FACTORS AFFECTING VENTRICULAR REMODELING POST MYOCARDIAL INFARCTION

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fulfillment of the requirements for the
Degree of Doctor of Philosophy

by

Udit Agarwal

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The term ventricular remodeling refers to the series of events that lead to the orchestration of the biological processes that determine left ventricular size, shape, and function following acute myocardial infarction (AMI). The present study focuses on defining the mechanisms of two distinct phenomena that are known to regulate this process; namely, extracellular matrix (ECM) degradation via protease inactivation and cardiac myocyte death.

AMI is followed by degradation of ECM of the heart leading to thinning and rupture of the left ventricular wall. A critical regulator of ECM degradation is plasmin, a protease. The generation of plasmin in turn is regulated by the oxidation-sensitive enzyme Plasminogen Activator Inhibitor -1 (PAI-1). PAI-1 is an inhibitor of urokinase-like plasminogen activator (uPA), which converts plasminogen into plasmin. PAI-1 deficient (Pai-1−/−) mice die of ventricular rupture within 7 days following infarction. It has been previously reported that the activity of PAI-1 is sensitive to oxidation and hence inhibited by leukocyte-derived oxidant-generating systems, such as Myeloperoxidase (MPO) and inducible nitric oxide synthase (iNOS). Furthermore, the expression of ceruloplasmin (CP), another oxidant-generating enzyme, is increased in the blood post-AMI. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity regulates the oxidant-generating capacity of MPO, iNOS, and CP. However, the relative contributions of each of these leukocyte-derived oxidant producing enzymes in PAI-1
oxidation is not known. We hypothesized that leukocyte-derived NADPH oxidase oxidizes PAI-1 and hence participates in ventricular rupture post-AMI. To address this issue, we transplanted Pai-1^{−/−} mice with Pai-1^{+/−}, wild type (WT), p47phox^{−/−} (a subunit of NADPH oxidase complex, also known as neutrophil cytosolic factor-1), Mpo^{−/−}, iNOS^{−/−} and Cp^{−/−} bone marrow, performed Left Anterior Descending (LAD) artery ligation in all the groups, and measured their survival for 21 days. Data demonstrate Pai-1^{−/−} mice were dead by day 7 due to ventricular rupture and WT marrow partially rescued Pai-1^{−/−} mice survival. Interestingly, p47phox^{−/−} marrow-transplanted mice had a significantly better survival compared to WT marrow-transplanted Pai-1^{−/−} mice suggesting that, NADPH oxidase is critical in inhibiting PAI-1-induced protease inactivation and ventricular rupture. PAI-1 activity was higher in p47phox^{−/−} marrow-transplanted Pai-1^{−/−} animals compared to WT marrow-transplanted Pai-1^{−/−} animals, indicating that leukocyte-derived NADPH oxidase inhibit PAI-1 activity in this model of ventricular remodeling.

Another determinant of ventricular remodeling is cardiac myocyte death. Several studies have identified the stromal cell-derived factor-1α (SDF-1α)/CXCR chemokine receptor 4 (CXCR4) axis to be important for the homing and survival of stem cells at the site of injury post-AMI. It has also been reported that Sdf-1^{−/−} and Cxcr4^{−/−} mice exhibit defects in hematopoiesis, neurogenesis, vasculogenesis, and ventricular septum formation and die within a few days of birth. Multiple reports claim that overexpression of SDF-1 at the infarcted region improves ventricular function by preservation of cardiac myocytes and increased vasculogenesis. Since the role of cardiac myocyte-derived CXCR4 was not
well-defined in this context, we hypothesized that normal cardiac development is dependent on cardiac myocyte-derived CXCR4 expression. In the adult heart, SDF-1α and its receptor, CXCR4 do not get expressed at the same time. Post-AMI, there is a short and immediate period of SDF-1α expression, which is followed by CXCR4 expression in cardiac myocytes. Therefore, we postulated that cardiac myocyte-derived CXCR4 expression is not critical in adulthood post-AMI. To address these issues, we developed congenital and conditional cardiac myocyte-specific Cxcr4−/− mouse models. Our results demonstrate that congenital deletion of CXCR4 has no effect on cardiac function and septal defect. Furthermore, the ventricular function of conditionally deleted cardiac myocyte-specific CXCR4 was not significantly different from their WT littermates.

Collectively, our observations suggest that leukocyte-generated NADPH oxidase-derived free radicals participate in ventricular rupture post-AMI by oxidizing PAI-1 and cardiac myocyte-derived CXCR4 has no major role in cardiogenesis during development and ventricular remodeling post-AMI due to mismatch in the timing of expression of SDF-1 and CXCR4 in the heart.
1.0 BACKGROUND

1.1 Acute Myocardial Infarction

AMI is the leading cause of death for both men and women throughout the world. In the United States, almost 1 million patients of AMI and 400000 new cases of chronic heart failure (CHF) are diagnosed annually(1). AMI is caused by ischemia of the heart muscle. Cardiac ischemia results when an atheromatous plaque (comprised of a deposition of lipids and white blood cells on the arterial wall) is dislodged due to plaque rupture, hemorrhage or ulceration leading to blockade of a coronary artery. Following this, platelets get activated by subendothelial collagen, form microthrombi, and release mediators that cause vasospasm. Tissue factor activates the coagulation pathway and within minutes a thrombus is formed that occludes the whole vessel and causes ischemia to the heart muscle. When left untreated, a cardiac ischemia can cause significant damage to the heart muscle and to cardiac function resulting in death (Figure 1). The classic symptoms of AMI include sudden onset of piercing chest pain radiating to left arm, back, chin, or epigastric region with associated nausea, vomiting, shortness of breath, palpitations and anxiety. If the patient survives the initial insult to the myocardium, the complications of AMI can cause significant morbidity. Various complications of AMI include CHF, contractile dysfunction, infarct extension and expansion, myocardial
rupture, arrhythmias, mural thrombus, ventricular aneurysm pericarditis, cardiogenic shock and embolism.
Figure 1. Figure depicting AMI.

AMI is caused by occlusion of a coronary artery following the rupture of a vulnerable atherosclerotic plaque, which if left untreated causes ischemia and death of cardiac myocytes.
Plaque build up in the coronary artery blocking blood flow and oxygen to the heart.

Damage and death to heart tissue shown in purple.

Source: http://www.nlm.nih.gov/medlineplus

Figure 1
1.2 Pathophysiology of post infarction left ventricular remodeling

Ventricular remodeling can be categorized into physiological and adaptive. Physiological ventricular remodeling occurs during normal growth and development, whereas adaptive ventricular remodeling occur post-AMI(2-3). Following AMI, mechanical, chemical, neurohormonal, and genetic changes result in alterations in the left ventricular size, shape, and functions that are collectively referred to as ventricular remodeling.

The myocardium is comprised of cardiac myocytes, ECM, and cardiac vasculature. The ECM is composed of collagen, and it provides a structural organization of the myocytes and the microcirculation, thereby ensuring functional integrity of the myocardium. AMI result in an inflammatory response at the infarct zone, characterized by infiltration of leukocytes that provide proteases such as plasmin and matrix metaloproteinases (MMPs), which degrade the ECM at the site of infarction.

Adaptive ventricular remodeling (post-AMI) has been divided into 2 phases: an early phase (within 72 hours post-AMI) and late phase (after 72 hours post-AMI). Although repair of injured tissue occurs mainly in the late phase, early phase events, such as myocyte death, disruption of ECM, and microcirculation are the major determinants of the ensuing remodeling. After occlusion of the coronary artery and subsequent hypoxia to cardiac myocytes, a chain of events follow. ATP depletion starts within seconds and reduces to 10% of normal levels within 40 minutes. Loss of cardiac myocyte contractility
occurs within 2 minutes, and irreversible cell injury happens within 20-40 minutes. This is followed by necrosis of cardiac myocytes, which starts in the subendocardial region and becomes transmural within 12-24 hours through a process known as infarct expansion (Figure 2). Following cardiac myocyte death, acute inflammation ensues, which results in the secretion of chemokines (interleukin (IL)-8, leukotrienes B4 and C5a, SDF-1α) and cytokines (interferon-γ, Tumor Necrosis Factor-α (TNFα), IL-1) at the site of injury. Various cellular forms, including white blood cells (WBCs) and stem cells, migrate to the site of injury. WBCs release MMPs, which break down the ECM by protease activation and free oxygen radicals, which oxidize proteins and lipids on the cell surface by oxidative damage (Figure 3). This mechanism is beneficial in removing dead cells from the tissue; however, in the process, it causes injury and subsequent death of viable cardiac myocytes via apoptosis. Furthermore, ECM breakdown decreases the tensile strength, making the myocardium more prone for complications such as myocardial rupture and arrhythmias. Therefore, the early phase of ventricular remodeling requires therapeutic intervention in order to decrease myocardial insult and subsequently improve ventricular function. The foci of this study are (i) to define one of the mechanisms targeted against preventing ECM breakdown by protease inactivation, and (ii) to define the mechanism that prevents cardiac myocyte death by upregulating antiapoptotic factors.

During the late phase of ventricular remodeling, the anterior wall of the left ventricle thins out and stimulates posterior wall hypertrophy, leading to decreased
ventricular function. After AMI, the acute loss of cardiac myocytes results in the formation of the infarcted border zone and the remote non-infarcted myocardium. Cardiac tissue necrosis and subsequent increase of load on the left ventricle triggers signaling cascades leading to reparative processes that include dilatation, hypertrophy, and collagen formation. These changes continue for months following AMI until the mechanical forces resulting in dilatation are counterbalanced by the tensile strength of collagen scar laid down by fibroblast cells(4).
**Figure 2.** The figure shows the area at risk for infarction. The infarct starts at the subendocardial region and becomes transmural within 12-24 hours of coronary artery obstruction.
Figure 2

**Figure 3.** Events post-AMI.

Normal Myocardium consists of cardiac myocytes, ECM and capillaries. After ischemia, acute inflammation ensues. Various chemokines (IL-8, Leukotreines B4 and C5a, SDF-1α) and cytokines (interferon-γ, TNFα, IL-1) are secreted at the site of injury. WBCs migrate and release MMPs, free oxygen radicals that cause protease activation, ECM degradation, cellular death, and capillary destruction. The process leads to infarct expansion and compromised ventricular function.
Adapted from: Cardiovascular Research 69 (2006) 604-613

Figure 3
1.3 Protease activation and plasminogen activator inhibitor-1 oxidation post-AMI

Multiple studies have demonstrated the critical role of protease degradation of infarcted myocardial tissue in the LV remodeling process(5-8). Heyman et al.(9) studied the role of multiple proteases by inducing LAD ligation in murine models deficient in specific proteases, including tissue plasminogen activator (tPA), uPA, and MMP 3, 9, and 12. These studies demonstrated a central role for uPA in LV remodeling. uPA cleaves the propeptide of plasminogen to convert it to its active form, plasmin (Figure 4). Plasmin itself can degrade ECM proteins (mainly fibrin, fibronectin, thrombospondin, and laminin), but its effects are augmented by its ability to convert pro-MMP-9 to MMP-9(10). The activity of uPA can be regulated by its inhibitor, PAI-1.

PAI-1 belongs to the serine protease inhibitor supergene family and is the natural inhibitor of not only tPA and uPA, but also of other proteases, such as matriptase-3, thrombin, activated protein C and differentially expressed squamous cell carcinoma-1 (DESC-1). PAI-1 is a secretory protease inactivator that is secreted in its active form from neutrophils, mast cells, cardiac myocytes, platelets, endothelial cells, and smooth muscle cells within the myocardial environment(11). The highly unstable active form spontaneously converts into the inactive (latent) form with a half-life of 1–2 hours at 37°C. The active form of PAI-1 blocks uPA activity by irreversibly binding to the
plasminogen binding site of uPA. The reactive center loop (RCL) located at amino acids 320–351 of active PAI-1 reacts very rapidly with uPA at Arg-346 to form an inactive uPA-PAI-1 complex. This complex is internalized by the LDL receptor related protein-1 (LRP-1) and degraded in lysosomes. Therefore, uPA inhibition by PAI-1 inhibits the proteolytic cleavage of plasminogen, thereby, inhibiting the plasminogen-plasmin axis.

