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New approaches in cardiac regeneration for heart failure treatment

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Alla mia famiglia

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Preface

Cardiovascular diseases are the primary cause of death in industrialized world. Despite significant advances in the management of heart disease over the last two decades, there remains an obviously unmet clinical need in treating this large and expanding patient population. The development and progression of cardiovascular diseases are complex, dynamic and time dependent processes that involve a variety of pathophysiological modifications that contribute to adverse cardiac remodelling. The most common manifestations are hypertension, coronary artery disease, heart failure and stroke. Heart failure is a leading cause of mortality. Heart failure is dictated by an uncompensated reduction in the number of viable and fully functional cardiomyocytes. The prevalence of heart failure in industrialized nations has reached epidemic proportions; in the U.S., it is nearly 6 million and continues to rise *(Roger VL et al., 2011).* The number of patients is steadily increasing as a consequence of an aging population and/or enlarging prevalence of cardiovascular risk factors such as diabetes *(Gilbert RE and Krum H, 2015)* and improved survival rates after acute myocardial infarction (MI) putting a greater number of patients at risk of developing a late left ventricular dysfunction. The poor prognosis of symptomatic heart failure is likely associated with the limited long-term efficacy of conventional therapeutic strategies on the underlying ongoing loss of cardiomyocytes, which is followed by the deleterious formation of a fibrotic scar in the failing heart. Unlike other tissue, the heart has been regarded until recently as an organ in which cardiomyocytes were unable to proliferate. Therefore, repair of damaged myocardium leads to replacement by fibrotic tissue, which disrupts contractile function resulting in decreased cardiac performance. Long-term survival has improved with recent medical therapies aiming at reducing cardiac overload and neurohumoral activation, as well as mineralcorticoid deregulation. Significant advances have also been achieved through surgical revascularization strategies including percutaneous coronary angioplasty and coronary artery bypass grafting. More than 50% of heart failure patients die in 4 years and 40% of them perish or are readmitted to hospital within the first year. In the last years efforts have been performed to promote cardiac tissue regeneration with stem cell-based therapy *(Leri A et al., 2011).*

Over the last decade, the classical paradigm that the human heart is a post-mitotic and terminally developed organ with no cell renewal capability has been undermined with the demonstration that cardiomyocytes turnover can occur in adult mammals, including humans. Experimental and clinical evidences of the regenerative potential of the adult human heart has accumulated over the years. However, such inherent capability of human to regenerate myocardium with aging or after injury in adulthood is entirely insufficient to fully compensate for the loss of function associated with these conditions. One of the major therapeutic goals of modern cardiology is to design strategies aimed at

minimizing myocardial necrosis and optimizing cardiac repair following myocardial infarction. However, a sound understanding of the biology is necessary before a specific intervention is pursued on a therapeutic basis.

1. Myocardial infarction and inflammatory response

1.1. Tissue damage and response to injury

Mammals respond to organ damage through either compensatory growth of the remaining tissue, by activating resident precursor cell proliferation, or by the formation of a scar. Successful mammalian regeneration requires precise coordination of multiple processes, which include scavenging cellular debris, proliferation and activation of progenitor cells, immune modulation, angiogenesis, and innervation of the newly forming tissue. While the involvement of immune cells in tissue repair has been appreciated since Metchnikoff observed that macrophages play a role in tissue repair in the late 1800s, recent advances highlight new mechanisms that the immune system employs to regulate regeneration.

Pathologists define two normal kinds of repair in response to injury: healing and regeneration (*Kumar V et al., 2005*). Healing is a tissue response to a wound or to inflammatory processes in internal organs due to necrosis or infection, and consists of variable proportions of cellular regrowth and fibrous tissue production or scar formation. Regeneration refers to the growth of cells or tissues that replace both form and function in damaged organs of mammals or amputated appendages of amphibians or teleosts (*Goss RJ, 1992*). Unlike the normal healing response, functional organ regeneration typically involves minimal scarring and requires an intact or a reconstituted connective tissue scaffold (*Kumar V et al., 2005*). Innate immune mechanisms, driving the early inflammatory phase of both wound healing and organ regeneration, help activate signaling pathways required for nuclear programming of local cells (*Antonio et al., 2015*). Various receptors that sense cellular damage or microorganisms, including toll-like receptors (TLRs) expressed by fibroblasts and keratinocytes (*Portou et al., 2015*), stimulate signaling pathways that increase epigenetic plasticity and render cells more responsive to microenvironmental cues capable of changing the cells phenotype (*Cooke et al., 2014; Lee et al., 2012*). Phenotypic change and reprogramming of gene expression in cells of injured tissues are generally more important during organ regeneration than in tissue repair (*Jessen et al., 2015; Knapp and Tanaka, 2012*). Other cues that impinge on cells undergoing reprogramming in regenerating systems direct these cells in forming new tissues and include factors from signaling centers which establish the pattern of the regenerated organ.

Myocardial infarction is associated with an inflammatory reaction, which is a prerequisite for healing and scar formation (*Entman ML et al., 1994; Frangogiannis NG et al. 1997; Frangogiannis NG et al., (a) 1998*). Coronary artery occlusion critically reduces blood flow to the portion of the myocardium subserved, markedly impairing the energy

metabolism. Ischemia of significant duration to induce infarction does result in an inflammatory response; this response is both accelerated and augmented if the ischemic tissue is reperfused. The first experimental evidence that inflammation can extend myocardial injury came as the result of implementing anti-inflammatory strategies in animal models of myocardial ischemia and reperfusion. Subsequent investigation suggested that corticosteroids inhibit the inflammatory process decreasing the number of infiltrating leukocytes, but also delay healing and collagen deposition (*Kloner et al., 1978).* This process is dependent on a complex interaction between a variety of pleiotropic inflammatory mediators.

1.2. Humoral inflammatory response

Complement activation

Hill and Ward were the first to demonstrate that ischemic myocardial injury can activate the complement cascade in a rat model of myocardial infarction (*Hill JH and Ward PA, 1971)*. Subsequently Pinckard and colleagues (*Pinckard RN et al., 1975*) suggested that myocardial cell necrosis results in the release of subcellular membrane constituents rich in mitochondria, which are capable of triggering the early acting components (C1, C4, C2 and C3) of the complement cascade. Rossen and colleagues (*Rossen et al., 1994*) have suggested that during myocardial ischemia, mitochondria, extruded through breaks in the sarcolemma, unfold and release membrane fragments rich in cardiolipin and proteins. By binding C1 and supplying sites for the assembly of later acting complement components, these subcellular fragments provide the means to disseminate the complement-mediated inflammatory response to ischemic injury. mRNA and proteins for all the components of the classical complement pathway are upregulated in areas of myocardial infarcts (*Vakeva AP et al., 1998; Yasojima K et al., 1998)*. Complement activation may have an important role in mediating neutrophils and monocytes recruitment in the injured myocardium.

Reactive oxygen species

Reactive oxygen species (ROS) are molecules with unpaired electrons in their outer orbit. They have the potential to directly injure cardiac myocytes and vascular cells and may be involved in triggering inflammatory cascades through the induction of cytokines (*Lefer DJ t al. 2000*). ROS have been shown to exert a direct inhibitory effect on myocardial function in vivo and have a critical role in the pathogenesis of myocardial stunning. In

addition, Granger and colleagues (*Granger DN et al., 1988*) have provided evidence for a potential role of reactive oxygen in leukocyte chemotaxis. Potential mechanisms through which reactive oxygen intermediates may generate a leukotactic stimulus include complement activation (*Shingu M et al., 1984*), induction of P-selectin expression (*Akgur FM et al., 2000; Patel KD et al., 1991*), chemokine upregulation *(Lakshminarayanan V et al. 1997; Lakshminarayanan V et al., 1998),* and increase in the ability of endothelial ICAM-1 to bind to neutrophils (*Sellak H et al., 1994*).

1.3. The immune system in myocardial infarction

Activation of the immune system is a hallmark of myocardial infarction (MI). After 15-20 minutes of severe ischemia cardiomyocytes exhibit irreversible alterations that ultimately result in their death. Subendocardial cardiomyocytes are more susceptible to ischemic injury; thus, a "wavefront" of necrosis is noted that extends from the subendocardial area to the subepicardium (*Reimer KA et al., 1977*). Ischemia and necrotic cardiomyocytes trigger a reparative process that can be divided into three distinct but overlapping phases. During the inflammatory phase, activation of innate immune signals results in induction of chemokines and cytokines leading to infiltration of the infarct with neutrophils and pro-inflammatory monocytes that clear the infarct from dead cells and matrix debris. Apoptotic death of neutrophils marks the end of the inflammatory phase and may activate signals that inhibit inflammation and induce resolution of the leukocyte infiltrate. The proliferative phase follows, as reparative monocytes are recruited in the infarct and macrophages may acquire an angiogenic or fibrogenic phenotype that promotes differentiation and growth of myofibroblasts and endothelial cells. Activated myofibroblasts contribute to wound contraction and produce structural matrix proteins forming a scar. Maturation of the infarct follows and is characterized by progressive apoptotic death of the cellular elements and by collagen cross linking. As the infarct heals, the ventricle dilates, while non-infarcted segments become hypertrophic and exhibit increased interstitial fibrosis. The extent of adverse remodeling is dependent not only on the size of the infarct, but also on the qualitative characteristics of the wound.

Initiation of the immune response in the infarcted myocardium

Acute sudden death of cardiomyocytes in the infarcted heart rapidly activates the immune system triggering an intense, but transient, inflammatory reaction. In the infracted myocardium, cellular necrosis and matrix fragmentation generate alarm signals that activate the innate immune response; these molecules are known as danger-

associated molecular patterns (DAMPs) (*Timmers L et al., 2012*). The nuclear chromatin-binding protein High-mobility group box-1 (HMGB1) plays a crucial role in initiation of the inflammatory response through actions that may involve Toll-Like Receptor (TLR) and RAGE signaling (*Andrassy M et al., 2008*). Heat shock proteins (HSPs), ATP and matrix fragments (including low molecular weight hyaluronan and fibronectin fragments) also serve as "danger signals" activating the immune response (*Arslan F et al., 2011; Huebener P et al., 2008; Dobaczewski M et al., (a) 2010)* The immune response following MI involves signaling through the TLRs, a family of transmembrane receptors that activate downstream pro-inflammatory cascades. TLRs recognize conserved motifs in pathogens, thus contributing to protection from infection, but are also activated by DAMPs, initiating immune responses that clear injured tissues from dead cells and matrix debris and activate reparative signals. 13 mammalian TLRs have been identified; in the infarcted myocardium, loss-of function approaches have identified TLR2 and TLR4, both localized in the cell surface, as important mediators of the inflammatory reaction. Impaired TLR4 signaling attenuated the inflammatory reaction following MI and reduced adverse remodeling; complete loss of TLR4 was also associated with a reduction in the size of the infarct (*Agostini et al., 2004; Colotta et al., 1993*). TLR2 signaling has also been reported to mediate inflammatory cardiomyocyte injury following coronary ischemia and reperfusion (*Cullinan et al., 1998*). DAMPs also activate the complement system, another essential cascade in transducing the immune response in the infarcted heart. Hill and Ward demonstrated C3 cleavage in the infarcted myocardium and documented a role for the complement system in leukocyte infiltration (*Hill JH and Ward PA, 1971*). Moreover, complement inhibition consistently attenuated leukocyte recruitment following MI (*Lugrin J et al., 2015*) highlighting the critical role of the complement cascade in triggering inflammation in the ischemic myocardium. Generation of ROS also activates immune cells in the infarcted heart. ROS promote leukocyte chemotaxis (*Granger DN et al., 1988*) by activating complement, by stimulating expression of P-selectin and by upregulating cytokine and chemokine synthesis through activation of the Nuclear Factor (NF)-κB system (*Hensleyn K et al., 2000*). Although reactive oxygen generation is important for cardiac repair following infarction, overactive ROS-mediated signaling has injurious potential by promoting cell apoptosis and by inducing degradation of the extracellular matrix. The normal heart possesses substantial ability to counterbalance the generation of ROS through inhibitory enzymatic pathways (such as catalase, glutathione peroxidase and the superoxide dismutases) and through the actions of intracellular antioxidants. Following MI, the antioxidant defenses are overwhelmed, resulting in net generation of free radicals that may suppress myocardial function and activate immune cells inducing inflammatory injury. Because of the pleiotropic effects of ROS, both beneficial and detrimental actions have been reported after administration of free radical scavengers in myocardial infarction.

Cytokine and chemokine signaling in the infarcted heart

TLR-mediated, complement-activated and ROS-induced pathways in the infarcted myocardium converge on activation of NF-κB that drives induction of adhesion molecules in endothelial cells and production of pro-inflammatory cytokines and chemokines by fibroblasts, leukocytes and vascular cells. As the prototypical pro-inflammatory cytokine, Interleukin (IL)-1 mediates synthesis of chemotactic mediators in the infarct and stimulates leukocyte recruitment (*Bujak M et al., 2008; Bujak M et al., 2009*). Processing and generation of active IL-1β in inflamed tissues requires formation of specialized molecular platforms, called "inflammasomes" (*Schroder K et al., 2010*). Generation of active IL-1β is primarily mediated through processing of the precursor protein by the intracellular cysteine protease caspase-1; however, caspase-1-independent mechanisms of IL-1β activation have also been described (*Netea MG et al., 2014*). Activation of caspase-1 requires formation of a multi-component platform termed the inflammasome; one of the components of the inflammasome, Nucleotide-binding oligomerization domain-Like Receptor with a Pyrin domain 3 (NLRP3), plays an important role in generation of active IL-1β (*Agostini et al., 2004*).

Two investigations have demonstrated the activation of the inflammasome in the infarcted myocardium. Inflammasome activation in the infarct is localized in leukocytes, resident fibroblasts and border zone cardiomyocytes (*Mezzaroma E et al., 2011; Kawaguchi M et al., 2011*) and drives IL-1-mediated inflammatory cell infiltration and cytokine synthesis. ROS production, ATP and potassium efflux may play a role in inflammasome activation in infarcted cells. Activation of the inflammasome in cardiac fibroblasts and in cardiomyocytes has been extensively documented in experimental models of MI and may contribute to the post-infarction inflammatory reaction extending injury (*Sandanger O et al., 2013; Mezzaroma E et al., 2011; Kawaguchi M et al., 2011*) The pro-inflammatory cytokine Tumor Necrosis Factor (TNFα) is also released in the infarcted myocardium and may play a role in stimulating cytokine synthesis by mononuclear cells infiltrating the infarct (*Frangogiannis NG et al., (b) 1998*). In addition to its pro-inflammatory actions, TNFα may also exert cytoprotective effects (*Kurrelmeyer KM et al., 2000*) and may regulate post-infarction cardiac remodeling through divergent actions involving TNFR1 and TNFR2 receptors (*Hamid T et al., 2009*). TNFR1 signaling stimulates cardiomyocyte apoptosis and enhances inflammatory activity in the infarcted heart, whereas TNFR2 reduces TNF-induced NF-κB activation and attenuates cardiac injury. Members of the IL6 family of cytokines (including IL6, cardiotrophin-1, oncostatin-M and leukemia inhibitory factor) are also consistently upregulated in experimental models of MI and may modulate the inflammatory and reparative response, signaling through receptors that share the transmembrane glycoprotein (gp)130 (*Fischer P et al., 2008*). Genetic loss of IL6 did not affect infarct size, ventricular function and cardiac

remodeling in nonreperfused infarcts (*Fuchs M et al., 2003*); in the absence of IL6 other gp130 cytokines may act in a compensatory manner, activating JAK/STAT signaling and maintaining STAT3 phosphorylation. On the other hand, experiments targeting IL6 through administration of an anti-IL6 receptor antibody attenuated adverse remodeling (*Kobara M et al., 2010*). Timely activation and suppression of gp130 signaling may play an important role in the regulation of post-infarction remodeling. In an experimental model of myocardial infarction, failure to suppress gp130/STAT3 signaling resulted in prolonged and accentuated inflammation predisposing to cardiac rupture (*Hilfiker-Kleiner D et al., 2010*). Several members of the chemokine family are also prominently involved in the postinfarction inflammatory response (*Frangogiannis NG, 2007*) and provide key directional signals for recruitment of leukocyte subpopulations into the infarct. CC chemokines that serve as mononuclear cell chemoattractants, ELR^+ CXC chemokines that function to attract neutrophils in sites of injury, and ELR-negative CXC chemokines that recruit lymphocytes and may exert antifibrotic and angiostatic actions are rapidly, but transiently, upregulated in the infarcted myocardium. The chemotactic actions of chemokines are dependent on their immobilization to glycosaminoglycans on the endothelial surface, or on the extracellular matrix; as leukocytes are captured by activated endothelial cells and roll on the endothelial surface (through selectin-mediated actions) they "sense" the bound chemokines, and exhibit integrin activation, thus engaging adhesive interactions with vascular endothelial cells. Various leukocyte subpopulations exhibit distinct chemokine receptor profiles and interact with different chemokines; thus selective activation of chemokine/chemokine receptor pairs orchestrates recruitment of leukocyte subpopulations with distinct properties (*Nahrendorf M et al., 2007; Dobaczewski M et al., (a) 2010; Proudfoot AE et al., 2003*). Activated leukocyte β2 integrins interact with endothelial Intercellular Adhesion Molecule (ICAM)-1 resulting in firm adhesion of leukocytes to the endothelial layer. Transmigration of activated leukocytes follows, and is dependent on several adhesion molecules, including ICAM-1, members of the Junctional Adhesion Molecule (JAM) family and Vascular-endothelial (VE)-cadherin (*Corada M et al., 2005; Williams MR et al., 2011; Woodfin A et al., 2011;*).

1.4. The cellular effectors of the post-infarction inflammatory reaction

Both resident and bone marrow-derived cells participate in the post-infarction inflammatory reaction; understanding the relative contribution of inflammatory activation of various cell types is limited by the absence of studies using cell-specific loss-of-function approaches *in vivo*.

Cardiomyocytes: Necrotic cardiomyocytes release a variety of danger signals, thus actively contributing to activation of innate immune cascades. Moreover, border zone cardiomyocytes may exhibit activation of the inflammasome, thus serving as a source of bioactive IL-1β.

Endothelial cells: Endothelial cells actively participate in the post-infarction inflammatory reaction. Cytokine release in the infarct zone induces adhesion molecule expression on the endothelial cell surface, thus promoting adhesive interactions with circulating leukocytes. Endothelial cells are also a major source of pro-inflammatory chemokines (such as MCP-1 and IP-10) in the infarcted myocardium (*Frangogiannis NG et al., 2001; Bujak M et al. 2009*).

Platelets: In addition to their hemostatic role, platelets modulate inflammatory responses and may link tissue injury with the activation of reparative pathways (*von Hundelshausen P et al., 2007)*. In the healing infarct activated platelets aggregate in the site of injury and may contribute to the formation of a fibrin-based provisional matrix, necessary to support influx of hematopoietic cells and migration of reparative mesenchymal cells (*Dobaczewski M et al., 2006)*. Platelets may also release chemokines, cytokines and growth factors in the infarcted tissue; however, their relative significance in the post-infarction inflammatory reaction remains unknown.

Neutrophils: Following either cardiac ischaemic injury or pressure overload, neutrophils are the first innate immune cells recruited to the myocardium in large numbers (*Dreyer WJ et al., 1992*). Activation of the complement cascade, ROS generation, and ELR^+ CXC chemokines have been implicated in recruitment of neutrophils in the infarcted myocardium. By generating ROS and through the release of proteolytic enzymes, neutrophils infiltrating the infarcted myocardium contribute to the clearance of the infarct from dead cells and matrix debris. In addition, neutrophils are capable of secreting pro-inflammatory mediators that may amplify recruitment of mononuclear cells. Experimental evidence, derived primarily through neutrophil depletion strategies, suggested that neutrophils may directly injure ischemic, but viable, cardiomyocytes increasing the size of the infarct through adhesive interactions involving neutrophil integrin activation and cardiomyocyte ICAM-1 expression (*Albelda SM et al., 1994*). However, the *in vivo* significance of neutrophil-induced cardiomyocyte injury has been challenged by loss-of-function experiments in genetically targeted mice. ICAM-1 null mice had no significant difference in infarct size and scar formation after 1-3 weeks of reperfusion (*Metzler B et al., 2001*). Moreover, animals with a combined deficiency in both ICAM-1 and P-selectin and animals with disrupted IL-1 signaling showed no reduction in infarct size following

ischemia and reperfusion, despite a marked impairment in neutrophil trafficking (*Briaud SA et al., 2001*).

Mast cells: In large animals, the heart contains a significant number of resident mast cells predominantly located in close proximity to vessels; the population of cardiac mast cells is smaller in mice, leading perhaps to underestimation of their role in heart disease when murine models of cardiac injury are used (*Gersch C et al., 2002*). In a canine model of experimental infarction, resident cardiac mast cells in the ischemic territory showed rapid and impressive degranulation, releasing large amounts of preformed pro-inflammatory mediators, including histamine and TNFα (*Frangogiannis NG et al., (b) 1998*).

1.4.1.Fibroblasts

The role of fibroblasts has been intensively studied in both homeostatic heart and cardiac repair. Fibroblasts are the most abundant non-cardiomyocytes in the adult mammalian heart and are strategically located in the interstitial and perivascular space. In the infarcted heart, resident cardiac fibroblasts undergo inflammatory activation and activate the inflammasome platform (*Kawaguchi M et al., 2011*), thus serving as a source of bioactive IL-1β. Activated fibroblasts may also secrete chemokines; however, their relative contribution to the activation and progression of the post-infarction inflammatory reaction remains unknown. After MI, fibroblasts undergo phenotypic changes involved in the post-MI inflammatory response and repair.

Animal models of MI showed that cardiac extracellular matrix (ECM) response to injury can be divided to three stages: acute early response, proliferation, and late maturation. In rodents, the duration of early response is up to 2 days; the proliferative stage lasts 2–5 days, and the maturation stage takes up 1 month. In large mammals, this time is prolonged for several days (early response), up to two weeks (proliferation), and 1–2 months and longer (maturation) (*Frangogiannis et al., 2008; Dobaczewski et al., (a) 2010*). Each of these stages is distinct but overlapping. In each stage, cardiac fibroblasts have a distinct phenotype. The inflammatory early post-MI response stage is characterized by acute reaction of innate immunity to massive death of heart muscle cells.

In the proliferative stage, multiple fibroblasts and vascular cells migrate to the injured site to heal the damage. Activated fibroblasts transform to myofibroblasts that produce ECM proteins and generate the scar. Stimuli that inhibit pro fibrotic/angiogenic responses and mediate transition to the maturation stage are incompletely understood. The scar maturation is accompanied by apoptosis of cell elements with remaining cross-linked collagen matrix that constitute the mature scar (*Chen W and Frangogiannis NG, 2013*).

During the post-MI repair, the left ventricle is subjected to unfavorable morphological and functional changes. After MI, ventricular remodeling leads to the hypertrophy of survived myocardium, dilation and increased volume of the chamber (*Pfeffer MA and Braunwald E, 1990*). Geometrical alterations are associated with induction of arhythmias and higher risk of heart failure and sudden death. The process of ventricular enlargement is mainly influenced by the infarct size. The large infarct typically leads to the abnormal remodeling. However, other factors also significantly contribute to adverse consequences of post MI ventricular remodeling. For example, excessive matrix formation induces rigidity of ventricle and diastolic heart failure (*Yu CM et al., 2007*). Prolonged post-MI inflammatory response leads to higher matrix metalloprotease (MMP) activity, impaired ventricular remodeling, and worse cardiac dilation (*Dobaczewski M et al., (a) 2010*). Cardiac fibroblasts play the most prominent role in the proliferative stage of heart repair but significantly contribute to each phase of healing process.

Compared to cardiomyocytes, cardiac fibroblasts are significantly less sensitive to acute hypoxic conditions induced by MI. Due to their abundant presence in the heart interstitium and tight contacts with muscle cells, fibroblasts are among the first cardiac cells who sense and respond to heart injury. In cell culture experiments, fibroblasts demonstrate inflammatory activation and inflammatory responses (*Heim A et al., 2000*; *Lafontant PJ et al., 2006*).

However, the actual impact of fibroblasts to the acute post-injury inflammation is hindered by the contribution of other resident non-immune cells such as vascular endothelium and mast cells, which are involved in the post-MI inflammatory mechanism and can produce a broad spectrum of proinflammatory factors (*Frangogiannis NG et al., (a) 1998; Ruiz-Villalba A et al., 2015; Rohrbach S et al., 2015*). The exact assessment of the inflammatory role of fibroblasts is hampered by the lack of specific markers of cardiac fibroblasts. Vimentin usually used as a fibroblast marker in cardiac investigations is not restricted to fibroblasts since it is expressed by other mesenchymal cells including endothelial cells (ECs) and smooth muscle cells (SMCs). α-Smooth muscle actin (α-SMA) employed as a marker of fibroblast transition to cardiomyocytes is abundantly produced by SMCs. Fibroblast-specific protein 1 (FSP1) that was suggested as a fibroblast-specific marker is widely produced by hematopoietic cells, ECs, and vascular SMCs (*Kong P et al., 2013*). However, periostin, a secreted ECM protein, can serve as a specific marker of activated fibroblasts because its expression is greatly up-regulated in cardiac fibroblasts after MI and is almost absent in the normal heart (*Oka T et al., 2007*).

Activation of cardiac fibroblasts after myocardial injury

Proinflammatory activation of cardiac fibroblasts is associated with the inflammasome induction that leads to caspase stimulation and release of IL-1β (*Kawaguchi M et al., 2011; Sandanger O et al., 2013*). Inflammasome activation in cultured fibroblasts is induced by ROS-dependent signaling and K^+ efflux (*Kawaguchi M et al., 2011*). Mouse myocardial fibroblasts primed *in vitro* with lipopolysaccharide (LPS), secrete IL-1β and IL-18 (both are proinflammatory cytokines) in response to ATP (*Sandanger O et al., 2013*), a danger signal molecule released by fibroblasts after cardiac injury (*Lu D et al., 2012*). Pro-inflammatory activation of cardiac fibroblasts leads to functional changes. In addition to the production of proinflammatory mediators (*Sandanger O et al., 2013*), fibroblasts up-regulate matrix-degrading properties through increased release of MMPs (*Fan D et al., 2012*). In cultured rat heart fibroblasts, higher gelatinase activity was observed in response to oxidative stress and stimulation with IL-1β and TNFα (*Siwik DA et al., 2000*; *Siwik DA et al., 2001*). In cultured cardiac fibroblasts, IL-1β and TNFα also differentially regulated production of tissue inhibitors of metalloproteinases (TIMPs) and activated a desintegrin and metalloproteinase domain-containing protein 10 (ADAM10) (*Li J et al., 1999*). Activation of matrix-degrading proteases is essential to facilitate migration of leukocytes in the site of injury and promote clearance of dead cells and ECM debris. Concomitantly, exposure to inflammatory stimuli led to the inhibition of collagen synthesis in cardiac fibroblasts (*Siwik DA et al., 2000; Li J et al., 2002*). Inflammatory cytokines also stimulate induction of the proinflammatory phenotype in fibroblasts and production of inflammatory mediators such as CXCL1, CXCL5 (*Lafontant PJ et al., 2006*), CXCL8 (*Seino Y et al., 1995*), and CXCL10 (*Zymek P et al., 2007*). CXCL1, CXCL5, and CXCL8 serve as attractants for neutrophils while CXCL10 helps to recruit monocytes. Indeed, in response to inflammatory signals, fibroblasts loose the quiescence and show principal functional switch from the matrix-producing activity to the matrix-degrading activity along with secretion of inflammatory factors and chemoattractants needed for cardiac recruitment of leukocytes. IL-1β seems to play a key role in mobilization of cardiac fibroblasts after heart injury. After ischemia-reperfusion-induced heart injury, IL-1 receptor 1-deficient mice had decreased accumulation of neutrophils and macrophages in infarcted myocardium and diminished early inflammatory and pro fibrotic responses (*Saxena A et al., 2013*) suggesting for down-regulation of cardiac repair in a case of disrupted IL-1-dependent signaling. IL-1β inhibits proliferation of fibroblasts by inducing cell cycle arrest during G1/S transition (*Palmer JN et al., 1995; Koudssi F et al., 1998*). IL-1β also prevents fibroblast-myofibroblast transdifferentiation (*Saxena A et al., 2013*). Indeed, IL-1β too early suppresses the induction of a matrix-synthetic contractile phenotype in fibroblasts until the injury is cleared from dead cells and matrix. However, extended exposure of cardiac fibroblasts to inflammatory cytokines such as IL-1β

observed in prolonged inflammatory phase of post-MI healing may have worse effects. Long-term action of IL-1β on cardiac fibroblasts delays or prevents transition from the proinflammatory stage to the proliferative phase of heart repair that may induce adverse remodeling and heart failure through reducing heart contractility, promoting cardiomyocyte apoptosis, and supporting prolonged production of matrix-degrading proteases (*Bujak M and Frangogiannis NG, 2009*). In contrast to IL-1β, TNFα may indirectly induce the profibrotic activity of fibroblasts through increasing expression of type 1 angiotensin II (AT1) receptors (*Gurantz D et al., 1999* ; *Peng J et al., 2002*). However, TNFα and IL-1β have synergistic effect on the type 2 angiotensin II receptor through suppression of its activity including inhibition of collagen synthesis in rat cardiac fibroblasts (*Tamura M et al., 1999*). Indeed, some inflammatory cytokines that cooperate in pro-inflammatory activation of cardiac fibroblasts can demonstrate opposed effects on the fibrotic fibroblast activity.

Cardiac fibroblasts during the inflammation resolution

The acute inflammatory response should be dampened in a timely manner to avoid further exacerbation of cardiac inflammation and propagation of heart damage (*Frangogiannis NG, 2012*). Down-regulation of inflammation is triggered by gradual activation of anti-inflammatory mechanisms mediated by IL-10 and TGF β along with induction of intracellular inhibitors of innate immunity such as interleukin receptor associated kinase M (IRAK-M) (*Maekawa Y et al., 2009 ; Chen W et al., 2012*). IRAK-M serves as a functional decoy preventing excessive TLR/IL-1-mediated responses. IRAK-M suppresses IL-1-dependent signaling that leads to the prevention of adverse post-MI remodeling, down-regulation of in flammatory effects of leukocytes, and inhibition of the matrix-degrading activity of fibroblasts (*Chen W et al., 2012; Cochain C et al. 2012*) showed an important role of the chemokine decoy receptor D6 that specifically binds and scavenges inflammatory chemokines limiting heart inflammation and avoiding adverse cardiac remodeling after MI. The transmembrane receptor CD44 may also be involved in the resolution of inflammation and trafficking of fibroblast to the infarcted region. In a mouse model of reperfused infarction, CD44 deficiency was associated with extended heart inflammation, attenuated collagen deposition and decreased fibroblast proliferation/migration (*Huebener P et al., 2008*). A role of cardiac fibroblasts in the resolution of inflammation is poorly understood. It is unclear whether fibroblasts are among cell inducers of dampening cardiac inflammation or their role is limited to be effector cells. Cell mechanisms that control transition from inflammation to proliferation and heart repair should be further studied to identify key molecular modulators responsible for this process.

The role of fibroblasts in the proliferative stage

In this stage, fibroblasts acquire the proliferative myofibroblast phenotype due to induction of the growth factor signaling that initiates synthesis of contractile and ECM proteins and cell mobility. Due to increased synthetic and secreting capacity, myofibroblasts develop a large endoplasmic reticulum network (*Hinz B et al., 2007*). Expression of α-SMA, non-muscle myosin, and other contractile proteins is a characteristic of myofibroblasts. Myofibroblasts do not produce adult smooth muscle-specific proteins including smooth-muscle isoforms of myosin SM1 and SM2, but abundantly express the embryonal isoform of myosin heavy chain (SMemb), a marker of dedifferentiated SMCs (*Shiojima I et al., 1999; Frangogiannis NG et al., 2000*). SMemb production may indicate the flexibility of myofibroblast phenotype in injured heart that can change depending on the physiological needs.

In the proliferative repair stage, myofibroblasts occupy the infarct zone as well as the infarct border zone. In early stages of differentiation from fibroblasts, myofibroblasts form stress fibers from cytoskeletal actin and focal adhesions containing β- and γ-actin micro filaments, which link to non-muscle myosin. These cells were called 'protomyofibroblasts ' (*Hinz B et al., 2007*).

Signaling mechanisms triggering transition of fibroblasts to myofibroblasts

Transdifferentiation of heart fibroblasts to myofibroblasts is a complex process that is induced and mediated by multiple signaling mechanisms that cooperate to each other. TGF β is a key regulator of fibrosis (*Meng XM et al., 2016*). This growth factor induces α-SMA expression in fibroblasts via SMAD3-mediated signaling and by activation of nuclear trafficking of myocardin-related transcription factor (MRTF)-A (*Bujak M et al., 2007; Small EM et al., 2010*). MRTF-A is a coactivator of the serum-response transcription factor (SRF) that drives α-SMA transcription. TGF β stimulates SRF expression in a p38 MAPK-dependent manner (*Penke LR et al., 2014*). p38 MAPK signaling plays an important role in providing TGF β-dependent stimuli through activating many downstream molecular substrates that control myofibroblast differentiation (*Hashimoto S et al., 2001; Sousa AM et al., 2007)*. ECM mediates conversion of fibroblasts to myofibroblasts. Type I and III collagens activate cardiac fibroblast proliferation, while type VI collagen promotes myofibroblast differentiation (*Naugle JE et al., 2006*). Mechanical forces contribute to the transition from cardiac fibroblasts to myofibroblasts through several signaling mechanisms that involve selective activation of MAPKs (such as p38 MAPK and Erk1/2) and Rho kinases (*Wang J et al., 2003; Wang J et al., 2005*) regulating expression of α-

SMA and other contractile proteins. Mechanotransuction and TGF β can cooperate in the regulation of fibroblast transdifferentiation (*Escudé M et al., 2014*). Fibroblast-to-myofibroblast conversion involves many effector pathways, whose activity and interaction should be strictly controlled during myocardial repair. Otherwise, extensive matrix-building activity and proliferation of activated fibroblasts and myofibroblasts can cause adverse effects such as cardiac fibrosis and hypertrophy.

Cardiac fibroblast migration can be induced by growth factors such as TGF β and FGFs (*Detillieux KA et al., 2003; Stawowy P et al., 2004*) and cytokines (IL-1β, TNFα, cardiotrophin-1) (*Mitchell MD et al., 2007; Freed D et al., 2011*). Stimulation of fibroblasts by IL-1β and TNFα leads to the activation of MAPKs such as p38 MAPK, Jnk, and Erk1/2 and subsequent induction of matrix-degrading proteases such as MMP2, MMP3, and MMP9. However, in cultured rat primary cardiac fibroblasts, TNFα exhibited less prominent migration-inducing capacity and up-regulated only MMP-9 production compared with IL-1β (*Mitchell MD et al., 2007; Brown RD et al., 2007; Xie Z et al., 2004*) showed that IL-1β-induced expression of MMP2 and MMP9 in rat cardiac fibroblasts is primed by NF-kB. Erk1/2, Jnk, and PKCα/β1 are involved in IL-1β-induced expression of MMP9 not MMP2. Angiotensin II was shown to induce migration of murine heart fibroblasts through Erk-dependent stimulation of transcription factors AP-1, NF-kB, and Sp1 followed by increased expression of MMP2, MMP7, MMP9, and MMP14 (*Siddesha JM et al., 2013*). Matrix-degrading activity of MMPs is essential for migration of activated fibroblasts (*Nong Z et al. 2011*); reported that, collagen type 1 cleavage is required for efficient post-MI tissue repair. Fibroblast locomotion is highly varied depending on the local environment.

When formation of collagen-rich scar is finished, the heart repair goes to the final stage in which scar maturates. In this stage, myofibroblasts transit to the quiescent state. This mechanism requires action of factors that inhibit proliferation and migration. The maturation stage is characterized by matrix cross-linking, decellularization, and vascular regression. Gradual depletion of resident myofibroblasts is observed during scar maturation. The mechanism of scar maturation is poorly understood. Apoptosis was suggested as a major mechanism driving removal of myofibroblasts from the mature scar (*Takemura G et al., 1998*). However, Kisseleva and colleagues (*Kisseleva et al., 2012*) showed that not all myofibroblasts undergo apoptotic death during fibrosis regression in the liver repair.

1.4.2.Monocytes and macrophages

Monocyte subpopulations are sequentially recruited in the infarcted myocardium and regulate inflammatory and reparative cascades. Infiltrating monocytes are primarily derived from the circulating blood; however, the contribution of alternative sources, such as the spleen is increasingly recognized (*Swirski FK et al., 2009; Jung K et al., 2013)*. During the first few hours after reperfusion, complement activation and induction of the CC chemokine CCL2/monocyte chemoattractant protein (MCP)-1 play an important role in recruitment of pro-inflammatory monocytes (*Denwald O et al., 2005*); these cells exhibit potent pro inflammatory and phagocytotic properties. At a later stage, recruitment of reparative mononuclear cell subsets may play an important role in activation of fibroblasts and ECs, promoting formation of a scar. Understanding of the time course, phenotype and mechanism of recruitment of specific monocyte subsets in the healing infarct is limited. In response to the dynamic alterations in cytokine and growth factor expression in the infarct, infiltrating monocytes undergo phenotypic changes. Maturation of monocytes into mature macrophages involves the local upregulation of growth factors, such as Macrophage Colony Stimulating Factor (M-CSF) (*Frangogiannis NG et al., 2003)*. Considering the remarkable potential of macrophages to acquire unique phenotypic characteristics in response to microenvironmental cues, differentiation of several distinct subpopulations of macrophages is likely to orchestrate the inflammatory and reparative response. Thus, macrophage subsets may play multiple roles in the healing infarct. First, they phagocytose dead cells and matrix debris and clear the infarct from apoptotic neutrophils and cardiomyocytes. Second, they may serve as a source of cytokines and growth factors regulating inflammatory activity, fibroblast growth and activation, and neovessel formation and maturation. Third, they may contribute to extracellular matrix remodeling by producing MMPs and their inhibitors.

