^X-collagen (1.7±0.3, n=6) treated groups, with collagen treated groups also showing non-significantly higher proliferation (1.4±0.3, n=6), compared to PBS (1.0±0.4, n=6) (Figure 3.11, *p*=0.008 and *p*=0.07, respectively).

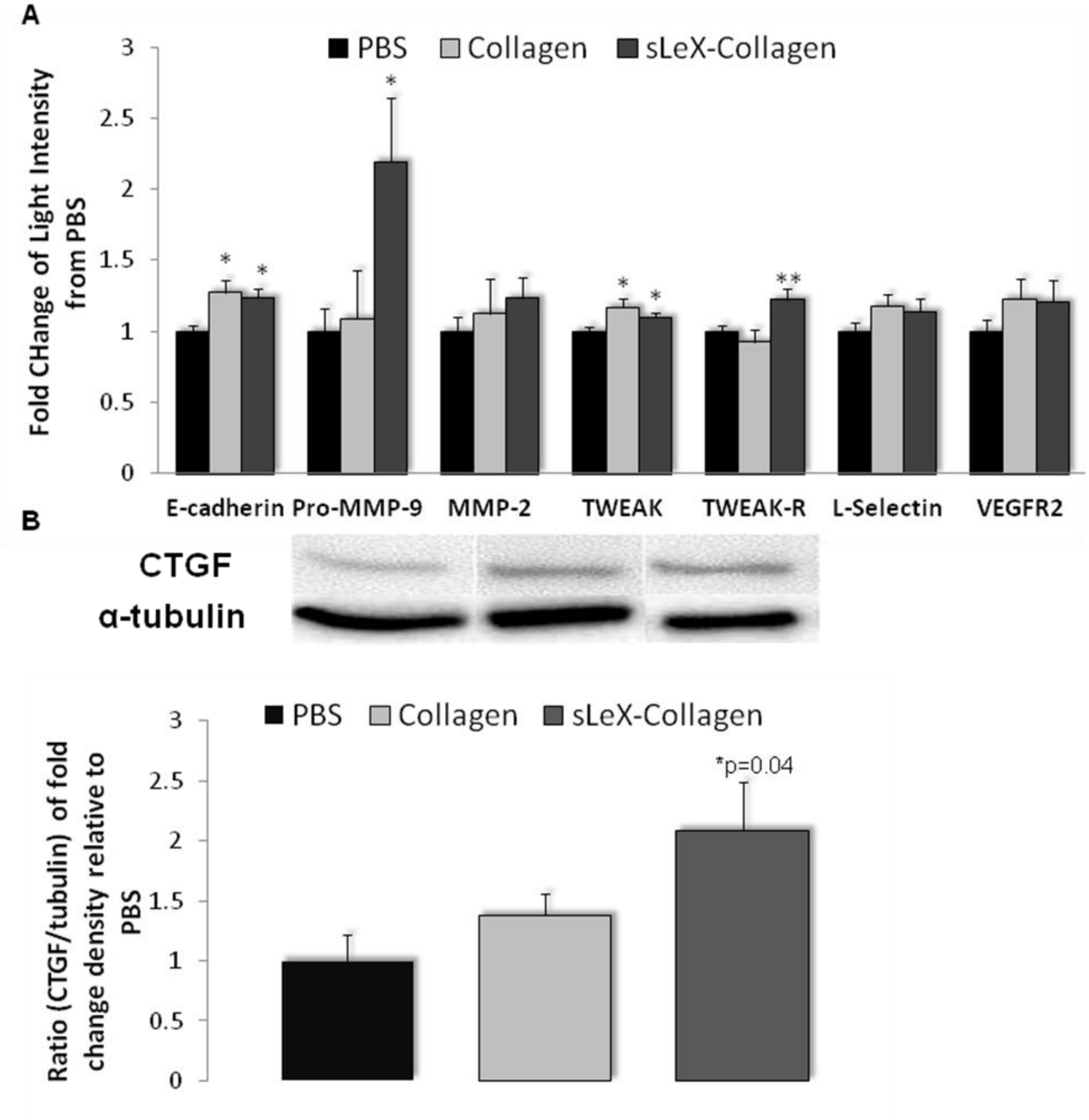

Figure 3.8. Collagen based matrices enhance cytokine expression. (**A**) Protein extracted from MI tissue was analyzed by cytokine array for factors: E-cadherin, pro-MMP-9, MMP-2, TWEAK, TWEAK-R, L-selectin and VEGFR2. The results were measured by light intensity variations and are represented as a fold change relative to the PBS treated group, with each group having 5-7 animal samples. (**B**) Protein samples were run on a Western blot and immuno-probed for CTGF with α-tubulin as a loading control. Densitometry was conducted on the blots and the representative graph of the data is depicted. (* indicates statistical significance of $p \leq 0.05$ compared to PBS, ** indicates statistical significance of $p \leq 0.05$ compared to PBS and collagen, $5 \leq n \leq 7$).

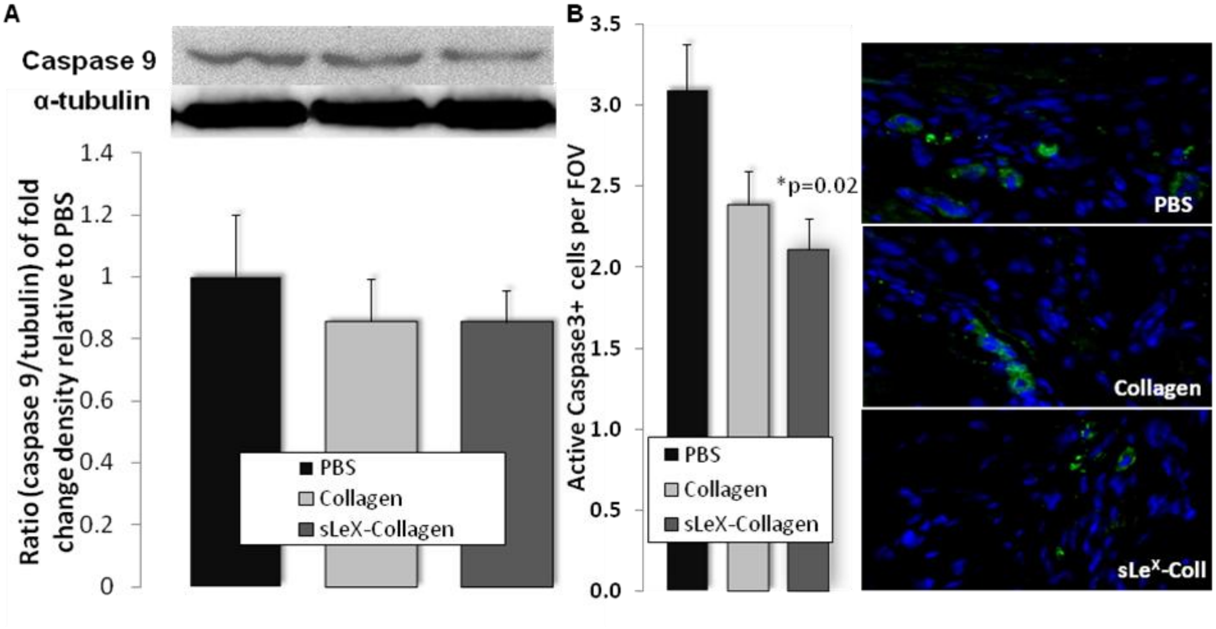

Figure 3.9. sLe^{X}-Collagen based matrix decreases apoptosis in MI mice. Western blots were conducted on samples of infarcted tissue protein and immuno-blotted for caspase 9, with α-tubulin as a loading control. Densitometry was conducted on the blots and the representative graph of the data is depicted (**A**). Infarcted tissue sections were stained for active caspase 3 (green) and DAPI (blue) and the average number of positive cells was counted per FOV at a magnification of 60x (**B**). (* indicates statistical significance when compared to PBS, n=6).