During the process of inflammation, PAI-1 secretion is increased. Leukocytic migration results in a respiratory burst catalyzing various enzymes, including NADPH oxidase, MPO, iNOS, and CP, which form reactive oxygen species (ROS) at the site of injury. Therefore, at the site of inflammation, the surrounding environment becomes amenable for the ROS to oxidize the amino acid residues of the RCL of active PAI-1, making it inactive. This conformational change in PAI-1 leads to the uninterrupted action of uPA, causing excessive ECM breakdown by plasmin. Several reports have documented that the ability of PAI-1 to inhibit uPA activity is redox-sensitive(12-13). In vitro models of oxidation, such as exposure to caustic oxidants/chemical agents, have reported a reduction in PAI-1 activity with accompanying alterations in PAI-1 confirmation(12). It has been suggested that the oxidative modification of methionine(s) to methionine sulfoxide is required during PAI-1 inactivation. Furthermore, a recent study by our lab explored a critical link between MPO-generated oxidants and PAI-1 oxidation(14). The increased PAI-1 activity in MPO null mice and improved ventricular function suggested that MPO-generated oxidants are involved in PAI-1 oxidation. These studies point towards the growing evidence of oxidative inhibition of PAI-1; however,
the relative contribution of ROS-generating enzyme systems in oxidizing PAI-1 has not been studied so far.
Figure 4. Protease activation post-AMI – Plasminogen-Plasmin axis.

The proteolytic cleavage of plasminogen by uPA converts it into plasmin. Plasmin initiates ECM breakdown by augmenting its action through activation of MMP-9. The inhibitor of this axis, PAI-1, binds with uPA in a 1:1 ratio and forms an inactive complex. PAI-1 is an oxidant-sensitive protease inhibitor, and various pathways generating ROS may oxidize PAI-1.
Figure 4
1.4 Enzyme systems generating reactive oxygen species and their role following AMI

Various studies have observed a reduction in infarct size upon depletion of leukocytes such as neutrophils (15-16), mast cells (17-19), and macrophages (20-21), suggesting their involvement in LV remodeling. One of the mechanisms by which leukocytes combat any injury involves the generation of free radicals. Naturally, these free radicals play critical roles in innate host defenses, by confronting the invasion of parasites and pathogens through oxidative modification of lipids and proteins on their membranes. Although this process makes parasites and pathogens more susceptible to phagocytosis, it also causes deleterious effects on the surrounding healthy environment, resulting in decreased biological function. Four major pathways are employed by leukocytes in generating free oxygen radicals: (i) the NADPH Oxidase complex, (ii) MPO (iii) iNOS (iv) CP. A simplified schematic of the oxidants generated by these enzymatic pathways is depicted in Figure 5.

**NADPH Oxidase Complex:** NADPH oxidase is expressed by a variety of cells including leukocytes, cardiac myocytes, and endothelial cells at the site of infarction. NADPH oxidase complex in phagocytes is comprised of five subunits. Two subunits, gp91phox and p22phox, form the heavy and light chains of cytochrome b558 (CYBB), located on the membranes of secretory vesicles and granules. The other 3 subunits,
p40phox, p47phox, and p67phox, exist as a complex in the cytosol. Upon leukocyte activation, the cytosolic complex migrates and assembles with CYBB and in the presence of NADPH, produces superoxide ions(22-25). Recently a study by Frantz et al on gp91phox deficient mice demonstrated no significant difference in LV remodeling and dysfunction post-AMI(27). Notably, these authors did not observe ROS depletion after AMI in the myocardium. However, they did observe an upregulation of other NADPH oxidase subunits suggesting a compensatory mechanism in these mice. In contrast to this study, another study by Doerries et al demonstrated that p47phox deficiency has a protective role on LV remodeling after AMI(26). The study demonstrated that in p47phox−/− mice, ROS formation was severely depleted and LV cavity dilatation, dysfunction, cardiomyocyte hypertrophy, apoptosis, and interstitial fibrosis were reduced after AMI as compared to WT. These contrasting results by deletion of different subunits of NADPH oxidase suggest that the superoxide generation is highly compartmentalized to specific subunits and cell types in the myocardial environment post-AMI.

Multiple cell types including cardiac myocytes, endothelial cells, and fibroblast cells express p47phox subunit; leukocytes are highly dominant in its expression. Although global deletion of ROS and decreased MMP-2 activity was attributed to the observed protective effect on LV remodeling, the relative contribution of ROS derived from leukocytes and the mechanistic link between ROS and MMP-2 activity was not studied.
**Inducible Nitric Oxide Synthase (iNOS):** iNOS is one of the nitric oxide synthase (others being endothelial NOS (eNOS) and neuronal NOS (nNOS)), which is expressed in leukocytes post-AMI(30). The activity of this enzyme is independent of the level of calcium in the cell, in contrast with eNOS and nNOS(31). The production of nitric oxide (NO) from iNOS lasts much longer, and it reacts with the superoxide ions to form peroxynitrite ions during the respiratory burst. Recently, studies have been done on ventricular remodeling in iNOS−/− murine model(30, 32-33) where decreased mortality and improved LV function were shown. Although deleterious effects of peroxynitrite ions (NOO−) have been reported to be responsible, the precise mechanism is still not known.

**Myeloperoxidase:** This is a heme-containing lysosomal protein stored in azurophilic granules of neutrophils. During the respiratory burst, it is released from the granules and generates hypochlorous acid (HOCl) from hydrogen peroxide (H₂O₂) and chloride ions. Furthermore, it oxidizes tyrosine to tyrosyl radical using H₂O₂ as an oxidizing agent. HOCl and tyrosyl radical are cytotoxic and neutrophils use them to kill bacteria and other pathogens(34). Recent study by Askari et al have demonstrated a decreased leukocyte infiltration, significant reduction in LV dilation, and marked preservation of LV function in Mpo−/− murine model(14). This study demonstrated for the first time that after AMI, MPO-derived oxidants oxidize PAI-1 as PAI-1 activity was found to be more in Mpo−/− mice as compared to WT mice.
**Ceruloplasmin**: CP, also known as ferro-oxidase, is an acute phase reactant and has been known to increase in patients after AMI(35). Various cells that express CP include macrophages and hepatocytes(36). CP carries 90% of the total body’s copper, which is highly oxidant sensitive. In the presence of superoxide ions (O$_2^-$) the Cu$^{+1}$ ions get converted into Cu$^{+2}$ ions which have a very high oxidative capacity(35). Although studies have demonstrated increased expression of CP in plasma of patients with AMI(35), its role in ventricular remodeling is still not known.

**AIM 1** - The first aim of this study is an attempt to define one of the missing links in the improved ventricular remodeling observed in these specific oxidant-generating enzyme systems. The aim focuses on determining the relative contribution of each enzyme system in oxidizing PAI-1 and subsequent activation of uPA driven plasminogen/plasmin axis resulting in adverse ventricular function and remodeling. We hypothesized that NADPH oxidase, as an upstream free radical-generating pathway, is the major contributor in oxidizing PAI-1. To address this issue, we developed chimeric models of *Pai-1*−/− mice that will help us understand the biology of various oxidant-generating enzyme systems in oxidizing PAI-1.
**Figure 5.** Enzymatic pathways generating oxidants: NADPH oxidase acts as a superoxide (O$_2^-$) feeder molecule for MPO, iNOS and CP. The oxidants generated from these enzyme systems oxidize various proteins, glycoproteins, lipids, and glycosaminoglycans present on the cell membrane.
Figure 5


1.5 Infarct expansion post-AMI - SDF-1 α and CXCR4

During the initial stages following AMI, infarct expansion and infarct extension are events with extreme short- and long-term consequences. Infarct expansion refers to acute localized disproportionate dilatation and thinning of the infarct segment. Following AMI, cardiac myocyte necrosis starts in the sub-endocardial region, which progresses and becomes transmural within hours (Figure 2). The process peaks by 7-14 days post-AMI, resulting in adverse effects on infarct structure and function for several reasons. Studies have revealed that the extent of transmural necrosis and not the infarct size determines the ensuing infarct expansion. Various mechanisms that contribute in infarct expansion include: (i) cell necrosis, (ii) stretching of viable cardiac myocytes resulting from cellular necrosis, and (iii) cardiac myocyte slippage due to ECM breakdown, resulting in cardiac myocyte apoptosis. Finally, infarct expansion results in increased infarct size, myocardial thinning and over-all dilatation of the left ventricle. Therefore, patients with infarct expansion develop worse exercise tolerance, a greater propensity for congestive heart failure and higher mortality compared to those without expansion. The chances of developing myocardial rupture and ventricular aneurysm also increase in these patients. Various strategies have been implemented to restrain this pathophysiological phenomenon in an attempt to decrease the mortality and morbidity associated with this condition. These strategies mainly include decreasing ECM breakdown, preventing cardiac myocyte death via delivery of various cytokines and growth factors, and
regeneration of injured myocardium. One of the strategies to decrease cardiac myocyte apoptosis has been attributed to the SDF-1α/CXCR4 signaling pathway within cardiac myocytes. Therefore, another focus of this study targets in defining the biology of the SDF-1α/CXCR4 system responsible for decreasing cardiac myocyte apoptosis following AMI.

SDF-1α, also known as CXCL12, belongs to the subfamily of CXC-chemokine. These are small secreted proteins that serve as chemotactic cytokines. The SDF-1 gene encodes two small proteins, SDF-1α (89 amino acids) and SDF-1β (93 amino acids) that are alternatively spliced (37). Both proteins were initially purified from the supernatant of mouse bone marrow stromal cells (38-39). SDF-1α is a chemo-attractant for stem cells at the site of injury. A high level of SDF-1α in the bone marrow forms a concentration gradient that helps to retain the stem cells in the bone marrow. However, after an injury, SDF-1α levels are increased, and the gradient shifts towards the site of injury (40). This physiological process is instrumental in mobilization and migration of stem cells from the bone marrow to the site of the lesion. SDF-1α signaling is initiated by binding of the chemokine with its receptor, CXCR4. CXCR4, previously known as LESTR or fusin, is a seven transmembrane G-protein coupled receptor GPCR. Various studies have demonstrated that SDF-1α/CXCR4 initiates its signaling via the PI3 kinase pathway, and the beneficial effects of the SDF-1α/CXCR4 axis in preserving the myocardium post-AMI. Recently, CXCR7 (also called RDC1 and Cmkor1) has been identified as a novel receptor for SDF-1α. Upon SDF-1α binding, CXCR7 forms a heterodimer with CXCR4.
and transduces the signal downstream (41). Although the biology behind SDF-1α/CXCR7 signaling is in its preliminary stage and is still highly debatable, the effects of the SDF-1α/CXCR4 axis has been widely tested in various disease processes but poorly defined.

1.6 SDF-1α/CXCR4 axis following myocardial ischemic injury

Ischemia is a condition of inadequate blood supply that leads to cell death and degeneration of the tissue. It has been reported that ischemic injury stimulates stem cell mobilization and engraftment at the site of the lesion, resulting in improved function. Multiple groups as well as our lab have reported that SDF-1α levels are elevated immediately after AMI (42). AMI causes left ventricular remodeling, which is characterized by myocardial thinning, left ventricular dilation and reduced cardiac function resulting in congestive heart failure (43). Without artificial interference, there is a slow mobilization of stem cells from the bone marrow secondary to AMI for regeneration of cardiac tissue; however, it is not adequate to prevent left ventricular remodeling (44). In an attempt to enhance cardiac function following AMI, various cell-based therapeutic strategies have been investigated. Reestablishment of SDF-1α in the cardiac tissue by injecting skeletal myoblasts (SKMB) expressing the chemokine in the heart 7 days post-AMI, lead to homing and engraftment of bone marrow-derived stem
cells at the site of injury resulting in regeneration of the cardiac tissue microenvironment (42). Although the initial studies were based on the assumption that SDF-1α mediates stem cell homing followed by vasculogenesis and regeneration of infarcted tissue, subsequent experiments elucidated that cardiac myocyte preservation via SDF-1α/CXCR4 signaling also has a major role to play in improved ventricular function post-AMI. These observations prompted us to further investigate the role SDF-1α/CXCR4 plays in preserving cardiac myocyte integrity and function.