Recent studies of macrophage activity in various mammalian models of tissue repair and regeneration make clear that this cell population includes cells with a range of distinct functional phenotypes, with proinflammatory M1 macrophages associated with the first phases of acute inflammation gradually being replaced under the control of complex environmental stimuli by anti-inflammatory M2 cells (*Chazaud B, 2014; Das A et al., 2015*). The emerging M2 macrophages not only promote the resolution of inflammation, but also exert many salutary effects on the parenchymal cells of damaged organs, promoting recovery of tissue homeostasis, stimulating stem and progenitor cell development, and contributing to conditions necessary for tissue repair and regeneration (*Chazaud B, 2014*). Resolution of inflammation, an active process involving changes in the local population of macrophages and in gene expression in other immune cells, must occur in a timely manner for a successful regeneration. Macrophages are key players in several aspects of inflammation as well as resolution, and their participation is required

during the inflammatory response leading to regeneration, likely owing to their importance for resolution.

Cardiac macrophages

The adult mammalian heart, which lacks regenerative capacity, contains different cardiac macrophage subsets, with diverse functions, developmental origins, and mechanisms of homeostasis. The four populations, segregated by expression levels of CCR2, Ly6C, and MHC class II, perform different functions. Upon injury such as a MI, a biphasic monocyte response occurs to promote an initial inflammatory phase followed by a reparative phase mediated by $Ly6C^{hi}$ or $Ly6C^{low}$ splenic monocytes, respectively. The two systems are linked by the ability of splenic $Ly6C^{hi}$ monocytes to replenish all four subsets in phase I. In the neonatal mouse heart, which can fully regenerate after MI, there is also a biphasic splenic monocyte response, although the characterization of subsets of resident cardiac macrophages has yet to be investigated. Interestingly, neonatal cardiac macrophages differ from those of the adult in their localization, abundance, and gene expression profile after injury and are required for regeneration by promoting angiogenesis. The regenerative subtype in the neonate has yet to be defined.

The adult mammalian heart is notoriously resistant to regeneration after injury, due primarily to the irreversible withdrawal of postnatal cardiomyocytes from the cell cycle. After injury of the adult heart, the inflammatory response and specifically the monocyte/macrophage response has a dual function in scar formation. During the later phase, $Ly6C^{low}$ and M2 macrophages are necessary for mediating angiogenesis concomitantly with fibrosis to form a scar (*Nahrendorf M et al., 2007*). In contrast, before postnatal day (P) 7, neonatal mice efficiently regenerate up to 20% of the mass of the heart after surgical ablation or myocardial infarction (*Porrello ER et al., 2011; Porrello ER et al., 2013*). Recent studies showed that the immune response to cardiac injury differs quantitatively and qualitatively during regeneration in comparison to the profibrotic response mentioned (*Aurora AB et al., 2014*). Also, depletion of macrophages in P1 mice subjected to myocardial infarction impaired heart regeneration, at least in part, due to a lack of neoangiogenesis. Given that the neonatal mouse does not mount a robust fibrotic response after ischemic cardiac injury, the data suggest that in mammalian heart regeneration, macrophages have the potential to promote angiogenesis without activating fibroblasts. The relative distribution in the neonate of the four cardiac macrophage populations found in the adult and the potential contribution of each population to the process of neonatal heart regeneration and angiogenesis remain to be defined. The unique gene expression profile of early neonatal macrophages is of future interest for developing therapies that target the immune system to promote angiogenesis and tissue regeneration.

There are two main subsets of circulating monocytes in mice, Ly6C^{hi} monocytes and Ly6C^{low} monocytes. Ly6C^{+} monocyte progenitors give rise to Ly6C^{hi} monocytes and, through a nuclear receptor subfamily 4 group A member 1 (NR4A1) dependent transcriptional programme, LY6C^{hi} monocytes differentiate into Ly6C^{low} monocytes (*Yona, S. et al., 2011*) Global transcriptional profiling has shown that these monocyte subsets are conserved in humans (*Ingersoll, M. A. et al. 2010)* and they have very different roles *in vivo*. Ly6C^{low} monocytes adhere to and move along the endothelium, both clearing damaged cells and triggering inflammatory responses without entering tissue (*Carlin, L. M. et al., 2013; Frangogiannis, N. G. et al., 2007)*. During cardiac stress, Ly6C^{hi} monocytes are the primary subset recruited to the heart, either following ischaemic injury or hypertensive stress; whereas Ly6C^{low} monocytes do not seem to be directly recruited into the myocardium *(Hashimoto, D. et al., 2013; Jakubzick, C. et al., 2013)*. Monocytes recruited into ischaemic myocardium are found in the blood, but the spleen is also a monocyte reservoir that can be used when blood and bone marrow stores are insufficient (*Leuschner, F. et al. 2010)*. Splenic monocytes also seem to have a protective role, as splenectomy leads to impaired infarct healing. Monocyte recruitment is in part dependent on innate B cells, which are also recruited to the ischaemic myocardium, and these B cells drive monocyte population expansion through a CC chemokine ligand 7 (CCL7) dependent process (*Zouggari, Y. et al., 2013)*. Once monocytes enter the tissue, they begin to differentiate into macrophages. For many years, it has been very challenging to separate recruited monocytes from resident macrophages and, as a result, they have been analysed as a single population. However, these populations have different ontological lineages, and this has important functional implications.

Cardiac macrophage subsets

Recently, studies using genetic fate mapping, parabiosis and adoptive transfer techniques have defined resident cardiac macrophage populations in much more detail. Rather than being a homogenous population, resident cardiac macrophages comprise three discrete subsets that have different origins and functions (*Epelman S et al., 2014*). These three macrophage subsets are defined by their differential expression of MHC class II and CC-chemokine receptor 2 (CCR2). MHC class II^{hi} and MHC class II^{low} cardiac macrophages are both CCR2^{-} and are, numerically, the dominant subsets in the heart (*Epelman S et al., 2014*). These macrophage subsets are primarily derived from embryonic progenitors, with a substantial number arising from embryonic yolk sac precursors, and they renew through *in situ* proliferation, rather than through monocyte input. After birth, there is some dilution of embryonically-derived macrophages by recruited monocyte-derived

macrophages in the heart, but in adult mice the majority of resident cardiac macrophages remain of embryonic origin (*Molawi, K. et al., 2014)*. The third cardiac macrophage subset is made up of $CCR2^+$ macrophages, which are derived from, and slowly replenished by, circulating blood monocytes. Detailed studies during hypertensive stress suggest that embryonically derived macrophage populations expand solely through *in situ* proliferation, whereas monocyte derived macrophages require monocyte input prior to proliferative expansion in the tissue. $CCR2^+$ macrophages express high levels of pro-inflammatory genes, including those associated with the NLPR3 inflammasome, which is required to process and deliver IL-1β to the heart during cardiac stress (*Epelman S et al., 2014*). Inflammasome activation promotes adverse cardiac remodeling following ischaemic injury, genetic cardiac hypertrophy and hypertensive cardiac disease (*Bracey NA et al. 2014; Bracey NA et al. 2013)*; it is likely that $CCR2^+$ cardiac macrophages are involved in inflammasome activation in each of these settings. The robust pro-inflammatory gene signature of $CCR2^+$ cardiac macrophages suggests that they are important for immune protection against invading pathogens, but their inappropriate activation in the setting of sterile inflammation may lead to unwanted pathology (*Dunay, IR et al., 2008; Kim YG et al., 2011)*. Prior studies have used dichotomous expression of two classical myeloid cell markers (F4/80 and CD11b) in order to discern embryonic versus adult monocyte origin (*Schulz C. et al., 2012).* However, different cardiac macrophage lineages (embryonic and adult) cannot be distinguished by these cell surface markers alone and, as such, genetic fate mapping remains the most accurate method for lineage discrimination of cardiac macrophages in adult mice (*Molawi, K. et al., 2014).*

1.5. Resolution of the inflammatory response and activation of fibrogenic and angiogenic pathways

Optimal tissue repair is dependent on activation of endogenous signals that restrain the inflammatory reaction and stimulate reparative cascades. These STOP signals are particularly important in the repair of the injured heart. In the myocardium, normal function is dependent on optimal preservation of structure; even subtile alterations in cardiac morphology may induce severe dysfunction. Thus, in the injured heart unrestrained, prolonged or extended inflammatory activity could have catastrophic consequences, leading to extensive matrix degradation, adverse dilative remodeling and progressive dysfunction. Because myocardial infarction activates several distinct inflammatory cascades in all cell types involved in cardiac repair, multiple inhibitory

signals may co-operate to restrain and suppress inflammatory signaling. Recent studies have identified specific cellular processes and STOP signals involved in negative regulation of the post-infarction inflammatory reaction; in their absence, remodeling of the infarcted heart is accentuated.

Clearance of apoptotic neutrophils and removal of matrix debris

Neutrophils participate in resolution of the inflammatory response through their death. Neutrophils infiltrating injured tissues are programmed to undergo apoptosis (*Haslett C., 1999*); ingestion of apoptotic neutrophils by macrophages exerts potent anti-inflammatory and immunosuppressive effects (*Savill J et al., 2002*), mediated by macrophage-derived secretion of TGF β, IL-10 and lipid-derived pro-resolving mediators (such as lipoxins). Clearance of apoptotic cells by macrophages may function as an important endogenous inhibitory mechanism; however, its role in resolution of inflammation following MI has not been investigated. Removal of matrix fragments generated in the injured heart also appears to play an essential role in restraining the post-infarction inflammatory response (*Dobaczewski M et al., (a) 2010)*. Evidence suggested that clearance of low molecular weight hyaluronan fragments generates important inhibitory signals in the infarcted heart (*Huebener P et al., 2008).* Recent findings indicate that defects in debris clearance can prevent effective regeneration. Macrophages, the professional phagocytes of the immune system, mount a polarized, biphasic response to tissue injury. Macrophages and the monocytes from which they are derived exhibit considerable heterogeneity that is not yet fully understood. After conditioning by the inflammatory milieu including local growth factors and cytokines, macrophages polarize into classically activated (M1) or alternatively activated (M2) subtypes based on their markers, function, and cytokine profiles (*Gordon S and Martinez FO, 2010*). Typically, M1 cells produce high levels of proinflammatory cytokines and nitric oxide that aid in host defense but can also damage healthy tissue, while M2 macrophages mediate wound healing, tissue repair, and the resolution of inflammation. However, M1 cells can also play a positive role in tissue regeneration. In the heart and skeletal muscle, early infiltration by M1 macrophages facilitates clearance of necrotic tissue (*Arnold L et al., 2007; Nahrendorf M et al., 2007*), and disrupting macrophage polarization impairs healing and regeneration, respectively (*Perdiguero E et al., 2011*). While macrophages influence multiple facets of muscle regeneration, it appears that in some instances, debris clearance may supersede their roles in satellite cell proliferation, myofiber growth, and endothelial cell activation. For example, HIF-1α, the master transcriptional regulator of the hypoxia response, is dispensable in satellite cells during skeletal muscle regeneration. Surprisingly, myeloid-specific deletion of HIF-1α leads to

decreased activation of Cox-2, decreased macrophage phagocytosis, and a subsequent delay in skeletal muscle regeneration (*Scheerer N et al., 2013*).

Regulatory T cells (Tregs) and inhibitory monocytes/macrophages

Mononuclear cell subpopulations with inhibitory properties are ideally suited as negative regulators of the post-infarction inflammatory reaction. The sequential recruitment of inflammatory and reparative monocytes in the healing infarct (*Nahrendorf M et al., 2007)* suggests that early activation of MCP-1/CCR2-dependent chemotaxis may be followed by late activation of chemotactic pathways that specifically attract inhibitory cells. The chemokine/chemokine receptor pairs that may be involved in recruitment of inhibitory monocytes remain poorly understood. Differentiated macrophages may also play an important role in suppression of the post-infarction inflammatory reaction. In the dynamic and complex microenvironment of the infarct, cytokine stimulation may drive differentiation of macrophage subpopulations with inhibitory properties that may serve as key effectors in negative regulation of the inflammatory reaction. Inhibitory lymphocytes are also recruited in the infarcted myocardium and may play an important role in restraining the post-infarction inflammatory response. Regulatory T cells (Tregs) infiltrate the healing infarct and may suppress macrophage and lymphocyte inflammatory activity through contact-dependent interactions and by secreting soluble inhibitory mediators, such as IL-10 and TGF β. Interactions involving the chemokine receptor CCR5 are crucial for recruitment of Tregs in the infarct and play an important role in negative regulation of the inflammatory reaction (*Dobaczewski M et al., (b) 2010*).

Molecular signals involved in suppression and resolution of inflammation

Suppression and resolution of the post-infarction inflammatory reaction involves activation of a complex network of endogenous STOP signals that may co-operate to inhibit inflammatory activity in all cell types involved in cardiac repair. Secretion of soluble inhibitory mediators (such as IL-10 and TGF β), activation of endogenous cascades that inhibit innate immune response, increased expression of decoy receptors and deposition of matricellular proteins that modulate inflammatory signaling may play an important role in termination and resolution of the post-infarction inflammatory reaction.

IL-10

IL-10 expression shows a late and prolonged time course in the infarcted myocardium and is localized in T cells and in a subset of macrophages (*Frangogiannis NG et al.,*

2000). Associative *in vivo* studies and *in vitro* experiments suggested that IL-10 may suppress pro-inflammatory cytokine synthesis and may contribute to stabilization of the matrix by inducing synthesis of protease inhibitors, such as Tissue Inhibitor of Metalloproteinases (TIMP)-1, by macrophages. Experiments in IL-10 null mice have produced contradictory results on the role of IL-10 in negative regulation of the immune response following MI. Yang and coworkers suggested that IL-10 -/- mice had markedly increased mortality and exhibited an enhanced inflammatory response following reperfused MI (*Yang Z et al., 2000)*. In contrast, Zymek P et al. found no effects of IL-10 disruption on survival of mice undergoing reperfused infarction protocols and showed only subtle differences between IL-10 null and wildtype animals (*Zymek P et al., 2007)*. Although IL-10 -/- mice had higher peak myocardial TNFα and MCP-1 mRNA levels following infarction than wildtype animals, both groups had timely repression of pro-inflammatory cytokine and chemokine mRNA synthesis, exhibited a similar time course of resolution of the neutrophil infiltrate and had comparable adverse remodeling (*Zymek P et al., 2007*). Recent findings suggested that IL-10 signaling plays a non-critical role in suppression of inflammatory mediators, resolution of the inflammatory response, fibrosis and matrix metabolism following MI.

TGF β

The TGF βs are pleiotropic cytokines, which are implicated in a wide variety of cell functions, critically regulating inflammation, ECM deposition, cell proliferation, differentiation and growth. Three structurally similar isoforms of TGF β (TGF β1, 2 and 3), encoded by three distinct genes, have been identified in mammalian species (*Schiller M et al., 2004)*. TGF β1 is the prevalent isoform and is found almost ubiquitously, whereas the other isoforms are expressed in a more limited spectrum of cells and tissues. Although the three isoforms have similar *in vitro* properties, their *in vivo* effects are distinct. TGF β is produced by many cell types and is secreted as a latent complex, unable to associate with its receptors. Activation of TGF β signaling pathways is primarily regulated by release of active TGF β from the latent complex that is stored in significant amounts in most tissues.

TGF β may play an important role in suppression of the immune response following infarction, orchestrating the transition from inflammation to fibrous tissue deposition. TGF β suppresses cytokine and chemokine synthesis by macrophages and ECs, reduces adhesion molecule expression and promotes Treg differentiation *(Bujak M et al., 2007)*. On the other hand, TGF β activates fibroblasts, inducing transdifferentiation to myofibroblast, stimulating synthesis of extracellular matrix proteins (including collagens,

fibronectin, matricellular proteins and proteoglycans) (*Bassols A et al.,1988)*, and suppressing matrix degradation by inducing expression of protease inhibitors (such as Plasminogen Activator Inhibitor (PAI)-1 and TIMP-1) (*Laiho M et al., 1986)*. In healing infarcts, TGF β may serve as a "master switch" that links repression of the inflammatory reaction with activation of a fibrogenic program. Unfortunately, the complex biology of TGF β activation, its pleiotropic and context dependent actions, and its multiple cellular targets have hampered our efforts to understand its role in modulating the immune response in the infarcted heart. A crucial role for TGF β in resolution of post-infarction inflammation was suggested in inhibition studies through injection of an adenovirus harboring soluble TGF β type II receptor in the hindlimb muscles. Late TGF β inhibition attenuated left ventricular remodeling by reducing cardiac fibrosis (*Okada H et al., 2005)*; however, early TGF β inhibition significantly increased mortality and exacerbated left ventricular dilation enhancing cytokine synthesis. These findings suggested that TGF β signaling may play an important role in suppression of inflammatory mediator synthesis following MI (*Ikeuchi M et al., 2004*). TGF β exerts its cellular effects by binding to the constitutively active type II receptor (TβRII); subsequently the complex transphosphorylates the cytoplasmic domain of the type I receptor (TβRI). Activation of TβRI propagates downstream intracellular signals, through the Smad proteins, essential components of the TGF β signaling pathway (*Shi Y et al., 2003)*, but also activates Smad-independent pathways; the Smad3 cascade is responsible for the fibrogenic, but not for the anti-inflammatory actions of TGF β in the infarcted myocardium (*Bujak M et al., 2007; Dobaczewski M et al., (c) 2010).*

Inflammation or innate defensive and reparative response (IRR)

Inflammation is a tissue/cell response to a noxious agent and/or to a cell damage to protect, defend, repair and limit the harm of the organism. Acute inflammation was already well known to Hyppocrates and thereafter precisely described by Galeno as an acute phenomenon with prevalent vascular involvement. The modern picture is more complex and detailed due to the knowledge of pathways and molecular players for its local activation, specific tissue reactions, production of effectors, recruitment of distant cells, systemic involvement, production of damage, fine tuning regulation, and inhibition of progression for resolution and repair. Inflammation and reparative response (IRR) may result inadequate (that is ineffective in protecting and repairing), chronic (that is prolonged), inopportune (when activated untimely and awkwardly), excessive (when is not rightly controlled) during one or all its phases (*Strowig, T et al., 2012)*. In these situations IRR is clinically chronic and contributes substantially to host damages, causing

multiorgan dysfunctions. In the progression of IRR it is possible to recognize typical signs and symptoms such as the accumulation of exudate, fever, leukocyte infiltration, excess of ROS production, damage to surrounding tissue, and a continuous fibrotic repair which leads to fibrosis and to organ insufficiency (*Strowig, T et al., 2012)*. Inflammation is triggered by exogenous damaging agents such as viruses, bacteria, fungi, other pathogens and extraneous agents, presenting on their surface characteristic chemical pattern (PAMPs), or by endogenous signals (DAMPs) (*Bianchi ME, 2007)*, which are usually released by damaged cells (necrosis, membrane shedding, exosomes, apoptosis). Collectively PAMPs and DAMPs are also known as alarmins (*Iwasaki A and Medzhitov R, 2004; Marshak-Rothstein A, 2006; Fritz JH et al., 2006)*. These pleiomorphic molecules activate, with different specificity, a number of receptors belonging to highly conserved families with diverse signaling pathways, converging in the activation of NF-kB, AP-1 and other TFs which are recognized as the major players in the inflammation, controlling all the genes participating to this response (*Grivennikov SI et al., 2010)*. Alarmin receptors (ARs) were firstly localized in tissue resident leukocytes (*Iwasaki A and Medzhitov R, 2004)*, but at present they have been described in many other cells and tissues. In fact, there are evidences that ARs can be induced and expressed *de novo* in resident or recruited stem cells and pluripotent undifferentiated progenitors from many tissues, under stressing conditions such as acute or chronic hypoxia and cellular ROS increase (*Ryan BJ et al., 2014; Reuter S et al., 2010)*. ARs, after sensing alarmins, generate a signal cascade that activates NF-kB (or other TFs), leading to the expression of genes belonging to the large families of the IRR, all involved in defence, protection and repair of cell and tissues. Another molecular component of the innate response is inflammasome constituted by large protein complexes assembled by cytosolic alarmin receptors (NOD-like receptors or NLRs) activated into the cytosol (*Martinon F et al., 2002; Tabas, I et al., 2013)*. While surface membrane ARs interact with signals coming from extracellular space (of exogenous origin or released by damaged cells), inflammasomes are activated by intracellular molecules already present in damaged, stressed or aged cells. Examples of these molecules include monosodium urate crystals (gout), cholesterol crystals (hypercholesterolemia and atherosclerosis), islet amyloid polypeptide (stored in the pancreatic beta cells in type 2 diabetes) *(Tabas I et al., 2013),* viral and bacterial DNA and simpler molecules such as ATP, HMGB1 and membrane phosphatidylserine which become available in damaged or dying cells as membrane debris. Activated NLRs respond rapidly to different cytosolic PAMPs and DAMPs, recruiting and activating pro-caspase-1 and other pro-inflammatory caspases (*Martinon F et al., 2002)*. Caspase-1 activates the major pro inflammatory cytokines (IL-1 and IL-18) and is also responsible for degradation of many target proteins, contributing to cell degradation (*Robbins GR et al., 2014)*. Defects in the structure and activity of inflammasomes can be responsible for a large number of chronic human diseases, including chronic arthritis, Crohn's disease, irritable

bowel and other inflammatory diseases, diabetes, metabolic syndrome, cancer and ageing (*Rastrick J and Birrel M, 2014; Walsh JG et al., 2014)*. Targeting these proteins represents an additional new therapeutic strategy for these pathologies (*D'Elia RV et al., 2013)*.

Effectors and Cytokine Storm

Inflammation or innate immunity involve signaling pathways leading to the production of inflammatory mediators or effectors in a cell-specific manner. Examples are classical mediators such as histamine, prostaglandins, leukotrienes, lipoxins, plasmalogens, cytokines, chemokines, reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), and anti-inflammatory molecules (IL-10, A20, IL-1RA). Sometimes a massive production of pro-inflammatory cytokines (cytokine storm) occurs leading to multiorgan irreversible damage and shock (*D'Elia RV et al., 2013)*. As a matter of fact, all the effectors are a double-edge sword, having from one side an exquisite protective role to defeat invaders and limiting the harm of the host, from the other hand, when their action is excessive or improper, they can be the driving force responsible for tissue/organ damages. During inflammation, a production of numerous ROI, RNI and chlorine species occurs, in addition to the products of lipid and sugar oxidation (*Mangge H et al.,2014; Asghar MN et al., 2014)*. Most of these products are capable of chemically modifying protein amino acids, changing the structure, folding and function of proteins. Increasing evidence demonstrates that such oxidative post translational modifications may generate neo-epitopes capable of eliciting both innate and adaptive immune responses giving origin to autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, chronic bowel diseases and others (*Ryan BJ et al., 2014; Reuter S et al., 2010*). Some new epitopes generated by oxidation are conserved along evolution as PAMPs (*Ryan BJ et al., 2014; Reuter S et al., 2010).*

IRR Regulation

Usually inflammatory response is self-limiting and well controlled, but in the presence of improper activation, aberrant amplification, mutated alarmin receptors or inflammasomes, inhibited or delayed resolution results in chronic diseases. Much attention has been focused on proinflammatory signaling but the precise mechanisms leading to the inhibitory control of inflammation is still unclear (*Vallabhapurapu S and Karin M, 2009)*. The IkB kinase (IKK) complex contains two catalytic subunits, IKKα and IKKβ, and controls the activation of NF-kB transcription factors, the pivotal actor/controller of the inflammation *Napetschnig J et al., 2013)*. NF-kB activation and

activity persistence are tightly controlled by a number of endogenous mechanisms that limit the excessive and prolonged production of pro-inflammatory mediators and the associated tissue damage (*Napetschnig J and Wu H, 2013)*. NF-kB is a transcription factor that controls and modulates the expression of genes involved in both the innate and adaptive immune response (*Vallabhapurapu S and Karin M, 2009)*. The regulatory ability of NF-kB is controlled by post transcriptional modifications such as phosphorylation and complex interactions with other cytosolic and nuclear proteins. Upon activation of the NF-kB signaling pathway, IkB kinases target IkBs for degradation. This allows NF-kB to translocate and accumulate in the nucleus, where it binds to DNA, resulting in the expression of target genes *(Tafani M et al., 2013)*. One of the genes activated by NF-kB is that one encoding IkB. Newly synthesized IkB binds to NF-kB and attenuates the pathway of response to alarmin receptors, thereby creating a negative feedback loop within this signaling pathway. SOCS gene family is another major regulator of IRR once activated. Seven SOCS members have been identified but in particular SOCS-1 inhibits the cytokine signaling via Janus kinase (JAK) and STAT, efficiently negatively regulating NF-kB-directed pro-inflammatory expression. Most of the times, the IRR starts locally at the damaged site, and then rapidly eliminate the invading pathogen and/or repair the damaged cell and tissues. However, if the local activation is strong and the release of mediators (such as eicosanoids and cytokines) is abundant, distant leukocytes and cells are recruited, thus amplifying the response at systemic level. The necrosis of damaged tissues, such as in hypoxic tumours, contributes to the IRR amplification further activating resident leukocytes and recruiting the peripheral ones (*Ahmed TJ et al., 2014; Pruessmeyer J et al., 2014)*. In summary IRR is a complex response to a pathogen or to a cell damage in which inflammatory cells (chiefly leukocytes and ECs, but also tissue cells) are activated and express a specific set of genes named "proinflammatory genes" acting to defend against pathogens, protecting and repairing damaged cells. Proinflammatory gene expression has been observed also in hypoxic tissue cells, especially stem cells and less differentiated progenitors, and appears basically similar to that of activated leukocytes.

2. Stem cells

The overall clinical cardiac regeneration experience suggests that stem cell therapy can be safely performed, but it also underlines the need for reproducible results for their effective use in a real-world scenario. One of the significant challenges is the identification and selection of the best suited stem cell type for regeneration therapy. Bone marrow mononuclear cells, bone marrow-derived mesenchymal stem cells, resident or endogenous cardiac stem cells, endothelial progenitor cells and induced pluripotent stem cells are some of the stem cell types which have been extensively tested for their ability to regenerate the lost myocardium. While most of these cell types are being evaluated in clinical trials for their safety and efficacy, results show significant heterogeneity in terms of efficacy.

Preliminary efficacy studies indicate that stem cells have the potential to enhance myocardial perfusion and/or contractile performance in patients with heart failure, (a) by transdifferentiation into cardiomyocytes or vascular cells and (b) through paracrine effects by secreting growth factors which stimulate the repair and growth of host cells and the recruitment of endogenous stem cells (*Gnecchi M et al., 2008).*

2.1. Stem cells from different sources in the treatment of cardiovascular disease

For regenerative therapy in the treatment of cardiovascular disease, various cell types and stem cells from different sources at different developmental stages, including embryonic, foetal, and adult cells, have been considered for transplantation into the heart.

Human embryonic stem cells

Embryonic stem cells (ESCs) have the capacity to divide indefinitely and differentiate into any cell type. Although ESCs are incapable of spontaneous differentiation into cardiomyocytes, they can be directed to differentiate into cardiomyocytes or cardiac progenitor cells using various induction methods (*Manuilova ES et al., 2001)*. One established advantage of the use of ESCs is the ability of ESC-derived cardiomyocytes (ESC-CMs) to electrically integrate with the heart muscle. In a swine model of an atrioventricular block, transplanted human ESC-CMs showed electrical coupling and a reversal of the block *(Li R et al., 2005)*. One of the initial technical challenges faced in ESC research was the attainment of high purity and a large yield of differentiated cells belonging to a single lineage type (*Chen A et al., 2014)*. Various approaches, such as

genetic modification, specialised culture methods, and treatment with chemical and biological factors, have been used to enrich, purify, and select homogeneous and functionally intact populations of ESC-CMs generated from heterogeneous ESCs (*Bernstein HS, 2012; He W et al., 2012)*. Recently Chong and colleagues (*Chong JJ et al., 2014)* succeeded in generating cardiomyocytes from ESCs on a large scale. These ESC-CMs were able to successfully engraft and repair the injured myocardium in a primate model of MI. These results are encouraging, since only very few cell types have shown efficacy in large animals. Importantly, ESCs are pluripotent cells, which give them an advantage over other adult stem cell types with limited differentiation potential. Despite the evidence of ESCs' efficacy in larger animal models, their clinical use has been hampered by important limitations, including their genetic instability, potential tumorigenic and immunogenic properties, and ethical considerations related to the origin of these cells (*Robertson JA, 2001).* In addition, few European nations, for example, have strict laws prohibiting 'destructive embryo research' , while federal laws in the USA permit the use of embryos which have been discarded after *in vitro* fertilisation (*Dhar D et al., 2009)*.

Skeletal myoblasts

Skeletal myoblasts, also referred to as skeletal muscle satellite cells, were one of the few cell types first considered for cardiac regeneration (*Marelli D et al., 1992)*. These cells are a type of progenitor cell with myogenic capacity, and they are abundantly expressed in the human body. Isolated skeletal myoblasts can be made to proliferate and expand in vitro. The other advantages of these cells are their ability to contract and their capacity to withstand ischaemic insult (*Reinecke H et al., 2002; DerSimonian H et al., 2003)*. The evidence of efficacy in animal models facilitated the use of skeletal myoblasts in clinical trials. Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) was the first randomised placebo-controlled study of myoblast transplantation. However, this study did not demonstrate an incremental improvement in left ventricular function over that provided by CABG (Coronary Artery Bypass Grafting) alone (*Menasche P et al., 2008)*. Later, multiple experiments were aimed towards trans-differentiating these cells into cardiomyocytes, but none of the attempts were successful, suggesting that skeletal myoblasts are committed towards a skeletal muscle fate (*Reinecke H et al., 2002)*. Moreover, myofibres derived from the transplanted skeletal myoblasts fail to integrate electromechanically with the host myocardium due to a lack of adhesion proteins. This results in the failure to develop the intercalated discs required for electrical integration between adjacent myofibres. The excitement around skeletal myoblasts was further reduced when clinical studies reported the occurrence of ventricular arhythmias following

transplantation in patients: failure to electrically couple these cells with the existing tissue may be a reason for myofibres' arrhythmogenicity. Genetic modification to introduce the expression of connexin 43, a gap junction protein, was considered as a strategy for overcoming this limitation (*Roell W et al., 2007)*. However, a later study by Fernandes and colleagues (*Fernandes S et al., 2009)* found that this modification was not sufficient to reduce the arrhythmogenic potential of these cells.

Bone marrow stem cells

The first research on stem cells was conducted on bone marrow as early as 1950, which found at least two kinds of stem cells within bone marrow: hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). The ability of transplanted bone marrow-derived cells (BMCs) to regenerate the infarcted myocardium was first shown by Orlic and colleagues in 2001 (*Orlic D et al., 2001)*. They demonstrated that HSCs marked by the surface protein cKit were account able for the trans-differentiation of BMCs into mature cardiomyocytes (CMs), smooth muscle cells (SMCs), and ECs in a murine model of MI. In support of the above results, injection of isolated $cKit^+$ cells into the peri-infarct regions resulted in improved left ventricular function in the infarcted heart (*Orlic D et al., 2001)*. However, their claim regarding the ability of HSCs to transdifferentiate into cardiovascular cells has been questioned by several other studies (*Murry C t al., 2005; Nygren JM et al., 2004; Balsam LB et al., 2004)*. Nevertheless, the important outcome was that these cells showed a significant improvement in cardiac function after engraftment. Apart from cKit, many other cell surface markers have also been identified that define populations enriched for freshly isolated human HSCs, including the $CD133^+$ and $CD34^+$ hematopoietic cells (*Yin AH et al., 1997)*. Interestingly, $cKit^+$ HSCs have never been tested clinically, which is required to truly compare their efficacy with other cell types. Similar to the $cKit^+$ cells, $CD34^+$ cells have also been considered for cardiac regeneration. $CD34^+$ cells are routinely used clinically to reconstitute the deficient hematopoietic system after radiation or chemotherapy (*Sidney LE et al., 2014)*. In addition to being a resident population in the bone marrow, these cells were identified in the peripheral blood by Körbling and colleagues (*Körbling M et al., 2002)*. Evidence has suggested that endothelial progenitor cells (EPCs) and differentiated ECs also express CD34, leading to studies testing the angiogenic capacity of bone marrow and peripheral blood-derived $CD34^+$ cells. These cells also showed the ability to differentiate into CMs and smooth muscle, in addition to ECs, after transplantation into an infarcted heart (*Yeh ET et al., 2003)*. However, this approach was questioned by Norol and colleagues (*Norol F et al., 2007)*, who did not find any cardiac phenotype following transplantation of $CD34^+$ cells into non-human primates. Despite these controversial findings, most clinical trials to date

have used total bone marrow mononuclear cells, which comprise HSCs, MSCs, and monocytes (*Arnous S et al., 2012)*. A review by the Cochrane Heart Group summarised 33 clinical trials (1,765 patients) on the effectiveness of BMCs for cardiac regeneration following acute MI and concluded that while no significant improvement was observed in the mortality and morbidity of the patients who received BMCs, they demonstrated a significant and sustained improvement (12 to 61 months follow-up period) in left ventricular ejection fraction (LVEF) was demonstrated (*Clifford DM et al., 2012)*. Further, in a recent meta-analysis (23 clinical trials and 1,255 patients) the same group concluded that, in addition to the improvement in LVEF, BMCs were also able to improve the morbidity and mortality in patients with chronic ischaemic heart disease (IHD) and congestive heart failure (*Fisher SA et al., 2014)*. Despite these promising results, debate continues about whether the therapeutic potential of BMCs in improving left ventricular function might be attributed mostly to their paracrine effects (*Uemura R et al., 2006)*. The conclusions from the Cochrane Heart Group were optimistic but caveats included the high degree of heterogeneity observed in the results. A recent study utilised weighted regression and meta-analysis to compare the results from 49 trials using BMCs for cardiac regeneration (*Nowbar AN et al., 2014)* : this analysis identified 600 discrepancies in 133 reports from these trials. Interestingly, the trials with the highest number of discrepancies also showed the maximum increment in LVEF in patients. The studies that failed to show any benefit from BMCs had the lowest number of discrepancies (*Nowbar AN et al., 2014)*. With such differing data available from clinical trial results, it is extremely difficult to draw conclusions on the efficacy of BMCs (*Abbott A, 2014).*

Mesenchymal stem cells

MSCs have been typically considered as the cells with the capacity for self-renewal and differentiate into the mesenchymal lineages, including skeletal myoblasts, chondrocytes, and adipose tissue (*Pittenger MF, 1999)*. This classical view is now challenged as they have been shown to differentiate into neural (non-mesenchymal) tissues as well *(Long X et al., 2005)*. The adherent MSC population is shown to express cell surface markers CD73, CD105, CD29, CD44, and CD90 and lack CD34 and CD45, which are mainly expressed by HSCs (*Dominici M et al., 2006)*. Considering that these cells can be isolated from a variety of tissues, including bone marrow, adipose tissue, and cord blood, it makes them a more practical option for regenerative therapy. The second advantage with MSCs is that they lack major histocompatibility complex II and B7 co-stimulatory molecule expression; hence, they are able to evade immune responses and have an innate ability to overcome the rejection. This opens the possibility of non autologous transplantation in patients (*Kuraitis D et al., 2011)*. MSCs have been shown to

differentiate into CMs as well as vascular endothelial cells *in vitro*. Conversely, experimental evidence suggests that when transplanted *in vivo*, MSCs contribute to neo-vascularisation and CM protection, mainly through the activation of paracrine factors. They may persist within the myocardium in a differentiated state, although substantial evidence for their ability to attain cardiac cell phenotype *in vivo* is still needed (*Miyahara Y et al., 2006)*. Currently, clinical trials are being conducted to investigate the therapeutic effects of human MSCs in many cardiovascular and neurodegenerative disorders (*Kalladka D and Miur KW, 2014*; *Mastri et al., 2014*). As previously said, in addition to their multilineage differentiation potential, MSCs may exert their regenerative effect via the production of multiple paracrine factors (*Kalladka D and Miur KW, 2014*; *Mastri et al., 2014*). Production of IL-6, vascular endothelial growth factor (VEGF), hepatocytes growth factor (HGF), brain-derived neurotrophic factors (BDNF), glial-derived neurotrophic factor (GDNF), neurotrophin-3 (NT3), fibroblast growth factor (FGF), and thrombospondins can be promoted by MSCs.

Summarizing, MSCs have shown great promise in ischemic tissue repair and have the following advantages: 1) No ethical issues, as they are a self-derived bone marrow derivative 2) high proliferation rate, and so can be amplified in a short period of time; 3) no transplant rejection occurs so no immune-suppressants are required.

Induced pluripotent stem cells

The cellular differentiation process was once believed to be an irreversible process. In 2006, however, Takahashi and Yamanaka (*Takahashi K and Yamanaka S, 2006)* successfully induced pluripotency in somatic cells through retroviral transduction of several factors involved in the self-renewal of ESCs. The combination of transcriptional factors commonly used for cellular reprogramming are Krüppel-like factor 4 (Klf-4), sex determining region Y-box 2 (Sox-2), c-Myc or octamer-binding transcription factor 4 (Oct3/4), Nanog, and Lin-28 (*Takahashi K and Yamanaka S, 2006).* Since then, several studies have demonstrated the wide differentiation potential of induced pluripotent stem cells (iPSCs), which includes their ability to differentiate into cell types from any of the three germ layers (*Lee J-H et al., 2014)*. iPSCs have been found to differentiate into CMs, ECs, and SMCs *in vitro*. When injected into the infarcted heart of mice, iPSCs can differentiate into the cardiac phenotype (*Martens A et al., 2012; Yu SP et al., 2013)*. One of the initial problems with using iPSCs was the poor experimental efficiency in the successful induction of pluripotency to somatic cells. The use of genetic factors, chemical inhibitors, and signalling molecules that can either replace core reprogramming factors or enhance reprogramming efficiency has now been investigated. Recently, Rais and

colleagues (*Rais Y et al., 2013)* found that the Mbd3/NuRD (nucleosome remodelling and de-acetylation) repressor complex is the predominant molecular block preventing the deterministic induction of ground-state pluripotency, and hence by depleting the Mbd3 gene, they could successfully synchronise all cells to attain pluripotency. This does overcome a chief barrier to the clinical use of iPSCs (*Rais Y et al., 2013).* Due to their ESC-like properties, however, they were also found to be tumorigenic (*Riggs JW et al., 2013)*. Hence, to overcome the problem of tumorigenesis, Martens and colleagues (*Martens A et al., 2012)* differentiated iPSCs into cardiomyocytes *in vitro* before transplanting them into the infarcted heart. Transplanted cells not only improved the cardiac functions, but also localised to the host myocardium (*Martens A et al., 2012)*. Further, biosynthetic tissues are also created from cardiac cells derived from iPSCs (*Christoforou N et al., 2013)*. Several bioengineering strategies are being explored to improve the efficacy of iPSC-derived transplants to improve their engraftment, survival, and functionality in tissues (*Fisher MB et al., 2013)*. The *in vivo* safety and functionality of these cells need to be assured before their clinical translation is considered.