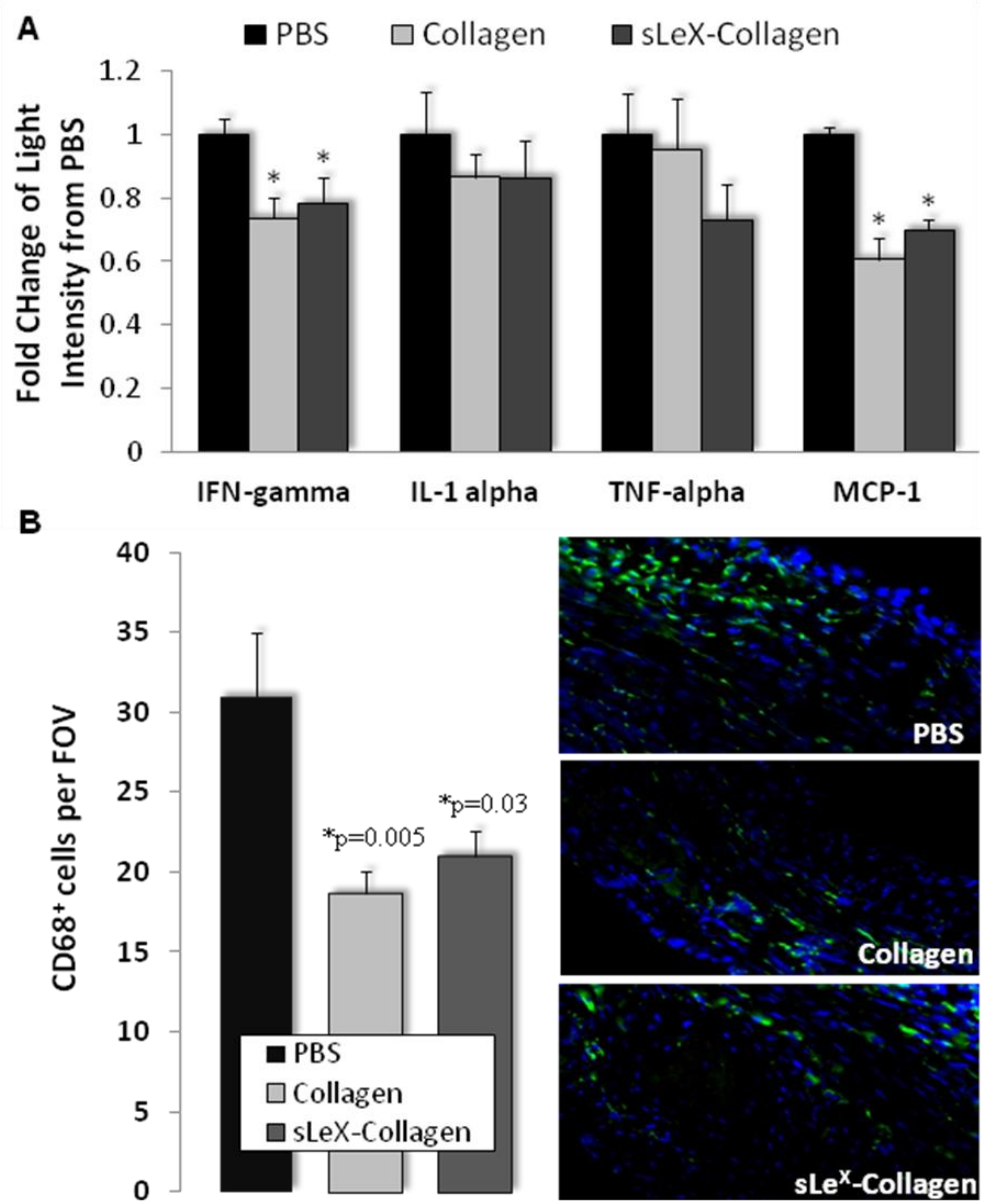

Figure 3.10. Collagen matrices decrease the inflammatory response in the MI mouse heart. Protein extracted from MI tissue was analyzed by cytokine array for inflammatory cytokines: IFN-γ, IL-1α, TNF-α and MCP-1, measured by light intensity variations and represented as a fold change relative to the PBS treated group, n=5-7 (**A**). (**B**) Infarcted tissue was stained for macrophage marker CD68 (green) and DAPI (blue). The number of positive cells was counted per FOV. (* indicates statistical significance of $p \leq 0.05$ compared to PBS, $5 \leq n \leq 7$)

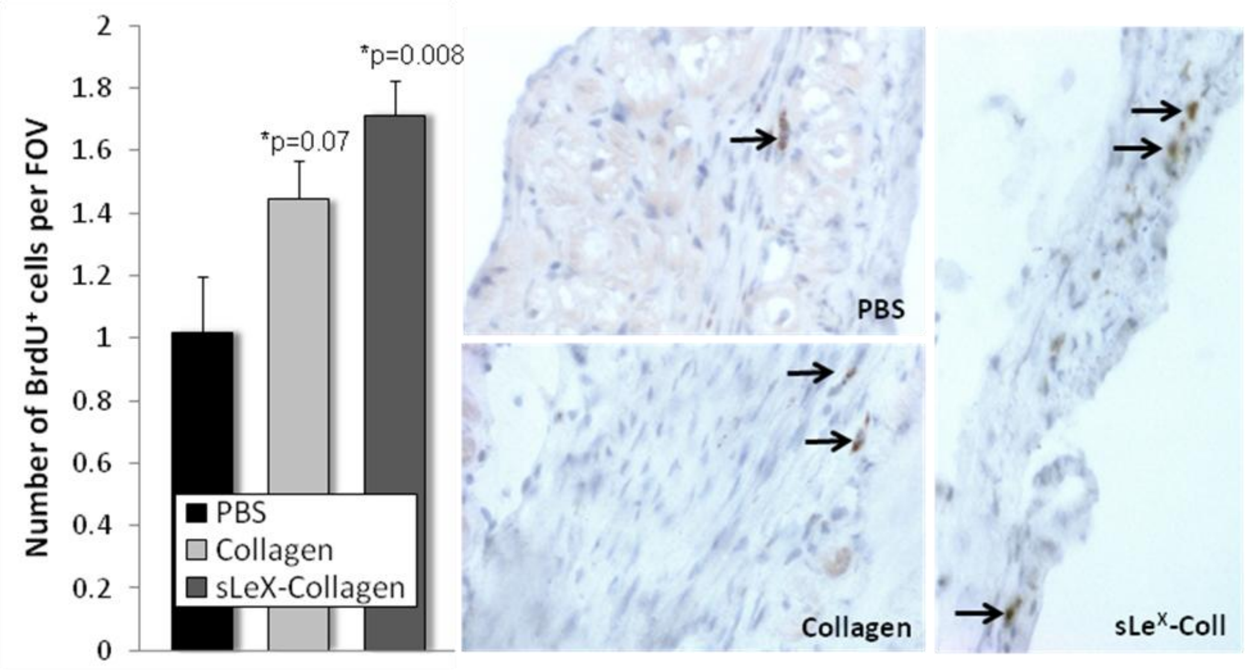

Figure 3.11. sLe^X-Collagen based matrix enhanced proliferation in infarcted tissue. MI mice were fed with BrdU-infused water for 4 weeks after the injection of the treatments. Following sacrifice, sections of the MI tissue was stained with BrdU antibody (brown) and hematoxylin (blue) to visualize the nucleus of proliferating cells. The number of positive cells was counted per FOV at 40x magnification and graphed as an average. Representative images are shown with arrows pointing to BrdU$^+$ cells. (*indicates statistical significance when compared to PBS, n=6)

3.2 RESULTS PART B: Cryopreservation of PBMCs

3.2.1 Viability of PBMCs and generated CACs is not reduced after cryopreservation

Cell viability was analyzed using $7AAD^-$ exclusion stain and quantified using flow cytometry. Fresh samples of PBMCs and CACs showed about 99.7±0.08% and 96.4±0.07% viability, respectively. Following cryopreservation, PBMCs sustained a non-significant increase in cell death with the trend of lower viability being more apparent with the late-thaw cells (viability 93±1.5% at day 1; 85±4.3% at day 28, n=7) (Figure 3.12, p_B=0.22). Viability of CACs remained stable over time under cryopreservation at 91±1.4% on day 1 and 94±3.8% on day 28 (Figure 3.12 A, p_B=1).

The thawed cells preserved their fresh counterpart sample's morphology as observed under a light microscope at 10× magnification (Figure 3.12 B).

3.2.2 Cryopreservation affects PBMC phenotype

The phenotype of the fresh and frozen cells was analyzed by staining the cells for surface markers: CD31, CD34, KDR (VEGFR2) and L-selectin and their appropriate isotype matched IgGs. The cells were quantified using flow cytometry and are shown as a percentage of total cells. The IgGs were used qualitatively (Hulspas et al. 2009) as there were no significant differences observed between the different sample time points. Expression of the endothelial marker CD31 was not affected significantly at day 1 and day 28 of cryopreservation, and was also not different when comparing total PBMCs versus the viable population (Figure 3.13 A, n=7). PBMC expression of VEGFR2 was increased after cryopreservation, when compared to the fresh sample (p_B=0.005). VEGFR2 levels increased in total PBMCs from 10±1.6% to 27±1.8% (Figure 3.13 B, p=0.0005) in 1-day frozen cells and remained elevated at day 28 (21±3.5%, n=7) (Figure

3.13 B, *p*=0.01). The $7AAD^{-}VEGFR2^{+}$ cells (viable cells) follow a similar increase; however, there was also a significant difference between day 1 and day 28 cryopreservation time points (Figure 3.13 B, *p*=0.05). L-selectin, an adhesion molecule, is reduced in the cryopreserved PBMC samples when compared to the fresh PBMC samples, regardless of whether total or viable populations are examined (Figure 3.13 C, $p\leq0.01$). The population of PBMCs expressing the progenitor marker CD34 increased from 36±1.4% to 48±2.7% (*p*=0.005) after one day cryopreservation with the levels staying elevated at 44±2.6% (*p*=0.007) after being frozen for 28 days (Figure 3.13 D, n=7), with no significant difference between day 1 and day 28 cryopreservation time points.

3.2.3 Cryopreservation does not affect CAC phenotype

The phenotype of the fresh and frozen cells was analyzed by staining the four day cultured CACs for surface markers: CD31, CD34, KDR (VEGFR2) and L-selectin and quantified using flow cytometry. The phenotype of CACs generated from PBMCs after 1 and 28 days of cryopreservation did not significantly differ from CACs generated from fresh PBMC samples, whether selecting for total or viable populations (Figure 3.14; n=7). In addition, CD31, VEGFR2, L-selectin and CD34 expression was not significantly different between early- and late-thaw time points (Figure 3.14 A-D).

