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Ahmed Abdel-Latif
University of Kentucky, akabde2@uky.edu

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Ahmed Abdel-Latif, Student

Dr. Donald A. Cohen, Major Professor

Dr. Joseph McGillis, Director of Graduate Studies

ROLE OF THE IMMUNE SYSTEM AND BIOACTIVE LIPIDS IN TRAFFICKING BONE MARROW-DERIVED STEM CELLS IN PATIENTS WITH ISCHEMIC HEART DISEASE

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

Ahmed Abdel-Latif
Lexington, Kentucky
Director: Donald A. Cohen, Ph.D.
Professor of Microbiology, Immunology, and Molecular Genetics
Lexington, Kentucky
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ABSTRACT OF DISSERTAION

ROLE OF THE IMMUNE SYSTEM AND BIOACTIVE LIPIDS IN TRAFFICKING BONE MARROW-DERIVED STEM CELLS IN PATIENTS WITH ISCHEMIC HEART DISEASE

Acute myocardial infarction (AMI) triggers the mobilization of stem/progenitor cells from bone marrow (BMSPCs) into peripheral blood (PB). The underlying mechanisms orchestrating this mobilization and subsequent homing of BMSPCs to the myocardium are poorly understood. While the role of traditional chemokines in the mobilization and homing of hematopoietic stem cell (HSCs) to BM niches is undisputed, their role in directing BMSPCs to the highly proteolytic environment of the ischemic myocardium is debatable and other redundant mechanism may exist. Based on our observation that bioactive lipids, such as sphingosine-1 phosphate (S1P) and ceramide-1 phosphate (C1P), play an important role in regulating trafficking of HSCs; we explored if they also direct trafficking of BMSPCs in the setting of myocardial ischemia. While BMSPCs expressed S1P receptors regardless of the source, the expression of S1P receptor 1 (S1PR1) and receptor 3 (S1PR3), which are responsible for migration and chemotaxis, was elevated in BMSPCs in naïve BM cells and was reduced following mobilization. This expression correlated to differential response of BMSPCs to S1P in chemotaxis assays. By employing flow cytometry analyses, we observed an increase in circulating PB $CD34^+$, $CD133^+$ and $CXCR4^+$ lineage negative (Lin^-)/$CD45^-$ cells that are enriched in non-HSCs ($P < 0.05$ vs. controls). This corroborated our mass spectrometry studies showing a temporal increase in S1P and C1P plasma levels. At the same time, plasma obtained in the early phases following AMI strongly chemoattracted human BM-derived $CD34^+$/Lin^- and $CXCR4^+$/Lin^- cells in Transwell chemotaxis assays in an S1P dependent fashion. We examined other mechanisms that may contribute to the homing of BMSPCs to the infarcted myocardium due to the reduction of S1PRs upon mobilization. We observed that hypoxia induced higher expression of cathelicidins in cardiac tissues. Indeed, PB cells isolated from patients with AMI migrated more efficiently to low, yet physiological, gradient of SDF-1 in Transwell migration assays compared to SDF-1 alone. Together, these observations suggest that while elevated S1P plasma levels early in the course of AMI may trigger mobilization of non-HSCs into PB, cathelicidins appear to play an important role in their homing to ischemic and damaged myocardium.

Ahmed Abdel-Latif
January 10th, 2013

ROLE OF THE IMMUNE SYSTEM AND BIOACTIVE LIPIDS IN TRAFFICKING BONE MARROW-DERIVED STEM CELLS IN PATIENTS WITH ISCHEMIC HEART DISEASE

By

Ahmed Abdel-Latif

Dr. Donald A. Cohen

Director of Dissertation

Dr. Joseph McGillis

Director of Graduate Studies

January 10th, 2013

DEDICATION

This dissertation is dedicated to my wife, mother, siblings, and mentors, who instilled in me the moral virtues of hard work and diligence and who were unequivocally supportive throughout my scientific journey.

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Chapter 1

INTRODUCTION

The prevalence of ischemic heart disease and acute myocardial infarction (AMI) has increased to alarming rates in the United States and the western world (1). Patients who survive the initial AMI suffer ischemic cardiomyopathy (ICM) which is often complicated by high mortality and poor overall prognosis (2, 3). Despite significant advances in medical therapy and revascularization strategies, the prognosis of patients with AMI and ischemic cardiomyopathy remains dismal (4, 5). The last decade has demonstrated significant progress and rapid translation of myocardial regenerative therapies particularly those utilizing stem cells isolated from adult tissues (6).

Studies examining the potential therapeutic use of bone marrow (BM)-derived cells in myocardial regeneration have overshadowed the growing evidence of innate cardiac reparatory mechanisms. Up until early 2000s it was believed that the human heart was a post-mitotic organ that is not capable of self-renewal, and therefore the AMI-damaged myocardium could not be regenerated. However, this dogma has been continuously challenged in the last decade. Follow up of cardiac transplantation patients have demonstrated continuous replenishment of cardiomyocytes by recipient derived cells through poorly understood mechanisms (7). Repeated biopsies showed an increasing number of recipient derived cells that have differentiated to fully functional

cardiomyocytes, vascular smooth muscle cells and endothelial cells. In one study of a male heart transplant into a female recipient, a fraction of the cardiomyocytes were Y-chromosome-positive, providing direct evidence that these cells originated from the host to the myocardium of the grafted heart. The number of recipient-derived cardiomyocytes, vascular smooth muscle cells and endothelial cells increased significantly chronologically after the transplantation. Furthermore, these primitive cells, which probably originated in the bone marrow (BM), expressed stem cell antigens including c-kit, and MDR1. The migration of primitive cell populations to the grafted heart resulted in the loss of stem-cell markers, active proliferation, and acquisition of the mature phenotype followed by cell colonization and de novo formation of cardiomyocytes, coronary arterioles, and capillaries (7). Thus, all three major lineages of the heart are being renewed by cells from the recipient.

To address the question of BM origin of chimeric cardiomyocytes, a follow-up investigation analyzed hearts of patients who have undergone gender-mismatched BM transplantation (8). The key findings suggest that BM acts as a reservoir of “off-site” tissue committed stem cells that contribute to cardiomyocyte formation. Interestingly, the potential origin and phenotype of marrow myocyte precursors included lineage-restricted mesenchymal, hematopoietic, and multipotent adult progenitor cells (9). Together, these data established human bone marrow as a source of bone marrow stem/progenitor cells (BMSPCs) capable of de novo cardiomyocyte formation and possibly repair. However, the

mechanisms governing the mobilization of BM cells from their niches to the myocardium are poorly understood.

Animal studies have confirmed cardiomyocyte chimerism to be a dynamic process responding to significant injury such as myocardial infarction and which is most apparent in the peri-infarct zone (10). Hsieh and colleagues describe the renewal of cardiomyocytes at baseline and after cardiomyocyte loss such as following AMI or pressure overload. While cardiomyocyte renewal was minimal under physiological conditions, the rates of newly formed cardiomyocytes increased significantly after AMI especially at the peri-infarct border suggesting a dynamic response to injury. The rate of diffuse chimerism was also dynamic during pressure overload conditions albeit at a lower rate than that seen in ischemic injury. Although this process appears to be robust enough to achieve the renewal of approximately 50% of all cardiomyocytes in the normal life span, very little is known about its underpinnings (11).

Complex innate reparatory mechanisms are initiated by myocardial ischemia interacting with different elements of the immune system, the infarcted myocardium and bone marrow stem cells, culminating in BM-SPCs mobilization as we and others have demonstrated (12, 13). However, very little is known about the mechanisms and clinical significance of this mobilization. Animal studies show that mobilized BM-derived cells (BMCs) repopulate the infarct border, however the significance of this mobilization is unclear given the low rate of their differentiation into cardiomyocytes (14).

Adult bone marrow contains populations of pluripotent stem and primitive cells

The bone marrow acts as a reservoir for a heterogeneous pool of tissue-committed and non-committed stem cells. These populations contain progenitors that aid in the chimerism and cellular turnover of different organs as well as very rare populations of pluripotent and non-committed stem cells. The old dogma that adult tissues lack pluripotent stem cells (PSCs) has been continuously challenged during the last decade through multiple studies that isolated PSCs from adult humans' and animals' tissues. These populations were distinguished based on their morphology with small cell size, large nucleus demonstrating euchromatin and large nucleus to cytoplasm ratio. Furthermore, cell surface markers as well as nuclear transcription factors, such as SSEA1/4, Oct4 and Nanog, have been deployed.

Very small embryonic like stem cells (VSELs) represent a rare yet pluripotent population of adult stem cells. They have been initially described by Dr. Ratajczak's group in the murine BM based on their expression of Sca1 (murine stem cell marker) and lack of expression of CD45 (pan–leukocytic marker) and differentiated lineage (Lin) markers (15). Following their isolation from murine tissues, VSELs were subsequently isolated from human BM, umbilical cord blood (CB) and peripheral blood based on the lack of expression of Lin/CD45 and the expression of the stem cell markers CD133, CXCR4 and CD34. **Figure 1** illustrates the flow cytometry protocol for identifying and isolating VSELs from murine and human samples. VSELs were further characterized

using a multi-dimensional approach comprising molecular, protein and cell imaging techniques to confirm their pluripotent features (16). VSELs are morphologically similar to embryonic stem cells demonstrating small diameter compared to more committed progenitors/stem cells with large nucleus containing open-type chromatin surrounded with thin rim of cytoplasm and multiple mitochondria (16). VSELs exhibit multiple embryonic and pluripotent surface and nuclear embryonic markers such as Oct4, SSEA1/4, Nanog, and Rex1. In vivo and in vitro studies have demonstrated the capability of VSELs to differentiate into multiple cell lineages across germ lines including cardiomyocytes (15).

The bone marrow harbors other multi- and pluri-potent stem cell populations such as the mesenchymal stem cells (MSC) (17, 18), multipotent adult progenitor cells (MAPC) (19), and marrow-isolated multilineage inducible cells (MIAMI) (20). Similar populations with cardiac differentiation potential have been also isolated from skeletal muscle and other tissues (21). However, it is conceivable that different investigators have isolated, using different methods, the same or very similar populations and named them differently. It is also possible that these populations at least in part contain VSELs that might explain their pluripotent potential.

BM-derived stem cells are mobilized in the peripheral circulation following myocardial ischemia in animal models and humans

Myocardial ischemia, particularly large myocardial infarction, produce multiple stimuli include various chemokines, cytokines, kinins, bioactive lipids and members of the complement cascade, that lead to the mobilization and subsequent homing of BMSPCs. Indeed, several reports have confirmed that mobilization of committed stem cells originating from the BM occurs in response to myocardial ischemic injury (22-27) and heart failure (28). Similar observations were noted in patients with acute neurological ischemia (29) and patients with extensive skin burn (30).

The first evidence for the mobilization of CD34+ mononuclear cells in AMI was demonstrated by Shintani *et al* (27). The authors demonstrated successful in vitro differentiation of circulating BMSPCs into endothelial cells that expressed CD31, VE-cadherin and the kinase insert domain receptor (KDR) (27). Leone *et al* demonstrated that the levels of circulating CD34+ cells in the setting of AMI were higher when compared to patients with mild chronic stable angina and healthy controls. The magnitude of CD34+ cell mobilization correlated with the recovery of regional and global LV function recovery as well as other functional LV parameters (23). Similarly, Wojakowski *et al* demonstrated the mobilization of multiple BMSPC populations in patients with AMI (31). In their following publication, the authors demonstrated the correlation between circulating BM-SPCs and ejection fraction at baseline and lower brain natriuretic peptide (BNP) levels (24). Interestingly, the successful mobilization of multiple BMSPCs correlated with improved recovery of left ventricular (LV) functional parameters suggesting the clinical significance of this mobilization (32).

Figure 1.

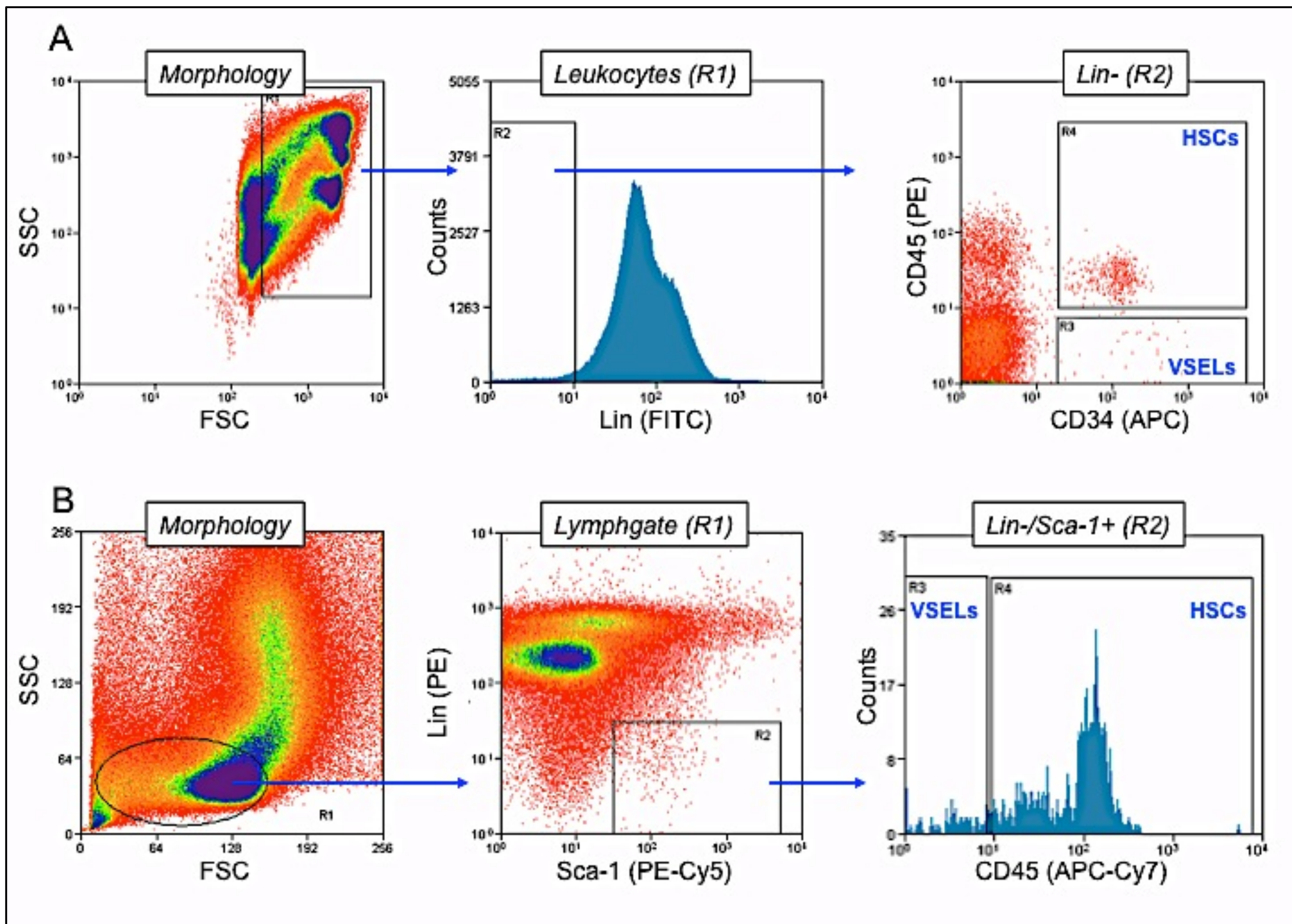

Figure 1. Strategy for flow cytometric analysis of human and murine Very Small Embryonic-Like and hematopoietic stem cells. Nucleated cells are isolated by lysis of red blood cells and cells are then gated based on the cell size (>2 μm) using beads to set up the size gates.

Panel A – Gating strategy for isolating human cord blood (CB)-derived VSELs. Morphology of total CB-derived nucleated cells is shown on dot-plot representing FSC and SSC parameters related to cell size and granularity/complexity, respectively. All objects larger than 2μm are enclosed in region R1 and further visualized on histogram showing the expression of markers of mature hematopoietic cells (lineage markers; Lin). Cells not expressing differentiated hematopoietic markers (Lin- in region R2) are then analyzed for CD34 and CD45 expression. VSELs are identified as CD45-/Lin-/CD34+ cells (region R3), while hematopoietic stem cells (HSCs) as CD45+/Lin-/CD34+ cells (region R4).

Panel B – Sorting of murine bone marrow (BM)-derived VSELs. Morphology of total murine BM-derived nucleated cells is shown on dot-plot presenting FSC and SSC parameters and all objects in range of 2-10μm in diameter are included in region R1. Lymphocytic cells including stem cell fraction is further analyzed for Sca-1 and differentiated hematopoietic lineages markers (Lin) expression and only Sca-1+/Lin- cells are included in region R2. Cells from this region are further seperated based on CD45 expression. Murine VSELs are distinguished as CD45-/Lin-/Sca-1+ cells (region R3), while HSCs as CD45+/Lin-/Sca-1+ cells (region R4).

Stem cell mobilization was initiated by stimuli from the infarcted myocardium and this mobilization was reduced by the successful revascularization of the culprit vessel in AMI that reduced the resultant damage (33). However, the majority of the above mentioned studies have focused on the mobilization of partially committed stem cells such as hematopoietic stem cells (HSPCs) and endothelial progenitor cells (EPCs).

Multipotent Mesenchymal stromal cells (MSCs) are often isolated from the bone marrow, but have been identified in a number of tissues, including fetal and umbilical blood, lung, liver, kidney and adipose tissue (34). It has recently been shown that cells surrounding epithelial cells in capillaries and microvessels and cells residing in the tunica adventitia share antigenic markers and behave similarly to MSC in culture (35, 36). Therefore, it has been proposed that the natural MSC niche is perivascular both within bone marrow and other tissues (35). The defining characteristics and the isolation procedure of MSC differ among investigators due to lack of agreed-upon specific markers. While historically MSCs were often isolated by plastic adherence and a fibroblastic appearance, more stringent guidelines for MSC identifications were recently released. In 2006 The International Society for Cellular Therapy recommended three minimal criteria for defining MSCs: (i) plastic-adherent when maintained in standard culture conditions, (ii) Specific surface phenotype (must express CD105, CD73, CD90 and must lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, HLA-DR), and (iii) In vitro differentiation into osteoblasts, adipocytes and chondroblasts (37). MSCs express a number of chemokine

receptors allowing for their migration in response to chemokine gradients in damaged tissue (38). While the data regarding the kinetics of MSC mobilization are not consistent and need confirmation (39), the SDF-1/CXCR4 signaling axis seems to be responsible for MSC migration which potentially leads to their homing to the infracted myocardium (40-42). Given the above findings and their immune-privileged status, MSCs might be the optimal cells for cardiac repair. In fact, numerous studies have described a positive effect of MSC therapy in tissue regeneration, specifically increased capillary density in infracted area or reduced scar formation after myocardial infarction (43-46).

Hematopoietic stem/progenitor cells (HSCs) are multipotent cells that can differentiate into all the blood cell types, both in the myeloid and the lymphoid cell lineage and have unlimited capacity of self-renewal. HSCs are identified in the BM and PB fractions as being CD34+ and/or CD133+. The number of CD34+ cells predicts hematopoietic recovery after blood stem cell transplantation and are thus used to assess the numbers of HSCs in the peripheral blood (47). Wojakowski et al demonstrated that HSC mobilization peaks within the first 12 hours after acute MI, followed by a steady decrease in HSC plasma levels over the following 7 days (31). Conversely, others have shown that maximum HSC efflux occurs 5 days after acute MI (23), suggesting a significantly delayed chemotactic signaling cascade.

The mechanisms governing BMSPC mobilization after ischemic myocardial injury are still debatable. While a role for the SDF-1/CXCR4 axis in retention of BMSPCs in bone marrow is undisputed, its exclusive role in their

mobilization and homing to a highly proteolytic microenvironment, such as the ischemic/infarcted myocardium, is less established and redundant mechanisms may exist (48-50). The limited contribution of traditional peptide chemokine mobilization of BMSPCs to cardiac tissue maybe explained by their active degradation at the sites of inflammation and myocardial infarction by metalloproteinases (50-52). On the other hand, bioactive lipid mediators such as sphingosine-1 phosphate (S1P) and ceramide-1 phosphate (C1P) are resistant to proteases and exhibit potent chemotactic effects on SPCs. There is evidence that the levels of lysophosphatidic acid (LPA) and S1P are modulated at the sites of myocardial infarction and thrombosis where they have been implicated in preconditioning and myocardial protection (53-55). Moreover, recent evidence implicates a pivotal role for S1P in the mobilization of hematopoietic stem cells (HSCs) (56, 57).

The homing of mobilized BMSCs is however a less understood phenomenon. Stimuli responsible for the mobilization and homing of BMSPCs in the setting of myocardial ischemia show similarities and differences with those involved in hematopoietic stem cells (HSCs) homing to the BM. Due to the above mentioned reasons, traditional chemokines play a limited role in the homing of BMSPCs to the infarcted myocardium and the peri-infarct zone where most of the regeneration begins. Therefore, more studies are needed to better define the pathways that are initiated by AMI and examine their therapeutic implications.

Figure 2.

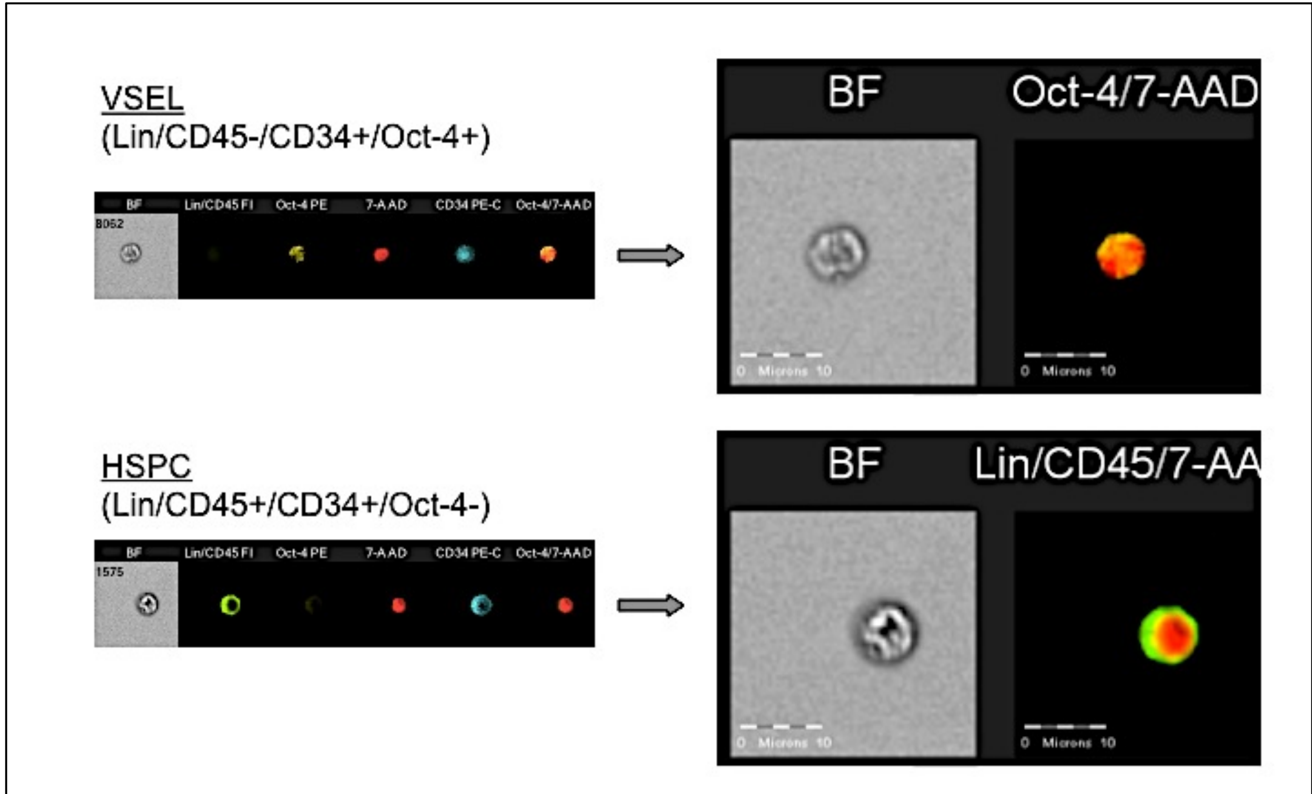

Figure 2. Representative ImageStream images of VSEL and hematopoietic stem/ progenitor cell (HSPC) circulating in peripheral blood following acute ST-elevation myocardial infarction. Cells were stained against: 1) hematopoeitic lineges markers (Lin) and CD45 to be detected in one channel (FITC, green), 2) marker of pluripotency Oct4 (PE, yellow) and 3) stem cell antigen CD34 (PE-Cy5, cyan). Nuclei are stained with 7-aminoactinomycin D (7-AAD, red). Scales represents 10 μm. VSELs are identified based on the lack of expression of both Lin and CD45 markers and positive staining for CD34 antigen and nuclear appearence of Oct-4 transcription factor (**Upper Panel**). HSCs are identified as cell expressing Lin and/or CD45 markers as well as CD34 antigen; however, negative for Oct-4 (**Lower Panel**).

The above evidence suggests an innate, yet poorly understood, reparatory mechanism that culminates in the mobilization of BMSPCs following acute myocardial injury. However, the mobilization of pluripotent stem cells, which carry higher regenerative potential in IHD, has not ben examined before. Future studies aiming at selective mobilization of PSCs rather than the non-selective actions of agents such as granulocyte colony stimulating factor (G-CSF) may prove beneficial in the field of myocardial regeneration. Furthermore, the mechanisms of homing of mobilized cells are poorly understood and represent one of the cornerstones of future BM-based myocardial regenerative strategies.

Sphingolipids: background and signaling

The above findings directed the investigation towards proteolysis-resistant sphingolipids, specifically sphingophospholipids (sphingosine 1-phosphate and ceramide 1-phosphate), which were shown to be potent chemoattractants for BMSPCs. Sphingolipids were first identified by a German neurochemist, J.L.W. Thudichum in ethanolic brain extracts in the 1870s and he named them after a mythological creature, the Sphinx (58). In Greek mythology, the Sphinx is a treacherous and merciless human-animal hybrid: those who cannot answer her riddle are eaten whole and raw. Initially, sphingolipids were believed to be sheathing nerves, and the interest in their research remained confined to a small group of scientists. As the evidence for pathophysiological importance of sphingolipids grew, so did their research field. As of today sphingolipids are shown to be involved in a wide variety of biological responses in a diversity of cell

types including stimulation of cell proliferation, inhibition of apoptosis and regulation of cell shape and cell motility (59-61).

Sphingolipids are important structural components of cell membranes and are derived from ceramide, the proverbial 'core' of sphingolipid metabolism. Ceramide can be deacylated to sphingosine which is then phosphorylated by sphingosine kinases (SPHK1 or SPHK2) to yield sphingosine 1-phosphate (S1P) (**Figure 3**). Both transcripts of SPHK1 and SPHK2 are subject to alternative splicing resulting in multiple isoforms for each kinase (62). Transgenic mouse studies have demonstrated partial redundancy of SPHK1 and SPHK2 since $SPHK1^{-/-}$ or $SPHK2^{-/-}$ mice were phenotypically normal while elimination of both genes resulted in embryonic death (63, 64) indicating that S1P is produced exclusively by SPHKs in vivo. Ceramide 1-phosphate (C1P) can be generated by phosphorylation of ceramide (N-acyl sphingosine) by ceramide kinase (65). Both S1P and C1P have limited half-lives and their levels are kept in check by numerous enzymes. S1P is irreversibly degraded by S1P lyase, and is also regulated by lipid phosphate phosphatases (LPP1–3) and S1P-specific phosphatases (SPP1 and SPP2) (66-69), C1P is regulated by LPP1–3 (66, 68). The major source of plasma S1P are red blood cells, activated platelets, albumin, high-density lipoproteins, and extracellular SPHK1 derived from vascular endothelial cells (70, 71); while the primary contribution to C1P plasma levels comes from intracellular C1P which has been released or 'leaked' from damaged cells (72).

Upon their release, both S1P and C1P interact with a variety of G protein–coupled seven-transmembrane receptors. There are 5 S1P receptor subtypes (S1PR1-5) that are widely expressed throughout mammalian tissues (**Figure 3**). S1PR4 and S1PR5 are expressed and function in the immune and nervous system, respectively, S1PR1-3 are most abundant throughout the cardiovascular system and are expressed on BMSPCs. S1PR1 is coupled exclusively via G_i to Ras-MAP kinase, phosphoinositide (PI) 3-kinase-Akt pathway and phospholipase C pathway. S1PR2 and S1PR3 are coupled to multiple G proteins, such as G_q, $G_{12/13}$ and G_i to activate phospholipase C pathway and Rho pathway (59-61). The signaling cascade activated by S1P binding to either S1PR1 or S1PR3 is responsible for HSPC migration (73, 74). Activation of S1PR2, however, yields an opposite effect - negatively regulating HSPC mobilization (75). While the receptor for C1P is yet to be identified, its signaling is sensitive to pertussis toxin, thereby implicating a G_i protein coupled receptor (76, 77).

Sphingosine 1-phosphate is a potent BMSPCs chemoattractant

Once S1P receptors were discovered on BMSPCs, they were immediately characterized as G protein–coupled seven-transmembrane receptor thereby placing them in the same class as chemokine receptors. This observation raised one important question: can S1P act as a direct chemoattractant for BMSPCs? Initially, Seitz et al. demonstrated a dose-dependent chemotactic effect of S1P on human HSPCs in a modified Boyden chamber assay (57).

Figure 3.

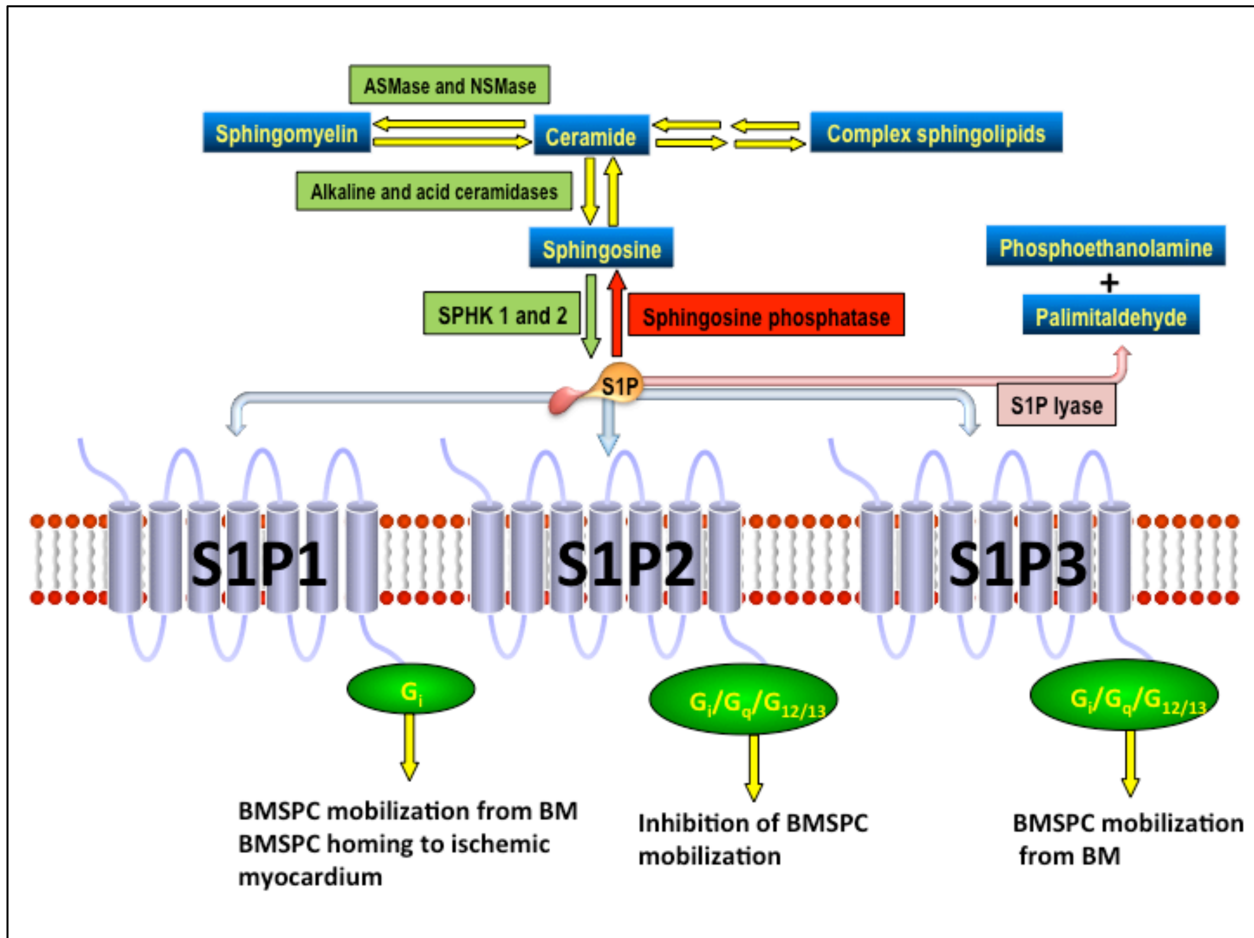

Figure 3. Sphingosine 1-phosphate (S1P) metabolism and signaling in bone marrow stem and progenitor cells (BMSPCs). Interconversion of membrane sphingolipids and final phosphorylation of sphingosine by SPHKs results in formation of S1P which signals through S1PR1, S1PR2 and S1PR3 receptors in the BMSPCs. These three receptors activate a distinct set of pathways through G_i, G_q, or $G_{12/13}$ proteins which results in BMSPCs mobilization from the bone marrow niches (S1PR1 and S1PR3); inhibition of BMSPC mobilization from the bone marrow (S1PR2); and BMSPC homing to ischemic myocardium (S1PR1).

It is possible that polarizing doses of S1P promote signaling through the S1PR2 receptor, which in contrast to S1PR1 inhibits HSPC chemotaxis (56). Subsequent studies established that the gradient of S1P between BM and PB is a major determining factor in HSPCs egress. While SDF-1 still has a significant role in HSPCs mobilization, it was demonstrated that plasma derived from normal and mobilized PB strongly chemoattracts murine BM HSPCs independent of plasma SDF-1 levels (56). This was especially evident when removal of lipids from plasma by charcoal stripping abolished HSPCs chemotaxis but did not affect responsiveness towards SDF-1 (56). Ratajczak et al. further showed that steady state S1P plasma levels create a gradient favoring HSPCs egress from the BM. As previously described, HSPCs are actively retained in BM via SDF-1-CXCR4 and VLA4-VCAM1 interactions. Ratajczak et al. corroborated the significance of S1P in HSPCs chemotaxis by demonstrating that disruption of these interactions via CXCR4 antagonist AMD3100 or triggering a proteolytic environment in the BM would release HSPCs form their niches and therefore free them to follow the bioactive lipids' gradient to PB. Furthermore, Ratajczak et al showed that a robust innate immune response during G-CSF mobilization is responsible for increased plasma S1P levels. G-CSF is currently the most frequently used mobilizing agent that efficiently mobilizes BMSPCs after a few consecutive daily injections (78).

Figure 4.