2.2. Stem cell types obtained from the heart

In spite of the numerous conflicting reports concerning the regenerative capacity of myocardium, the majority of researchers agree that there is a profound difference in the regenerative capacity of skeletal and cardiac muscles. In the mammalian heart, it is generally accepted that adult CMs do not possess the ability to proliferate. Hence, they had long been classified as *elementi perenni* or cells of static cell populations *(Rumyantsev PP, 1977)*. Based on this view, the participation of a pool of structurally undifferentiated myoblasts or "myogenic stem cells" in cardiac myogenesis had been hypothesized many decades ago (*Rumery RE and Rieke WO, 1967; Shafiq SA et al., 1968)*. Only recently, however, the identity of such a heart specific stem cell pool emerged. In 2007, Hsieh and colleagues provided strong evidence that stem cells renew adult mammalian CMs (*Hsieh PC et al., 2007)*. Although this study was preceded by the discovery and characterization of different cardiac progenitor cell populations, including $cKit^+$ cardiac stem cells (CSCs), it provided the first clear evidence for the presence of an undifferentiated cell population that directly contributes to myocardial renewal upon injury.

Evidence for existence of cardiac stem/progenitor cells

Different groups have shown the presence of different cell populations in the adult heart that share some characteristics with stem cells. These cells, called CSCs, would be involved in the replacement of senescent or apoptotic cardiac cells, allowing the maintenance of cardiac homeostasis. They are self-renewing, clonogenic and multipotent, being able to differentiate into CMs, SMCs and ECs under *in vitro* stimulation. CSCs would also contribute to coronary vessels regeneration, in addition to CMs. During ischemic processes, several paracrine signals stimulate these CSCs to divide. However, these divisions are insufficient to replace the cell loss caused by a MI (*Jezierska-Wozniak K et al., 2011)*. In several studies, CSCs have been isolated and expanded from human myocardium biopsies, offering a new source of stem cells for cardiovascular regenerative medicine (*Sanz-Ruiz R et al., 2011)*. The different populations of CSCs have been classified mainly according to the expression of different surface markers.

Identity of resident cardiac stem/progenitor cells

The cKit$^+$CSCs were originally considered a population apart from other progenitor cells as they did not appear to express endothelial or bone marrow progenitor markers, such as CD34 or CD45, expressed in hematopoietic stem cells. They were also negative for the transcription factors and cytoplasmic proteins found in mature CMs, ECs and SMCs, including Isl1 (*Leri A et al., 2009)*. However, other studies have documented the presence of different subpopulation of cKit$^+$CSCs, some of them expressing mesenchymal and endothelial markers (*Bearzi C et al., 2009; Gambini E et al., 2011).*

So far, the best-characterized and well-studied CSC population is the cKit$^+$/Lin$^-$ cells. cKit$^+$/Lin$^-$ cells with properties of CSC were first described in the rat heart by Beltrami et al. in 2003 (*Beltrami AP et al., 2003)*. The authors initially found rare cKit$^+$/Lin$^-$ cells present in the rat myocardium. First described by Yarden et al. in 1987 as a proto-oncogene (*Yarden Y et al., 1987)*, cKit is a tyrosine kinase receptor expressed at high levels in hematopoietic stem cells, multipotent progenitors, common myeloid progenitors and less abundantly in other cell types. The ligand for this receptor has been described as stem cell factor (a substance that causes certain types of cells to grow), also known as steel factor or cKit ligand. The signaling pathways downstream of the cKit receptor are essential for regulating proliferation, survival and other vital functions in early hematopoietic cells (*Edling CE and Hallberg B*, *1995*). Based on the presence of a stem cell marker cKit (*Morrison SJ et al., 1997; Weissman IL et al., 2001)* and the absence of pan leukocyte marker CD45 and endothelial/hematopoietic progenitor marker CD34, it was proposed that cKit$^+$/Lin$^-$ cells represent a unique stem/progenitor population resident

in the heart (*Beltrami AP et al., 2003).* They were usually present in small clusters, most of which contained cells at early stages of cardiac myogenic differentiation, as demonstrated by the expression of the transcription factors Gata4, Nkx2.5, and Mef2, together with low levels of sarcomeric proteins in the cytoplasm. When isolated and grown in culture, $cKit^+/Lin^-$ cells exhibited extensive self renewal capacity and clonogenicity, being able to proliferate for more than 19 months without reaching growth arrest or senescence. In addition, when induced to differentiate *in vitro*, they gave rise to CMs, smooth muscle, and ECs, although *in vitro* differentiated cells were morphologically and functionally immature. Furthermore, when injected into an ischemic heart, they reconstituted differentiated myocardium with new vessels and myocytes. Since then, $cKit^+/Lin^-$ CSCs have been described in multiple mammalian species, including human (*Bearzi C et al., 2007; Linke A et al., 2005; Urbanek K et al., 2006)*. The $cKit^+/Lin^-$ cardiac cell population likely represents a heterogeneous cell population that contains lineage-committed progenitor cells along with true stem cells. Anversa and colleagues reported that $cKit^+/Lin^-$ cardiac cells included subpopulations that appeared to be more committed along the cardiac differentiation (i.e., lineage restricted) based on co-expression of cKit and transcription factors of cardiovascular lineages, such as Gata4, Mef2c, Gata-6 , and Ets1 (*Linke A et al.2005; Urbanek K et al., 2006)*. Accordingly, a hierarchy among $cKit^+$ cardiac cells was proposed, in which $cKit^+$ cardiac cells are further categorized into different subpopulations of progenitor cells with variable growth and differentiation characteristics (*Anversa P et al., (a) 2006)*. Recently, the same group also identified a coronary vascular progenitor cell (VPC) population in the human heart (*Bearzi C et al., 2009)*. VPCs were found in specialized niches in epicardial coronary arteries, arterioles, and capillaries, and characterized by co-expression of cKit and KDR/VEGFR2, an early marker of angioblast precursors (*Yang L et al., 2008)*. They also expressed low levels of the endothelial cell adhesion molecule CD31 and the smooth muscle cell marker TGF β1 receptor, while being negative for hematopoietic markers (e.g., CD45 and CD34) and α-sarcomeric actin. Upon differentiation, VPCs preferentially gave rise to the cells of vascular lineages (i.e., endothelial and smooth muscle cells) both in vitro and in vivo (*Bearzi C et al., 2009)*, indicating that they are more committed to the vascular lineages. Collectively, the $cKit^+/Lin^-$ cardiac cell population may represent several different lineage-committed progenitor cell populations along with true stem cells. Such finding emphasizes the need for more rigorous characterization of the $cKit^+/Lin^-$ cardiac cell population, which is used in both preclinical and clinical settings. It is uncertain if the proportions of different progenitors remain relatively constant or vary among individuals, especially depending on the disease state or other physiological variables. It will be of great interest to study how the changes in cellular composition of the $cKit^+/Lin^-$ cardiac

cell population translate into differences in proliferative and differentiation potentials, and ultimately, in the therapeutic efficacy of patient-specific cKit$^+$CSC populations.

Origin of cKit$^+$/Lin$^-$ CSCs

The developmental origin, as well as the origin of adult cKit$^+$/Lin$^-$ CSCs have been controversial topics. As mentioned above, the initial characterization of adult cKit$^+$/Lin$^-$ CSCs revealed that they are negative for CD45 and CD34, suggesting that they are not of hematopoietic origin (*Beltrami AP et al., 2003)*. Also, the presence of specialized CSC niches within the heart argues for their cardiac origin. The CSC niches contain clusters of undifferentiated cKit$^+$CSCs and early cardiovascular lineage-committed cells (i.e., cKit and Gata4, Mef2c, or Ets1 double-positive cells) (*Urbanek K et al., 2006)*. Moreover, within the CSC niche, CSCs and early lineage-committed cells are connected to neighboring myocytes and fibroblasts via gap and adherens junctions (*Urbanek K et al., 2006).* Taken together, these observations strongly suggest that CSCs not only reside stably in the heart, but are also specifically committed to give rise to multiple cardiac cell types. Recently, Ferreira-Martins et al. provided evidence that cKit$^+$CSCs originate from the developing heart itself by using transgenic mice that express GFP under the control of the cKit promoter (cKit-GFP). The authors utilized two photon microscopy to directly monitor *in situ* the temporal and spatial changes in the GFP$^+$ (i.e., cKit$^+$) putative CSC population in the heart of developing cKit-GFP transgenic embryos (*Ferreira-Martins J et al., 2012)*. Starting at E6.5, a cluster of cKit$^+$ cells was detected in the cardiogenic mesoderm, and by E8.0, cKit$^+$ cells in the heart tube were observed migrating towards the apex. Importantly, the authors reported that migration of extra-cardiac GFP$^+$ (i.e., cKit$^+$) into the heart was not observed during the window of observation, arguing for the cardiac origin of cKit$^+$CSCs. However, it should be noted that rigorous and continuous monitoring of GFP$^+$ cells in a developing embryo is technically challenging , and thus the possibility that a small number of extra-cardiac (e.g., hematopoietic) stem cells contributing to the cardiac cKit$^+$ cell population and to the developing myocardium could not be completely ruled out by the study. In contrast, several studies have suggested that cKit$^+$ cardiac cells may not represent a stable resident cell population but have an extra-cardiac origin, such as bone marrow (BM). This view is, in part, supported by the intriguing observations that CMs of non-cardiac origin are detected in patients who have received gender-mismatched heart (*Quaini F et al., 2002; Muller P et al., 2002)*, BM (*Thiele J et al., 2004; Deb A et al., 2003)*, and peripheral blood stem cell (*Thiele J et al.,2004)* transplants. Fazel and colleagues (*Fazel S et al., 2006)* have shown that cKit$^+$ cells are mobilized from the BM and recruited to the heart following MI, and serve cardio-protective functions. Interestingly, the majority of them downregulated and no longer expressed CD45.

Similarly, others have shown that hematopoietic stem cells are recruited into the damaged myocardium and contribute to cardiac repair (*Fujita J et al., 2007; Wang Y et al., 2006)*. These studies raised an interesting possibility that the cKit$^+$CSCs may not originate from the heart, but from the BM (*Segers VF et al., 2008)*. A recent study tested this idea by creating chimeric mice whose BM had been reconstituted, with one constitutively expressing GFP (*Ellison GM et al., 2013)*. Using these BM chimeric mice, one can easily identify BM-derived cells in different organs based on the GFP expression. The chimeric mice were subjected to isoproterenol-induced heart injury, and the presence of BM-derived GFP$^+$ cells in the regenerated heart was examined at 28 days of recovery. The authors were unable to find any GFP$^+$ cardiomyocytes in the restored myocardium, clearly demonstrating that BM-derived cells do not directly contribute to myocyte renewal. Interestingly, they also noted that a small fraction of cKit$^+$/CD45$^-$ cardiac cells was GFP$^+$ (i.e., of BM origin) in both control and isoproterenol-treated hearts (*Ellison GM et al., 2013)*. It is not clear, however, if these BM-derived cKit$^+$/CD45$^-$ cardiac cells eventually acquire the role of resident CSCs and contribute to the maintenance and renewal of myocardium. It appears that a genetic fate mapping study is needed to resolve the issues surrounding the developmental as well as adult origin(s) of resident cKit$^+$ CSCs. Such studies would involve introducing permanent reporter gene expression (e.g., LacZ or GFP), specifically in cardiac mesoderm or early cardiac progenitor cells in the developing embryo, and tracking the reporter gene expression to the cardiac cKit$^+$/CD45$^-$ cell population in the adult heart.

Numerous studies have demonstrated that cKit$^+$/Lin$^-$ CSCs isolated and expanded in culture exhibit all the properties of bona fide stem cells, and when injected into the injured myocardium, they are capable of restoring (to a variable extent) the cardiac structure and function in various animal models, as well as in patients suffering from ischemic cardiomyopathy (*Bolli R et al., 2011; Tang XL et al., 2010)*. Moreover, CSC dysfunction has been implicated in development and progression of cardiac pathologies associated with diabetes and anthracycline toxicity (*Rota M et al., 2006; De Angelis A et al., 2010)*. However, the precise role of endogenous c-kit$^+$ CSCs in maintenance and renewal of the heart has remained poorly understood.

Sca-1$^+$ progenitor cells

In 2003, Oh et al. (*Oh H et al., 2003)* reported the isolation of a 'myocyte depleted' fraction of adult cardiac cells expressing Sca-1 and CD31, but negative for blood cell lineage markers (CD34, CD8, B220; Gr-1, Mac-1 and TER119). These cells were also positive for cKit, Flt-1, Flk-1, vascular endothelial cadherin, von Willebrand factor and hematopoietic stem cell markers CD45. They showed telomerase activity and were able

to differentiate into mature CMs when cultured in the presence of 5-azacytidine (*Oh H et al., 2003)*. Sca-1 was first reported as a cell surface marker of hematopoietic stem cells belonging to the Ly-6 antigen family (*van de Rijn M et al., 1989)*. Recently, many reports have demonstrated that multipotent stem cells derived from BM and skeletal muscle also express Sca-1. These cells are able to differentiate *in vitro* into different lineages (hepatocytes, CMs, skeletal muscle, neural cells, ECs, adipocytes and myocytes) if they are submitted to the appropriate stimuli (*Matsuura K et al., 2004)*. Sca-1 plays a role in myoblast differentiation, proliferation, fusion and cell-cycle exit. It appears to downregulate muscle cell proliferation, thereby maintaining a pool of functional progenitor cells for muscle homeostasis and repair (*Holmes C et al., 2007)*.

Side population cells

Side population (SP) cells were first described by Goodell et al. as a subpopulation of murine bone marrow cells able to block the Hoechst 33342 dye efflux activity (*Goodell MA et al., 1996)*. Some years later, the existence of a cardiac SP was reported by Pfister et al. (*Pfister O et al., 2005).* These cells were $CD31^-/Sca\text{-}1^+$ and they were capable of cardiomyogenic differentiation into mature CMs through a process mediated by cellular coupling with adult CMs (*Pfister O et al., 2005)* or in the presence of cardiomyogenic agents such as 5-azacytidine (*Oh H et al., 2004)*, oxytocin or trichostatin A (*Oyama T et al., 2007).* This was the first population isolated directly from digested heart tissue. Cardiac SP cells are highly enriched for Sca-1, suggesting a possible relationship between $Sca\text{-}1^+$ cells and SP cells in the heart (*Goichberg P et al., 2014)*. It has also been described a $Sca^+/CD31^+$ subset in the isolated SP cells that seem to have lower cardiogenic potential than $Sca^+/CD31^-$ cells. *In vitro*, SP cells can differentiate into CMs, SMCs and ECs, and also into glial cells and neurons, suggesting that the origin of these cells could be in the neural crest (*Tomita Y et al., 2005)*. More than 93% of adult cardiac SP cells are $Sca\text{-}1^+CD45^-CD34^-$, exhibit levels of telomerase expression similar to neonatal myocardium and express cardiogenic transcription factors. Moreover, these cells do not express cardiac structural genes until stimulated to differentiate *in vitro* (*Holmes C et al., 2007)*. However, these cells are relatively rare in the adult tissue, and their ability to contribute to the regeneration and functional repair of damaged cardiac muscle remains to be determined (*Barile L et al., (b) 2007)*.

$Isl1^+$ progenitor cells

Isl1 is a LIM-homeodomain transcription factor that controls cardiomyocyte specification and differentiation. Isl1 is a marker of myocardial lineage during mammalian

cardiogenesis and identifies a common population of progenitor cells in the heart that can differentiate into CMs, SMCs and ECs. Two-thirds of the cells within the entire heart originate from Isl1-positive progenitor cells (*Fonoudi H et al., 2013)*. This transcription factor promotes postnatal angiogenesis and vasculogenesis by improving the angiogenic properties of ECs and MSCs (*Barzelay A et al., 2010)*. Isl1 is a characteristic marker of an abundant population in the embryonic heart. Some of these cells remain undifferentiated even after the complete heart formation but its number decreases gradually (*Leri A, 2009)*. In the adult heart, some $Isl1^+$ cardioblasts can be found in atria and ventricles as isolated cells. They also express early cardiac differentiation markers such as Nkx2.5 and Gata4 but lack mature CMs transcription factors, Sca-1 and cKit (*Vanelli A et al., 2011)*. These cells, however, have only been described to date in neonatal specimens, so their application for cardiac regeneration may be limited (*Barile L et al., (a) 2007)*.

Cardiospheres and Cardiosphere-derived cells

In 2004, Messina et al. described a new isolation technique for cardiac resident progenitor cells from murine hearts as well as from human atrium and ventricle subcultures. After enzymatic digestion of the tissue and seeding in culture flasks, a monolayer of adherent cells with fibroblastic morphology grew in the flasks. Some days later, some small, round and bright cells started to migrate over this monolayer, being in suspension. When these cells were seeded in poly-D-lysine-coated flasks, they gave rise to cluster formations that were called cardiospheres (CS). These cells were clonogenic and expressed endothelial progenitors and pluripotency markers, showing the same properties of cardiac progenitor cells (CPCs). They were able to self-renew and differentiate into cardiac specialized cells as CMs and vascular cells, either *in vivo* and *in vitro* (*Messina E et al., 2004)*. These CSCs growing as CS represent per se an actual cardiac microtissue, where the most differentiated cells are localized in the outer layers, while those with a higher differentiation potential and expression of stem cell and angiogenesis markers are in the inner core (*Gaetani R et al. 2010)*. Three years after their first report, Smith et al. used CS to obtain cardiosphere-derived cells (CDCs) from human and porcine tissue. They compared both phenotypes, finding several similarities and slight differences *(Smith RR et al., 2007)*. Human CS are $cKit^+$ and $CD105^+$ (one of the regulators for the TGF β receptor, important for angiogenesis and hematopoiesis processes). CS also show a high expression of connexin 43, a component of the tight junctions between cells. CDCs also express connexin 43, which suggests an electric coupling potential between these cells as well as between them and cardiac myocytes. CDCs are also positive for CD90, CD34 and CD31 and negative for CD133, CD45 and blood antigens (*Messina E et al., 2004)*.

3. Stem cell therapy

Stem cells (SCs) have the potential to restore cellular function in individuals suffering from incurable diseases by their capacity to self-renew and to differentiate into one or more specialized cell types. Stem cells from various origins have, in addition to their regenerative power, the capacity to induce endogenous *in situ* regeneration/repair. Therefore, a paracrine effect is considered as an important therapeutic element in addition to the regenerative one. The combined regenerative and paracrine effects are therefore two items that should be considered as intrinsic characteristics of any stem cell to be translated into valid therapy.

A majority of the cardiovascular cell therapy clinical trials have used autologous cell populations, which obviate immunologic mediated cellular rejection. The mode of delivery has been primarily intracoronary, but has also included intramyocardial and endocardial routes. Potential side effects associated with cell therapy include arrhythmogenesis, tumorigenesis, myocardial injury, infection or immunologic responses. Nevertheless, although these complications are possible, very few side effects have been observed till now and cell therapy for cardiovascular disease is relatively safe; it may modulate remodeling and may yield a modest improvement in cardiac function.

3.1. Challenges in identifying the best source of stem cells for cardiac regeneration

Before translation of stem cell use from preclinical studies to the clinic, one significant challenge is the identification and selection of the best suited stem/progenitor cell types. The primary task is to obtain clear evidence on what truly drives the improvement in heart function after the administration of stem cells. The mechanism(s) underlying the observed functional improvement in the heart remains unclear and is an issue for debate. Classically, it is believed that an ideal stem cell should differentiate into CMs that integrate both mechanically and electrically with innate myocytes and should be able to form blood vessels to boost the blood supply to the scar zone. On the other hand, recent studies suggest that paracrine factors secreted by the stem cells may play a more important role in the improvement of cardiac function (*Chimenti I et al., 2010)*. This has changed the belief that the stem cells must differentiate into cardiac cell types to improve cardiac function. Hence, stem cell types which are not multi-potent themselves can still improve cardiac function comparable to stem cells committed to cardiac lineages (*Urbich C et al., 2004)*. The next step now is to systematically characterise these cells by their ability to differentiate into cardiac cell types and their ability to improve cardiac health by paracrine mechanisms (*Gnecchi M et al., 2008)*.

3.1.1.Practical and technical challenges

Many of the cell types considered are pluripotent. The stem cells that are committed to the myocardial lineage can be selected using different reporter systems linked to the endogenous activation and expression of cardiogenic or myogenic genes. For cardiac regeneration, it is essential that the selected population of cells shows high cardiogenic potential, since the injection of highly proliferative uncommitted pluripotent stem cells can lead to carcinogenicity. Due to the large amount of heterogeneity in the number and quality of adult stem cells in patient tissue samples, selecting and isolating a pure population of cardiac lineage committed stem cells is essential for the safety as well as efficacy of stem cell therapy (*Kamp TJ, 2011)*. The number and efficacy of stem cells have been shown to change depending on several factors such as age, gender, treatments, and pre-existing conditions. Sanada and colleagues (*Sanada F et al., 2014).* reported a marked decline in the $cKit^+$ population with an increase in age. This was supported by another study showing a loss of cardio-protective effects in CSCs isolated from older patients, which also achieved early senescence during *in vitro* culture (*Avolio E et al., 2014)*. In addition to age, CSCs isolated from patients with pre-existing morbidities, such as diabetes and hypertension or end-stage heart disease, also showed marked differences in gene expression, cell survival properties, and functional ability compared with those isolated from healthy individuals (*Itzhaki-Alfia A et al., 2009; Seewoodhary J et al., 2013)*. Similarly, mobilisation of $CD34^+$ cells by granulocyte colony stimulating factor (G-CSF) was found to be completely impaired in diabetic patients, whereas the levels of $CD34^+$ cells were increased 2.2-fold after mobilisation in non-diabetics (*Fadini GP et al., 2013)*. However, some important questions remain unanswered regarding, for example, the effect of the severity of the disease, the time since the acute event, and the duration of pre-existing conditions such as diabetes and hypertension on the number and efficacy of stem cells. In addition to these factors, stem cells from the same organ source might show functional differences depending on the location. For example, CSCs isolated from the atria and the ventricles might have differential gene expression and functional efficacies similar to CMs from these two regions (*Ng SY et al., 2010)*. Further, gender differences in the aging process of the human heart might also occur in terms of differences in CSC quality (*Olivetti G et al., 1995)*. This evidence suggests the need for more research to develop a personalised evaluation protocol in order to identify optimal stem cell sources depending on patient need.

Challenges in isolation and expansion of stem cells

The next challenge in translating stem cell therapy from a preclinical to a clinical setting is the practicality of its use, such as the ease and efficiency of stem cells isolation and expansion. Peripheral blood samples are easy to collect, although the relative expansion abilities of the EPCs from the peripheral blood are limited (*Itzhaki-Alfia A et al., 2009)*. In contrast, endomyocardial biopsies are difficult to obtain, but CSCs can be isolated and expanded from tiny (approximately 5 mg) endomyocardial biopsies (*D'Amario D et al., 2011)*. Within the heart, CSCs from a particular location may be more effective (CSCs from ventricular biopsies) in myocardial regeneration but can be difficult to procure. Another tricky task in stem cell therapy is marker-based selection. Selection of the right marker for isolation is a critical decision because of the heterogeneity in stem cells such as CSCs (*Mishra R et al., 2011)*. In addition, stem cells from the same source can express heterogeneous markers. Some stem cell markers represent a 'moving target', which means that cells retain stem cell-like properties even after losing the expression of these markers following subsequent passages in culture. Hence, capturing stem cells based on these markers becomes illusive at times (*Fadini GP et al., 2008)*. As above, the severity of the disease-associated pre-existing conditions and age also change the expression of stem cell surface markers and the overall number of stem cells expressing a particular marker. For example, the percentage of $cKit^+$ cells was found to be higher in patients with end-stage heart failure compared with patients without end-stage heart failure, whereas the number of circulating endothelial progenitor cells was found to decrease with age (*Scheubel RJ et al., 2003; Ballard VL et al., 2008)*. The percentage of cKit was found to be very low in unfractionated CDCs, while almost 90% of these cells were found to be positive for MSC markers (*Tateishi K et al., 2007)*. While the suitability of cKit to identify CSCs has been questioned, the above evidence implies that a single marker may not be sufficient for identifying an effective cell population (*van Berlo JH et al., 2014)*. This is further supported by a study from Li and colleagues (*Li TS et al., 2012)*, who demonstrated the superior effect of unsorted CDCs compared with a purified cKit subpopulation. Further studies are required to understand the best marker(s).

In addition to source and marker, isolation technique and culture conditions also offer a major challenge in the expansion of stem cells. Studies suggest that differences in the intensity of enzymatic digestion may affect the type of cells isolated (*Leinonen JV et al., 2013)*. Different cell types, growth conditions, passaging methods, and number of cell passages also influence the outcomes. Pfister and colleagues attributed the low expression of cKit in CDCs to enzymatic cleavage during the digestion process, as treatment of $cKit^+$ bone marrow cells with a cardiac digestion regimen resulted in a significant reduction in $cKit^+$ cells (*Pfister O et al., 2005)*. One other study showed that $CD34^+$ HSCs undergo epigenetic changes involving DNA methylation, which leads to the

loss of stemness over subsequent *in vitro* culture passages (*Weidner CI et al., 2013)*. The need for increased numbers of appropriate cells continues to limit the clinical development of cell therapy. This has led to a considerable number of studies focussing on *ex vivo* stem cell expansion. Different strategies have been used for the ex vivo expansion of stem cells from different sources. A high degree of logistic support may be required for the large scale expansion of stem cells. Open system configurations such as culture dishes and flasks and closed-systems such as gas-permeable bags, stirred/spinner flasks, flatbed perfusion bioreactors, and three dimensional scaffolds have been used for culturing and expanding stem cells on a large scale (*Madlambayan GJ et al., 2006)*. Amplification of a cell's proliferation potential is also required. For example, HSCs can be expanded *ex vivo* with the use of specific growth factors and a serum-free media. Some of these factors include thrombopoietin, stem cell factor, G-CSF, IL-6, and Fms-related tyrosine kinase 3 ligand (*Douay L et al., 2001)*. EPCs are expanded by culturing them on fibronectin in the presence of growth factors favouring endothelial cell growth (*Peplow PV et al., 2014)*. Fibroblast growth factors have been used for expanding MSCs and CSCs (*Walenda T et al., 2011)*. The use of growth factors and favourable culture conditions to expand stem cells may also have an effect on their functional properties (*Rodrigues M et al., 2010)*. Reports suggest that the use of basic fibroblast growth factor to culture MSCs can extend the doublings of these cells up to 80 population doublings (*Yanada S et al., 2006)*. But as the cells reach senescence, there is a down-regulation of growth factor receptors, and hence they may become resistant to the proliferation stimuli (*Tran KT et al., 2003; Shiraha H et al., 2000)*. While different combinations of growth factors have been used to improve the proliferation and functional efficacy of stem cells, due to the effect of cell culture conditions on cell physiology it is difficult to compare and interpret the results from these studies (*Weidner CI et al., 2013)*.

Stem cell preconditioning strategies

"Preconditioning" refers to any pharmacological, environmental or genetic modification of the cells for the amplification of their potency. Preconditioning promotes stem cell survival and may promote proliferation, differentiation, or its resistance against oxidative stress *in vitro* and *in vivo* (*Mohsin S et al., 2011)*. While several studies have exclusively confirmed the differentiation and proliferative ability of stem cells in *in vitro* settings, these effects are barely seen after transplantation *in vivo*, with less than 3% of injected cells surviving 1 week after transplantation (*Malliaras K et al., 2011)*. To overcome this challenge, combinations of molecular approaches, such as chemical and hypoxic preconditioning and genetic engineering, have been used in an attempt to boost the ability of transplanted cells to withstand the adverse microenvironment, thereby

improving their regenerative capacity *in vivo* (*Yu SP et al., 2013)*. Some of the preconditioning techniques include exposing the stem cells to hypoxia, treatment with growth factors and anti-aging compounds, irradiation, and modification of the cells using microRNAs (*Mohsin S et al., 2011)*. Katare et al. showed that transplanted human pericyte progenitor cells repair the infarcted heart through the activation of an angiogenic programme involving miR-132 (*Katare R et al., 2011)*. Hence, targeting the expression of microRNAs can be a novel possible approach for enhancing the angiogenic potential of stem cells. Most of these approaches are aimed at salvaging depleted stem cell function. In one of these studies, preconditioning of diabetic MSCs with cardiomyocyte conditioned medium markedly improved their efficacy after transplantation into the diabetic heart (*Khan M et al., 2013*). In another study, exposing MSCs to SDF-1 significantly enhanced cell survival, proliferation, and engraftment of the transplanted cells into the infarcted myocardium via SDF-1/CXCR4 signaling (*Pasha Z et al., 2008)*. Of note, most of the autologous transplantations are performed in elderly patients with pre-existing diseases; the functional efficacy of stem cells in these cases are often impaired. Hence, a cell rejuvenation strategy in the form of preconditioning might be required to salvage the therapeutic potential of these cells (*Itzhaki-Alfia A et al., 2009).* In support of this notion, stem cell factor was recently shown to reverse the senescence of cardiac stem cells in the aging myocardium (*Sanada F et al., 2014).* Data from individual studies aimed at modulating a single target protein or a set of target proteins within a stem cell are inadequate to select the perfect preconditioning strategy. There is thus a need for a high throughput screening plan that can be utilised to identify the whole array of pro-survival and angiogenic target proteins which can be used as targets for stem cell preconditioning.

Hypoxia and hypoxia adaptation: Hypoxia is a reduction of normal level of tissue oxygen tension, occurring for several reasons in some physiological processes such as early embryonic growth or stem cell niche maintenance and in a number of pathological conditions such as infarction, tumors, diabetes, obesity, pathological hypertrophy, neurodegenerative diseases and many others (*Eltzschig HK and Carmeliet P, 2011)*.

Oxygen tension represents a critical component in the maintenance of the stem/progenitor state. *In vitro*, hypoxic conditions promote proliferation and prevent apoptosis of stem/progenitor cells (*Studer L et al., 2000*) while preserving or enhancing their multipotency (*Felling RJ et al., 2006; Santilli G et al., 2010*).

Hypoxia has two effects:

1 -necrosis of cells in the region more distant from tissue vessels.

2-adaptation of cells in the region along the hypoxic gradient close to the vessels.

The O_2 shortage causes inhibition of ATP production, rapid fall of the energy charge, and loss of ionic gradients with the alteration of cytosolic calcium homeostasis (*Schanne FA et al., 1979; Russo MA et al., 1981)*. The increase of cytosolic Ca^{++} concentration above 10^{-6} M, dramatically activates the peroxidative metabolism (*Schanne FA et al., 1979)*, an irreversible contraction and degradation of the cytoskeleton (*Russo MA et al., 1981)* and the activation of Ca^{++}-dependent cytosolic proteases (calpains) and DNA-ases (*Smith MA and Schnellmann RG, 2012; Nagamalleswari E et al., 2012)*, all leading to a rapid and irreversible cell degradation. Plasmamembrane rupture due to lipoperoxidation is responsible for the definitive loss of the ionic gradients and for the release of intracellularly segregated molecules, many of which are called alarmins for their ability to signal the cell damage to specific receptors on adjacent cells. Adaptation of cells surviving to the hypoxia (when they are closer to the vessels) occurs through the activation of HIF-1α and the expression of a number of genes, such as VEGF, Glut1 and HKII, TERT (*Semenza GL, 2014)*, stemness genes and endogenous alarmin receptors, such as RAGE, P2X7 (*Tafani M et al., (b) 2011)*, TLRs (*Sandholm J et al., 2014)*, NOD-like receptors (*Jones HD et al., 2014)*, etc. . The beneficial effects of these genes can be observed in multiple cell or tissue functions and include: vasodilatation and neoangiogenesis to increase the local blood supply, metabolic shift from oxidative mitochondrial metabolism to anaerobiotic glycolysis, activation of telomerase in stem cells, reprogramming somatic cells to stem cells (*Tafani M et al., 2014)*. Importantly, alarmins released by necrotic cells (HMGB1, ATP/ADP, membrane debris, nucleic acids, etc.) bind their receptors and activate NF-kB with the expression of hundreds of genes of the IRR. Therefore, non leukocytic cells in hypoxic tissue microenvironment can present a NF-kB-dependent proinflammatory gene response similar to that seen in activated leukocytes.

Hypoxia increases several secreted factors and their receptors, such as VEGF, C-X-C motif chemokine receptor (CXCR) 4, CXCR7, C-X-3C chemokine receptor (CX3CR) 1 and hepatocyte growth factor receptor (c-Met), which play a vital role in chemotaxis, viability, and homing of stem cells both *in vitro* and *in vivo* (*Haque N et al., 2013*); Stem/progenitor cells cultured in hypoxia might therefore have an increased efficiency for migrating to and repairing damaged tissue.

HIF and Oxygen Sensors: Cellular adaptation to hypoxia rely on the transcription factor HIF, which is inactive when oxygen is abundant but is activated in hypoxic conditions *(Semenza GL et al., 2007)*. Oxygen-dependent hydroxylation of prolyl residues in HIF-1α or HIF-2α in the HIF heterodimer by prolyl hydroxylaes (PHDs) creates a binding site for the von Hippel–Lindau (VHL) gene product, which is a component of the E3 ubiquitin ligase complex; the binding of the VHL gene product to HIF-1α (or HIF-2α) culminates in

the destruction of the α subunit in proteasomes *(Kaelin WG, 2007)* . In addition, hydroxylation of asparagyl residues in HIF-1α (or HIF-2α) by factor-inhibiting HIF — an oxygen dependent asparagyl hydroxylase — reduces the transcriptional activity of HIF (*Coleman ML and Ratcliffe PJ, 2009)* The functions of both hydroxylases (PHDs and factor-inhibiting HIF) depend on oxygen. *(Semenza GL et al., 2007; Kaelin WG Jr and Ratcliffe PJ, 2008)* . Germline mutations in the PHD2 gene have been found in association with familial erythrocytosis and with a syndrome of familial erythrocytosis with paraganglioma (*Ladroue C et al., 2008)*; inactivating mutations of both copies of the VHL gene cause Von Hippel–Lindau disease (which is characterized by hemangioblastomas, clear-cell renal carcinomas, and pheochromocytomas) *(Kaelin WG, 2007).* HIF can be activated under normoxic conditions, which allows the initiation of an inflammatory response before tissues become hypoxic. Examples of this mechanism are the increase in HIF-1α transcription by bacterial lipopolysaccharide (*Blouin CC et al., 2004)* and the stabilization of HIF-1α when ROS and reduced cellular iron inhibit PHD (*Cash TP et al., 2007; Knowles HJ et al., 2006).* The phenotype of mice with HIF-1α deficiency differs from that one of mice with HIF-2α deficiency, which implies that these components of the HIF transcription-factor polypeptides have different target genes *(Fraisl P et al., 2009).* The HIF-2α gene in certain forms of familial erythrocytosis has a gain-of-function mutation, which probably causes normoxic stabilization of the HIF-2α protein *(Percy MJ et al., 2008).*

HIF-1α activity is induced by hypoxia through changes in HIF-1α mRNA and protein levels in brain, heart, kidney, lung and skeletal muscle. The regulation of HIF-1α mRNA levels is not well understood because it is not observed in most cell lines, whereas O_2-dependent prolyl hydroxylation and asparaginyl hydroxylation of HIF-1α negatively regulate its protein stability and transcriptional activity, respectively (*Kaelin WG Jr and Ratcliffe PJ, 2008).* HIF-1α activates gene transcription by binding to the core DNA sequence 5'-RCGTG-3' (R = A or G), which is embedded within a hypoxia response element (HRE) (*Semenza GL et al., 1996)*. HIF-1α directly regulates the expression of more than 1.000 human genes. Whereas the expression of a subset of HIF-1α target genes is induced by hypoxia in most or all cell types, the vast majority of these genes are induced by hypoxia in a cell type-specific manner.

Among the HIF-1α target genes are those encoding secreted factors and cell surface receptors that control O_2 delivery by regulating angiogenesis and vascular remodeling. HIF-1α functions as a master regulator in this process because it coordinately regulates the expression of a large number of genes whose protein products play critical roles in mediating vascular responses to hypoxia and ischemia. In addition to promoting O_2 delivery, HIF-1α also activates the transcription of genes encoding enzymes,

transporters, and mitochondrial proteins that decrease O_2 utilization, again functioning as a master regulator to switch cells from oxidative metabolism to glycolitic metabolism.