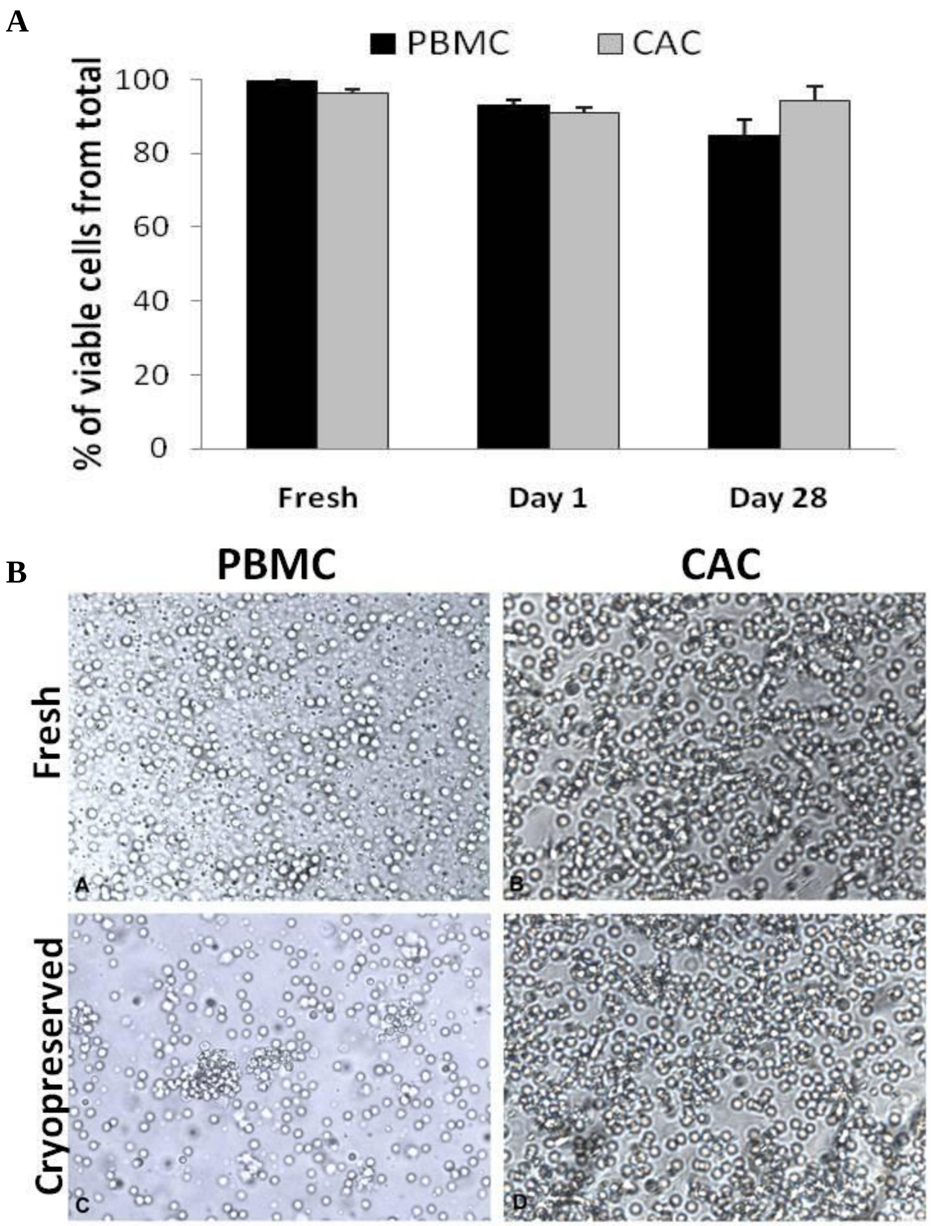

Figure 3.12. PBMC and CAC viability and morphology is unchanged following cryopreservation. $7AAD^-$ exclusion staining was used to enumerate the number of viable cells for fresh, early and late samples via flow cytometry. The proportion of viable cells is represented as a percentage (**A**). Images of fresh and cryopreserved PBMCs and CACs were taken by light microscopy at 10× magnification (**B**) (n=7).

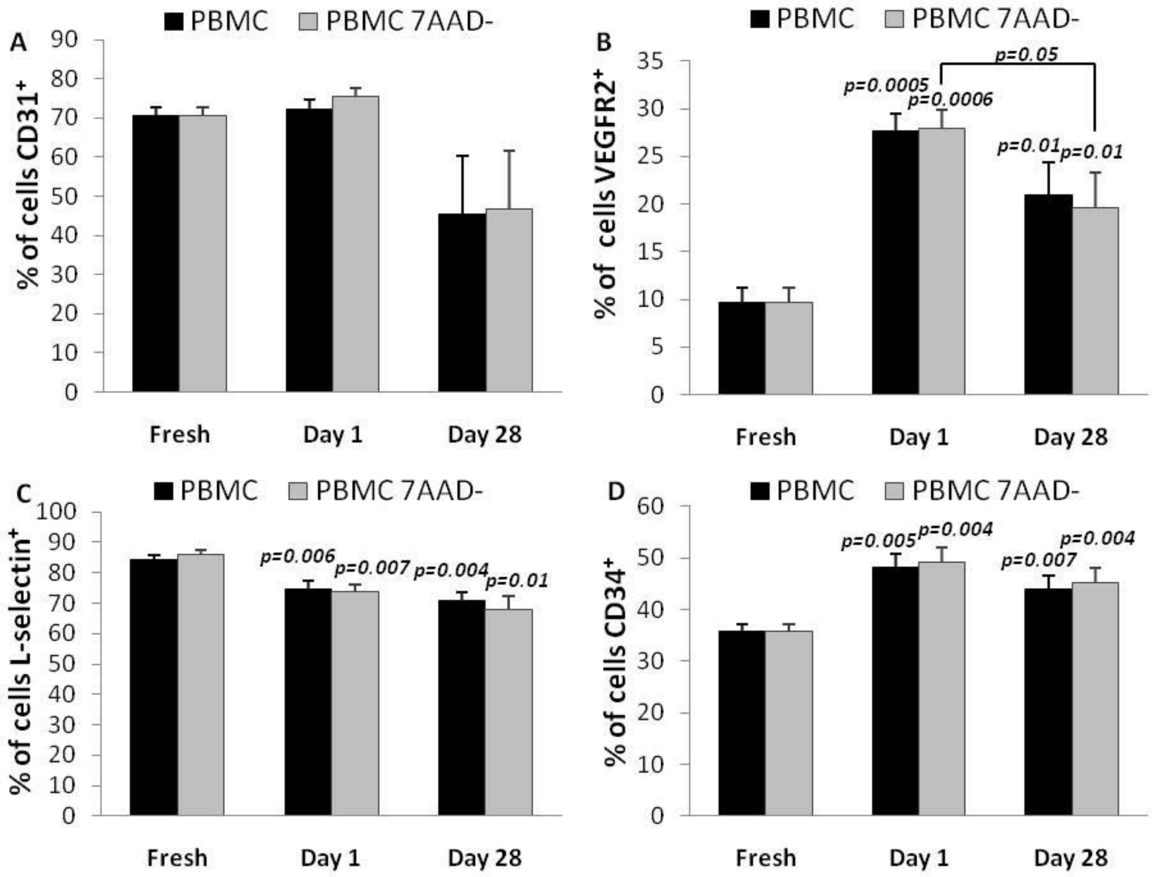

Figure 3.13. PBMC phenotype is affected by cryopreservation. PBMCs were assessed for viability using 7AAD as well as stained for markers: **A**) CD31, **B**) VEGFR2, **C**) L-selectin and **D**) CD34. The *p*–values represent a significant difference when compared to the corresponding fresh sample, unless otherwise indicated (n=*7*).

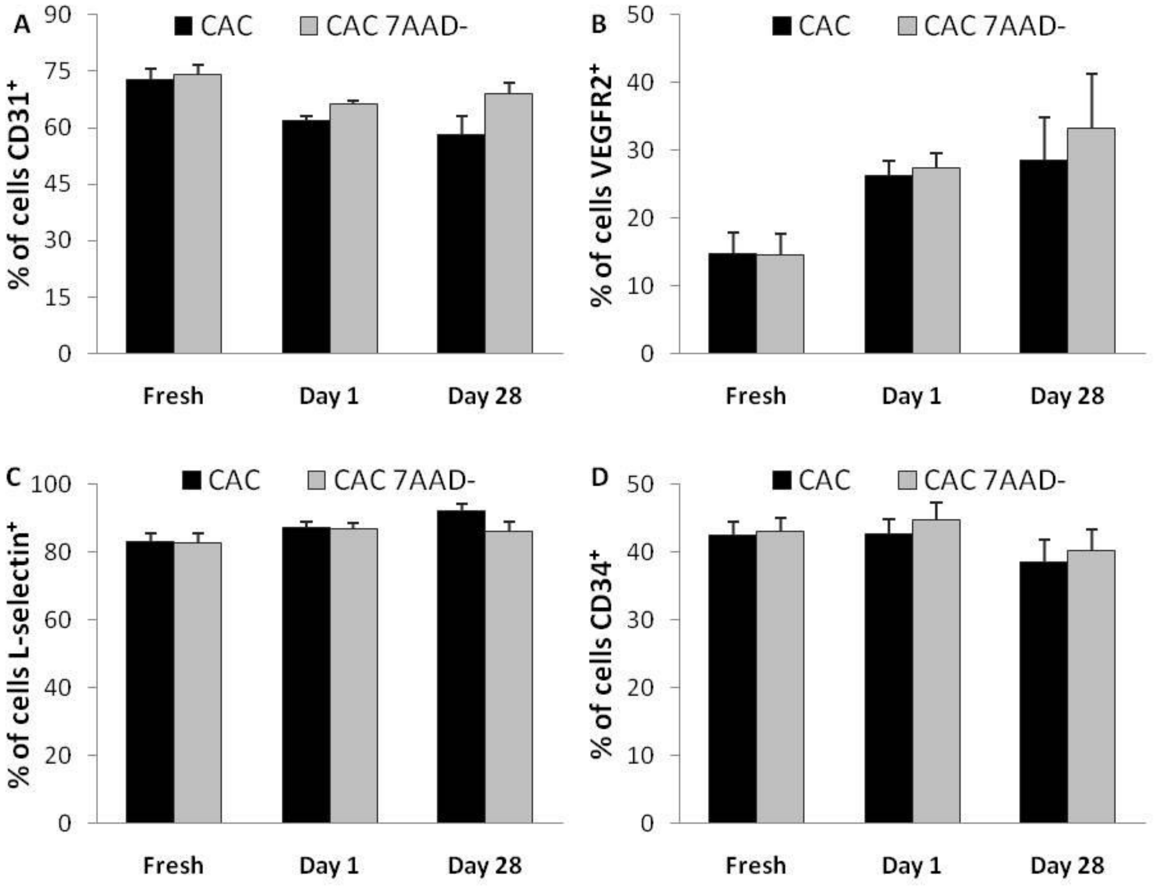

Figure 3.14. CAC phenotype is unaffected by cryopreservation. CACs were generated from 4-day plated PBMCs and assessed for viability using 7AAD, as well as additionally stained for markers: **A**) CD31, **B**) VEGFR2, **C**) L-selectin and **D**) CD34. No significant difference was observed between the cryopreservation time points (n=*7*).