Our group has recently demonstrated that mesenchymal stem cells (MSCs) home to the infarcted region post-AMI, and MSCs overexpressing SDF-1α home better leading to improved cardiac function(45). MSCs engineered to overexpress SDF-1α (SDF-1α:MSC’s) were injected into the tail vein of mice that were induced with AMI by LAD ligation. The SDF-1α:MSC’s migrated to and engrafted at the infarct region, providing survival support to the cardiac myocytes. No regeneration of cardiac myocytes was evidenced, suggesting a chemokine-mediated preservation of cardiac tissue by decreased cell death and increased angiogenesis. Furthermore, the expression of SDF-1α by these MSCs recruited small cardiac myosin-positive cells to the infarct zone. These small cells, presumably cardiac stem cells, were highly proliferative and depolarized in vivo, however, they did not differentiate into cardiac myocytes (46). These observations contribute to the growing evidence of cardiac myocyte preservation following AMI by
the SDF-1α/CXCR4 axis, however, debates the issue of cardiac myocyte regeneration from stem cells.

In the normal pathophysiological process following AMI, SDF-1α expression increases immediately, peaks within 24 hours, and returns to baseline within 72 hours (Figure 6). However, CXCR4 expression in cardiac myocytes, as demonstrated by our group, starts at 48 hours and peaks at 72-96 hours post-AMI (47). This physiological mismatch was aligned in the studies done with overexpression of SDF-1α at the site of injury; however, the role of CXCR4 in this scenario is still not known. Recent studies in small cell carcinoma of lung revealed that CXCR4 can be upregulated via the epidermal growth factor-dependant PI3 kinase pathway, suggesting a strategy to increase CXCR4 expression in vivo. However, CXCR4 biology in cardiac myocytes is not fully understood yet. Therefore, it becomes intriguing to understand the role that CXCR4 derived from cardiac myocytes plays in preserving cardiac myocytes post-AMI.

**AIM 2** - The second aim of this study focuses on defining the biology of cardiac myocyte-derived CXCR4 post-AMI. We hypothesized that cardiac myocyte-derived CXCR4 has no major role to play in the normal pathophysiological response to myocardial infarction. To address this hypothesis, *Ccr4*−/− mice would have been an appropriate model to study; however, they die within few days of birth due to inadequate hematopoiesis, neurogenesis, and angiogenesis. Therefore, we generated congenital and
conditional cardiac-specific CXCR4-deficient mice in order to understand the role cardiac myocyte-derived CXCR4 plays after myocardial infarction.
Figure 6. Schematic diagram of SDF-1α and CXCR4 expression in the myocardium following AMI.

SDF-1α expression increases immediately and peaks within 24 hours after AMI. CXCR4 expression starts after 48 hours and peaks in 72 hours post-AMI.
Figure 6

SDF-1 and CXCR4 Expression Post-AMI

Hours Post-AMI

24 hours  48 hours  72 hours
2.0 MATERIALS AND METHODS

Bone Marrow Transplantation

Preparation of Total Bone Marrow Cells – 4-6 week old male mice of each Pai-1<sup>−/+</sup>, WT, p47phox<sup>−/+</sup>, Mpo<sup>−/+</sup>, iNOS<sup>−/+</sup> and Cp<sup>−/+</sup> genotype were sacrificed by intraperitoneal injection of xylazine and ketamine. Femur and tibia were stripped of the muscles and cut at both ends. The bone marrow cells were flushed in a 50 ml falcon tube with α-MEM. The total bone marrow cells were subjected to red blood cells lysis buffer (155 mM NH<sub>4</sub>Cl 12 mM NaHCO<sub>3</sub>, 0.1 mM EDTA) to lyse the RBC’s. Finally the total bone marrow cells were resuspended at a concentration of 3 million cells/10μl of PBS. 10 μl of this mix was transplanted in each femur of the radiated mouse.

Preparation of Animals for Bone Marrow Transplantation (BMT) -6-8 week old Pai-1<sup>−/+</sup> mice were radiated with 2 divided doses of 425 rads (total dose 850 rads) each 4 hours apart after keeping them on acidified water (pH = 2.5-3) for 7 days supplemented with neomycin (1.1g/l) and Polymixin (1 million units/l). Various groups of mice for the study were generated by transplanting the Pai-1<sup>−/+</sup> mice with 3 million total bone marrow cells of Pai-1<sup>−/+</sup>, WT, p47phox<sup>−/+</sup>, Mpo<sup>−/+</sup>, iNOS<sup>−/+</sup> and Cp<sup>−/+</sup> mice respectively via intra-femoral route as described (48). The transplanted mice were always kept on acidified water (pH=2.5-3.5) with antibiotics (in order to decrease toxicity caused by gastrointestinal tract bacteria) for another 4 weeks in cages which were changed every second day.
ELISA for PAI-1 Activity

Total PAI-1 and active PAI-1 levels in tissue were compared among two groups – *Pai-1*<sup>−/−</sup> mice transplanted with WT bone marrow and *Pai-1*<sup>−/−</sup> mice transplanted with *p47phox*<sup>−/−</sup> bone marrow. The animals were sacrificed at Day 3 post-AMI and the heart was perfused with 10 ml of saline to remove blood from the tissue. The infarcted tissue of the left ventricle was cut with scissors just below the ligation of LAD artery. Tissue from these groups for total PAI-1 and active PAI-1 ELISA assays were homogenized in PBS with 0.1% Triton X-100 supplemented with PMSF (100 mM), leupeptin (10 µg/ml) and aprotinin (10 µg/ml) (49). The solutions were then centrifuged at 5000 rpm for 10 min. The supernatant was collected and frozen at -80°C until used. 150 µg of protein was loaded in each well of total and active PAI-1 ELISA plates coated with PAI-1 antibody and uPA respectively. Both the ELISA plates were run simultaneously. The PAI-1 antibody binds with the total PAI-1 and active PAI-1 binds with uPA coated on ELISA plates from Molecular innovations. The PAI-1antibody-PAI-1 complex and uPA-PAI-1 complex on ELISA plate was subjected to primary PAI-1 antibody for 30 minutes followed by secondary antibody incubation for 30 minutes. The final reaction was quenched using H<sub>2</sub>SO<sub>4</sub>. The absorbance was measured at 450nm and the Active PAI-1/Total PAI-1 ratio was analyzed.
Generation of cardiac myocyte–specific Cxcr4<sup>−/−</sup> mice and in vivo analysis of CXCR4 deletion

Previous studies have reported the generation of mice having CXCR4 flox allele (Figure 7), in which exon 2 (2.2kbp) is flanked by two loxp sequences (50). We crossed Cxcr4<sup>−/−</sup> mouse with the mice bearing a transgene of α-Myosin heavy chain (α-MyHC) promoter driven –MerCreMer<sup>+/+</sup> and Myosin ventricular light chain promoter driven Cre (MLC2v-Cre<sup>+</sup>) (Figure 14). α-MyHC promoter driven –MerCreMer<sup>+</sup> mice were bought from Jacksons laboratory as heterozygotes and were crossbred to yield MerCreMer<sup>+/+</sup> mice. The homozygosity of cre was assessed using quantitative PCR and the mice with duplicate copies of cre were used in the crossing for faster results. The progenies of the first cross between Cxcr4<sup>−/−</sup> and MerCreMer<sup>+</sup> and Cxcr4<sup>−/−</sup> and MLC2v-Cre<sup>+</sup> were crossbred to yield Cxcr4<sup>−/−;MerCreMer+</sup> and Cxcr4<sup>−/−;MLC2v-Cre+</sup> mice. 40mg/kg of tamoxifen in corn oil was administered intra-peritoneally (IP) in 6 weeks old Cxcr4<sup>−/−;MerCreMer+</sup> male mouse continuously for 5 days. The genomic DNA from the heart of 8 week old mice from both types was isolated and subjected to PCR to determine CXCR4 deletion. The location of the primers used to detect CXCR4 deletion is depicted in Figure 15A. The primers used are – forward 5’-CACTACGCATGACTCGAAATG-3’ and reverse – 5’ CCTCGGAATGAAGAGAGATTATG-3’.
**Figure 7.** Floxed *Cxcr4* allele - Schematic representation of the wild-type *Cxcr4* locus, the targeting vector, the targeted allele, the *Cxcr4*-floxed, and the deleted locus. B, BamHI; C, ClaI; P, PvuII; S, SalI.
Source: J Exp Med. 2004 November 1; 200(9): 1145–1156

Figure 7
Immunostaining

A mixed cellular population (containing cardiac myocytes, endothelial cells and fibroblast cells) was isolated from neonatal hearts (1-3 days old) of Cxcr4<sup>f/f</sup>;MLC2v-Cre<sup>+</sup> parents with Neomyts Kit (Cellutron Life Technology Cat # nc-6031) and plated on the 35mm plate. The cells were cultured for 24 hours and then fixed in 2% paraformaldehyde, blocked with 1% BSA,1% donkey serum solution and then stained for CXCR4 (primary – 1:100 from Abcam Catalog#7199 overnight at 4°C, secondary 1:1000- anti rabbit from Abcam for 1 hour at room temperature).

Immunostaining in tissue was performed on the hearts harvested 48 hours post-AMI and 21 days post-AMI from Cxcr4<sup>f/f</sup>;MerCreMer<sup>+</sup> mice with and without tamoxifen treatment. The hearts were fixed in formalin and embedded in paraffin blocks. 6μm sections were made and immunostained. Forty-eight hour post-AMI tissue were stained for CXCR4 (Primary - NB100-74396 (Novus Biologicals), secondary- donkey anti rabbit IgG Alexa Fluor 488 from molecular probes) and 21 days post-AMI tissue was stained to calculate vessel density with isolectin (Cat # FL-1201 from Vectorlabs). Wheat germ agglutinin was used to outline the morphology of the tissue section (Cat#1022, Vectorlabs).
Western Analysis

For Nitrotyrosine and PAI-1

Day 3 post-AMI, infarcted hearts of Pai-1+/mice transplanted with Pai-1+/, WT and p47phox−/− bone marrow was homogenized in PBS with 0.1% Triton X-100 supplemented with PMSF (100 mM), leupeptin (10 µg/ml) and aprotinin (10 µg/ml) (49). Total protein (50 µg) from each sample was prepared in 4x laemmli buffer (200 mM Tris HCl (pH 6.8), 8% SDS, 0.1% bromophenol blue, 40% glycerol), subjected to 10% SDS PAGE and transferred on PVDF membrane. A single lane from one end was incubated with primary antibody to PAI-1 (1:200, SC-6644; Santa Cruz Biotechnology, Inc) and the remaining membrane was incubated with primary antibody to nitrotyrosine (1:400 Cayman chemicals – 189542) in 5% milk in 1X TBST (Tris base, 2.42 g; NaCl, 8 g; 1M HCl, 3.8 ml with pH to 7.5; water, 1 L; Tween 20, 2 ml) overnight at 4°C. The membranes were then incubated in peroxidase conjugated anti mouse (for nitrotyrosine) and anti goat (for PAI-1) secondary antibody for 1 hour (1:4000), The peroxidase was visualized on Xray film using enhanced chemiluminescence (Amersham Biosciences UK Limited, Buckinghamshire, UK). Microtek scanner was used to scan the blots and and the density of the bands was analysed using the NIH software, Image J.

For CXCR4

Western blotting was done as described above on the 48 hours post infarcted tissue homogenate of Cxcr4−/−;MerCreMer+ mice (with and without tamoxifen
administration). The blot was finally probed with primary antibody (1:500 in 5% milk in 1xTBST) against CXCR4 (Abcam, cat# 2074) followed by incubation with peroxidase-conjugated anti-mouse secondary antibody (1:4000 in 5% milk in 1xTBST). Chemiluminescence (Amersham Biosciences) was used to visualize the bands.