Ischemia refers to the state in which perfusion in not adequate to supply adequate O_2 and nutrients or to remove toxic metabolites. HIF-1α activity activates transcription of genes encoding angiogenic factors. These factors stimulate the remodeling of collateral blood vessels, leading to increased blood flow. HIF-1α coordinately activates the transcription of genes encoding glucose transporters and glycolitic enzymes. Increased glycolytic flux allows cells to maintain ATP levels under hypoxic conditions. HIF-1α also inhibits mitochondrial oxidative metabolism, thereby reducing the generation of ROS under conditions of hypoxia or hypoxia-reoxygenations, by multiple strategies. Such strategies include: 1) a regulatory subunit switch in cytochrome c oxidase, in which COX4-1 is degradated by LON protease and replaced by COX4-2 (*Fukuda R et al., 2007)*; 2) induction of lactate dehydrogenase A, which converts pyruvate to lactate (*Semenza GL et al., 1996),* 3) induction of pyruvate dehydrogenase kinase 1 (PDK1) or PDK3, which inactivates pyruvate dehydrogenase and thereby shunts pyruvate away from the mitochondria (*Kim JW et al., 2006);* 4) induction of miR-210, which inhibits the expression of ISCU, an iron-sulfur cluster assembly factor that is required for activity of aconitase and electrone transport complex I (*Favaro E et al., 2010)*, 5) induction of BNIP3 and BNIP3L, which trigger mitochondria-selective autophagy (*Zhang H et al., 2008)*.

Hypoxia Signaling and NF-κB: Members of the nuclear factor κB (NF-κB) family of transcription factors regulate inflammation and orchestrate immune responses and tissue homeostasis (*Naugler WE et al., 2008; Vallabhapurapu S and Karin M, 2009; Pasparakis M, 2008*). Members of this family interact with members of the PHD–HIF pathway in ways that link inflammation to hypoxia (*Taylor CT, 2008*). Additional interactions between hypoxia and inflammation are seen in the IκB kinase complex, a regulatory component of NF-κB (*Cummins EP et al., 2006*) and in the regulation of HIF-1α transcription by NF-κB before and during inflammation (*Bonello S et al., 2007; Rius J et al., 2008*). Hypoxia amplifies the NF-κB pathway by increasing the expression and signaling of TLRs, which enhance the production of antimicrobial factors and stimulate phagocytosis, leukocyte recruitment, and adaptive immunity (*Kuhlicke J et al., 2007*).

3.1.2.Stem cell delivery to the patient

Finally, when it comes to the delivery of stem cells to a patient, three important factors need to be considered: first is the type and nature of the injury, second the timing of the therapy, and third the ability of the cells to engraft to the host myocardium. The decision

to select a particular cell type should be influenced mainly by the nature of the injury. For example, in cases of chronic ischaemia with a functional myocardium, the purpose is to rescue the non-necrotic ischaemic myocardium and improve the blood supply to it. Hence, it is desirable to use a cell type that secretes pro-angiogenic factors, such as BMCs, or cells that induce vascular regeneration, such as EPCs. On the other hand, if the goal is to regenerate an infarcted myocardium with severe loss of functional myocardial tissue, it would be more suitable to use a progenitor cell type, such as CSCs with cardiomyogenic and vasculogenic capability, or consider the delivery of functional ESC-CMs or iPSC-derived cardiomyocytes (*Timmers L et al., 2008)*.

As in other cases, a huge dilemma still remains regarding the best time for stem cell delivery to patients. A comparison of two large clinical trials (LateTIME and Reinfusion of Enriched Progenitor Cells and Infarct Remodelling in Acute Myocardial Infarction (REPAIR-AMI)) in which patients underwent intracoronary infusion of BMCs either 3 to 5 days (REPAIR-AMI) or 2 to 3 weeks (LateTIME) after an acute event showed significant enhancement of LVEF only in patients who received BMCs within 5 days after the acute event (*Mills JS and Rao SV, 2007; Traverse JH et al., 2011)*. This suggests that cell therapy may be efficacious only if administered early after acute MI, which is impossible in the case of autologous transplantation using CSCs. However, the SCIPIO and CADUCEUS trials have demonstrated benefits in patients even when cell therapy was initiated 4 months and 2 to 4 weeks, respectively, after an acute event, although it is not clear if this benefit overweighs the benefit of the REPAIR-AMI trial (*Chugh AR et al., 2012; Makkar RR et al., 2012)*. The researchers who designed the SCIPIO trial argue that cell therapy was initiated after 4 months to separate the effects of CSCs from those of surgical revascularisation immediately after therapy, as in many patients LVEF is known to improve spontaneously during the first few months after coronary artery bypass graft (CABG) surgery (*Shivalkar B et al., 1996)*. However, due to the lack of immune reactions with CSCs, they can be embraced as an off-the shelf product if future studies confirm that an early time point could be ideal for stem cell transplantation (*Torella D et al., 2005)*. In addition to choosing the best stem cell type and the best timing for the treatment, the most important factor after transplantation is the ability of the cells to engraft into the host myocardium. Several studies have reported that cellular engraftment after transplantation into damaged tissues is inadequate and that transplanted cells are susceptible to the hostile ischaemic environment and tend to disappear within a few days (*Cho HJ et al., 2012)*. Several ongoing research studies have used different methods to precondition stem cells prior to transplantation in order to increase their potential to withstand the adverse microenvironment following transplantation, with the aim of improving their engraftment and survival. Direct cell delivery into the myocardium has been shown to have disadvantages in terms of meagre

cell engraftment and poor mechano-electrical coupling. Cardiac regeneration approaches are evolving from cell therapy to advanced tissue engineering. Cardiac tissue engineering is an alternative strategy in which cell transplantation is accompanied by the support of biomaterials (called scaffolds) and regulatory factors (for example, growth factors) (*Le Huu A et al., 2013)*. The *in vitro* engineering of beating cardiomyocyte containing tissue constructs and the engineering of stem cell-containing tissue constructs are the two strategies currently used for cell implantation. The cardiac tissue constructs are formed by seeding cardiomyocytes in three-dimensional scaffolds and culturing under appropriate conditions to develop cell alignment, electrical communication, and spontaneous beating *in vitro* (*Zimmermann WH et al., 2002)*. Apart from using the tissue constructs of beating cardiomyocytes, engineered stem cell sheets are also an attractive choice for therapeutic delivery, owing to their paracrine effects and plasticity (*Narita T et al., 2013)*. In the future, these bio-engineered cardiac patches or cell sheets may become the preferred approach for delivering stem cells into the diseased heart (*Zhang G et al., 2008).*

3.1.3.Survival and retention of CSCs following transplantation

A number of studies have reported that stem cells of various sources suffer low viability, and only few persist several weeks following transplantation into the injured myocardium (*Tang YL et al.,2005; Laflamme MA et al., 2005; Reinecke H et al., 1999; Toma C et al., 2002; Robey TE et al., 2008)*. Similarly, one of the problems with the current therapy with $cKit^{+}$CSCs is the poor survival and retention of the injected cells in the heart (*Li Q et al., 2011)*. It is thought that myocardial ischemia/reperfusion and infarction create a hostile environment for stem cells, because of the presence of inflammatory cells and cytokines/mediators, lack of ECM and supporting cells, and poor supply of oxygen and nutrients, all of which conspire to promote death of the grafted cells (*Forrester JS et al., 2007; Penn MS et al., 2008)*. For this reason, increasing survival and retention of the transplanted CSCs in the heart currently constitutes one of the major challenges in the field of CSC therapy. Troubleshooting this issue would require close and accurate monitoring of the number, distribution, and fate of the transplanted donor cells, and correlation of these variables with changes in functional parameters.

Several studies have tested different strategies to overcome the problem of poor survival or retention of the cells in the hostile environment of the infarcted heart. For example, Mohsin and colleagues tested the effect of *ex vivo* gene delivery of a pro-survival gene, Pim-1 kinase on survival/engraftment and reparative potential of human CSCs using a mouse model of ischemic cardiomyopathy (*Mohsin S et al., 2012)*. The same group had previously identified Pim-1 as a kinase responsible for cardioprotection downstream of

pro-survival Akt signaling (*Muraski JA et al., 2007; Wang Z et al., 2001)*. Human CSCs engineered to overexpress Pim-1 were superior over the control cells in terms of cellular engraftment and differentiation. Bioluminescence imaging analysis of luciferase-expressing donor cells (*Wu JC et al., 2003)* showed that the presence of Pim-1 CSCs persisted up to 8 weeks post transplantation, whereas control cells became undetectable by 1 week. Moreover, the persistence of cells in the recipient heart was accompanied by improvement in vasculature and reduction in infarct size, which were coupled with increased hemodynamic performance at 20 weeks post-transplantation (*Mohsin S et al., 2012)*. Interestingly, a subsequent report by the same group showed that the increases in proliferation and telomere lengths observed in Pim-1-expressing CSCs are only transient, suggesting that Pim-1 overexpression does not lead to immortalization or oncogenic transformation of CSCs (*Cottage CT et al., 2012)*. The results are promising and indicate that *ex vivo* delivery of a pro-survival gene prior to transplantation may serve a viable option in overcoming current limitations in the field. Such strategy also has proven fruitful in terms of promoting survival and therapeutic efficacy of other stem cell sources, including MSCs (*Xu M et al., 2012; Huang W et al., 2012)*. One of the first attempts to improve post-transplantation donor MSC survival was reported by Mangi and colleagues (*Mangi AA et al., 2003)*. They genetically engineered rat MSCs *ex vivo* using retroviral transduction to overexpress the pro-survival gene Akt1 (*Datta SR et al., 1999; Fujio Y et al., 2000)*. At 24h following intramyocardial injection of MSCs into the rat MI heart, they observed a greater number of donor cells and a lower rate of apoptosis with MSCs overexpressing Akt (Akt-MSC) compared to the control GFP-expressing MSCs (*Mangi AA et al., 2003)*. Subsequently, transplantation of MSCs into the ischemic heart significantly reduced intramyocardial inflammation, fibrosis and myocyte hypertrophy, and normalized cardiac function. The study reported that Akt-MSCs restored fourfold greater myocardial volume compared to the control MSCs. Another study tested the utility of stably introducing heme oxygenase-1 (HO-1) expression in MSCs (*Laflamme MA et al., 2005)*. HO-1 is known to exert a potent anti-apoptotic, anti-oxidant and cytoprotective activity in an ischemic environment (*Melo LG et al., 2002; Vulapalli SR et al., 2002)*. When they injected HO-1-MSCs into acute MI heart, their survival was fivefold greater than that of the control MSC group (expressing lacZ) at 7 days of implantation, and this was consistent with a significantly reduced rate of apoptosis among HO-1-MSCs. Importantly, HO-1 MSCs were also superior over the control cells in attenuating left ventricular remodeling and enhancing the functional recovery of injured hearts. Similarly, the pro-survival or cardio-protective effects of various candidate genes, including GATA4 (*Xu M et al., 2012)*, heat shock protein 27 (*McGinley LM et al., 2013)*, miRNA-1, and protein kinase G1α (*Wang L et al., 2013)*, have been recently exploited to promote survival and efficacy of MSCs in the context of hypoxia-reoxygenation injury.

Pharmacologic activation of innate cytoprotective mechanisms seems to be another attractive option to enhance the survival and engraftment of CSCs. In a recent study, Cai et al. showed that treatment of human cKit$^+$CSCs with cobalt protoporphyrin (CoPP), a well known HO-1 inducer (*Shan Y et al., 2000)*, promoted cell survival after increased oxidative stress *in vitro* (*Cai C et al., 2012)*. The cytoprotective effects of CoPP were dependent on the upregulation of HO-1, cyclooxygenase-2 (COX-2), and nuclear factor-like 2 (NRF2). Interestingly, preconditioning CSCs with CoPP also led to a global increase in release of a variety of cytokines, and the conditioned medium from cells pretreated with CoPP conferred naive CSCs remarkable resistance to apoptosis, demonstrating that cytokines released by preconditioned cells also play a major role in the pro-survival effects of CoPP (*Cai C et al., 2012)*. Another study by Zafir and colleagues examined the role of protein O-GlcNAcylation (β-O-linkage of N-acetylglucosamine, O-GlcNAc), a universal cytoprotective pathway (*Ngoh GA et al., 2010)* in the protection of mouse CSCs against hypoxia-reoxygenation injury (*Zafir A et al., 2013)*. Protein O-GlcNAcylation was significantly elevated in post-hypoxic CSCs upon reoxygenation. Reduction in the O-GlcNAc signal via pharmacological inhibition sensitized CSCs to post-hypoxic injury, whereas increasing O-GlcNAc levels enhanced cell survival. The study has an important implication because pharmacologic augmentation of protein O-GlcNAc levels can be effective in priming CSCs for survival within the unfavorable environment of the infarcted myocardium.

Likewise, preconditioning of cells prior to transplantation or co-treatment with different agents has been shown to be a successful strategy for reinforcing viability and therapeutic efficacy of the donor MSCs (*Ye L et al., 2013; Iso Y et al., 2013; Herrmann JL et al., 2010; Penna C et al., 2013; Pendergrass KD et al., 2013)*. Preconditioning or priming the stem cells with pharmacological agents prior to autologous transfer is likely to be clinically viable, because it is relatively easy to implement and does not involve direct genetic manipulation of the cells. Undoubtedly, identification of important cytoprotective mechanisms within CSCs and potential pro-survival candidate genes will be critical in developing novel strategies for augmenting post-transplantation survival and engraftment of donor CSCs.

Lack of robust, direct differentiation of transplanted CSCs

Although a variety of stem cell populations, including cKit$^+$CSCs, have been shown to play regenerative and/or protective functions following transplantation in animal models of acute and chronic MI, it has been widely accepted that therapeutic benefits of stem cell therapy are mainly contributed by paracrine factors secreted by the donor cells, rather than by direct differentiation of transplanted stem cells into cardiac cell types (*Gnecchi M*

et al., 2011; Mirotsou M et al., 2011). Regardless of the cause, there is a disconnection between functional benefits of CSC therapy and the lack of robust differentiation of transplanted cells. Based on this, one can argue that the contribution of direct cardiovascular differentiation of the donor stem cells to the salutary effects is negligible. However, previous studies on transplantation of non-cardiac stem cells suggests that direct cardiac differentiation of the donor stem cells is essential for the functional benefits they provide. Yoon and colleagues investigated the contribution of cardiovascular differentiation of BM-derived mononuclear cells (BMMNCs) to the renewal of myocardium following acute MI by using a system in which the differentiated progenies of the donor cells can be selectively ablated in a temporally controlled manner (*Yoon CH et al., 2010)*. They engineered the donor cells to express a vector that encoded a prodrug-activated suicide gene (herpes simplex virus thymidine kinase; HSV tk) under the control of endothelium (eNOS)$^-$, smooth muscle (SM22α)$^-$, or cardiomyocyte (α-MHC)-specific promoters, which permitted selective depletion of the individual cardiac lineage acquired by the donor cells via administration of a prodrug, ganciclovir. When each donor cell-derived lineage was ablated two weeks after transplantation of the engineered BMMNCs, they found that depletion of endothelium-committed or smooth-muscle–committed cells, but not cardiomyocyte-committed cells, induced a significant decline in ejection fraction (EF) (*Yoon CH et al., 2010)*. This demonstrated that vascular differentiation is one of the essential mechanisms by which BMMNCs contribute to the functional recovery of the injured heart. Another study by Behfar et al. also suggested that cardiogenic potential of the donor BM-derived MSC population dictates the functional outcome of stem cell therapy (*Behfar A et al., 2010)*. While the majority of patient-derived MSC failed to elicit significant improvements in EF (ejection fraction), stem cells from few individuals harbored a spontaneous capacity to improve cardiac performance in an animal model of chronic ischemic cardiomyopathy. Interestingly, the stem cells exhibiting the reparative activity expressed relatively high basal levels of early (e.g., NKX-2.5, TBX5, and MESP1) and late (e.g., MEF2C) cardiac transcription factors, compared to the non-effective MSC counterparts. Based on this observation, the authors formulated a "cardiogenic cocktail" (containing TGF β1, BMP-4, activin A, retinoic acid, IGF-1, FGF-2, α-thrombin, and IL-6) to stimulate cardiogenic potential/differentiation of naïve MSCs prior to transplantation. This strategy not only potentiated expression of cardiac transcription factors across different patient MSC populations, but also enhanced therapeutic efficacy of MSCs against chronic ischemic cardiomyopathy in mice (*Behfar A et al., 2010).* The study provides an interesting implication that enhancing differentiation potential or properties of donor stem cells is feasible, and is an effective strategy to further improve the efficacy of stem cell therapy.

The paracrine hypothesis

Stem cell therapy for tissue repair and regeneration holds great therapeutic potential (*Hodgkinson CP et al., 2010)*. It has been shown that injection of adult stem cells into the injured heart has beneficial effects. Originally it was thought that these stem cells engrafted into the damaged tissue and differentiated into cardiomyocytes, vascular or other cells (*Asahara T et al., 1997; Orlic D et al., 2001)*. However, it has been shown that after injection, adult stem cells had poor survivability (*Gnecchi M et al., 2008)*. Moreover, it has not been possible to reproduce the earlier studies which showed that bone marrow-derived stem cells differentiate into cardiac cells. Using a GFP mouse, Balsam et al. (*Balsam LB et al., 2004)* could not find any evidence that bone marrow-derived hematopoietic lineage negative/cKit positive cells differentiated into CMs when injected into infarcted myocardium. Instead, these cells adopted a typical hematopoietic fate. These findings, and others, have called into question the plasticity of bone marrow-derived stem cells and their direct role in tissue regeneration. On the basis of above studies, it is now clear that although engraftment can result in improved cardiac function (*Hatzistergos KE et al.,2010)*, the small number of adult stem cells engrafted cannot directly generate sufficient cardiomyocytes to account for the therapeutic benefits observed. Recent evidence suggests the importance of the paracrine mechanism of stem cell action. These observations have led to the proposal that adult stem cells exert their therapeutic benefits via the release of biologically active proteins, or paracrine factors, acting on resident cells. Indeed, there is now a large body of evidence supporting the hypothesis that paracrine factors are essential for the reparative effects of adult stem cells after delivery into the injured heart. Adult stem cells secrete a wide variety of growth factors and chemokines that can promote cardiac repair. Elevated levels of proteins such as vascular endothelial growth factor (VEGF), basic fibroblast factor (FGF), hepatocyte growth factor (HGF), and insulin-like growth factor-1 (IGF-1) are found in the heart after injection of adult stem cells (*Yoon YS et al., 2005).*

It has been demonstrated that paracrine factors promote cardiac regeneration through several mechanisms, including cardiomyocyte proliferation, cytoprotection, differentiation of resident stem cells, neovascularisation, and by limiting inflammatory and profibrotic processes.

It has been shown that cell culture medium conditioned by hypoxic MSCs reduces rat cardiomyocyte apoptosis and necrosis when exposed to conditions that promote cell death (*Gnecchi M et al., 2005)*. These studies validate that MSCs promote cardiomyocyte survival via paracrine factors and that Akt is crucial for this process (*Gnecchi M et al., 2006)*. Takahashi et al. (*Takahashi M et al., 2006)* showed that rat BMMNCs release proteins such as VEGF, platelet-derived growth factor, IGF-1, and IL-1β, some of which

were significantly enhanced by hypoxia. The conditioned media of bone marrow mononuclear cells strongly inhibited cardiomyocytes apoptosis and preserved their contractile capacity. Moreover, Uemura et al. (*Uemura et al., 2006*) demonstrated that bone marrow stromal cells, which showed upregulation of Akt after brief anoxia, prevented cardiomyocytes apoptosis in a cocultured model. This study went on to show that bone marrow stromal cells markedly inhibited LV remodeling after MI.

Adult stem cells when injected into myocardium dampen the inflammatory state associated with injury by downregulating expression of proinflammatory cytokines such as TNFα, IL-1β, IL-6, and monocyte chemoattractant protein-1 (*Ohnishi S et al., (a) 2007)*. These effects have a paracrine component; conditioned media prepared from cultured MSCs was found to inhibit damage to isolated adult CMs in response to monocyte chemoattractant protein-1 (*Ohnishi S et al., (b) 2007)*. MSCs posses immunomodulatory properties that affect a broad range of cells involved in the immune response. MSCs inhibit T-cell proliferation and cytoxicity, rendering the T cells unresponsive (*Glennie S et al.,2005)*. Paracrine factors released by MSCs, such TGF β, HGF, nitric oxide, indoleamine 2,3-dioxygenase and prostaglandin-E2 inhibit T-cell function (*Baraniak PR et al., 2010)*. Under certain conditions, MSCs release T-cells activators such as IL-6, IL-1 and RANTES (*Baraniak PR et al., 2010).* Depending on the strength of the stimulus, MSCs either promote or inhibit IgG production by B cells (*Rasmusson I et al., 2007)*. MSCs also prevent dendritic cell maturation and function via the release of IL-6 and prostaglandin-E2 (*Spaggiari GM et al., 2009)*. MScs also secrete interleukin 1 receptor antagonist, which inhibits the release of the proinflammatory cytokine TNFα from activated macrophages (*Ortiz LA et al., 2007)*.

Macrophages can promote angiogenesis and tissue healing through many secreted molecules (*Yano T et al., 2006)*. After transplantation of MSCs into infarcted tissue, large numbers of macrophages collect at the sites of injection despite an overall reduction in the population of these cells in the heart (*Amsalem Y et al., 2007).* Various reports have indicated that MSCs switch macrophages from the proinflammatory M1 phenotype to the anti-inflammatory M2 phenotype both *in vitro* and *in vivo* (*Zhang QZ et al., 2010)*, potentially through secreted factors such IGF-1 (*Santini MP et al., 2008)* and IL-10 (*Burchfield JS et al., 2008)*. These studies suggest that MSCs contribute to the overall recovery of cardiac function after MI, in part, via their effects on macrophages.

Cardiac injury with significant loss and functional impairing leads to cardiac remodeling, which is mediated by a significant change in the ECM including fibrosis, CM hypertrophy, and changes in ventricular dimension and function. Paracrine factors released by adult stem cells can alter the ECM and prevent post-infarction remodeling. Stem cells, such as MSCs, express many proteins that regulate the ECM such as MMPs, serine proteases and

serine protease inhibitors, suggesting that transplanted MSCs can inhibit fibrosis through a paracrine action (*Ohnishi S et al., (a) 2007)*. MSCs transplantation has been shown to inhibit post-MI increases in the expression of collagen-I and collagen-III as well as the tissue inhibitor of MMP1 (*Xu X et al.,2005)*. There is also evidence that the administration of adult stem cells promotes cardiac contractility. Takahashi et al. (*Takahashi M et al., 2006)* found that conditioned media from BMMNCs maintained fractional shortening and maximal rate of re-lengthening of adult rat ventricular CMs in culture. Conditioned media was more effective in preserving contractility if the BMMNCs were exposed to hypoxia. This suggests that the release of inotropic paracrine factors is increased by hypoxia. The identity of these inotropic paracrine factors is currently unknown, however it is well known that IGF-1, a growth factor released by MSCs, can promote cardiomyocyte contractility *in vitro* (*Freestone NS et al., 1996)*.

Another important effect of adult stem cells in the ischemic myocardium is neovascularisation. The molecular pathways that control angiogenesis are well characterized and involve proteins such as VEGF, basic FGF, HGF, and angiopoietin, among others. VEGF is an important proangiogenic paracrine factor as ablation of this gene significantly inhibits the ability of MSCs to promote functional recovery in the injured heart (*Hodgkinson CP et al., 2013)*. However, antibody targeting VEGF and FGF only partially attenuated the effect of conditioned media (*Kinnaird T et al., 2004)*, indicating that MSCs release other proangiogenic proteins besides these two growth factors. EPCs also promote angiogenesis via paracrine mechanism. VEGF and stromal-derived factor-1 are among the responsible bioactive molecules. Both proteins are secreted by EPCs and promote EC migration and capillary formation via differentiation independent mechanisms (*Urbich C et al.,2005)*.

The heart contains many resident stem cells, which are defined by several markers including cKit and Sca-1. The cKit cardiac progenitor cell (CPCs) are thought to be capable of promoting regeneration via mobilization into injured tissue and differentiation into mature cardiac cells (*Bolli R et al., 2011)*. However, in all studies, it was apparent that donor $cKit^+$ cell differentiation into mature cardiac cells, such as CMs, was too low to account for the functional benefits. The authors concluded that paracrine effect must be responsible for the regenerative effects of the injected $cKit^+$CSCs (*Tang XL et al., 2010)*. The nature of these paracrine factors remains to be identified.

What has become apparent is that paracrine factors released by stem cells are pleiotropic. This pleiotropic ability of stem cell-derived paracrine factors allows them to influence cardiac repair at multiple points after myocardial injury. Many paracrine factors, as IGF-1, FGF, platelet-derived growth factor and VEGF, posseses pleiotropic properties. Given the fact that response to cardiac injury involves complex, dynamic, and time-

dependent events, paracrine factors can influence myocardial patho-biology, a multifaceted temporal manner on different cell types and via different mechanisms.

3.2. Immunological barriers of stem cell-based therapies

The concept that stem cells may have an immune privilege status explains the initial lack of concern for the potential immunologic conflict between transplanted cells and a host. An exogenous cell delivered to a host is expected to encounter some form of host resistance. Recognition of foreign antigen initiates an immune response that involves the activation and proliferation of specific immune cells.

Immunogenicity is the recent concern of stem cell therapies whether using embryonic or adult, or autologous or allogeneic stem cells (*Zhao T et al., 2011; Charron D et al., 2009; Dhodapkar KM et al., 2010; Charron D et al., 2012).* The idea of acquired immunogenicity of autologous stem cells is disclosed as: too long-term culture periods, genomic instability, combination with matrix structures, genetic manipulation, epigenetic reprogramming, and impair stem cell immune privilege status. In the allogeneic scenario, the expression of immune relevant molecules, notably the polymorphic major histocompatibility complex (MHC) classes I and II antigens [human leukocyte antigen (HLA) class I and II in humans] is the backbone of rejection problems. The MHC antigens were originally recognized for their role in initiating T-cell responses that lead to the rejection of transplanted tissue. By binding to foreign antigens, MHC molecules form complexes that are recognized by specific T cells, thus initiating a cascade of events to identify and eliminate foreign invaders. The MHC class I antigens are traditionally associated with the activation of CD8 cytotoxic T lymphocytes, whereas MHC class II antigens are recognized by CD4 T lymphocytes. However, many stem cells express no or only low levels of MHC antigens and have been considered to be immunoprivileged, or lacking in the ability to induce an immune response. In fact, ESCs and MSCs have been presented as the prototype of the immunoprivileged cell for cell transplantation studies (*Menendez P et al., 2005; Yang XF et al., 2007)*. Human ESCs express low level of HLA class I that significantly increases after differentiation (*Drukker M et al., 2006)* and expanding MSC *in vitro* remarkably increases their MHC II. The MHC disparities are by far the most formidable immunological barrier to transplantation (*Erlich HA et al., 2001)*. Rejection occurs because of allelic differences between grafts and hosts mainly at polymorphic class I and class II MHC loci. Models of ESC transplantation using murine ESCs showing that the administration of these cells into the injured myocardium of allogeneic animals resulted in robust inflammatory responses and cellular infiltration by both the innate and adaptive composites of the immune system questioned their immune

privilege status (*Kofiis T et al., 2005)*. The cellular-based immune responses by innate natural killer (NK) and adaptive T cells upon recognition of allogeneic MHC are major mechanism of immune rejection (*Clarkson MR et al., 2005; Crespo M et al., 2015; Moreau A et al., 2013)*. Another mechanism widely recognized as an important component of allograft failure in organ transplantation is the antibody-mediated rejection (AMR) (*Terasaki PI et al., 2005; Lefaucheur C et al., 2013).*

MI leads to an intense inflammatory cell influx within the myocardium and the activation of local and systemic immune signaling pathways. Thus, stem cells are delivered into an immunologically activated milieu when applied after MI, which some investigators consider as a trigger for the up-regulation of stem cell MHC expression. Moreover, stem cells themselves can produce various immunomodulatory signaling factors, including both proinflammatory and anti-inflammatory molecules (*Patel SA et al., 2008; Rameshwar P et al., 2010)*.

Limitations of autologous cell therapy

Most clinical trials of cell therapy have been conducted using autologous cells. While autologous cell therapy carries no risk of immune rejection, it requires patient-specific tissue harvesting, cell manufacturing and quality control and is, therefore, associated with important limitations. First, autologous tissue harvesting is invasive and not risk-free. Second, culture of autologous cells may be complicated by technical manufacturing failures; third, expansion of autologous heart-derived cells to clinically relevant numbers poses significant timing constraints which prohibit administration of autologous heart-derived cellular products in acute MI or early post-MI. Fourth, donor age and comorbidities may (or may not) negatively impact upon cell quality, resulting in decreased cell potency. Finally, the technical, economic and logistic constraints associated with autologous cell therapy preclude broad adoption of cell therapy and limit its clinical application to few specialized centers.

The aforementioned limitations of autologous therapy could be overcome by the use of allogeneic cells. The obvious disadvantage of allogeneic therapy is the risk of immune rejection of transplanted cells, which could limit effectiveness of cell therapy (due to rapid rejection of transplanted cells), raise safety concerns (as it could theoretically result in immune related myocardial injury), and induce allosensitization of the recipient, which (if robust and persistent) could complicate future organ transplant. However, if allogeneic cell therapy were proven to be safe and effective, it would open up a new paradigm in cellular therapeutics. Large scale expansion of cells derived from allogeneic tissues could be performed in specialized manufacturing labs under strict quality control. In the case of

heart-derived cells, attractive sources of allogeneic tissue include hearts explanted from organ donors but not used for transplantation and surgical myocardial discards. Highly standardized, 'off-the-shelf' allogeneic cellular products would subsequently be shipped to hospitals throughout the world and banked for future use, thus enabling broad adoption and timely application of cell therapy in a cost-efficient manner. This attractive novel paradigm has motivated researchers to investigate the safety and efficacy of allogeneic therapy for myocardial repair. A critical step in the clinical translation of cell therapy, as mandated by regulatory authorities, is development of relevant potency assays (i.e., tests to measure potency of manufactured cellular products). This is particularly important for allogeneic cell therapy, as development of appropriate potency assays would enable prospective identification of donor cells with greater potential for clinical efficacy. However, the fact that cell therapy products appear to have complex, multifactorial and not fully characterized mechanisms of action presents difficulties in establishing which cellular attributes are most relevant to measuring potency. Given that, potency assays should ideally represent the cellular product's mechanism of action and multiple lines of evidence suggest that the mechanism of action of adult cells is indirect (i.e., is mediated by paracrine mechanisms), it appears logical that *in vitro* biological assays measuring paracrine factor secretion may serve as appropriate potency assays for cellular products.

Allogeneic MSCs are considered the prototypical 'universal donor' cells for cardiac applications. MSCs exhibit an immunologic profie that renders them attractive for allogeneic transplantation without concurrent immunosuppression: they express MHC class I, but lack expression of MHC class II surface antigens or costimulatory molecules. In addition, MSCs exert multiple immunosuppressive and immunomodulatory effects *in vitro*, as they have been shown to interact with virtually all immune cells involved in adaptive and innate immunity. More specifically, MSCs (through both paracrine and contact-dependent mechanisms) inhibit T-cell and B-cell proliferation, inhibit the maturation and cytotoxicity of NK cells, dampen the respiratory burst of neutrophils, inhibit the maturation of monocytes into dendritic cells and impair the antigen-presenting function of dendritic cells (*Uccelli A et al., 2008)*. Preclinical studies in large animal models of ischemic cardiomyopathy have demonstrated that allogeneic MSC transplantation without immunosuppression is safe and produces benefits that appear equivalent to those produced by autologous cells (*Jansen Of Lorkeers SJ et al., 2015)*. With regard to clinical translation, early-phase clinical testing of allogeneic MSCs in patients with heart disease has yielded promising results; allogeneic human MSCs have demonstrated a favourable safety profile (without eliciting a significant immune memory response in transplanted recipients) and have produced encouraging hints of efficacy (*Hare JM et al., 2009; Hare JM et al., 2012; Ascheim DD et al., 2014)*. It should be noted that even immunoprivileged cells like MSCs are eventually rejected after *in vivo*

transplantation without concurrent immunosuppression (*Eliopoulos N et al., 2005; Badillo AT et al., 2007; Poncelet AJ et al., 2007; Zangi L et al., 2009)*; however, since their mechanism of action is indirect (*Weil BR et al., 2015)*, rejection of MSCs may not be of therapeutic signifiance if it is delayed long enough to allow them to exert their reparative paracrine effects.

Undifferentiated ESCs are characterized by the basic traits of self-renewal, clonogenicity, and pluripotency (for differentiation into multiple cell types). Our current understanding of the immunoprivileged status of human ESCs is based on *in vitro* and *in vivo* xenogeneic transplantation studies (*Menendez P et al., 2005)*. These studies have collectively shown that human ESCs express low levels of MHC class I antigens but do not express MHC class II antigens or costimulatory molecules (*Drukker M et al., 2002; Fandrich F et al., 2002; Li L et al., 2004)*. Furthermore, human ESCs have been found to evade recognition by NK cells and inhibit T-cell–induced stimulation by antigen-presenting cells. These studies have also shown that human ESCs do not induce an inflammatory response, or presumably, an immune response upon injection into immunocompetent mice and are, thus, candidates for immunomodulation and tolerance induction (*Menendez P et al., 2005)*. In regenerative studies, ESCs have been reported to have salutary effects on organ repair, including the heart (*Caspi O et al., 2007; Kupatt C et al., 2005; Hodgson DM et al., 2004; Min JY et al., 2002)*. However, some studies contradict the idea of ESC immunoprivilege and suggest that ESCs are associated with immune rejection and teratogenicity (*van der Bogt KE et al., 2006; Lee AS et al., 2009; Nussbaum J et al., 2007; Dai W et al., 2007)*. Transplantation of undifferentiated allogeneic murine and human ESCs into mouse hearts has led to the formation of cardiac teratomas in immunodeficient animals (*Lee AS et al., 2008; Nussbaum J et al., 2007)*. Furthermore, treatment of immunocompetent animals with undifferentiated murine and human ESCs has resulted in immunologic rejection and intense inflammation after several weeks and the up-regulation of class I and class II MHC molecules (*Swijnenburg RJ et al., 2008)*. In a comparative experiment, mouse ESCs were injected into the injured myocardium of syngeneic, allogeneic, and severe combined immunodeficient (SCID) mouse recipients (*Kofidis T et al., 2005)*. In allogeneic mice, ESCs triggered an intense cellular inflammatory response consisting of T lymphocytes (CD3) and dendritic cells (CD11c), as well as a humoral response. However, fewer $CD3^+$ T cells were elicited in the syngeneic group, and no $CD3^+$ T cells were elicited in the SCID mice. Immunologic reactions and lymphocytic infiltration have also been observed after the transplantation of neural stem cells derived from ESCs (*Preynat-Seauve O et al., 2009)*. These observations indicate that up-regulation of MHC expression occurs when ESCs are used to treat conditions associated with ongoing inflammation (*Yang XF, 2007)*. This becomes of

particular consideration in the cardiac field as inflammation and immune cells play a prominent role in the early phases of MI.

Although the use of autologous stem cells is widely accepted from an immunological point of view, for a clinical use and to extend the therapeutic use of CSCs to a wide range of patients, the autologous administration of CSCs becomes very difficult (*Sanganalmath SK et al., 2013)*. Although new automated and high-throughput cell culture systems are being developed (*Kami D et al., 2013)*, for autologous therapies, growing the patient's own CSCs is likely to be time consuming, expensive and require sophisticated instrumentation, and therefore it would be available to a very limited number of patients. The immunogenicity and immunomodulatory properties of MSCs have been extensively described in the past (*Le Blanc K et al., 2008; Garcia-Castro J et al., 2008)* but less information is available regarding CSCs. Since the mechanism of action of these cells is still not fully understood, it would be difficult to fully elucidate the immunogenicity of CSCs in an allogeneic setting. Nowadays, there are different hypotheses concerning the mechanisms through which CSCs could provide a clinical benefit. In the past years, the transdifferentiation hypothesis attempted to explain the beneficial effect of CSC transplantation (*Anversa P et al., (b) 2006)*. However, the *in vivo* differentiation of adult stem cells into cardiac myocytes has not been demonstrated conclusively and, if it occurs, its frequency would be very low (*Gersh BJ et al., 2009)*. From an immunological perspective, the transdifferentiation of stem cells into cardiac or vascular cells does have an import on the immunogenicity of the implanted cells. *In vitro* experiments using MSCs have demonstrated that undifferentiated cells expressed very low levels of MHC class I and class II molecules. After differentiation toward myocytes, SMCs or ECs, these cells acquire an increased expression of MHC class Ia and II together with an increased susceptibility to allogeneic cytotoxic T cells (*Huang XP et al., 2010)*. The *in vivo* experiments using allogeneic MSCs in a rat model have clearly demonstrated that differentiated allogeneic cells are eliminated from the host tissue more rapidly than undifferentiated cells. Finally, it is important to note that very similar results have been found in allogeneic and xenogeneic settings where the transplanted cells were lost from the infarcted rat heart within 5 weeks (*Huang XP et al., 2010)* and 6 weeks *(Grinnemo KH et al., 2004)*, respectively. For these reasons, it is now accepted that transdifferentiation cannot be the main mechanism to explain improvements in cardiac function after stem cell therapy. The paracrine hypothesis is gaining adepts. Published evidence demonstrates that stem cells release soluble factors and extracellular vesicles with analogous effects in MSCs (*Timmers L et al., 2011)* and CSCs (*Ibrahim AG et al., 2014)*. The transplanted allogeneic CSCs have been proved to release paracrine factors that stimulate endogenous repair mechanisms, and experimental evidences in the infarcted rat model have shown that allogeneic cells survive in the myocardium only for a

short period (*Caspi O et al., 2007)*. According to the paper published by Marban's laboratory in 2012 using mismatched CDCs, this short period is long enough to stimulate regenerative pathways (*Caspi O et al., 2007)*. The administration of rat-derived CDCs in allogeneic recipients has demonstrated that these cells are hypoimmunogenic and did not induce a humoral response. However, it is important to note that, in these in vivo experiments, a cellular immune response was reported and alloreactive lymphocytes exhibited an enhanced proliferation against CSCs. If CSCs truly induce memory T cells for clinical trials, the fact that repeated administrations of these cells may induce a robust secondary immune response should be taken into account, since this will result in a rapid clearance of administered cells. Looking toward clinical translation, the allogeneic rejection risk of CSCs has been described in a recent paper from Al-Daccak et al. In this paper, the authors demonstrated that human CPCs shift their signaling capacities within the allogeneic setting toward signals that promote the development, maintenance and functioning of anti-inflammatory response (*Lauden L et al., 2013)*. Moreover, the authors demonstrated that programmed death-ligand 1 (PD-L1) plays a key role to identify low-risk allogeneic cardiac cells. Further studies are still needed to assure that allogeneic CSCs are safe and to explore the host response to this allogeneic therapy, but the evidence available so far is encouraging, and considering the enormous potential of cardiac-derived cellular products, Phase I clinical trials have been started using CDCs (ALLSTAR) and hCPCs (CAREMI). The ALLSTAR study is the first randomized, double-blind, placebo-controlled trial to determine the safety and efficacy of intramyocardial delivery of allogeneic CDCs in patients with ischemic left ventricular dysfunction following MI. The CADUCEUS study provided the foundation for the ALLSTAR trial with major difference between CADUCEUS and ALLSTAR being the source of the CDCs (autologous vs. allogeneic CAP-1002) (*Makkar RR et al., 2012; Malliaras K et al., 2014*) . The principal goal of ALLSTAR is to establish the safety of intramyocardial delivery of allogeneic CDCs. In parallel, signals of potential efficacy will be evaluated by a primary efficacy endpoint (infarct size by MI, similar to the CADUCEUS study) and other structural and functional measures.