3.2.4 Cryopreservation does not affect lectin binding and LDL uptake

Cells were incubated for an hour each with fluorescent labelled lectin and LDL to assess their binding and uptake of these factors, respectively. After the incubations, the number of the positively stained cells was quantified by flow cytometry. Lectin binding and LDL uptake are properties attributed to EPCs, a potent subset of CACs. There are no significant differences in the uptake of LDL and binding of lectin when day 1 and day 28 cryopreserved PBMCs are compared to fresh PBMCs (p_B=1). However, a non-significant (p_B =0.17) trend for increased LDL uptake is observed in CACs generated after cryopreservation: a 1.7±0.3 fold change at day 1 (p=0.03), and a 2.4±0.3 fold change at day 28 (p=0.05), compared to fresh CACs (1.0±0.2; Figure 3.15 A; n=7). There is also no difference in lectin binding for cryopreserved PBMCs and CACs (Figure 3.15 B; p_B =0.82).

3.2.5 Functional capacities of PBMCs and CACs after cryopreservation

Adhesion and migration capabilities were measured for both fresh and cryopreserved PBMCs and CACs. Adhesion was conducted by incubating the cells for an hour on fibronectin coated plates with media. The migration assay was conducted using a Boyden chamber to assess the cells' capacity to migrate towards VEGF as the chemokine factor. Cryopreserved PBMCs and CACs showed no difference in adhesion compared to the fresh samples (Figure 3.16 A; n=7). Cryopreserved PBMCs were unaffected in their ability to migrate, while CACs after 1 day of cryopreservation exhibited reduced migration (0.4±0.1 fold; p_{B}=0.04), compared to fresh CACs (1.0±0.2 fold; p=0.001). Migration of day 28 cryopreserved CACs was not significantly affected (Figure 3.16 B, p=0.12).

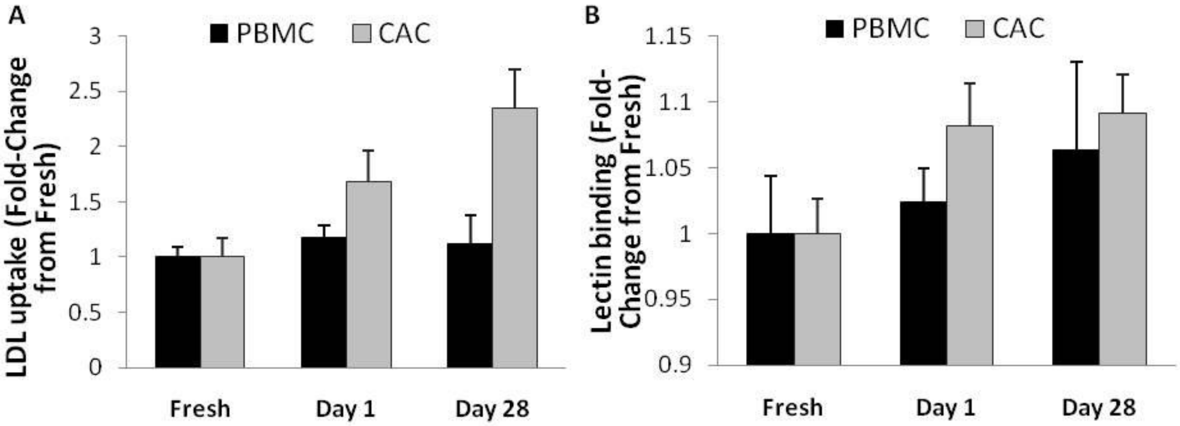

Figure 3.15. PBMC and CAC LDL uptake and lectin binding is unaffected following cryopreservation. The number of positive cells that took up fluorescent LDL (**A**) or that bound fluorescent lectin (**B**) were quantified using flow cytometry. Data is reported as fold change from donor-matched fresh samples. No significant difference was observed between the time points (n=7).

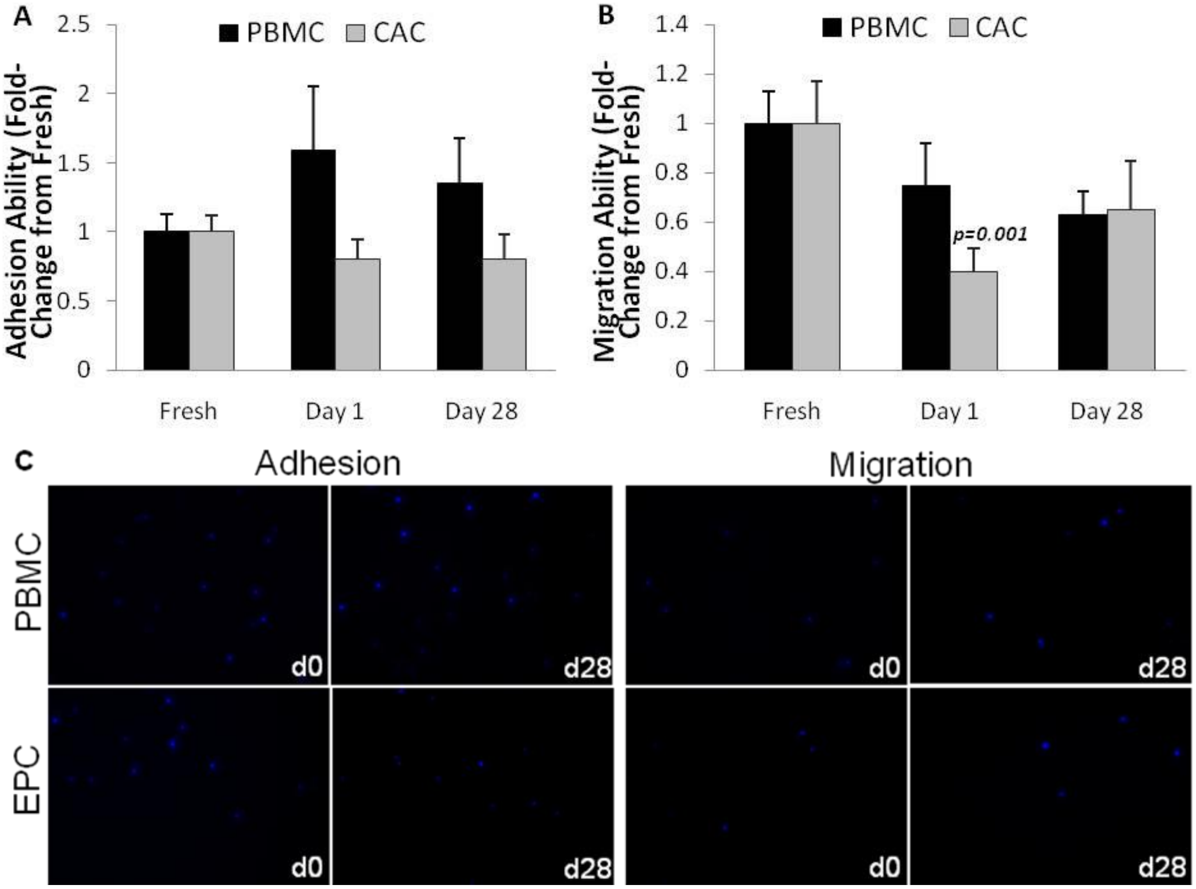

Figure 3.16. PBMC and CAC adhesive and migratory capabilities after cryopreservation. Cells were pre-stained with DAPI, which allowed for analyzing them under an electron microscope for their adhesive (**A**) and migratory (**B**) capabilities. 5 pictures were taken per well and the number of DAPI$^+$ cells were counted per FOV and representative pictures of each set of donors are shown (**C**). Data is reported as fold change from donor-matched fresh samples. The *p*–values represent significant difference when compared to the corresponding fresh sample unless otherwise indicated; (n*=7).*

DISCUSSION

4.0 Discussion

4.1 PART A: sLe^X-collagen matrix in the 1 week MI mouse model

The need for regeneration of vasculature and cardiomyocytes in the heart is obvious, as CAD is one of the most prevalent diseases of our era. While our expanding knowledge of stem cells and tissue engineering has shown some beneficial effects in the diseased heart, we are still a long way from optimizing stem cell therapy and fully regenerating the heart and vasculature after an infarct. This project's aim was to explore an ECM component, collagen, as a potential, minimally invasive treatment for improving heart function and reducing the damage that occurs due to an infarct. Our matrix can be administered with ease, which is one advantage that makes it an attractive strategy to explore. By injecting the liquid matrix via echo-guided injections, we diminish the need for any invasive procedures that carry greater risks of mortality and infection, as well as a longer and a more painful recovery.

The purpose of this section of the project was to investigate the beneficial effect of our tissue engineered sLe^X-collagen matrix on surgically-induced myocardial infarction in the mouse. In particular, the focus was on restoring cardiac function, as measured by echocardiography. The possible mechanisms of repair were considered and examined. They include: 1) neovascularization of the ischemic tissue to alleviate the blood and nutrient supply deficit and prevent further cardiomyocyte loss; 2) cardiomyogenesis and repair of the damaged heart tissue by replacing the cells with new ones; 3) cytokines and other factors released by the cells that could be involved in beneficial paracrine effects; and 4) alleviation of apoptosis and cell loss in the damaged tissue. The results were compared to MI mice treated with either collagen-only matrix or PBS, as our control. Injections of the sLe^X-collagen matrix into the infarcted heart improved heart function as measured via echocardiogram. We also observed that the sLe^X-collagen matrix had a regenerative effect on the heart through improved vascular density, augmented cardiomyogenesis, increased cell proliferation and cytokine production, and reduced inflammation and apoptosis.