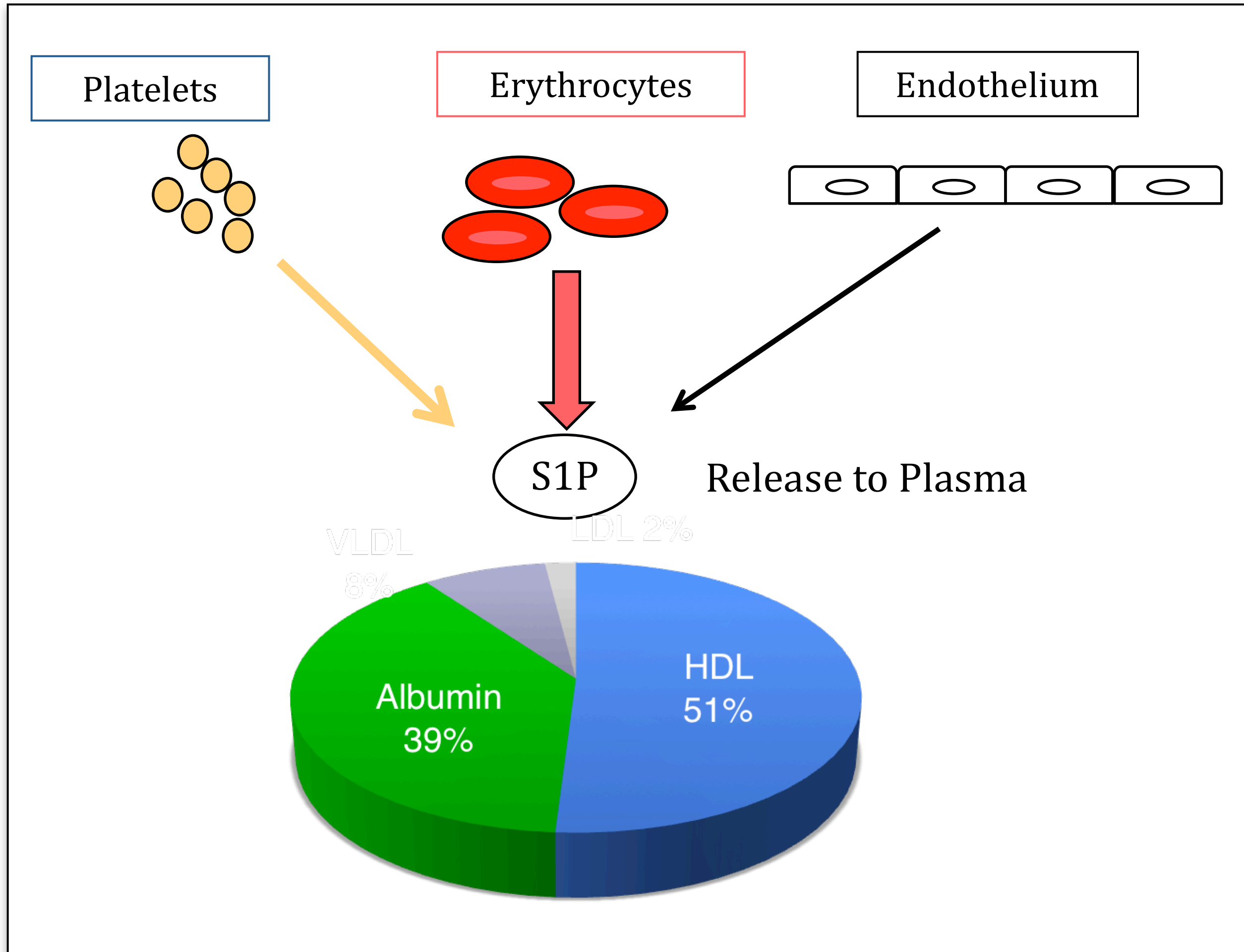

Figure 4. Sources of S1P in the plasma and its distribution in the peripheral blood. Sphingosine-1 phosphate is abundant in the circulation with concentrations approximately 25 times that in the tissues. Most of the S1P is stored in red blood cells, platelets and endothelial cells. S1P in the plasma is 90% bound to HDL and albumin with small portion present in the free form. The free form is presumed to be biologically active while the bound part of S1P is biologically inactive.

It has been established that G-CSF triggers complement complex activation which stimulates granulocytes to release proteolytic enzymes thereby perturbing SDF-1-CXCR4/VLA-4-VCAM1 interactions in BM niches and facilitating HSPCs release (79). Remarkably, the lasting effect of G-CSF promotes complement activation and formation of the membrane attack complex (MAC) that was shown to interact with erythrocytes (80). While erythrocytes serve as the major reservoir of S1P in the PB (81, 82), they are highly protected from MAC by CD59 and decay-accelerating factor (DAF) receptors (83). However, Ratajczak et al. demonstrated that expression of these receptors on erythrocytes does not give complete protection from activated MAC since G-CSF-induced MAC exposure resulted in plasma S1P levels sufficient for HSPCs egress (56).

While it has been established that S1P is responsible for HSPC trafficking, the mechanism to explain this regulation is still under investigation. Recent evidence suggests that SDF-1 and S1P work synergistically to facilitate migration of primitive murine hematopoietic progenitor cells out of the BM (84). Further in vitro studies on immature human CD34+ cells demonstrated that S1PR1 upregulation decreases their chemotactic activity towards SDF-1 due to reduced cell surface expression of CXCR4 suggesting a potential interaction between S1P and SDF-1 (85). These observations were recently corroborated by Golan et al showing that short-term inhibition of S1P/S1PR1 axis during steady state conditions or during CXCR4 inhibition (via AMD3100 administration) caused reduction of SDF-1 in the plasma (86). Interestingly, generation of reactive

oxygen species (ROS) via S1PR1 signaling were also implicated in HSPCs mobilization through the release of SDF-1 (87). Since previous studies showed that ROS inhibition reduces SDF-1 secretion during AMD3100-induced mobilization (88) it was hypothesized that ROS signaling might also contribute to SDF-1 secretion. Indeed, it was demonstrated that ROS signaling induced SDF-1 secretion thereby facilitating HSPCs egress (86).

S1P-SDF-1 interaction in HSPCs egress was further demonstrated with the help of FTY720, a potent S1PR1 desensitizing agent which causes S1P receptor internalization (89). Interestingly, administration of FTY720 for 24 hours resulted in increased plasma SDF-1 levels but had no effect on HSPCs egress. FTY720 treatment did reduce BM ROS signaling, due to S1PR1 downregulation, again pointing out the requirement of S1PR1 signaling in HSPC egress. Furthermore, mice that were treated with BM-specific S1P lyase inhibitor 4-deoxypyrodixne (DOP) (90) had increased BM ROS levels and decreased HSPC egress (86). Together these observations suggest that the increased concentrations of S1P and SDF-1 in the BM negatively affect HSPC egress, further highlighting the fact that both S1P and SDF-1 levels must be tightly regulated for balanced HSPCs mobilization. However, the role of S1P/S1PR1 axis in the mobilization of non-HSCs is poorly understood and more importantly, the role of this axis in BMSPCs mobilization during acute myocardial ischemic injury has not been explored.

Therapeutic mobilization of BM-derived stem cells in myocardial regeneration

Hematologists have used the concept of BM-derived stem cell mobilization using pharmacological agents such as G-CSF for a long time. Based on the available clinical experience and safety profile of these therapies, pharmacological stem cell mobilization in the setting of AMI has gained increasing enthusiasm. Multiple studies utilizing BM-derived stem cell mobilization in AMI have been conducted and demonstrated various degrees of success (91-98). Similar to BMC transplantation studies, the heterogeneous methodologies of the included studies confused the interpretations of the biological effects. The overall lack of efficacy with G-CSF BMC mobilization in the setting of acute myocardial infarction is somewhat incongruent with the salutary effects of BMC transplantation in humans and G-CSF therapy in animal models for myocardial regeneration.

The largest study utilizing G-CSF in the setting of acute myocardial infarction was the REVIVAL-2 trial that included 114 patients (99). The study randomized AMI patients to 10 μg/kg of G-CSF vs. placebo and left ventricular functional parameters were assessed using cardiac MRI (CMR). The study demonstrated no significant difference in the tested parameters between patients treated with G-CSF or placebo. However, baseline characteristics in the study population showed normal or near normal LV function and therefore the expected benefit is minimal. Patient selection was a methodological flaw that plagued some of the studies that utilized G-CSF. Indeed, with careful examination of the

available literature, patients with reduced LV function at baseline as well as those treated within the first 36 hours following AMI benefited the most (91, 100). On the other hand, safety concerns regarding a potentially increasing evidence of instent restenosis (101) and recurrent ischemia (102) have halted subsequent clinical trials. However, it is important to note that these safety concerns were not confirmed in large studies (99) or in the cumulative meta-analyses (91).

Beyond the methodological flaws encountered in human trials, this lack of efficacy can be explained by multiple factors. While G-CSF and similar therapies mobilize a wide array of BMSPCs in the peripheral blood, homing factors may not be sufficient to guide them to the myocardial peri-infarct zone. Indeed, the homing of c-Kit+ cells to the infarcted myocardium improved when G-CSF therapy was combined with local administration of SDF-1 (103). The myocardial levels of chemoattractants peak within 24-72 hours following injury (104-106) and therefore delayed therapy in some human trials may have missed the homing window to the infarct zone (91). Moreover, different cytokines are known to preferentially mobilize somewhat different subsets of BMCs (107, 108). Future studies investigating the characteristics of G-CSF-mobilized cells will be necessary to glean additional mechanistic insights in this regard.

Recently, a combined approach with stem cell mobilization and enhanced homing using therapies known to increase local SDF-1 or CXCR4 antagonists have been proposed and is currently being tested (109, 110). Going forward, the beneficial effects of BM-derived stem cell mobilization may be augmented by selective mobilization of undifferentiated BMSCs rather than differentiated

inflammatory cells. It is also important to remember that some of the G-CSF arbitrated effects can be mediated by its direct effect on cardiomyocytes which are known to express G-CSF receptor (111). G-CSF therapy may be inducing the proliferation of cardiomyocytes or the differentiation of resident cardiac stem cells. On a similar note, G-CSF therapy upregulates Akt (112) and may result in reducing apoptosis of ischemic cardiomyocytes if utilized early following the acute event.

BM-derived stem cell transplantation for myocardial repair

The use of BM-derived cells in myocardial regeneration has moved rapidly from the basic research lab to the clinical arena. The results from these studies varied widely probably secondary to the heterogeneous methodologies used with an overall marginal benefit with BM-derived cell transplantation compared to placebo. The underling mechanisms leading to the beneficial effect of transplanted BMCs are unclear. The observed benefits of BMCs transplantation is out of proportion with the observed rates of newly formed cardiomyocytes from BMCs' origin (113). Indeed, recent evidence suggests a primarily paracrine effect of BM-derived stem cells following their transplantation by recruiting and stimulating resident cardiac stem cells (CSCs) (114). Furthermore, human purified CD34+ cells are a source of several growth factors including VEGF, cytokines and chemokines that may prevent apoptosis of dying cardiomyocytes and promote angiogenesis in damaged myocardium (115). Cell membrane derived microvesicles or exosomes that are enriched in S1P may contribute to

regeneration of myocardium and its re-vascularization (116). Hence, transplanted CD34+ cells may contribute to regeneration of damaged heart by paracrine signals and released microvesicals (117) and was recently confirmed by others (118).

Multiple studies have utilized BM derived cells (BMCs) for myocardial regeneration. The majority of these studies, however, utilized unselected populations of BMCs and these studies provide the longest follow-up of up to 5 years (119-122). The first large study that utilized unselected BM mononuclear cells (BMMNCs) is the REPAIR-AMI trial that included more than 200 patients with acute STEMI. The study randomized 204 patients to BMMNCs or placebo and subjects were followed for 4 months to assess the recovery of LV functional parameters and clinical endpoints at 1 year. At 4 months follow-up, patients treated with BM-MNCs demonstrated significantly better recovery of cardiac functional parameters tested such as global LV ejection fraction (LVEF), regional wall motion at the infarcted zone and LV end systolic volume (LVESV). Clinical follow-up demonstrated significant reduction of the prespecified combined endpoint of death, recurrent myocardial infarction, re-hospitalization for heart failure and revascularization at 1 year. Patients with reduced cardiac function at baseline (left ventricular ejection fraction < 49%) and those treated at or after 4 days following the acute event benefited most from BM therapy.

Concurrently, multiple studies have examined therapy with BM-derived cells and they ranged widely in their methodologies regarding the cell type used from unfractionated BM cells to highly selected populations; the timing following

AMI which ranged from 1 day to few years; the route of delivery of cells; and the method of evaluating the LV function. Suffice it to say, the findings of the REPAIR-AMI study were negated with others who failed to demonstrate the same beneficial effects that could be related to the methodological differences noted above (123, 124). Nonetheless, the overall collective results of these small and methodologically heterogeneous studies demonstrate benefit with BM derived cells in patients when used in either acute MI or chronic ischemic heart disease (6, 125). Recent long-term follow-up studies demonstrated mixed results regarding the sustainability of the BMCs treatment benefit with 'catch-up' of the placebo treated patients (119, 126, 127). In patients with chronic ischemic heart disease (IHD), the evidence to support BMC therapy is less robust despite promising small studies (119, 122). Similarly, smaller studies have demonstrated the anti-anginal effects of BMC in patients with non-revascularizable severe coronary artery disease (128).

Selected BM-derived stem cell subpopulations represent an attractive substrate for cellular therapies since they lack the inflammatory cells, which contribute to the ongoing inflammatory response at the site of myocardial infarction, contained in the unselected BMCs populations. Furthermore, highly purified stem cell populations are more likely to induce myocardial regeneration through paracrine effects or by directly differentiating into cardiomyocytes. The largest study utilizing selected BM-derived stem cell population is the REGENT study which compared selected to non-selected populations of BMCs in patients with acute ischemic heart disease and reduced LV function at baseline (129).

While there were no significant differences between the groups, patients treated with selected CD34+/CXCR4+ cells showed trends of improvement in LV function when compared to controls.

Other studies have used a similar approach utilizing more primitive populations of BMSCs such as CD133+ cells with reported improvement of LV function and perfusion. In patients with severe and non-revascularizable coronary artery disease, Losordo and colleagues demonstrated the beneficial and sustained effects of BM-derived CD34+ cells (128). BM-derived mesenchymal stem cells are prominent candidates for myocardial regenerative therapies due to their anti-inflammatory, anti-apoptotic effects and immunosuppressive properties. Therefore, multiple studies have examined their role in patients with acute (46, 130) and chronic ischemic heart disease. Overall, the results of these small studies suggest a beneficial role of BM-MSCs particularly with high dose (6 x 10^{10}) cell therapy. Other studies utilizing primitive populations of BM-SPCs such as CD133+ cells also have reported improvement of LV function and perfusion (131, 132).

It is important to note that the above mentioned trials focused on surrogate endpoints rather than patient-important endpoints such as mortality, need for repeat revascularization, recurrent MI or hospitalization for congestive heart failure. While surrogate endpoints are important for mechanistic studies, patient-important endpoints are quintessential for a therapy to achieve mainstream status and more work is needed in this growing area of cell based myocardial regeneration trials. Long-term follow-up studies demonstrated a ‘catch-up

phenomenon' of the placebo treated patients, thus leading to mixed results regarding the sustainability of the BMCs treatment benefit (126, 127, 133). The benefit of BMC therapy is less robust among patients with chronic ischemic heart disease (IHD) (119, 134). Similarly, smaller studies have demonstrated the anti-anginal effects of BMCs in patients with non-revascularizable severe coronary artery disease (128, 135). Nevertheless and despite the disparity in the methodologies of the conducted studies, the overall collective effect of BMCs' transplantation suggests a small yet statistically significant benefit in myocardial regeneration (6, 125).

However, homing of the transplanted stem cell populations remains a limiting factor. Overall, most of the studies demonstrate very limited retention of the transplanted cells with rates of 2.5-10%. This important limitation reduces the effect of the transplanted cells and renders the therapy largely ineffective. Going forward, therapies aiming at improving the homing and retention of the transplanted cells are needed to achieve clinically relevant results.

Key Questions

The mobilization of committed BM-derived stem cells in tissue injury especially following acute myocardial infarction is well documented in the literature. The underlying mechanisms of this mobilization are poorly understood and some of the well-established chemokines are degraded at sites of myocardial infarction, thus, reducing their contribution to stem cell mobilization and homing. Therefore, other and poorly understood redundant mechanisms may exist. Bioactive lipids such as sphingosine-1 phosphate play an important role in the trafficking of mature lymphocytes between lymph nodes and peripheral blood. Their role in the mobilization of hematopoietic stem cells is increasingly appreciated. However, the role of bioactive lipids in stem cell mobilization during tissue injury is poorly understood. We hypothesize that pluripotent stem cells are mobilized to the injured myocardium during acute ischemic injury and that bioactive lipids play an important role in this mobilization and homing. We utilized a multidisciplinary approach to examine some of the different pathways orchestrating the mobilization and homing of BM stem cells during and after myocardial ischemia.

Our hypotheses will be tested in a well-established clinical/basic models using a broad multidisciplinary approach that will encompass diverse disciplines and techniques (cell biology, molecular biology, immunocytochemistry, flow cytometry, and protein chemistry on the basic research side). The clinical data will be collected via clinical follow up. Circulating primitive BMSCs, identified

based on surface and nuclear markers; and cellular morphology, will be isolated using fluorescent-activated cell sorting (FACS) and examined both on the nuclear and protein levels.

Examine the mobilization of pluripotent stem cells in acute myocardial ischemia and the clinical factors influencing this mobilization.

Previous studies have demonstrated the mobilization of committed BM-derived stem cells in myocardial ischemia. However, the mobilization of more primitive populations of BM-derived stem cells, which carry more regenerative potential, in the setting of tissue injury is poorly understood (136). PSCs express chemokine receptors and can respond to chemokine gradients. Moreover, the clinical factors that guide their mobilization and subsequent homing are not clear. We explored the mobilization of pluripotent cells at various time points following acute myocardial injury. We recruited a large sample size to enhance our statistical power which allowed us examine the different clinical factors that may influence this mobilization.

Examine the role of innate immunity and bioactive lipids in stem cell mobilization after acute myocardial infarction.

The complement cascade is activated in the setting of acute myocardial infarction. The terminal products of the complement cascade activation have been shown to play an important role in the mobilization and homing of hematopoietic stem cells from and to their BM niches (137-142). Furthermore,

the terminal products of the complement cascade activation and peripheral blood cells lead to the release of bioactive lipids and can potentially further contribute to the BMSPC mobilization. These pathways, which are activated in AMI, have not been explored and could present multiple therapeutic targets for myocardial regenerative therapies. We examined the changes in plasma bioactive lipids and their receptor expression in the setting of acute myocardial injury. We also examined the role of S1P receptor expression in guiding the mobilization and homing of BMSPCs.

Explore alternative homing mechanisms for mobilized bone marrow-derived stem cells to the infarcted myocardium.

There is sufficient data that negates an exclusive role of traditional chemokines in the homing of BMSPCs to injured myocardium in AMI. Bioactive lipids such as S1P and C1P contribute to the homing of HSCs to the myocardium after BM radiation, an environment that is similar to the infarcted myocardium. The literature also suggests a crucial role for the immune system in homing of BM-derived HSCs. Cathelicidins are important highly conserved antimicrobial proteins that are ubiquitous to multiple tissues. Cathelicidins have been shown to improve the response to low, yet physiological levels, of SDF-1 similar to levels seen in the irradiated BM or the infarcted myocardium. We examined the role of these and other pathways in the mobilization and more importantly homing of BMSPCs in the setting of acute myocardial injury.

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Chapter 2

RESULTS

Stem cell mobilization and homing in ischemic heart disease (12)

BM derived tissue committed stem cells play an important role in cardiomyocyte chimerism (8). The literature is scarce on the mobilization of primitive and pluripotent stem cells in tissue injury. We are particularly interested in populations of BM-derived stem cells that are enriched in very small embryonic like stem cells (VSELs) such as Lin-/CD45-/CXCR4+, Lin-/CD45-/CD133+ and Lin-/CD45-/CD34+ cells. Moreover, the pathways involved in stem cell mobilization following ischemic injury and the clinical factors that influence this mobilization are poorly understood. We examined the mobilization of BM derived pluripotent stem cells in different scenarios of myocardial ischemia using a multidisciplinary approach.

Traditional chemokines such as the SDF-1/CXCR4 axis are thought to be the universal mobilizing pathways for BM-derived stem cells. Using flow cytometry, we identified high numbers of circulating BM-derived stem cell with pluripotent surface markers during acute myocardial ischemia. The pluripotent nature of these cells was confirmed by imaging techniques such as ImageStream system and confocal microscopy. Gene expression for primitive, cardiac and endothelial genes was increased in circulating peripheral blood cells during the

early phases after myocardial injury. The increased gene expression was even more upregulated in enriched peripheral blood cells such as CD34+ cells.

We explored the different pathways that could potentially contribute to the mobilization of BM stem cells in cardiac ischemic injury. The literature suggests a limited role of traditional chemokines, such as SDF-1/CXCR4 axis, in stem cell mobilization and homing to the ischemic myocardium. The limited contribution of SDF-1 to myocardial regeneration may be explained by its active degradation at the sites of inflammation and myocardial infarction by metalloproteinases (50-52). The acute inflammatory changes accompanying ischemic cardiac injury result in the upregulation of metalloproteinases and proteases at the sites of myocardial infraction, a process that starts as early as few hours after the event and lasts for a few weeks (143). We did not find correlation between the levels of chemokines and mobilized BM stem cells in the peripheral blood.

The goal of these initial studies was to determine the mobilization of BM-derived pluripotent stem cells and the dynamics and factors of this mobilization. We enrolled a study population that consisted of 100 patients with acute ST-elevation myocardial infarction (STEMI) and age- and sex-matched subjects to the study population into the control (CTRL) group. The CTRL group is asymptomatic with no history of CAD but similar comorbidities to the study population (**Table 1**). Patients with STEMI were referred within 12 hours of symptom onset for primary percutaneous coronary intervention (PCI). Patients were excluded if they had a systemic inflammatory process, cancer, recent motor

Table 1. Demographic, clinical and laboratory characteristics of study population and controls.

	Controls (n = 12)	STEMI (n = 30)
Age (years)	47±9	61±11
Female	50%	23%
HTN	25%	72%
DM	8%	19%
Hyperlipidemia	25%	93%
Smoking	8%	60%
Peak Troponin	NA	62±39
Peak CK	NA	2877±3240
Peak CK-MB	NA	210±114

vehicle accident, recent surgery, active infection, history of MI or revascularization (coronary artery bypass graft, PCI), unsuccessful revascularization, or onset of the symptoms >12 hours. Peripheral blood (PB) samples were obtained at presentation in all patients. In STEMI patients, PB samples were collected at presentation [(BSL) on average 4.5±3.2 hours after the onset of chest pain] and 12, 24, 48 and 72 hours after PCI. The study protocol complies with the Declaration of Helsinki and was approved by the institutional Ethics Committee. All patients provided written informed consent.

Mobilization of pluripotent Oct-4+, SSEA4+ cells and VSELs in patients with myocardial ischemia

The mobilization of BM derived committed stem cells have been previously described as detailed above. However, these committed stem cells have limited regenerative potential and the mobilization of more primitive and pluripotent populations is poorly understood. Therefore, we went on to examine the mobilization of BMSC-populations that exhibit pluripotent features such as small cell size, large nucleus, and the expression of pluripotent markers such as the surface marker SSEA-4 and the transcription factor Oct4. In myocardial ischemia, the absolute circulating number of Oct4+ VSELs, as analyzed by Image Stream system (ISS), was significantly higher than controls. Representative images of Oct-4+ primitive cells obtained by ISS are shown in **Figure 5A**. In matched controls, the number of circulating VSELs was low (0.5 ± 0.1 cells/µl of PB). The absolute number of Lin-/CD45-/CD34+/Oct4+ cells was

higher among patients with STEMI (0.5 ± 0.1 vs. 2.2 ± 0.4 cells/μl of PB in controls vs. peak STEMI patients respectively, $P < 0.05$) (**Figure 5B**). In acute STEMI the number of Oct4+ VSELs reached peak at baseline (2.2 ± 0.4 cells/μl of PB) and decreased afterwards reaching a nadir of 0.3±0.1 cells/μl of PB at 48 hours (**Figure 5B**). Based on the unique capabilities of ISS technology, we were able to quantify PSCs accurately by distinguishing real intranuclear Oct-4 expression from false positives events. A similar pattern of mobilization was noted in the absolute numbers of circulating Lin-/CD45-/SSEA-4+ non-hematopoietic PSCs assessed by conventional flow cytometry (0.5 ± 0.2 vs. 1.1 ± 0.2 cells/μl of PB in controls vs. peak STEMI patients respectively, $P < 0.05$). The same pattern was also noted with Lin-/CD45+/SSEA4+ cells (0.9 ± 0.5 vs. 1.7 ± 0.7 cells/μl of PB in controls vs. peak STEMI patients respectively, $P < 0.05$) (**Figure 5C and 5D**).

Mobilization of non-hematopoietic stem cells (non-HSCs) in patients with acute myocardial ischemia

Following the documentation of pluripotent stem cell mobilization, we directed our efforts to examining the mobilization of BM-derived stem cell populations enriched in VSELs which have great regenerative potential based on their pluripotent features and their ability to differentiate into cardiomyocytes and endothelial cells in vitro and in vivo. Mobilization of Lin-/CD45-/CD133+, Lin-/CD45-/CD34+, and Lin-/CD45-/CXCR4+ stem cells enriched in VSELs was highest within the first 6 hours after presentation in STEMI patients (**Figure 6**).

Figure 5.

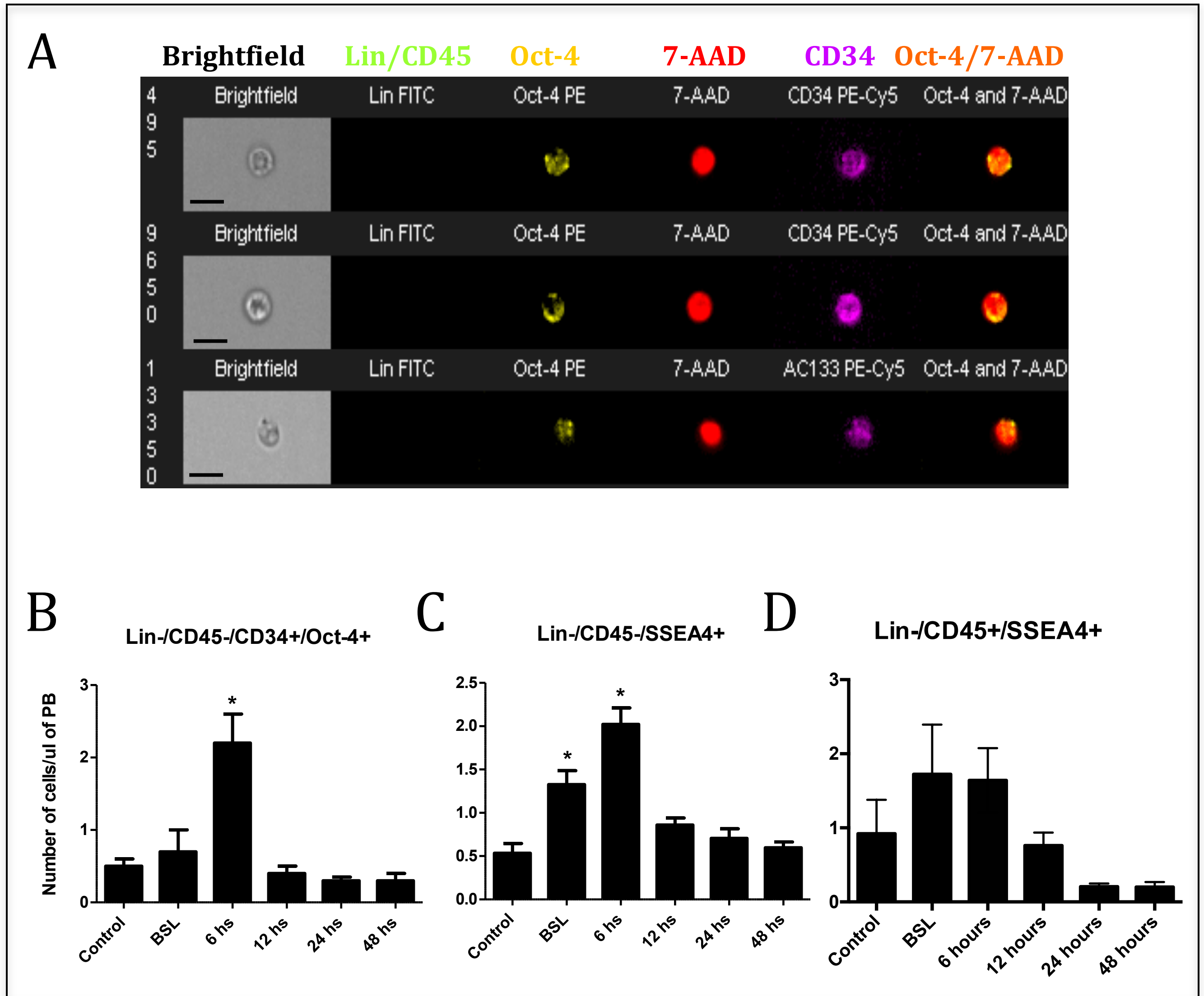

Figure 5. Mobilizations of pluripotent VSELs in ischemic heart disease patients and controls. Peripheral blood cells were isolated from STEMI patients at the pre-defined time points and total nucleated cells (TNCs) were isolated following lysing of red blood cells using Pharmlyse buffer. TNCs were stained against: 1) hematopoeitic lineages markers (Lin) and CD45 to be detected in one channel (FITC, green), 2) marker of pluripotency Oct4 (PE, yellow) and 3) stem cell antigen CD34 (PE-Cy5, cyan). Nuclei are stained with 7-aminoactinomycin D (7-AAD, red). Scales represents 10 μm. VSELs are identified based on the lack of expression of both Lin and CD45 markers and positive staining for CD34 antigen and nuclear appearence of Oct-4 transcription factor (**Panel A**). TNCs were also analyzed using flow cytometry to quantify the number of circulating Lineage (FITC), CD45 (PE-Cy7), CD34 (APC), SSEA4 (PE), and Oct4 (Alexaflour 780) cells. Lin-/CD45-/CD34+/Oct4+ cells (**Panel B**), Lin-/CD45-/SSEA4+ cells (**Panel C**), and Lin-/CD45+/SSEA4+ (**Panel D**) peaked in the early phase after STEMI. Data were analyzed using one-way ANOVA. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett as appropriate with control samples as the control category. (* $P < 0.05$ as compared to controls). BSL, baseline; PB, peripheral blood.

The absolute numbers of all three populations were significantly higher among STEMI patients at the time of presentation (BSL) and after 6 hours from revascularization as compared to control subjects (3-8 fold increase as compared to controls; $P < 0.01$). The consistent peak of non-HSCs mobilization point to endogenous mechanisms which are responsible for orchestrating this mobilization. This is the first report of non-HSCs enriched in VSELs in humans following myocardial ischemia.

Mobilization of hematopoietic stem cells (HSCs) in patients with myocardial ischemia

The literature suggests an important role HSCs in the post-ischemic myocardial repair. HSCs and endothelial progenitor cells (EPCs) have a common origin from the hemangioblast and therefore can be valuable in myocardial regeneration. Our flow cytometry analyses detected significant mobilization of Lin-/CD45+/CD133+, Lin-/CD45+/CD34+, and Lin-/CD45+/CXCR4+ HSCs in patients with myocardial ischemia when compared to controls (**Figure 7**). Lin-/CD45+/CXCR4+ but not Lin-/CD45+/CD133+ and Lin-/CD45+/CD34+ cells were significantly higher in STEMI patients as compared to other ischemic heart patients (2-4 fold increase; $P < 0.05$). The higher numbers of mobilized Lin-/CD45+/CXCR4+ cells early in STEMI patients can potentially be a reflection of the active recruitment by the infarcted myocardium via the SDF-1/CXCR4 axis. Similar temporal trends were noted with HSCs suggesting the involvement of similar mobilization pathways.

Figure 6.

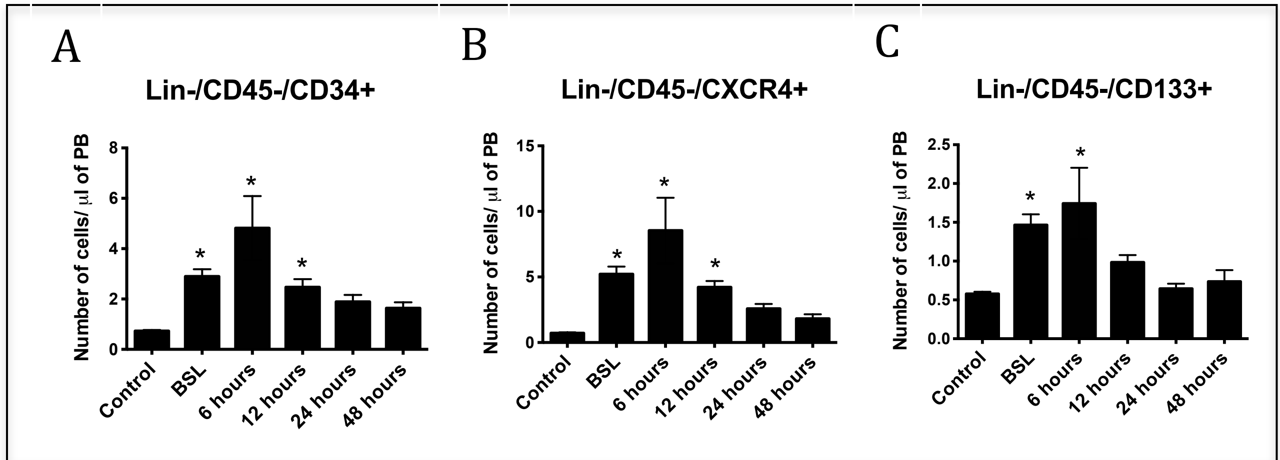

Figure 6. Mobilizations of non-hematopoietic BMSPCs enriched in very small embryonic like stem cell populations in ischemic heart disease patients and controls. Peripheral blood cells were isolated from STEMI patients at the pre-defined time-points and total nucleated cells (TNCs) were isolated following lysing of red blood cells using Pharmlyse buffer. TNCs were stained against: hematopoeitic lineages markers (Lin) (FITC), CD45 (PE-Cy7), CD34 (PE-Cy5), CD133 (APC), and CXCR4 (bioton-streptavidin-Alexaflour780). Populations enriched in VSELs are defined as events negative for lineage and CD45; and positive for CD34 (**Panel A)**, CXCR4 (**Panel B)** and CD133 (**Panel C)**. The figure represents bar graphs showing the absolute numbers of circulating BMSPC populations enriched in non-HSCs and VSELs in the peripheral blood of ischemic heart disease patients and controls; showing a peak mobilization early in STEMI patients. Data were analyzed using one-way ANOVA. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett test as appropriate with control samples as the control category. (* $P < 0.05$ as compared to controls). BSL, baseline; PB, peripheral blood.

Figure 7.

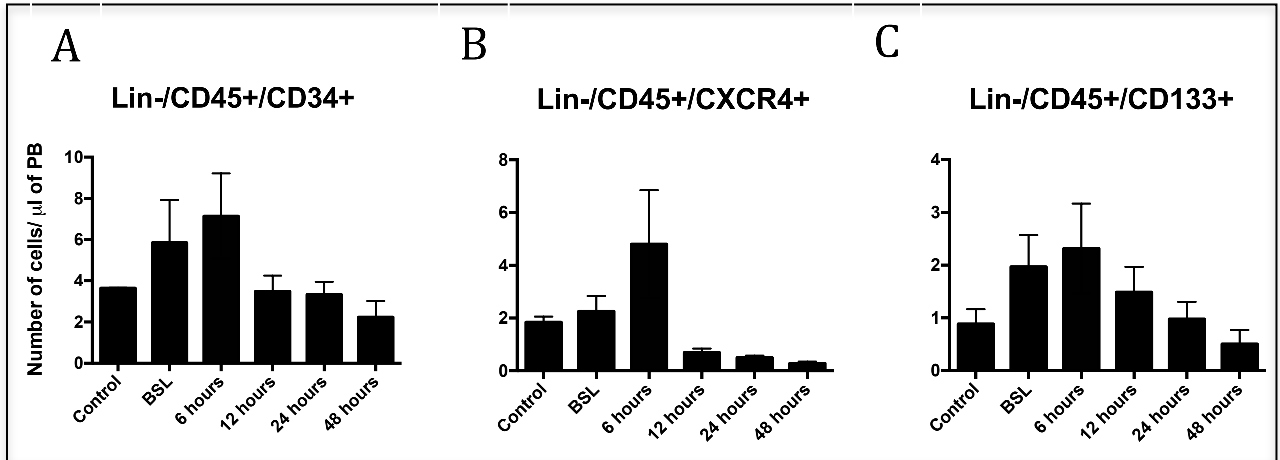

Figure 7. Mobilizations of BMSPCs enriched in hematopoietic stem cell populations in ischemic heart disease patients and controls. Peripheral blood cells were isolated from STEMI patients at the pre-defined time-points and total nucleated cells (TNCs) were isolated following lysing of red blood cells using Pharmlyse buffer. TNCs were stained against: hematopoeitic lineages markers (Lin) (FITC), CD45 (PE-Cy7), CD34 (PE-Cy5), CD133 (APC), and CXCR4 (bioton-streptavidin-Alexaflour780). Populations enriched in HSCs are defined as events negative for lineage markers, positive for CD45 and positive for CD34 (**Panel A)**, CXCR4 (**Panel B)** and CD133 (**Panel C)**. The figure represents bar graphs showing the absolute numbers of circulating BMSPC populations enriched in HSCs in the peripheral blood of ischemic heart disease patients and controls; showing a peak mobilization early in STEMI patients. Data were analyzed using one-way ANOVA. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett or Dunn tests as appropriate with control samples as the control category. (* $P < 0.05$ as compared to controls). BSL, baseline; PB, peripheral blood.