Rodent studies with allogeneic cardiac-derived products

The work performed so far with truly allogeneic cells is strictly limited since most studies in small rodents use either SCID mice and human cells (*Chimenti I et al., 2010; Messina E et al., 2004)* or syngeneic cells (*Monnet E et al., 2005)*. Most works describing a cellular type for the first time have also provided some rodent data to support their regenerative capabilities. Thus, the pioneering work performed by Beltrami et al. (*Beltrami AP et al., 2003)* describing $cKit^{+}/Lin^{-}$ cells demonstrated their capability to improve cardiac function

in rats, as later corroborated by other works (*Bearzi C et al., 2007)*. Similarly, the first study published using CS (*Messina E et al., 2004)* injects human CS in the peri-infarct area in SCID mice, using echocardiography to assess functional progress and reporting a preserved infarct wall thickness and percent fractional shortening, in the absence of changes to infarct size between treated and control animals. They describe the existence of bands of regenerating myocytes and neovascularization. Further down the road, works from the same laboratory (*Smith RR et al., 2007)* describe the first preclinical use of CDCs in a similar study, also using human cells in SCID mice. In this case, echocardiographic follow-up showed an improvement in global LVEF in CDC-treated animals versus controls, and while no differences in infarct size between groups were seen either, there were regions of viable myocardiocytes (stained red with Masson's Trichrome) within the infarct area in the treated hearts but not in the control animals. In an attempt to decrease the time needed for cell culture, cells directly grown from cardiac explants (outgrowth cells), that represents an intermediate step in the generation of CS, were compared to CDCs (*Davis DR et al., 2010)*. After a thorough characterization including in vitro differentiation, secretome and phenotyping, infarcted rats were injected with CDCs, outgrowth cells, fibroblasts or phosphate-buffered saline, and followed up using MRI, which is the gold standard for functional cardiac evaluation. These cells possessed genetic and surface markers of CPCs, were able to differentiate into a cardiac phenotype, secreted cardioprotective and angiogenic cytokines (including VEGF and IGF-1, but not HGF or PDGF) and could influence cardiac function, with LVEF 3 weeks after infarction being significantly higher in CDC- or outgrowth cells-injected animals compared to the phosphate buffered saline- or fibroblast-injected groups. Moreover, a trend toward decreased infarct size compared to controls was also evidenced in treated rats. Since no clear difference was seen between the two treatment groups, outgrowth cells may represent an alternative requiring less time-consuming processing prior to administration. Studies performed in small animals have also been used to determine the capabilities of cardiac-derived cellular products prior to advancing to more clinically relevant species. For example, the ability of CSCs to cross the vessel wall and reconstitute all cardiac tissues when delivered in a clinically relevant manner was demonstrated by Dawn et al. (*Dawn B et al., 2005)*. In this work, $cKit^{+}/Lin^{-}$ CSCs expressing enhanced green fluorescent protein (EGFP) were administered in infarcted rats via the coronary tree, and echocardiographic follow-up revealed a preservation of cardiac anatomy and function in CSC-treated animals compared to controls, where a clear deterioration of functional parameters was seen. $EGFP^{+}$ cells were found in clusters of small cells of an immature, neonatal appearance within the infarcted myocardium, whereas in non infarcted areas they were dispersed and of a much greater size (and mature phenotype). Independently of the different degree of maturation, which is a concern that still needs to be addressed in the field, this work proved that CSCs administered into the coronary tree could home

to the myocardium. Another interesting study performed with the same type of cells (*Rota M et al., 2008)* looked into the differential effect of the activation of endogenous CPCs with growth factors (GFs) or the direct implantation of CPCs, finding that both therapies had a salubrious effect on the scar, with the infarcted wall being significantly ($p > 0.001$) thicker and showing more viable myocytes in the treated groups compared to controls. They conclude that both treatments are able to salvage up to 45% of the infarcted tissue and replace it with functional myocardium. An interesting part of this study is that they also evaluated the ability of the injected or activated CPCs to migrate to the scar, demonstrating that the cells migrate via the expression of different MMPs, that have a marked catalytic effect easing the migration of CPCs from the border toward the center of the scar. Moreover, rodent studies are pivotal in discriminating the relative roles of different strategies or mechanisms of action, as proven by Chimenti et al. (*Chimenti I et al., 2010)*. This group injected human CDCs in the peri-infarct area of SCID mice after permanent coronary ligation to assess the direct and paracrine contributions of cardiac-derived cell therapy to the regeneration obtained after administration in an infarcted heart and quantify the relative contributions of each mechanism to the beneficial effects observed after therapy. They demonstrated that human CS and CDCs have the ability to secrete growth factors such as IGF-1, HGF and VEGF *in vitro* and *in vivo*. Since the cells are implanted in a mice model, and the secreted GFs come from human cells, they were able to prove the existence of *in vivo* secretion by finding the factors secreted by human cells at 1 and 3 weeks after administration. In terms of engraftment, they showed that at 1 week only about 30% of the signal intensity from luciferase-labeled CDCs remained and no cells could be detected by this nonquantitative method at 3 weeks. To calculate the relative contribution of paracrine and direct action of CDCs, they identified the new cells at both vascular structures and the myocardium, reporting that only about 20% of the new capillaries were human in origin. Since the paracrine effects can be exerted both on the myocardium and on endogenous stem cells, they studied the possible activation of endogenous CSCs by the injected hCDCs, finding an increase in endogenous c-kit positive cells around the human CDCs. This thorough study concludes that the mechanism of regeneration is threefold (direct differentiation, indirect paracrine effect on the myocardium and indirect paracrine effects on the endogenous CSCs), but the major contributors are represented by paracrine effects, in their two dimensions. A recent study aimed to explore repeated administration of CDCs. Considering that the inflammatory milieu of the heart immediately after reperfusion could be detrimental for cell engraftment, Carr et al. (*Carr CA et al., 2011)* administered CDCs labeled with GFP and micron-sized particles of iron oxide intramyocardially immediately after reperfusion and systemically via the tail vein at 2 days after the infarction, and the evolution of cardiac function demonstrated improved functionality in CDC-treated animals. An interesting finding is that cells positive for GFP and micron-sized particles of

iron oxide were found in these animals at 16 weeks after injection, thus pointing to greatly improved cell retention and survival with this administration regimen. To assess the feasibility and safety of an allogeneic therapy, Malliaras et al. (*Malliaras K et al., 2012)* aimed to establish the safety of allogeneic CDC administration in the absence of immunosuppression. Considering the evidence pointing toward a mainly paracrine effect, these authors consider that rejection may not be an issue for this therapy, since if the cells are cleared after exerting their beneficial paracrine effects their disappearance would not present a problem. However, the potential limitations of allogeneic therapy are not only related to their effectiveness but also to safety concerns. Therefore, they characterized the *in vitro* immunological properties of CDCs, monitoring the signs of immunorejection (such as inflammatory cytokine secretion, leukocyte infiltration and development of cellular or humoral memory response) and the survival of the cells as well as their functional effect. They injected CDCs of syngeneic, allogeneic and xenogeneic origin, as well as a control group, in the peri-infarct area in rats, quantifying the levels of circulating inflammatory cytokines to assess immunogenicity of the cells as well as performing a post-mortem pathological evaluation of the immune response. The baseline immunophenotype of the studied cells suggests that they may be useful as an allogeneic product: expression of MHC class I antigens confers the cells with protection against NK cell-mediated deletion, while the absence of MHC class II antigens allows CDCs to escape direct recognition from $CD4^+$ T helper cells. They found that during the first 3 weeks after injection, the clearance of syngeneic cells was slower than allogeneic cells, while cells of xenogeneic origin were rejected within 1 week. However, allogeneic and syngeneic transplantations exerted comparable and sustained beneficial effects on infarcted heart structure and function as evidenced by decreased infarct size, increased infarcted wall thickness and limited remodeling when compared to xenogeneic and control animals. These benefits persisted over time, up to 6 months post treatment, which supports the paracrine activation of endogenous cells as the mechanism of action. In terms of immunogenicity, there was a slight transient lymphohistiocytic infiltration at 3 weeks in the allogeneic group that was much lower than the one observed with xenotransplantation and that had completely subsided at 6 months. To further explore the effect of allotransplantation, researchers from the same laboratory (*Tseliou E et al., 2013)* performed intramyocardial injection of CS (not CDCs) in infarcted rats. First, they completed a characterization of the immunological profile of the CS, and, as happened with CDCs, showed that the outer cell layers expressed intermediate levels of MHC I in their surface, but no detectable MHC II. Interestingly, effects were very similar between allogeneic and syngeneic transplantation, with improvements in LVEF and ventricular volumes present as soon as 1 week and sustained for up to 6 months. Cell engraftment was low, as expected, with no cells being detected in the allogeneic transplantation group after 1 week. Despite this low engraftment, benefits were sustained over time. Pathology

showed no immune rejection signs in syngeneic or allogeneic groups, but a robust cell-mediated response was seen in the xenogeneic group. Curiously, inflammatory cytokines at day 7 were lower in the syngeneic and allogeneic groups (when compared with the control group), which points to an immunomodulatory action, consistent with reports from other groups (*Lauden L et al., 2013)* that could be responsible, at least in part, for the sustained effect seen over time. Considering the multitude of cell types currently underevaluation for cardiac therapy, Li et al. (*Li TS et al., 2012)* performed a direct head-to-head comparison of various stem cell types of human origin, namely, CDCs, bone marrow-derived MSCs, adipose tissue-derived MSCs and BMMNCs. These authors showed that cardiac-derived cells showed the greatest *in vitro* potential for myogenic differentiation and angiogenesis induction, along with a balanced and relatively high production of angiogenic and antiapoptotic factors. Similarly, cardiac-derived cells provided the greatest functional benefit in an SCID mice model of experimental infarction, enhancing cell engraftment and myogenic differentiation rates with the lowest number of apoptotic cells and the best preserved/repaired heart architecture. In a further study, they compared the effects of CDCs with that of a subpopulation of $cKit^+$ cells purified from CDCs and reported that CDCs performed better than c-kit+ in terms of functional benefit, which could be attributed to the fact that CDCs secreted higher amounts of paracrine factors than $cKit^+$ cells, which could account for the better results with these cells. In other direct comparison, Oskouei et al. (*Oskouei BN et al., 2012)* studied the relative therapeutic and biological efficacy of human $cKit^+$ CSCs of fetal and adult origin (36,000 CSCs of each kind per animal, respectively) versus two doses of bone marrow-derived MSCs (36,000 and 1,106 cells) in infarcted SCID mice. Eight weeks after administration, they reported a greater effect of fetal CSCs on preserving ventricular volumes and LVEF and decreasing infarct size. While adults CSCs performed on a level with the high-dose MSCs administered, the amount of cells needed for a similar effect was 30-times greater, which points to a greater potency of these cells. In terms of engraftment, as identified using human-specific DNA probes, CSC-treated hearts showed significantly higher incorporation of human stem cells, which was much lower in the high-dose MSC group and almost negligible in the low-dose MSCs (they report the appearance of only a few stained cells). Moreover, evidence of myocyte differentiation was seen in CSC-treated animals but not in MSC-treated animals with either dose. Their results indicate a greater potential of human CSCs compared to MSCs. However, not all rodent studies have reported good results. In an independent study, Li et al. (*Li Z et al., 2009)* used a transgenic mouse strain expressing firefly luciferase and EGFP as CSCs donor and followed up the fate of the cells after intramyocardial administration in an infarction model using bioluminescence imaging. Two days after injection, a robust signal was detected. However, this signal decreased dramatically over time, to 6.7% of baseline at day 7, and to just 0.4% after 8 weeks, indicating poor cell retention. Along these lines,

cardiac function, as monitored by multiple techniques (they used echocardiography, MRI, pressure–volume loops analysis, PET scan and pathological determination of the infarct size) deteriorated similarly in treated and control animals, while it remained stable in sham mice. These results, strikingly different from those reported by other groups with cardiac- derived cell products, could be attributed to differences in isolation techniques, donor cell sources, animal model (reperfused vs nonreperfused), etc. Similarly, van Berlo et al. (*van Berlo JH et al., 2014)* have recently reported that the contribution of $cKit^+$ cells to the heart's CMs is minimal, on the order of 0.03% or less, while they mostly gave raise to cardiac endothelial cells. For this study, they used a mouse model in which they used the Kit locus for tracing the cells and assess the frequency with which $c\text{-}kit^+$ cells generate CMs *in vivo*. This work, however, refers to the *in situ* $cKit^+$ cell performance, which may not reflect the regenerative potency of *ex vivo* proliferated cells. Along these lines, another group (*Ellison GM et al., 203)* has reported that these cells are necessary and sufficient for cardiac regeneration. After ablating the proliferating endogenous CSCs and their progeny with 5-fluorouracil, no regeneration or functional recovery was forthcoming in an isoproterenol induced damage in the murine model, while those animals that had only received isoproterenol, but not the 5-fluorouracil, slowly recovered cardiac function over 28 days, as expected from this model. Taken together, these conflicting results advise caution with the use of exogenous cardiac-derived cells in clinical trials. The amount of mechanistic studies available to elucidate the mechanisms by which this therapeutics could improve cardiac function is still scarce.

3.3. Clinical trials

Different studies have proven that CSCs are present in the heart of mammal species, including man, and can be used to help regenerate the infarcted myocardium in different ways. Human studies have been performed and even completed in some cases, with CSCs from autologous origin, with extremely good results.

SCIPIO (cardiac Stem Cell Infusion in Patients with Ischemic Cardiomyopathy) is a first-in-human, phase 1, randomized, open-label clinical trial designed to explore the effects of autologous c-kit-positive CSCs in patients with ischemic cardiomyopathy. The trial is still ongoing. C-kit-positive cells were isolated and grown from the right atrial appendage. Consequently, only patients undergoing "on-pump" CABG (coronary artery bypass grafting) surgery were enrolled in the trial, as the appendage is routinely removed in these cases to allow access for right atrial cannulation.

Interim results indicates that intracoronary infusion of autologous CSCs results in improved LVEF (assessed by 3D echocardiography), functional capacity (assessed by the NYHA class), and quality of life (assessed by the Minnesota Living With Heart Failure Questionnaire) (*Bolli R et al., 2011).* The salient findings of the study can be summarized as follows: 1) harvesting and processing the right atrial appendage in the operating room for subsequent isolation and expansion of CSCs was eminently feasible in all 33 patients enrolled in the study, yielding successful CSC cultures in each case despite the fact that these patients have severe coronary artery disease, severe heart failure, and multiple comorbidities; 2) this new method for obtaining autologous stem cells did not interfere with standard surgical procedures or with surgical outcomes, and thus appears to be feasible in most patients undergoing CABG; 3) CMR (cardiac magnetic resonance) (generally considered the most accurate technique for these analyses (*Soliman OI et al., 2008; Grothues F et al., 2002; Wu E et al., 2001; Kim RJ et al., 2000; Hendel RC et al., 2006)* demonstrated that intracoronary infusion of autologous CSCs ~4 months after CABG surgery resulted in a striking increase in LVEF (+ 7.7 EF units) 4 months later and an even greater increase (+13.6 EF units) 12 months later, accompanied by a parallel increase in regional contractile function in the infarcted regions that were infused with CSCs; 4) this improvement in LV function was coupled with a concomitant decrease in infarct size, which was consistently observed with three different CMR methods (reductions at 4 and 12 months after CSC infusion, respectively: -22.7 % and -30.2 % by manual delineation, -38.1% and -44.8% by a semi-automated method [FWHM], and -49.7% and -58.6% using non-viable mass), and was accompanied by an increase in LV viable mass (+11.6 g at 4 months and +31.5 g at 12 months after CSC infusion), implying robust regeneration of myocardial tissue. Taken together, these results, obtained with the best methodology currently available (CMR), indicate that administration of autologous CSCs obtained from a surgical specimen is effective in improving LV function and promoting cardiac regeneration in patients with chronic ischemic cardiomyopathy.

CADUCEUS (CArdiosphere-Derived aUtologous stem CElls to reverse ventricULar dySfunction) trial is based on previous preclinical studies that found that Cardiosphere-derived cells (CDCs) reduce scarring after MI, increase viable myocardium, and boost cardiac function. This study aimed to assess safety of such an approach in patients with left ventricular dysfunction after MI.

Percutaneous endomyocardial biopsies are used to obtain source tissue and the cardiosphere culture method (*Messina E et al., 2004*) to yield tens of millions of CDCs in a timely manner (Smith RR et al., 2007).

Six-month primary endpoint analysis of the trial demonstrated the feasibility of harvesting, expanding, and delivering autologous (*Smith RR et al., 2007*) by intracoronary infusion in post-MI patients.

CADUCEUS study showed an increase in viable myocardial tissue as a result of cell therapy. Although two clinical studies of BMMNCs have reported reductions in scar size with cell therapy, the effect was attributable only to reduced scar mass. Even when the discussion is restricted to scar reduction, CDC therapy is about 3–5 times more effective than BMMNCs. This study showed that CDCs have an unprecedented ability to reduce scar and simultaneously stimulate the regrowth of healthy myocardial tissue. The basis for the apparently improved efficacy of CDCs remains to be fully elucidated, but they have noted that CDCs outperform BMMNCs, MSCs, and $cKit^+$ cells in terms of paracrine potency, anti-apoptotic properties, tissue engraftment, and regenerative efficacy when the various cell types are compared directly in mice after MI *(Li TS et al.,2012*) . However, the CADUCEUS trial did not show any changes in this parameter compared to the control group after 1 year, despite demonstrating a 11.1% reduction in scar size (compared to unchanged scar size in control patients) and an increase in viable myocardium by 22.6 g in patients treated with CDCs versus 1.8 g in controls (*Malliaras K et al., 2014*).

Building on the preclinical experience discussed above, and considering the highly encouraging results of the autologous therapy trials, two clinical trials are underway with allogeneic heart-derived cellular products (ALLSTAR, CAREMI) for AMI. The first results from an allogeneic cardiac-derived stem cell product trial were advanced in the 63rd American College of Cardiology Meeting held in March 2014. In this meeting, the preliminary results (with a follow-up of at least 3 months after injection) from the Phase I ALLSTAR trial were reported. This trial aims to determine the safety profile of allogeneic CDCs administered via the infarct related coronary artery between 4 weeks and 12 months after infarction. Phase I was an open-label, dose-escalating study, and its results are being highly encouraging, meeting the first safety endpoint at 1 month after infusion: there were no acute myocarditis attributable to the infusion, no deaths due to ventricular tachycardia or fibrillation, no sudden deaths and no major adverse cardiac events. Phase II has been approved by the data safety monitoring board and enrollment is reported to be ongoing for the double-blind, randomized, placebo controlled Phase II (*Schwarzenberger P et al., 2000)*. On the other hand, the CAREMI trial has recently started its dose-escalating phase. Considering the preclinical and *in vitro* results reported by this Consortium (*Baez-Diaz C et al., 2014; Crisostomo V et al., 2014; Lauden L et al., 2013)*, the results from this trial will be awaited eagerly.

4. Aim of the study

The first aim of this study is to verify the response of cKit$^+$CSCs under hypoxic conditions focusing on the activation of specific pathways leading to an inflammatory and reparative response (IRR).

Then, the second aim of the present study is to verify if the hypoxia adaptation of cKit$^+$CSCs with activation of an IRR leads to a cardioprotective effect after transplantation in a mouse model of MI.

Third, this study wants to evaluate the immune response differences between an allogeneic and a syngeneic cKit$^+$CSC transplantation in a mouse model of myocardial infarction, to verify the safety and feasibility of allogeneic treatment with cKit$^+$CSCs.

5. Materials and Methods

5.1. Animal model and *in vivo* study

Myocardial infarction (MI) was induced by coronary artery ligation in 8 weeks old BalbC and C57BL6 female mice (20 gr body weight), as previously described (*Limana F et al., 2005*). Briefly, in mice under anesthesia (100 mg/kg ketamine and 1mg/kg acepromazine) and mechanically ventilated, thoracotomy via the third left intercostal space was performed. Then the left coronary artery was ligated and $5X10^4$ $cKit^+$CSCs cells in 10 µL phosphate buffer saline (PBS) solution were injected through a 32-gauge needle. Four injections (2.5 µL per injection) were performed in the ventricular wall bordering the viable myocardium. Control infarcted mice were injected with 200 ng of phosphate buffer saline (PBS). Sham operated mice were treated similarly, except that the ligature around the coronary artery was not tied. Animals were sacrified three and twenty one days after surgery. Hearts were excised and the border and infarcted regions of infarcted mice hearts were collected and stored at –80°C to be processed for protein extraction; specifically, the infarcted area was recognized as a pale zone caused by gross necrosis of the myocardium, due to interruption of the blood supply to the area while the border zone was identified as the area between the end of necrosis and the septum. The hearts of mice that did not undergo any surgery were used to isolate cardiomyocytes by collagenase digestion of the left ventricle (LV).

All experimental procedures complied with the Guidelines of the Italian National Institutes of Health, with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD) and were approved by the Institutional Animal Care and Use Committee.

5.2. Cell isolation and *in vitro* culture

Cardiac-derived cells were isolated from BalbC female mice at 2–3 months of age by collagen digestion (280 U/mL; Worthington, Lakewood, N.J., USA) through the coronary arteries and myocyte were removed by centrifugation. Mononuclear cells were cultured in F12:DMEM 1:1 supplemented with 10% fetal bovine serum and expanded. $cKit^+$ cells were selected with immunomagnetic CD117 microbeads, mouse (Miltenyi Biotec) and were expanded in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), penicillin-streptomycin 1X and Erythropoietin (Sigma-Aldrich) 2,5 mg in 500 ml (*Bearzi et al., 2007*)

5.3. Hypoxia

Hypoxic conditions were achieved by incubating cKit$^+$CSCs in a humidified incubator Galaxy 14S (Eppendorf) at 37°C with 5% CO_2 and 1% O_2 for 6, 24, 48 hours.

5.4. FACS analysis

For FACS analysis, cKit$^+$CSCs were fixed in 4% paraformaldehyde for 15 minutes at room temperature and incubated with anti-CD45 antibody (BD Pharmingen). Isotype control was performed (mouse polyclonal IgG; Santa Cruz Biotechnology). Specifically, cells were incubated with the primary antibody for 45 minutes at 37°C. Flow cytometry was performed with FACSAria instrument (Becton Dickinson, San Jose, CA). Cellular debris and aggregates were gated out based on forward and side scatter. Gating on the signal of the nuclear stain DAPI was employed to exclude additional artifacts. Isotype-matched negative controls were utilized to define the threshold for each specific signal and establish the appropriate gate for positive cells. Data were analyzed with Kaluza Flow Cytometry Analysis software (*D'Amario et al., 2011*).

5.5. mRNA isolation and quantification

Total RNA was extracted from cKit$^+$CSCs following 6, 24 and 48h of normoxia and hypoxia (n=3/group). Quality of RNA was checked using the Agilent 2100 Bioanalyzer and Nanodrop 1000. mRNAs levels were analyzed using the SYBR-GREEN qPCR method (5 ng/assay, Applied Biosystems). Then mRNA was quantified with ABI Prism 7000 SDS (Applied Biosystems). Relative expression was calculated using the comparative Ct method (2–[delta][delta]Ct) (*Livak et al., 2001*).

5.6. Real time RT-PCR

Real-time RT-PCR amplification was carried out in the 7900 Real-time PCR system (Applied Biosystems, CA, USA) using Real Time SyBr Green qPCR Superscript (Invitrogen, Milan, Italy).

All reactions were carried out in triplicate. The threshold cycle (CT), which correlates inversely with the levels of target mRNA, was measured as the number of cycles at which the reporter fluorescence emission exceeds the preset threshold level. The amplified

transcripts were quantified using the comparative CT method with the formula for relative fold change = $2^{-\Delta\Delta CT}$. The mean was calculated, and possible significant differences were analyzed using Student's *t* test. $P < 0.05$ was considered significant.

The primers used are reported in Table 1.

HIF1A_mm_fw	**gcactagacaaagttcacctgaga**
HIF1A_mm_rev	**cgctatccacatcaaagcaa**
VEGFa_mm_fw	**caggctgctgtaacgatgaa**
VEGFa_mm_rev	**gctttggtgaggtttgatcc**
EPO_mm_fw	**tctgcgacagtcgagttctg**
EPO_mm_rev	**cttctgcacaacccatcgt**
Glut1_mm_fw	**gaccctgcacctcattgg**
Glut1_mm_rev	**gatgctcagataggacatccaag**
NFkB_mm_fw	**ctggcagtccttctcaaagc**
NFkB_mm_rev	**tccaggtcatagagaggctca**
Rage_mm_fw	**agtcagaggaagcggagatg**
Rage_mm_rev	**aaggaggaattgggatggaatg**
Cox2_mm_fw	**caagcagtggcaaggcctcca**
Cox2_mm_rev	**ggcacttgcattgatggtggct**
Nos2_mm_fw	**ctttgccacggacgagac**

Nos2_mm_rev	**cattgtactctgagggctgac**
Tlr2_mm_fw	**accgaaacctcagacaaagc**
Tlr2_mm_rev	**agcgtttgctgaagaggact**
Tlr4_mm_fw	**ggactctgatcatggcactg**
Tlr4_mm_rev	**ctgatccatgcattggtaggt**
P2rx7_mm_fw	**aagtgcagacgctgtgtcc**
P2rx7_mm_rev	**gcagaactttaggccagctc**
Ptx3_mm_fw	**cgctgtgctggaggaact**
Ptx3_mm_rev	**gggaagaaaattgctgtttcac**
Cxcr4_mm_fw	**tggaaccgatcagtgtgagt**
Cxcr4_mm_rev	**gggcaggaagatcctattga**
MMP2_mm_fw	**taacctggatgccgtcgt**
MMP2_mm_rev	**ttcaggtaataagcacccttgaa**
MMP9_mm_fw	**acgacatagacggcatcca**
MMP9_mm_rev	**gctgtggttcagttgtggtg**
Timp3_mm_fw	**cacggaagcctctgaaagtc**
Timp3_mm_rev	**tcccacctctccacaaagtt**
Timp4_mm_fw	**agggagagcctgaatcatca**

Timp4_mm_rev	**gcactgcatagcaagtggtg**
GUSB_mm_fw	**ctctggtggccttacctgat**
GUSB_mm_rev	**cagttgttgtcaccttcacctc**

Table 1 : mRNA primers list.

5.7. Western blot analysis

Proteins from cardiac tissue or cKit$^+$CSCs were homogenized and extracted with RIPA buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 1% Deoxycolic acid, 0.1% SDS and 10% Glycerol) containing protease and phosphatase inhibitors (2 mM phenylmethylsulfonyl fluoride, 100 U/ml of aprotinin, 10 μg/ml of leupeptin and pepstatin, 10 mM sodium fluoride, 20 mM sodium vanadate). Equal amounts of total cellular proteins (100 μg/lane) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). Membranes were probed with rabbit monoclonal anti HIF-1α (Cell Signaling Technology, Danvers, MA), goat polyclonal anti HXK2 (Santa Cruz Biotechnology, Dallas, TX) rabbit polyclonal anti CAIX (Thermo Fisher Scientific, Whaltam, MA), rabbit polyclonal anti NF-kB (Santa Cruz Biotechnology), rabbit monoclonal anti COX2 (Cell Signaling Technology), goat polyclonal anti RAGE (Santa Cruz Biotechnology), goat polyclonal anti CXCR4 (Santa Cruz Biotechnology), goat polyclonal anti alfa-MHC and mouse monoclonal anti beta-MHC (Santa Cruz Biotechnology), mouse monoclonal anti CSRP3 (Santa Cruz Biotechnology), rabbit polyclonal anti collagen I (Abcam, Cambridge, UK), rabbit polyclonal anti LC3B (Novus Biologicals, Littleton, CO), rabbit polyclonal anti Cdk4 (Santa Cruz Biotechnology), rabbit monoclonal anti Atg7 (Cell Signaling Technology), rabbit polyclonal anti Bax (Santa Cruz Biotechnology), rabbit polyclonal anti Cleaved Caspase-3 (Cell Signaling Technology), mouse monoclonal anti Bcl-2 (BD Biosciences Pharmingen, Sparks, MD), mouse monoclonal anti CD45RA (Millipore, Darmstadt, Germany), rabbit monoclonal anti CD3 (Abcam, Cambridge, UK) and rabbit polyclonal anti CD68 (Abcam) followed by horseradish peroxidase-coupled secondary antibodies and developed by a chemiluminescence-based detection system (ECL, Amersham Pharmacia Biotech.).

5.8. Luminex Assay

Blood was collected in all animals just before sacrifice; the levels of circulating cytokines have been determined using: Bio-Plex Pro™ Mouse Cytokine Th1/Th2 Assay reagent kit (Bio-Rad, Hercules, CA) for pro-inflammatory cytokines (GMCSF, INFγ, IL-2, IL-12), anti-inflammatory cytokines (IL-4, IL-5, IL-10), TNFα and IL-17a and a Bio-Plex Pro™ TGF β Assay reagent kit (Bio-Rad) for TGF β1, TGF β2 and TGF β3. Data were acquired on the Bio-Rad Bio-Plex 200 reader equipped with a magnetic workstation and analyzed using Bio-Plex software version 6.0 (Bio-Rad).

5.9. ImmunoSorbent Assay-ELISA

ELISA analysis for mouse IGF-1 and HGF (R&D Systems, Minneapolis, USA) was performed following the manufacturer's instructions. Briefly, this assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibodies specific for IGF-1 or HGF has been pre-coated onto a microplate. Standards and samples (conditioned media) were pipetted into the wells and any IGF-1 or HGF present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for IGF-1 and HGF was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of IGF-1 and HGF bound in the initial step. The color development was stopped and the intensity of the color was measured. Optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm, with wavelength correction set to 540 nm or 570 nm.

5.10. Immunofluorescence analysis

Organs used in cryostat section were snap frozen in OCT-filled molds (Bio-Optica, Milan, Italy). The frozen organs were cut into sections with a thickness of 4 ·m in a cryostat at -20°C, mounted on glass slides and postfixed in ice cold acetone (1:1).

For immunofluorescence on tissue sections, tissue slides were stained with mouse monoclonal anti CD45RA (Millipore, Darmstadt, Germany), rabbit monoclonal anti CD3 (Abcam, Cambridge, UK) and rabbit polyclonal anti CD68 (Abcam). Nuclei were identified

using Hoechst staining or SYTO 82 orange fluorescent nucleic acid staining (Molecular Probes, USA). Coverslips were mounted on antifade mountant (ProLong Diamond Antifade Mountant, Life Technologies). Fluorescence intensity was visualized with an LSM 510 confocal microscope (Zeiss, Milan, Italy). Results were obtained by quantification of the number of positive cells/mm^2 for each marker.

5.11. Data collections and Statistics

Data shown as bar graphs are means ± error standard. Statistical significance between two measurements was evaluated by unpaired Student's *t* test. A probability value of $p<0.05$ was considered significant. Data were analyzed using Prism software (version 7, Graph Pad, San Diego, California).

6. Results

6.1. Hypoxia regulates expression of pro-inflammatory genes and proteins in cKit$^+$ CSCs

Several evidences suggest that in a hypoxic tissue, inflammation can be activated causing damage as well as repair. This response, named inflammatory and reparative response (IRR), can occur in many important human pathologies and involve chiefly distant and resident leukocytes. Nevertheless, HIF-dependent adaptation and expression of proinflammatory genes has been also observed in hypoxic stem cells and progenitors (*De Girolamo et al, 2013*).

To verify whether cKit$^+$CSCs are able to adapt to an hypoxic environment by the IRR, these cells were cultured and incubated either in normoxia and hypoxia for 6, 24, and 48 hours (h).

Importantly, in normoxic condition, cKit$^+$CSCs examined in our study did not show any significant presence of leukocytes as shown by CD45 FACS analysis (Fig. 1). Hypoxia clearly influenced the expression of pro-inflammatory genes and proteins. Specifically, mRNA expression for HIF-1α was significantly increased following 24h of hypoxia (Fig. 2A) while its target gene VEGF increased at all three time points (Fig. 2B). Among the other HIF-1α target genes, as showed by RT_PCR, the expression level of the glucose transporter GLUT1 gradually increased from 6 to 48h (Fig. 2C) while EPO expression strongly increased at all three time points showing a peak after 24h of hypoxia (Fig. 2D).

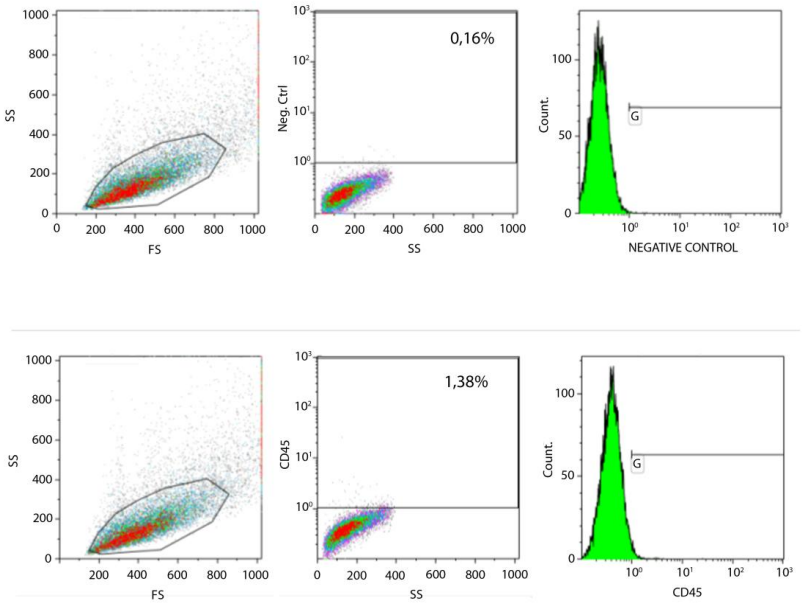

Fig.1 : Bivariate distribution of CSCs in forward and side light scatter. Expression of surface marker in Negative control (upper panel) is indicated. Distribution of CD45 (lower panel) in CSCs. CD45 expression is shown as percentage of positive cells.

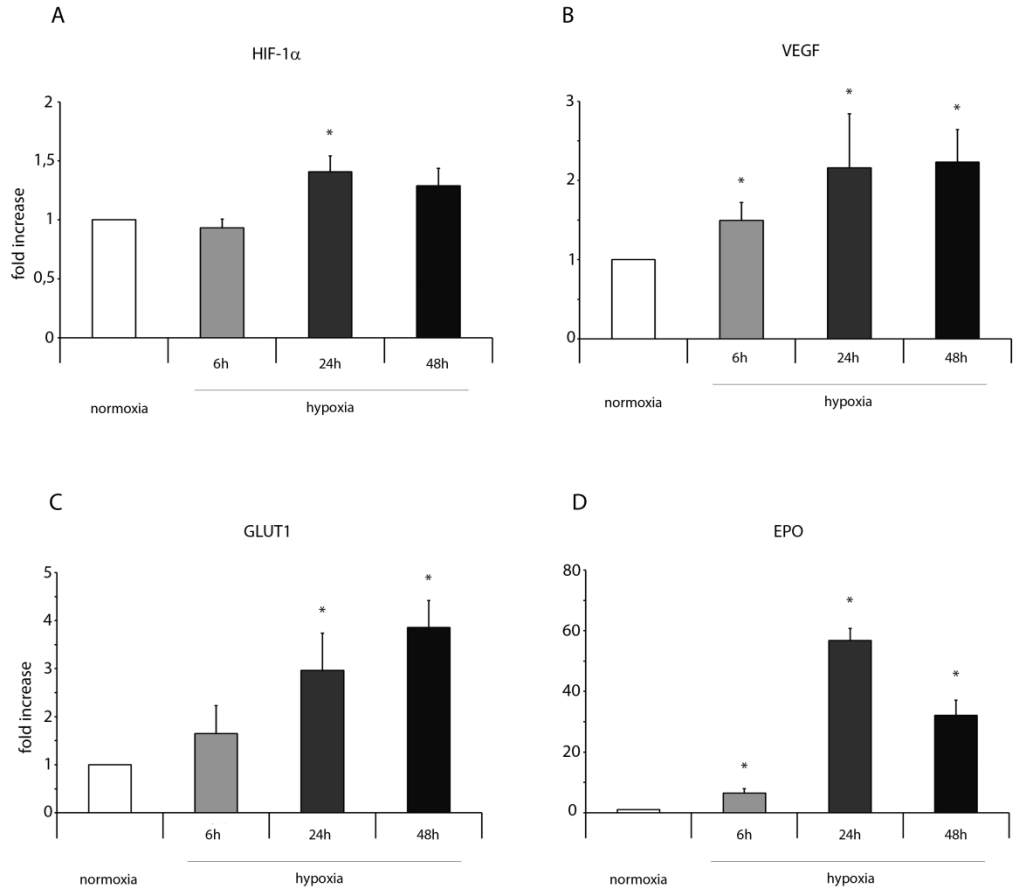

Fig.2 : Hypoxia enhances mRNA expression of HIF-1α and its target genes in cKit$^+$CSCs. cKit$^+$CSCs were incubated under normoxic and hypoxic conditions for 6, 24 and 48h. mRNA expression levels for (A) HIF-1α, (B) VEGF, (C) GLUT1 and (D) EPO were determined by real time PCR. The bar graphs show fold increases in expression of the studied genes in cKit$^+$CSCs with respect to control normoxic cells, set at 1. Data were normalized to GUSB, a housekeeping gene, and represent means ± SEM for three separate experiments, each repeated in triplicate. *, P < 0.05.