**Echocardiography**

Baseline 2D-echocardiography was performed on Cxcr4flo/flo;MLC2v-Cre+ , Cxcr4flo/flo;MerCreMer+ and Cxcr4flo/flo mice at 6 weeks of age using 15MHz linear array transducer interfaced with a Sequoia C256 and GE vision 7 without giving any anesthesia(45). 14 days post tamoxifen administration another echo was performed on Cxcr4flo/flo;MerCreMer+ mice to observe any difference in the baseline functions after CXCR4 deletion. Following myocardial infarction 3 days and 21days echocardiographic readings were also taken in these mice. Doppler and myocardial straining analysis was done on Cxcr4flo/flo;MLC2v-Cre+ mice at 6 weeks of age.

All animal protocols were approved by the Animal Research Committee and all animal were housed in the AAALAC-approved animal facility of the Cleveland Clinic.
Left Anterior Descending Artery Ligation

Anterior wall AMI was performed after 6 weeks of transplantation(51). AMI was induced in *Pai-1*^+/−* mice transplanted with *Pai-1*^+/−*, WT, *p47phox*^+/−*, *Mpo*^+/−*, *iNOS*^+/−* and *Cp*^+/−* bone marrow, *Cxcr4*^Δ/Δ*;MLC2v-Cre*^+/−*, *Cxcr4*^Δ/Δ*;MerCreMer*^+/−* and *Cxcr4*^Δ/Δ* mice by ligation of LAD. The animals were anesthetized with Xylazine/ketamine, intubated and ventilated with room air at 105 breaths per minute using a rodent ventilator (Harvard Apparatus). Sternotomy was performed and LAD was identified with the help of surgical microscope (Leica M500). LAD was ligated by using 7-0 prolene. Immediate blanching and anterior wall dysfunction revealed a successful ligation. The chest and skin were closed using 6-0 prolene. The animals were removed from the ventilator and kept under oxygen until they recover from anesthesia. Only the animals which survived first 24 hours of ligation were considered for the study.

Statistical Analysis

All numerical data are expressed as Mean ± SEM. Comparison among 2 groups was done by t-test. Survival curves were derived using the Kaplan-Meier method and compared using a log-rank test. The ventricular function was analysed with one-way ANOVA. The differences were considered significant at a p value of <0.05.
3.0 Critical role for white blood cell NADPH oxidase mediated Plasminogen Activator Inhibitor-1 oxidation and ventricular rupture following acute myocardial infarction

3.1 Abstract

PAI-1 plays a critical role in ventricular remodeling post-AMI. Pai-1−/− mice die within 7 days of myocardial infarction post-AMI due to increased plasmin activity leading to ventricular rupture. Similarly, leukocytes-derived oxidants play a central role in ventricular remodeling postAMI at least in part due to the recent observation that PAI-1 is an oxidant sensitive protease inhibitor that is inactivated by oxidation. The goal of this study was to assess the relevant pathways of leukocyte derived oxidants post-AMI that alter PAI-1 activity. Transplantation of WT bone marrow into PAI-1 null mice prolonged their survival after AMI (WT marrow in Pai-1−/− mice = 8.33% at day 21, 41.66% at day 7, Pai-1−/− marrow in Pai-1−/− mice = 0% survival at day 7 (p value at day 7 <0.02). We hypothesized that transplantation of marrow from mice with deletions of specific genes relevant to leukocyte derived oxidants (NADPH Oxidase, iNOS, MPO, and CP) would lead to decreased PAI-1 oxidative inactivation and further prolongation of life after AMI. Bone marrow from C57Bl/6 NADPH oxidase (p47phox subunit)−/−, iNOS−/−, Mpo−/−, Cp−/−, Pai-1−/− or WT mice was transplanted into lethally irradiated C57Bl/6 Pai-1−/− mice. Six weeks after the bone marrow transplantation, AMI was induced by ligation of the LAD and the onset of cardiac rupture was monitored. Pai-1−/− transplanted with
Mpo\textsuperscript{−/−}, iNOS\textsuperscript{−/−} or Cp\textsuperscript{−/−} marrow died within 9 days post-AMI and their survival was no different than mice that received WT bone marrow at day 9 (p value>0.05). Interestingly, Pai-1\textsuperscript{−/−} mice transplanted with p47phox\textsuperscript{−/−} marrow had a significant better survival at 21 days after AMI (30\%, p<0.03, n=10) compared to WT marrow (8.3\%, n=12). The Pai-1\textsuperscript{−/−} mice transplanted with WT bone marrow showed increased nitrination at 50 kDa position on western as compared to Pai-1\textsuperscript{−/−} mice transplanted with Pai-1\textsuperscript{−/−} bone marrow which was reduced to 2 fold in p47phox\textsuperscript{−/−} transplanted Pai-1\textsuperscript{−/−} mice. Furthermore, the Pai-1\textsuperscript{−/−} mice transplanted with p47phox\textsuperscript{−/−} marrow demonstrated greater myocardial PAI-1 activity compared to WT marrow. Collectively, these results demonstrate that leukocyte generated free radicals participate in PAI-1 oxidation, tissue degradation and ventricular remodeling after AMI. These data further suggest that modulating O\textsubscript{2}− generation by NADPH oxidase appears to be a therapeutically relevant target whereas downstream enzymes like MPO, iNOS and Cp are not.
3.2 Introduction

AMI and subsequent left ventricular remodeling is the most common cause of chronic heart failure. In the near-term post-AMI (<2 weeks) the remodeling process can lead to left ventricular wall rupture, papillary muscle rupture or ventricular septal defects. These complications, although rare, are lethal and account for 5-30% of in hospital mortality post-AMI (52). The pathways central to left ventricular remodeling, in order to minimize mechanical complications of myocardial infarction and optimize cardiac function, are under active investigation.

Several studies have emphasized on the critical role of plasminogen/plasmin/uPA axis, its inhibitor - PAI-1, and leukocyte derived oxidants in left ventricular remodeling following AMI (9, 53-54). PAI-1, a serine protease inhibitor, plays an important role in ventricular rupture as PAI-1 null mice die of ventricular rupture within 6 days of AMI(42). Studies have also reported that PAI-1 is an oxidant sensitive protease and inhibition of uPA, an activator of the plasminogen plasmin axis, by PAI-1 is redox sensitive(12, 42, 55).

Multiple studies have demonstrated an important contribution of various leukocyte derived oxidants producing enzyme systems (NADPH Oxidase, MPO, iNOS) in ventricular remodeling(26, 32, 42). CP, an enzyme having pro-oxidant properties is similarly elevated in patients following AMI(36, 56). While PAI-1 is known to be oxidant sensitive, the relative contribution of each of these leukocyte generating oxidants on PAI-1 function following AMI is unknown.
In the present study we systematically assessed the contribution of individual oxidant-generating pathways on PAI-1 activity and ventricular rupture. We demonstrate that NADPH oxidase being an upstream of MPO, iNOS and CP is required for PAI-1 inactivation and has a significant role in ventricular rupture. Since statin therapy inhibits the formation of NADPH oxidase(57), our findings suggest a potential mechanism for the improvement in ventricular function following myocardial infarction and further suggest that inhibition of downstream enzymes such as MPO, iNOS and CP is insufficient to significantly inhibit the consequences of oxidation in inflammation. The study design is explained in Figure 8.
Figure 8. Study design - 4-7 weeks old mice were started on antibiotic water. Bone marrow transplantation was performed on these mice at 5-8 week of age. 6 weeks after bone marrow transplantation, AMI was induced by LAD ligation. The survival was monitored for 21 days after AMI. All biochemical analysis were performed on the 3 day post infacted hearts of these mice.
Figure 8

- **Antibiotic Water treatment**
  - Neomycin
  - Polymyxin B
- **Bone Marrow Transplantation (Intra Femoral)**
  - 850 Rads,
  - 3-5 million cells
- **MI induction by left anterior descending artery ligation**
- **CBC to verify BM reconstitution**
- **PAI-1 activity ELISA assay**
- **Survival**

Timeline:
- **Day 0**
- **1 week**
- **2 weeks**
- **4 weeks**
- **Day 3**
- **Day 21**

**Males, 5-8 weeks of age**
3.3 Results

3.3.1 Effect of WT marrow in rescuing PAI-1 null mouse from myocardial rupture

We performed BMT with $Pai-1^{-/-}$ mice using bone marrow harvested from $Pai-1^{-/-}$ or WT mice. Four weeks post transplantation both groups of mice were found to have similar levels of bone marrow reconstitution based on complete blood counts. Six weeks after BMT, acute myocardial infarction was induced and survival was monitored for 21 days. All PAI-1 null mice transplanted with $Pai-1^{-/-}$ marrow died of ventricular rupture within 7 days of myocardial infarction (Figure 9). As in previous studies, ventricular rupture was defined as the animal appearing well the day prior to death and presence of blood in the chest on autopsy(14). Of the $Pai-1^{-/-}$ mice transplanted with WT marrow, 41.66% survived to 7 days and 8.33% of the mice survived to 21 days post-AMI (Figure 10).
Figure 9. PAI-1 deficient animals undergo ventricular rupture 3 days post myocardial infarction due to increased extracellular matrix degradation. The figure depicts the site of rupture in $Pai-1^{-}$ animals 3 days post myocardial infarction.
Ventricular rupture

Figure 9
3.3.2 Effect of leukocyte derived oxidant-generating enzyme systems in long-term rescue of PAI-1 null mice from myocardial rupture

To study the effect of leukocyte derived oxidant-generating system on PAI-1 oxidation after AMI we generated the PAI-1 deficient mice with bone marrow derived from $p47phox^{−/−}$ (Figure 10A), $Mpo^{−/−}$ (Figure 10B), $iNOS^{−/−}$ (Figure 10C) and $Cp^{−/−}$ (Figure 10D), respectively. These different groups of mice had white blood cell PAI-1 expression but lack NADPH Oxidase, MPO, CP or iNOS in their leukocytes. Following BMT, WBC reconstitution at 28 days was within normal limits of WT mice (Table 1) regardless of the source of bone marrow. After AMI induction we monitored the ventricular rupture of these mice for 21 days and compared it with the $Pai-I^{+/−}$ mice transplanted with WT marrow. We found that $Pai-I^{+/−}$ mice that received bone marrow from $p47phox^{−/−}$ (n=10) mice had a significant increase in survival to 30% at 21 days (Figure 10A) compared to an 8.3% survival at 21 days of $Pai-I^{+/−}$ mice that received WT marrow (n=12, p<0.03). MPO null marrow (n=11) (Figure 10B) and iNOS null marrow (n=6) (Figure 10C) transplanted mice died of ventricular rupture by day 9 and CP null marrow-transplanted $Pai-I^{+/−}$ animals (n=3) died of ventricular rupture by day 10 after AMI and were statistically no different than WT marrow (p>0.05).
Table 1. WBC reconstitution was achieved within 4 weeks of BMT in all the groups of transplanted PAI-1 KO mice
Figure 10. Mortality due to Ventricular rupture was monitored as a function of time after LAD ligation. The survival data for Pai-1/− mice transplanted with Pai-1/− bone marrow, open circles, n=7, and WT bone marrow, grey circles, n=12 are repeated on each figure to aid comparison. The black squares represent data from Pai-1/− mice transplanted with A.) NADPH oxidase/− bone marrow, n=10; B.) Mpo/− bone marrow, n=11. At day 21, NADPH oxidase/− the marrow-transplanted Pai-1/− animals have a significantly better survival (p<0.03) as compared to the WT bone marrow-transplanted Pai-1/− animals. All other groups were not statistically different than Pai-1/− mice that received WT bone marrow.
Figure 10 (A and B)
**Figure 10.** Mortality due to Ventricular rupture was monitored as a function of time after LAD ligation. The survival data for *Pai-1*−/− mice transplanted with *Pai-1*−/− bone marrow, open circles, n=7, and WT bone marrow, grey circles, n=12 are repeated on each figure to aid comparison. The black squares represent data from *Pai-1*−/− mice transplanted with C.) iNOS−/− bone marrow, n=6 and D.) *Cp*−/− bone marrow, n=3, respectively. No statistical difference was observed between the groups.
Figure 10 (C and D)
3.3.3 Effect of p47phox, MPO and iNOS in the initial post-infarct healing phase (<7 days)