Expression of pluripotent, cardiac and endothelial markers in circulating cells by RT-PCR

BM stem cells mobilized in the setting of acute myocardial ischemia represent a selected population that is destined to myocardial repair (106). Indeed, the expression of pluripotent, cardiac and endothelial markers by PB total nucleated cells (TNCs) was significantly higher in STEMI patients when compared to control subjects (**Figure 8**). The mRNA level of these genes peaked in STEMI patients at the time of presentation (BSL) and paralleled the peak mobilization of pluripotent stem cells. The fold change is as high as 15-fold difference between the early phase after STEMI compared to controls in primitive (Oct4 and Nanog) and cardiac (Nkx-2.5 and GATA4) markers.

Recently, Wojakowski *et al* demonstrated significant expression of these markers in sorted VSELs (13). In agreement with Wojakowski's data, the expression of pluripotent, cardiac and endothelial markers was significantly higher in sorted PB-derived CD34+ cells, isolated using magnetic beads, as compared to unfractionated PB-derived TNCs (**Figure 9**). This enrichment was confined to CD34+ cells sorted early after the acute injury. Cpompared to the 5-15-fold increase in primitive and cardiac genes, we noted 40-60-fold increase in the expression of primitive, cardiac and endothelial genes in the CD34+ subset. This data indicates that the fraction of CD34+ cells is enriched in PSCs and VSELs. The use of beads-isolated CD34+ population can provide a clinically feasible strategy for therapeutic use as it provides reduced and fast *ex vivo* processing.

Figure 8.

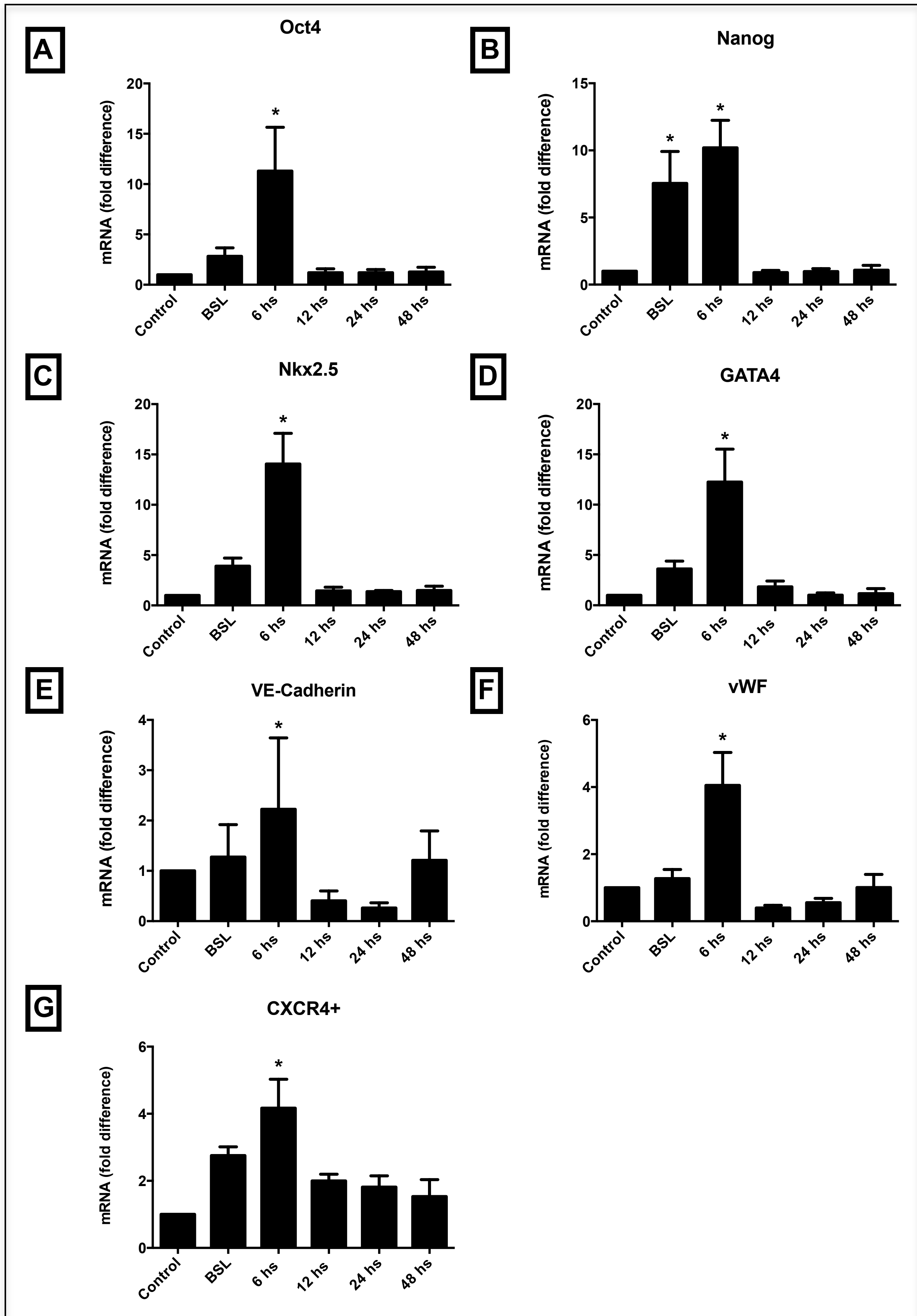

Figure 8. Bar graphs showing the mRNA expression of pluripotent markers - Oct-4 and Nanog (**Panel A** and **Panel B**, respectively), cardiac markers - Nkx2.5/Csx and GATA4 (**Panel C** and **Panel D**, respectively), endothelial antigens – VE-Cadherin (**Panel E**) and vWF (**Panel F**) and CXCR4 (**Panel G**) in PB total nucleated cells isolated from ischemic heart disease patients and controls**.** Total nucleated cells were obtained from peripheral blood samples at the pre-defined time points and RBCs were lysed using PharmLyse buffer. The relative quantification value of target gene, normalized to an endogenous control (β2-microglobulin gene) and relative to a calibrator, was expressed as $2^{-\Delta\Delta Ct}$ (fold difference), where $\Delta_C = C_t$ of target genes - C_t of endogenous control gene (β2-microglobulin), and $\Delta\Delta C_t = \Delta C_t$ of samples for target gene - ΔC_t of calibrator for the target gene. The expression of primitive, cardiac and endothelial genes was consistently higher in STEMI patients early after the acute event. Data were analyzed using one-way ANOVA. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett test as appropriate with control samples as the control category.

Figure 9.

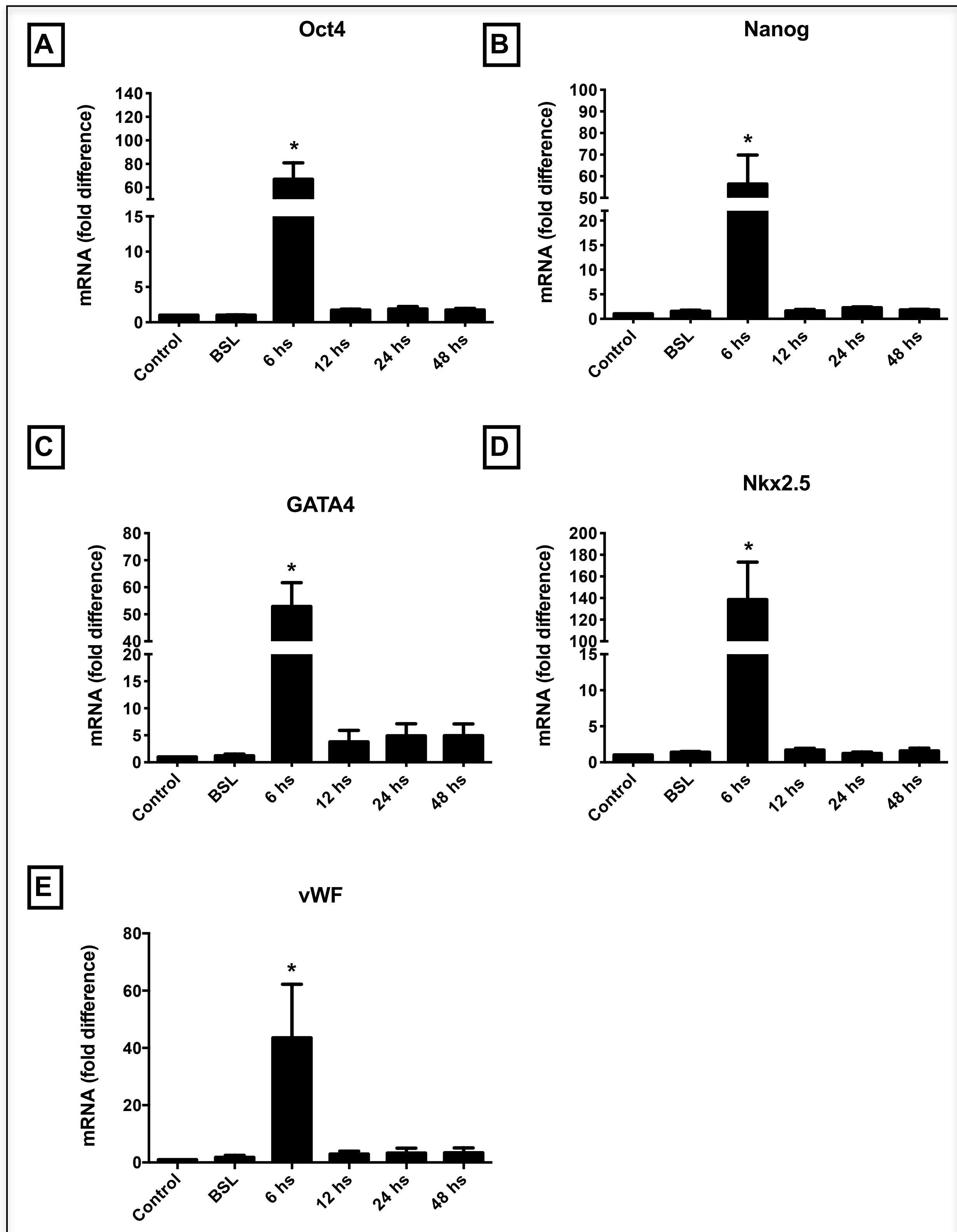

Figure 9. Bar graphs showing the mRNA expression of pluripotent markers - Oct-4 and Nanog (**Panel A** and **Panel B**, respectively), cardiac markers - Nkx2.5/Csx and GATA4 (**Panel C** and **Panel D**, respectively) and endothelial antigen - vWF (**Panel E**) in sorted CD34+ cells isolated from ischemic heart disease patients and controls**.** Total nucleated cells were obtained from peripheral blood samples at the pre-defined time points and RBCs were lysed using PharmLyse buffer. CD34+ cells were isolated using magnetic MACS beads sorting using positive selection technique. The relative quantification value of target gene, normalized to an endogenous control (β2-microglobulin gene) and relative to a calibrator, was expressed as $2^{-\Delta\Delta Ct}$ (fold difference), where Δ_C = C_t of target genes - C_t of endogenous control gene (β2-microglobulin), and $\Delta\Delta C_t = \Delta C_t$ of samples for target gene - ΔC_t of calibrator for the target gene. The expression of primitive, cardiac and endothelial genes was consistently higher in STEMI patients early after the acute event. Data were analyzed using one-way ANOVA. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett test as appropriate with control samples as the control category.

Cellular size and immunophenotype of mobilized PSCs

Some of the hallmarks of pluripotent and embryonic stem cell features are their morphological features such as small cells size, large nucleus and high nuclear to cytoplasm ratio (144). Sorted stem cell subpopulations enriched in VSELs were analyzed with ISS which confirmed their pluripotent phenotypic features such as their small size (7-8 μm on average) as well as their higher nuclear to cytoplasm ratio. Similarly, when assessed by confocal microscopy, VSELs appeared small in size with a large nucleus staining positive for Oct-4 (**Figure 10**) surrounded by a small rim of cytoplasm and staining positive for SSEA-4 on the surface. However, it is important to note that these characteristics are shared with multiple pluripotent stem cells as well as other stem cells and cannot be used solely to distinguish PSCs (16). Therefore, these features should be considered in conjunction with other distinguishing features such as surface markers and differentiation capacity. The embryonic morphological characteristics of VSELs isolated from STEMI patients are in line with VSELs populations isolated form murine and human BM and different organs and point to rare but very primitive populations of stem cells in adult tissues especially the BM (16).

Figure 10.

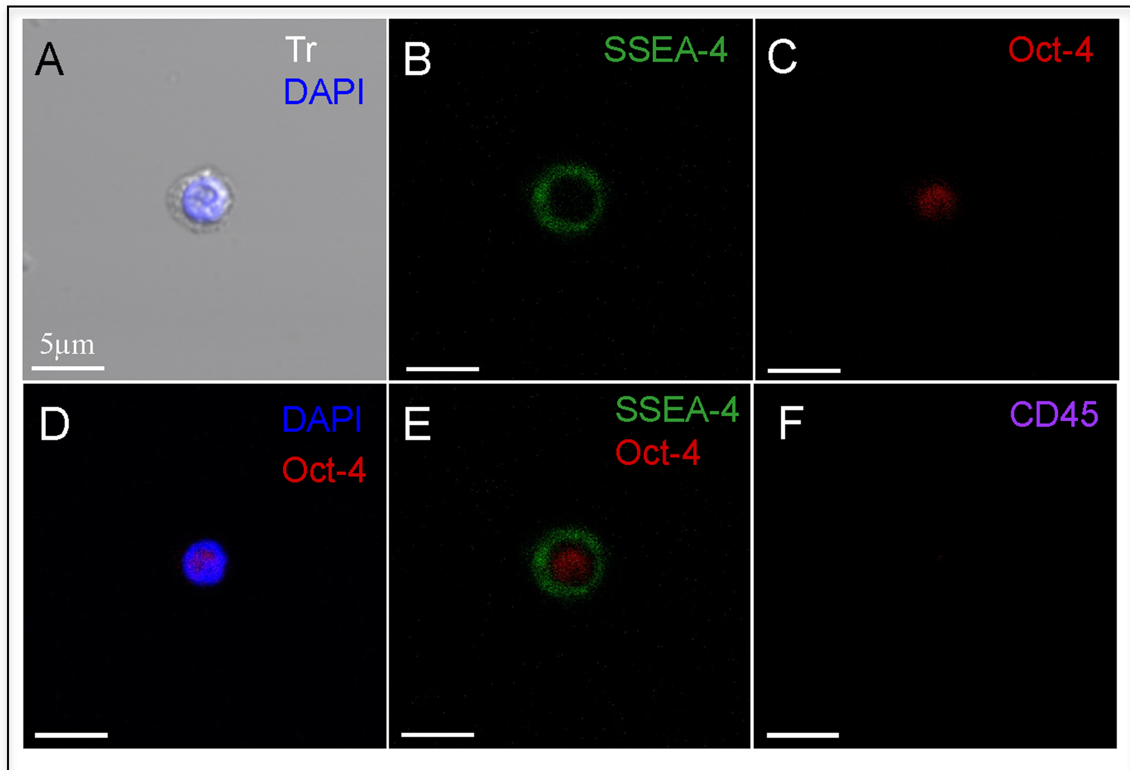

Figure 10. Representative confocal microscopic images documenting the expression of primitive markers in circulating very small embryonic like stem cells (VSELs). VSELs were sorted from peripheral blood samples from AMI patients at time points showing peak mobilization (~ 6 hours after the acute event). VSELs were sorted, using MoFlo cell sorter, based on the expression of CD34/CD133 and the lack of expression of lineage and CD45 surface markers. Sorted cells were plated on fibronectin-coated plates and stained for confocal microscopy as detailed in the methods section. Confocal images were then obtained at 40X using Zeiss-LSM confocal microscope. The pluripotent nature of circulating VSELs is evidenced by the positivity for the primitive surface marker SSEA-4 (FITC, green) and the nuclear marker Oct-4 (TRITC, red). Circulating VSELs are negative for the expression of CD45 (Cy5, Magenta). Nuclei are stained with DAPI (blue). The merged image demonstrates the co-localization of Oct-4 in the nucleus and SSEA-4 on the surface. The scale indicates 5 μm.

Plasma cytokine levels after myocardial ischemia

Traditionally, chemokines have been regarded as the primary mobilizing factors for BM-derived stem cells (145). However, the role of the SDF-1/CXCR4 axis in cardiac repair after AMI has been disputed by a growing line of evidence (50). We examined the levels of chemokines in the plasma of patients with STEMI in relation to the mobilization of BM-derived stem cell populations. The levels of G-CSF, VEGF, HGF, and SCF showed minimal dynamic changes after STEMI and did not correlate significantly with the mobilization of PSCs, non-HSCs or HSCs (**Figure 11 and Table 1**). This can be explained, at least in part, by the degradation of traditional chemokines at sites of myocardial injury and the subsequent upregulation of metalloproteinases (51, 143, 146). These data and other reports in the literature (49, 56, 147, 148) directed our attention to other possible pathways that could contribute to the BM-derived stem cell mobilization following myocardial ischemic injury.

Demographic correlations of VSELs and HSCs mobilization in STEMI patients

We examined the clinical factors that affect the mobilization of BM-derived stem cells in ischemic myocardial injury. We first examined the role of patient's age in the ability to mobilize BM-derived stem cells after ischemic injury. The literature suggests limited repair capability of stem cells in older individuals and indeed older individuals have lower cardiomyocyte chimerism rates than younger subjects (11). Our data suggest that all three populations enriched in VSELs (Lin-

/CD45-/CD133+, Lin-/CD45-/CD34+, and Lin-/CD45-/CXCR4+), PSCs and HSCs (Lin-/CD45+/CD133+, Lin-/CD45+/CD34+, and Lin-/CD45+/CXCR4+) in STEMI patients correlated negatively with patients' age (**Figure 12**). These data provides logical explanation to the lower chimerism rates in older individuals and support a growing consensus that aging is a "stem cell deficiency state". We also examined the influence of ischemic injury on the degree of stem cell mobilization response. Patients with larger ischemic injury as reflected by higher serum troponin T had significantly higher counts of circulating mobilized BM derived stem cells compared to those who suffered smaller injury ($P < 0.05$) (**Figure 13**). Furthermore, our exploration of other mobilizing factors that could contribute to the mobilization of BMSPCs lead us to the discovery of dynamic elevation of sphingosine-1 phosphate (S1P) which correlated temporally with the dynamics of BMSPC mobilization (**Table 2**). Therefore, we hypothesized that bioactive lipids could be playing an important role in stem cell mobilization in the setting of acute ischemic myocardial injury.

Figure 11.

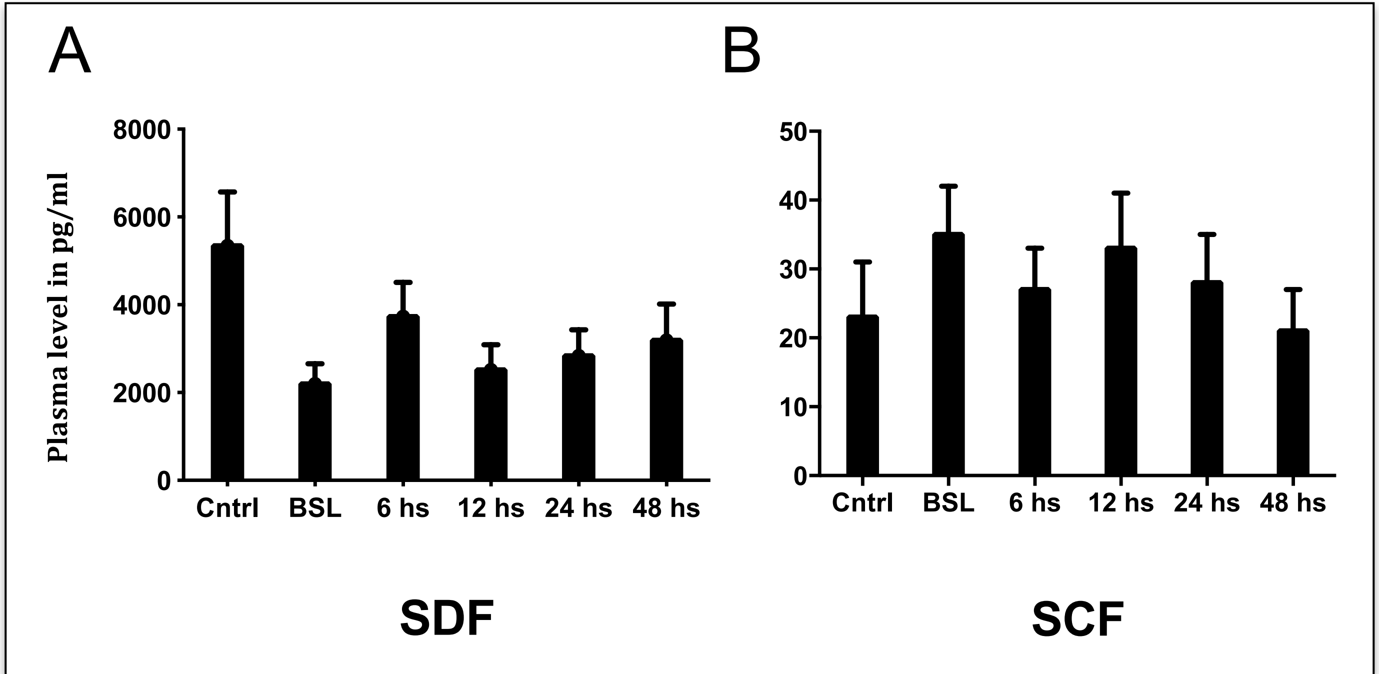

Figure 11. Plasma levels of chemokines in controls and during the first 48 hours following myocardial injury in STEMI patients. The level of cytokines was measured using an ELISA kit and the analysis was performed according to the manufacturer's protocol. Bar graphs of the levels of SDF-1 (**Panel A**) and stem cell factor (**Panel B**) in controls and patients with STEMI. Levels of both chemokines did not follow specific chronological pattern and did not correlate temporally with the mobilization of BM-derived stem cells described above. Data were analyzed using one-way ANOVA and did not reveal statistically significant differences between the STEMI and control groups or the different time points in the STEMI patients. SCF, stem cell factor; SDF, stromal cell derived factor.

Table 2. Plasma Cytokine Levels (pg/ml) in STEMI patients and Controls.

	Control	STEMI-BSL	STEMI-24 hours	STEMI-48 hours	STEMI-72 hours
G-CSF	118±28	95±18	166±38	78±15	60±23
VEGF	102±23	232±45	337±77	199±39	178±54
SDF-1α	5344±1226	2198±458**	2514±577*	2837±592	3190±824
HGF	1719±992	3866±1166	2327±776	2739±826	2486±939
SCF	23±8	35±7	33±8	28±7	21±6
S1P	78571±1786	137100±39903	143044±59712	119519±19159	68450±8991
DH S1P	10143±2227	18507±2832	23920±8860	16892±3158	7568±1757

** $P < 0.01$, * $P < 0.05$

Data were analyzed using one-way ANOVA and posthoc analyses if the initial one-way ANOVA was significant was performed using the Dunnett's method with the control arm used as the single control parameter.

Figure 12.

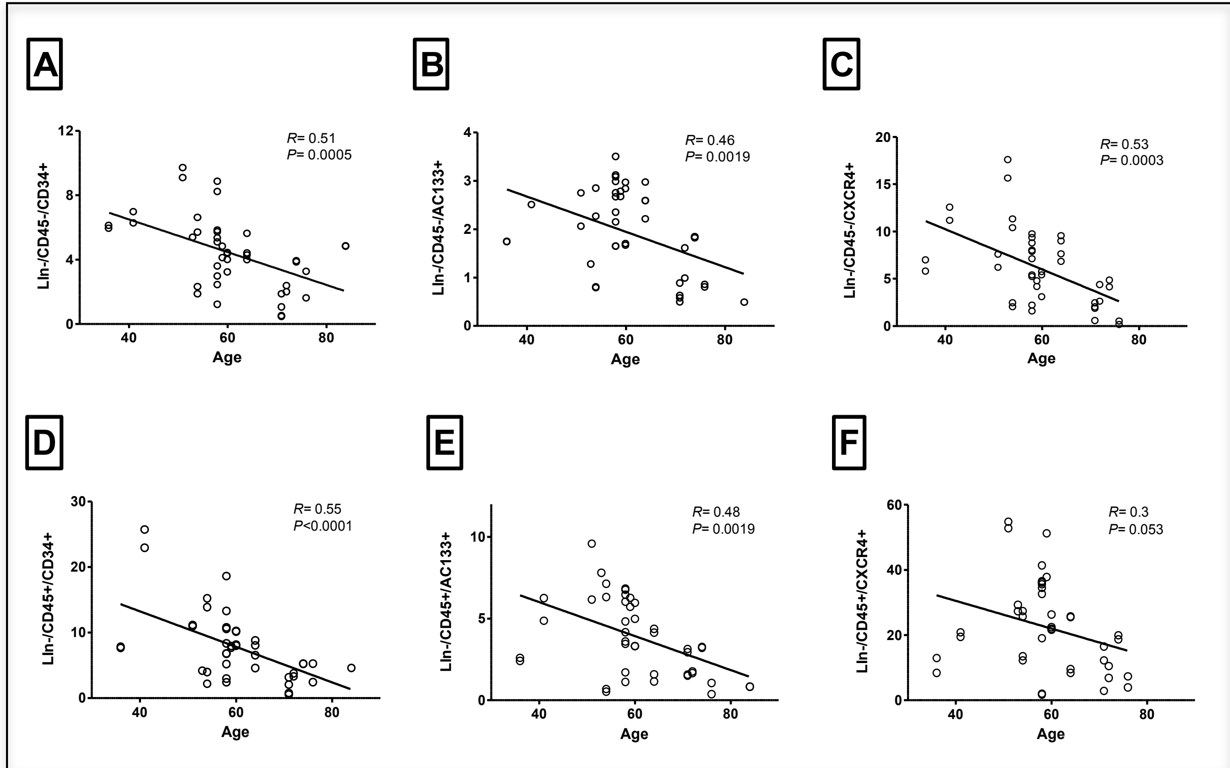

Figure 12. The negative correlation between the peak mobilization of different VSELs' subpopulations (**Panels A-C**) and HSCs (**Panels D-F**) in STEMI patients and patients' age. Total nucleated cells were obtained by lysis of RBCs and stained against the surface markers for VSELs and HSCs as detailed above. All three VSELs' subpopulations showed significant negative correlation with patient age ($P < 0.05$). HSCs showed statistically significant or strong trends towards negative correlation with patients' age. Data were analyzed using a simple linear regression analysis with the patient age on the Y-axis and separate analyses for each of the stem cell populations in the X-axis.

Figure 13.

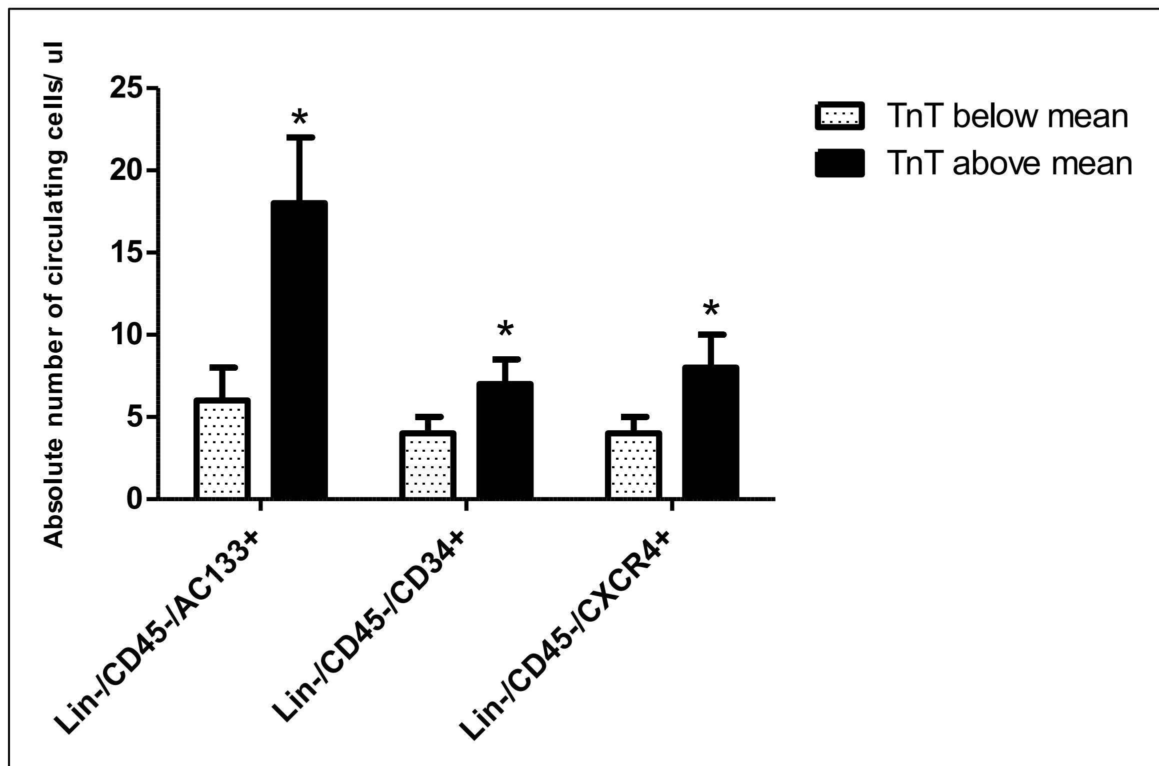

Figure 13. The relationship between degree of ischemic myocardial injury and effectiveness of BM-derived stem cell mobilization in patients with STEMI. Total nucleated cells were obtained by lysis of RBCs and stained against the surface markers for populations enriched in VSELs as detailed above. The degree of myocardial injury was quantified using the serum levels of troponin at the same time point of BMSPC evaluation. Patients with larger ischemic injury as reflected by troponin T higher than median (black bars) have significantly higher numbers of circulating BM-derived non-HSCs that are enriched in VSELs as compared to those with smaller myocardial ischemic injury (dotted bars). The data were analyzed using the Student's *t*-test (troponin T below vs. above the mean, $P < 0.05$). TnT, troponin T.

Conclusions and discussion

The ischemic myocardium releases a multitude of chemokines, growth factors and cytokines responsible for the dynamic mobilization, homing, and incorporation of BM-derived stem cells to the infarction zone (14, 149). Based upon the results presented here, we report the following findings: (i) BM-derived pluripotent stem cells (PSCs) are mobilized following acute myocardial infarction with an early peak, (ii) mobilized PSCs contain subpopulations enriched in VSELs that express multiple phenotypic and morphological properties of embryonic stem cells similar to their human and murine BM counterparts, (iii) mobilized BM-derived stem cells could be pre-destined to cardiac and endothelial fates and express corresponding genes, (iv) the mobilization of BM-derived stem cells correlate positively with the extent of myocardial injury and negatively with individuals' age, and (v) changes in plasma chemokines do not explain the dynamic mobilization of BM-derived stem cells. Bone marrow-derived stem cell populations enriched in VSELs have been shown to differentiate into cardiomyocytes *in vitro* (15) and regenerate the myocardium *in vivo* (150, 151), highlighting the clinical relevance of the presented data.

Adult bone marrow contains a multitude of non-committed, partially-committed, and committed stem cell populations that contribute to the regeneration of non-hematopoietic tissues (117). The most primitive of these stem cells in adults are the pluripotent non-hematopoietic stem cells such as VSELs that express various pluripotent markers including Oct-4, Nanog and

SSEA-1/4 (152, 153). VSELs isolated from adult BM exhibit morphological features characteristic of embryonic stem cells such as small size, large nucleus with open euchromatin and high nuclear to cytoplasm ratio when compared with HSCs and differentiated PB cells (16). Upon appropriate stimulation, VSELs are capable of differentiating *in vitro* into cells of all three germ layers (ecto-, endo- and mesoderm) including cardiac cells (15). When transplanted in infarcted myocardium, BM-derived stem cell populations enriched in VSELs give rise to cardiomyocytes and improve left ventricular function (150, 151).

The exclusive expression of Oct-4 in pluripotent and embryonic stem cells has been challenged by reports demonstrating its presence in differentiated PB cells (154). Therefore, in our studies, we examined the expression of Oct-4 and SSEA-4 with dual fluorescent immunostaining with the stem cell marker, CD34. We also excluded differentiated PB cells by excluding from our analyses cells staining positive for differentiated lineage antibodies. The influx of Lin-/CD45-/CD34+/Oct4+ and Lin-/CD45-/SSEA4+ cells in PB was significantly higher among STEMI patients as compared to controls and other ischemic heart disease patients. However, the peak mobilization of Oct-4+ cells preceded that of SSEA-4+ cells and the explanation for this is not readily apparent. It is possible that pathways responsible for SSEA-4+ and Oct-4+ cell mobilization are different and hence the differences in the timing of their peak mobilization. It may also be the case that the cells with a different expression of these two markers represent cells with different level of maturation/primitivity which may explain the response of these cells to mobilizing chemokines. Our experience with human umbilical

cord blood (UCB) indicates that SSEA-4+ VSELs are scarcer and exhibit more primitive morphology than Oct-4+ cells (155). This may indicate that primitive/pluripotent SSEA-4+ stem cells are anchored within their niches and/or more resistant to mobilization stimuli. Furthermore, we confirmed the pluripotent nature of isolated PB-derived VSELs on the morphological and phenotypic levels through our Image Stream and confocal microscopy analyses. PB VSELs isolated, based on their surface expression markers, from patients with myocardial ischemia are small in size (7-8 μm in diameter) and have a characteristically large nucleus surrounded by a narrow rim of cytoplasm similar to their BM and cord blood counterparts (**Figures 5 and 10**) (15, 156).

Our data are consistent with previous reports from our group and others demonstrating the mobilization of stem cell subpopulations in acute myocardial infarction. Although there are discrepancies in the reported literature about changes and absolute numbers of circulating stem cells in AMI patients, these discrepant findings can be explained by the differences in patient characteristics, flow cytometry protocols used, or timing of blood sampling. Nonetheless, the majority of the literature supports significant and consistent stem cell mobilization in the early phase of myocardial infarction. Our experiments extend these observations to Oct-4+ and SSEA-4+ pluripotent stem cells. It is however important to mention that the surface markers outlined in our study are not unique to any given population of stem or pluripotent cells. CD34+ and CD133+ cells isolated from the peripheral blood have been shown to differentiate into endothelial cells in vitro and home to ischemic limbs in animal in vivo models

(157, 158). Thus, these lineage negative cells represent multiple overlapping subpopulations that are capable of repopulating the injured heart and aid in its regeneration.

In conclusion, we present for the first time quantitative evidence of circulating Oct-4+ and SSEA-4+ cells in patients with various degrees of myocardial ischemia. The patients' capacity to mobilize these pluripotent stem/primitive cells is hampered in the elder patients with STEMI. Understanding the significance and underpinnings of this mobilization will be crucial in planning future studies examining the role of VSELs and other primitive cell populations in myocardial regeneration and may reveal the optimal therapeutic window suitable for pluripotent cellular therapies for myocardial regeneration.