4.1.1 Improved heart function after an infarct

Changes in heart function and/or structure can be assessed using various techniques such as magnetic resonance imaging (MRI), positron-emission tomography (PET) and echocardiography. Echocardiography is a non-invasive method that has been proven reliable for monitoring and measuring cardiac geometry and output post-MI (Litwin et al. 1994; Gao et al. 2000). In our one-week MI mouse study, echocardiogram analysis was used to measure parameters of heart function such as: 1) the LVEF, an important indicator of the heart's pumping ability; 2) FAC, which also allows us to quantify how well the heart contracts; and 3) the LV mass. Our focus was on the LV of the heart, since the LAD, which was ligated to induce the MI, is one of its main suppliers of blood. The mice treated with the ECM matrices showed a slight improvement in the %EF compared to their baseline measurements, while the PBS group showed a decline. Although the increase in ejection fraction is modest in the matrix treated groups, the difference between the PBS and matrix treated groups is approximately 6.5%, which is considered a significant improvement in other MI therapy studies and correlates with a survival benefit (Bax et al. 2004). FAC followed a similar pattern to what was observed with %EF as they both measure the heart's ability to contract and pump blood in the system. It has previously been stated that wall stress is inversely proportional to wall thickness and that thinning of the myocardial wall can lead to negative LV remodelling and expansion of the infarct border zone (Jackson et al. 2003). The injection of the ECM matrices showed a trend in increasing LV mass, which was further corroborated with histology analysis showing an increased infarct wall thickness, indicating that the matrices may negate further thinning of myocardium after a MI. These results are similar to a study done by *Dai* et al. who showed that thickening of the infarcted wall in rats after collagen treatment preserved cardiac function (Dai et al. 2005).

4.1.2 Preservation of the infarcted myocardium through neovascularization and progenitor cell mobilization

Immunohistochemistry analysis of the tissue for SMA and CD31 cells, which allows us to localize arterioles and capillaries, showed that the MI hearts injected with the ECM matrices had a higher blood vessel density compared to the groups treated with PBS. Neovascularization is partly mediated by the interactions between the endothelial cells and its progenitor cells with the ECM components (Francis et al. 2008). Therefore, it is not surprising that the use of collagen matrices has been successful in restoration of blood vessels in several studies (Huang et al. 2005; Suuronen et al. 2006), including our own. The restoration of blood flow is thought to help preserve viability of the remaining cardiomyocytes and prevent further loss of cardiac tissue. Studies that have seen an improvement in vasculature following a MI were also able to observe an improvement in cardiac function (Huang et al. 2005; van der Meer et al. 2005).

As CACs have been implicated in neovascularization (Asahara et al. 1999), we also looked at the cells positive for certain progenitor and angiogenic markers in the circulation of the MI mice at different time points post-treatment. After a MI, the body's innate response is to release cytokines that stimulate the mobilization and recruitment of progenitor cells for repair of the damaged tissue. Although no significant difference was observed between the three treatments in the cells positive for CD34, VEGFR2, CD45 or c-kit, the groups treated with the ECM matrices tended to have a higher number of these populations of cells compared to the PBS groups. However, after the first two weeks post-treatment this difference equalized. A subset of potent circulating angiogenic cells identified as $CD45^{-}CD34^{+}VEGFR2^{+}$ (Farace et al. 2007) were also analyzed but no significant difference was observed between the three treatments. The fact that we saw no differences between the three groups was surprising as our previous studies in the mouse hindlimb ischemia model have shown an increase in circulating progenitor cells in the groups treated with the collagen-based matrices (Suuronen et al. 2009). This may relate to the timing of matrix injection, which was at 1 week post-MI in the present study, versus injection at the time of injury in the previous hindlimb studies.

4.1.3 Preservation of the infarcted myocardium through reduction of apoptosis

Cell damage and death is a staple of a myocardial infarction. Although both apoptotic and necrotic pathways are activated during ischemia, apoptosis is the major contributor of cell death in both the infarct, bordering tissue and remote viable myocardium (Anversa et al. 1998) which contributes to the expansion of the infarct (Yaoita et al. 2000). The number of apoptotic cells in our animals was determined by staining the MI heart tissue for active caspase 3, one of the main effectors involved in this programmed cell death pathway. Treatment with the matrices reduced the number of apoptotic cells in the infarct compared to the PBS treated group. Previous studies have determined that a reduction of caspase 3 plays an important role in attenuating cardiac remodelling and function after a MI (Balsam et al. 2005) and that its over-expression has negative effects on the infarct size (Condorelli et al. 2001). The diminished number of caspase 3^+ cells in our sLe^X-matrix treated group, is likely a contributing factor to our other observations of improved ejection fraction and infarct wall thickness in these mice.

Apoptosis was further studied by conducting Western blots of heart tissue lysate samples for caspase 9. Caspase 9 is one of the activators of caspase 3 and is itself activated when complexed with apoptotic protease activating factor 1 (APAF-1) and cytochrome c released by the mithochondria (Li et al. 1997). Our results showed no significant difference between the three treatments, although there was a trend for reduction of caspase 9 in the matrix-treated groups compared to PBS. However, as caspase 9 is just one of the activators of caspase 3, it may be that the combined contribution of other pathways and factors that were not investigated but that also affect caspase 3, are causing the observed decrease in its activation.

4.1.4 Repair and regeneration of the infarcted myocardium

To further analyze the effects that our matrices had on the MI hearts, we stained the infarcted tissue for various factors important in maintaining healthy cardiomyocytes and cardiac regeneration. Connexin 43, a gap junction protein essential for normal cardiac functioning and the heart's electrical signals, tends to decrease and become disorganized after a MI (Peters 1995). This decrease in gap junctions has been previously associated with reduced electrical conduction leading to tachyarrhythmias, which is the foremost cause of death in patients after MI (Roell et al. 2007). In the sLe^X-collagen group, the expression of connexin 43 was significantly higher compared to the PBS and collagen-only groups. The increased expression of connexin 43, while not only measuring the health and functionality of cardiomyocytes, is also imperative in the integration of progenitor cells and newly formed cardiomyocytes (Bai et al. 2010). This is one of the major limitations of stem cell therapy, as cell-to-cell coupling in newly formed cardiomyocytes may not occur if the proteins important in this process such as connexin 43 are not expressed in the appropriate quantities needed for normal electrophysiological function (Mills et al. 2007). This can lead to death of newly differentiated, unintegrated cardiomyocytes or if the cardiomyocytes start beating asynchronously, it may lead to heart arrhythmias.

In order to investigate the potential for cardiomyogenesis in the infarct, we searched for newly formed cardiomyocytes or resident cardiac progenitor cells by staining the tissue for markers nkx2.5 and c-kit, respectively. As mentioned previously, nkx2.5 is an early marker of cardiac development and shows the cell's commitment to the cardiac and smooth muscle lineage (Wu et al. 2006), while c-kit is a surface marker used to identify resident CPCs and other stem cells. The number of nkx2.5$^+$ and c-kit$^+$ cells was observed to be significantly higher in the sLe^X-collagen treated groups compared to PBS, while the collagen treated groups tended to have numbers somewhere between the other two treatments. The fact that we observed more cells positive for these two markers indicates that there might be higher cardiomyocyte regeneration in the sLe^X-collagen treated tissue. Previous studies have confirmed that injections of c-kit$^+$ cells improve heart function through cardiomyogenesis (Dawn et al. 2005), stimulate the endogenous progenitor response and increase the expression of nkx2.5$^+$ cells (Loffredo et al. 2011).

Although nkx2.5 expression can indicate newly formed cardiac cells, other studies have correlated the expression of early fetal markers of cardiac development to hypertrophy of the heart. Ventricular hyperthrophy can be a maladaptive process that occurs due to the elevated mechanical stress placed on the heart causing cardiomyocytes to increase in size to compensate for damaged cardiac tissue which can ultimately lead to heart failure. This can usually be assessed by an increase in heart weight-to-body weight ratio. One of the implications of hypertrophy is that the cardiac cells revert back to their fetal stage and start expressing early markers of differentiation such as atrial natriuretic peptide, nkx2.5 and GATA4 (Thompson et al. 1998; Saadane et al. 1999). We analyzed the LV mass-to-body weight ratio in our mice to look for potential signs of hypertrophy prior to sacrifice. Mice treated with sLe^X-collagen showed no elevation in the left ventricle mass-to-body weight ratio compared to PBS, and in fact showed a trend for a decreased ratio. Therefore, although there was in increased expression of early cardiac marker nkx2.5 in sLe^X-collagen groups, the left ventricle mass-to-body weight ratio was comparable to PBS, indicating this increase in nkx2.5 was not due to greater hypertrophy in sLe^X-collagen treated groups.

The number of CXCR4$^+$ cells was also examined in the infarcted tissue, as previous studies have found that the number of CXCR4$^+$ cells correlated with vascular density (van Weel et al. 2007; Oh et al. 2010), and in addition, that the CXCR4$^+$ -SDF-1 axis plays a role in differentiation of cardiomyocytes (Chen et al. 2008) and cardioprotection after an injury (Saxena et al. 2008; Frangogiannis 2011). In our earlier mouse ischemic hindlimb study, administration of sLe^X-collagen increased the mobilization of CXCR4$^+$ cells in the circulation as well as their recruitment and retention in the ischemic hindlimb compared to PBS treated tissue (Suuronen et al. 2009). Interestingly, in the current study, the number of CXCR4$^+$ cells was not found to be different in the infarcted tissue between the three treatments. Similarly, we found that the number of circulating CXCR4$^+$ cells was also unchanged between the three treatments. As stated earlier, this may relate to the timing of matrix treatment. In the present study, the matrix was applied 1 week after the infarct was generated. Since the signals for cell recruitment are generated by the heart early, and return to baseline levels by 1 week (Ma et al. 2005), the opportunity for the matrix to optimally exert its effects on the mobilization and recruitment process may have already passed. In comparison, in previous ischemic hindlimb studies where an increased CXCR4$^+$ cell response was observed, the matrix was injected into the muscle at the time that ischemia of the tissue was created.