Importantly, alarmin receptors such as the Receptor for Advanced Glycation End products (RAGE), the purinergic receptor P2X ligand-gated ion channel 7 (P2X7R), the Toll-like receptors 2 and 4 (TLR2 and TLR4) were activated by hypoxia. The membrane receptor RAGE showed a transient induction by hypoxia at 6h and a low induction at 24 and 48h (Fig. 3A). The expression of the other membrane receptor P2X7R was increased only following 48h of hypoxia (Fig.3B). TLR2 and TLR4 showed a similar expression pattern: a decrease at 6h and a significant increase both at 24 and 48h (Figs. 3C-D).

The activation of these receptors resulted in the activation of NF-kB (Fig.3E) that led to the expression of genes belonging to the family of the IRR (all involved in defence, protection and repair of cells and tissues of the IRR as well) as the pro-inflammatory proteins cyclooxygenase-2 (COX2), pentraxin-3 (PTX3), chemokine (C-X-C motif)

receptor 4 (CXCR4) and nitric oxide synthase-2 (NOS2). In particular, hypoxia increased the expression of the inducible enzymes COX2 (Fig.3F) and NOS2 (Fig.3G) mainly at 48h but significantly at all three time points. Conversely, the expression of PTX3 was induced by hypoxic condition only at 6h (Fig.3I). Finally CXCR4 was induced by hypoxia at 48h (Fig.3H).

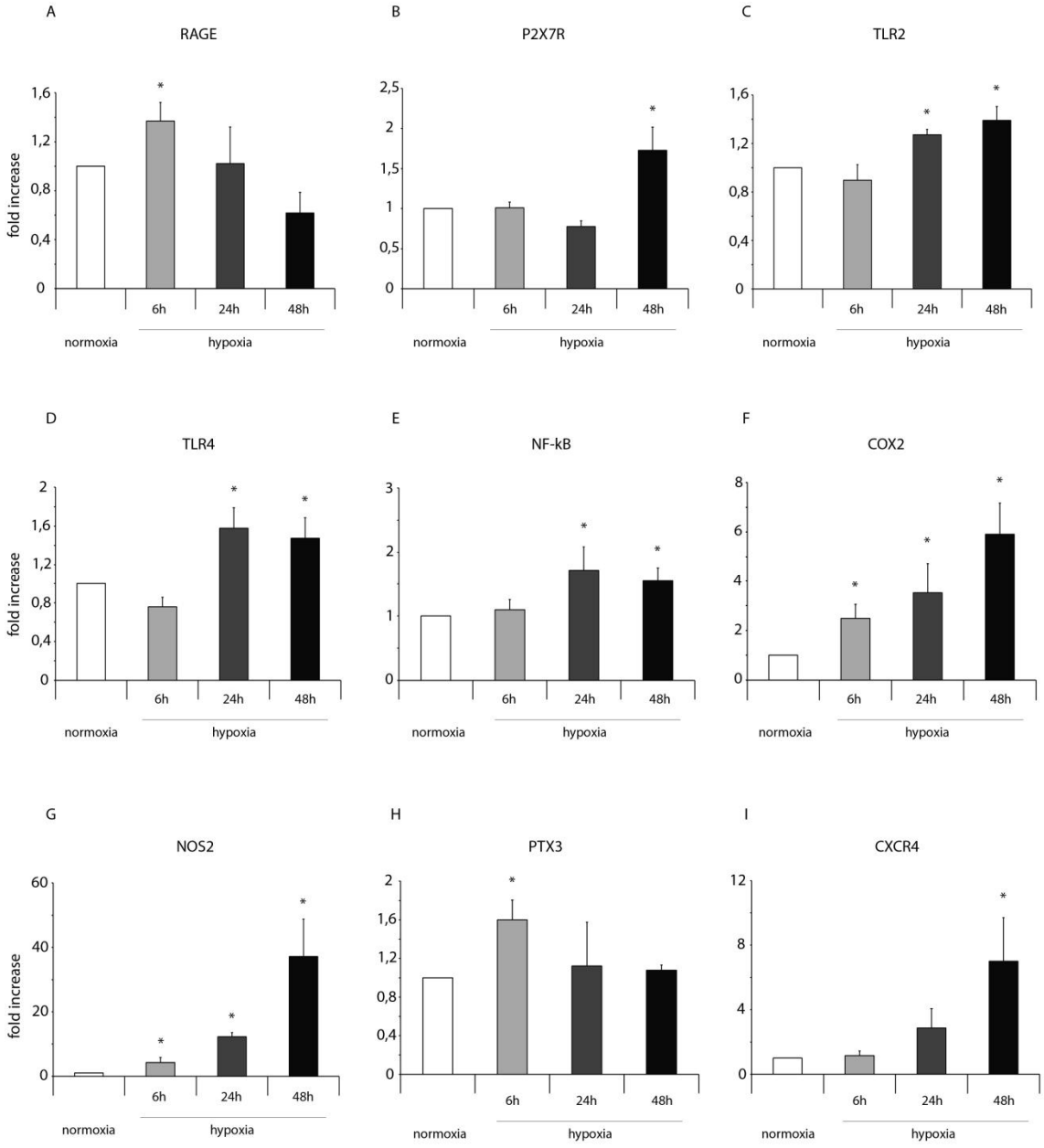

Fig.3 : Hypoxia enhances mRNA expression of NF-kB and its target genes in cKit$^+$ CSCs. cKit$^+$ CSCs were incubated under normoxic and hypoxic conditions for 6, 24 and 48h. mRNA expression levels for (A) RAGE, (B) P2X7R, (C) TLR2, (D) TLR4, (E) NF-kB, (F) COX-2, (G) NOS2, (I) PTX3 and (H) CXCR4 were determined by real time PCR. The bar graphs show fold increases in expression of the studied genes in cKit$^+$ CSCs with respect to control normoxic cells, set at 1. Data were normalized to GUSB, a housekeeping gene, and represent means ± SEM for three separate experiments, each repeated in triplicate. *, P < 0.05.

However, in all experimental conditions, we always observed an increased expression of the genes related to HIF-1α and NF-kB in CSCs under hypoxia compared to normoxia. For several markers, increased protein expression levels were obtained by WB analysis under normoxic and hypoxic conditions mainly at 24h (Figs. 4-5). Noteworthy, the protein expression of metabolic enzyme hexokinase 2 (HK2), regulated by HIF-1α, was increased both following 24h and 48h of hypoxia (Fig. 4).

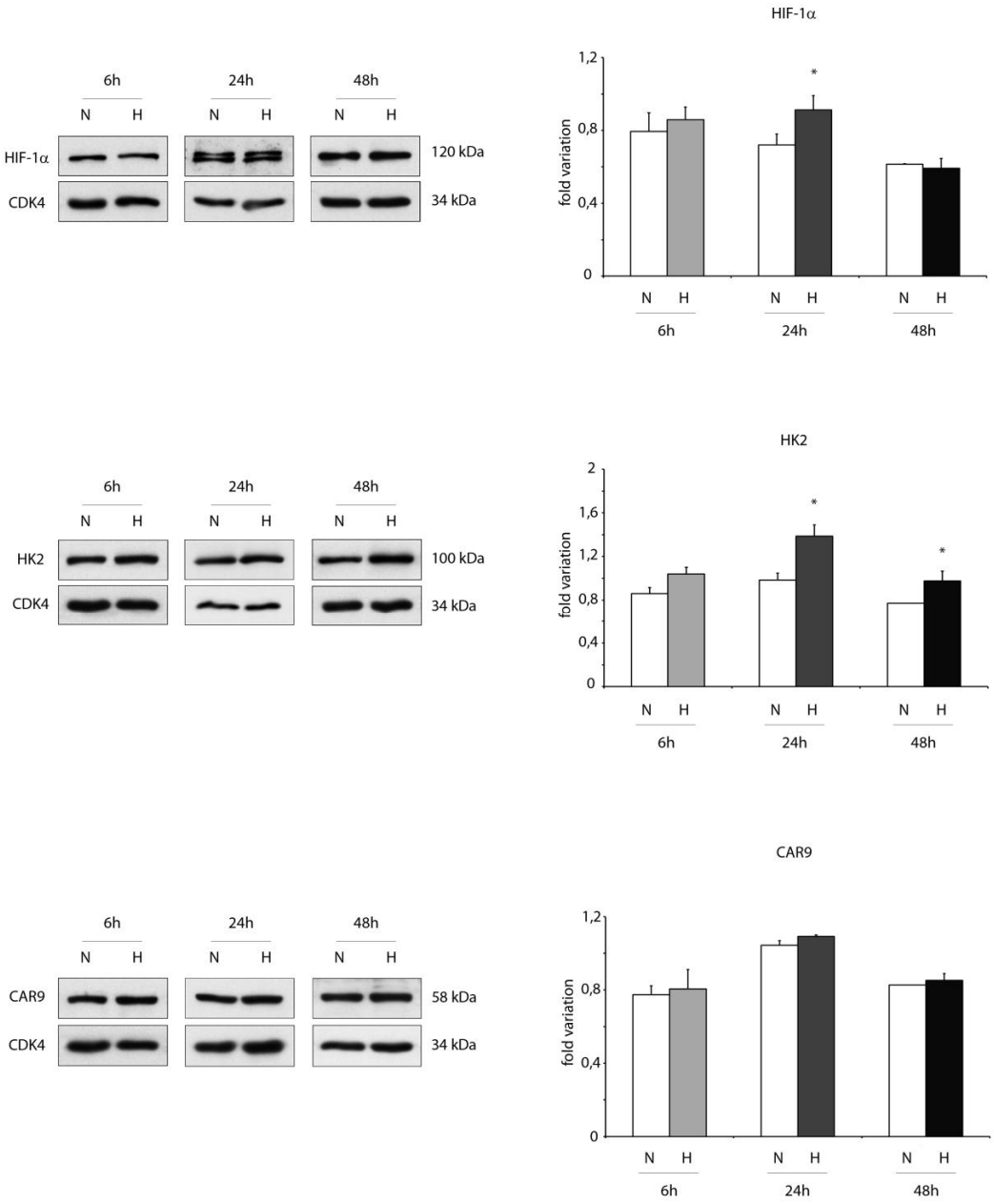

Fig.4 : Hypoxia enhances protein expression levels of HIF-1α and its target genes in cKit$^+$ CSCs. Western blot analysis showing the expression of (A) HIF-1α, HK2 and CAR9 in cKit$^+$ CSCs under hypoxic conditions (H) at 6, 24 and 48h compared to normoxic conditions (N). The same filter was probed with anti-CDK4 pAb to show the equal loading. Left panel: A representative Western blotting of three independent experiments is shown. Right panel: Densitometric analysis of Western blot. Data are shown as means ± SEM. *P < 0.05 vs normoxic conditions (N).

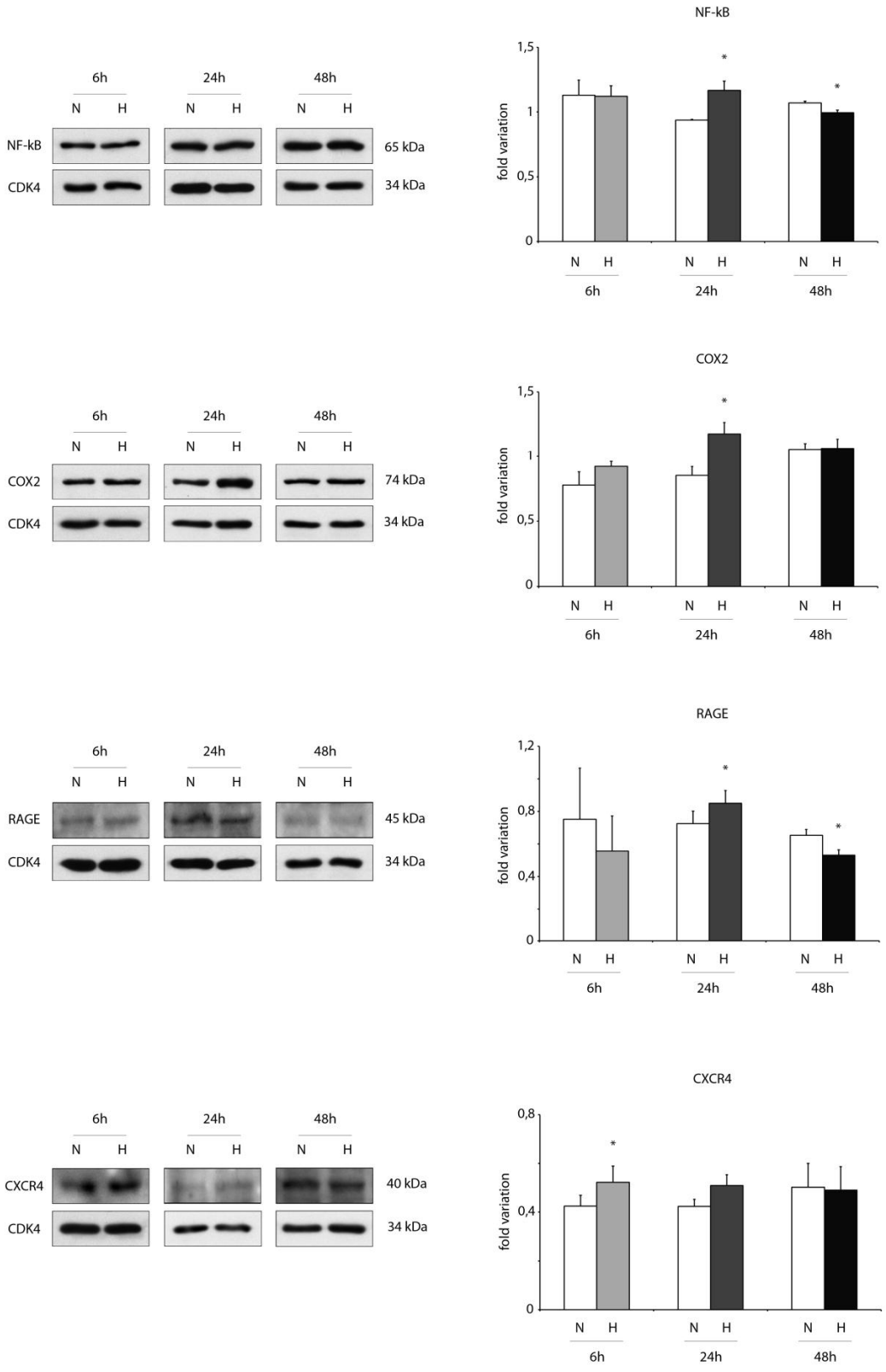

Fig.5: Hypoxia enhances protein expression levels of NF-kB, alarmin receptors and markers of the NF-kB-dependent inflammatory and reparative response in cKit$^+$CSCs. Western blot analysis showing the expression of RAGE, NF-kB, COX2 and CXCR4 in cKit$^+$CSCs under hypoxic conditions (H) at 6, 24 and 48h compared to normoxic conditions (N). The same filter was probed with anti-CDK4 pAb to show the equal loading. Left panel: A representative Western blotting of three independent experiments is shown. Right panel: Densitometric analysis of Western blot. Data are shown as means ± SEM. *P < 0.05 vs normoxic conditions (N).

6.2. cKit$^+$CSCs reduce apoptosis and induce autophagy following acute MI

Since activation of the IRR leads to protection and repair of damaged cells, we hypothesized that following transplantation in a infarcted myocardium, cKit$^+$CSCs would exert a cardioprotective effect. To test this hypothesis, cKit$^+$CSCs were transplanted into the damaged heart of an ischemic mouse. Firstly, by Luminex assay, we detected circulating levels of different pro- and anti-inflammatory cytokines three days following surgery: we did not find differences among infarcted injected hearts and controls except for IL-10, a potent anti-inflammatory cytokine, that resulted significantly up regulated (Fig.6).

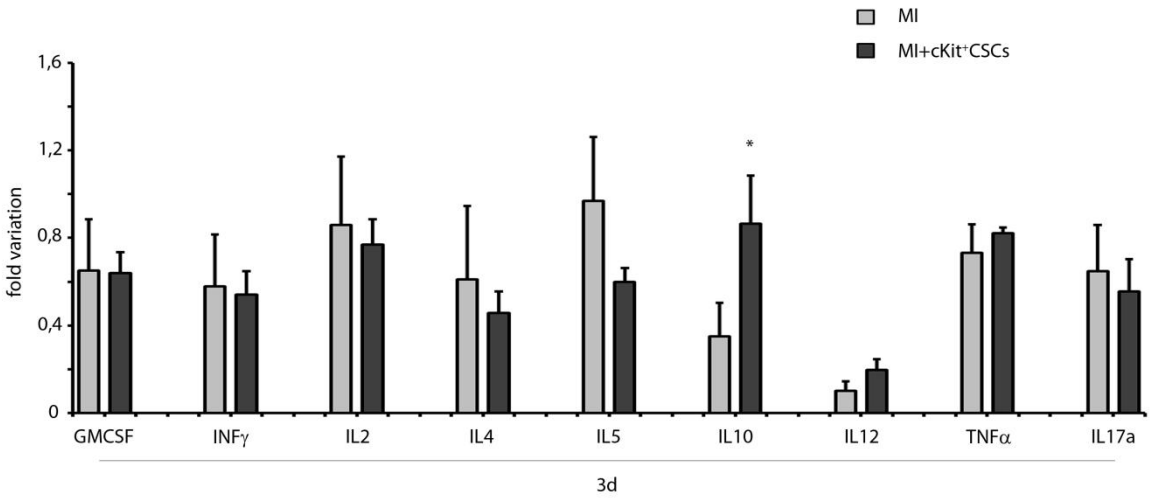

Fig. 6 : Circulating levels of pro- and anti-inflammatory cytokines detected by Luminex assay three days following surgery in the plasma of PBS treated infarcted mice used as controls (MI, n=4) and cKit$^+$CSC transplanted infarcted mice (MI+cKit$^+$CSCs, n=4), all normalized for their controls. Data were obtained using samples from 4 mice/each group. Data are shown as means ± SEM. *P<0.05 vs MI.

At the same time point, we verified the antiapoptotic effect of these cells. Specifically, we evaluated caspase-3 cleavage by WB analysis in the border zone of infarcted hearts (Fig. 7A). Treated hearts showed lower expression levels of cleaved caspase-3 compared to controls. Further, we also measured the expression levels of apoptotic downstream targets, including the anti-apoptotic protein Bcl-2 as well as the pro-apoptotic protein Bax (Figs 7B-C). Results showed that the protein expression of Bcl-2 was significantly increased in the border zone of three-day infarcted hearts by cKit$^+$CSC transplantation (Fig.7B). Conversely, cell treatment significantly inhibited Bax expression compared to the control group (Fig.7C).

Since recent studies have indicated a protective role of the autophagic process in ischemic heart disease (*Schiattarella and Hill, 2015; Nishida and Otsu, 2015)*, we tested whether reduced apoptotic rate in the peri-infarct region of transplanted hearts was associated to increased autophagy. Firstly, we analyzed the expression of the ubiquitin-like modifier LC3 by WB analysis (Fig.7D). Results showed that, three days following MI, there was a significant increase in LC3II formation in the border zone of $cKit^{+}$CSC transplanted hearts compared to controls indicative of autophagosome formation. Further, the infarcted hearts were also characterized, following cell transplantation, by upregulation of the protein levels of the autophagy-related gene 7 (Atg7), another important protein for autophagosome formation (Fig.7E).

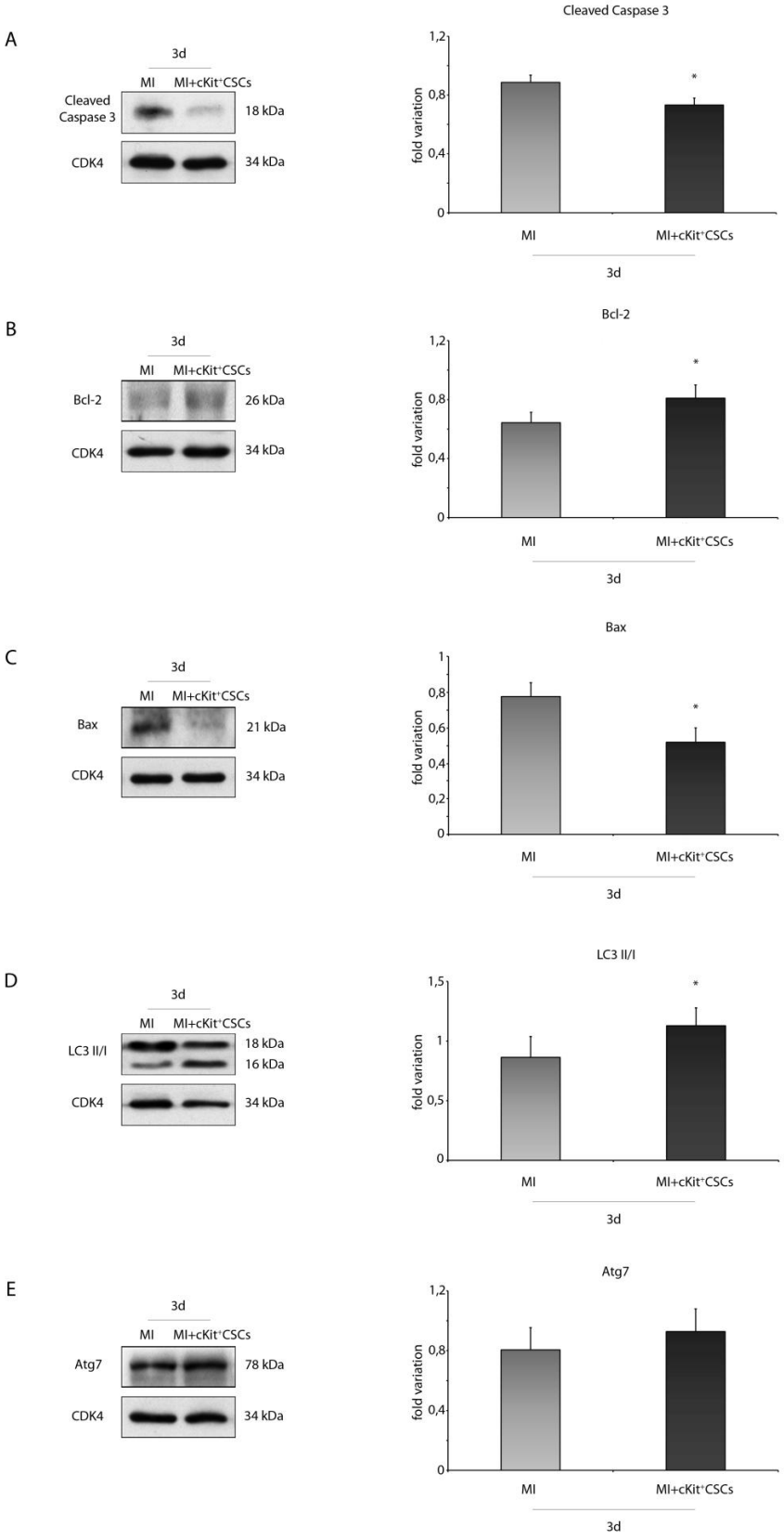

Fig. 7 : Western blot analysis showing the expression of (A) cleaved-caspase 3, (B) Bcl-2, (C) Bax, (D) LC3 and (E) Atg7, in the border zone of three days infarcted hearts following cKit$^+$ CSC transplantation (MI+cKit$^+$CSCs, n=3). Infarcted hearts treated with PBS were used as control (MI, n=3). Left panel: A representative Western blotting of three independent experiments is shown. Right panel: Densitometric analysis of Western blot. Data are shown as means ± SEM. *P < 0.05 vs MI.

6.3. cKit$^+$CSCs attenuate cardiac remodeling (hypertrophy) in the infarcted murine heart

Muscle LIM Protein (MLP), also known as cysteine rich protein 3 (CSRP3, CRP3), has a significant impact on cardiac muscle physiology and pathology. Specifically, in mice, reduced expression of CSRP3 leads to myocardial hypertrophy followed by dilated cardiomyopathy and heart failure (*Buyandelger B et al., 2011*). In our experimental model, we detected, by WB analysis, a higher expression level of CSRP3 in the border zone of 21 day-infarcted hearts following cKit$^+$CSC transplantation compared to controls (Fig. 8A).

Cardiac hypertrophy is characterized by a switch of expression from α-myosin heavy chain (α-MHC), the mature isoform, to β-MHC, the embryonal isoform (*Izumo et al., 1987, Frustaci et al., 2006*). 21 days after cell therapy, we observed, in the border zone of infarcted hearts, the presence of lower levels of β-MHC and of higher levels of α-MHC, as showed by WB leading to a marked decrease in MHC β-α ratio (Fig.8B).

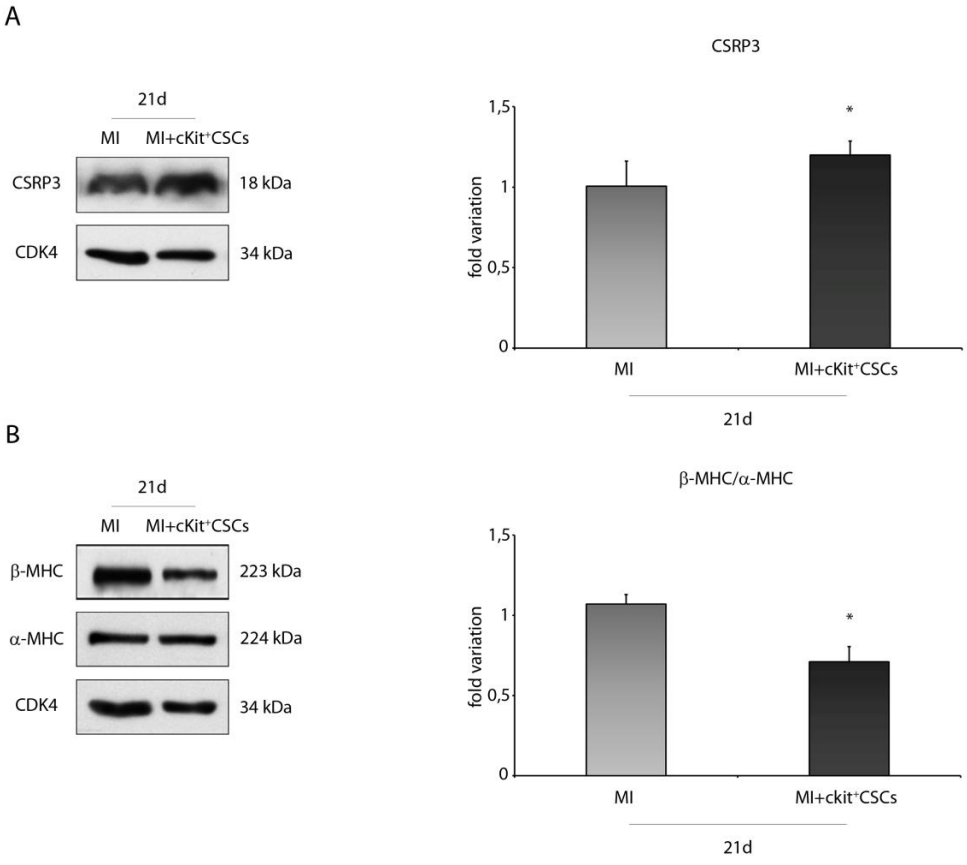

Fig. 8 : The relative levels of (A) CSRP3 and (B) β-MHC/α-MHC were investigated in the border zone of twenty one days infarcted control hearts (MI, n=3) or cKit$^+$CSC transplanted hearts (MI+cKit$^+$CSCs, n=3) by Western blot (left panel) and relative densitometry of three independent experiments (right panel). Data are shown as means ± SEM. *P < 0.05 vs MI.

6.4. cKit$^+$CSCs reduce cardiac fibrosis following acute MI

TGF β is an indicator of cardiac fibrosis that plays an important role in the pathogenesis of cardiac remodeling. In our experimental model, 3, 7 and 21 days following cKit$^+$CSC treatment, infarcted mice were characterized by circulating levels of TGF β1, detected by LUMINEX assay, significantly lower than in controls suggesting an attenuation in adverse remodeling (Fig.9A).

In the infarcted murine heart, TGF β promotes myofibroblast transdifferentiation and matrix synthesis. Accordingly to our results on circulating TGF β1, we detected lower expression of collagen I protein in the infarcted hearts 21 days following stem cell transplantation compared to control hearts (Fig.9B). Further, in order to account for these results, we evaluated whether hypoxic cKit$^+$CSCs had an effect on the expression of matrix matalloproteinases (MMPs) and their inhibitors (TIMPs). 24 h of hypoxia had no significant effect on MMP2 mRNA while it markedly enhanced MMP9 mRNA in cKit$^+$CSCs (Fig.9C). Further, when we examined the mRNA expression of the MMP inhibitors, we found that TIMP4 was significantly lower in hypoxic cKit$^+$CSCs than in normoxic cells (Fig.9D). In contrast, there was no effect on TIMP3 expression (Fig.9D).

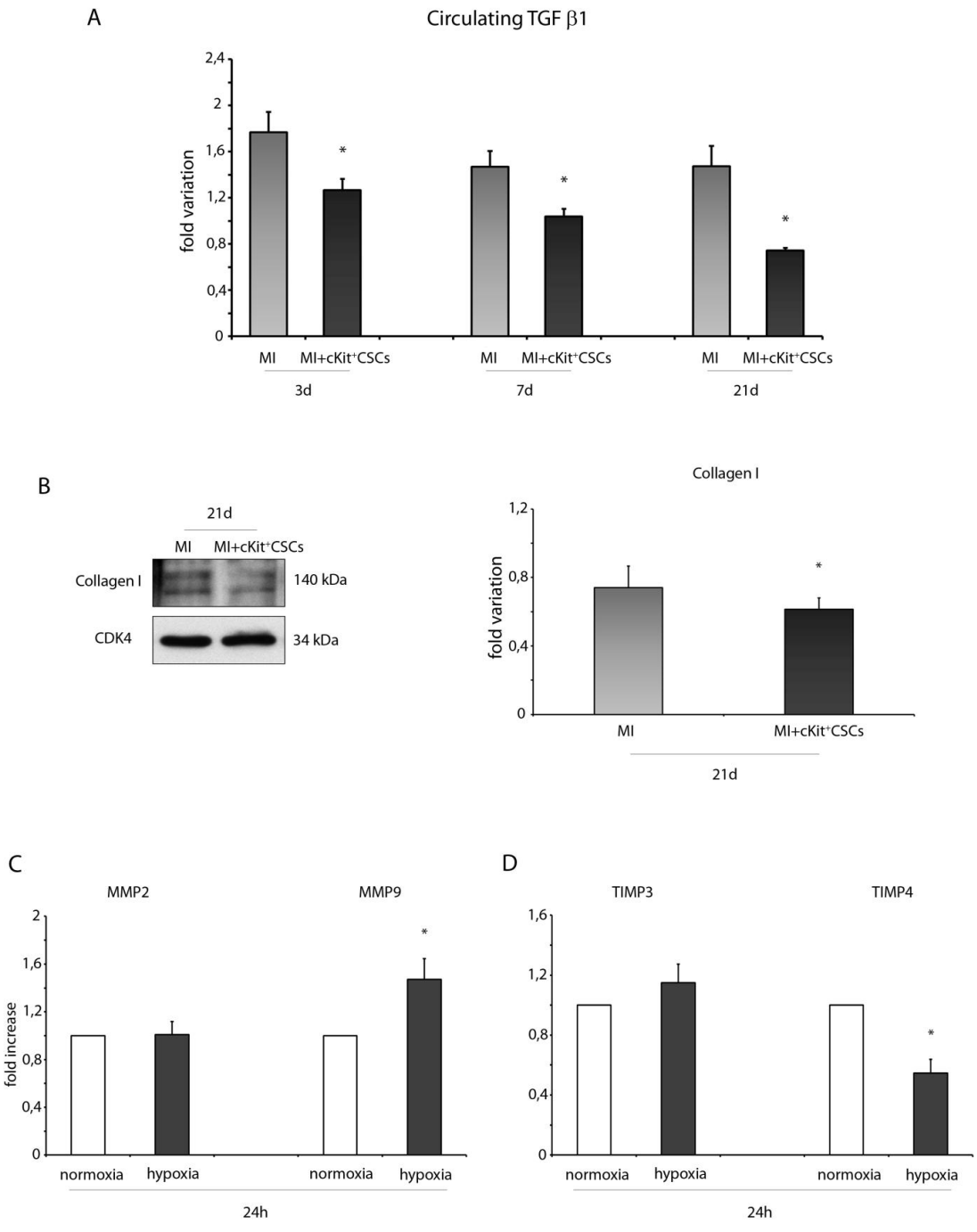

Fig. 9 : cKit+ CSCs reduces the expression of fibrotic markers. (A) Circulating levels of TGF β1 detected by Luminex assay 3,7 and 21 days following surgery in the plasma of PBS treated infarcted mice used as controls (MI, n=4) and cKit+ CSC transplanted infarcted mice (MI+cKit+ CSCs, n=4), all normalized for their controls. Data were obtained using samples from 4 mice/each group. Data are shown as means ± SEM. *P<0.05 vs MI. (B) Collagen I expression in twenty one day infarcted control hearts (MI, n=3) or cKit+ CSC transplanted hearts (MI+cKit+ CSCs, n=3) by Western blot (left panel) and relative densitometry of three independent experiments (right panel). Data are shown as means ± SEM. *P < 0.05 vs MI. (C) MMP2 and MMP9, (D) TIMP3 and TIMP4 mRNA expression by real time PCR in normoxic and hypoxic cKit+ CSCs at 24h. Data were normalized to GUSB, a housekeeping gene and represent means ±SEM; Data were obtained from three independent experiments. *p< 0.02 vs normoxic cKit+ CSCs.

6.5. cKit$^+$CSCs enhances the production of IGF-1 and HGF under hypoxia conditions

ELISA assays were performed to measure the levels of two growth factors, both involved in cardioprotection, in the conditioned medium of normoxic and hypoxic cKit$^+$CSCs. After 48h of hypoxia, we detected a significant increase in IGF-1 (Fig.10A) and HGF levels (Fig.10B) in conditioned media of cKit$^+$CSCs compared to normoxia.

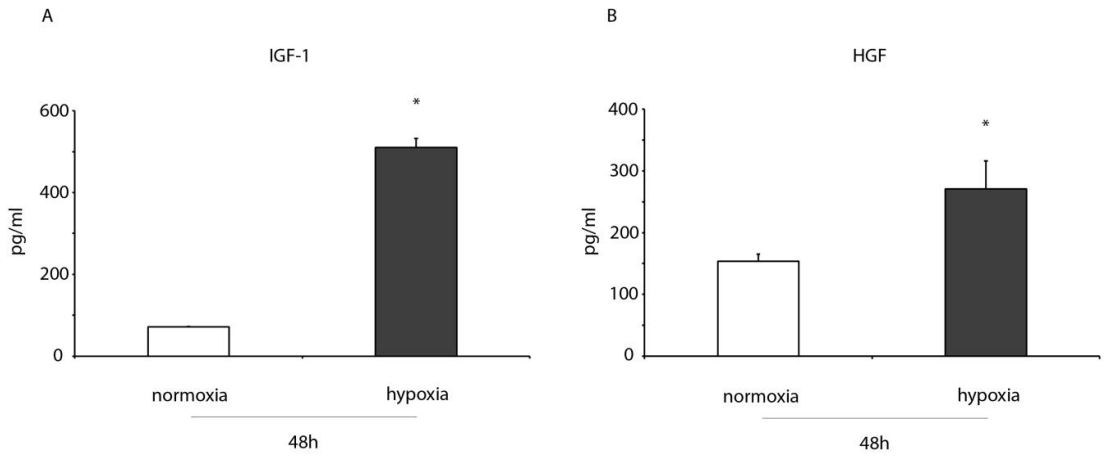

Fig. 10 : IGF-1 and HGF levels increase in the conditioned medium of hypoxic cKit$^+$CSCs. ELISA detection of (A) IGF-1, (B) HGF in in the conditioned medium of normoxic and hypoxic cKit$^+$CSCs (n= 3 independent experiments). Results are presented as means ± SD. *P < 0.001 vs normoxic conditions.

6.6. Allogeneic cKit$^+$CSC transplantation doesn't increase immune response in mouse after MI

Most clinical trials of cell therapy to-date have been conducted using autologous cells. While autologous cell therapy carries no risk of immune rejection, it requires patient-specific tissue harvesting, cell manufacturing and quality control and is, therefore, associated with important limitations, that could be overcome by the use of allogeneic cells. The obvious disadvantage of allogeneic therapy is the risk of immune response, which could limit effectiveness of cell therapy, raise safety concerns and induce allosensitization of the recipient, which could complicate future organ transplant. If allogeneic cell therapy were proven to be safe and effective, the possibility to use allogeneic CSCs to repair infarcted hearts would be an alternative of paramount importance to autologous procedures. This possibility has been already demonstrated for cardiospheres, another source of CSCs, but not for cKit$^+$CSCs (*Baez-Diaz C et al., 2014; Crisostomo V et al., 2014; Lauden L et al., 2013).*

Then, to verify whether allogeneic cKit$^+$CSC transplantation could exert an increase in the immune response, we decided to isolate and expand cKit$^+$CSCs from Balb C murine hearts and then to transplant them into the hearts of Balb C mice (to have a syngeneic transplant) or in the hearts of C57 BL6 mice (to have an allogeneic transplant). At three different time points (3,7 and 21 days) hearts were collected from mice and used for immunohistochemistry and WB analyses. Specifically, using these techniques, we detected and quantified different markers of primary and secondary inflammation among the different groups of mice to verify the presence of an immune response: CD45RA to identify B monocytes, CD3 for lymphocytes and CD68 for macrophages.