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Chapter 3

RESULTS

The role of bioactive lipids and the immune system in myocardial ischemia induced stem cell mobilization (159)

The mobilization of different stem cell populations that have been shown to contribute to myocardial regeneration presented in the previous chapter can carry important therapeutic implications. However, the pathways involved in this mobilization are poorly understood and the traditional chemokines seem to play minimal role in this process. Therefore, we explored other pathways that may contribute to this mobilization in an attempt to find therapeutic targets for myocardial regeneration.

Cardiomyocytes undergo continuous renewal, maintained at least in part by bone marrow (BM)-derived non-hematopoietic stem cells (non-HSCs) that include populations such as of CD34+, CD133+ and CXCR4+ lineage negative (Lin-)/CD45- cells which are enriched in VSELs (7, 9, 160). While the mechanisms of cardiomyocyte renewal are poorly understood, this process is capable of renewing up to half of the cardiomyocytes during the normal life span (11). In rodents, this phenomenon is dynamic and responds to myocardial injury (10) and is maintained, at least in part, by BM derived cells (8). Acute myocardial infarction (AMI) in patients presented with STEMI initiates innate reparatory mechanisms through which non-HSCs are mobilized from bone marrow (BM) into peripheral blood (PB) and chemoattracted to the ischemic myocardium, a

process that can potentially contribute to myocardial regeneration as we have shown in the previous chapter and as has been reported in the literature (12, 13, 24, 26, 27, 161). Nevertheless, very little is known about the underlying mechanism and clinical significance of this mobilization phenomenon. Clinical studies investigating stem cell mobilization as a strategy to augment repair of the infarcted myocardium have achieved limited success probably as result of a low number of mobilized non-HSCs homing to damaged heart tissue (91, 97-99).

Similarly, the process of recruitment of stem cells from BM into PB itself is still not fully understood. The α-chemokine stromal derived factor-1 (SDF-1) has been identified as a potent stem cell chemoattractant present in PB plasma (145). Recently however, other factors such as bioactive lipids - sphingosine-1 phosphate (S1P) and ceramide-1 phosphate (C1P) have been identified in PB as major HSCs chemoattractants that enhance their egress from BM into PB (48, 56, 162, 163). SDF-1 has been also reported to become upregulated in a hypoxia inducible factor-1α (HIF-1α) dependent manner at sites of organ tissue injury (e.g., in infarcted myocardium) (164-166). While a role for the SDF-1/CXCR4 axis in stem cell trafficking is undisputed, its exclusive role in homing to a highly proteolytic microenvironment, such as the ischemic/infarcted myocardium, is less established and redundant mechanisms may exist (48-50). The limited contribution of SDF-1 to myocardial regeneration may be explained by its active degradation at the sites of inflammation and myocardial infarction by metalloproteinases (50-52). However, as recently demonstrated, despite SDF-1 degradation by proteases, the chemotactic responsiveness of stem cells to even

low SDF-1 gradient could be significantly enhanced by members of the family of cationic antimicrobial peptides (CAMPs), products of complement cascade activation (anaphylatoxin C3a) (137, 138, 141, 167, 168) and fibroblast- and leukocyte-derived (cathelicidin and β-2 defensin) (169). Thus, an increase in level of CAMPs at sites of injury enhances responsiveness of stem cells to even very low level of SDF-1. The expression of S1P receptors is crucial for the cell response to S1P gradients and we examined the expression of various S1P receptors on BM-derived stem cells from different sources. We also explored the factors orchestrating the dynamic expression of S1P receptors and how to manipulate them to enhance this expression and the response to S1P gradients in various physiological and pathological conditions.

Based on the above literature, we hypothesized that bioactive lipids (S1P and C1P) and elements of the innate immune system (cathelicidin and β2-defensin) are upregulated during STEMI and can potentially contribute to non-HSCs mobilization from BM into PB followed by their homing to the ischemic myocardium.

Concurrent temporal elevation of plasma bioactive lipid levels and non-HSCs numbers in peripheral blood following acute myocardial infarction

Our recent studies demonstrated the critical role of bioactive lipids such as S1P ands C1P in the mobilization and homing of BM-derived HSCs (48, 49, 56). Since we expect that similar mechanisms are involved in the release of non-HSCs from their BM niches in STEMI, we examined the changes in S1P levels in PB plasma following acute myocardial injury and as compared to controls.

Figure 14 shows that the levels of the bioactive lipid S1P were elevated following STEMI particularly in the early phases following myocardial ischemia and started decreasing following successful revascularization. The level of S1P tripled to 0.31 ± 0.02 μM as compared to 0.14 ± 0.02 in controls ($P < 0.01$). S1P levels then decreased 6 hours after the acute event but continued to be elevated at 48 hours compared to controls. The levels of ceramide-1 phosphate (C1P) followed similar trends but with earlier peak with C1P.

The largest reservoirs of bioactive lipids in the peripheral blood are red blood cells (RBCs), platelets and endothelial cells (170). Our experiments confirmed these data and in whole blood, RBCs contain significantly higher concentration of S1P and C1P compared to plasma (**Figure 15A**). Release of S1P and CIP from RBCs could therefore account for higher plasma levels in the setting of STEMI. Activation of the complement cascade and the resultant generation of C5b-C9 (membrane attack complex) may enhance release of S1P from RBCs. The complement cascade is activated in STEMI patients (171-175), and in our STEMI population, C5b-C9 levels were elevated in serum in the early phases after AMI and continued to be elevated at 48 hours after the acute ischemic event (**Figure 16**). Upon incubation with *in vitro* activated complement components, RBCs released S1P and C1P in a pattern similar to the elevated plasma levels of S1P and C1P in plasma of AMI patients (**Figure 15B and 15C**). We believe that part of the release is secondary to hemolysis of RBCs upon their incubation with C5b-C9 given the elevated level of free hemoglobin in our samples after their incubation with activated complement and the

Figure 14.

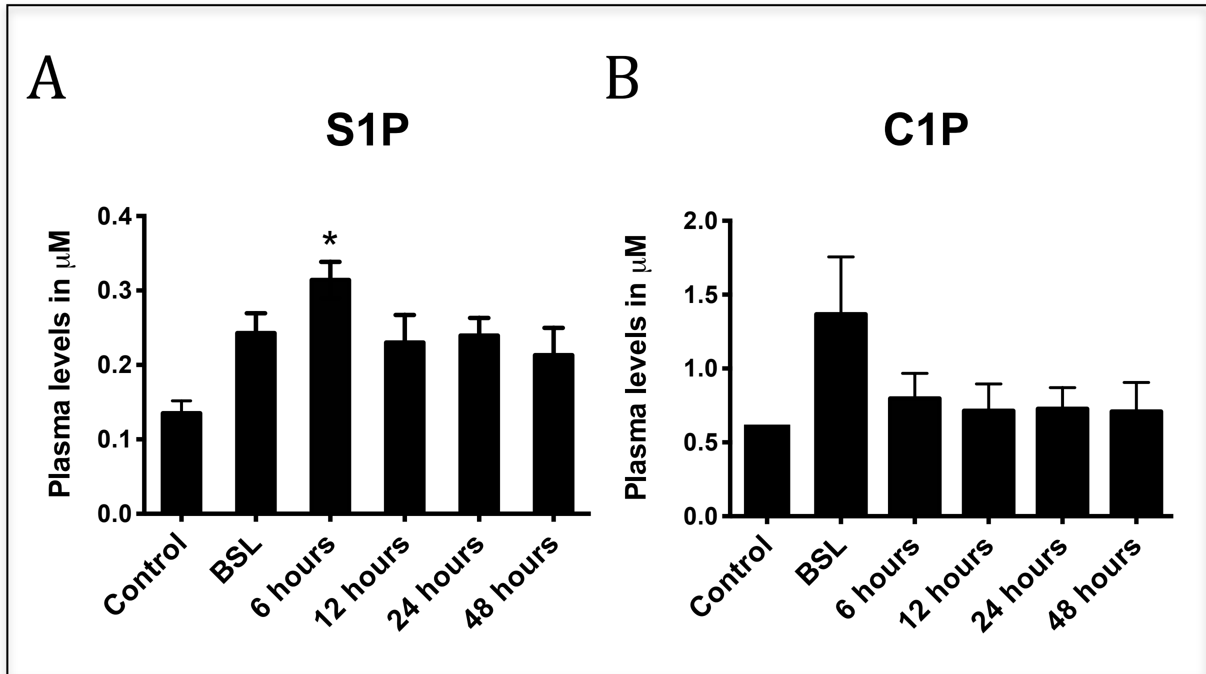

Figure 14. Elevated levels of bioactive lipids at early stage following acute myocardial infarction. Bar graphs showing the plasma levels of sphingosine-1 phosphate (**Panel A**) and ceramide-1 phosphate (**Panel B**) in patients with ST-elevation myocardial infarction and controls. Plasma isolated from patients admitted with STEMI and controls were analyzed using mass spectrometry for the concentrations of bioactive lipids. The graphs show an early peak of both members of the bioactive lipid family occurring at 6 hours with S1P and at baseline in the case of C1P. Data were analyzed using one-way ANOVA. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett test with control samples as the control category. BSL, baseline denoting the time of arrival to the hospital; S1P, sphingosine-1 phosphate; STEMI, ST-elevation myocardial infarction.

Figure 15.

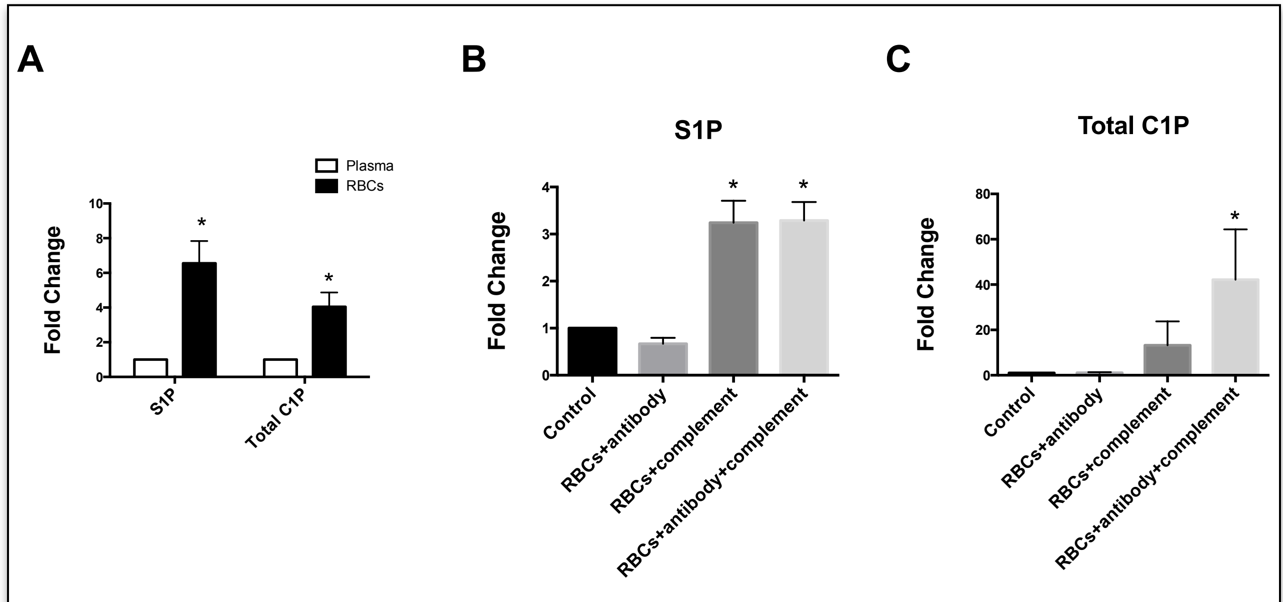

Figure 15. Red blood cells contain high concentrations of bioactive lipids that are released upon complement activation. PB samples were obtained from healthy donors. Plasma and red blood cells were isolated using 600g centrifugation. The content S1P and C1P were assessed in these fractions using mass spectrometry. RBCs were incubated with multiple components of the complement cascade, which are activated in vitro, alone or in conjunction with specific antibody against RBCs (CD235a). Bar graphs showing the content of S1P and C1P in the plasma and purified red blood cells (RBCs) (**Panel A**). Content of S1P and C1P is significantly higher in the RBCs compartment compared to plasma. Upon incubation with activated complement and activated complement together with antibodies against RBCs, cells release both S1P (**Panel B**) and C1P (**Panel C**) with a 3-fold increase in their free levels corresponding with the elevated plasma levels noted with STEMI. The content of S1P and C1P in different PB compartments were analyzed using the Student's *t*-test while the levels of S1P and C1P in Panels B and C were analyzed using one-way ANOVA. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett test with control samples as the control category. (* $P < 0.05$ as compared to controls). C1P, ceramide-1 phosphate; RBCs, red blood cells; S1P, sphingosine-1 phosphate.

Figure 16.

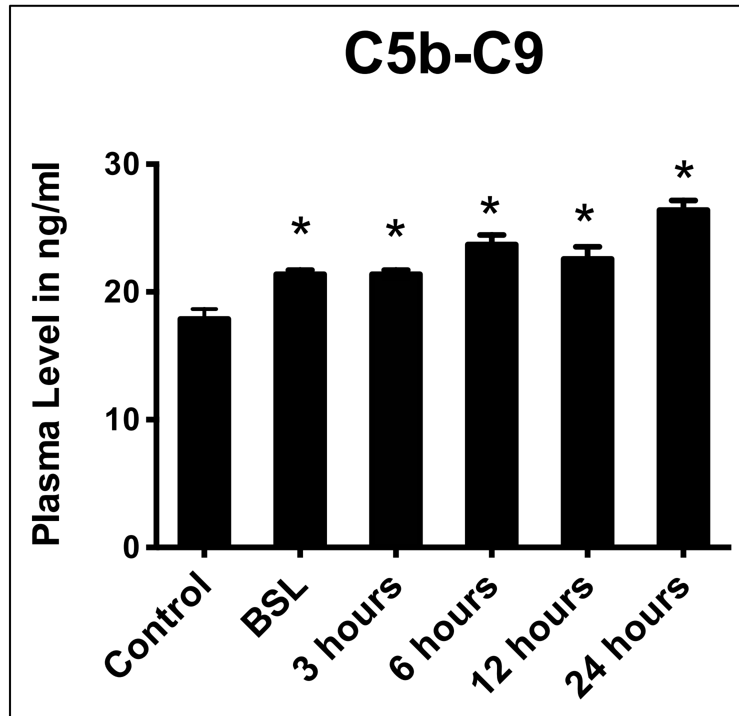

Figure 16. Complement cascade component levels in the plasma following acute myocardial infarction. Bar graphs showing the increased plasma level of C5b-C9, one of the terminal products of the complement cascade activation in patients with acute myocardial infarction as compared to controls. The levels were significantly higher at the time of presentation and continued to increase for 48 hours following revascularization. Data were analyzed using one-way ANOVA. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett test with control samples as the control category.

Figure 17.

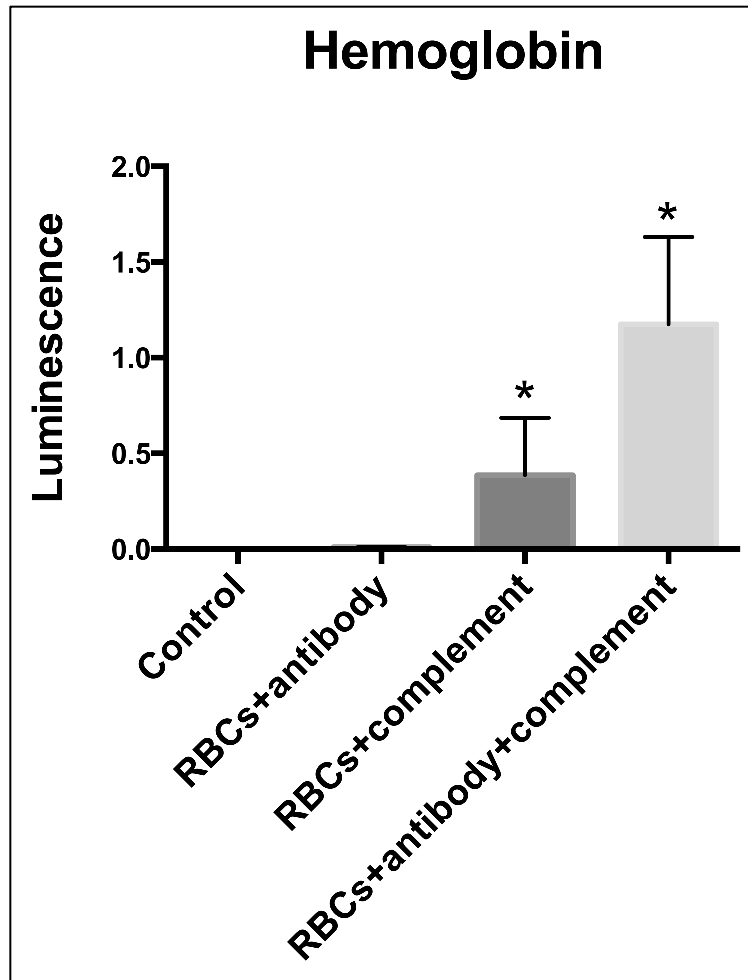

Figure 17. Incubation of red blood cells with activated complement results in their hemolysis that can explain the release of bioactive lipids. PB samples were obtained from healthy donors. Red blood cells were isolated using 600g centrifugation. RBCs were incubated with multiple components of the complement cascade, which are activated in vitro, alone or in conjunction with specific antibody against RBCs (CD235a). The free hemoglobin levels, are elevated when RBCs are incubated with complement or complement + specific antibody against RBCs (CD235a) as compared to controls and RBCs incubated with antibody alone. Data were analyzed using one-way ANOVA. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett or Dunn tests as appropriate with control samples as the control category (* $P < 0.05$ as compared to controls). RBCs, red blood cells.

Figure 18.

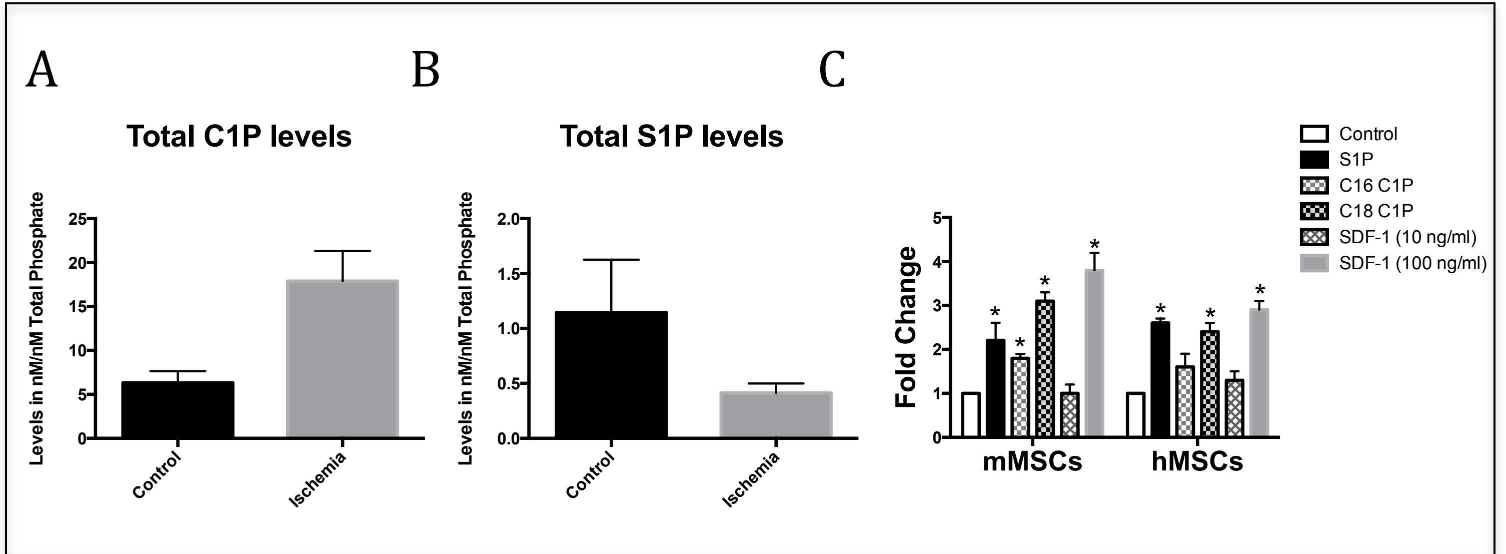

Figure 18. Levels of C1P are elevated in cardiac tissues after ischemic injury and C1P is capable of chemoattracting murine and human MSCs. The levels of S1P and C1P were assessed in the myocardium at baseline and after 40 minutes hypoxia followed by 40 minutes reperfusion using the Langendorf apparatus. The heart was then isolated and snap frozen using liquid nitrogen. Tissue was homogenized and levels of S1P and C1P were examined using mass spectrometry. There was a reduction in the level of S1P with ischemia reperfusion. On the other hand, the level of C1P as noted in the above bar graph increases to 5-7 fold compared to controls. Data were analyzed using Student's *t*-test but no significant differences noted. Human and murine MSCs were obtained following culture of adherent BM cells for 3 passages in DMEM supplemented with 10% FBS. MSCs were seeded at a density of 3×10^4 cells/well into the upper chambers of Transwell inserts (Costar Transwell; Corning Costar). The lower chambers were filled with SDF-1 (10 or 100 ng/mL), sphingosine-1-phosphate (0.1 μM), C16-ceramide-1-phosphate (1 mM), or C18-ceramide-1-phosphate (0.1–10 mM) in 0.5% BSA DMEM or EBM (control). After 24 hours, cells in the lower chambers were isolated and stained against lineage, CD45, CD34 (humans cells) and Sca-1 (murine) surface markers. C18-C1P was capable of chemoattracting primitive populations of BM-derived mesenchymal stem cells, particularly the C18-C1P sub-fraction, at a rate similar to that of S1P and high concentrations of SDF-1. Data were analyzed using one-way ANOVA. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett test with control samples as the control category.

corresponding release of bioactive lipids (**Figure 17**). The higher C1P plasma levels at BSL may also reflect the release of C1P from damaged cardiomyocytes as we have recently shown and C1P is capable of chemoattracting murine and human BM-derived primitive cells such as mesenchymal stem cells (MSCs) (**Figure 18**). On the other hand, we noted reduction of S1P in the ischemic cardiac tissue that can be due to elevated activity of S1P lyase as previously described (176).

We observed temporal correlation between the elevation in plasma levels of bioactive lipids and the peak mobilization of PSCs, non-HSCs and HSCs following acute myocardial injury as detailed in the previous chapter. The absolute numbers of non-HSCs populations such as Lin-/CD45-/CD34+, Lin-/CD45-/CXCR+, and Lin-/CD45-/CD133+ cells peaked at the early stages following myocardial ischemia (4.8 ± 1.3 vs. 0.7 ± 0.04 cells/μl of PB, 8.6 ± 2.5 vs. 0.7 ± 0.06 cells/μl of PB, and 1.7 ± 0.5 vs. 0.6 ± 0.03 cells/μl of PB; in PB samples obtained 6 hours after presentation in STEMI patients vs. control samples respectively, $P < 0.05$). This mobilization correlated with the early elevation of plasma levels of S1P and total C1P thus suggesting a chemotactic role for bioactive lipids. These data are in agreement with the increasingly recognized role of bioactive lipids in guiding HSCs mobilization and homing (49, 56).

<u>BM-derived stem cells express S1P receptors</u>

Given the potential role of bioactive lipids in the mobilization of non-HSCs, we examined the expression of various S1P receptors (S1PRs) on the surface of

BMSPCs isolated from normal individuals and found high expression of type 1 and type 3 S1P receptors - S1PR1 and S1PR3 respectively (**Figure 19**). These receptors play an important role in trafficking of lympho/hematopoietic cells (177, 178). They have also been shown to orchestrate the mobilization, chemotaxis and homing of BM-derived HSCs in animal and human models (86, 163, 179). We focused on the subset of BM-derived cells Lin-/CD34+ cells that are enriched in stem and progenitor cells and flow cytometry analysis revealed that Lin-/CD34+ express both S1PR1 (14.7±1.4%) and S1PR3 (9.9±0.9%) (**Figure 20**). Furthermore and as described with lympho/hematopoietic cells (180, 181), this expression was dynamic in that both receptors were internalized in the presence of S1P (**Figure 20**). The expression of S1PRs on peripheral blood lineage negative cells in the early phases following AMI was significantly lower than their counterparts isolated from the BM. The increased level of plasma S1P could explain the possible internalization of S1P receptors on circulating mobilized BM-derived stem cells (182). The literature suggests the internalization of S1P receptor 1 and 3 with exposure to higher concentration of S1P and this process may be crucial for guiding the trafficking of lymphocytes between the peripheral blood and lymph nodes (183, 184). Indeed, in our hands the expression of S1PR1 and S1PR3 on the surface of Lin-/CD34+ BM-derived stem cells was significantly reduced after exposure to physiological levels of S1P (**Figure 20**). Of note, since C1P receptors are not identified yet, we could not perform similar receptor expression studies for this bioactive lipid.

Figure 19.

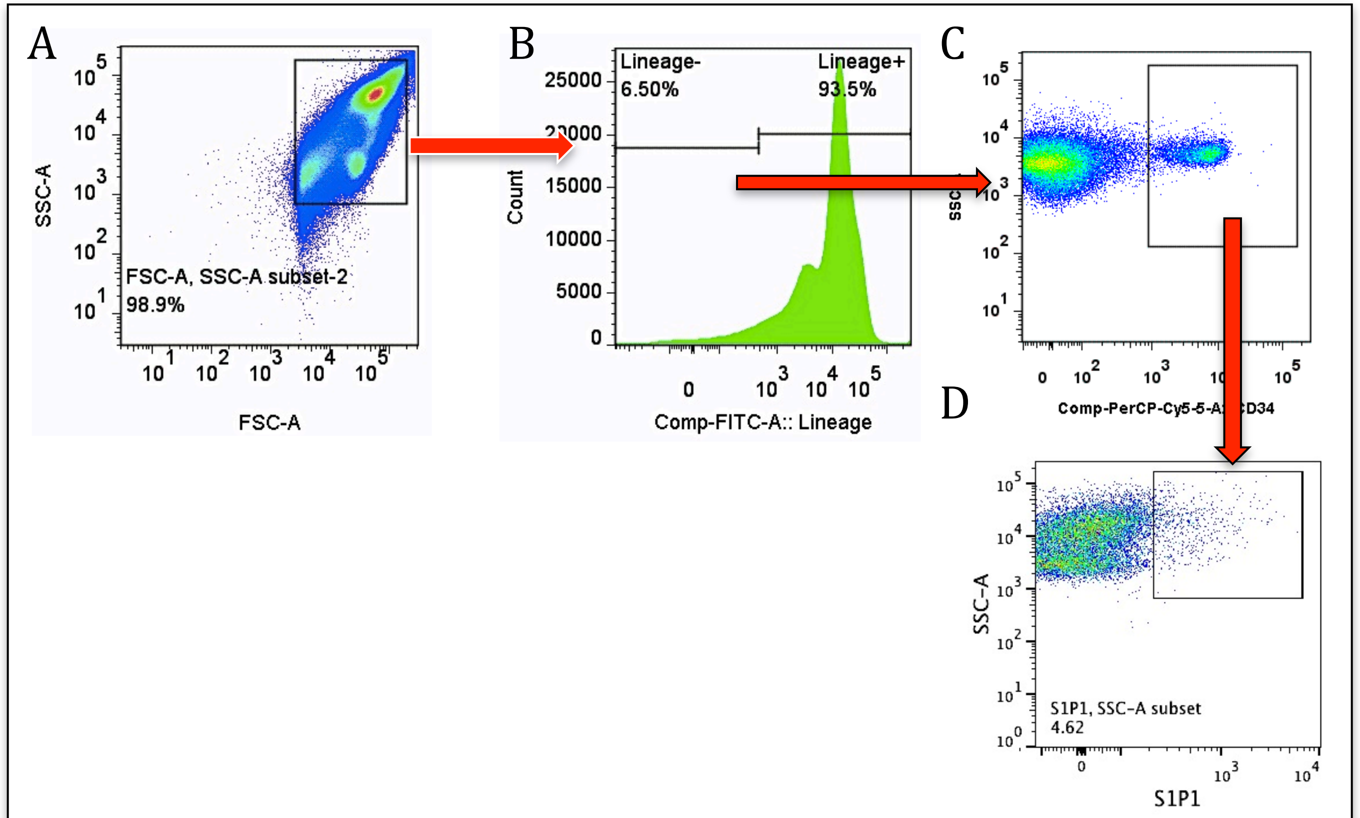

Figure 19. Experimental protocol showing the flow cytometry analysis of BM-derived stem cells for the expression of S1P receptors. Total nucleated cells (TNC) obtained after RBCs lysis were analyzed using side and forward scatter to gate the population of interest (**Panel A**). TNC were then analyzed based on the expression of lineage commitment markers (**Panel B**) and lineage negative cells were analyzed for the expression of the stem cell marker (CD34+) (**Panel C**). Cells negative for the commitment lineage markers and positive for the CD34 marker were examined for the expression of S1P receptor 1 (**Panel D**).

Figure 20.

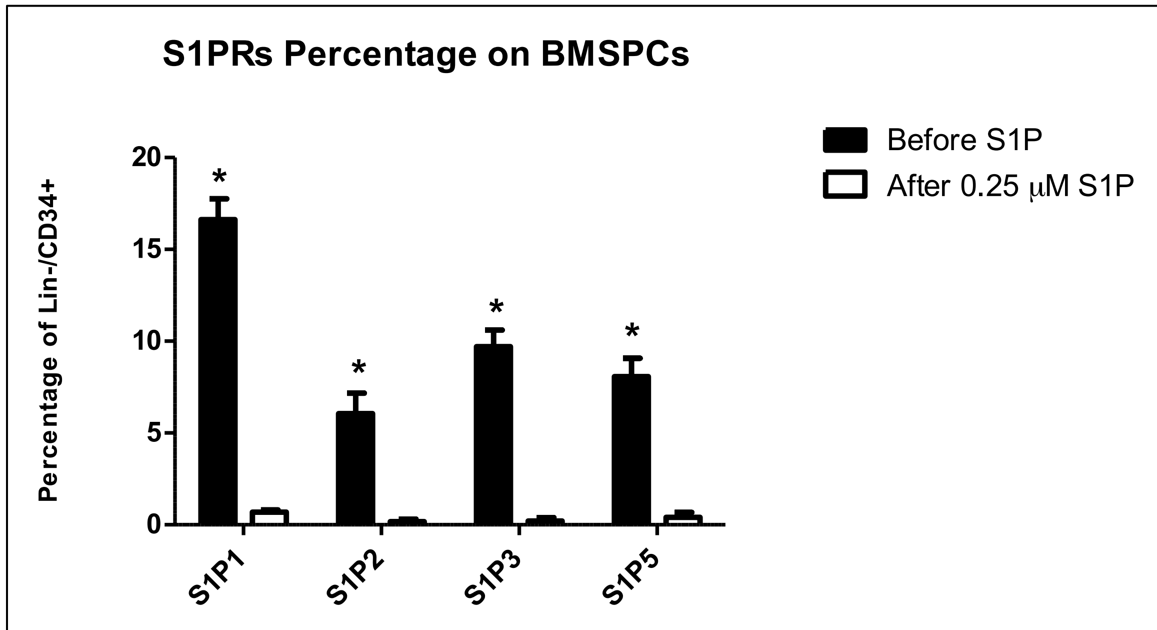

Figure 20. S1P receptors are expressed on naïve BMSPCs and surface expression is reduced following exposure to S1P. Bar graphs showing the expression of S1P receptor 1, 2, 3 and 5 on the surface of BM derived Lin-/CD34+ stem cells. BM cells were obtained from healthy donors and RBCs were lysed using PharmLyse buffer. Cells were the stained against lineage markers (FITC), CD34 (PerCP), S1P1 (PE), S1P2 (APC), S1P3 (goat primary antibodies followed by secondary anti-goat antibody labeled with PE-Cy7), and S1P5 (PE). Using flow cytometry (LSR II), we gated on the Lin-/CD34+ population and assessed the expression of S1PRs on these cells. The graphs show relatively higher expression of S1P receptors on the BM stem cells that are reduced significantly following 2 hours exposure to S1P (250 nM). Data were analyzed using the Student's *t*-test. BM, bone marrow; S1P, sphingosine-1 phosphate.

The expression of S1PR1 and S1PR3 depends on surrounding S1P concentration

Given the variable response of BM-derived stem cells from different sources to mobilization stimuli in transplantation studies, we examined the expression of S1P receptors especially S1PR1 and S1PR3 on the surface of stem cells isolated from naïve BM, mobilized peripheral blood cells (mPBCs) and cord blood cells from human donors. In these experiments we utilized human samples and the mPBCs were isolated from patients after 3-5 days of G-CSF therapy. We simultaneously examined the levels of S1P in their surrounding microenvironment. The expression of S1PR1 and S1PR3 was highest amongst naïve BM-derived Lin-/CD34+ cells followed by mPBCs and finally cord blood cells. This differential expression of S1P receptors was inversely proportional to the concentration of S1P in the surrounding microenvironment of the cells suggesting its possible role in internalization of these receptors (**Figure 20**). This can explain the differential response of BM-derived stem cells from different sources to S1P gradients and their different homing potentials after BM transplantation. These findings have important clinical implications; the BM reconstitution capabilities of human mPBCs are variable and influence the success of BM transplantation. Using the expression of S1P receptors as an indicator for the success of BM transplantation can be of clinical importance and more clinical studies are needed to examine the clinical correlation between S1P receptor expression and the success of mPBCs transplantation.

Figure 21.

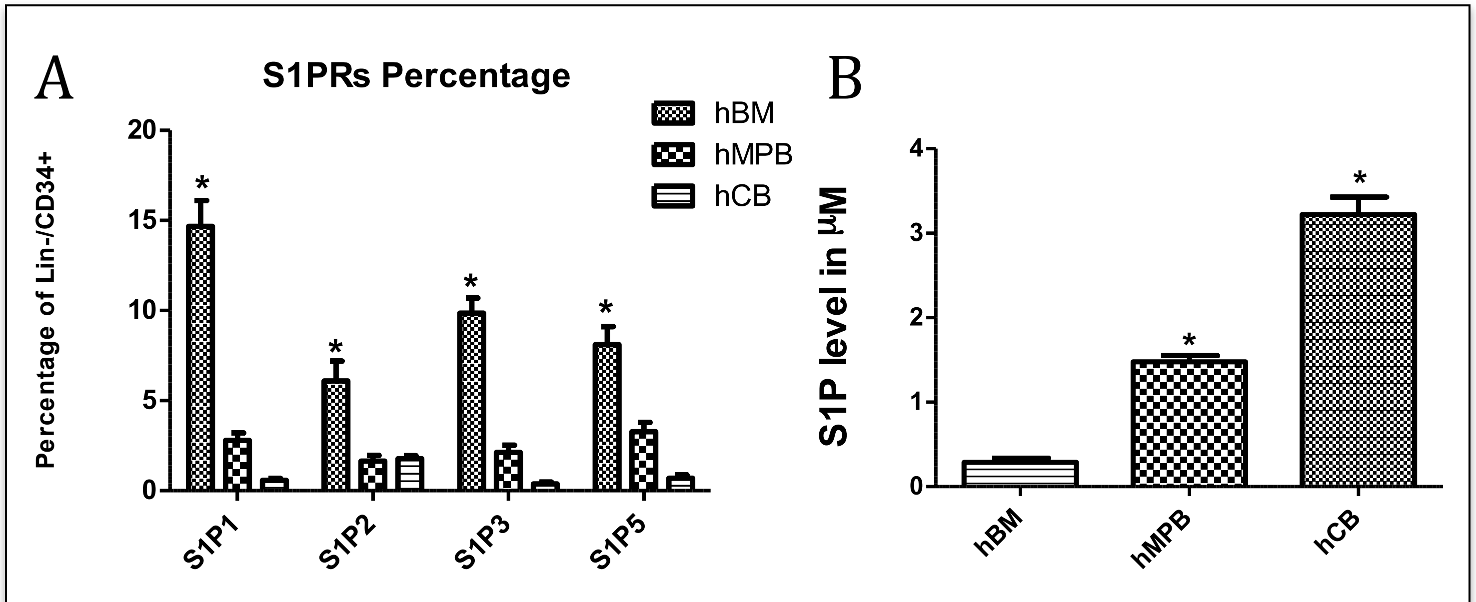

Figure 21. Differential expression of S1P receptors on stem cells isolated naïve BM, mobilized peripheral blood cells and cord blood cells. The expression of S1P receptors 1, 2, 3 and 5 on the surface of similar populations of BM-derived stem cells isolated from human naïve BM, G-CSF mobilized peripheral blood cells, and cord blood cells. BM and mPB cells were obtained from healthy donors and cord blood cells from human sample bank. Supernatant was isolated and examined for bioactive lipid content using mass spectrometry and RBCs were lysed using PharmLyse buffer. Cells were the stained against lineage markers (FITC), CD34 (PerCP), S1P1 (PE), S1P2 (APC), S1P3 (goat primary antibodies followed by secondary anti-goat antibody labeled with PE-Cy7), and S1P5 (PE). The expression of S1PR1 and S1PR3, which are responsible for cell mobilization and homing, were highest among naïve BM cells that coexisted in an environment with relatively low levels of S1P. The expression of S1P receptors was lower among mPBCs and cord blood stem cells and correlated inversely with the levels of surrounding S1P. S1P was measured in the supernatant of BM, hMPB and hCB samples using mass spectrometry. Data were analyzed using one-way ANOVA. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett test with control samples as the control category. hBM, hCB, human cord blood cells; human naïve BM cells; hMPB, human G-CSF mobilized peripheral blood cells; Lin, lineage combination of antibodies.