Another way to repopulate the damaged tissue in the heart is through proliferation of the progenitor cells and the already existing cardiac cells. The ability for cardiomyocytes to proliferate is still a debated topic (Buja et al. 2008) although some studies have shown that it is possible to expand adult cardiomyocytes *in vitro* (Engel et al. 2005). Evidence also points to the fact that the myocardium contains niches of resident CPC which are self-renewing and clonogenic (Beltrami et al. 2003). In fact, it has been observed that a depleted side-population of resident CPCs after a MI, reconstitutes itself to near baseline levels mostly by self-proliferation and in part by selective homing of BMCs (Mouquet et al. 2005). In this study, we administered BrdU-laced water to the MI mice for 4 weeks post-treatment. The number of BrdU$^+$ cells in the sLe^X-collagen treated groups was significantly higher compared to PBS, indicating higher numbers of proliferating cells in the sLe^X-collagen animals. Nevertheless, it is unknown as to which cells in the myocardium are proliferating and further studies need to be done to identify these subsets of cells.

4.1.5 Paracrine effects in the infarcted myocardium

Cytokines and growth factors released after an injury are important contributors to tissue repair. We investigated the relative expression of certain factors through cytokine arrays and Western blots. Cytokine arrays were conducted on MI tissue samples sacrificed 28 days post-injection. Several cytokines were found to be higher in matrix-treated groups compared to PBS. E-cadherin, important in cell adhesion and migration, was significantly higher in collagen and sLe^X-collagen treated groups compared to PBS. Additionally, TWEAK was significantly higher in both the matrix treated groups, however its receptor, TWEAK-R, was only higher in the sLe^X-collagen treated mice. A recent study by Novoyatleva et al. has shown that TWEAK plays an important role in neonatal cardiomyocyte proliferation, although this effect was reduced in adults due to decrease in the expression of TWEAK-R by the cardiomyocytes (Novoyatleva et al. 2010). The fact that we observed an increase in both TWEAK and TWEAK-R in sLe^X-collagen treated mice may contribute to the observed increase in proliferation (Brdu$^+$ cells) in the tissue of these mice.

Another factor that was significantly enhanced in the sLe^X-collagen groups was pro-MMP-9, an inactive form of a protein responsible in breaking down the ECM. Although not significantly changed, MMP-2 is also responsible for ECM remodelling, and followed a trend of higher expression in the matrix-treated groups. MMPs are important contributors of LV remodelling and ECM degradation. After a coronary event, chronic deposition of collagen can lead to fibrosis and scar formation which makes it hard for stem cells to engraft and repair the damaged tissue. MMP-9 and MMP-2 levels naturally increase in the tissue (Kai et al. 1998; Thompson et al. 2002) and are responsible for attenuating collagen deposits to prevent accumulation. In fact, knock-out

MMP-2 mouse studies show that MMP-2 plays a predominant role in preventing collagen deposits (Mias et al. 2009), while MMP-9 has been observed to play an important role in BMC mobilization through the release of soluble kit ligand (Heissig et al. 2002). MMP-9 release by MSCs overexpressing CXCR4 increased cell migration into the infarcted area and improved remodelling of post-MI tissue, ultimately leading to enhanced LV function (Huang et al. 2011). Since the amplification of pro-MMP-9 is only observed in sLe^X-collagen groups and not collagen-only treated groups, it is likely that this increase is not triggered by matrix injection but perhaps is due to other processes such as possibly higher recruitment of MSCs and other MMP-9 secreting cells to the tissue treated with sLe^X-collagen.

Another factor analyzed was CTGF, also implicated in extracellular matrix remodelling, as well as in endothelial cell function and angiogenesis (Takahashi et al. 1999; Brigstock 2002). There was a significant increase observed in the sLe^X-collagen matrix administered group. This can be tied into the higher pro-MMP-9 levels seen in the same groups of mice as well as augmented neovascularization. VEGFR2, an important receptor in cell migration and recruitment, and L-selectin, a ligand of sLe^X, were not greatly changed in the tissue between the three treatments.

4.1.6 Inflammatory processes in the infarcted myocardium

The inflammatory process is one of the first responses that occur after an infarct. In the rodent model, within the first hours to 1 day post-MI there is a substantial up-regulation of cytokine such as TNF-α and interleukins in the myocardium (Deten et al. 2002). Inflammatory cells are important in initiating wound healing and removing the dead and damaged cells as well as matrix debris (Nah et al. 2009). This augments and clears the microenvironment in order for tissue repair to commence. However, timely resolution of inflammatory responses is a crucial factor in cardiac repair, otherwise adverse LV remodelling may occur. The expression of inflammatory cytokines is normally abated to baseline levels by 1 week after infarction occurs unless there is a large MI or considerable stress placed on the myocardium (Ono et al. 1998). In our study the cytokine levels of IFN-γ and MCP-1 were found to be significantly reduced in the matrix treated groups 5 weeks after the induction of the infarct. Although not significant, there was also a trend observed for the reduction of other inflammatory factors: IL-1α and TNF-α. MCP-1 is a factor involved in the recruitment of inflammatory monocytes and macrophages. While MCP-1 is important in wound healing, prolonged expression can lead to adverse LV remodelling and impaired cardiac function (Xia et al. 2007). To further investigate the inflammatory response, staining for macrophage invasion of the infarcted tissue was conducted by looking at the number of $CD68^+$ macrophages. The presence of these cells was found to be significantly reduced in both matrix groups compared to the PBS treatment, which coincides with the observed decrease of its chemokine MCP-1.

There are thought to be several mechanisms that contribute to regenerating cardiac tissue and restoring its function after a MI. These mechanisms are: neovascularization and reduction of apoptosis to preserve remaining cardiac tissue, cardiomyogenesis and cell proliferation to replace the lost tissue, and paracrine effects to enhance stem cell mobilization and repair. In this study, we investigated these mechanisms to identify how our collagen-based matrix may support the repair and regeneration of cardiac tissue. As these mechanisms are all interconnected and are affected by one another, it was not surprising that we saw contributing processes from multiple pathways and mechanisms in myocardial repair post-MI, which ultimately led to a positively augmented environment of the infarcted tissue. Overall, our study and other such studies using collagen-based matrices for infarcted tissue were successful in showing the beneficial effect of such treatments in improving cardiac function.

4.1.7 Optimal Matrix Delivery Time

Thus far, numerous studies have been conducted in MI animal models; however, the optimal delivery time of the treatment has yet to fully be established. In this project we treated our MI-induced mice with our matrices at both 1 week and 2 weeks post-LAD ligation surgery. Originally, a 2-week time point was chosen in our model as there was a PET imaging component planned. Two weeks was deemed adequate to allow inflammatory processes in the infarct to abate, as they can cause visual impairment during the imaging scans. However, the imaging component was removed from the study, and the 2-week results were not found to be as beneficial as predicted based on previous studies. Therefore, a 1-week time point was implemented for treatment administration, before the scar sets in and the innate stem cell responses subside. Although both time points showed a similar improvement for our sLe^{X}-collagen groups, the collagen treatments had a much greater impact on cardiac function when administered in the first week after a MI. While, there were no noteworthy differences between circulating progenitor cells or the infarct thickness between the two time points, a significant difference was observed for the arteriole density. MI animals treated with the collagen-based matrices at the 1-week time point had a significantly larger vascular network compared to the PBS treated groups, while the same results were not detected at the 2-

week time point. Due to these observations, we decided to continue our analyses only with the 1-week time point.

The few studies on optimal delivery of matrix and/or stem cells have shown mixed results; some studies observed that injections of stem cells during the acute inflammatory period in the first 24-72 hours post-MI showed no beneficial effect and that delivery at around 1 week was the most beneficial (Nakamuta et al. 2009; Zhang et al. 2009) while another study observed that the cardiac function was only improved if the stem cells were delivered in the first 4 days (Ma et al. 2005). The one thing that is agreed on is that in order for the stem cell transplant to be beneficial (using non-embryonic source cells), it is important for the myocardium to be reversibly damaged as opposed to irreversibly dead and scarred (Bel et al. 2003). This means that the myocardium must still retain its ability to provide signals and cytokines to drive the differentiation, engraftment and function of the transplanted or recruited cells. Even fewer studies have been conducted on the optimal time for tissue-engineered scaffold implantation after an infarct. The current theory right now is that it is important to deliver the scaffold to coincide with the natural peak of pro-angiogenic and repair cytokines and innate mobilization of stem cells, which is usually within the first week or two post-MI (Takahashi et al. 1999; Hojo et al. 2000; Ma et al. 2005; Wang et al. 2006). However, further studies need to be conducted on this topic.