The transplantation of WT marrow into \textit{Pai-1}^{-/} mice led to a delay in the onset of myocardium rupture from 3 to 4 days after AMI (Figure 11A). Therefore, we wanted to determine if transplantation of p47phox, MPO, iNOS or CP null bone marrow led to delay of the onset of myocardium rupture. Transplantation of p47phox, MPO and iNOS null marrow delayed the onset of myocardial rupture to 6 days after AMI (Figure 11B, 11C, 11D), and the transplantation of CP null marrow delayed the onset of rupture to 7 days after AMI (Figure 11E). Furthermore at day 7, when all \textit{Pai-1}^{-/} mice were dead, 90% of p47phox\textsuperscript{-/-} bone marrow-transplanted, 54.5% of Mpo\textsuperscript{-/-} bone marrow-transplanted, 66.6% of Cp\textsuperscript{-/-} bone marrow-transplanted and 33.3% of iNOS\textsuperscript{-/-} bone marrow-transplanted \textit{Pai-1}^{-/} animals were living as compared to 41.66% of WT bone marrow-transplanted \textit{Pai-1}^{-/} animals (table 2). At day 7 post-AMI among all the groups, when compared to WT marrow-transplanted \textit{Pai-1}^{-/} mice, only p47phox null bone marrow-transplanted \textit{Pai-1}^{-/} mice had a significant difference in survival (p value < 0.03). These results suggest that all these leukocyte derived oxidant-generating enzymes participate in oxidizing PAI-1. However, the effect of p47phox (NADPH oxidase) in PAI-1 oxidative inactivation is more pronounced in the initial as well as in the later stages of infarct healing.
Figure 11. Effect of free oxygen radicals on PAI-1 oxidation during initial phase of MI. The onset of ventricular rupture was delayed as compared to Pai-1+ marrow-transplanted Pai-1+ mice and WT marrow-transplanted Pai-1+ mice (A) in p47phox- marrow-transplanted Pai-1+ mice (B), Mpo- marrow-transplanted Pai-1+ mice (C), iNOS- marrow-transplanted Pai-1+ mice (D) and Cp- marrow-transplanted Pai-1+ mice (E).
Figure 11
Table 2: Survival (%) at day 7 post-AMI. p47phox⁻/⁻ marrow rescued 90% of Pai-1⁻/⁻ mice at day 7 post-AMI as compared to 41.66% from Wt marrow and 0% from Pai-1⁻/⁻ marrow. Survival between WT and p47phox⁻/⁻ bone marrow-transplanted Pai-1⁻/⁻ mice (p<0.03).
3.3.4 Oxidation of PAI-1 in transplanted mice

We wanted to confirm that the PAI-1 derived from the blood cells in WT transplanted $Pai-1^{-/-}$ mice is oxidized in vivo 3 days post-AMI. Therefore we estimated the change in total nitration at the level of PAI-1 protein by western blotting in $Pai-1^{-/-}$ and WT bone marrow-transplanted $Pai-1^{-/-}$ mice (Figure 12). Probing for PAI-1 in adjacent lane confirmed the position of PAI-1 band on the gel. We found that the level of nitration was 4.6 fold higher in WT bone marrow-transplanted $Pai-1^{-/-}$ mice ($n=5$) as compared to $Pai-1^{-/-}$ marrow-transplanted $Pai-1^{-/-}$ mice. This suggests that, PAI-1 being an extra protein in WT transplanted $Pai-1^{-/-}$ animals, was oxidized in these mice. On transplanting the $p47phox^{+/}$ bone marrow in $Pai-1^{-/-}$ mice this nitration was reduced by almost 2.5 folds. These results suggest that PAI-1 oxidation takes place in these animals and $p47phox$ has a significant role in oxidizing PAI-1.
Figure 12. Nitrotyrosine levels were determined by Western blot at day 3 post-AMI at the level of PAI-1 protein (50 KD), Figure shows 4.6 folds increase in total nitration at the level of PAI-1 protein in WT bone marrow-transplanted Pai-1−/− mice (n=5) as compared to Pai-1−/− bone marrow-transplanted Pai-1−/− mice (p<0.02). The total nitration was significantly reduced in p47phox−/− bone marrow transplanted Pai-1−/− mice (n=6) as compared to WT marrow-transplanted Pai-1−/− mice (p<0.01) and 2 X higher than the Pai-1−/− marrow-transplanted into Pai-1−/− mice. Data represents Mean ± SEM.
Bone marrow transplanted in Pai-1^{-/-} KO mouse

<table>
<thead>
<tr>
<th>Pai-1^{-/-}</th>
<th>Wt</th>
<th>p47phox^{-/-}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrotyrosine</td>
<td></td>
<td></td>
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<tr>
<td>β Actin</td>
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</table>

PAI-1 -- + +

Fold change in nitration relative to Pai-1^{-/-} mice

Wt marrow in Pai-1^{-/-} mice (n=5)
p47phox^{-/-} marrow in Pai-1^{-/-} mice (n=6)

Figure 12
3.3.5 **PAI-1 activity in vivo**

To further confirm our hypothesis, we wanted to verify that the *Pai-1*−/− mice transplanted with *p47phox*−/− marrow had increased PAI-1 activity as compared to *Pai-1*−/− mice that received WT marrow. Therefore, we examined the PAI-1 activity in the infarcted region of heart in both the groups 3 days after AMI using ELISA and activity assays. The total PAI-1 in both the groups was found to be similar with no statistical difference (Figure 13B), however active PAI-1 was 2 fold higher in *p47phox*−/− transplanted *Pai-1*−/− mice as compared to WT transplanted *Pai-1*−/− mice (Figure 13A). The relative PAI-1 activity (Active PAI-1/Total PAI-1) in *p47phox*−/− marrow-transplanted animals was 2.2 times greater than that measured in the heart of *Pai-1*−/− mice that received WT marrow (Figure 13C). As shown in Figure 13, there was significantly greater PAI-1 activity in those animals lacking WBC NADPH oxidase compared to WT WBC.
**Figure 13.** ELISA was performed on 3 day post-AMI infarcted portion of heart from Wt marrow in *Pai-1^{−/−}* mice and *p47phox^{−/−}* marrow in *Pai-1^{−/−}* mice.  

A. Total PAI-1  

B. Total Active PAI-1  

C. Fold change in mean Active PAI-1/Total PAI-1 ratio in the hearts of *Pai-1^{−/−}* mice transplanted with Wt total bone marrow (n=5) vs *p47phox^{−/−}* bone marrow (n=6) 3 days after LAD ligation.  

Data represents Mean ± SEM, (p value < 0.03)
Figure 13
3.4 Discussion:

This study adds to the growing evidence for a role for leukocyte derived oxidants in regulating ventricular rupture after myocardial infarction. In addition these data further refine the critical enzyme systems that regulate protease activation, and suggest the relevant enzymes systems that could be targets for therapeutic benefit.

The propeptide of zymogen-plasminogen is cleaved and results in plasmin by uPA/tPA (58). PAI-1 regulates the activation of uPA(59) and as we show here this reaction is redox sensitive. In the setting of uninhibited uPA activity, plasmin is generated from plasminogen which activates MMP-9, leading to degradation of the extracellular matrix by itself as well as the synergistically by activation of MMP-9(60-61). The increased protease activity can increase the risk of ventricular rupture as evidenced by the inevitable myocardial rupture in PAI-1 deficient mice(60-61). Our observation that transplantation of WT marrow into PAI-1 null mice led to the delay and inhibition of myocardial rupture allowed us to develop a system through which we could systematically investigate the relative oxidant-generating enzyme systems involved in modulating PAI-1 activity. To achieve our goal we used the *Pai-1−/−* model as a host to study the relative contribution of each oxidant-generating enzyme in oxidizing PAI-1 and subsequent prevention of ventricular rupture in *Pai-1−/−* mice.

We tested the relative importance of NADPH Oxidase, MPO, CP and iNOS in inactivating PAI-1 activity following AMI. We rationalized that using the *Pai-1−/−* mouse as a host and knocking the specific oxidant-generating enzyme system out of the white
blood cells that expressed PAI-1(62-64) at the site of inflammation in the heart, we could determine those enzyme systems that had a significant role in the functional consequences of PAI-1 oxidation. After complete bone marrow reconstitution the blood of these mice had PAI-1 but lacked the specific oxidant-generating enzyme system. Interestingly, only the deletion of NADPH oxidase led to any significant prolongation of survival following AMI. These results suggested that NADPH oxidase derived free radicals have a major rate limiting role in oxidizing PAI-1.

Leukocyte infiltration following AMI begins within hours of AMI and peaking at day 2-4 following AMI(4). In PAI-1 deficient mice, the volume of neutrophil infiltration is increased at day 3 post-AMI(65) and their hearts begin to rupture at an earlier time point than WT mice. Our results demonstrated that PAI-1 deficient mice begin to rupture their ventricles 3 days after AMI compared to 4 days in the PAI-1 null mice that received WT bone marrow. Interestingly, ventricular rupture was delayed to at least 6 days after AMI in the Pai-1−/− mice transplanted with marrow deficient in any of the oxidant-generating enzymes. Although ventricular rupture was delayed during the initial phase of inflammation, long-term survival was only affected by deletion of NADPH oxidase. This finding may be attributed to the fact that NADPH oxidase is upstream of MPO, iNOS and CP. Therefore in the setting of depletion of NADPH oxidase derived superoxide ions, the downstream effects of MPO, iNOS and ceruloplasmin are also inhibited, leading to a more complete inhibition of leukocyte generated oxidants. Our data further suggest that, in the setting of the specific lack of MPO, iNOS or CP but presence of NADPH oxidase, there is sufficient PAI-1 oxidation from the remaining enzyme systems and tissue
degradation is not inhibited and ventricular rupture still occurs.

There are limitations associated with the methodologies implemented in our studies that should be mentioned. Specifically, LV function and remodeling in these mice was not assessed in our studies. Handling of mice induces stress which can be associated with increased cardiac rupture. Therefore, to minimize potentially confounding variables these mice were not manipulated following the LAD ligation protocol. Another limitation is that multiple cell types are transplanted following whole bone marrow transplantation including stem cells. Therefore, it is theoretically possible that diminished stem cell function secondary to deletion of oxidant-generating systems could be responsible for some of our observations. Finally other proteases and protease inhibitors are oxidant sensitive. For example, inhibition of NADPH oxidase has been shown to decrease MMP-2 activity(66), which preserves ventricular function. Thus, our principle finding that NADPH oxidase is the most relevant oxidant-generating system for ventricular rupture, may serve as an important starting point for future studies on the effects of oxidation on the function of other proteases and protease inhibitors.

Consistent with our findings, recent studies suggest that short term treatment with statins in patients improve ventricular remodeling after AMI(57, 67-68). The subsequent decrease in oxidative stress at the site of inflammation has been shown to be a potential reason. However, our findings demonstrate a plausible mechanism for these observations of improved LV remodeling in these patients due to NADPH oxidase inhibition, preserved PAI-1 oxidation and decreased protease activation and tissue degradation.

In conclusion, our study demonstrates that PAI-1 has an important role to play in
ventricular remodeling and rupture following AMI and offers a novel mechanistic link between NADPH oxidase and PAI-1 oxidation. These data further suggest inhibition of PAI-1 oxidation and protease activation as a potential target for the early administration of statin therapy following AMI.
4.0 Role of cardiac myocyte CXCR4 expression in cardiac development and post-AMI left ventricular remodeling

4.1 Abstract:

SDF-1/CXCR4 axis has an instrumental role during cardiac development and has been shown to be a potential therapeutic target for optimizing ventricular remodeling post-AMI and in ischemic cardiomyopathy. The importance of the SDF-1/CXCR4 axis in cardiac development is highlighted by the fact that the SDF-1 and CXCR4 null species are not viable and demonstrate ventricular septal defects. The specific role of cardiac myocyte CXCR4 expression in the cardiogenesis and survival of cardiac myocyte and left ventricular remodeling post-AMI is unknown. We hypothesized that cardiac myocyte-derived CXCR4 is critical for cardiac development. We further postulated that cardiac myocyte CXCR4 expression may not be critical in adulthood secondary to the short transient expression of SDF-1 and the delayed cardiac myocyte expression of CXCR4 in the heart following AMI. To address this issue we developed congenital and temporally induced cardiac myocyte-specific Cxcr4<sup>−/−</sup> mouse models.