Expression of S1PR1 and S1PR3 receptors dictates the migration response of BM stem cells to S1P gradients

Based on the above data confirming the significant role of surrounding environment on the expression of S1P receptors, we examine the migratory response of BM-derived stem cells isolated from different sources to S1P gradients. For these experiments we utilized naïve BM cells, G-CSF mobilized peripheral blood cells and human cord blood cells. We used S1P gradients of 0.01, 0.1 and 1 μM in the lower chamber as our chemoattractant. By correlating the expression of S1P receptors with the chemotaxis response, we could identify the role of different S1P receptors in this process. Naïve BM-derived stem cells had the highest response to S1P gradients with a peak mobilization at 0.01 μM. This correlates with our previous and published data confirming the bell-shaped response of BM-derived stem cells to S1P gradients (162). However, BM-derived mPBCs and cord blood cells did not show significant chemotaxis to S1P gradients compared to vehicle controls (**Figure 22**). The response to S1P at 0.01 and 1 μM was similar among all groups. The response at 0.1 μM was highest in naïve BM cells followed by mPBCs and finally cords blood cells. This is in strong correlation with the expression of S1P receptors, particularly S1PR1, on the surface of these cells.

Expression of S1P receptors is dynamic and can be enhanced by a S1P free microenvironment leading to improved BM stem cell chemotaxis

The BM-derived stem cells examined in the above experiments have the

Figure 22.

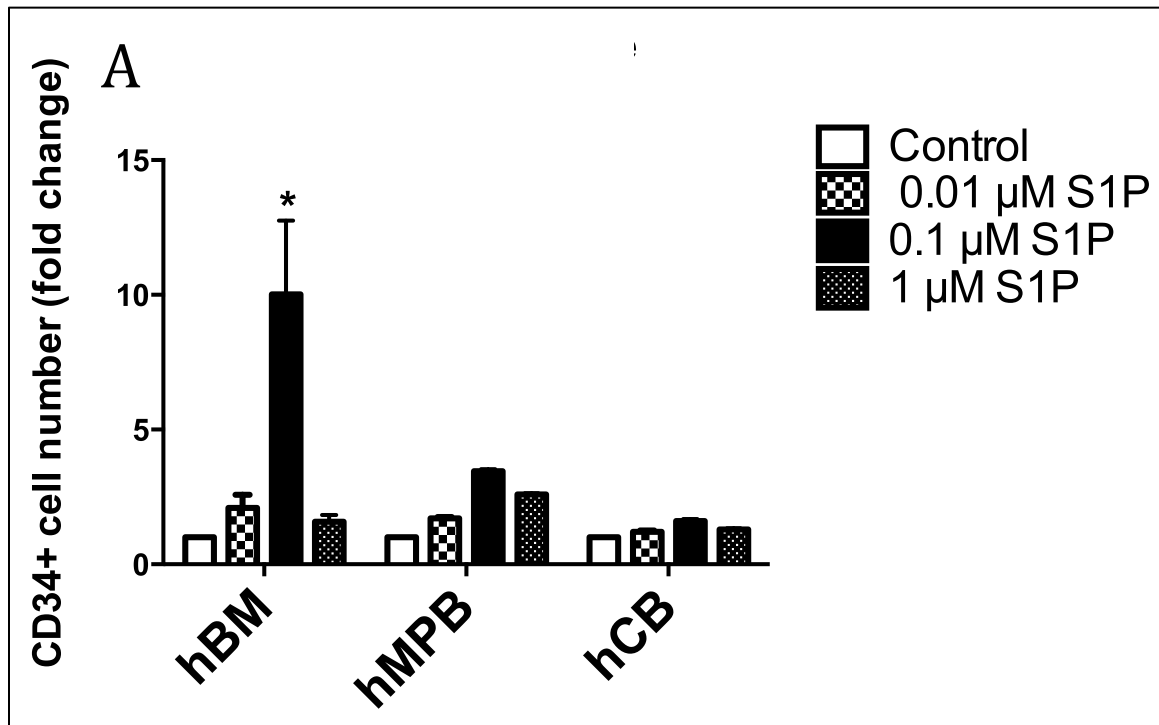

Figure 22. Differential chemotaxis of BM-derived stem cells isolated from naïve BM, mobilized peripheral blood cells and cord blood cells to S1P gradients. Bar graphs demonstrating the chemotaxis of BM-derived stem cells isolated from naïve BM, G-CSF mobilized peripheral blood cells and cord blood cells. RBCs were lyse and BM, hMPB and hCB cells were seeded at a density of 1×10^6 cells/well into the upper chambers of Transwell inserts (Costar Transwell; Corning Costar). The lower chambers were filled with regular DMEM medium supplemented with sphingosine-1-phosphate (at concentrations of 0.01, 0.1 and 1 μM). After 6 hours, cells in the lower chambers were isolated and stained against lineage, CXCR4 and CD34 surface markers. The chemotaxis was evident in naïve BM cells particularly towards 0.1 μM of S1P. On the other hand, chemotaxis was significantly reduced in mobilized peripheral blood and cord blood cells that demonstrated low expression for S1PR1 and S1PR3 receptors. Data were analyzed using one-way ANOVA. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett test with control samples as the control category.

same origin from the BM, yet have significantly different expression of S1P receptors and response to S1P gradients. We hypothesized based on our earlier data that the expression of S1P receptors especially S1PR1 is dynamic and responds to S1P levels in the surrounding microenvironment. Therefore, we examined mPBCs immediately after their isolation and at 6 hours time intervals thereafter for the expression of S1P receptors and their response to S1P gradients in chemotaxis chambers. We used mPBCs in these experiments for their clinical implications as they are widely used in human BM reconstitution studies. We found that the expression of S1PR1 and S1PR3 is rather dynamic and incubation of mPBCs in S1P free medium (DMEM supplemented with 0.1% FBS) resulted in the re-expression of S1PR1 and S1PR3 on the surface of mPB Lin-/CD34+ stem cells (**Figure 23**). Starting at 12 hours after incubation, we noticed a dramatic increase in the percentage of Lin-/CD34+ cells expressing S1PR1 and S1PR3. The cell viability did not drop significantly with these long incubations. Furthermore, chemotaxis experiments demonstrated a significant increase in the mPB Lin-/CD34+ stem cell response to 0.1 μM S1P gradient in mPB cells incubated in S1P free medium as compared to cells form the same sample that were freshly isolated and in correlation with the re-expression of S1PR1 and 3 (**Figure 23**). This is strong evidence supporting a crucial role of S1PR1 and S1PR3 receptor expression in the mobilization and chemotaxis of BM-derived stem cells. The data presented herein has important implications for enhancing the efficiency of human BM transplantation practices.

Figure 23.

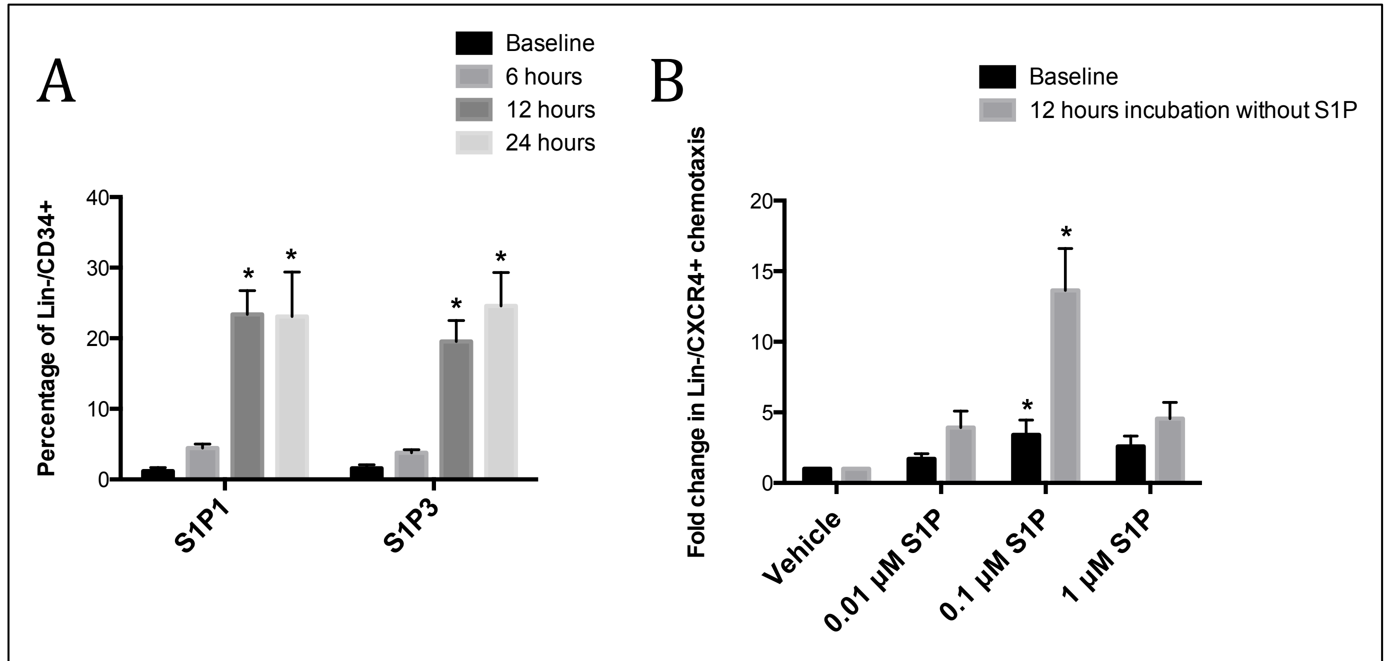

Figure 23. Dynamic expression of S1P receptors on mPB stem cells and its effect on their response to S1P gradients. Bar graphs demonstrating the percentage of G-CSF mobilized peripheral blood Lin-/CD34+ stem cells that express S1P receptors 1 and 3 immediately after isolation and at 6-hour intervals thereafter culture in DMEM supplemented with low concentration of FBS (0.1%) (**Panel A**) and the migration response of mPB stem cells immediately after isolation or 12 hours after incubation in S1P free medium (**Panel B**). G-CSF mobilized peripheral blood cells were lysed using PharmLyse buffer. Cells were then stained, at different time intervals after culture in DMEM supplemented with 0.1% FBS, against lineage markers (FITC), CD34 (PerCP), S1P1 (PE), S1P2 (APC), S1P3 (goat primary antibodies followed by secondary anti-goat antibody labeled with PE-Cy7), and S1P5 (PE). Starting 12 hours after incubation, cells re-express the S1P receptors 1 and 3 at significantly high levels (**Panel A**). hMPB cells immediately after their isolation or after 12 hours of culture on DMEM with 0.1% FBS were lysed and seeded at a density of 1×10^6 cells/well into the upper chambers of Transwell inserts. The lower chambers were filled with sphingosine-1-phosphate (at concentrations of 0.01, 0.1 and 1 μM). After 6 hours, cells in the lower chambers were isolated and stained against lineage, CXCR4 and CD34 surface markers. The re-expression of S1PR1 and S1PR3 was paralleled by increased responsiveness of mPB stem cells to S1P gradients in chemotaxis chambers (**Panel B**). Data were analyzed using one-way ANOVA. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett test with control samples as the control category.

BM-derived stem cells migrate towards plasma isolated from acute myocardial ischemia patients in an S1P dependent manner

Since the plasma levels of S1P were elevated simultaneously with the peak mobilization of non-HSCs at 6 hours following revascularization, we sought to examine the role of bioactive lipids in the migration of BMSCs using in vitro migration assays. Using plasma isolated from patients with STEMI at time points corresponding with the peak mobilization of BM-derived stem cells and S1P levels, we noted that plasma from STEMI patients was able to chemoattract BM stem cells at levels 6-12 folds higher than vehicle control. **Figure 24A and 24B** show that Lin-/CD34+ and Lin-/CXCR4+ BMSPCs migrate towards intact plasma isolated from STEMI patients at the peak mobilization of non-HSCs. This migration was inhibited by delipidation of the plasma (charcoal stripping-CSP), which removed > 90% of the S1P (**Figure 24A, 24B and 24C**). Additionally, pretreatment of BM cells with the selective S1PR1 antagonist, W146 (10 μM), or the S1PR1/S1PR3 receptors antagonist, VPC23019 (10 μM) also significantly reduced the migration of BM cells towards plasma from AMI patients. Neither W146 nor VC23019 altered cell viability (**Figure 24D**). Although we cannot isolate the effects of other factors present in the plasma in chemoattracting BMSCs, the significant reduction of this migration with delipidation of the plasma and S1P receptor blockers point to an important role of bioactive lipids in this process. This data in total supports a potential role for bioactive lipids present in the plasma of STEMI patients in the mobilization and egress of stem cells from the BM to the PB.

Figure 24.

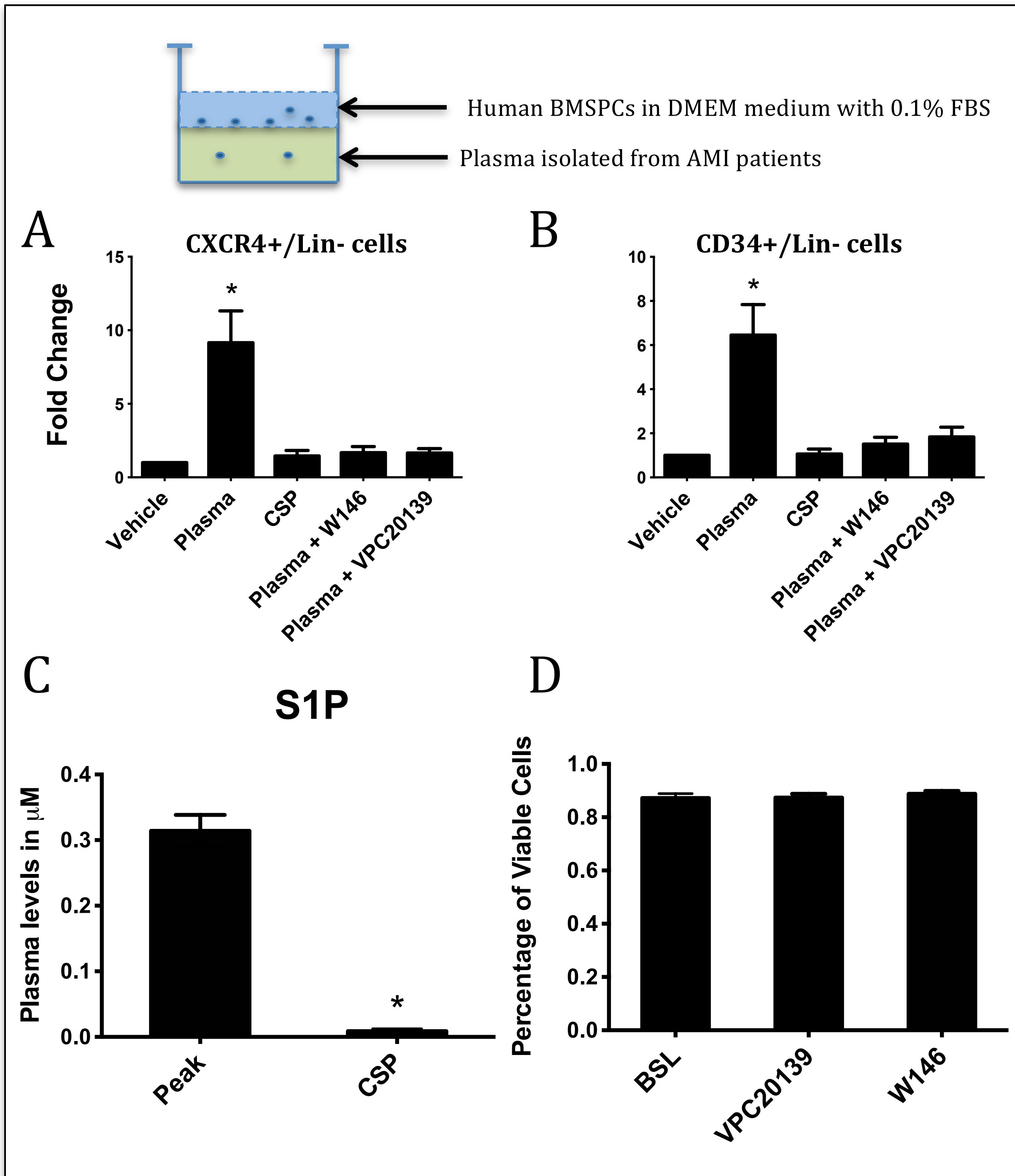

Figure 24. BMSPCs migrate towards plasma from AMI patients in an S1P/S1PR dependent fashion. Bar graphs showing the migration of lineage negative (Lin-)/CD34+ and CXCR4 positive cells towards plasma isolated from patients with acute myocardial infarction. As shown, Lin-/CD34+ (**Panel A**) and Lin-/CXCR4+ (**Panel B**) cells migrate towards plasma isolated from AMI patients at peak stem cell mobilization. Data were analyzed using one-way ANOVA. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett test with control samples as the control category. BM cells were lysed and seeded at a density of 1×10^6 cells/well into the upper chambers of Transwell inserts. Some BM cells were incubated with 10 μM W146 or VPC20139 for 2 hours before the chemotaxis experiment. The lower chambers were filled with plasma isolated form STEMI patients at 6 hours following the acute event or DMEM with 0.1% FBS (vehicle). After 6 hours, cells in the lower chambers were isolated and stained against lineage, CXCR4 and CD34 surface markers. This mobilization was blunted by charcoal stripping of the plasma (CSP) and the use of selective S1P receptor 1 blocker (W146) and S1P receptor 1 and 3 selective blocker (VPC20139); where the number of migrated BM stem cells was not significantly different from vehicle control. Delipidation using activated charcoal significantly reduces the levels of bioactive lipids (**Panel C**). Data were analyzed using Student's *t*-test (* $P < 0.05$ as compared to controls). Incubation with S1P receptor blockers does not influence BM cell viability as assessed by trypan blue as analyzed by two-way ANOVA (**Panel D**). CSP, charcoal stripped plasma; Lin-, lineage negative cells.

Migrated BM cells are enriched in populations expressing primitive, cardiac and endothelial genes

We have previously shown that mobilized BMSCs in the setting of STEMI are enriched in primitive, cardiac and endothelial genes (Chapter 2) in agreement with other data in the literature (185). It is unclear whether the mobilization is selective for tissue specific stem cells that are enriched in these markers or if the mobilization stimuli/process initiates the differentiation of mobilized cells that respond to them. Nonetheless, the expression of Oct4, Nkx-2.5, GATA4, vWF and VE-Cadherin are elevated in mobilized cells at the peak mobilization in STEMI patients. We examined the expression of genes of cardiac and endothelial differentiation in migrated BM cells isolated from the lower chambers of the chemotaxis assays. The expression of various primitive, cardiac and endothelial genes were significantly higher in cells isolated from the lower chambers as compared to the total population of cells placed in the upper chambers as examined by qRT-PCR (**Figure 25**). We also noted increased expression of CXCR4 in migrated cells that correlates with previous data supporting the interaction of S1P and CXCR4 in enhancing stem cell mobilization and homing (163, 179). According to these studies, the activation of S1PR1 leads to the initiation of downstream pathways that increase the response of BMSCs to SDF-1 gradients in G-CSF mobilized peripheral blood CD34+ stem cells (179). This data also corroborates our overall hypothesis that mobilized stem cells with increased CXCR4 expression can home to the myocardium that has elevated SDF-1 expression after myocardial ischemic injury.

Figure 25.

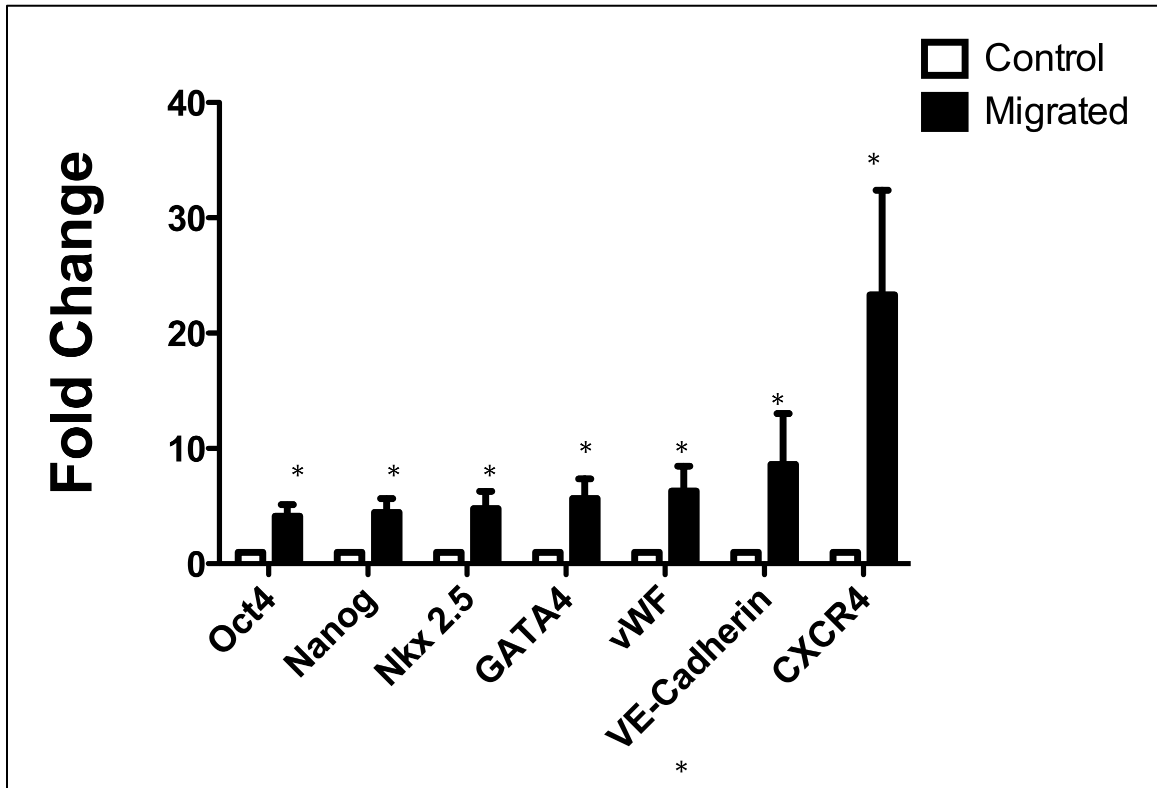

Figure 25. Migrated BM cells have higher expression of primitive, cardiac and endothelial genes examined by qRT-PCR compared to input cells. Bar graphs depicting the expression of primitive, cardiac, endothelial and CXCR4 genes in BM cells isolated form the lower chamber of the previously described chemotaxis chambers (Figure 21) (black bars) compared to input cells (white bars). The real time RQ-PCR was performed as detailed above. The figure demonstrates significantly higher expression of primitive (Oct4 and Nanog), cardiac (Nkx-2.5 and GATA4), endothelial (vWF and VE-Cadherin) and CXCR4 genes in migrated cells compared to input. Data were analyzed using Student's *t*-test and all differences expressed in fold change were significant with P value less than 0.05.

Ischemic cardiac tissues express cathelicidins that enhance responsiveness of circulating PB cells to an SDF-1 gradient

Our data highlighted the potential role of S1P and the dynamic expression of its receptors in the chemotaxis of non-HSCs from the BM to the PB in the setting of STEMI. However, the reduced expression of S1PR1 and S1PR3 following exposure to S1P levels similar to those encountered in the peripheral blood (~250 nM) in the early phases following AMI (**Figure 20**) suggest that other mechanisms than those involving bioactive lipids may be involved in their homing from PB to the myocardium. Moreover, the increased expression of CXCR4 in migrated cells point to possible contribution of the SDF-1/CXCR4 axis in the homing of mobilized cells to the infarcted myocardium. To support this further, previous experiments have indicated that S1P lyase activity is increased in the ischemic myocardium thus leading to S1P degradation, lower local levels of S1P and myocardial injury (176). Therefore, it is unlikely that S1P will play a major role in the homing of circulating non-HSCs to myocardium.

Several reports suggests the involvement of the SDF-1 that is upregulated in an hypoxia inducible factor-1α (HIF-1α) manner in infarcted myocardium in homing of stem cells to damaged heart tissues (186). However, since the injured myocardium is enriched in proteolytic enzymes (e.g., metalloproteinases - MMPs) (143) that degrade SDF-1 (51, 52), its actual chemotactic gradient is usually low. As we have recently demonstrated, despite SDF-1 degradation by MMPs, the chemotactic responsiveness of stem cells to even low SDF-1 gradient could be significantly enhanced by members of family of cationic antimicrobial

peptides (CAMPs) including products of complement cascade activation – anaphylatoxin C3a (187) and fibroblast- and leukocyte-derived cathelicidin and β-2 defensin (169). Given the above data, we investigated the expression and potential role of CAMPs in homing of non-HSCs into infarcted myocardium.

Our assessment of the complement cascade indicates that the time course of activation correlated with our observation of the peak elevation of the S1P (**Figure 16**). Thus, activation of complement may simultaneously release C3a in the plasma that enhances responsiveness of BM cells to low SDF-1 gradient. Next, we investigated whether the myocardium expresses two other CAMPs (cathelicidin and β2-defensin) of which expression is regulated by hypoxia. To address this question, we first employed the Langendorff apparatus (*ex vivo* cardiac reperfusion model) and murine cardiac ventricular tissues were subjected to ischemic/reperfusion injury (30 minutes ischemia followed by 20 minutes reperfusion). **Figure 26A** shows increased expression of both CAMPs in myocardial tissue following hypoxia/reperfusion as compared to control myocardium. We then examined cardiac fibroblasts subjected to hypoxia using a hypoxic chamber, and the expression of cathelicidin and β2-defensin was measured in parallel with expression of HIF-1. **Figure 26B** shows that both CAMPs were upregulated in hypoxia. Finally, we employed Transwell chemotactic assays to test the hypothesis that human PBSPCs respond to low gradients of SDF-1 in the presence of cathelicidin similar to our previous observations with HSCs (169). **Figure 26C** shows that human cathelicidin (LL37) enhanced the migration of PBSPCs towards low SDF-1 gradients.

Figure 26.

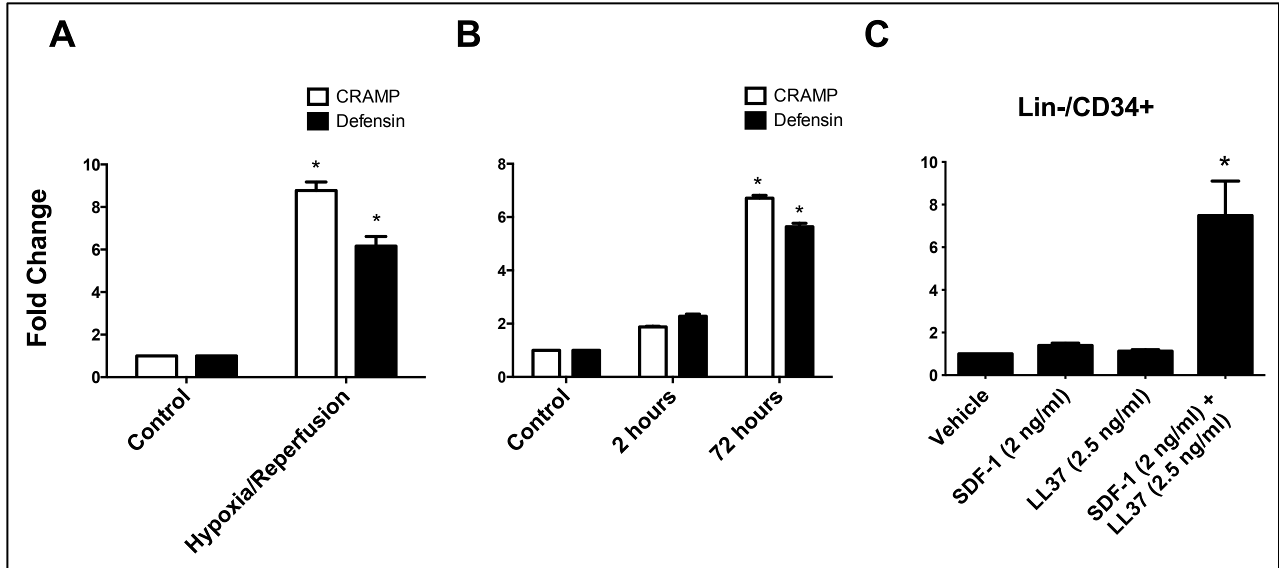

Figure 26. Cardiomyocytes increase the expression of cathelicidins following ischemic injury and cathelicidins prime BMSPCs to lower levels of SDF1. The gene expression of cathelicidins was assessed in the myocardium at baseline and after 40 minutes hypoxia followed by 40 minutes reperfusion using the Langendorf apparatus as well as cultured cardiac fibroblasts isolated using enzymatic digestion of WT cardiac tissues. Real time RQ-PCR was performed as previously detailed. Bar graphs showing the increased mRNA expression of murine cathelicidins related antimicrobial protein (CRAMP) and β2-Defensin in murine cardiac tissues with hypoxia/reperfusion (**Panel A**). The expression of CRAMP and defensin was also increased in cardiac fibroblasts subjected to 72 hours of hypoxia in hypoxic chambers followed by reperfusion as compared to 2 hours of hypoxia and controls (**Panel B**). Data were analyzed using the Student's *t*-test showing significant increase in cathelicidin expression. Peripheral blood cells were isolated form STEMI patients at 6 hours after the acute event and utilized in migration chambers. **Panel C** demonstrates the migration of human lineage negative (Lin-)/CD34+ positive cells towards RPMI medium supplemented with 0.1% FBS alone (Vehicle), or supplemented with SDF-1 (2 ng/ml) or LL37 (2.5 ng/ml) alone or the combination of SDF-1 and LL37 in the same concentrations. Data were analyzed using one-way ANOVA. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett test with appropriate with control samples as the control category (* $P < 0.05$ as compared to controls).

The role of bioactive lipids in BMSCs mobilization under physiological conditions and in disease

Elevated bioactive lipids in the plasma during metabolic syndrome and its effects on stem cell mobilization

Obesity is approaching epidemic levels in the western world (188) and is a well documented risk factor for coronary artery disease, myocardial infarction and sudden cardiac death both in men and women independent of other well documented known CAD risk factors (189). Interestingly, stable outpatients as well as hospitalized obese and overweight individuals with cardiomyopathy appear to have better survival compared to lean individuals (190-192). This finding is paradoxical because obesity has been shown to increase the risk of and worsen prognosis of other cardiovascular diseases. The underlying mechanisms behind this protective effect are poorly understood but have been postulated to relate to greater metabolic reserve and possibly different pathophysiology of the congestive heart failure. Adipose tissue has an increasingly recognized role as a paracrine and endocrine organ secreting multiple angiogenic factors such as VEGF and HGF as well as cytokines and chemokines (193). Furthermore, stem cells have been isolated from animal and human fat and their capability to differentiate into cardiomyocytes has been proven (194). There is also growing evidence that the metabolism of bioactive lipids is altered in adipocytes secondary to insulin resistance resulting in the elevation of serum sphingosine and S1P (**Figure 27**) (195). However, the role of these changes in altering the S1P gradient is not understood.

Figure 27.

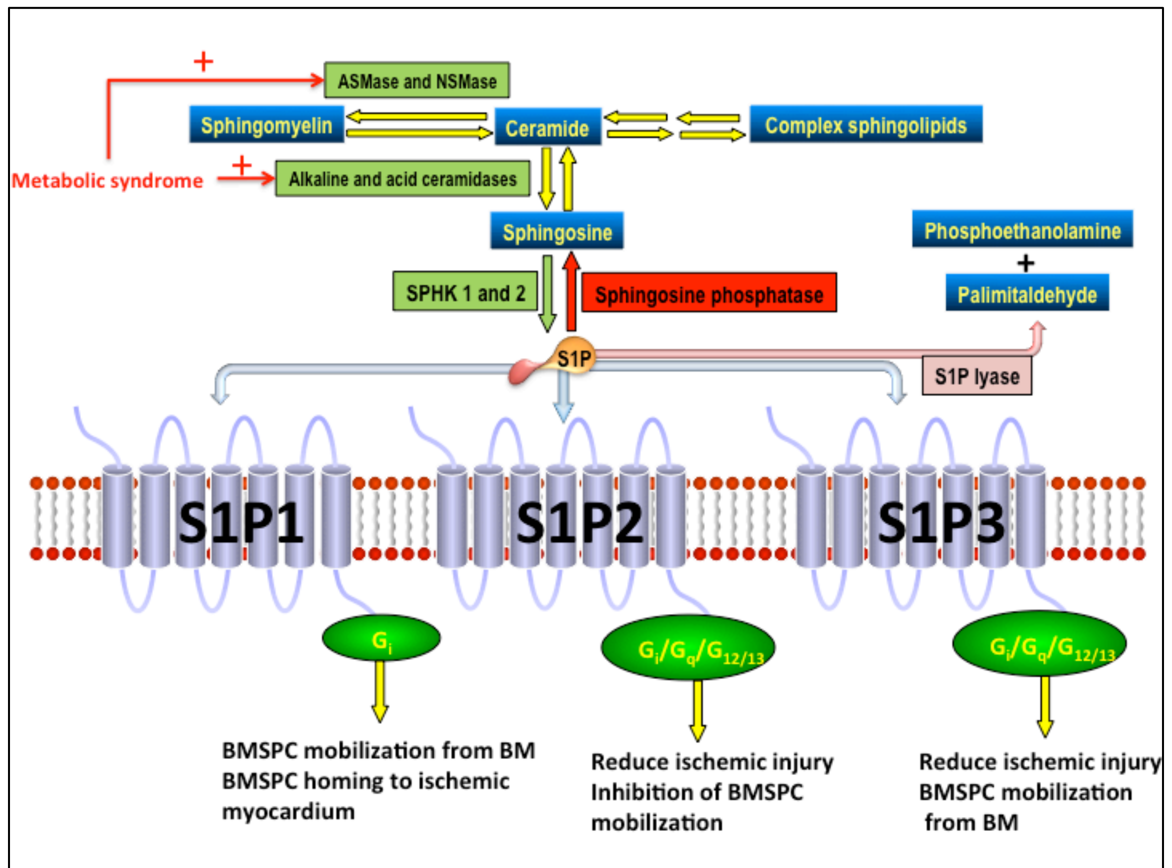

Figure 27. The effect of metabolic syndrome on the metabolism of bioactive lipids. Metabolic syndrome influences key regulatory enzymes involved in the metabolism of bioactive lipids. By stimulating acid and neutral sphingomyelinases as well as alkaline and acid ceramidases, substrates for the production of sphingosine and eventually sphingosine-1 phosphate are increased. This results in the elevation of S1P in adipose tissue and plasma (195).