For western blot analysis, we collected the entire left ventricle (LV) of sham mice while we separated the infarcted zone from the survived zone of the border zone of infarcted mice.

First, we did not detect significative differences in the expression levels of the three inflammatory markers between sham BalbC and C57 BL6 mice (Fig.11 and Fig.12).

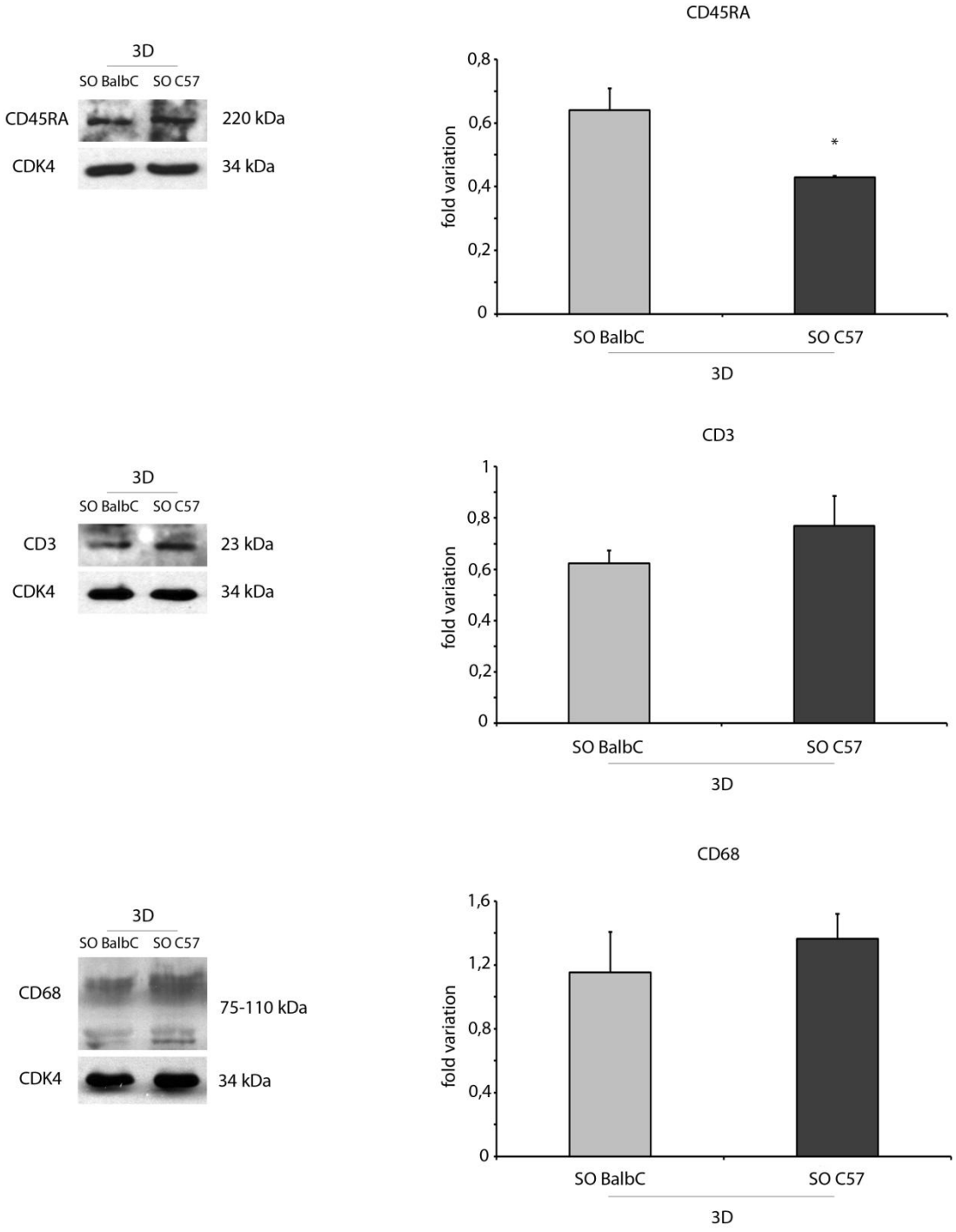

Fig. 11 : Western blot analysis showing the expression of CD45RA, CD3 and CD68 in the heart of Sham BalbC and C57 mice 3 days following operation (SO, n=3). Left panel: A representative Western blotting of three independent experiments is shown. Right panel: Densitometric analysis of Western blot. Data are shown as means ± SEM. *P < 0.05 vs SO BalbC.

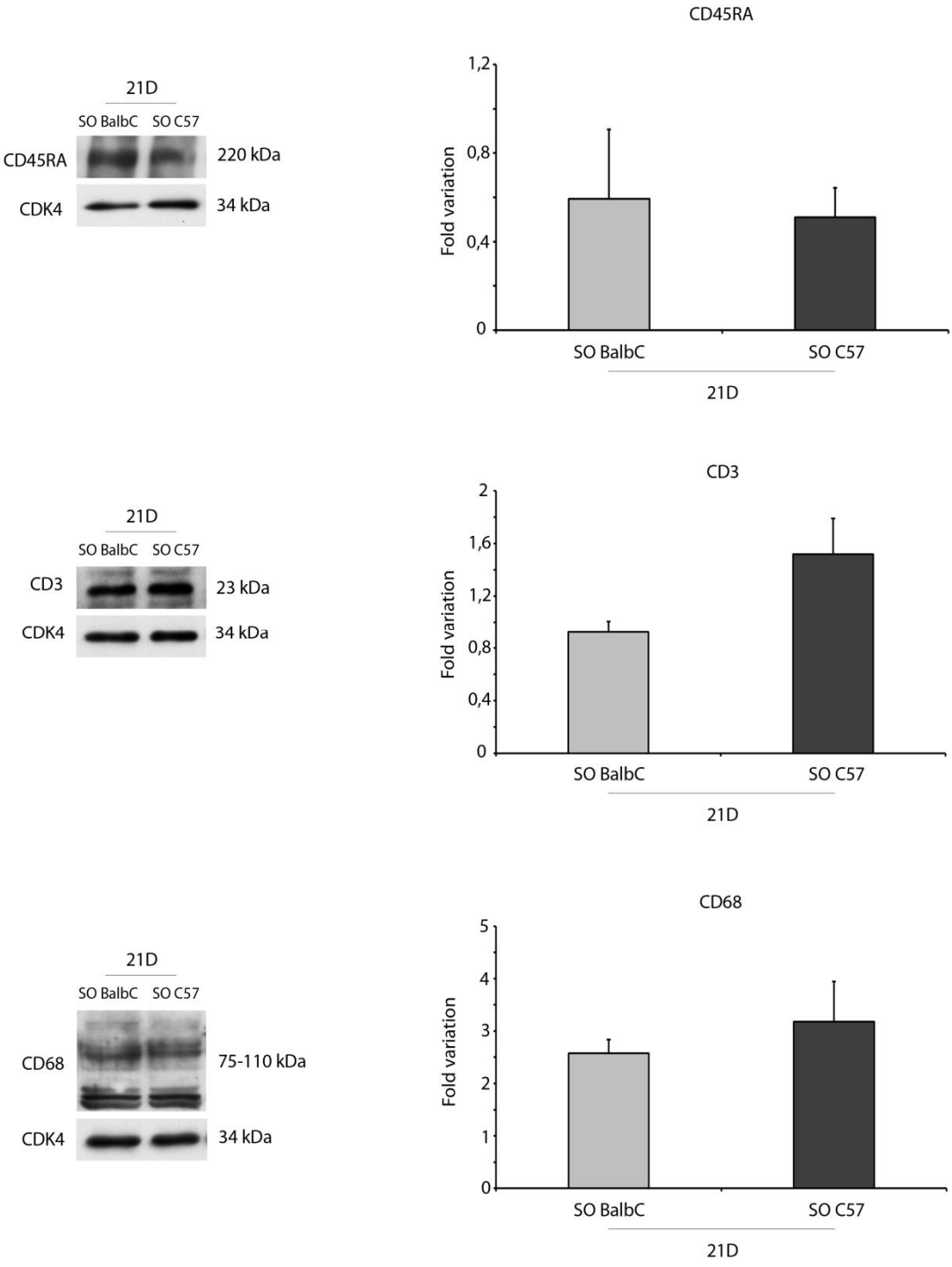

Fig. 12: Western blot analysis showing the expression of CD45RA, CD3 and CD68 in the heart of Sham BalbC and C57 mice 21 days following operation (SO, n=3). Left panel: A representative Western blotting of three independent experiments is shown. Right panel: Densitometric analysis of Western blot. Data are shown as means ± SEM. *P < 0.05 vs SO BalbC.

By western blot, the same results were obtained comparing sham BalbC and C57 BL6 mice both transplantated with cKit^{+}CSCs at all three time points (Fig.13, Fig.14, Fig.15)

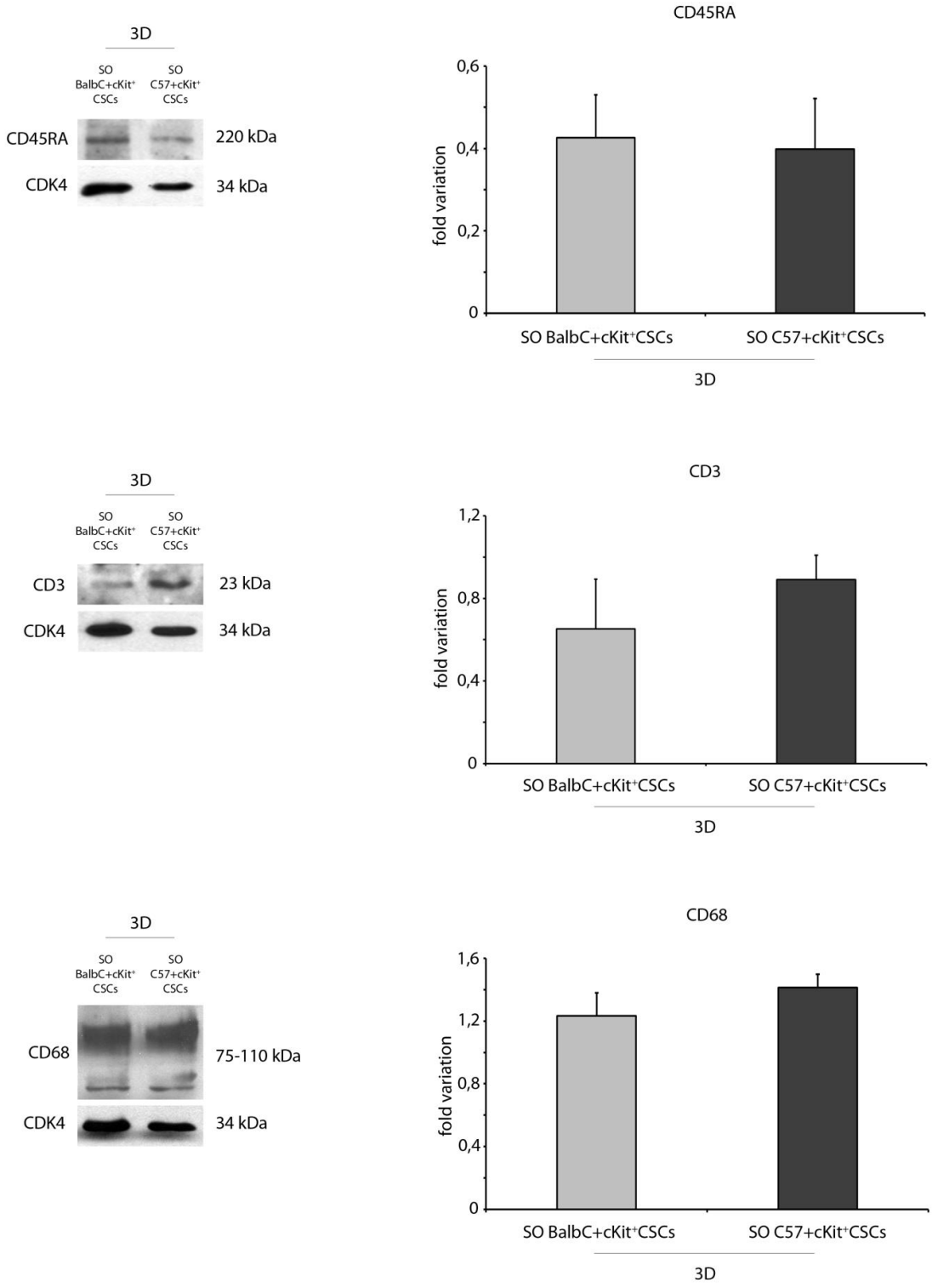

Fig. 13: Western blot analysis showing the expression of CD45RA, CD3 and CD68 in the heart of transplanted Sham BalbC (syngenic) and C57 (allogeneic) mice 3 days following operation (SO+ cKit$^+$ CSCs, n=3). Left panel: A representative Western blotting of three independent experiments is shown. Right panel: Densitometric analysis of Western blot. Data are shown as means ± SEM. *P < 0.05 vs SO BalbC+cKit$^+$ CSCs.

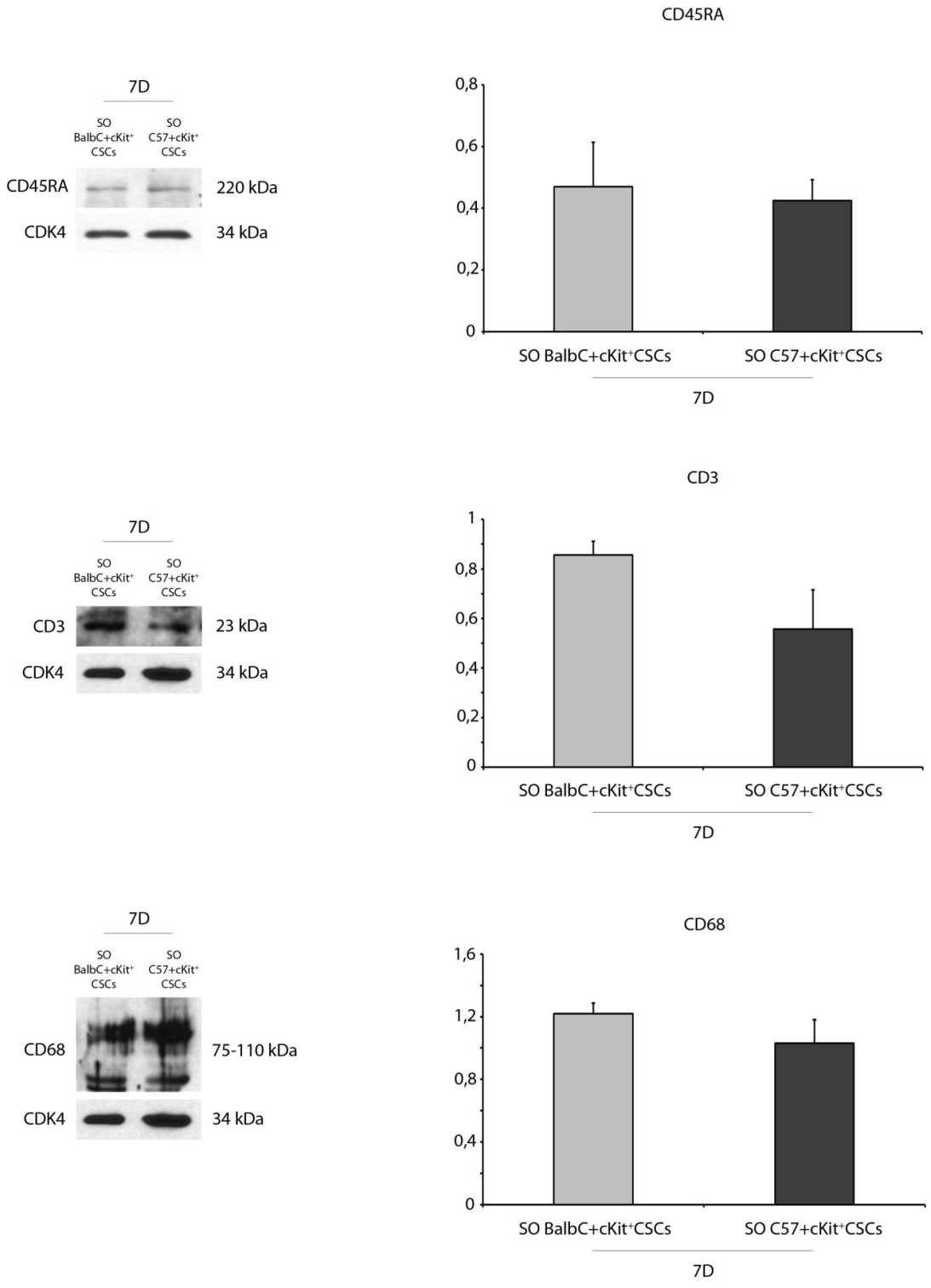

Fig. 14: Western blot analysis showing the expression of CD45RA, CD3 and CD68 in the heart of transplanted Sham BalbC (syngenic) and C57 (allogeneic) mice 7 days following operation (SO+ cKit$^+$CSCs, n=3). Left panel: A representative Western blotting of three independent experiments is shown. Right panel: Densitometric analysis of Western blot. Data are shown as means ± SEM. *P < 0.05 vs SO BalbC+ cKit$^+$CSCs.

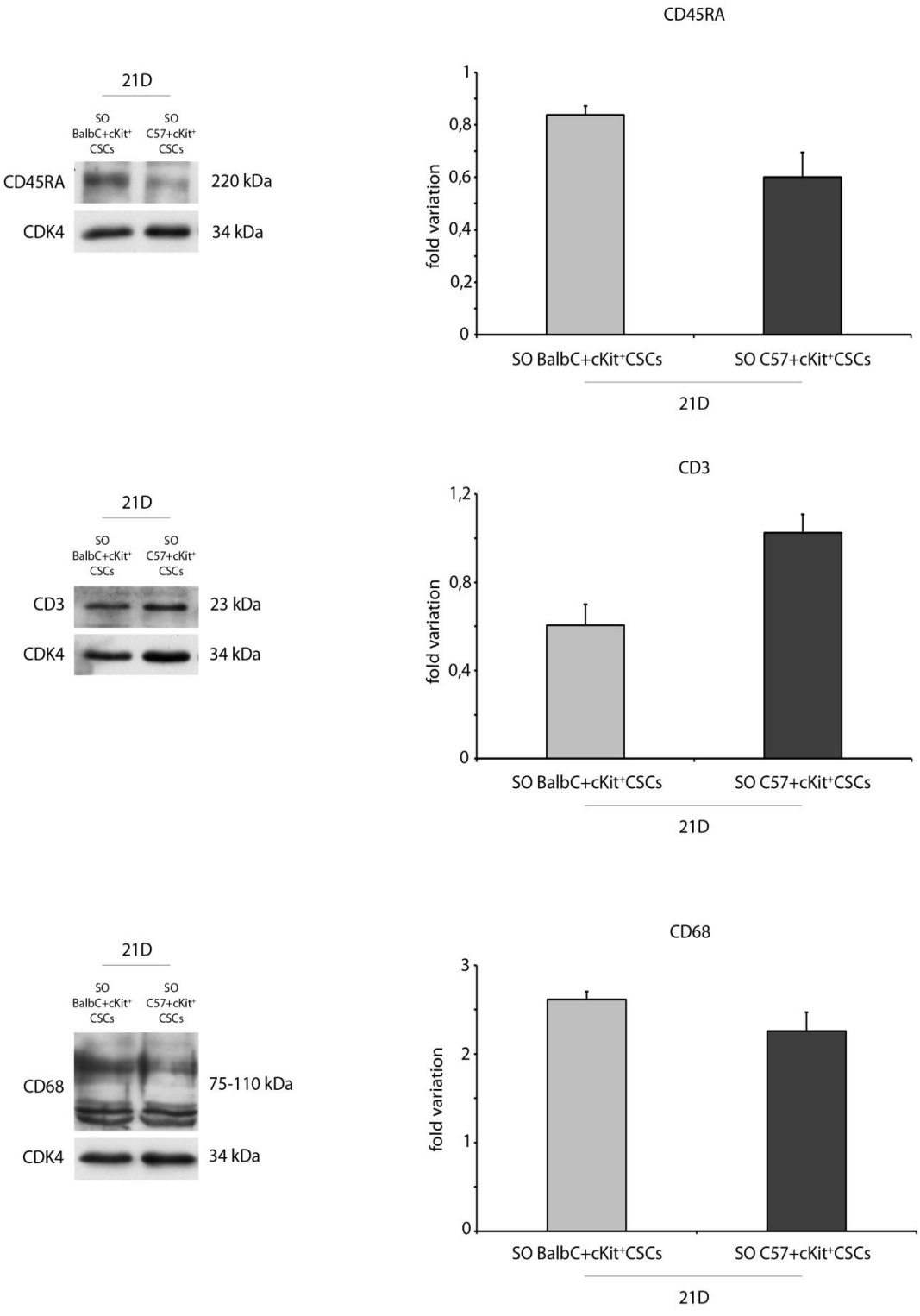

Fig. 15: Western blot analysis showing the expression of CD45RA, CD3 and CD68 in the heart of transplanted Sham BalbC (syngeneic) and C57 (allogeneic) mice 21 days following operation (SO+ cKit$^+$CSCs, n=3). Left panel: A representative Western blotting of three independent experiments is shown. Right panel: Densitometric analysis of Western blot. Data are shown as means ± SEM. *P < 0.05 vs SO BalbC+ cKit$^+$CSCs.

A similar situation was detected in infarcted hearts from the two strains of mice both in the absence and in the presence of transplanted cKit$^+$CSCs (syngeneic transplant versus allogeneic transplant) at all three time points: We did not detect significative differences in the expression levels of the three inflammatory markers between MI BalbC and C57 BL6 mice and between syngeneic and allogeneic transplant (Fig.16, Fig.17, Fig.18, Fig.19, Fig.20, Fig.21).

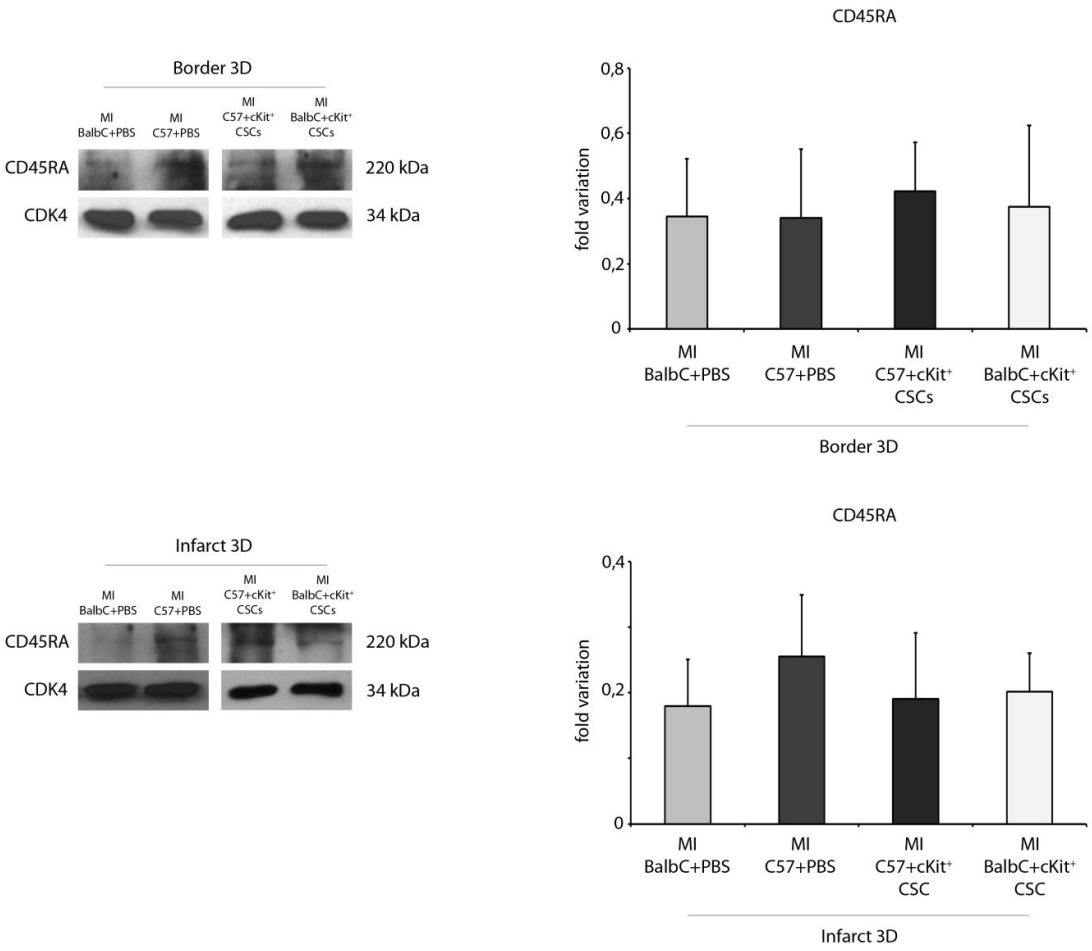

Fig. 16: Western blot analysis showing the expression of CD45RA in the border zone and in the infarcted region of three days infarcted hearts following syngeneic (MI BalbC+cKit$^+$CSCs) and allogeneic (C57+cKit$^+$CSCs) transplantation (MI+cKit$^+$CSCs, n=3). Infarcted hearts treated with PBS were used as control (MI+PBS, n=3). Left panel: A representative Western blotting of two independent experiments is shown. Right panel: Densitometric analysis of Western blot. Data are shown as means ± SEM. *P < 0.05.

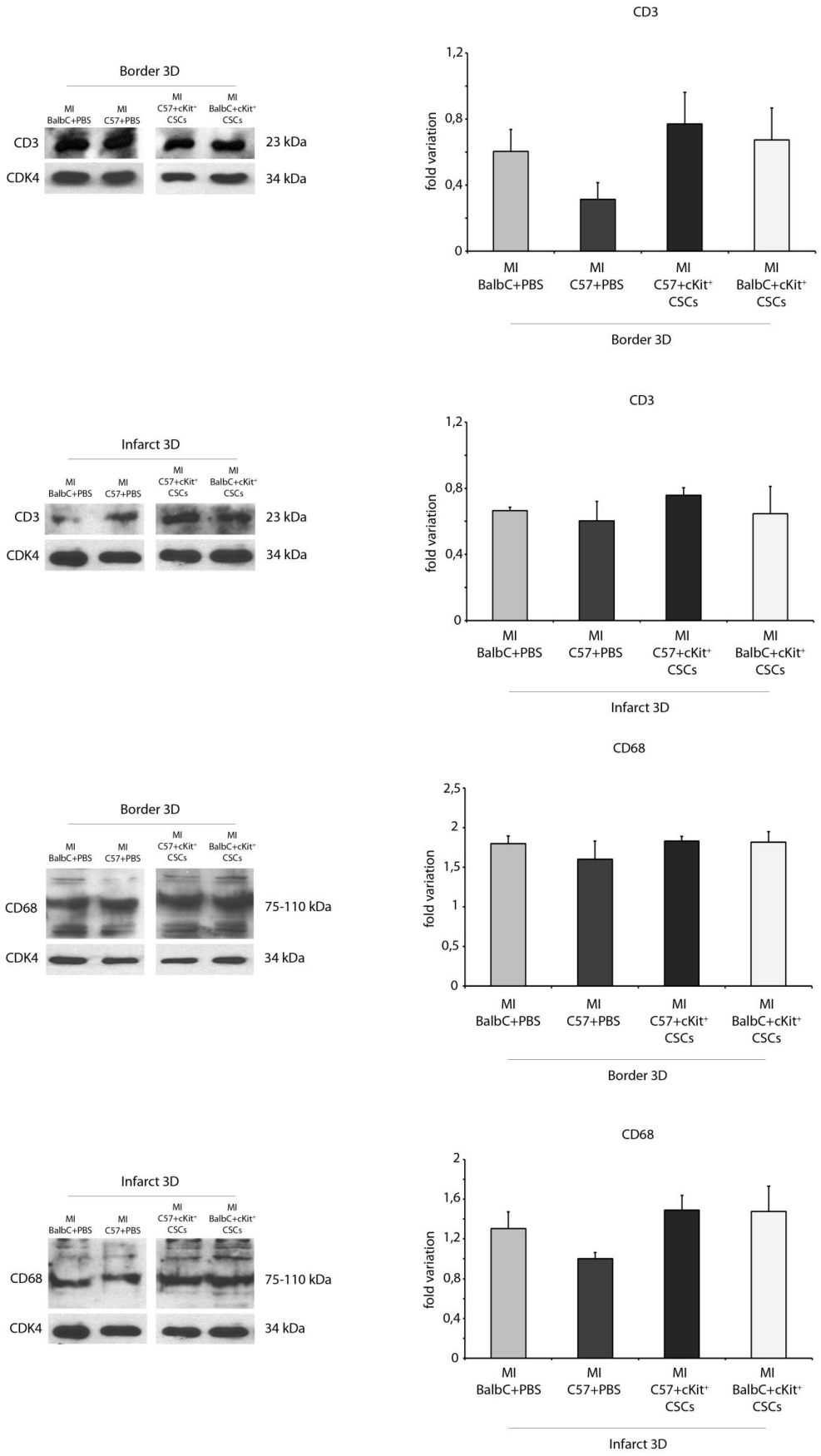

Fig. 17: Western blot analysis showing the expression of CD3 and CD68 in the border zone and in the infarcted region of three days infarcted hearts following syngeneic (MI BalbC+cKit$^+$CSCs) and allogeneic (C57+cKit$^+$CSCs) transplantation (MI+cKit$^+$CSCs, n=3). Infarcted hearts treated with PBS were used as control (MI+PBS, n=3). Left panel: A representative Western blotting of four independent experiments is shown. Right panel: Densitometric analysis of Western blot. Data are shown as means ± SEM. *P < 0.05.

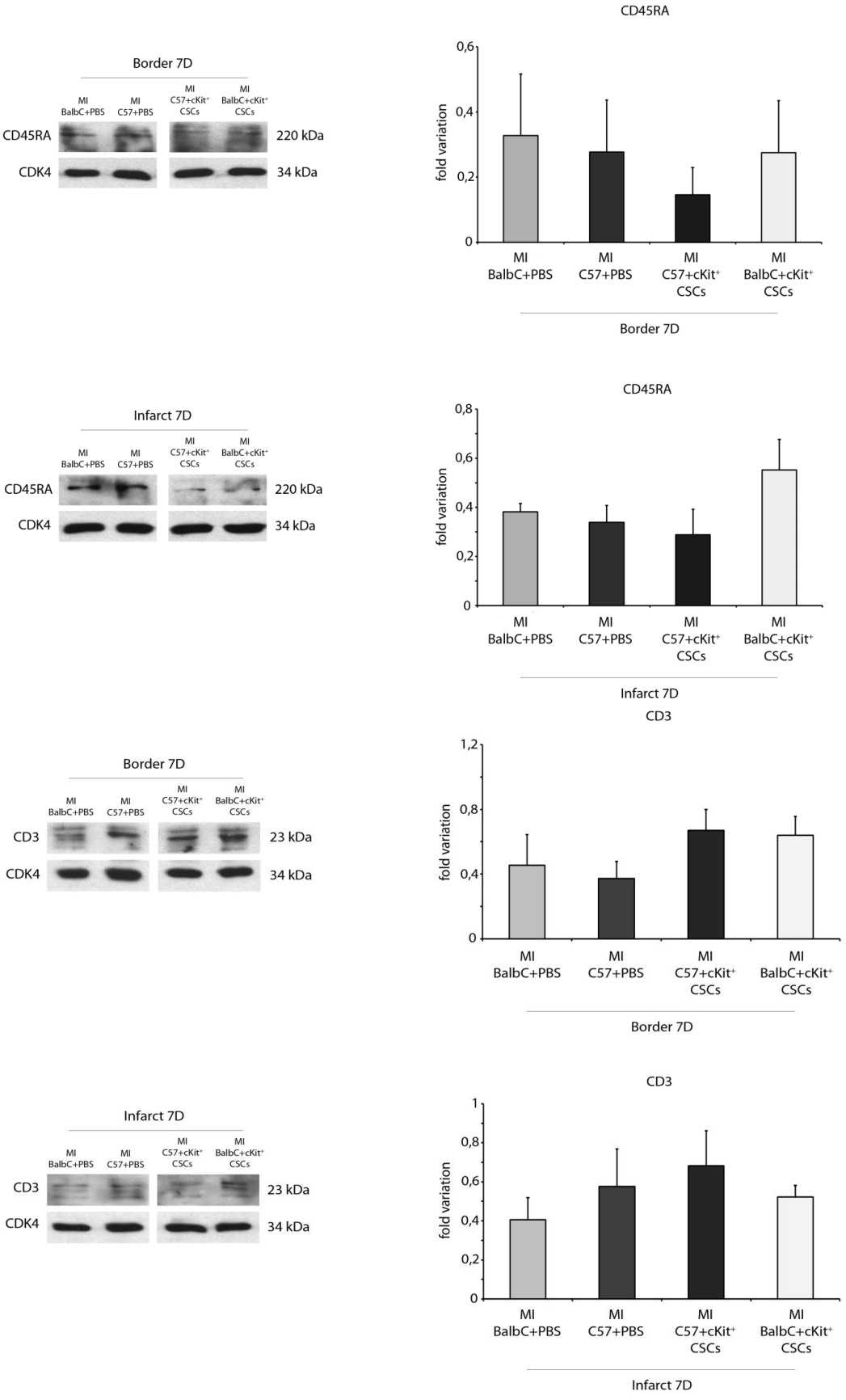

Fig. 18: Western blot analysis showing the expression of CD45RA and CD3 in the border zone and in the infarcted region of seven days infarcted hearts following syngeneic (MI BalbC+cKit$^+$CSCs) and allogeneic (C57+cKit$^+$CSCs) transplantation (MI+cKit$^+$CSCs, n=3). Infarcted hearts treated with PBS were used as control (MI+PBS, n=3). Left panel: A

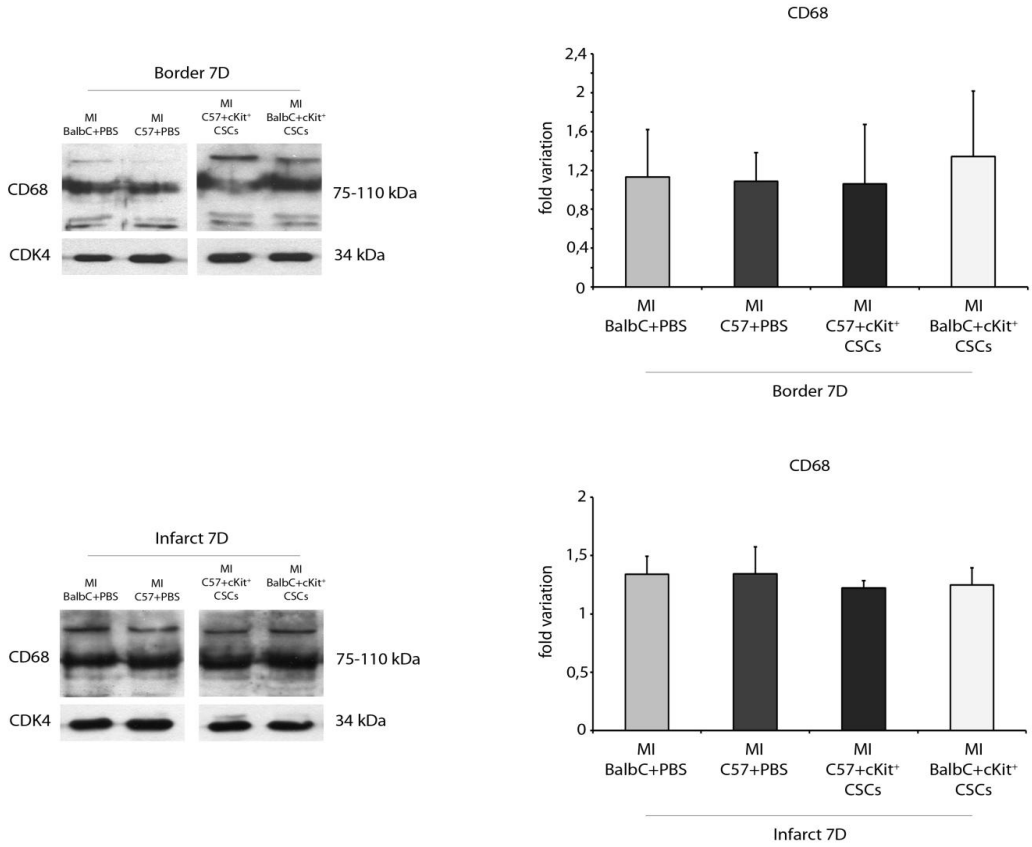

Fig. 19: Western blot analysis showing the expression of CD68 in the border zone and in the infarcted region of seven days infarcted hearts following syngeneic (MI BalbC+cKit$^+$CSCs) and allogeneic (C57+cKit$^+$CSCs) transplantation (MI+cKit$^+$CSCs, n=3). Infarcted hearts treated with PBS were used as control (MI+PBS, n=3). Left panel: A representative Western blotting of two independent experiments is shown. Right panel: Densitometric analysis of Western blot. Data are shown as means ± SEM. *P < 0.05.