Mice were generated by crossing Cxcr4<sup>−/−</sup> mice with MerCreMer<sup>+</sup> mouse and MLC2v-Cre<sup>+</sup> mouse on C57BL/6J background. Breeding continued to yield
Studies demonstrated recombination in both models-congenitally in the MLC2v-Cre+ mice and following tamoxifen in the MerCreMer+ mice. Surprisingly the Cxcr4fl/fl;MLC2v-Cre+ are viable, had normal cardiac function and no evidence of ventricular septal defect. Cxcr4fl/fl;MerCreMer+ treated with tamoxifen 2 weeks prior to AMI demonstrated 90% decrease in cardiac CXCR4 expression 48h post-AMI. Twenty-one days after LAD ligation, echocardiography revealed no statistically significant difference in the wall thickness, LV dimensions or ejection fraction (40.86±7.49 vs. 34.42±2.62%) in Cxcr4fl/fl mice vs. Cxcr4+/+ mice regardless of strategy of Cre expression. There was also no difference in vascular density 21 days post-AMI (2369±131vsls/mm² vs. 2471±126vsls/mm²; Cxcr4fl/fl vs. cardiac myocytes-specific Cxcr4+/ mouse).

We conclude that cardiac myocytes derived CXCR4 is not essential for cardiac development and potentially due to the mismatch in timings of peaks of SDF-1 and CXCR4, plays no major role in ventricular remodeling post-AMI in normalcy.
4.2 Introduction:

Stem cell and gene therapy based strategies are being pursued in an attempt to decrease infarct size, optimizing ventricular remodeling and prevent in the onset of chronic heart failure in patients following acute myocardial infarction. While benefits have been demonstrated in several clinical trials and recent meta-analyses(69-71), the mechanism responsible for the benefits seen are under investigation. One potentially important pathway that has been demonstrated to be important by multiple laboratories is the SDF-1/CXCR4 signaling pathway(42, 72-75). This pathway has been implicated in stem cell survival following transplantation, homing of stem cells to the heart, and cardiac myocyte survival in both acute myocardial infarction and chronic heart failure. The SDF-1/CXCR4 axis has been demonstrated to be critical in cardiac development(76-77); however its role in myocardial repair in adulthood is less clear since SDF-1 expression is immediate and transient and cardiac myocyte CXCR4 expression is delayed and persistent following AMI. More recently the hypothesis has been forwarded that the basis of benefit associated with stem cell therapy following acute myocardial infarction is the restoration of the temporal alignment of SDF-1 and CXCR4(47).

CXCR4, a G protein-coupled seven-transmembrane receptor, together with its ligand SDF-1α, plays a crucial role during embryonic development and in maintaining the stem cell niche, homing of stem cells at the site of injury and preservation of the injured tissue in an adult mouse. During embryogenesis, CXCR4 expression starts as
early as blastocysts formation and is expressed in various differential stages of variety of cells throughout(78). The importance of CXCR4 during developmental process is evidenced from the observations noted in CXCR4 deficient mice. In these mice, the deficiency of CXCR4 proves to be lethal as the developing embryo acquires various developmental anomalies including defective hematopoiesis (β-lymphopoiesis and myelopoiesis), neurogenesis (abnormal cortex formation), angiogenesis and cardiogenesis (ventricular septal defect)(76-77). Cardiac neural crest participate in the formation of ventricular septum during cardiogenesis and express CXCR4(79), however the contribution of cardiac myocyte-derived CXCR4 in ventricular formation is not known.

Following AMI, SDF-1 expression is elevated immediately and peaks within 24 hours. However cardiac myocyte CXCR4 expression starts late and peaks between 72 and 120 hours after AMI(45). This physiological mismatch in the peaks of the ligand and receptor has led various researchers to over-express SDF-1 in infarcted regions. The prolonged expression of SDF-1 in infarcted tissue leads to re-establishment of stem cell homing, neoangiogenesis(80-83), myocardial preservation and increased ventricular function. Although SDF-1-CXCR4 axis has been shown to be critical in myocardial reparative process post-AMI, the exact role of CXCR4 derived from cardiac myocytes in ventricular remodeling is not known.

To address this question directly, we have developed a congenital (MLC-2Vcre) and conditional (MerCreMer⁺) deletion of cardiac myocyte CXCR4 using the Cxcr4floxed mouse (Figure 14). We have characterized these mice pre and post-AMI.
Figure 14. Breeding pattern to generate congenital and temporal deletion of cardiac myocyte-specific Cxcr4<sup>−/−</sup> mice.
Figure 14
4.3 Results:

4.3.1 Generation of congenital cardiac myocyte-specific CXCR4 deficient mice

CXCR4 deficiency is embryonically lethal and is known to exhibit defective hematopoiesis, neural development and ventricular septal defects. The ventricular septum begins to form in a mouse at day 11 and completes by day 12.5 post conception (pc)(84). However MLC2v expression starts at day 9 pc in the primitive heart tube(85). Therefore to assess the role of cardiac myocyte-specific CXCR4 expression in cardiac development we generated a mouse with congenital deletion of CXCR4 specifically in cardiac myocytes using the MLC2v-Cre mediated expression. We successfully crossed MLC2v-Cre\textsuperscript{+} (kind gift from Ken Chien, MD) mouse with Cxcr4\textsuperscript{f/f} mouse (kind gift from Yong-Rui Zou, PhD) and Cxcr4\textsuperscript{f/f} to generate Cxcr4\textsuperscript{f/f};MLC2v-Cre\textsuperscript{+} mouse and Cxcr4\textsuperscript{f/f};MLC2v-Cre\textsuperscript{+} mouse respectively (Figure 15B). All these mice were viable with litter sizes similar to that observed with MLC2v-Cre\textsuperscript{+}. A successful recombination of cre to the lox p sites was determined by genomic PCR on total ventricular homogenate (Figure 15A and 15B). The lox p sites surrounds the exon 2 of CXCR4 gene (encodes >90% of protein) and we used the primers that bind before and after the lox p sites. The extension time for the qPCR was limited to 60 sec that would not allow sufficient extension to generate product associated with the 2.2 kb Exon 2. However, 60 seconds is
sufficient to allow extension and product following deletion of Exon 2 following Cre recombination. Immunofluorescence for CXCR4 in neonatal cardiac myocytes showed an absence of CXCR4 staining in Cxcr4^{f/f};MLC2v-Cre^+ mice. Importantly, non-cardiac myocytes as defined by phase contrast microscopy from the myocardium of Cxcr4^{f/f};MLC2v-Cre^+ remained positive for CXCR4 expression ((Figure 15C)).

Based on echocardiography and autopsy no detectable ventricular septal defect or valvular defects were observed, Figures 11D and 11E, respectively. Echocardiography revealed normal cardiac function in Cxcr4^{f/f};MLC2v-Cre^+ mice compared to littermates that were Cxcr4^{f/f} or Cxcr4^{f/-} but did not have the MLC2v-Cre^+ allele. Ejection fraction (EF) was 94.38% ± 1.35% versus 94.6% ± 2.23%, fractional shortening (FS) was 67.0 ± 5.7% versus 63.6 ± 2.6% and left ventricular end diastolic dimension (LVEDd) was 2.95 ± 0.17 cm versus 2.75 ± 0.15 cm in the presence and absence of MLC2v-Cre, respectively (Table 3). We also observed no difference in myocardial contractility as measured by myocardial strain imaging: radial strain: 23.4 ± 13.3 versus 21.9 ± 9.8 and circumferential strain: -11.1 ± 3.9 versus -9.8 ± 4.1 in the presence and absence of MLC-2v-Cre in Cxcr4^{f/f} or Cxcr4^{f/-} mice (Figure 15F). These data suggest that cardiac myocyte CXCR4 expression is not required for normal heart development.
Figure 15. Characterization of Cxcr4\textsuperscript{ff}; MLC2v-Cre\textsuperscript{+} mouse: A.) Exon 2 of CXCR4 gene is flanked by lox p site. The primers bind before and after the lox p sites to detect cleaved CXCR4 B.) A PCR analysis on genomic DNA from tail and heart was performed on 8 weeks old Cxcr4\textsuperscript{ff}, Cxcr4\textsuperscript{ff};MLC2v-Cre\textsuperscript{+} and Cxcr4\textsuperscript{ff+};MLC2v-Cre\textsuperscript{+} mice.
Figure 15 (A and B)
Figure 15. Characterization of $Cxc4^{ff}$; MLC2v-Cre$^+$ mouse C) CXCR4 staining (Red) and phase contrast pictures of neonatal cardiac myocytes from $Cxc4^{ff}$ and $Cxc4^{ff}$;MLC2v-Cre$^+$ mice shows deletion of CXCR4 from neonatal cardiac myocytes of $Cxc4^{ff}$;MLC2v-Cre$^+$ mice. The white arrows point towards the cardiac myocytes and the green arrow points towards non cardiac myocyte cells in culture. The cells were distinguished morphologically with phase contrast pictures.
Figure 15 (C)
Figure 15. Characterization of $\text{C}x\text{cr}4^{+/+};\text{MLC}2\text{v}-\text{Cre}^+$ mouse: D.) The doppler shows normal outflow and inflow of blood during systole and diastole of $\text{C}x\text{cr}4^{+/+};\text{MLC}2\text{v}-\text{Cre}^+$ mouse with no obvious septal defect. E) The right ventricle cut open to reveal normal thick Inter Ventricular Septum (IVS) in $\text{C}x\text{cr}4^{+/+};\text{MLC}2\text{v}-\text{Cre}^+$. 
Figure 15 (D and E)
Figure 15. Characterization of $Cxcr4^{ff}$; MLC2v-Cre$^+$ mouse: F.) Circumferential and radial strain in $Cxcr4^{ff}$;MLC2v-Cre$^+$ and $Cxcr4^{ff}$ mice shows no significant difference (n=4 in each group). G.) Representative normal m-Mode recording of $Cxcr4^{ff}$ and $Cxcr4^{ff}$;MLC2v-Cre$^+$ at 6 weeks of age.
Figure 15 (F and G)
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<th>$Cxcr4^{+/+}$</th>
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<td>IVS</td>
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<td>1.08 ± 0.06</td>
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<td>1.71 ± 0.07</td>
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<td>LVPW</td>
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<td>LVED</td>
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<td><strong>Ejection Fraction</strong></td>
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<td>91.29 ± 2.45</td>
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<td>83.14 ± 1.53</td>
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**Table 3.** Baseline left ventricular function (n=7 in each group)
4.3.2 Generation of conditionally regulated cardiac myocyte-specific CXCR4 deficient mice:

To determine the role of cardiac myocytes derived CXCR4 in ventricular remodeling post-AMI we also generated conditional cardiac specific CXCR4 deficient mice. We crossed Cxcr4\(^{0/}\) and Cxcr4\(^{1/-}\) mice with MerCreMer\(^{+}\) mice (from Jacksons Laboratories) to generate Cxcr4\(^{0/-}\);MerCreMer\(^{+}\) mouse and Cxcr4\(^{1/+}\);MerCreMer\(^{+}\) mice respectively (Figure 16A). To verify recombination we quantified CXCR4 deletion by performing qPCR on the mice treated daily with 40 mg/kg of tamoxifen dissolved in corn oil, intraperitoneally for 2, 4, 6 and 8 days. Using the same PCR strategy described above, we observed no further deletion of CXCR4 following tamoxifen administration beyond 4 days (Figure 16B).