Obesity in mice is associated with changes in bioactive lipid levels and corresponding changes in circulating BM-derived stem cells

Given the available literature suggesting the alteration in bioactive lipid metabolism and levels in obese mice, we examined their plasma levels in relation to circulating BM-derived stem cells. Male C57BL/6 mice were placed on high fat (HF, defined as 60% fat) or low fat (LF, defined as 10% fat) diet for 6 months and HF fed mice were significantly more obese than LF fed mice. At 6 months, mice were euthanized and blood samples were obtained from the renal vein. Plasma examination using mass spectrometry demonstrated significantly higher levels of S1P and ceramide-1 phosphate (C1P). This correlated with significantly higher numbers of circulating peripheral blood Sca1+/cKit+/Lin- (SKL) cells (**Figure 28**). This supports our data regarding the role of bioactive lipids in the mobilization of BM-derived stem cells. The implications of these findings are not currently understood. Interestingly, obese individuals have better prognosis following AMI and whether this is related to the effect of bioactive lipids on the myocardium or better regeneration due to higher levels of circulating stem cells is still to be examined.

Bioactive lipids and circulating BM-derived stem cells are higher in obese individuals after STEMI

Our data demonstrate higher levels of bioactive lipids and consequently circulating BMSCs under physiological conditions. However, changes in plasma bioactive lipids in the setting of STEMI have not been explored before. Plasma collected from obese and lean patients admitted with STEMI demonstrated

Figure 28.

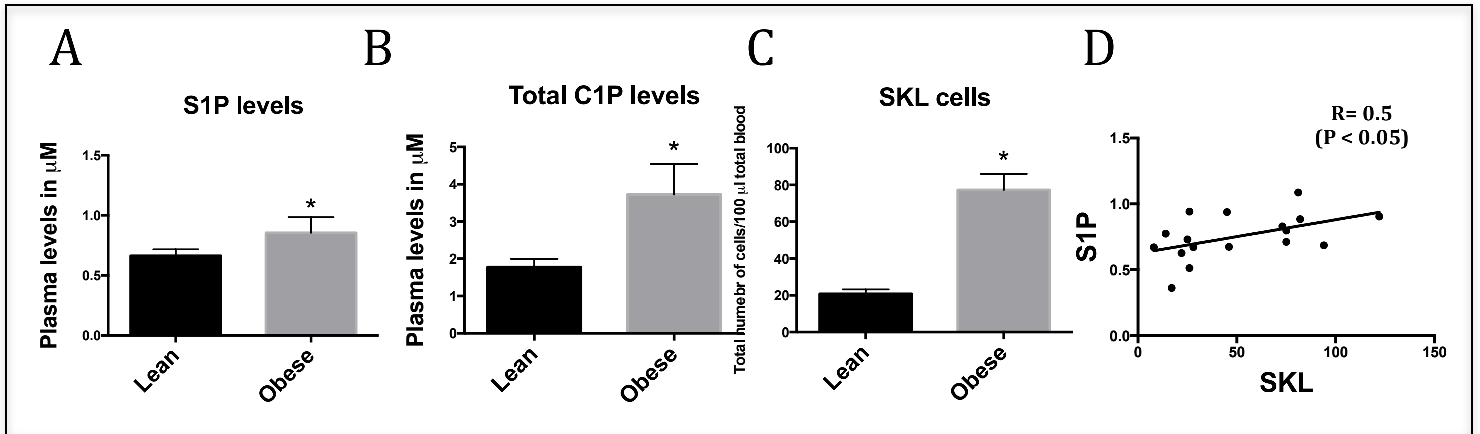

Figure 28. Elevated levels of bioactive lipids in obesity and their correlation with circulating murine stem cells. Mice were fed with high fat and low fat diet for 6 months and PB samples were obtained from obese and lean mice. Plasma was isolated after centrifugation at 1800 RPM and cells were lysed as detailed above. Plasma levels of bioactive lipids were assessed using mass spectrometry. PB cells were stained against lineage (FITC), Sca-1 (PE-Cy7) and cKit (PerCP) surface markers and analyzed using flow cytometry. Bar graphs demonstrating the plasma level of sphingosine-1 phosphate (**Panel A**) and ceramide-1 phosphate (**Panel B**) in lean and obese WT mice. The differences are more prominent in C1P than they are in the S1P levels. This correlates with significantly higher number of circulating Sca1+/cKit+/Lin- stem cells in obese mice (**Panel C**). Data were analyzed using the Student's *t*-test showing significant increase in bioactive lipids as well as circulating SKL cells in obese mice. The number of circulating Sca1+/cKit+/Lin- stem cells correlated with plasma levels of sphingosine-1 phosphate as assessed using simple linear regression analysis. C1P, ceramide-1 phosphate; S1P, sphingosine-1 phosphate.

Figure 29.

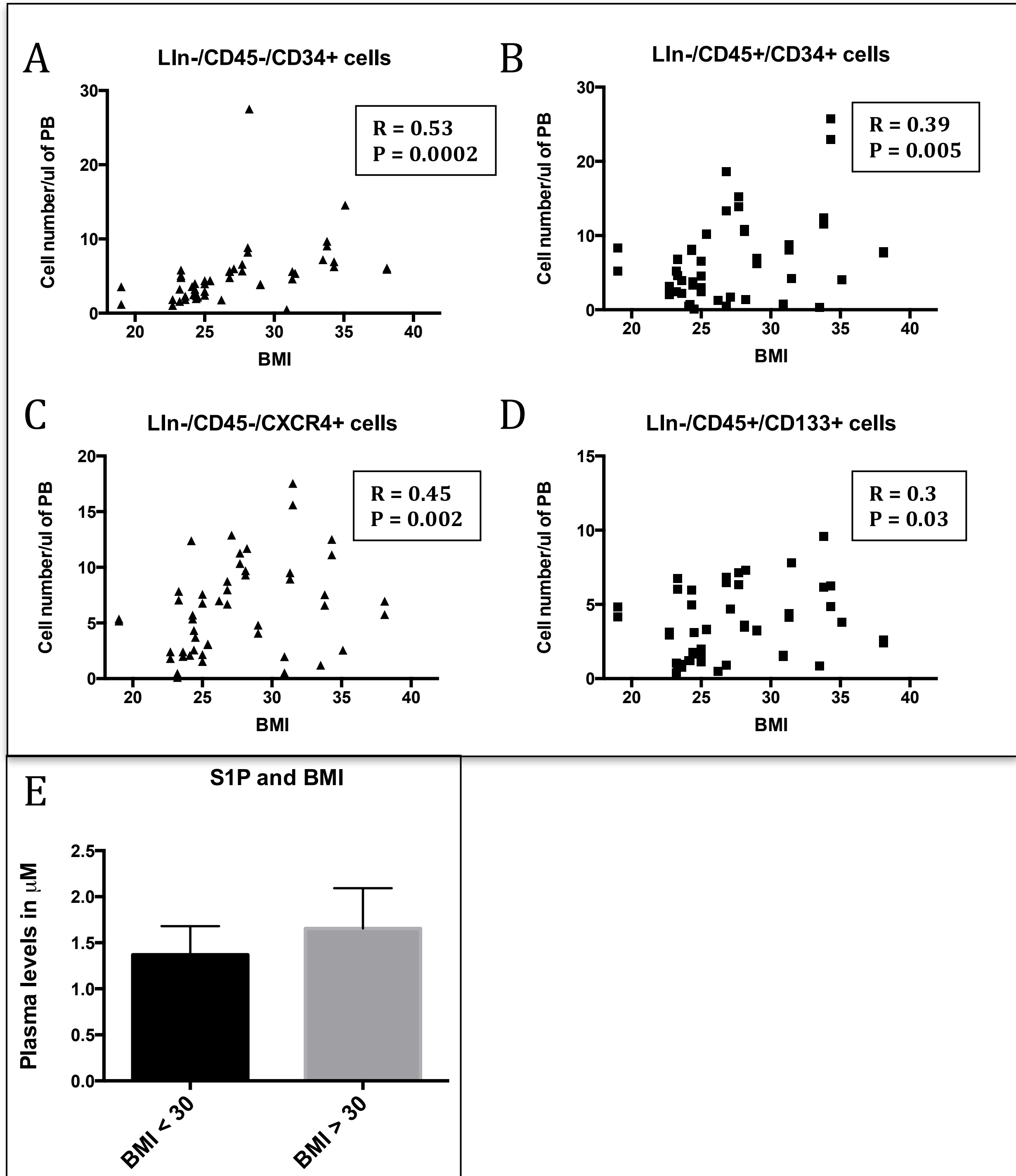

Figure 29. Circulating BMSCs correlate with body mass index in patients admitted with acute myocardial infarction. Circulating numbers of multiple populations of BMSCs correlate with the patients' body mass index (BMI) in patients admitted with STEMI. We isolated PB samples from patients with AMI with different BMI. RBCs were lysed using PharmLyse as detailed above and TNCs were stained against lineage markers, CD45 and multiple stem cell markers as previously described. The number of circulating Lin-/CD45-/CD34+, Lin-/CD45+/CD34+, Lin-/CD45-/CXCR4+ and Lin-/CD45+/CD133+ stem cells correlated with patient BMI as assessed using simple linear regression analysis. Overall, plasma levels of sphingosine-1 phosphate is higher among obese individuals, defined based on a BMI > 30, as compared to lean individuals with BMI < 30. We use the Student's *t*-test to assess the significance of this difference but it was not statistically significant. BMI; body mass index.

dynamic S1P levels. Obese individuals had higher peak plasma levels of S1P compared to lean individuals. The peak levels of S1P, and other bioactive lipids, were noted early after the acute event (around 6 hours following the emergency room presentation). This temporal trend correlates with our published data demonstrating the early mobilization of different hematopoietic and non-hematopoietic stem cells in the peripheral circulation (12). Moreover, the peak number of circulating BMSPCs following AMI correlated positively with body mass index (BMI) as shown in **Figure 29**. Obese individuals, with higher S1P levels had significantly higher number of circulating BMSPCs following AMI (**Figure 29**).

Ability of S1P lyase inhibitors to alter plasma levels of S1P and their effect on stem cell mobilization

The data presented in the above chapters indicate an important role of bioactive lipids, especially the more critically studied S1P in the mobilization of BM-derived stem cells in the setting of acute myocardial ischemia. Furthermore, current therapies targeting BM cell mobilization failed to demonstrate efficiency in treating ischemic heart disease due to multiple factors including their lack of specificity in the populations mobilized. The field of bioactive lipids has experienced many advances with emerging pharmacotherapies and designed molecules that target bioactive lipid metabolism and receptor expression. Based on our previous data, we hypothesized that increasing S1P levels in the plasma will create a strong chemotactic gradient that will increase the egress of BMSCs and the number of circulating PBSCs. For these experiments, we utilized

tetrahydroxybutylimidazole (THI) at a concentration of 25 mg/l in drinking water of wild strain (WT) mice for 24 hours. THI has been shown to inhibit S1P lyase, one of the major enzymes responsible for the irreversible degradation of S1P (**Figure 29**). At 24 hours after treatment, WT mice demonstrated elevated levels of plasma S1P in comparison to pre-treatment levels and controls in agreement with published literature (176) (**Figure 31**). A separate group treated with THI and AMD3100 to enhance the mobilization of BMSCs did not show significant differences compared to the group treated with THI alone in terms of plasma S1P levels. We included AMD3100 for its ability to create a proteolytic environment in the BM and reduce the interaction between BMSCs and osteoclasts in the BM niches. We have shown that this is an important step in initiating mobilization (86).

We simultaneously examined the number of circulating SKL cells in conjunction with the elevated levels of plasma S1P. Indeed, the number of circulating SKL cells were significantly higher at 24 hours after initiation of treatment in comparison with pre-treatment levels and controls. In contrast to our expectation, treating mice simultaneously with AMD3100 did not enhance the mobilization of SKL cells and the reasons behind this are not currently understood (**Figure 31**).

Bioactive lipids play a role in the differentiation of BM-derived stem cells

The role of bioactive lipids in the physiology of BM-derived stem cells extends beyond their mobilization and homing. We examine the role of S1P in the differentiation of mobilized stem cells isolated from the peripheral blood. Zhao

Figure 30.

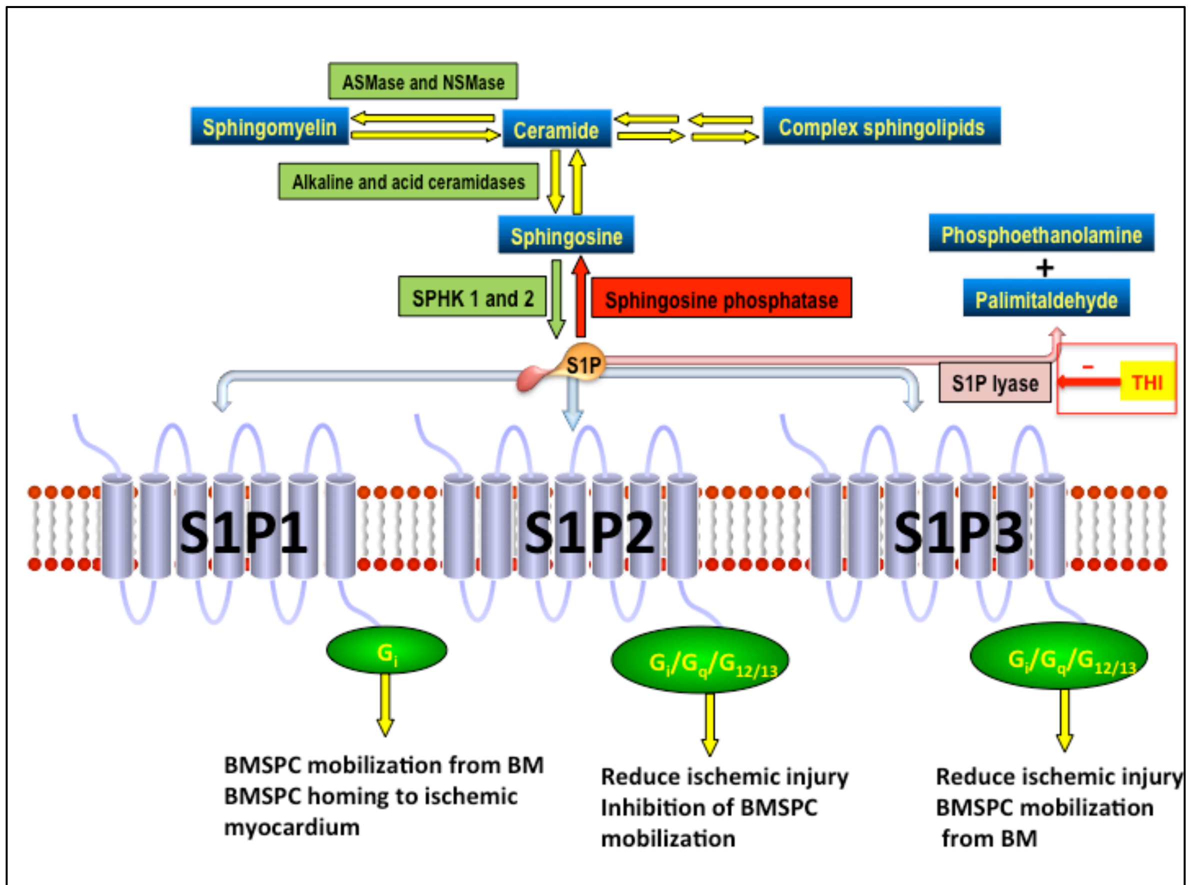

Figure 30. The effect of THI on the metabolism of bioactive lipids. Tetrahydroxybutylimidazole (THI) inhibits S1P lyase (SPL), a rate-limiting enzyme in the degradation of S1P. S1P lyase is responsible for the irreversible degradation of S1P. Due to the differential expression of SPL in PB and BM cells, systemic inhibition of SPL results in the elevation of S1P in adipose tissue and plasma (176).

Figure 31.

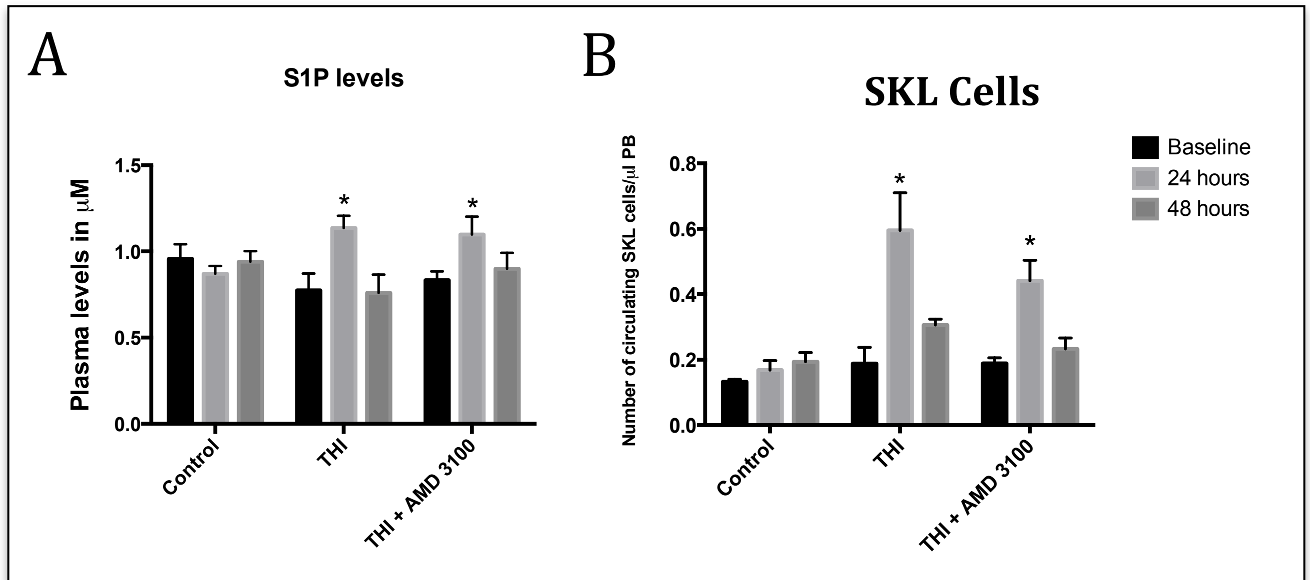

Figure 31. Inhibiting S1P lyase using THI increases plasma levels of S1P and the number of mobilized SKL BM stem cells. Bar graphs demonstrating the plasma levels of S1P in control, THI treated and THI+AMD3100 treated mice (**Panel A**) and the corresponding number of circulating SKL cells (**Panel B**). WT mice were given THI (25 g/L) in drinking water for 24 hours and PB samples were collected for assessment of bioactive lipid levels (using mass spectrometry) and number of circulating stem cells (assessed by flow cytometry). AMD treated mice were given 1 dose of AMD3100 (25 mg) subcutaneously at the beginning of THI therapy. Control mice were treated with regular water. S1P levels were significantly elevated at 24 hours after THI and THI+AMD3100 therapies and returned to baseline levels 24 hours after stopping therapy (48 hours time point). Elevated levels of S1P correlated with an increase in the number of circulating SKL cells in THI and THI+AMD3100 treated groups. Data were analyzed using one-way ANOVA. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett test with control samples as the control category.

et al examined the role of S1P in the differentiation of cord blood MSCs (196). After 10 days of culture in medium obtained from conditioned cardiomyocyte cultures and enriched with 1 μM of S1P, the authors noticed significantly higher numbers of MSCs expressing cardiac differentiation proteins and exhibiting the phenotypic characteristics of cardiomyocytes. Moreover, the authors were able to demonstrate the ability of the differentiated cells cultured in conditioned medium supplemented with S1P to generate electrical action potentials characteristic of cardiomyocytes, a hallmark of cardiomyogenic differentiation. Given the fact that cord blood cells are technically mobilized BMCs that are mobilized during the stress of late pregnancy and delivery, we examined similar populations of BM stem cells, namely mPB cells. After short incubation with S1P supplemented medium, mPB cells expressed cardiac and endothelial genes at the mRNA level (**Figure 32**). The fold increase ranged from 4-7 fold and was statistically significant compared to mPB cells cultured in DMEM medium supplemented with 10% FBS alone. The gene expression at 48 hours paralleled protein expression of cardiac and endothelial proteins at 4 weeks of culture. Moreover, cells acquired the characteristic phenotype of cardiomyocytes and endothelial cells (**Figure 32**). We further evaluated endothelial differentiation using the matrigel assay for endothelial tube formation. mPB cells, cultured in medium supplemented with S1P, were plated on matrigel for 6 hours and the ability to form endothelial tubes was assessed microscopically. Cells cultured with S1P supplementation showed significantly higher ability to form capillary tubes compared to controls.

Figure 32.

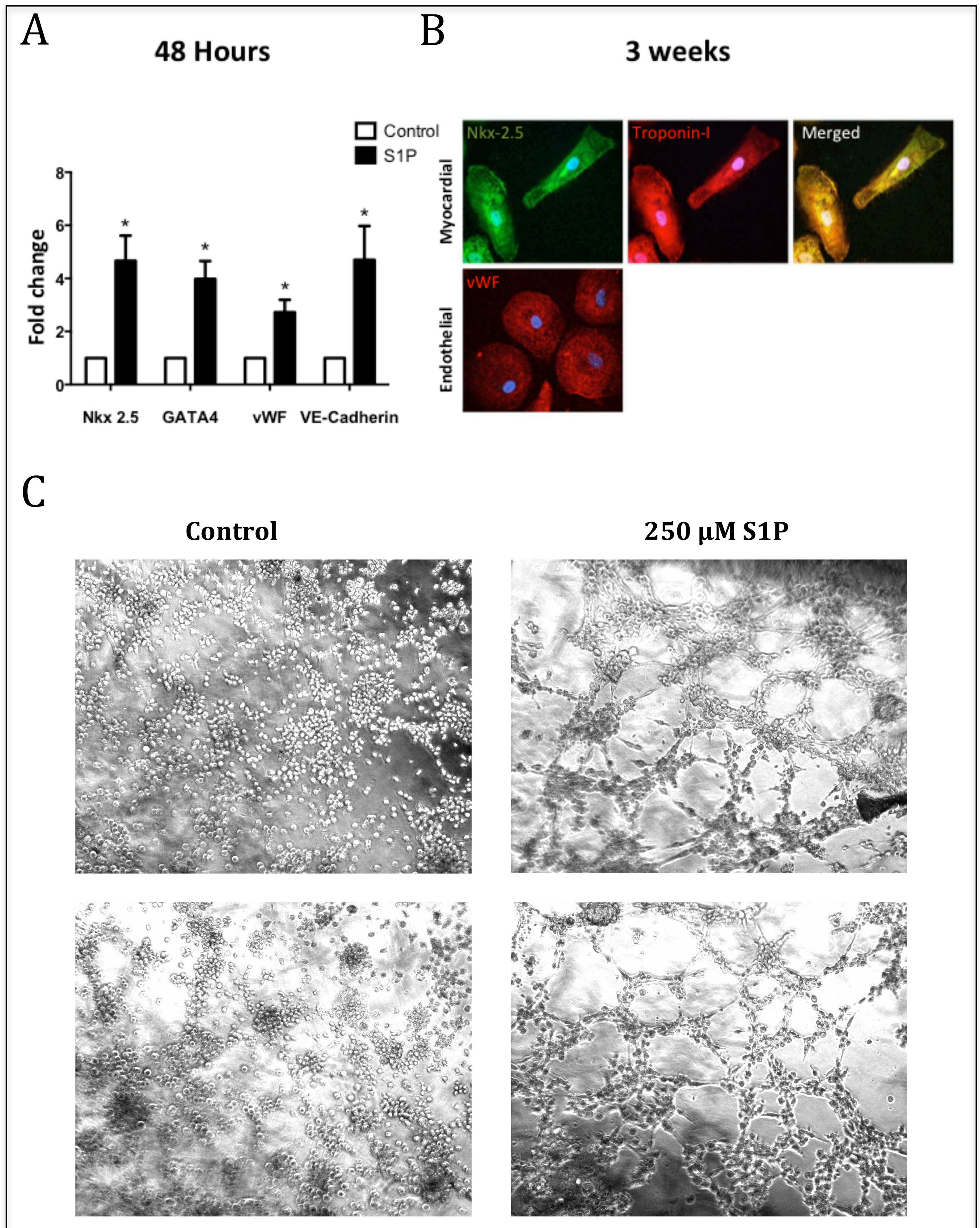

Figure 32. S1P plays an important role in the differentiation of mobilized peripheral blood cells. mRNA was isolated mobilized peripheral blood cells (mPBCs) at baseline and after 48 hours of culture in medium supplemented with 1 μM of S1P for 48 hours (**Panel A**). Cells were continued in culture for 4 weeks with S1P supplementation every 48 hours and at 4 weeks were stained for various cardiac and endothelial specific proteins. The mRNA upregulation was associated with cells acquiring the cardiac and endothelial phenotype and expressing specific cardiac and endothelial proteins at 4 weeks of culture in S1P supplemented medium (**Panel B**). mPBCs were also trypsinized and plated on matrigel to assess endothelial differentiation. The endothelial differentiation of mPBCs was confirmed by their ability to form capillary like tubular structure when cultured on matrigel for 6 hours (**Panel C**).

Conclusion and discussion

Acute myocardial infarction (AMI) initiates multiple innate reparatory mechanisms, including the activation of the complement cascade, that is responsible for the release of bioactive lipids such as sphingosine-1 phosphate (S1P) and ceramide-1 phosphate (C1P) from their natural reservoirs in red blood cells, platelets and local endothelial cells. In this chapter, we identified an important role of bioactive lipids in the mobilization of BMSPCs into the peripheral blood following acute myocardial infarction. Our experiments on human BM-derived stem cells isolated from naïve BM cells, mobilized peripheral blood and cord blood cells demonstrated dynamic expression of S1P receptors and the ability to respond to an elevated S1P gradients in the plasma. In our relatively large human sample, we observed elevation in the plasma levels of S1P and C1P early after the onset of AMI. S1P and its receptors play an important role in the mobilization of BMSPCs to the PB from their niches in the BM. We also demonstrated that AMI results in increased expression of cathelicidins in the myocardium demonstrating a potential role in priming circulating mobilized peripheral blood SPCs to physiological SDF-1 levels and hence potentially improving their homing to the ischemic myocardium. Taken together, these data suggest that while the levels of SDF-1 in the myocardial tissue may be influenced by the elevated levels of proteases, an increase in the level of CAMPs enhances the responsiveness of non-HSCs to a SDF-1 gradients and may potentially be aiding in their homing. Additional homing mechanisms for

mobilized BMSCs include locally elevated levels of C1P that act as a chemoattractant for BMSCs. These findings underscore the therapeutic potential of strategies targeting the modulation of bioactive lipids, cathelicidins and their receptors in BMSPCs based myocardial regenerative studies.

It is well known that S1P is transported in PB mainly by erythrocytes and is also associated with albumin and HDL (81, 197). Our data showed a 4-7 fold higher content of S1P and C1P in erythrocytes compared to plasma (**Figure 14**). Erythrocytes can take up and release S1P and this buffering function likely explains the ~25x higher concentration of S1P in PB as compared to tissues. Innate immune system activation following ischemic myocardial injury, including the complement cascade (171-175), may play an important role in the release of S1P from blood components such as activated platelets (198-200), red blood cells (81, 201) and endothelial cells (56, 202). We demonstrated for the first time that plasma levels of S1P and C1P are significantly elevated following AMI (**Figure 14**). The elevated plasma levels corresponded with activation of the complement cascade as evidenced by elevated plasma levels of C5b-9 (**Figure 16**). Furthermore, exposure of erythrocytes to activated complement ex-vivo resulted in the release of bioactive lipids (**Figure 15**), which could explain the correlation between elevated plasma levels of S1P in the setting of AMI and systemic complement activation. We also noted that at least some of the bioactive lipid release from RBCs is mediated by hemolysis as we have shown in **Figure 17**.

We have shown in the previous chapter the mobilization of BM non-HSCs following acute ischemic injury, which is in agreement with multiple reports in the literature (13, 22, 23, 25-27). Although the mobilization process has been postulated to be directed by a decrease in SDF-1–CXCR4 and VLA-4–VCAM-1 interactions in BM, we did not find corresponding dynamic changes in plasma levels of cytokines or correlation between these factors and the number of circulating BMSPCs. The complement cascade is activated locally at sites of myocardial infarction with elevated levels of C5b-C9 both in the myocardium and plasma (175) and we have shown elevated plasma levels following STEMI (**Figure 16**). Recent evidence suggests a role for C5b-C9 and other members of the complement cascade in the mobilization and homing of BM stem cells (56, 137, 138). Our experiments also indicate that the exposure of peripheral RBCs to activated complement results in the release of bioactive lipids and thus may explain the temporal correlation between the elevated levels of C5b-C9 and bioactive lipids in the plasma of patients with AMI (**Figure 16**). Taken together, these data support our hypotheses that AMI activates the complement cascade that in turn activates the release of bioactive lipids from RBCs.

The literature suggests an important role for bioactive lipids in the mobilization and homing of HSCs that express S1P receptors. However, there is no data on the role of bioactive lipids in the mobilization of BMSPCs in ischemic heart disease. The data shown herein supports an important role of bioactive lipids in the mobilization of BMSPCs following MI. We noted an elevated level of S1P and C1P in the plasma of MI patients shortly after the onset of myocardial

ischemia. These levels showed temporal correlation with the increased numbers of circulating BMSPCs suggesting a role for bioactive lipids in this mobilization. Furthermore, plasma isolated from AMI patients at peak BMSPCs mobilization was capable of chemoattracting BMSPCs in migration assays, a phenomenon that was blocked by delipidation of the plasma and selective S1PR1 and S1PR3 antagonists (**Figure 24**). Thus, our findings extend the role of bioactive lipids in myocardial ischemia to the mobilization and homing of BMSPCs.

Recently, S1P and C1P have been shown to be important mediators in the signaling cascades involved in apoptosis/survival, proliferation, stress responses and cell trafficking (203, 204). The majority of these actions are achieved through the interaction between S1P and one of its five receptors. We demonstrated here that non-HSCs such as Lin-/CD34+ stem cell populations enriched in VSELs express S1P receptors on their surface in a dynamic fashion (**Figures 19 and 20**). S1P receptor expression, especially that of S1PR1 and S1PR3, dictated the ability of stem cells to migrate towards S1P gradients (**Figure 19**). This expression was heavily influenced by the levels of S1P in the surrounding microenvironment and could be manipulated to enhance the migratory response of BM non-HSCs to S1P gradients (**Figure 20**). Indeed, mPB cells that were exposed to high levels of S1P in their microenvironment and did not express S1PRs could re-express S1PR1 and S1PR3 following incubation in S1P free medium which correlated with their improved chemotaxis to S1P in migration chambers. These findings can have very important implications in the fields of

clinical mPB and BM cell transplantation that clinically result in variable degrees of engraftment and success.

Studies mobilizing BM derived cells using G-CSF or transplanting BM-derived cells following ischemia-induced damage have faced limited engraftment and modest clinical success (6, 91, 93, 99, 119, 205, 206). Evidence from animal studies demonstrate that BM cells mobilized in the setting of AMI home to the myocardium but differentiate at very low rates to cardiomyocytes (14). Further evidence suggests that paracrine factors released from BMSCs such as cKit+ cells recruit and stimulate resident cardiac stem cells to proliferate, differentiate and repair the myocardium after ischemic injury (114). Regardless of the mechanisms of benefit, better engraftment of the transplanted cells is needed. Following myocardial infarction, there is elevation in the matrix metalloproteinases (MMPs) at the site of infarction as early as a few hours after the acute event (143). Elevated MMPs have been shown to degrade traditional chemokines such as SDF-1 (51) and Monocyte Chemoattractant protein (MCP) (52) among others thus lowering their chemoattractant activity. Recent evidence suggests a role for cathelicidins in priming BM-derived HSCs migration to lower levels of SDF-1 and their contribution to HSCs homing to the BM following irradiation injury (169). We demonstrated that CAMPs are overexpressed following myocardial ischemia in cardiac tissues as well as cardiac fibroblasts. In addition, the human CAMP-LL37 primed mobilized PBSPCs isolated from patients following AMI to low, yet physiological, levels of SDF-1 (2 ng/ml) (**Figure 26**). This is in agreement with other studies which showed that pre-incubating

endothelial progenitor cells with LL37 enhanced their homing and recruitment to areas of hind limb ischemia and the process of neovascularization (207). Taken together, these data support a potential role for CAMPs in the homing of BMSPCs to the ischemic myocardium by enhancing their sensitivity to lower levels of SDF-1. These findings may have important therapeutic implications in planning future BMSPCs based myocardial regenerative studies.

We further examined the levels of bioactive lipids and their effect on stem cell mobilization under physiological and pathological conditions as an extension to our work on their role in acute ischemic heart disease. Obesity and metabolic syndrome have been shown to alter the metabolism of bioactive lipids and increase the level of S1P in plasma and various tissues. We confirmed these findings in our experiments and demonstrated enhanced stem cell mobilization and a corresponding increased numbers of circulating SKL stem cells in obese mice compared to lean mice (**Figure 28**). Similar unique findings were also noted in obese individuals admitted with STEMI. The elevated plasma level of bioactive lipids and circulating stem cells in obese individuals may provide a mechanistic basis for the paradoxically enhanced survival of obese individuals with cardiomyopathy. The compelling data regarding a potentially beneficial role of bioactive lipids in acute ischemic heart disease led us to explore pharmacological pathways to increase their levels in plasma. Using tetrahydroxybutylimidazole (THI), we were able to temporarily increase the plasma levels of S1P. We demonstrate an elevated number of circulating SKL stem cells correlating with the elevated S1P levels. This confirmed its chemotactic role and opens the door

for future therapeutic manipulation in the management of patients with acute ischemic heart disease.

In conclusion, experiments detailed in this chapter highlight the potential role of bioactive lipids and cathelicidins in the mobilization and homing of BM derived cells to the ischemic myocardium with their potential role in cardiomyocyte chimerism. Multiple new therapies that modulate the plasma levels of S1P or S1P receptor expression are approved by the FDA and can be utilized in improving the mobilization of BM derived stem cells in myocardial ischemia. Similarly, priming of BM-derived cells with the human cathelicidin, LL37, can be used to improve their homing to the ischemic myocardium and thus overcome a major hurdle in stem cell regenerative myocardial therapies. We are currently examining both strategies in our laboratory to improve the mobilization and homing of BMSPCs to the ischemic myocardium.

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Chapter 4

Discussion

Chronic diseases or non-communicable diseases such as cardiovascular diseases and stroke account for a significant portion of mortality worldwide with high mortality among people under the age of 60. Out of all the chronic diseases, cardiovascular disease (CVD) remain the leading cause of death in the United States and the western hemisphere (1). While CVD encompasses a myriad of diseases including cardiomyopathy, hypertensive heart disease, heart failure, cor pulmonale, cardiac dysrhythmias, inflammatory heart disease, valvular heart disease, cerebrovascular disease (stroke), peripheral arterial disease, and ischemic heart disease (or coronary artery disease); ischemic heart disease (IHD) remains the biggest cause of morbidity and mortality worldwide being responsible for 12.2% of all deaths. Interestingly, US CVD death rates have declined from 1997 to 2007 by 27.8%, yet the burden of IHD or myocardial ischemia remains high. IHD caused 1 of every 6 deaths in the United States in 2007 (1). It has been estimated that 785,000 Americans will have a new IHD event every year, and ≈470,000 will have a recurrent attack. Approximately every 25 seconds, an American will suffer from a myocardial infarction (MI) brought on by IHD, and approximately every minute, someone will die of an MI (1).