4.2 PART B: Cryopreservation of PBMCs

The cryopreservation experiment was first begun to look into whether it is possible to freeze PBMCs isolated for flow cytometry, without affecting the cells' phenotype and function. This could be useful in limiting the loss of experimental data, since the flow cytometer may not always be available or working on the days that samples need to be analyzed. Additionally, it was thought that being able to freeze a patient's own stem cells would allow their future combination with other treatment options, such as tissue engineered matrices, as the next treatment step to further enhance repair and regeneration in the infarcted heart. Cryopreservation is an appealing method for storing an individual's own autologous stem and progenitor cells until needed for therapy; however, the effects of freezing cells for storage have not been fully characterized. In particular, there is limited data regarding cryopreserved PBMC-generated CACs, which have been a major focus of vascular regeneration research. Our data demonstrated that cryopreservation does not adversely affect the viability of PBMCs and CACs. Cryopreservation does, however, cause phenotypic changes in PBMCs. Despite this, cryopreserved PBMCs maintained their ability to generate the same therapeutic CACs that fresh PBMCs can generate, as determined by their phenotype and *in vitro* function.

The maintenance of PBMC viability following cryopreservation observed in the present work is in accordance with other studies that also did not find considerable cell death after cryopreservation of PBMCs (Kleeberger et al. 1999; Weinberg et al. 2000); however, it is believed that the cell viability achieved after cryopreservation is largely dependent on the expertise/technique of the laboratory that performs the cryopreservation (Kleeberger et al. 1999; Weinberg et al. 2000). In particular, one report showed cell viability post-cryopreservation ranging from 1% to more than 90% (Weinberg et al. 2000).

The effect of cryopreservation on CAC viability has not been studied in great detail (Lu et al. 2008; Mieno et al. 2008), especially when considering PBMC-derived

CACs. One investigation using 7-AAD, examined the viability of CACs from cryopreserved umbilical cord blood (UCB) and found a significant level of apoptosis (Lu et al. 2008). In contrast, our results showed no significant decline in CAC viability after short (1 day) and long (28 days) periods of cryopreservation. It is possible that this is attributable to differences between the cells under study or the cryopreservation techniques. The importance of cell viability after cryopreservation is imperative as CACs make up a small fraction of the total PBMCs. The ability to generate a large number of viable cells allows for a better stem cell therapy outcome. A study by Iwasaki et al. showed that there is a dose-dependent correlation of injected CACs and improvement in myocardial infarction. The hearts treated with the largest doses of CACs were found to have greater capillary density, better cardiac output and higher gene expression of cardiomyocyte and endothelial cells' markers (Iwasaki et al. 2006).

The cells' phenotype was analyzed by staining for cell surface markers CD31, L-selectin, CD34 and VEGFR2 using flow cytometry. CD34 and VEGFR2 are markers used to characterize the CACs, although they are not exclusive to this population and can be expressed by other hematopoietic lineages (Peichev et al. 2000). CD31 is a marker of endothelial cells and L-selectin is an adhesion molecule found on CAC, leukocytes and other PBMCs. The expression of CD31 by PBMCs was unchanged by cryopreservation; whereas the frequency of L-selectin$^+$ PBMCs was decreased. This result was expected as previous studies have also found down-regulation of L-selectin in PBMCs following cryopreservation (Costantini et al. 2003), which may be a cellular response to stress (Griffin et al. 1990). Regarding progenitor markers CD34 and VEGFR2, we observed increased expression following cryopreservation of PBMCs. Ketheesan *et al.* showed that the proportion of CD34$^+$ cells in frozen cord blood did not change after freezing

(Ketheesan et al. 2004), while Lanza *et al*. demonstrated that 90-day cryopreserved PBMCs derived from patients with non-Hodgkin's lymphoma had an increase in CD34$^+$ cells compared to fresh samples, attributed to a decrease in mature myeloid cells (Lanza et al. 1999). It remains unclear why there is an increase in CD34 and VEGFR2 expression in our set of cryopreserved cells.

In our investigation, CACs generated from cryopreserved PBMCs showed no difference in expression of all investigated surface markers. Although studies have shown that L-selectin decreases in immunomagnetically-purified CD34$^+$ cells, immediately after thawing, the cells do recover their expression of L-selectin after a short period of culture (Hattori et al. 2001). This may explain why in our study we observed a decrease in PBMC's L-selectin expression, which were analyzed immediately, while the CACs were generated by a 4-day culture protocol before analysis, and demonstrated no change in L-selectin. This could also explain the increase in expression of CD34 and VEGFR2 as a potentially transient consequence of cryopreservation.

We also assessed the function of the populations' capacity for lectin-binding and LDL uptake, which are further characteristics used to assess EPCs, a potent subset of the CAC population (Hirschi et al. 2008; Yoder et al. 2009). As expected, a higher percentage of cells in the CAC sample showed lectin binding and DiI-LDL uptake, compared to PBMCs. In both populations, there was no significant difference between the number of fresh and cryopreserved cells staining positive for either one of these factors. An investigation by Mieno *et al.* has also shown that LDL uptake and lectin-binding of CACs is unaffected by cryopreservation (Mieno et al. 2008).

The cells' function was further assessed by their adhesion and VEGF-mediated migration capabilities. The adhesion of PBMCs was unaffected by cryopreservation, which is somewhat contradictory to the observed decrease in L-selectin after cryopreservation. Binding of L-selectin inside the cell results in the activation of ß1 and ß2 integrins which promotes adhesion of leukocytes to fibronectin (Hwang et al. 1996). It is possible that while the loss of L-selectin in PBMCs is statistically significant, it is not physiologically relevant, as compensatory mechanisms may be in action (e.g. other selectins may assume similar functions (Sperandio 2006)).

The migration potential of PBMCs using VEGF for chemotaxis was also unaffected by cryopreservation. This was surprising as PBMCs showed a higher expression of one of the VEGF receptors, VEGFR2, after cryopreservation. Therefore, it was expected for the migration capabilities of the cryopreserved cells to be augmented; however, that was not the case. The migrative capabilities of the frozen PBMCs were unchanged compared to fresh samples and in fact showed a slight decreasing trend. The fact that there was no change in VEGF-mediated migration even though there was an increase in its receptor expression has not been further investigated by us. One theory could be attributed to the newly expressed receptor proteins being non-functional and therefore not being able to contribute to the cells' migration. Further studies need to be conducted in order to investigate this data.

CAC adhesion and migration capacities were mostly unaffected by cryopreservation. There is a significant decrease in CAC migration between fresh and 1-day frozen samples, but no significant difference between fresh and 28-day frozen samples. We are unsure as to why we saw a decrease in the migration capabilities for the 1 day cryopreserved cells; whereas the maintenance of migratory potential in the 28-day cells was not significantly different from the procured fresh samples. The loss of migratory function in the 1-day samples may be attributed to shock that the cells underwent during the short succession between freezing/thawing procedures or some other set-up procedure. Other studies investigating cryopreservation have observed that the migrational capabilities of the cells are unaffected, similar to our 28 day cryopreserved CACs (Vanneaux et al.; Mieno et al. 2008).

The extraction and cryopreservation of PBMCs is a well established protocol, already in use for treatment of patients suffering with bone marrow cancers and other blood diseases. While the amount of serum used during our freezing step is on the low side at 6%, whereas other protocols have gone up to storage solution containing 90% serum (Makino et al. 1997; Zhou et al. 1997), we did not observe a sizeable loss of viability. This is an important aspect as a study investigating cryopreservation of MNCs has noticed that cells with a viability of <70% have compromised proliferative responses to antigens and mitogens, and are not suitable for cytokine production studies, flow cytometric analyses, and immunomagnetic cell separation (Reimann et al. 2000; Weinberg et al. 2007). A concentration of 5% cryoprotectant, DMSO, was also used to diminish the negative effects it has on the cells upon thawing and freezing. DMSO is toxic to cells at room temperature, as it is hypertonic and can cause osmotic injury. 5% concentration of DMSO has been observed to be close to ideal for freezing the PB progenitor cells (Bakken 2006), as it is a low concentration that does not damage the cells severely upon thawing but it is a high enough concentration to prevent freeze damage. Also as DMSO has clinical side-effects that includes: nausea, vomiting, cramps and other more severe consequences such as renal, respiratory and cardiovascular complications (Zambelli et al. 1998; Berz et al. 2007) it is beneficial for the patients undergoing stem cell therapy to be exposed to minimal amount of this cryoprotectant.

5.0 Conclusion

As cardiovascular disease is the leading cause of death and morbidity in the world, restoring the blood supply to the ischemic tissue by rebuilding of vessels through neovascularization as well as cardiomyocyte regeneration remain important objectives in eradicating this ailment. In this study, we have seen that use of injectable collagen materials improved cardiac function as measured by ejection fraction as well as the myocardial infarct thickness. Both collagen-based matrices showed an improvement in vascular density and a decrease in inflammatory responses compared to PBS. Additionally, sLe^X-collagen matrix treated groups showed greater connexin 43 levels as well as more cells expressing the early cardiomyocyte marker Nkx2.5 and the CPC marker c-kit, which is indicative of active cardiomyogenesis. The sLe^X-collagen matrix furthermore, decreased the number of apoptotic cells, increased the number of proliferative cells and enhanced the expression of regenerative cytokines. In conclusion, it seems that injection of the sLe^X-collagen matrix and the collagen matrix, although to a lesser extent, has a beneficial effect on the mouse MI heart through contributions of several repair mechanisms.

An additional project looked at the ability to generate functional CACs from cryopreserved PBMCs, as a potential stem cell therapy method that could be incorporated with the tissue engineered collagen matrices for improvement in heart function post-MI. Our data indicate that the function of therapeutic CAC populations is generally preserved after short- and longer-term cryopreservation, with the cells retaining their adhesion and migratory capabilities, as well as their capacity for lectin binding and low-density lipoprotein metabolism. Despite the phenotypic changes in PBMCs following cryopreservation, these cells can still generate CACs that are of the same phenotype and function as fresh CACs. There is also no significant effect on viability of the cells after cryopreservation, allowing for close to a maximum retention of the cells for stem therapy even after a month-long storage. These observations lead us to conclude that the PBMCs obtained from the circulation of human donors can be safely stored until needed and the cryopreservation procedure does not affect the CAC subpopulation.