We have previously demonstrated that cardiac myocyte CXCR4 expression is upregulated beginning 48 h after acute myocardial infarction (45, 86). Therefore to quantify the degree of cardiac myocyte CXCR4 recombination, Cxcr4\(^{0/-}\);MerCreMer\(^{+}\) and Cxcr4\(^{1/+}\);MerCreMer\(^{+}\) mice were treated with tamoxifen for 5 days, allowed to recover cardiac function for 14 days and then underwent induction of AMI by LAD ligation. Two days following myocardial infarction, immunohistochemistry demonstrated down regulation of cardiac myocyte, but not endothelial CXCR4 expression after LAD ligation in the tamoxifen treated group (Figure 16C). Based on Western blots of whole heart homogenates, we achieved >90% reduction in myocardial CXCR4 protein compared to littermates that did not receive tamoxifen (Figure 16D). The baseline ventricular function-pre and post tamoxifen of was within normal limits as shown in table 3.
Figure 16: Characterization of $\text{Cxcr}^{4/-};\text{MerCreMer}^+$: A) A PCR analysis on genomic DNA from tail and heart was performed on 8 weeks old $\text{Cxcr}^{4/-}$; $\text{Cxcr}^{4/-};\text{MerCreMer}^+$ and $\text{Cxcr}^{4/+};\text{MerCreMer}^+$ mice. The deleted band for CXCR4 was detected after tamoxifen administration.
Figure 16 (A)
Figure 16: Characterization of Cxcr4^{0.5};MerCreMer\textsuperscript{+}: B.) A dose response curve for tamoxifen shows maximum deletion after 4 days of tamoxifen injection. Cleaved CXCR4 band was quantitated by qPCR. As shown in the bottom panel.
Figure 16 (B)
Figure 16: Characterization of $\text{Cxcr}^{\text{f/f}};\text{MerCreMer}^+$: C.) Immunofluorescence for CXCR4 48 hours post-AMI shows knockdown of CXCR4 in cardiac myocytes of tamoxifen treated $\text{Cxr}^{\text{f/f}};\text{MerCreMer}^+$ and endothelial cells positive for CXCR4 (Green)
Figure 16 (C)
Figure 16. Characterization of $\text{Cxcr}^{0\%};\text{MerCreMer}^{+}$: D.) Western analysis of CXCR4 48 hours post-AMI on infarcted region shows 90% deletion of CXCR4 in whole heart homogenate.
Figure 16 (D)
Figure 16: Characterization of Cxcr4\textsuperscript{ff}; MerCreMer\textsuperscript{+}: E.) Representative echocardiographic m-Mode recording of Cxcr4\textsuperscript{ff} and Cxcr4\textsuperscript{ff}; MerCreMer\textsuperscript{+} after tamoxifen administration.
Figure 16 (E)
4.3.3 Effect of CXCR4 on ventricular remodeling and function before and after AMI

*Cxcr4<sup>fl/fl</sup>;MerCreMer<sup>+</sup>* mice were treated with tamoxifen (40 mg/kg) for 5 days at 6 week of age. As recently reported in studies with the *MerCreMer<sup>+</sup>* mice, following tamoxifen administration we observed a transient decrease in cardiac function that resolved 10-14 days later(87), therefore, we did not perform any procedures on the MCM mice until 14 days after tamoxifen administration. Mice that did (*Cxcr4<sup>fl/fl</sup>;MerCreMer<sup>+</sup>) or did not receive tamoxifen (*Cxcr4<sup>fl/fl</sup>;MLC2v-Cre<sup>+</sup> and *Cxcr4<sup>fl/fl</sup>* ) underwent left anterior descending artery ligation to induce AMI and their cardiac function was monitored by echocardiography. We hypothesized that because SDF-1 expression is transiently expressed for a short period of time immediately after AMI and begins to return to baseline by the time cardiac myocyte CXCR4 expression is significantly up-regulated, the deletion of cardiac myocyte CXCR4 expression would not alter cardiac function or cardiac remodeling. Consistent with our hypothesis, 3 days after AMI the ejection fraction, fractional shortening, anterior wall thickness and posterior wall thickness of the cardiac myocyte-specific *Cxcr4<sup>+</sup>* mice(*Cxcr4<sup>fl/fl</sup>;MerCreMer<sup>+</sup> and MLC2v-Cre<sup>+</sup>;Cxcr4<sup>fl/fl</sup>) was 57.8 ± 5.0 %, 28.0 ± 3.7%, 0.34 ± 0.09mm and 0.31 ± 0.09 mm respectively and statistically not different from mice that did not receive tamoxifen (EF: 50.1 ± 5.0%, SF: 22.3 ± 3.2%, AW thickness: 0.27 ± 0.10mm and PW thickness: 0.37 ± 0.08mm respectively)(Figure 17A-17F). Twenty one days after myocardial infarction the
ventricular function and remodeling was not statistically different between the absence and presence of cardiac myocyte CXCR4 expression: EF: 34.4 ± 2.6% vs 40.9 ± 7.5%, SF: 14.0 ± 1.2% vs 19.3 ± 4.8%, anterior wall thickness (AWT): 0.14 ± 0.02mm vs 0.24 ± 0.10mm and posterior wall thickness (PWT): 0.45 ± 0.06mm vs 0.48 ± 0.11mm respectively (Figure 17A-17F). These results suggest that cardiac myocytes expression of CXCR4 in normal pathophysiological process after AMI is not essential for cardiac remodeling and function of heart.

We evaluated vascular density 21 days after AMI to determine if cardiac myocyte CXCR4 may have had any effect on vascular density. Using isolectin staining (Figure 17G), we measured vascular density in the infarct borderline zone and found similar levels of vascular density in the presence and absence of cardiac myocytes CXCR4 expression (2471 ± 126 vs 2369 ± 131 vessels/mm², respectively).
**Figure 17.** Echocardiographic analysis of 8-10 weeks old Cxcr4\(^{ff}\) and cardiac specific Cxcr4\(^{+/-}\) mice as a function of time after AMI. A.) AWT (mm) B) PWT (mm) C.) LVEDd (mm) at 0,3 and 21 days post-AMI (n=7 for Cxcr4\(^{ff}\) and n=12 for Cxcr4\(^{+/-}\) at each time point). There was no significant difference between the measured parameters at day 3 and 21 days post-AMI. Data represents mean ± SEM.
Figure 17 (A-C)
Figure 17. Echocardiographic analysis of 8-10 weeks old Cxcr4<sup>f/f</sup> and cardiac specific Cxcr4<sup>−/−</sup> mice as a function of time after AMI. D.) EF (%) E.) FS (%) at 0, 3 and 21 days post-AMI (n=7 for Cxcr4<sup>f/f</sup> and n=12 for Cxcr4<sup>−/−</sup> at each time point). There was no significant difference between the measured parameters at day 3 and 21 days post-AMI. Data represents mean ± SEM.
Figure 17 (D and E)
Figure 17. Echocardiographic analysis of 8-10 weeks old Cxcr4<sup>−/−</sup> and cardiac specific Cxcr4<sup>−/−</sup> mice as a function of time after AMI. F.) Representative m-mode recording of Cxcr4<sup>−/−</sup> and cardiac specific Cxcr4<sup>−/−</sup> mice after 21 days of MI.
21 Days Post-AMI

Figure 17 (F)
Figure 17. G.) Vascular density 21 days post-AMI in Cxcr4<sup>ff</sup> (n=4) and cardiac myocytes specific Cxcr4<sup>ff</sup> (n=6) mice showed no statistically significant difference among the groups. (Green – Isolectin, Red – Wheat Germ Agglutinin, Blue-Dapi).
Figure 17 (G)
4.4 Discussion

As significant body of literature has been developed that has demonstrated a critical role for the SDF-1:CXCR4 axis in cardiac development and myocardial response to cell therapy, the goal of this study was to analyze the importance of cardiac myocytes derived CXCR4 during development and myocardial repair following injury. To address this question we generated a congenital cardiac deletion of CXCR4 mouse and a conditional tamoxifen inducible cardiac myocytes specific CXCR4 deletion mouse. We characterized the baseline function of these animals and then studied the myocardial response to injury in these mice.

Cardiac Myocyte-derived CXCR4 in cardiac development

SDF-1 and CXCR4 are ubiquitously expressed during embryogenesis. However the timing and the stage specific expression of the cytokine and ligand orchestrate the migration of stem cells and development of various organs. CXCR4 and SDF-1 deficient mice present with similar phenotypes and exhibits ventricular septal defect in the membranous part of the septum. Studies have demonstrated that CXCR4 is expressed in the outflow tract and descending part of bulbus cordis by cardiac myocytes, endothelial cells and cardiac neural Crest cells during the septum formation(41, 88). However, the contribution of specific cell type expression of CXCR4 during in the septum formation is still not known. MLC2v is expressed in the outflow tract at day 9pc, much before septum
formation which starts at around day 11 pc. We chose to MLC2v as a promoter to regulate cre expression in order to remove cardiac myocyte CXCR4 expression before formation of the septum in order to determine if the lack of cardiac myocyte CXCR4 expression would aid in the development of VSD formation. Our results demonstrate that the CXCR4 derived from the cardiac myocytes does not play a role in the formation of ventricular septal defects observed in Cxcr4-/- mice. However, due to the limitation of MLC2v expression in the endocardial cushions and muscular septum(89), the contribution of non-cardiac myocyte CXCR4 derived from the cells of the descending aortopulmonary (AP) septum still remains a matter of investigation.

**Cardiac Myocyte-derived CXCR4 in myocardial repair**

We generated the conditional deletion of CXCR4 mouse because we initially hypothesized that the congenital deletion of cardiac myocyte CXCR4 would potentially be lethal or result in cardiac anomalies or dysfunction. Therefore, we wanted to allow for normal cardiac development in the presence of cardiac myocyte CXCR4 and then conditionally delete cardiac myocyte CXCR4 following the administration of tamoxifen.

Of note, as has been recently reported28 that tamoxifen administration results in a transient decrease in cardiac function in the MCM mice. Consistent with these observations, we observed significant degrees of cardiac dysfunction following the administration of tamoxifen to the mercremer mouse that resolved 14 days after the final dose. Thus all our studies commenced 14 days after the final dose of tamoxifen.

Our initial hypotheses were that the lack of cardiac myocyte CXCR4 would prove
lethal secondary to abnormal cardiogenesis and at the very least VSD formation. Our findings with the MLV2v-Cre mouse would suggest this hypothesis was false. We further hypothesized that the absence of cardiac myocyte CXCR4 would not adversely affect left ventricular remodeling or function following AMI because SDF-1 is rapidly and transiently expressed following AMI before cardiac myocyte CXCR4 is up-regulated. This hypothesis proved to be true.

Recent studies have demonstrated that mesenchymal stem cells (MSC) with or without SDF-1 over expression have beneficial effects on ventricular function post-AMI(45). Various mechanisms including stem cell homing, neoangiogenesis and decreased myocytes death have been suggested. Studies have also demonstrated that SDF-1 over expression initiates CXCR4 signaling in hypoxic cardiac myocytes and induce anti-apoptotic pathway by Akt phosphorylation. CXCR4 has further roles following AMI as it is involved in the recruitment of endothelial progenitor cells and mesenchymal stem cells to the site of injury(90-92). Furthermore the role of SDF-1/CXCR4 signaling is questionable in recruitment and differentiation of cardiac stem cells into cardiac myocytes(93).

The Cxcr4flo;MerCreMer+ mouse we developed for these studies will serve as a tool to dissect out the mechanisms responsible for the beneficial effects associated with the administration of SDF-1. In particular, since baseline and post-AMI cardiac function is the same in the presence and absence of cardiac myocyte CXCR4 expression, differences in response to stem cell therapy will be able to be attributed to the absence of cardiac myocyte CXCR4. We will further be able to determine the importance and
relative contribution of CXCR4 on strategies associated with enhancing cardiac myocyte survival such as ischemic preconditioning.

In conclusion, deletion of cardiac myocyte CXCR4 does not alter cardiac development or function. Similarly, deletion of cardiac myocyte CXCR4 prior to AMI does not alter myocardial response to injury. These data taken together would suggest no role for cardiac myocyte CXCR4 expression in development, normal physiology or response to injury. Future studies will need to determine whether there is a role for cardiac myocyte CXCR4 expression in the modulation of the myocardial response to injury mediated by SDF-1 and/or stem cell therapy.
5.0 DISCUSSION AND FUTURE PERSPECTIVES

Although novel therapies for AMI have reduced the incidence of mortality, the morbidity associated with the disease still has a significant impact on the patient’s condition. Our study targets ventricular remodeling post-AMI with 2 different approaches, i) preventing ECM degradation by protease inhibitor-mediated protease inactivation, and ii) preservation of myocardium.