Acute myocardial infarction (AMI) and the resultant IHD are often complicated with high mortality and poor overall prognosis (2, 3). The loss of cardiomyocytes is the hallmark of IHD followed by replacement with fibrous tissue, resultant cardiac remodeling and reduced heart function. All available therapies for IHD are symptomatic and/or palliative and there are no available therapies that can replace the dead myocardium except for heart transplantation that is hampered by multiple inherent limitations including the very limited availability of donor organs. Cardiomyocyte chimerism is an emerging concept indicating the capability of cardiomyocyte renewal (7, 9, 160); a process that is maintained, at least in part, by BM-derived stem cells (8). The underlying mechanisms of cardiomyocyte chimerism are poorly understood but this process is dynamic (10) and is capable of renewing up to half of the heart's population of cardiomyocytes during the normal life span (11). On the other hand, current myocardial regenerative therapies using transplantation of BM stem cell or their mobilization have achieved limited success in contrast to the success observed with innate mechanisms (11, 91, 97-99). Therefore, understanding the pathways involved in innate cardiomyocyte reparatory mechanisms would help establish a strong scientific framework for their utilization in successful human myocardial regenerative clinical trials.

AMI initiates innate reparatory mechanisms through which BMSPCs are mobilized towards the ischemic myocardium and contribute to myocardial regeneration as we and others have demonstrated (12, 13, 24, 26, 27, 161). However, very little is known about the underlying mechanism and clinical

significance of this mobilization. The role of the SDF-1/CXCR4 axis and other traditional chemokines in the mobilization and homing of BMSPCs during AMI is disputed and is believed to be less important than its role in HSCs mobilization and homing during physiological conditions (48-50). This can be explained, at least in part, by the active degradation of chemokines at the sites of inflammation and myocardial infarction by metalloproteinases (50-52). On the other hand, bioactive lipid mediators such as sphingosine-1 phosphate (S1P) and ceramide-1 phosphate (C1P) are resistant to proteases and exhibit potent chemotactic effects on SPCs. S1P lyase is upregulated in inflammation in cardiac tissue resulting in reduced cardiac levels. However, ceramidases are not influenced by MI and C1P levels are elevated locally following MI leading to a homing gradient for stem cells. In our studies, we demonstrated an important role for members of the inflammatory system, bioactive lipids and their receptors in the mobilization and homing of BM-derived stem cells in general and after acute myocardial infarction in particular.

Bone marrow pluripotent stem cells are mobilized during myocardial ischemia

Several reports have confirmed the mobilization of partially committed and committed stem cells originating from the BM in response to myocardial ischemic injury (24, 26, 27, 31, 106). Upon appropriate stimuli, BM-derived cells are mobilized in the circulation and migrate to the injured myocardium in a dynamic fashion following what was previously thought to be a cytokine gradient of SDF-1,

LIF and HGF (22, 208). In our initial experiments, we demonstrate consistent mobilization of BM pluripotent stem cell populations enriched in VSELs in peripheral blood of patients with acute myocardial ischemia (**Figures 5-7**). Mobilized PB cells strongly exhibit markers of pluripotency, cardiac and endothelial lineages (**Figure 8**) and hence can potentially contribute to the repair of the injured myocardium. Indeed, previous studies in humans and animals demonstrate the commitment of mobilized BM-derived stem cell populations enriched in VSELs and PSCs for myocardial and endothelial lineages (13, 150). Moreover, we observed the expression of these markers to be further up-regulated in sorted CD34+ cells only in the very early phase after the acute injury (**Figure 9**). The temporal reduction in the expression of these markers may be related to “back homing” of CD34+ and other selected subpopulations not incorporated in the myocardium. Of note, multiple human studies have successfully utilized BM-derived CD34+ cells in myocardial regeneration (128, 209).

Losordo and colleagues examined the role of G-CSF-mobilized CD34+ cells in the treatment of patients with severe refractory non-revascularizable coronary artery disease (128). Patients received CD34+ cell injections via the intramyocardial route with guided delivery in ischemic yet viable segments of the myocardium to maximize the benefit. Patients treated with CD34+ cell therapy had fewer angina symptoms, required less antianginal medications and had relevant improvement in exercise time compared to controls treated with standard of care therapy alone. In patients with acute ischemic heart disease,

Pasquet et al demonstrated the efficiency of G-CSF-mobilized CD34+ cells as well (209). Patients with AMI were treated with G-CSF mobilized CD34+ cells which expressed cardiac and endothelial cell characteristics in vitro. When transplanted through the intracoronary route, patients experienced significant improvement of multiple cardiac functional parameters. Importantly, there were no associated side effects with either CD34+ cell mobilization or transplantation confirming the safety of this approach.

The mobilization of PSCs appears to be related to the extent of myocardial ischemia and the degree of myocardial damage. The number of circulating VSELs enriched populations was highest in patients with ST-elevation myocardial infarction (STEMI), particularly in the early phases following the injury, when compared to patients with lesser degrees of ischemia such as non-STEMI (NSTEMI) and those with chronic ischemic heart disease (12). Moreover, the ability of patients to mobilize PSCs in the peripheral circulation in response to AMI decreases with age, reduced global LV ejection fraction (LVEF) and diabetes supporting the notion of an age/comorbidity related decline in the regenerative capacity (12, 13). Indeed, animal models confirm the reduction of number as well as pluripotent features of BM-derived VSELs with age (16). Similarly, studies have demonstrated a reduction in number as well as functional capacity of endothelial progenitor cells in diabetic patients (210).

The pluripotent features of mobilized VSELs, including the presence of octamer-binding transcription factor-4 (Oct4) and stage specific embryonic antigen-4 (SSEA4), were confirmed both on the RNA and protein levels. Utilizing

the capabilities of the ImageStream system, we demonstrated that circulating VSELs during AMI have very similar embryonic features similar to their BM and CB counterparts including the small size (7-8 μm), large nucleus and high nuclear-to-cytoplasm ratio (**Figure 5**). Furthermore, circulating VSELs during AMI express markers of early cardiac and endothelial progenitors which suggests that the mobilization is rather specific and that circulating VSELs are destined to aid in myocardial regeneration following injury (12, 13, 22). Indeed, there is evidence that the mobilization of CXCR4+ cells in the setting of AMI is correlated with LVEF recovery as well as myocardial reperfusion when assessed with cardiac MRI in humans (32). The pluripotent features observed in mobilized VSELs are similar to their counterparts isolated from the BM, cord blood and other organs in humans and animals suggesting a common origin.

Studies in sex-mismatched heart- and bone marrow-transplantation demonstrate the role of BM-derived cells in the chimerism of cardiomyocytes reaching 50% of the total cardiomyocyte count during the normal human life span (7, 11, 160). In a seminal paper, de Weger *et al*. demonstrated that donor BM-derived cells contribute to the chimerism of the recipient's myocardium as well as other organs such as the liver (8). Mobilized BM-derived cells can potentially be contributing to the reparatory mechanisms by reducing apoptosis and stimulating the resident cardiac stem cells rather than differentiating into cardiomyocytes (211). Fukuhara et al showed that BM cells mobilized following AMI home to the ischemic myocardial border with 10% of the cells residing in the infarction border being mobilized BM cells. The authors found a significant increase in newly

formed cardiomyocytes and dividing cells where the BM derived cells resided. Interestingly, a very small portion of these newly formed cardiomyocytes and actively dividing cells were derived from the mobilized BM cells. This data whcoh is also supported by other studies indicate that the majority of beneficial effects with BMSPCs are paracrine in nature rather than transdifferentiation (14).

The beneficial mechanisms for mobilized and transplanted stem cells in cardiomyocyte regeneration are poorly understood. The direct differentiation of BM cells into cardiomyocytes and vascular cells represent the logical explanation for the observed beneficial effects (212). However, there is limited evidence to support the capability of BM cells to trans-differentiate into major cardiac lineages in vivo (213, 214). The literature however supports other beneficial mechanisms such as inflammatory modulation, paracrine stimulation of angiogenesis or endogenous cardiac stem cells, reduction of apoptosis and ultimately inhibition of ventricular remodeling. Loffredo et al. (114) examined the effect of transplanting c-Kit positive BM cells from wild type mice into the infarct zone after myocardial infarction in a genetic fate-mapping model where only cardiomyocytes express the GFP protein. The authors observed spontaneous cardiomyocyte chimerism with cells from non-GFP progenitors. This process was further enhanced by transplanting BM-derived c-Kit positive cells resulting in improved regeneration, improved cardiac function and reduced scar size. Interestingly, the beneficial effects of c-Kit positive cells was not observed in the early phase after AMI, but rather required 8 weeks to be realized. The authors performed extensive analyses to track the fate of transplanted c-Kit positive cells and found no

evidence of trans-differentiation into cardiomyocytes. Furthermore, the survival of transplanted cells in the myocardium beyond 3 weeks was limited suggesting other mechanisms other than trans-differentiation to account for the beneficial effects. Similarly, the authors ruled out cell fusion as a mechanism for the beneficial effects. We observed similar findings following the transplantation of VSELs after AMI in our murine model. While there was significant attenuation in left ventricular systolic dysfunction and hypertrophy, reduction in adverse remodeling and scar size, and increased viable myocardium; the numbers of transplanted cells in the infarct and peri-infarct zones was small and does not explain the observed benefit. Thus, the beneficial effect of VSELs transplantation following AMI could be due to paracrine effects of VSELs such as reduction of apoptosis and/or activating resident cardiac stem cells (150).

Traditional chemokines do not explain BM stem cell mobilization

The number of circulating HSPCs increases in PB in response to systemic or local inflammation, strenuous exercise and stress, tissue/organ injury, and pharmacological agents. We have shown in the previous chapter the mobilization of BM non-HSCs following acute ischemic injury, which is in agreement with multiple reports in the literature (13, 22, 23, 25-27). Overall, the mobilization process has been postulated to be directed by a decrease in SDF-1–CXCR4 and VLA-4–VCAM-1 interactions in BM, reversal of the trans-endothelial chemotactic gradient between the BM microenvironment and plasma, activation of the coagulation cascade, and finally, as recently postulated, activation of the

complement cascade (CC) (49, 56, 168). Interestingly, many of the above mentioned mechanisms and cells are activated in the setting of acute myocardial infarction.

Lately, however, this paradigm was challenged by numerous observations supporting SDF-1–CXCR4-independent mobilization and homing of BMSPCs. Studies have shown that the plasma SDF-1 level does not always correlate with mobilization of BMSPCs (56, 57, 81, 215). While some in vivo studies have observed chemotaxis of BMSPCs in response to an increased SDF-1 gradient, the SDF-1 was administered in these assays at supraphysiological concentrations (100-300 ng/ml) (216, 217), which is about 100 times higher than the SDF-1 concentrations measured in human or murine biological fluids (218). Furthermore, BM preconditioning for BM transplantation such as after irradiation therapy as well as AMI induce upregulation of several proteolytic enzymes, such as metalloproteinase 2 (MMP-2), MMP-9, cathepsin G and neutrophil elastase, thereby proteolytically inactivating SDF-1 and CXCR4, effectively neutralizing the chemotactic activity of SDF-1 towards BMSPCs (51, 52). It is important to note that this proteolytic environment would promote HSPC mobilization by decreasing SDF-1–CXCR4-mediated retention (as well as attenuating VLA-4-CD106 interaction) in the BM, however SDF-1 homing would be impaired due to enhanced proteolytic degradation of SDF-1 (219-221). Together these observations imply that other, possibly protease-resistant chemoattractants are involved in HSPC mobilization in order to make up for the deficiency of the SDF-1 gradient between the BM and PB.

These data have important therapeutic implications. Studies examining the mobilization of BM stem cells have achieved limited success. This can be explained by the non-selective mobilization of BM stem cells and the limited role of SDF-1 and chemokines in their homing to the infarction border. Indeed, multiple animal studies have demonstrated a limited role of the SDF-1/CXCR4 axis in myocardial regeneration following AMI (50, 114). It is prudent to explore and utilize alternative mechanisms that may be involved in BMSPCs mobilization and homing to design successful BM based regenerative therapies.

BM stem cell mobilization is maintained by complex interplay between the immune system, bioactive lipids, chemokines and the BM niches

The complement cascade (CC) is an evolutionarily conserved regulatory mechanism for sensing and responding to inflammation and organ injury. Multiple mechanisms including tissue hypoxia/injury and exposure to microorganisms can activate the CC through the classical, lectin or alternative pathways. Activation of the complement cascade has been documented with the use of several BM HSCs mobilizing agents such as G-CSF, zymosan and AMD 3100 (139, 141, 222). Following CC activation, multiple fragments such as C3a, C5a, or the membrane attack complex (MAC) are produced, which modulate the mobilization and homing of BM HSCs albeit with variable effects (56). For instance, C3 (C3a or ${}_{desArg}C3$) cleavage fragments contribute to BM HSPC retention in BM niches. On the other hand, C5 cleavage fragments (C5a or ${}_{desArg}C5$) are involved in BM HSC egress from the BM, as evidenced in multiple animal studies (140, 168,

187, 223). C3 fragments are shown to enhance the integration of CXCR4 into lipid rafts thus increasing the responsiveness of stem cells to SDF-1. Furthermore, C3 fragments increase the cross talk between different proteins such as the guanine nucleotide triphosphates (GTPases) Rac1 and Rac2 which are crucial for engraftment/homing of stem cells. On the other hand, C5 fragments and the generation of the C5b-C9 membrane attack complex (MAC) activate BM myeloid cells which secrete proteolytic enzymes that are then responsible for degrading the SDF-1/CXCR4 and VCAM/VLA4 bonds between HSCs and their BM niches. The effect is to increase the permeability of the endothelial-PB barrier, releasing S1P from RBCs in the PB increasing the S1P gradient. All these actions culminate in the release of HSCs from the BM and their migration to the PB.

The complement cascade is activated locally as well as systemically following myocardial infarction with elevated levels of C5b-C9 both in the myocardium and plasma (175). We confirmed the activation of the complement cascade in the plasma following acute myocardial infarction. Our experiments also indicate that the exposure of peripheral RBCs to activated complement results in the release of bioactive lipids which may explain the temporal correlation between the elevated levels of C5b-C9 and bioactive lipids in the plasma of patients with AMI in agreement with the available literature (**Figure 17**). In addition to the role of CC activation in elevating plasma S1P levels, a potential role of increased myocardial tissue levels of C3a and C5a in homing BMSPCs to the ischemic myocardium need to be further explored. Taken

together, these data support our hypothesis that AMI activates the complement cascade that in turn activates the release of bioactive lipids from RBCs.

The resultant S1P gradient chemoattracts BM derived stem cells including non-HSCs populations enriched in VSELs from their BM niches to the PB. In agreement with this hypothesis, we noted correlation between the peak levels of S1P and peak mobilization of non-HSCs in our patient population. Moreover, plasma from AMI patients chemoattracted BMSPCs in an S1P/S1PR1 dependent fashion. However, homing of mobilized BMSCs follow different and more complex pathways. Our experiments and other studies have shown that S1P levels are reduced in the ischemic myocardium due to the activation of S1P lyase (176). In addition, exposure of BMSPCs to S1P levels comparable to plasma levels after AMI resulted in the reduced surface expression of S1PRs. Therefore, other mechanisms are responsible for BMSPCs homing and we think there are multiple and redundant pathways contributing to this phenomenon: i) elevated levels of C1P which we have shown to be a potent chemoattractant of BM stem cells such as MSCs, ii) elevated levels of cathelicidins and ß2-defensins enhance the response of BMSCs to SDF-1 levels by incorporating CXCR4 into lipid rafts of the stem cell membrane, and iii) locally elevated complement cascade fragments also enhance the response of BMSCs to even low levels of SDF-1. We therefore propose a new paradigm in which complex interactions between the complement cascade, immune system members and bioactive lipids orchestrate the mobilization and subsequent homing of BMSCs following AMI (**Figure 33**).

The role of bioactive lipids in stem cell mobilization was further confirmed by our experiments on S1P receptor expression. We confirmed the dynamic expression of S1PRs which responded very quickly to S1P levels in the surrounding microenvironment. Similar to the dynamic changes in the expression of S1PRs and their role in lymphocyte trafficking, S1PR expression on BMSPCs was reduced with elevated surrounding S1P levels. Thus, the expression of S1PRs and their response to S1P may play an important role in BMSPC mobilization and homing. However, we could re-express S1PRs by removing surrounding S1P and thus enhancing the response of BMSPCs to S1P gradients. These results may have very important implications in clinical BM and mPB transplantation therapies which show variable degrees of success. By enhancing the expression of S1PRs, we may be able to enhance the engraftment of BMSCs to the BM. Alternatively, examination of S1PR expression on BMSCs before transplantation may serve as a clinically relevant and easy to perform marker for the transplantation success.

Retention of transplanted BMSCs in the myocardium is poor with the average rate ranging from 2 to 7.5%. This may explain the limited success of clinical studies to demonstrate clinically relevant results. Our results point to possible pathways that can be further explored therapeutically to improve the outcomes of BM regenerative studies. Incubating stem cells with LL37 (cathelicidins) significantly enhanced their chemotaxis to low, yet physiological, levels of SDF-1 and this phenomenon could be further explored in clinical settings. Indeed, homing of EPCs towards ischemic hind limbs and the resultant

neovascularization has been enhanced by incubating EPCs with LL37 prior to their transplantation. This pathway has not been explored before in the myocardial infarction model and could provide a solution to poor engraftment. Further pathways that include byproducts of the activated CC system activation such as C3 fragments also could be explored in this setting (140, 168, 187, 223).

Figure 33.

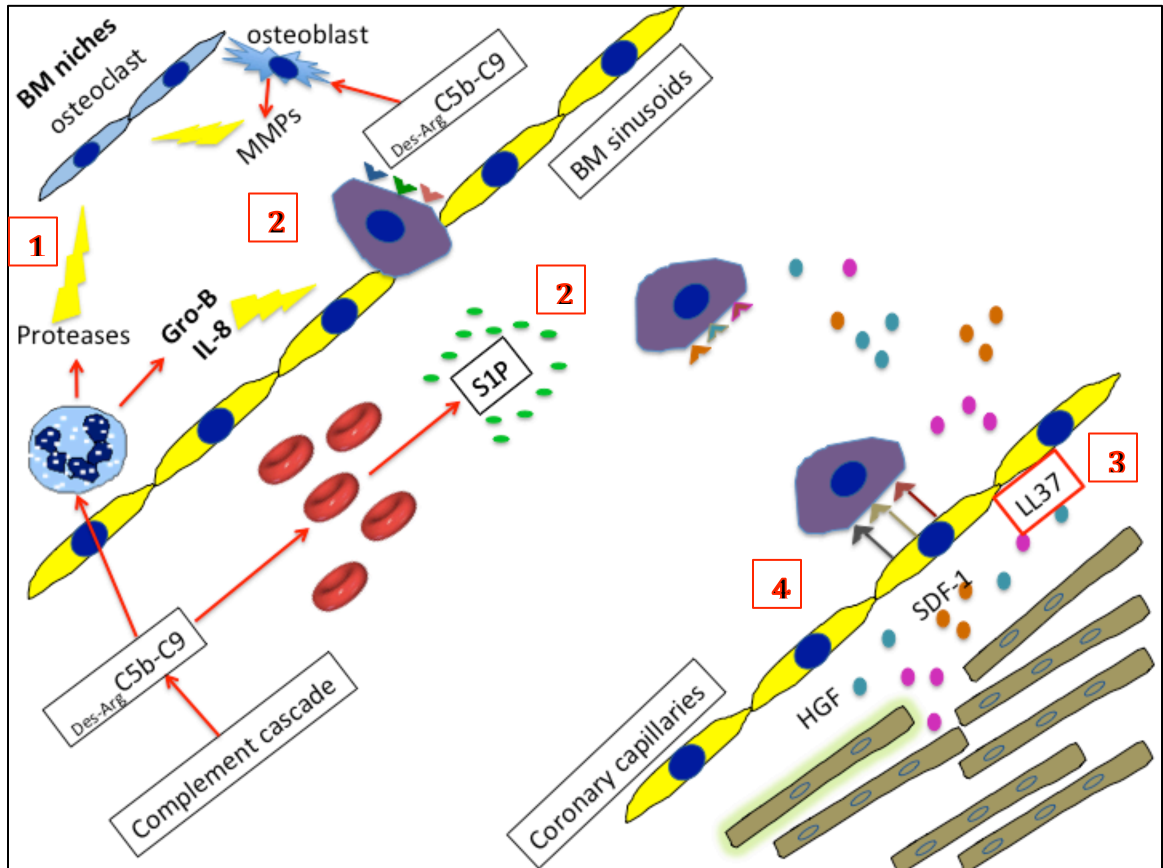

Figure 33. Sequence of events in BMSPC mobilization from the BM towards ischemic myocardium during MI. Acute MI initiates an inflammatory response resulting in release of proteases (by granulocytes and osteoclasts) in the BM (1) which proteolytically inactivate the SDF-1-CXCR4 interaction between BM osteoclasts and BMSPCs (2). The now released BMSPCs follow an increasing SDF-1 and S1P/C1P gradients to exit the BM niches into the PB. Acute inflammation also promotes release of cathelicidins (LL-37) which facilitate clustering of CXCR4 into lipid rafts thereby increasing their sensitivity towards lower levels of circulating SDF-1. Together, the increased sensitivity towards SDF-1 and S1P gradients facilitate BMSPCs homing towards ischemic myocardium.

Future directions

The above-mentioned data supporting the mobilization of PSCs and VSELs, enriched in cardiac and endothelial markers, in ischemic heart disease have multiple clinical implications. Circulating PSCs can be regarded as markers of ischemic injury in humans and as predictors for myocardial recovery following large ischemic damage. Indeed, studies in humans have demonstrated a clinical correlation between the number of circulating stem cells and recovery of left ventricular parameters after AMI (32). However, the clinical outcomes relevance of this mobilization is not fully understood. On the other hand, the therapeutic application of VSELs in myocardial regeneration has proven beneficial in animal models although the beneficial mechanisms remain elusive and are probably mainly paracrine in nature (150). Nonetheless, smaller numbers of the pluripotent VSELs (10,000 cells per mouse) have proven to be more beneficial than larger numbers of the more committed HSCs (100,000 cells per mouse) indicating their greater therapeutic potential (150). Moreover, ex-vivo expansion and priming of VSELs have proven to be a successful strategy in animal models and their clinical applications are pending (150, 224).

The role of bioactive lipids in BMSC mobilization can be further exploited using readily available FDA-approved therapeutics. The use of agents such as FTY720 in manipulating S1PR expression and response to S1P gradients may prove beneficial in myocardial regeneration following AMI. It is possible that enhancement of S1PR1/S1PR3 receptor expression can lead to enhanced mobilization of BM-derived stem cells including non-HSCs and the more potent

PSCs following AMI. We are currently planning animal studies utilizing specific receptor agonists for S1PR1 to enhance the mobilization and homing of BMSPCs to the injured myocardium following AMI. We will employ the selective S1PR1 agonist SEW2871 [5-(4-phenyl-5- trifluoromethylthiophen-2-yl)-3-(3-trifluoromethylphenyl)-(1,2,4)-oxadiazole (Calbiochem, La Jolla, CA)] at 20 mg/kg twice daily intra-peritoneal injection starting third day following AMI (225). Since S1PR2 inhibits chemotactic responsiveness of cells to S1P gradient (226, 227) and in order to increase BMSPC egress from BM in response to elevated plasma S1P gradient during AMI, we will block S1PR2 receptor on BMSPC by employing the S1PR2 antagonist, JTE-013 starting the third day after acute injury at 250 μM dose (228, 229). Dose response studies will be conducted to establish safety and timing for this compound by examining the peripheral blood cell count and circulating SKL cells as well as monitoring fro side effects at daily intervals for 1 week. The rationale for using JTE-013 is supported by *in vivo* strategies to increase migration of neural progenitor cells toward the area of ischemia (229) and the fact that S1PR2$^{-/-}$ macrophages accumulate at higher level in sites of inflammation (75). We envision that this antagonist will improve signaling of S1P through S1PR1 and S1PR3 receptors and therefore enhance mobilization and homing of BMSPCs to the myocardium in the setting of acute myocardial ischemia.

S1P lyase (SPL) is the rate-limiting step in S1P degradation leading to irreversible conversion of S1P into phosphoethanolamine and palimitaldhyde. Hence, SPL is a major regulator of the levels of S1P in the plasma as well as

different tissues (230). Interestingly, there is differential expression of SPL in BM cells, peripheral blood cells such as lymphocytes, endothelial cells and the lymphatic tissue. Borowsky et al described relatively low expression of SPL among hematopoietic cells in the BM in comparison to the higher expression in circulating lymphocytes and granulocytes (231). This differential expression suggests that systemic inhibition of SPL can result in higher gradients of S1P in the plasma thus facilitating the mobilization of BMSPCs. We have shown an almost 2-fold increase in plasma levels of S1P after 24 hours of tetrahydroxybutylimidazole (THI) (ST. Louis, MO) treatment. This correlated with a significant increase in circulating SKL stem cells at physiological conditions. Therefore, to better address the role of SPL inhibition in enhancing the mobilization and homing of BMSPCs to the injured myocardium, we will treat mice with at a dose of 25 mg/L THI + 10 g/L glucose in drinking water for 24 hours following AMI (Glucose is added to improve palatability of THI solution). We will examine the role of THI in enhancing the mobilization and homing of BMSPCs to the myocardium and its effect on myocardial functional recovery following myocardial infarction. Beside the above-described role of bioactive lipids in BMSCs mobilization, S1P plays a role in the differentiation of stem cells into cardiac and endothelial lineages which can enhance the regenerative potential of mobilized cells.

Improving the engraftment of mobilized as well as transplanted BMSPCs would be of great benefit in regenerative studies. Increasing the expression of cathelicidins and ß-defensins in the myocardium through gene therapy could

improve the homing of mobilized stem cells to the injured myocardium where the levels of SDF-1 are relatively elevated. Although this seems feasible, its clinical implications are limited due to the unpredictable nature of AMI. On the other hand, pre-incubating BMSCs with cathelicidins or C3a fragments of the CC pathway may be of great importance in increasing their retention in the infarction border following their transplantation and thus increasing their therapeutic potential. This approach has been successfully used in BM transplantation studies and has improved the engraftment of BM-HSCs to the BM stroma after transplantation into lethally irradiated mice (223). We are currently examining the engraftment of BMSPCs in the ischemic myocardium after incubation with various members of the immune system such as CC proteins (C3a and C5a) and cathelicidins in animal models.

Oxidative stress has been shown to impair the functional capacity of EPCs (232) and studies are underway examining the efficacy of factors mitigating oxidative stress such as eNOS overexpression in BMSC therapy for myocardial regeneration (233). Nuclear reprogramming to convert differentiated adult cells such as fibroblasts into induced pluripotent cells (iPS) has opened the door for creating patient-specific autologous pluripotent stem cells with multiple therapeutic opportunities (234). Further studies are needed to examine the feasibility as well as the safety of iPS particularly their tumorigenicity and immunogenicity before they can be explored in human studies.

On the biotechnology frontier, multiple modifications of the transplanted cells (priming) and the host environment are being tested in humans to improve

the efficiency of BMSCs' regenerative capacity. Researchers now have the tools to design three dimensional constructs that can be transplanted in the heart and provide a safe haven for attracting and homing transplanted and native resident stem cells to the sites of myocardial infarction and thus improving engraftment and retention (reviewed in Mooney et al (235)). These constructs can be supplemented with various cytokines and chemokines to enhance the engraftment and promote the survival and differentiation of stem cells. Most importantly, the concept of multiple doses of stem cells to repair a complex environment such as the myocardium after myocardial infarction is gaining more traction. While the field of stem cell regenerative therapy for ischemic heart disease is still in its infancy, the accelerated advances in a wide array of biological and biotechnological areas have rapidly propelled the field from the bench to clinical applications.

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Chapter 5

Materials and Methods

Human subjects

The study population consisted of 60 patients with acute ST-elevation myocardial infarction (STEMI). We enrolled 30 age- and sex-matched subjects into the control (CTRL) group, which is asymptomatic with no history of CAD but with a similar risk factor profile to the STEMI group. Patients with STEMI were referred within 12 h of symptom onset for primary percutaneous coronary intervention (PPCI). Patients were excluded if they had a systemic inflammatory process, cancer, recent motor vehicle accident, recent surgery, active infection, history of MI or revascularization (coronary artery bypass graft, PCI), unsuccessful revascularization, or onset of the symptoms >12 h. Peripheral blood (PB) samples were obtained at presentation in all patients (BSL) followed by samples at 6, 12, 24, and 48 after PCI and only PPCI patients were enrolled. BM samples were obtained from normal individuals and the BM cells were examined by smear and flow cytometry for any pathological findings before being utilized in the chemotaxis experiments. The study protocol complies with the Declaration of Helsinki and was approved by the institutional Ethics Committee. All patients provided written informed consent.

Flow cytometric analysis and sorting of circulating primitive stem cells from PB

Erythrocytes were lysed twice using BD PharmLyse lysing buffer (BD Biosciences, San Jose, CA) at room temperature for 10 min and subsequently washed in phosphate-buffered saline (PBS) to yield total nucleated cells (TNCs). TNCs were subsequently stained for hematopoietic lineages markers (Lin) using the following fluorescein isothiocyanate (FITC) conjugated antibodies (Abs) against human: CD2 (clone RPA-2.10); CD3 (clone UCHT1); CD14 (clone M5E2); CD16 (clone 3G8); CD19 (clone HIB19); CD24 (clone ML5); CD56 (clone NCAM16.2); CD66b (clone G10F5) and CD235a (clone GA-R2). These antibodies were purchased from BD Biosciences. The cells were simultaneously stained for the panleukocytic marker - CD45 (PE-Cy7 conjugated Abs, clone HI30; BD Biosciences) and one of the following antigens: CXCR4 (APC conjugated Abs, clone 12G5, BD Biosciences), CD34 (APC conjugated Abs, clone 581, BD Biosciences), CD133 (CD133/1; APC conjugated Abs, Miltenyi Biotec, Auburn, CA), S1P receptor-1 (PE conjugated Abs, clone 218713 RnD systems, Minneapolis, MN, USA), SSEA-4 (PE conjugated Abs, clone E025016, eBioscience, San Diego, CA), and S1PR3 (Biotinylated antibody with a streptavidin secondary antibody labeled with PE-Cy7, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Staining was performed in PBS with 2% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), at 4^0 C for 30 min. Cells were subsequently washed, re-suspended and analyzed using an LSR II (BD Biosciences). At least 10^6 events were acquired from each sample. The absolute numbers of PSCs and VSELs were calculated (individually for each patient) per 1µl of PB based on the percentage content of these cells detected by flow

cytometry and the absolute number of white blood cells (WBCs) per 1 μl of PB. FlowJo software was used for analysis (Tree Star, Ashland, OR).

Following lysis of erythrocytes, the populations of PB cells enriched in VSELs (Lin-/CD45-/CD133+, Lin-/CD45-/CD34+ and Lin-/CD45-/CXCR4+) were sorted using a multiparameter fluorescence-activated cell sorting (FACS) with a MoFlo cell sorter (Beckman Coulter, Fullerton, CA) according to previously published protocol (136) and used for immunocytofluorescence analyses. Similarly, total fractions of CD34+ cells were sorted for real-time RT-PCR (RQ-PCR) analysis of gene expression.

Murine experiments were conducted using similar techniques. Peripheral blood samples were obtained from the retro-orbital route and cells were stained against a lineage antibody mixture (FITC), CD90.1 (PerCP), CD45 (PE), CD117 (APC), and Sca-1 (PECy7). All antibodies were obtained from BD Biosciences. Staining was performed at 4^0 C for 30 min following by one-step lysis-fixation using BD Biosciences lysing-fixing buffer. Cells were subsequently washed, re-suspended and analyzed using an LSR II (BD Biosciences).

Imaging flow cytometric analysis with Image Stream system

PB-derived TNCs were isolated as detailed above. TNCs were fixed with 4% paraformaldehyde (Sigma Aldrich, St. Louis, MO) for 20 min, permeabilized with 0.1% Triton X-100 solution (Sigma Aldrich, St. Louis, MO) for 10 min and washed twice with PBS. TNCs were subsequently stained for intranuclear transcription factor Oct-4 using anti-mouse/human Oct-4 antibody (purified, clone

9E3, Millipore, Billerica, MA) for 2h at 37°C, followed by washing the incubation with the secondary anti-mouse IgG antibody conjugated with PE (BioLegend, San Diego, CA) for 2h at 37°C. Cells were further washed and stained for CD45 (FITC conjugated Abs; clone HI30, BD Biosciences), hematopoietic lineages markers (Lin, as detailed above) and CD34 (PE-Cy5 conjugated Abs; clone 581, BD Biosciences) or CD133 (biotin conjugated Abs, clone CD133/1, Miltenyi Biotec). Staining with biotinylated antibodies was followed with staining with streptavidin conjugated with PE-Cy5 (BD Pharmingen, San Jose, CA) to visualize the CD133 or CD34 expression. 7-aminoactinomycin D (7-AAD) was added for 10 minutes before analysis (BD Pharmingen; 40μM) to visualize nucleated objects. Samples were run directly on the Image Stream System (ISS) 100 (Amnis Corporation, Seattle, WA). Signals from FITC, APC, 7-AAD and PE-Cy5 were detected by channels 3, 4, 5 and 6, respectively, while side scatter and brightfield images were collected in channels 1 and 2, respectively.

Real-Time RT-PCR

To study mRNA levels for PSCs antigens PSCs (Oct-4, Nanog) as well as early myocardial (Nkx2.5/Csx, GATA4) and endothelial (vWF) markers, total mRNA was isolated using RNeasy Mini Kit (Qiagen Inc., Valencia, CA) and reverse-transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Measurements of mRNA levels of PSC, cardiac, and endothelial markers and β2-microglobulin were performed by RQ-PCR using an ABI PRISM 7000 Sequence Detection System (ABI, Foster City, CA). The 25

ul of reaction mixture contained SYBR Green PCR Master Mix, forward and reverse primers for specific gene. Primers were designed with Primer Express software. All of the primer sequences are provided in **Table 3**. The threshold cycle (Ct), i.e., the cycle number at which the amount of amplified gene of interest reached a fixed threshold, was subsequently determined. The relative quantification of Oct-4, Nonog, Nkx2.5/Csx, GATA4, vWF, VE-Cadherin and CXCR4 mRNA expression was performed with the comparative Ct method. Briefly, the relative quantification value of target gene, normalized to an endogenous control (β2-microglobulin gene) and relative to a calibrator, was expressed as $2^{-\Delta\Delta Ct}$ (fold difference), where Δ_C = C_t of target genes - C_t of endogenous control gene (β2-microglobulin), and $\Delta\Delta C_t$ = ΔC_t of samples for target gene - ΔC_t of calibrator for the target gene. To avoid the possibility of amplifying contaminating DNA: 1) all of the primers for RQ-PCR were designed with an intron sequence inside cDNA to be amplified, 2) reactions were performed with appropriate negative controls (template-free controls), 3) uniform amplification of the products was rechecked by analyzing the melting curves of the amplified products (dissociation graphs), 4) the melting temperature (T_m) was 57°C to 60°C, the probe T_m was at least 10°C higher than primer T_m.

Immunohistochemistry

Immunofluorescence identification of pluripotent specific transcription factors and intracellular proteins was performed on sorted stem cell populations enriched in VSELs. Briefly, cells were fixed with 4% paraformaldehyde for 10

minutes (Sigma Aldrich) then washed with PBS (Sigma Aldrich). Cells were permeabilized following fixation with 0.1% Triton-X 100 (Sigma Aldrich) for 10 minutes, blocked with 2% donkey serum (Jackson Immunoresearch laboratories, West Grove, PA) for 30 min and then stained with primary antibodies against Oct-4 (clone 9E3, Millipore) for 16 hours at 4^0 C. Primary antibodies were washed three consecutive times with PBS before secondary antibodies were added at a concentration of 1:100. Staining with secondary anti- mouse IgG antibodies conjugated with TRITC (Jackson Immunoresearch laboratories) was performed at 37^0 C for 2 hours and then cells were washed three times with PBS. Cells were additionally stained for SSEA-4 (FITC conjugated Abs, clone MC-813-70; BioLegend) and CD45 (biotin conjugated Abs, clone HI30, BD Biosciences) followed by incubation with streptavidin conjugated with Cy5 (BioLegend) and finally nuclei were stained with DAPI (Molecular Probes, Carlsbad, CA). All immunofluorescence photomicrographs were acquired using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Thornwood, NY).

Measurement of Blood Cytokine Levels

Blood samples were collected in the above mentioned time points both in STEMI patients and controls in EDTA tubes. Tubes were centrifuged at 2000 RPM for 15 minutes. Plasma was divided into aliquots and stored at -80°C. Plasma levels of stroma-derived-factor 1 (SDF-1α), granulocyte-colony-stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and stem-cell factor (SCF) were quantified using

the Luminex platform system: Milliplex Human Cytokine Kit (Millipore, Billerica, MA, USA) according to the manufacturer's protocols.