6.0 Clinical Perspectives and Future Experiments

As with several other diseases, CAD patients do not experience the same symptoms and recovery after a MI and, therefore, may need different therapy strategies. Tissue engineered matrices offer a way to augment the body’s natural repair mechanisms and allow for a better engraftment of stem and progenitor cells for enhanced regeneration of the injured tissue. The matrices can be delivered with minimal invasiveness and unlike the potential for transplanted stem cells, they rarely illicit a foreign-body response and other inflammatory processes. In addition, if the infarct is large or if the patient’s autologous stem cells are diseased, scaffold systems may be combined with stem cell therapy to not just enhance the endogenous repair mechanisms but to improve the delivery, engraftment and survival of the transplanted stem cells.

PBMCs and CACs are already being used in clinical trials of ischemic and infarcted tissue. Their beneficial angiogenic properties have made them an ideal cell candidate not just for alleviating ischemic disease but for neovascularizing grafts, allowing for better outcomes of such surgeries. However, as with all cells, aging and disease can affect how beneficial CACs can be. It has already been observed that people with heart disease have reduced capabilities of their CACs. Being able to cryopreserve these therapeutic cells without any loss of function for a patient’s future use will allow better stem cell therapy outcome as well as allowing a patient to replenish their diseased and dwindling supply of these cells.

A combination of our sLe^X-collagen matrix and the cryopreserved CACs might synergistically improve the heart function more so then the two treatments administered on their own. The matrix would be beneficial by improving the engraftment and retention of the transplanted CACs, while CACs would enhance the neovascularization and paracrine effects in the infarcted tissue. A current study being conducted in our laboratory is exploring this theory and its possible contributions to augmented heart function after an infarct. With the expanding prevalence of CVD, tissue-engineered matrices with a combination of stem cell therapy can offer another potential treatment option for regenerating and repairing the damaged vasculature and myocardium.

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8.0 Appendix

ACE	**GM-CSF**	**IL-6**	**SDF-1 α**
ALK-1	**ICAM-1**	**L-Selectin**	**TIMP-2**
bFGF	**IFN-γ**	**MCP-1**	**TNF α**
CT-1	**IGFBP-2**	**M-CSF**	**TWEAK**
Decorin	**IGFBP-5**	**MMP-2**	**TWEAK R**
E-Cadherin	**IGF-I**	**Pro-MMP-9**	**VCAM-1**
EGF	**IL-10**	**P-Selectin**	**VEGF**
Endoglin	**IL-13**	**RAGE**	**VEGF R1**
Galectin-3	**IL-1 α**	**Resistin**	**VEGF R2**
GCSF	**IL-1 β**	**SCF**	**VEGF D**

Table 2. List of cytokines quantified in the tissue array. A custom cytokine array was purchased from RayBiotech to quantify the above cytokines. Tissue from the infarcted region of the MI mice hearts was harvested and 80ug of protein lysate was incubated with the array according to the manufacturer’s protocol. The light produced at each cytokine spot (proportional to the amount of cytokine bound) was quantified using AlphaEaseFC.

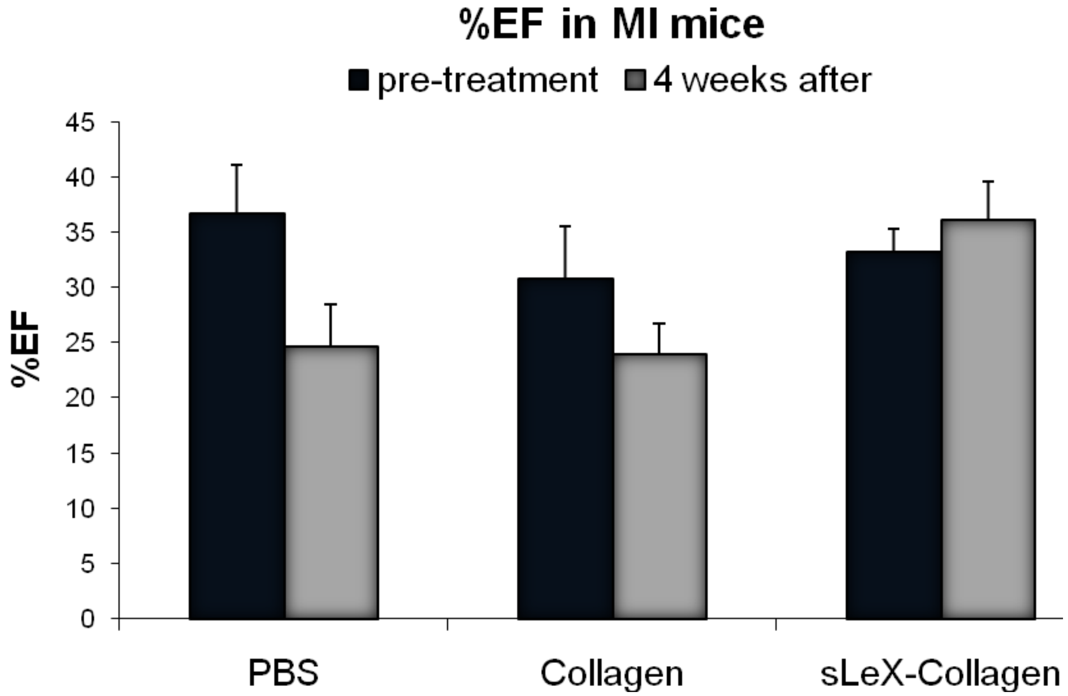

Figure 8.1. %Ejection fraction in 2 week MI mice. The mouse heart function was measured pre-treatment (baseline at 2-week post-MI) and again 4 weeks later via echocardiogram. The average data for each group is shown (5≤n≤7).

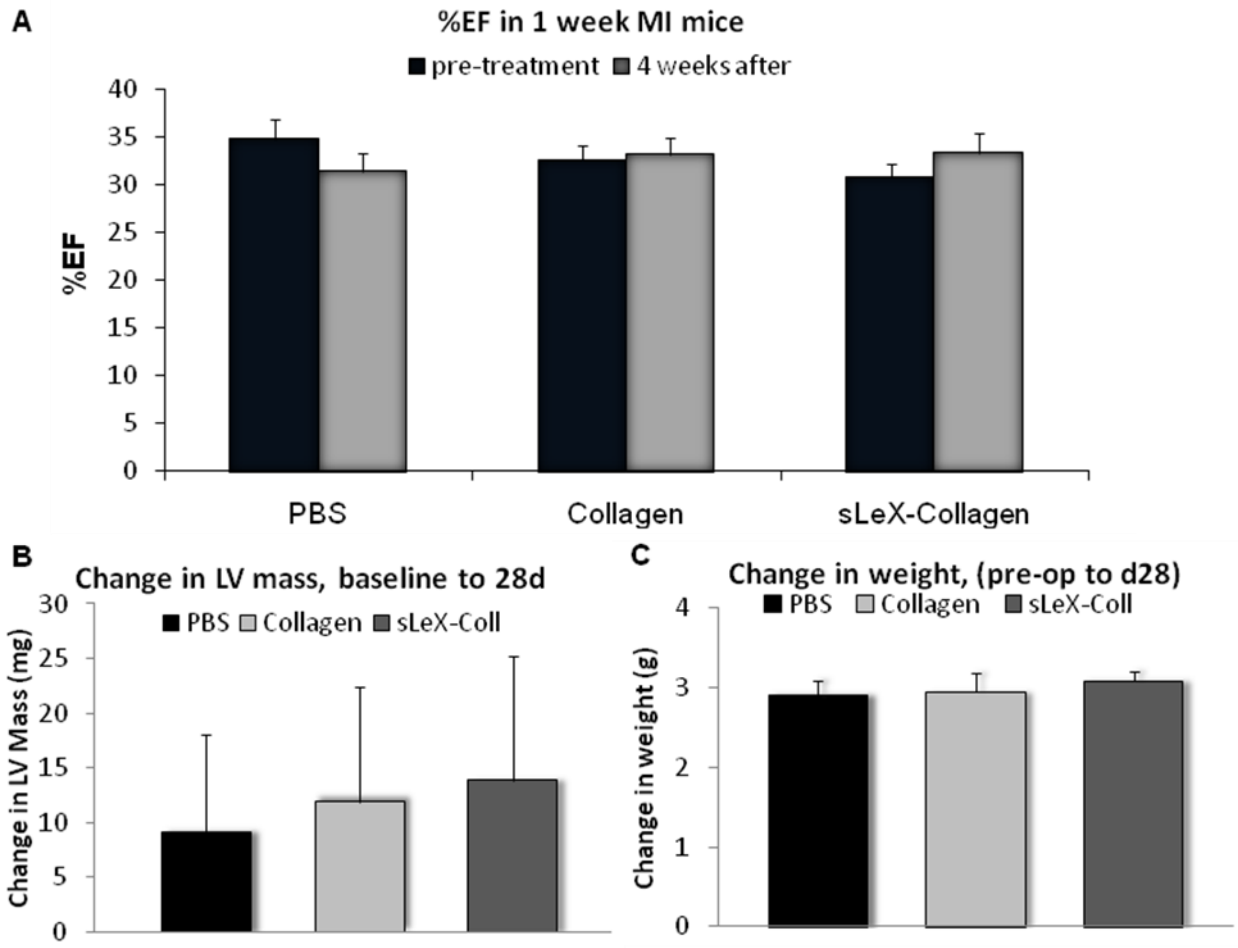

Figure 8.2. %Ejection fraction, change in LV mass and weight in MI mice treated 1-week after infarct surgery. (**A**) %EF and (**B**) change in LV mass were measured using an echocardiogram. (**C**) Change in weight of the mice was measured using a scale and is presented in milligrams. The difference between the 1 week post-MI surgery (pre-treatment) and 4 weeks post-treatment (d28) were calculated and are presented as an average change between the three treatments (**B,C**) (18≤n≤20).