Aim 1 of this study focused on regulation of PAI-1 activity via oxidant-generating enzyme systems after AMI. We generated various chimeric Pai-1−/− animals and determined their survival post-AMI. The results demonstrated delayed myocardial rupture in Pai-1−/− mice transplanted with NADPH oxidase, MPO, iNOS, and CP deficient bone marrow. These observations indicated that NADPH oxidase, MPO, iNOS, and CP participate in oxidizing PAI-1. Furthermore, the Pai-1−/− animals transplanted with NADPH oxidase deficient bone marrow had prolonged survival suggesting NADPH oxidase plays a major role in oxidizing PAI-1. NADPH oxidase is an upstream enzyme that provides functional support to MPO, iNOS and CP. Although, xanthine oxidase is another enzyme system in human body which also generates superoxide ions, its ability to cause sufficient damage to myocardial tissue is still debatable. A recent study on the LAD ligation model of AMI in dogs showed that allopurinol, a xanthine oxidase inhibitor, did not have any effect on ventricular remodeling post-AMI, suggesting a very limited oxidative role of xanthine oxidase following AMI.
Upon depletion of NADPH oxidase from the leukocytes, the downstream enzymes MPO, iNOS, and CP become non-functional, at least in terms of their free radicals-generating capacity. Although, deletion of individual enzyme systems delayed myocardial rupture (compared to the WT bone marrow-transplanted \(Pai^-1^+\) mice), they did not rescue the phenotype. These observations can be explained by the assumption that in these animals, PAI-1 was getting oxidized from other active enzymatic pathways that were functionally supported by oxidants generated by NADPH oxidase. For example, \(Pai^-1^-\) animals transplanted with \(Mpo^-\) bone marrow had NADPH oxidase, iNOS, and CP actively generating oxidants post-AMI. Therefore, with respect to PAI-1 oxidation at the site of injury, our findings suggest that near-to-complete global deletion of free oxygen radicals or superoxide ions \textit{per se}, had a greater impact as compared to deletion of downstream specific individual oxidants generating enzymes.

The mechanistic models we designed in our laboratory to study PAI-1 oxidation \textit{in vivo} suggest a new target to potentiate therapies directed against ventricular remodeling and myocardial rupture. Clinically, thrombolytic therapy is initiated in the patient post-AMI, if they are brought to the hospital within 3 hours of onset, to lyse the clot that has clogged the artery(94-97). A clot mostly consists of platelets accumulated in a fibrin mesh. Plasmin, a protease, acts as an enzyme to degrade the fibrin mesh. Drugs like alteplase, and urokinase potentiate the conversion of plasminogen into plasmin; therefore, lyses the clot and restores blood supply to the ischemic myocardium. Restoration of blood supply to the heart within 3 hours proves to be beneficial, as suggested by various studies(94-97); however, after 3 hours, the benefit of thrombolytic
therapy is still a matter of debate. PAI-1 can act as an inhibitor to the mode of action of thrombolytics, as it inhibits tPA or uPA but can also prevent plasmin and MMP-mediated excessive ECM degradation. Therefore, it would be crucial to determine the timing of administration of the drugs directed against uPA/tPA post-AMI. Following infarction, various cytokines start to attract neutrophils at the site of injury, peaking in 3 days. This migration requires ECM breakdown and thus disrupts the myocardial architecture. The subsequent result of this damage causes myocytic slippage, inevitable death of potent cardiac myocytes, and expansion of infarct size, thereby further increasing injury to myocardium. By administering a drug that can inhibit the plasminogen/plasmin axis or that can potentiate PAI-1 action may have therapeutic benefits in this scenario. uPA axis inhibitors can also be useful in counteracting the growth, spread and metastasis of various malignant tumours. One such inhibitor, WX-UK1, has already been successfully tested in phase 1 clinical trial for metastatic tumors(98-100).

Recently, statin therapy has demonstrated significant improvement in the ventricular functions of patients suffering from AMI. Studies done on rats post-AMI revealed improved ventricular function and NADPH oxidase inhibition following fluvastatin administration. Our finding that NADPH oxidase mediates inactivation of PAI-1 may provide a plausible explanation to these observations. In conclusion, our findings from the first aim of this study provide a mechanistic basis of ventricular remodeling post-AMI and offer a novel therapeutic target to prevent it.
The second aim of this study is focused on the role of cardiac myocyte-derived CXCR4 in ventricular remodeling following AMI. We hypothesized that cardiac myocyte-derived CXCR4 is not critically involved in the normal response to AMI. This hypothesis stems from the fact that the peak expression levels of SDF-1α and CXCR4 post-AMI differ temporally. To test this hypothesis, we developed congenital and conditional cardiac myocyte-specific CXCR4 deficient mouse models. These transgenic mice demonstrated no developmental defects. We observed no statistical difference in ventricular function and remodeling in these transgenic mice post-AMI. Normally, after AMI, SDF-1α levels peak within 24 hours and return to baseline by 72 hours. However, CXCR4 expression starts at 48 hours and peaks at 72 hours post-AMI. Therefore, due to this physiological temporal mismatch, the presence or absence of cardiac myocyte-derived CXCR4 should not alter the ventricular remodeling. Indeed, we observed no statistical difference in ventricular function and remodeling, even after knocking out CXCR4 from the cardiac tissue post myocardial infarction.

These transgenic mice can serve as a better tool to study the mechanism underlying the beneficial effects of SDF-1α/CXCR4 signaling. Strategies to overexpress SDF-1α within 24 hours post-AMI, have already demonstrated improved ventricular function, although CXCR4 overexpression studies are yet to be done. As shown in Figure 18, SDF-1α overexpression using MSC’s or SDF-1α expression vector-mediated delivery at the site of infarction prolongs the SDF-1α bioavailability.
**Figure 18.** Temporal expression profiles of SDF-1α and CXCR4 post-AMI.

SDF-1α expression starts immediately after AMI and peaks within 24 hours; however, CXCR4 expression initiates late and peaks at around 72 hours. This mismatch can be therapeutically fixed by the strategies shown in the lower panel by overexpressing SDF-1α or upregulating CXCR4 at an earlier time point.
Figure 18
When co-expressed, cardiac myocyte-derived CXCR4 binds to its ligand, SDF-1α, and initiates signaling via PI3K/Akt pathway, that imparts anti-apoptotic effects. Consistent with this, SDF-1α overexpression at the site of injury have been attributed to increased cardiac myocyte preservation and vasculogenesis. Therefore, MSCs overexpressing SDF-1α-mediated or SDF-1α expression vector-mediated delivery in the mouse model we developed can be instrumental in dissecting the relative contribution of cardiac myocyte-derived CXCR4 in cardiac remodeling post-AMI.

Although SDF-1α has been widely tested, CXCR4 upregulation at an early time point in this mismatch may evolve as a novel strategy to improve ventricular function. CXCR4 is not normally expressed in adult heart. CXCR4 expression is induced in the myocardium only after ischemic injury. Recent studies have demonstrated that delivery of various growth factors such as fibroblast growth factor-2 (FGF-2), platelet derived growth factor-BB (PDGF-BB), insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF) and epidermal growth factor (EGF), post-AMI improves ventricular function and remodeling. Other reports claim that, under hypoxic conditions, CXCR4 expression synergistically increases following the delivery of EGF and IGF-1. Since growth factors are known to preserve cardiac myocyte via PI3K/Akt signaling (Figure 19), it has been recently proposed that the cardiac myocyte preservation is dependent on CXCR4 upregulation. The cardiac myocyte-specific CXCR4 deficient mouse model will be useful in further understanding the involvement of CXCR4 in growth factor-mediated cardiac myocyte preservation. Following growth factor delivery in cardiac specific CXCR4 deficient mice, we can investigate the role of cardiac myocyte-derived CXCR4.
In conclusion, the murine model we generated and characterized in our laboratory can serve as an important tool to dissect out various mechanisms underlying improved ventricular function resulting from SDF-1α/CXCR4 signaling events and paves a way to develop novel therapeutic strategies for AMI.
Figure 19. CXCR4 expression is upregulated following AMI.

Binding of SDF-1α with CXCR4 initiates expression of anti-apoptotic factors via PI3k/Akt pathway. Growth factors such as PDGF, IGF or bFGF also act via PI3K/Akt signaling. Therefore, it has been hypothesized that growth factor-mediated cardiac myocyte preservation is dependent on upregulation of CXCR4 expression.
Figure 19
APPENDIX I:

List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AMI</td>
<td>Acute Myocardial Infarction</td>
</tr>
<tr>
<td>AWT</td>
<td>Anterior Wall Thickness</td>
</tr>
<tr>
<td>αMHC</td>
<td>Alpha Myosin Heavy Chain</td>
</tr>
<tr>
<td>BMT</td>
<td>Bone Marrow Transplantation</td>
</tr>
<tr>
<td>CHF</td>
<td>Congestive Heart Failure</td>
</tr>
<tr>
<td>CP</td>
<td>Ceruloplasmin</td>
</tr>
<tr>
<td>Cp&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Ceruloplasmin deficient</td>
</tr>
<tr>
<td>CYBB</td>
<td>Cytochrome B558</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Chemokine(C-X-C) receptor 4</td>
</tr>
<tr>
<td>Cxcr4&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Chemokine(C-X-C) receptor 4 deficient</td>
</tr>
<tr>
<td>DESC-1</td>
<td>Differentially Expressed Squamous Cell Carcinoma-1</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra Cellular Matrix</td>
</tr>
<tr>
<td>EF</td>
<td>Ejection Fraction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Fibroblast Growth Factor-2</td>
</tr>
<tr>
<td>FS</td>
<td>Fractional Shortening</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
</tr>
<tr>
<td>HOCl</td>
<td>Hypochlorous Acid</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin like Growth Factor-1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>iNOS⁻/⁻</td>
<td>Inducible Nitric Oxide Synthase deficient</td>
</tr>
<tr>
<td>IP</td>
<td>Intra-peritonial</td>
</tr>
<tr>
<td>IVS</td>
<td>Inter Ventricular Septum</td>
</tr>
<tr>
<td>LAD</td>
<td>Left Anterior Descending Artery</td>
</tr>
<tr>
<td>LVEDd</td>
<td>Left Ventricular End Diastolic dimension</td>
</tr>
<tr>
<td>MLC2v</td>
<td>Myosin Light Chain-2 ventricular</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>MLC2v-Cre</td>
<td>Myosin Light Chain-2 ventricular-promoter driven Cre</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MPO</td>
<td>Myeloperoxidase</td>
</tr>
<tr>
<td>Mpo&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Myeloperoxidase deficient</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal Stem Cells</td>
</tr>
<tr>
<td>NADPH oxidase</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate-oxidase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOO&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Peroxynitrite ions</td>
</tr>
<tr>
<td>O&lt;sub&gt;2&lt;/sub&gt;−</td>
<td>Superoxide Ions</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen Activator Inhibitor -1</td>
</tr>
<tr>
<td>Pai-1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Plasminogen Activator Inhibitor -1 deficient</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>Platelet Derived Growth Factor-BB</td>
</tr>
<tr>
<td>PWT</td>
<td>Posterior Wall Thickness</td>
</tr>
<tr>
<td>p47phox&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>NADPH oxidase p47phox subunit deficient</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>Stromal cell-derived Fator-1α</td>
</tr>
<tr>
<td><strong>Sdf-1/−</strong></td>
<td>Stromal cell-derived Fator-1 deficient</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>SDF-1α:MSCs</td>
<td>MSCs overexpressing SDF-1α</td>
</tr>
<tr>
<td>SkMB</td>
<td>Skeletal Myoblasts</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue Plasminogen Activator</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase like Plasminogen Activator</td>
</tr>
<tr>
<td>WBCs</td>
<td>White Blood Cells</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
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