Mass spectrometry measurements of C1P and S1P

PB samples were obtained in EDTA tubes and plasma was isolated by centrifuging whole blood for 10 minutes at 800 x G. Supernatant was then removed and centrifuged at 9400 x G for 10 minutes to remove platelets and supernatant was then used for lipid measurements. RBCs were isolated using the leukocyte depletion kit (Pall Inc., East hills, NY, USA) and purified by centrifuging at 600 x g for 10 minutes followed by washing in normal saline at the same speed. To assess the effect of activated complement on bioactive lipids release, RBCs were incubated for 3 hours at 37^0C with saline, antibody against RBCs alone (BD Biosciences), normal human serum complement alone at 1:5 dilution (Quidel, Santa Clara, CA), or antibody and complement together. Lipids were extracted from plasma, supernatant and RBCs using acidified organic solvents, as we have previously described (236). Analysis of S1P and C1P was carried out using a Shimadzu UFLC coupled with an AB Sciex 4000-Qtrap hybrid linear ion trap triple quadrupole mass spectrometer in multiple reaction monitoring (MRM) mode. Detailed LCMSMS conditions for analysis of S1P were previously described in Selim et al (170). Various C1P species were separated using an Agilent Zorbax Eclipse XDB C8 column, 5 um, 4.6 X 150 mm column. The mobile phase consisted of 75/25 of methanol/ water with formic acid (0.5%) and 5 mM ammonium formate (0.1%) as solvent A and 99/1 of methanol/ water

with formic acid (0.5%) and 5 mM ammonium formate (0.1%) as solvent B. For the analysis of various C1P species the separation was achieved by maintaining 75% of solvent B for 3 min, then increasing to 100% B over the next 3 min and maintaining at 100% B for the last 18 minutes. The column was equilibrated back to the initial conditions in 3 min. The flow rate was 0.5 mL/min with a column temperature of *60^0C*. The sample injection volume was 10 uL. The mass spectrometer was operated in the positive electrospray ionization mode with optimal ion source settings determined by synthetic standards with a declustering potential of 46 V, entrance potential of 10 V, collision energy of 19 V, collision cell exit potential of 14 V, curtain gas of 30 psi, ion spray voltage of 5500 V, ion source gas1/gas2 of 40 psi and temperature of 550^0C. MRM transitions monitored were as follows: 562.436/264.1 for C12-C1P, 618.565/264.2 for C16-C1P, 730.8/264.2 for C24-C1P, 422.3/264.4 for C2-C1P, 590.4/264.4 for C14-C1P, 620.5/266.4 for DH16-C1P, 644.5/264.4 for C18-1 C1P, 646.5/264.4 for C18-C1P, 674.6/264.1 for C20-C1P, 702.7/264.4 for C22-C1P, 728.6/264.4 for C24-1 C1P, 784.7/264.4 for C26-1 C1P and 758.7/264.4 for C26-C1P.

Measurement of the complement cascade activation in plasma of AMI patients

Blood samples were collected at the above-mentioned time points both in STEMI patients and controls in EDTA tubes and placed immediately on ice. Tubes were centrifuged for 2000 RPM for 15 minutes. Plasma was divided into aliquots and stored at -80°C. Plasma levels of C5b-C9 were quantified using the

Luminex platform system: Complement quantification kit (BD Biosciences) according to the manufacturer's protocols.

Measurement of the hemoglobin level

RBCs were isolated using the leukocyte depletion kit (Pall Inc., East hills, NY, USA) and purified by centrifuging at 600 x g for 10 minutes followed by washing in normal saline at the same speed. To assess the effect of activated complement on bioactive lipids release, RBCs were incubated for 3 hours at 37^0C with saline, antibody against RBCs alone (BD Biosciences), normal human serum complement alone at 1:5 dilution (Quidel, Santa Clara, CA), or antibody and complement together. Hemolysis levels were analyzed by measuring the absorbance of plasma at 540nm as described elsewhere (237) and calculated as the percentage of hemolysis expressed as fold increase of hemoglobin in mobilized plasma as compared with normal plasma.

Chemotaxis assays

Cell migration assays were performed using the chemotactic (Boyden) chamber (Neuroprobe, Gaithersburg, MD). BM- and PB-derived cells were lysed as described above. Cells were then suspended in S1P free medium (RPMI with 0.1% FBS) for 3 hours prior to the migration assays. The lower chambers were loaded with controls or the testing agents. The cell suspension ($1x10^6$ cells/100 μl) was loaded into the upper chambers on a 5 μm membrane, and the chambers were incubated (37 ºC, 95% humidity, and 5% CO_2) for 3 hours, and subsequently cells in the lower chambers were harvested, stained against the

lineage markers, CD34 and CXCR4 as detailed above and counted by flow cytometry. The lower chambers contained no chemoattractant medium-vehicle (RPMI medium with 0.1% FBS, i.e. control) or plasma isolated from STEMI patients during the peak mobilization of stem cells. To examine the role of bioactive lipids in inducing BM-derived stem cell migration, simultaneous experiments utilizing charcoal-stripped plasma in the lower chamber were performed as previously described (238). Similarly to examine the role of S1PR1 in this mobilization, BM-derived cells were incubated with 10 μM of the selective S1PR1 receptor antagonist W146 at 10 μM (Cayman Chemicals, Ann Arbor, MI) or VPC23019 at 10 μM (Avanti Polar Lipids, Alabaster, AL) for 1 hour prior to the migration assay. To examine the role of LL37 on PBC migration, PB cells isolated from patients with AMI ($1x10^6$ cells/100 μl) were loaded in the upper chambers. The lower chamber was loaded with RPMI medium with 0.1% FBS supplemented with SDF-1 at 2 ng/ml (PeproTech, Rocky Hills, NJ) alone or LL37 at 2.5 ng/ml (AnaSpec, Fremont, CA) alone or the combination of both. Chemotaxis in these experiments was conducted as detailed above. All migration results are reported as fold change in migration compared to controls. To assess the migration of BM-derived stem cells and MSCs isolated from normal BM isolated from normal human donors and WT mice, BM cells were cultured in DMEM with 10% FBS for 3 passages then cells were detached with Trypsin-EDTA, washed in DMEM (or EBM), resuspended in DMEM (or EBM) with 0.5% BSA, and seeded at a density of 3×10^4 cells/well into the upper chambers of Transwell inserts (Costar Transwell; Corning Costar). The lower chambers were

filled with SDF-1 (2 or 300 ng/mL, R&D systems, Minneapolis, MN, USA), Sphingosine 1-phosphate (0.01. 0.1 or 1 μM, Cayman Chemical, Michigan, USA), C16-Ceramide 1-phosphate (1 μM, Avanti Polar Lipids, Alabaster, Alabama, USA), or C18-Ceramide 1-phosphate (0.1~10 μM, from bovine brain, Sigma, Sonicated in distilled water. 0.5% BSA DMEM or EBM (control). After 3 hours for BM cells and 6 hours for MSCs, cells from the lower chamber were collected, stained and quantified by flow cytometry.

Myocardial ischemia and cardiac fibroblast isolation experiments

Six C57/B6 mice were utilized in the myocardial hypoxia experiments. Hearts were excised and cannulated for the Langendorff apparatus, using perfusion buffer containing 4.7mM K^+ and 1.8mM Ca^{++}. The controls were allowed to beat for 50 minutes in warmed and oxygenated perfusion buffer then removed and left ventricles were flash frozen in liquid nitrogen. The ischemic hearts were hung and allowed to beat for 5 or 6 minutes with flow in the warmed and oxygenated perfusion buffer. The flow was stopped for 30 minutes and the hearts were bathed in the warmed buffer. Following ischemia, flow was restarted and the hearts started beating in about 0.5 to 1 minute after onset of flow and continued beating for 20 minutes to simulate reperfusion injury. The left ventricles from ischemic hearts were then flash frozen as detailed above. Frozen myocardial samples were utilized for the RQ-PCR experiments.

Cardiac fibroblasts were isolated from isolated hearts of euthanized C57/B6 mice. Left ventricular tissues were minced into small pieces (less than 1

mm in diameter) using razor blades. The minced left ventricular tissues were incubated with HBSS solution (Invitrogen, Carlsbad, CA) containing glucose, NACL, KCL and NaHCO3; and supplemented with dispase and collagenase B (Roche, Indiannapolis, IN) for 30 minutes followed by washing twice. The cell pellet was incubated overnight in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Thermoscientific, Waltham, MA) overnight. Floating cells were washed and adherent cells were allowed to grow to 70-80% confluence. Hypoxia experiments were conducted in hypoxia incubators where cells were maintained at <1% O2, 5% CO2 and 37^0C for either 2 hours followed by 1 hour reperfusion or 72 hours hypoxia followed by reperfusion. Cells were then harvested and flash frozen for RQ-PCR.

Animal models

The Institutional Animal Usage Committee of the University of Kentucky (IACUC) has approved the animal protocols utilized in this project. All protocols included in this dissertation comply with the PHS policies on humane care and use of laboratory animals (PHS). Wild type (WT) mice were fed low fat (LF, D12450B) or high fat (HF, 12492) diets for 6 months leading to obese and lean mice. Both diets were protein matched.

For experiments examining THI and its role in altering plasma S1P levels and SKL cell mobilization, THI was added to drinking water at 25 mg/l. 5.5 mmol/l glucose was added to the drinking water to improve palatability. AMD3100 was given subcutaneously at 5 mg/kg 24 hours prior to examining S1P levels and

circulating SKL numbers.

Statistical Analysis

Data are expressed as mean ± SEM. Differences were analyzed using the unpaired Student *t*-test or ANOVA (one way or multiple comparisons) as appropriate. Post hoc multiple comparison procedures (MCP) were performed using 2-sided Dunnett or Dunn tests as appropriate with control samples as the control category. The significance level throughout the analyses was chosen to be 0.05. All statistical analyses were performed using the SPSS (version 16) statistical software (SPSS Inc., Chicago, IL).

Table 3. Primers used for the qRT-PCR experiments.

	Forward	Reverse
Oct4	5'-GAT GTG GTC CGA GTG TGG TTC T-3'	5'-TGT GCA TAG TCG CTG CTT GAT-3'
Nanog	5'-GCA GAA GGC CTC AGC ACC TA-3'	5'-AGG TTC CCA GTC GGG TTC A-3'
Nkx2.5/Csx	5'-CCC CTG GAT TTT GCA TTC AC-3'	5'-CGT GCG CAA GAA CAA ACG-3'
GATA4	5'-GTT TTT TCC CCT TTG ATT TTT GAT C-3'	5'-AAC GAC GGC AAC AAC GAT AAT-3'
VE-Cadherin	5'-CCG ACA GTT GTA GGC CCT GTT-3'	5'-GGC ATC TTC GGG TTG ATC CT-3'
β2-microglobulin	5'-AAT GCG GCA TCT TCA AAC CT-3'	5'-TGA CTT TGT CAC AGC CCA AGA TA-3'

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CURRICULUM VITAE

Ahmed Abdel-Latif, MD, MS

Education

Jan 2009-Present	PhD student, Department of Immunology and Molecular Biology *University of Kentucky, Lexington, KY*
Aug 2005-May 2007	Masters of Science, School of Public Health, Clinical Investigator Track *University of Louisville, Louisville, KY*
Sept 1991-Jun 1998	Medical Degree, Medical School *Ain Shams University, Cairo, Egypt*
Jun 1990-Jun 1991	General Certificate of Education, Human Biology *University of London, England*

Professional Experience

Sept 2009-Present	**Assistant Professor,** Division of Cardiology, Department of Medicine *University of Kentucky, Lexington, KY*
Sept 2007- Sept 2009	**Interventional Cardiology Fellow** *University of Kentucky, Lexington, KY*
Sept 2004- Sept 2007	**Chief Fellow**, Division of Cardiology, Department of Medicine *University of Louisville, Louisville, KY*
Jan 2004- Sept 2004	**Research fellow**, Division of Vascular Medicine *The Cleveland Clinic Foundation, Cleveland, OH*
Jan 2000-Jan 2004	**Resident**, Categorical Internal Medicine Program *The Cleveland Clinic Foundation, Cleveland, OH*
Jul 1998-May 1999	**Resident**, Internal Medicine *Ministry of Health Teaching Hospitals, Cairo, Egypt*
Oct 1997-Nov 1997	**Clinical Clerkship**, Internal Medicine Department *Heidelberg University Hospitals, Heidelberg, Germany*
May 1997-Jun 1998	**Intern** *Ain Shams University Hospitals, Cairo, Egypt*

Community and Civic Experience

Sept 1999-Dec 2000	Medical Volunteer

The American Red Cross, Philadelphia, PA

May 1999-Sept 1999 Research Fellow, Cardiovascular Research
Illinois Masonic Medical Center, University of Illinois, Chicago, IL

Honors and Awards

Nov 2007 American Heart Association Travel Award for best abstracts

Jun 2007 Fellow of the Year Award
Division of Cardiology, University of Louisville

Jun 2007 Graduate Dean Citation Award for academic excellence
School of Public Health, University of Louisville

Mar 2007 American College of Cardiology/ Bristol-Meyers Squibb Travel Award

Jun 2004 Cash Memorial Award for best clinical research project
"Cross sectional study of the predictors of anticoagulation in nursing home residents with atrial fibrillation"
The Cleveland Clinic Foundation

Jun 1995 Pediatrics Department Prize for Student Research

Certifications and Licensure

Aug 2010 Registered Physician Vascular Interpretation (RPVI) certification

Nov 2008 American Board of Cardiovascular Diseases

Sept 2008 American Board of Cardiac and Vascular CT

Jun 2007 American Board of Echocardiography

Oct 2006 American Board of Nuclear Cardiology

Jun 2006 DEA certification

Jun 2005 Registered Vascular Technology (RVT) certification

Mar 2004 Permanent license to practice medicine, Kentucky

Aug 2004 American Board of Internal Medicine

Nov 2003 Scored in the 99th percentile in the in-service exam

Nov 2002 Scored in the 99th percentile in the in-service exam

Nov 2001 Scored in the 99th percentile in the in-service exam

Apr 2000 USMLE Step III

Oct 1998 USMLE certificate, valid indefinitely

Jun 1998 USMLE Step II

Oct 1997	USMLE Step I
Jun 1998	Permanent License to practice medicine, Cairo, Egypt

Presentations

- **Abdel-Latif A**, El Kabarity H. "Habitual Abortion: Review" Presentation in the annual student meeting of the department of Obstetrics and Gynecology. Cairo, Egypt, 1995.

- **Abdel--Latif A "**A case of Primary Pulmonary Hypertension." Presented in the Cleveland Clinic Internal Medicine Board Review, Cleveland, OH, 6, 2001.

- **Abdel-Latif A,** Jneid H, Braun W, Tubbs R. " A Case of Cyclosporin Induced Thrombotic Microangiopathy in a Renal Transplant Patient." Presented in the American College of Physicians (ACP) meeting, Cleveland, OH, 10, 2001.

- **Abdel-Latif A**, Messinger Rapport B. "Predictors of anticoagulation therapy in nursing home residents with atrial fibrillation". Presented in the American Geriatric Society meeting. Las Vegas, NV, 2004.

- **Abdel-Latif A**, Zuba-Surma E, Dawn B. "TGF-β1 Enhances Cardiomyogenic Differentiation Potential of Adult Primitive Cells". Presented in the ACC meeting, Atlanta, GA, 2006.

- Zuba-Surma EK, **Abdel-Latif A,** Kucia M, Hunt G, Ranjan S, Ratajczak MZ, Bolli R, Dawn B. Effects of Culture Conditions on Cardiomyogenic Differentiation and Expansion of Bone Marrow-Derived Tissue-Committed Stem Cells. Presented in the AHA meeting, Chicago, IL, 2006.

- **Abdel-Latif A**, Bolli R, Tleyjeh IM, Montori VM, Perin EC, Hornung CA, Zuba-Surma EK, Al-Mallah M, Dawn B. "Adult Bone Marrow-Derived Cells for Cardiac Repair: A Systematic Review and Meta-Analysis". Presented in the SCAI annual scientific sessions, Orlando, FL, 2007.

- **Abdel-Latif A**, Zuba-Surma E, Kucia M, Ziada KM, Syed MA, Mukherjee D, Hamdalla H, Smyth SS, Ratajczak MZ. "Evidence of mobilization of pluripotent and very small embryonic-like (VSEL) stem cells in patients with myocardial ischemia- a potential therapeutic target". Presented in the AHA meeting, New Orleans, LA, 2008.

- **Abdel-Latif A**, Mesgarzadeh P, Mukherjee D, Ziada KM. "Drug eluting stents in patients with end-stage renal disease: A systematic review and meta-analysis". Presented in the SCAI meeting, San Diego, CA, 2010.

- **Abdel-Latif A, Karapetyan AK, Kim C, Sunkara M, Morris AJ, Ratajczak MZ. "**Differential Responsiveness of HSPCs Harvested From Bone Marrow , Mobilized Peripheral Blood and Umbilical Cord Blood to the BM Homing Factors Stromal-Derived Factor-1, Sphingosine-1 Phosphate and Ceramide-1 Phosphate Is Related to the Desensitization of mPB and UCB HSPCs by S1P and C1P Present in mPB and UCB Plasma". Presented in the American Society of Hematology annual meeting, San Diego, CA, December 2011.

- **Abdel-Latif A**, Thomas J, Charnigo R, Imran S, Ziada KM. "Incidence of macular degeneration in veterans with ischemic heart disease". Presented in the AHA meeting, Orlando, FA, 2011.

- **Abdel-Latif A**, Karapetyan AK, Sunkara M, Selim S, Ziada KM, Smyth SS, Ratajczak MZ, Morris AJ. "Role for bioactive lipids and the immune system in the mobilization and homing of bone marrow derived stem cells in acute myocardial infarction". Presented at the ACC, Chicago, IL, March, 2012.

Publications

A. Peer-reviewed Manuscripts

1. **Abdel-Latif A,** Jneid H, Isada C, Francis G. "A 34 years old man with facial droop and dysarthria: A case of acute complicated infective endocarditis". **Cleve Clin J Med**. 2003; 70(7): 602-610.

2. **Abdel-Latif A**, Messinger-rapport B. "Should nursing home residents with atrial fibrillation be anticoagulated?". **Cleve Clin J Med**. 2004;71(1):40-44.

3. Wazni OM, Martin DO, Marrouche NF, **Abdel-Latif A**, et al. "Plasma B-type natriuretic peptide levels predict postoperative atrial fibrillation in patients undergoing cardiac surgery". **Circulation**. 2004; 110(2): 124-127.

4. Bolli R, **Abdel-Latif A**. "No pain, no gain. The useful function of angina". **Circulation**. 2005; 112: 3541-3543.

5. **Abdel-Latif A**, Peng X, Messinger-Rapport B. "Predictors of anticoagulation prescription in nursing home residents with atrial fibrillation". **J Am Med Dir Assoc**. 2005. 6(2):128-131.

6. Almahameed A, **Abdel-Latif A**, Graham LM. "Management of abdominal aortic aneurysms: treat the aneurysm and the risk factors". **Cleve Clin J Med.** 2005;72(10):877-888.

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10. **Abdel-Latif A**, Almahameed A, Lauer M. "Should we screen for abdominal aortic aneurysms?" **Cleve Clin J Med**. 2006; 73(1): 9-22.

11. Tarakji K, Bruncken R, McCarthy PM, Al-Chekakie O, **Abdel-Latif A**, et al. "Myocardial viability testing and the effect of early intervention in patients with advanced left ventricular systolic dysfunction". **Circulation**, 2006; 113:230-237.

12. Banchs JE, Dawn B, **Abdel-Latif A**, Stoddard M. "Acquired aortic cusp fusion following chronic left ventricular assist device support". **J Am Soc Echocardiog**. 2006;19:1401.e1-e3.

13. Zuba-Surma EK, **Abdel-Latif A,** Case J, Tiwari S, Hunt G, Vincent RJ, Ranjan S, Srour EF, Bolli R, Dawn B. Sca-1 expression is associated with decreased cardiomyogenic differentiation potential of adult primitive cells. **J Mol Cell Cardiol.** 2006; 41 (4):650-660.

14. Tleyjeh IM, **Abdel-Latif A**, Rahbi R, Scott CG, Bailey KR, Steckelberg JM, Wilson WR, Baddour LM. "A systematic review of population-based studies of infective endocarditis". **Chest.** 2007**;** 132 (3):1025-1035.

15. **Abdel-Latif A**, Bolli R, Tleyjeh IM, Montori VM, Perin EC, Hornung CA, Zuba-Surma EK, Al-Mallah M, Dawn B. "Adult Bone Marrow-Derived Cells for Cardiac Repair: A Systematic Review and Meta-Analysis". **Arch Intern Med**. 2007; 167 (10): 989-997.

16. Bhatt DL, Chew DP, Grines C, Mukherjee D, Leesar M, Gilchrist IC, Corbelli JC, Blankenship JC, Eres A, Steinhubl S, Tan WA, Resar JR, AlMahameed A, **Abdel--Latif A**, Hazen SL, Topol EJ. "Peroxisome proliferator-activated receptor γ agonists for the prevention of adverse events following percutaneous coronary revascularization – results of the **PPAR** study". **Am Heart J.** 2007; 154 (1): 137-143.

17. Zuba-Surma E, Kucia M, **Abdel-Latif A**, Lillard JW, Jr., Ratajczak MZ. "The ImageStream System: A Key Step to a New Era in Imaging". **Folia Histochem Cytobiol**. 2007; 45 (4): 279-290.

18. Zuba-Surma E, Kucia M, **Abdel-Latif A**, Dawn B, Brian Hall, Singh R, Lillard JW, Jr., Ratajczak MZ. "Morphological characterization of very small embryonic- like stem cells (VSELs) by image stream system analysis". **J Cell Mol Med**. 2008; 12 (1): 292-303.

19. Flaherty MP, **Abdel-Latif A**, Li Q, Hunt G, Ranjan S, Ou Q, Tang X, Johnson RK, Bolli R, Dawn B. "Noncanonical wnt11 signaling is sufficient to induce cardiomyogenic differentiation in unfractionated bone marrow mononuclear cells". **Circulation,** 2008; 117 (17); 2241-2252.

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30. Wiisanen ME, **Abdel-Latif A**, Mukherjee D, Ziada KM. "Drug eluting stents in saphenous venous graft interventions: A systematic review and meta-analysis". **JACC Cardiovasc Interv**. 2010; 3 (12): 1262-1273.

31. **Abdel-Latif A,** Moliterno DJ. "Protein C and S deficiency as a risk factor for stent thrombosis-When a rare disorder can predispose to rare events". **J Interven Cardiol**. 2010; 23 (6), 565-568.

32. **Abdel-Latif A**, Zuba-Surma E, Kucia M, Ziada KM, Cohn D, Kaplan A, Van Zant G, Selim S, Smyth SS, Ratajczak MZ. "Evidence of mobilization of pluripotent and very small embryonic-like (VSEL) stem cells in patients with myocardial ischemia". **Experiment Hematol.** 2010; 38 (12), 1131-1142.

33. **S**angalanamath SK, **Abdel-Latif A**, Bolli R, Xuan YT, Dawn B. "Hematopoeitic cytokines for cardiac repair: mobilization of bone marrow cells and beyond". **Basic Res Cardiol**. 2011 (Epub ahead of print).

34. Ratajczak MZ, Kim CH, **Abdel-Latif A**, Schneider G, Kucia M, Morris AJ, Laughlin MJ, Ratajczak J. "A novel perspective on stem cell homing and mobilization: review on

bioactive lipids as potent chemoattractants and cationic peptides as underappreciated modulators of responsiveness to SDF-1 gradients". **Leukemia**. 2011 (Epub ahead of print).

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37. **Abdel-Latif A**, Zuba-Surma EK, Ratajczak MZ. "Bone marrow derived pluripotent stem cells in ischemic heart disease: bridging the gap between basic research and clinical applications". Springer Book, 2011. (Epub ahead of print)

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39. Ruiz-Rodriguez E, Ziada KM, **Abdel-Latif A**. "Understanding stent thrombosis in the era of drug eluting stents". **Treatment Strategies-Thormbosis**, 2011. (Epub ahead of print)

40. Wallace EL, **Abdel-Latif A**, Charnigo R, Moliterno NJ, Brodie B, Matnani R, Ziada KM. Meta-analysis of long term outcomes for drug-eluting stents versus bare-metal stents in primary percutaneous coronary interventions for ST-elebvation myocardial infarction. **Am J Cardiol**, 2012; 109 (7): 932-940.

41. Yang F, Dong A, Mueller P, **Abdel-Latif A**, Smyth SS. "Coronary artery remodeling in model of left ventricular pressure overload is influenced by platelets and inflammatory cells". **PLos One**, 2012; 7 (8), e40196.

42. Jeevanantham V, Butler M, Saad A, **Abdel-Latif A**, Zuba-Surma EK, Dawn B. "Adult bone marrow cell therapy improves survival and induces long-term improvement in cardiac parameters: A systematic review and meta-analysis". **Circulation,** 2012: 126 (5): 551-560.

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44. Gross AK, Dunn SP, Feola DJ, Martin CA, Charnigo R, Li Z, **Abdel-Latif A**, Smyth SS. "Clopidogrel treatment and the incidence and severity of community acquired pneumonia in a cohort study and meta-analysis of antiplatelet therapy in pneumonia and critical illness. **J Thromb Thrombolysis**, 2012 (Epub ahead of print).

45. Kim CH, Schneider G, **Abdel-Latif A**, Sunkara M, Morris AJ, Kucia M, Ratajczak J, Ratajczak MZ. "Ceramide-1-phosphate regulates migration of multipotent stromal cells (MSCs) and endothelial progenitor cells (EPCs) – implications for tissue regeneration". **Stem Cells**, 2012 (In Press).

46. Klyachkin YM, **Abdel-Latif A**, Ratajczak MZ. “Novel aspects of stem cell mobilization during myocardial ischemia”. **US Cardiology**, 2012 (In press).

47. Jeevanantham V, Butler M, Saad A, **Abdel-Latif A**, Zuba-Surma EK, Dawn B. “Adult bone marrow cell therapy improves survival and induces long-term improvement in cardiac parameters: A systematic review and meta-analysis”. Response to the letter to the editor. **Circulation,** 2012: 126 (5): 551-560.

48. Karapetyan AK, Klyachkin Y, Zuba-Surma E, Kucia M, Ziada KM, Smyth SS, Ratajczak MZ, Morris AJ, **Abdel-Latif A**. “Bioactive lipids and cationic antimicrobial peptides as new potential regulators for trafficking of bone marrow derived stem cells in patients with acute myocardial infarction”. Submitted to **Stem Cells Dev**, 2013 (Epub ahead of print). PMID: 23282236

49. Schneider G, Bryndza E, **Abdel-Latif A**, Ratajczak J, Morris AJ, Ratajczak MZ. “Bioactive lipids sphingosine 1 phosphate and ceramide 1 phosphate are novel pro-metastatic factors in human rhabdomyosarcoma and their tissue levels increase in response to radiation and chemotherapy. Submitted to **Cancer Research**, 2012.

50. Bolli R, Tang XL, Sanganalmath SK, Rimoldi O, Mosna F, Loredo M, Gatti A, **Abdel-Latif A**, Jneid H, Dawn B, Bearzi C, Kajstura J, Leri A, Anversa P. “Intracoronary delivery of autologous cardiac stem cells improves cardiac function in a porcine model of chronic ischemic cardiomyopathy”. Submitted to **Circulation**, 2012.

B. Abstracts

1. Tleyjeh I, Ziada KM, Almahameed A, **Abdel-Latif A**, Lytle W, Yared JP, Topol E. “Perioperative Creatine Kinase Elevation is a strong Predictor of Early and Late Mortality after Coronary Bypass Grafting.” **J Am Coll Cardiol** 2002; 39(5)(Suppl A):436A.

2. Khot U, Alkotob L, **Abdel-Latif A**, Bruncken R, McCarthy P, Smedira N, Starling R. “Low Mortality in Ischemic Cardiomyopathy Treated with Coronary Artery Bypass Grafting Despite negative Viability Study.” **J Am Coll Cardiol** 2002; 39(5)(Suppl A):319A.

3. **Abdel-Latif A**, Jneid H, Tleyjeh I, Braun W. “Cyclosporine induced thrombotic microangiopathy in a renal transplant patient”. **J Gen Int Med** 2002;17(1)(Suppl 1):27.

4. Jneid H, **Abdel-Latif A**, Wolf J, Sneider D, Richter J. “A mentally retarded man with eighteen months of wasting and diarrhea”. **J Gen Int Med** 2002;17(1)(Suppl 1):50.

5. Ellis K, Ziada K, Viveacanathan D, **Abdel-Latif A**, Shaurraoui M, Martin D, Grimm R. “The negative predictor value of transthoracic echocardiography for left atrial appendage thrombus”. **Circulation** 2003; 108(17):482.

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8. **Abdel-Latif A**, Messinger Rapport B. "Predictors of anticoagulation therapy in nursing home residents with atrial fibrillation". Presented in the American Geriatric Society meeting. Las Vegas, NV, 2004.

9. Zuba-Surma EK, **Abdel-Latif A,** Case J, Tiwari S, Hunt G, Vincent RJ, Ranjan S, Srour EF, Bolli R, Dawn B. Sca-1 expression is associated with decreased cardiomyogenic differentiation potential of adult primitive cells. **Circulation** 2005;112(Suppl II):II-36.

10. Dawn B, Tiwari S, Hunt G, Guo Y, Stein AB, Zuba-Surma EK, **Abdel-Latif A,** Huang Y, Levent C, Thomas PD, Vincent RJ, Ildstad ST, Bolli R. Myocardial regenerative benefits of postinfarct cytokine therapy are attenuated in the absence of selectins. **Circulation** 2005;112(Suppl II):II-129.

11. Tiwari S, Guo Y, Hunt G, Zuba-Surma EK, **Abdel-Latif A,** Vincent RJ, Levent C, Bolli R, Dawn B. Enhanced recruitment of endothelial progenitor cells following ischemic preconditioning promotes angiogenesis, improves LV function, and attenuates adverse remodeling after myocardial infarction. **Circulation** 2005;112(Suppl II):II-228.

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15. Zuba-Surma EK, **Abdel-Latif A,** Kucia M, Khayat M, Hunt G, Ranjan S, Ratajczak MZ, Bolli R, Dawn B. Effects of Culture Conditions on Cardiomyogenic Differentiation and Expansion of Bone Marrow-Derived Myocardial Tissue-Committed Stem Cells. **Circulation** 2006; 114: II-170.

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27. Jeevanantham V, Butler M, Saad A, **Abdel-Latif A**, Zuba-Surma A, Dawn B. "Impact of Bone Marrow Cell Therapy on Left Ventricular Function, Structure, and Clinical Outcomes in Patients with Ischemic Heart Disease: A Meta-Analysis". **Circulation**. 2011; 124 (21 S).

28. Thomas J, Mohammed SI, **Abdel-Latif A**, Ziada KM. "Age related macular degeneration and coronary artery disease: Evidence of an association in the US veterans population". **Circulation**. 2011; 124 (21 S).

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C. Book Chapters

1. **Abdel-Latif A,** Jeha L, Tsao B. "A 78 years Old Female with Acute Progressive Lower Extremity Weakness: A Case of Guillain Barre Syndrome." **The Cleveland Clinic Book of Clinical Vignettes**. William and Wilkins, 2002. p. 860-873.

2. **Abdel-Latif A,** Ziada KM. "Vascular Ultrasound in the diagnosis and management of patients with peripheral vascular disease." **Manual of peripheral vascular intervention**. Lippincot, Williams, and Wilkins. 2010 (In Press).

3. **Abdel-Latif A**. "Multivessel percutaneous coronary interventions". **CathSAP 4**. American College of Cardiology, 2012 (In Press).

Multicenter Clinical Trials

Sub-Investigator for the following

- **AQUARIUS -** A 104 week, randomized, double-blind, placebo-controlled, parallel-group, multicenter study to evaluate the efficacy of aliskiren on the progression of atherosclerosis in patients with coronary artery disease when added to optimal background therapy

- **CYPRESS -** A Prospective, Randomized, Multi-Center, Double-Blind Trial to Assess the Effectiveness and Safety of Different Durations of Dual Anti-Platelet Therapy (DAPT) in Subjects Undergoing Percutaneous Coronary Intervention with the CYPHER® Sirolimus-eluting Coronary Stent (CYPHER® Stent

- **dal-OUTCOMES** - A phase III, double-blind, randomized placebo-controlled study, to evaluate the effects of dalcetrapib on cardiovascular (CV) risk in stable CHD patients, with a documented recent Acute Coronary Syndrome (ACS)

- **INNOVATE-PCI** - A Randomized, Double-Blind, Active-Controlled Trial to Evaluate Intravenous and Oral PRT060128, a Selective and Reversible P2Y12-Receptor Inhibitor, vs. Clopidogrel, as a Novel Antiplatelet Therapy in Patients Undergoing Non-Urgent Percutaneous Coronary Interventions

- **Jomed Jostent** - Use of the Jomed Jostent for Management of Coronary Perforation During Catheter Interventional Coronary Procedures

- **PVD Database** - Outcome Assessment in Peripheral Vascular Disease

- **The PARIS Registry** - Patterns of Non-Adherence to Anti-platelet Regimens In Stented Patients An Observational Single Arm Study

- **REVEAL** - Randomized, Multi-Center, Double-blind, Placebo Controlled Trial of the Effects of Erythropoietin on Infarct Size and Left Ventricular Remodeling in Survivors of Large Myocardial Infarctions

- **SATURN:** Study of Coronary Atheroma by Intravascular Ultrasound: Effect of Rosuvastatin Versus Atorvastatin - A 104-week, randomized, double-blind, parallel group, multi-center Phase IIIb study comparing the effects of treatment with rosuvastatin 40mg or atorvastatin 80mg on atherosclerotic disease burden as measured by intravascular ultrasound in patients with coronary artery disease

- **SOLSTICE**: A Study Of LoSmapimod Treatment on Inflammation and InfarCt SizE. A randomized, double-blind, placebo-controlled study to evaluate the safety of 12 weeks of dosing with GW856553 and its effects on inflammatory markers, infarct size, and cardiac function in subjects with myocardial infarction without ST-segment elevation

Completed Research Support

Microarray Pilot Project Jan 2010 - Jan 2011
Title: "Gene Expression of Mobilized Bone Marrow-Derived Stem Cells"
Objective: Understand the gene expression profile of circulating bone marrow-derived stem cells following myocardial ischemia
Role: Principal Investigator

Center for Clinical and Translational Science Pilot Project Jul 2008 - Jun 2010
Title: "Mobilization of Bone Marrow-Derived Very Small Embryonic Like Stem Cells in Myocardial Ischemia"
Objective: Understand the mobilization pattern of very small embryonic like stem cells (VSELs) in different myocardial ischemic scenarios
Role: Co-Principal Investigator. PI: Khaled Ziada

Center for Clinical and Translational Science Pilot Project Jul 2010 - Jun 2012

Title: "Role of bioactive lipids and their receptor expression in the mobilization of bone marrow derived stem cells after myocardial ischemia"
Objective: Understand the role of sphingosine-1 phosphate and its receptors in orchestrating the mobilization and homing of bone marrow derived stem cells after cardiac ischemic injury
Role: Principal Investigator

Ongoing Research Support

UK College of Medicine Clinical Scholar Program Jul 2011 - Jun 2014

Title: "Examining the dynamics and clinical significance of stem cell mobilization in patients with ischemic heart disease"
Objective: Understand the stimuli, dynamics and significance of stem cell mobilization in humans with ischemic heart disease
Role: Principal Investigator

Society and Professional Memberships

1998 Egyptian Medical Syndicate

2001 American College of Physicians

2004 American College of Cardiology

2004 American Heart Association

Editorial Activity-Reviewer

- Stem Cells
- Cell
- Journal of Molecular and Cellular Cardiology
- Journal of Interventional Cardiology
- Catheter and Cardiovascular Interventions
- Journal of the American Medical Directors Association

Editorial Activity-Assistant Reviewer

- Circulation
- Circulation Research

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