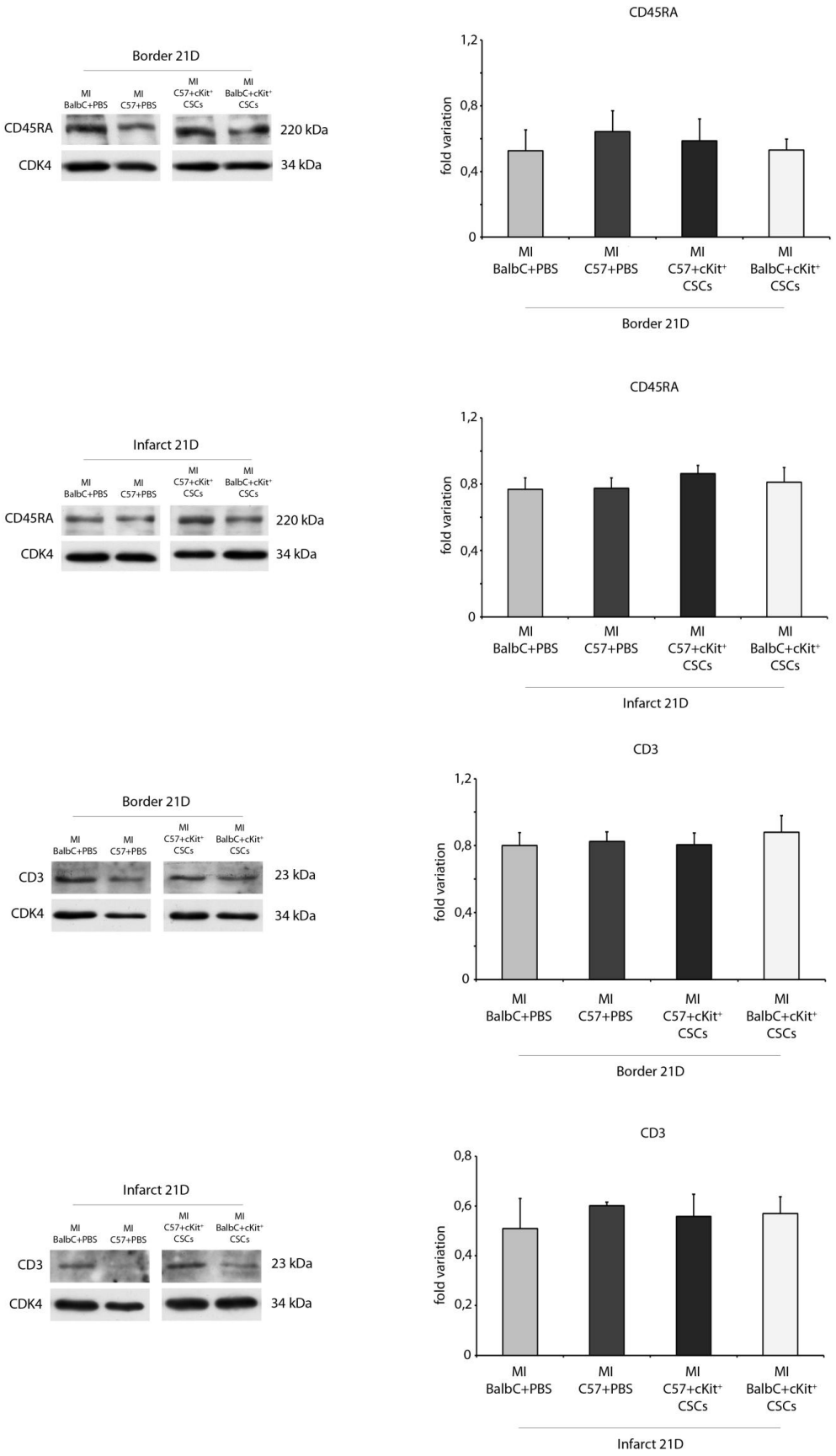

Fig. 20: Western blot analysis showing the expression of CD45RA and CD3 in the border zone and in the infarcted region of twenty one days infarcted hearts following syngeneic (MI BalbC+cKit$^+$CSCs) and allogeneic (C57+cKit$^+$CSCs) transplantation (MI+cKit$^+$CSCs, n=3). Infarcted hearts treated with PBS were used as control (MI+PBS, n=3). Left panel: A representative Western blotting of four independent experiments is shown. Right panel: Densitometric analysis of Western blot. Data are shown as means ± SEM. *P < 0.05.

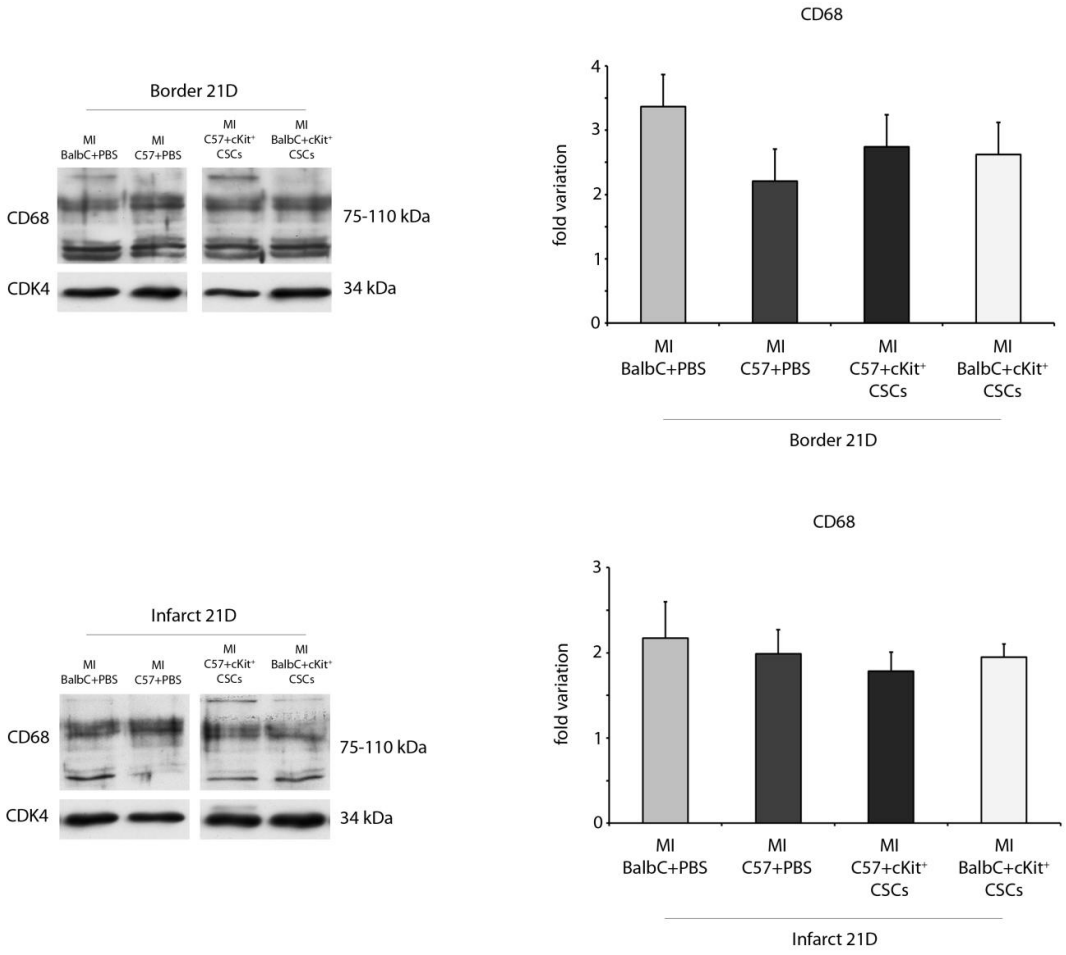

Fig. 21: Western blot analysis showing the expression of CD68 in the border zone and in the infarcted region of twenty one days infarcted hearts following syngeneic (MI BalbC+cKit$^+$ CSCs) and allogeneic (C57+cKit$^+$ CSCs) transplantation (MI+cKit$^+$ CSCs, n=3). Infarcted hearts treated with PBS were used as control (MI+PBS, n=3). Left panel: A representative Western blotting of four independent experiments is shown. Right panel: Densitometric analysis of Western blot. Data are shown as means ± SEM. *P < 0.05.

Immunoflorescence was performed to evaluate the presence of the same three markers on myocardial frozen sections. The number of positive cells/mm^2 for each marker has been considered. Results showed that, in each mouse strain, comparing the number of labeled cells in infarcted and transplanted hearts with the number of labeled cells in infarcted and PBS-treated hearts, we obtained similar increments in syngeneic and allogeneic groups. This lack of difference between the two groups was detected for all the three markers of inflammation at 3 and 21 days following MI and transplantation (Fig.22).

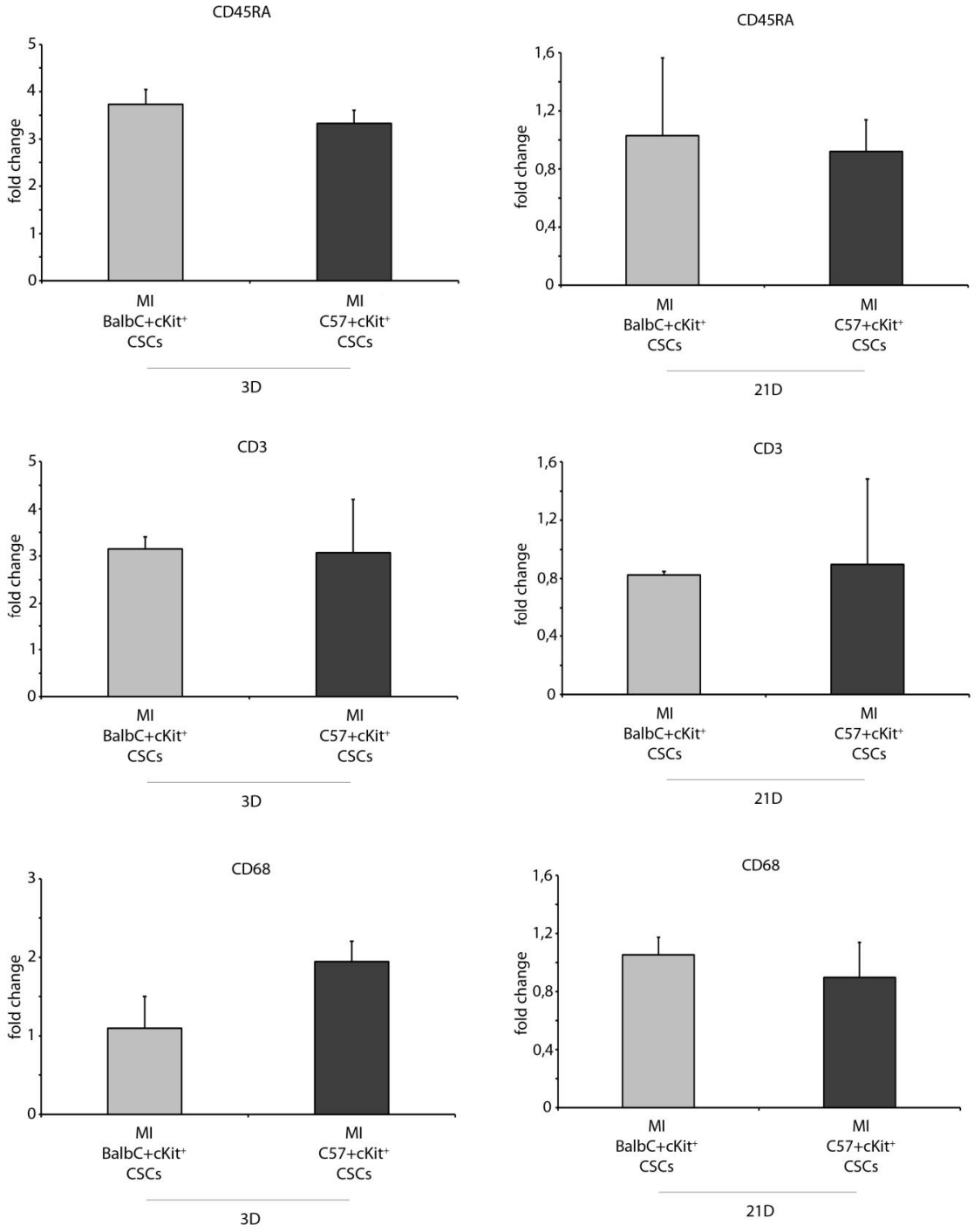

Fig. 22: Immunofluorescence analysis and CD45RA$^+$, CD3$^+$ and CD68$^+$ cell counts in mice infarcted hearts three and twenty one days following syngeneic (MI BalbC+cKit$^+$CSCs) and allogeneic (C57+cKit$^+$CSCs) transplantation (MI+cKit$^+$CSCs, n=3). Values are expressed as fold change compared to the relative control (MI+PBS, n=3). Data are shown as means ± SEM. *P < 0.05.

Therefore, we did not find a different immune response comparing the syngeneic group to the allogeneic group both by WB analysis and by immunofluorescence. Specifically,

the presence of monocytes, lymphocytes and macrophages was similar in the two groups at 3, 7 and 21 days following infarction and transplantation.

6.7. Allogeneic and syngeneic transplantations show comparable levels of pro-inflammatory and anti-inflammatory cytokines in mouse after MI

In our experimental model, 3,7 and 21 days following cKit$^+$CSC treatment, blood was collected in all animals just before sacrifice to determine the levels of circulating cytokines using the LUMINEX Assay. We used a panel (Th1_Th2 panel) for pro-inflammatory cytokines (IFNγ, TNFα, IL-2, IL-12, GMCSF) and anti-inflammatory cytokines (IL-4, IL-5, IL-10) to identify and quantify the immune response mediated by T helper lymphocytes (Th). Furthemore, we also quantified the expression level of the cytokine IL-17A, involved in inflammation and defense mechanisms.

Plasma quantification of these pro- and anti-inflammatory cytokines at 3,7 and 21 days following MI and transplantation, showed that the differences between the syngeneic and allogeneic groups are not statistically significant, thus confirming the data obtained by WB analysis and immunofluorescence (Fig.23 and Fig.24).

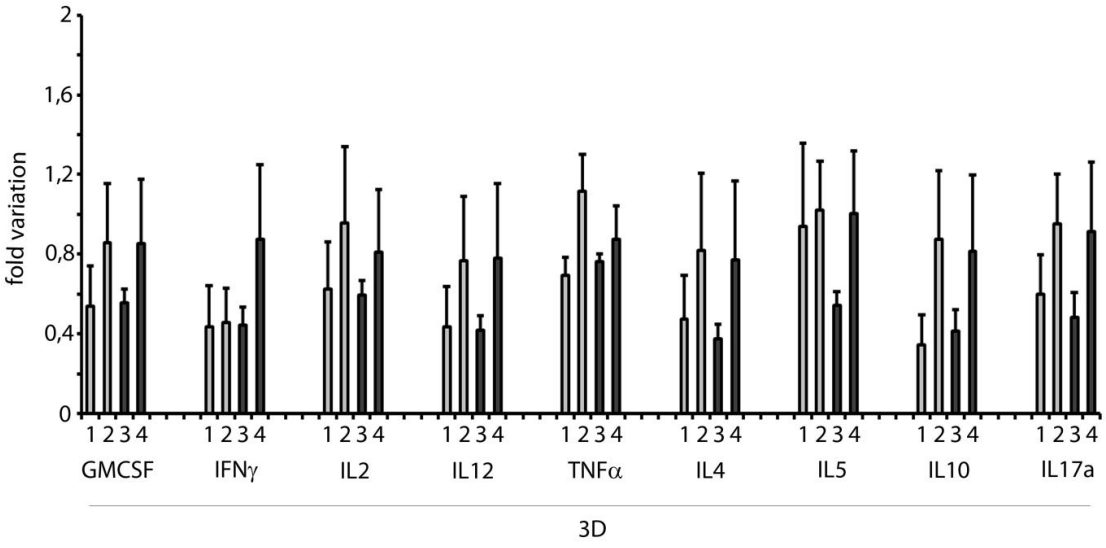

Fig. 23: Circulating levels of pro- and anti-inflammatory cytokines detected by Luminex assay in the plasma of treated mice three days following treatment (n=4/ each group). Data are shown as means ± SEM. *P < 0.05.

1: SO BalbC+cKit$^+$CSCs/SO Balbc
2: SO C57+cKit$^+$CSCs/SO C57
3: MI BalbC+cKit$^+$CSCs/MI BalbC+PBS
4:MI C57+cKit$^+$CSCs/MI C57+PBS

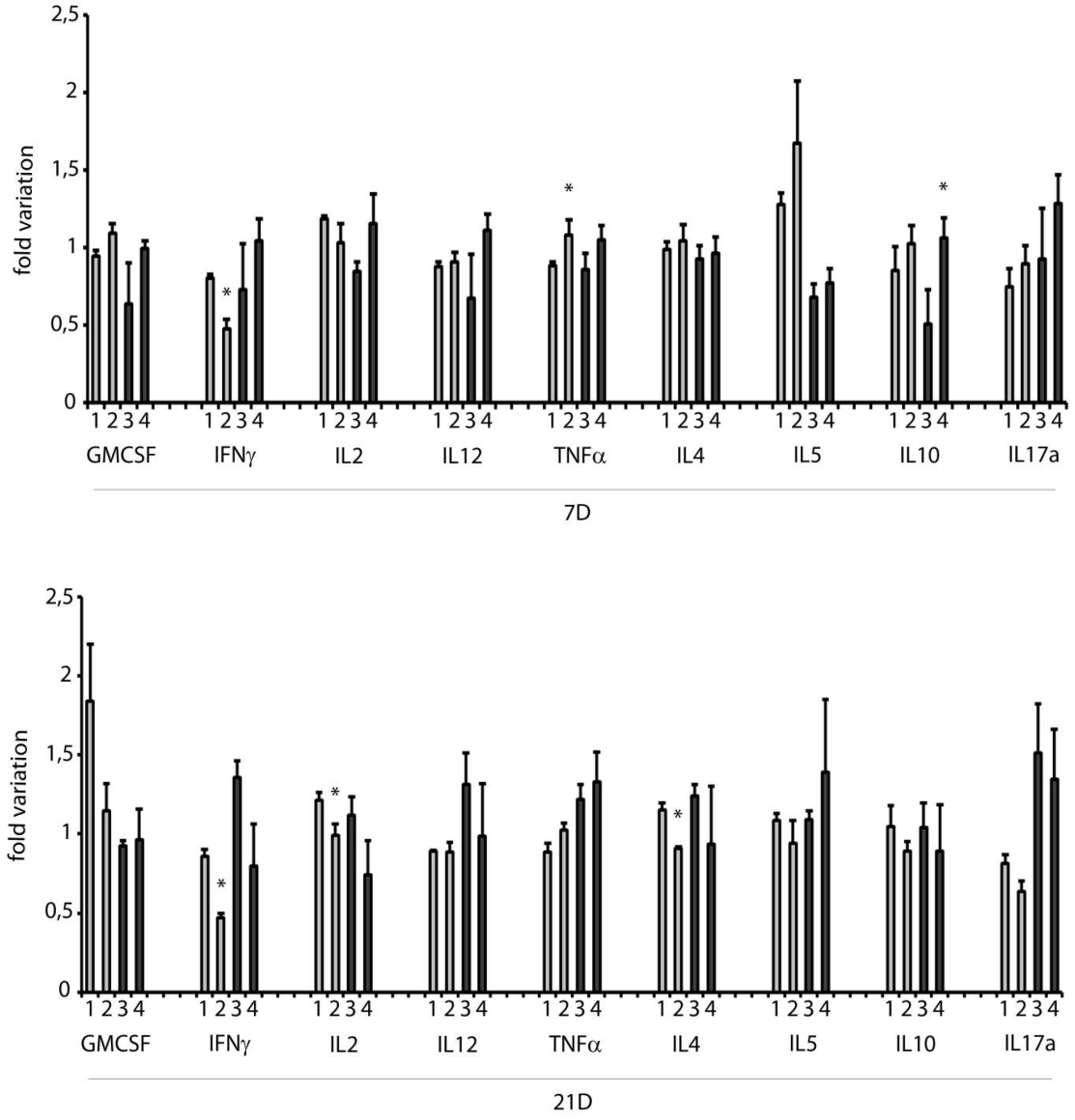

Fig. 24: Circulating levels of pro- and anti-inflammatory cytokines detected by Luminex assay in the plasma of treated mice seven and twenty one days following treatment (n=4/ each group). Data are shown as means ± SEM. *P < 0.05.

1: SO BalbC+cKit$^+$ CSCs/SO Balbc
2: SO C57+cKit$^+$ CSCs/SO C57
3: MI BalbC+cKit$^+$ CSCs/MI BalbC+PBS
4:MI C57+cKit$^+$ CSCs/MI C57+PBS

In conclusion, we demonstrated the feasibility of an allogeneic transplantation using CSCs as demonstrated by comparable expression levels of inflammatory cytokines between the syngeneic and allogeneic groups both in the cardiac tissue, following MI and transplantation, and in the bloodstream.

7. Discussion

In the present study, we have demonstrated that cardiac cKit$^+$CSCs adapt to hypoxia and acquire an inflammatory and reparative phenotype through the expression of HIF-1α and NF-kB target genes. In vivo, following intramyocardial injection of these cells, hypoxia adaptation led to cardioprotection of the infarcted myocardium. Specifically, cKit$^+$CSC transplantation after acute MI lowered apoptosis, induced autophagy and attenuated adverse remodeling by reducing hyperthophy and fibrosis.

Following myocardial injury, necrotic cardiomyocytes release DAMPs that activate innate immune signaling pathways in leukocytes (*Frangogiannis NG et al., 2015*). In particular, DAMPs bind their receptors, i.e. RAGE, P2X7R (*Tafani M et al., 2011*), Toll-like receptors (*Sandholm J et al., 2014*), NOD-like receptors (*Jones HD e al., 2014*), and activate NF-kB that leads to the expression of genes that are involved in the protection and repair of the injured tissue, known as IRR. Therefore, leukocytes play a critical role in regulating the inflammatory and reparative response that follows MI. In our *in vitro* model, we found that cardiac cKit$^+$CSCs can activate a gene response similar to that present in activated leukocytes. Accordingly, hypoxic conditions induced in our cells the activation of HIF-1α allowing them to adapt to the reduced oxygen level. Hypoxia-mediated activation of HIF-1α determined the transcription of genes encoding angiogenic factors (*Semenza GL, 2014*). Indeed, we detected an increased mRNA expression of VEGF and EPO in cardiac cKit$^+$CSCs following 6, 24 and 48 hours of hypoxia. Another important target gene of HIF-1α is represented by the glucose transporter GLUT1. The switch from oxidative to glycolytic metabolism represents one of the most important intracellular adaptation to hypoxia mediated by HIF-1α (*Iyer NV et al., 1998*). In a recent study, it has been demonstrated that *in vitro* treatment of bone marrow-derived angiogenic cells (BMDACs) with an inducer of HIF resulted in a metabolic reprogramming of these cells as reflected by the increased expression levels of glucose transporters, metabolic enzymes and pH regulators (*Rey S et al., 2011*). These alterations in metabolism allowed the survival of pretreated BMDACs in the ischemic hindlimb. In our *in vitro* experiments, we found that HIF-1α induction in hypoxic cardiac cKit$^+$CSCs determined higher expression of the glucose transporter GLUT1 indicative of a potential switch from oxidative to glycolytic metabolism that may promote their survival in the infarcted heart. Several recent reports suggest that metabolic and cell survival pathways are closely related (*Roberts DJ et al., 2014*). HK2 regulates glucose metabolism by glucose phosphorylation. Nevertheless, it is increasingly recognized that it functions also as a protective signaling molecule. Specifically, in the heart, cardiac-specific HK2 overexpression prevents maladaptive hypertrophy by decreasing ROS accumulation (*McCommis KS et al., 2013*). Interestingly, hypoxia enhanced protein expression levels of HK2 in our cells.

The receptors for DAMPs were firstly localized in tissue resident leukocytes (*Iwasaki A and Medzhitov R, 2004; Bianchi ME, 2007*), but have been also described in many other cells and tissues. In particular, under stress conditions, these receptors can be induced and expressed *de novo* in resident or recruited stem cells and pluripotent undifferentiated progenitors from many tissues (*Tafani M et al., 2011; Ryan BJ et al., 2014; Reuter S et al., 2010*). For instance, mesenchymal stem cells (MSCs) express TLR (*DelaRosa O et l., 2010; Feng Y et al., 2011; Faure E et al., 2000*) and upon their stimulation, paracrine factors are released and may boost or reduce their immunosuppressive capacities (*van den Akker F et al., 2013*). In the setting of I/R injury, two different studies suggested that TLR2 presence and activation on injected MSCs could be essential for myocardial recovery (*Abarbanell AM et al., 2010*) while TLR4 activation would have deleterious effects (*Wang Y et al., 2010*). Nevertheless, it should be noted that since both studies used a I/R injury in an isolated rat heart model, no immune system was present and therefore the effect of TLR2 and TLR4 activation on immunomodulation by MSC remains unclear. In our experimental setting, we found that TLR2 and TLR4 were both activated by hypoxia in cKit$^+$CSCs at 24 and 48h. On the other end, RAGE, another important DAMPs receptor, showed a significant mRNA up-regulation mainly after 6h of hypoxia and increased expression of protein levels at 24 and 48h. Interestingly, in a prior study, we have already demonstrated that cardiac cKit$^+$ cells express RAGE and their regenerative responses, elicited by intramyocardial injection of HMGB1 following acute MI, are mediated, at least in part, by the interaction HMGB1-RAGE (*Limana et al., 2013*). Adipose-derived stem cells, bone marrow stromal cells (*Forostyak O et al., 2016; Li W et al., 2015*) and MSCs (*Peng H et al., 2016*) express the purinergic P2X7R and its activation is involved in their migration and differentiation. Additionally, it has been also demonstrated a key role for P2X7R as an essential pro-survival signal in embryonic stem cells (*Thompson BA et al., 2012*). In a model *ex vivo* of rat heart, agonists of this receptor are responsible for the release of cardioprotectants induced by ischemic pre- and post-conditioning (*Vessey DA et al., 2011*). Our findings have shown a significant up-regulation of P2X7R mRNA in cKit$^+$CSCs 48h following hypoxia. The precise role of the up-regulation of all these receptors in our hypoxic cells is not clear but, as already mentioned at the beginning if this section, it should be noted that following MI, necrotic cardiomyocytes release DAMPs that, through the activation of these receptors in leukocytes, trigger a specific gene expression NF-kB-dependent, responsible for the IRR (*Tafani M et al., 2011*).

The mRNA expression levels of the inducible enzymes COX2 and NOS2 were up-regulated by hypoxia at all three time points and the increased COX2 mRNA was

associated with an increase in COX2 protein expression following 24h of hypoxia. Interestingly, Dr. Bolli's group, using a molecular genetic approach and a model of MI, has demonstrated that the cardioprotection afforded by NOS2 gene therapy is mediated by COX2 upregulation via NF-kB activation (*Li Q et al., 2007*). In particular, upregulation of COX2 determines an increase in myocardial production of two cytoprotective prostanoids (*Bolli R et al., 2002*).

The long pentraxin PTX3 is expressed in the heart under inflammatory conditions and has a cardioprotective role in acute myocardial infarction in mice (*Salio M et al., 2008*). Specifically, PTX3-deficient mice showed larger infarcts compared to wild type mice and the use of recombinant PTX3 reduced heart damage and inflammation. More recently, it has been demonstrated that PTX3 deficiency is associated with increased atherosclerosis, macrophage accumulation and inflammation in the atherosclerotic lesions suggesting a modulation by PTX3 of the vascular associated inflammatory response (*Norata GD et al., 2009*). Together these observations support the possibility that PTX3 may exert a cardiovascular protective effect through the modulation of the immunoinflammatory balance. Our results showed that PTX3 expression is influenced by hypoxia only at the earliest time point following hypoxia.

Finally, we detected a significant up-regulation of the receptor for CXC chemokines CXCR4 in hypoxic cKit$^+$CSCs after 48h of hypoxia. Hypoxic preconditioning of cKit$^+$ cardiac progenitor cells improves their survival following MI (*Yan F et al., 2012*) by inducing CXCR4 expression. Further, CXCR4 overexpression in MSCs attenuates cardiac remodeling after MI and this cardioprotective effect is mediated by the release of the antifibrotic enzyme MMP9 (*Huang W et al., 2012*). It is noteworthy that in our *in vitro* experiments, we detected a significant increase in the mRNA expression of MMP9 and a significant decrease in the mRNA expression of the MMP inhibitor TIMP4 in hypoxic cKit$^+$CSCs compared to normoxic cells.

The activation of NF-kB mounts a regenerative response by inducing genes that encode growth factors with the potential to mediate different cardioprotective effects. Our cells secreted high levels of IGF-1 and HGF under hypoxia conditions. These secreted paracrine factors have been demonstrated to exert beneficial effects on cell survival and cardiac remodeling. For instance, myocardial injury induced by cardiotoxin injection in transgenic mice with cardiac-restricted mIGF-1 expression determined restoration of cardiac function. This effect was in part mediated by modulation of the inflammatory response. Specifically, mRNA IL-10, a potent anti-inflammatory cytokine, was found to

rapidly increase in transgenic hearts 24 hours after cardiac injury and this increase was even higher at 1 week (*Santini MP et al., 2008*). It should be noted that IL-10 has been showed to attenuate left ventricular remodelling after MI by reducing inflammation-mediated fibrosis (*Krishnamurthy P et al., 2009*). Most importantly, transplantation of bone marrow mononuclear cells in infarcted mouse hearts led to the secretion of significant amounts of IL-10 that were associated to decreased reactive hypertrophy and myocardial collagen deposition. These effects resulted in a significant improvement in cardiac function (*Burchfield JS et al., 2008*). Interestingly, in our *in vivo* model, we detected higher levels of circulating IL-10 in mice at 3 days following cKit^{+}CSC transplantation compared to controls. In the same mice, at 3 days but also at later time points, we also found decreased levels of circulating TGF β1 and, at 3 weeks, a reduced expression of collagen I in the border zone of treated infarcted hearts. Reduction in LV remodeling was also supported by a decrease in hypertrophy in transplanted infarcted hearts compared to controls as evidenced by down modulation of the β-MHC/α-MHC ratio and up-regulation of CSRP3. Expression of α- and β-myosin heavy chain (MHC), the two functionally distinct cardiac MHC isoforms is species-dependent and tightly controlled by developmental and hormonal factors (*Everett AW et al., 1984; Allen DL et al., 2001*). Relative expression levels of these isoforms can be altered in disease states such as cardiac failure or hypertrophy (*Nadal-Ginard B et al., 1989*). β-MHC is characterized by lower adenosine triphosphatase activity and lower filament sliding velocity, but can generate cross-bridge force with a higher economy of energy consumption than α-MHC (*Holubarsch C et al., 1985; Sugiura S et al., 1998*). This suggest that a shift from α- to β-MHC might be an adaptive response in order to preserve energy. On the other hand, depressed contractile function can promote disease progression (*Kiriazis H et al., 2000; Fatkin D et al., 2000*). Therefore, it is conceivable that the decrease in contractile function due to increased β-MHC might outweigh the benefits of improved economy and ultimately dictate clinical outcome. MLP, the protein encoded by CSRP3, belongs to the LIM-only domain family, a large protein family with diverse functional roles, including transcriptional regulation, cell fate determination, cell adhesion and motility, cytoskeleton organization and signal transduction (*Schmeichel KL and Beckerle MC, 1997; Kadrmas JL and Beckerle MC, 2004; Zheng Q and Zhao Y, 2007*). Its diverse functional roles have significant impact on cardiac and skeletal muscle physiology and pathology. At the nucleus level, CSRP3 serves as a positive regulator of myogenesis, promoting myogenic differentiation (*Arber S et al., 1994*). Accordingly, various studies have demonstrated that CSRP3 overexpression results in enhanced myotube differentiation (*Arber S et al., 1994; Kong Y et al., 1997; Vafiadaki E et al., 2014*).

IL-10 negatively affects cardiac remodelling also by attenuating apoptosis (*Krishnamurthy P et al., 2010*). These data are in accordance with our results that showed the induction of autophagy and the attenuation of apoptosis in infarcted hearts three days after cKit$^+$CSC transplantation. Importantly, an antiapoptotic effect on cardiomyocytes is also exerted by HGF (*Deuse T et al., 2009*) and transplantation of MSCs overexpressing HGF in a mouse model of MI was associated with less cardiomyocyte apoptosis (*Zhao L et al., 2016*).

Growing evidences suggest that stem cell therapy following MI could improve cardiac function not by differentiation into cardiomyocytes and vascular cells but most likely by the production of paracrine factors which have beneficial effects (*Hodgkinson CP et al., 2016*). In recent years, the immune system represents one of the most studied system that could be influenced by these paracrine factors. In particular, it has been demonstrated by several reports that stem cell therapy could activate a subset of reparative macrophages (*Weirather J et al., 2014*). For instance, transplantation of MSCs into the infarcted heart contribute to the recovery of cardiac function, in part, by switching macrophages from a pro-inflammatory to an anti-inflammatory and reparative phenotype through secreted factors such as IGF-1 and IL-10 (*Dayan V et al., 2011; van den Akker F et al., 2013; Ben-Mordechai T et al., 2013; de Couto G et al., 2015*) Thus, a possible scenario is that cKit$^+$CSCs, following transplantation in a hypoxic environment as the infarcted heart, exert cardioprotective effects by modulating the activation of macrophages via a paracrine mechanism. Indeed, in a very recent paper, it has been postulated that cardiac cKit$^+$ progenitors originate from a cardiac myeloid lineage, i.e. macrophage progenitor cells (*Leinonen JV et al., 2016*) suggesting the potential of cKit$^+$ progenitors to display functional characteristics of macrophages.

Immunogenicity is the recent concern of stem cell therapies whether using embryonic or adult, or autologous or allogeneic stem cells (*Charron D et al., 2009; Dhodapkar KM et al., 2010; Zhao T et al., 2011; Charron D et al., 2012*). An exogenous cell delivered to a host is expected to encounter some form of host resistance. Recognition of foreign antigen initiates an immune response that involves the activation and proliferation of specific immune cells. The MHC disparities are by far the most formidable immunological barrier to transplantation (*Erlich HA et al., 2001).* Cardiosphere-derived cells (CDCs) were the first therapeutic modality to demonstrate a reduction in scar tissue associated with an increase in the presence of viable, functional tissue in the randomized, placebo controlled CADUCEUS trial (*Makkar RR et al., 2012; Malliaras K et al., 2014*). The intracoronary (IC) administration of autologous CDCs also improved regional function of infarcted myocardium with an excellent safety profile (*Makkar RR et al., 2012; Malliaras K et al., 2014*). However, widespread applicability of autologous

CDC therapy, as for others cardiac-derived stem cells, is challenged by the need for patient-specific tissue harvesting with myocardial biopsy, cell processing and quality control resulting in delays to therapy and imposing significant logistic and economic constraints (*Malliaras K et al., 2014*). In addition, cell potency has also been reported to be affected by age and presence of comorbidities (*Zhuo Y et al., 2010*). Autologous CSCs can be induced to proliferate and differentiate *ex vivo* before transplanting them into the damaged heart. However, several limitations exist in the isolation and expansion of this pool of cells in short periods and in quantities that can be employed therapeutically. Thus, the use of an allogeneic CSC approach would be of primary clinical relevance. The use of allogeneic stem cells, if proven safe and effective, has the potential to overcome the limitations of autologous cardiac stem cell therapy with the ability to provide a ready to use, "off-the-shelf" product for widespread clinical usage. The safety and feasibility has been already demonstrated for CDCs in rat (*Malliaras K et al., 2012; Makkar RR et al., 2012; Malliaras K et al., 2014*). In a "proof-of-concept" study of allogeneic CDCs in a rat MI model, allogeneic CDC transplantation without immunosuppression was noted to be safe and resulted in cardiac regeneration and improvement in cardiac function (*Malliaras K et al., 2012*). Similarly, administration of allogeneic cardiospheres in a rat model resulted in increased viable myocardium, decreased scar, and improved cardiac function with attenuation of adverse remodeling (*Tseliou E et al., 2013*). The cardioprotective effects of allogeneic CDCs and cardiospheres were durable for at least 6 months, although allogeneic cells disappeared within 4 weeks (*Malliaras K et al., 2012; Tseliou E et al., 2013*). Allogeneic MSCs delivered IM during left ventricular assist device implantation in patients with severe heart failure were well-tolerated with a trend towards improvement in wearing parameters (*Ascheim DD et al., 2014*). The ALLSTAR study is the first randomized, double-blind, placebo-controlled trial to determine the safety and efficacy of intramyocardial delivery of allogeneic CDCs in patients with ischemic left ventricular dysfunction following MI. The results from the ALLSTAR Phase 1 trial demonstrated that IC infusion of allogeneic cardiosphere-derived cells was safe and feasible (*Chakravarty T et al., 2016*). We performed allogeneic cKit^{+}CSC transplantation in our mouse MI model to evaluate immune response to transplant. We analysed, by WB and immunofluorescence, the expression of three markers of primary and secondary inflammation, respectively CD45RA to identify B monocytes, CD3 for lymphocytes and CD68 for macrophages. After 3, 7 and 21 days, we detected comparable levels of immune cells infiltration between allogeneic and syngeneic transplantation. Moreover, we detected comparable circulating levels of pro- and anti-inflammatory cytokines. Our results showed safety and feasibility of an allogeneic transplantation using cKit^{+}CSCs for MI treatment in a MI mouse pre-clinical model. Moreover, our results agree with others clinical studies, which have used different stem cells as MSCs, for cardiac repair (*Hare*

JM et al., 2009; Hare JM et al., 2012; Ascheim DD et al., 2014; Perin EC et al., 2015). However, if allogeneic cell therapy were proven to be safe and effective, it would open up a new paradigm in cellular therapeutics. Large scale expansion of cells derived from allogeneic tissues could be performed in specialized manufacturing labs under strict quality control. In the case of heart-derived cells, attractive sources of allogeneic tissue include hearts explanted from organ donors but not used for transplantation and surgical myocardial discards. Highly standardized, 'off-the-shelf' allogeneic cellular products would subsequently be shipped to hospitals throughout the world and banked for future use, thus enabling broad adoption and timely application of cell therapy in a cost-efficient manner.

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