Stem Cell Interactions with Murine Heart following Ischaemia-Reperfusion Injury

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Abstract

Haematopoietic stem cell (HSC) migration to injured cardiac tissue has shown to aid in cardiac tissue repair after acute myocardial infarction. Hesitation towards clinical trials however remain; due to the poor understanding of molecular mechanisms by which they are recruited to injured cardiac tissue. In this study the adhesive properties of HSCs to ischemia-reperfused (IR) cardiac tissue was investigated *in vitro*. Following injury, adhesion of HSCs was significantly increased to cardiac tissue compared to sham controls. H_2O_2 pre-treatment of HSCs did not enhance adhesion to both sham and injured cardiac tissue *in vitro*. Blockade of CD31, CD44 and CD49d did not significantly attenuate HSC adhesion *in vitro*. A change in adhesive properties of cardiac tissue after remote organ injury led to significantly increased adhesion of neutrophils *in vitro* to mice with liver injury induced by concanavalin A (ConA). This is the first study to show enhanced adhesion of neutrophils and HSCs to murine cardiac tissue *in vitro* following IR injury. If the mechanisms by which HSCs adhere to injured cardiac tissue can be identified and enhanced, it can help optimize the use of cellular therapy to reduce cardiac tissue damage after acute MI.

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Contents

CHAPTER 1:

INTRODUCTION

(1) Introduction

1.1 Myocardial Infarction

Acute myocardial infarction (MI) has become increasingly prevalent and is a prominent cause of mortality and morbidity in developed countries. Coronary heart disease (CHD) is known to contribute to 12.8% of fatalities worldwide and remains a high cause of acute MIs (Hausenloy & Yellon, 2013). They are the leading cause of death in the US and one of the highest in the UK (Frank *et al,* 2012). The British Heart Foundation (BHF) report that there are 103,000 acute MIs each year in the UK, with 14.1% fatality rates within 30 days of patients being admitted into hospitals (Townsend *et al*, 2012). Some clinical treatment and management strategies such as ensuring early reperfusion have led to a decline in the rate of mortality but the burden of this disease still remains a problem (Werns & Lucchesi, 1987; Boersma *et al*, 2003; White & Chew, 2008).

Acute MI, otherwise known as a 'heart attack', occurs as a result of cardiac ischemia and injury is characterised by necrosis of myocardial tissue. Ischemia is governed by reduced blood flow to tissue. When blood flow is impaired in scenarios such as acute MIs, an injury is established (Buja, 2005). Levels of MI related cell death are dependent on the severity of myocardial ischemia and the length of time it is established for. The most susceptible area of heart tissue following ischemia is the subendothelium and as the duration of occlusion increases, damage of ischemia progresses towards the subepicardial layer (Braunwald & Kloner, 1985). After 15-20 minutes of coronary flow occlusion the damage to myocardium becomes irreversible (Orlic *et al*, 2002). The World

Heart Federation (WHF) formed a universal definition in 2012 for diagnosis and prognosis of MIs in a clinical setting (Thygesen *et al*, 2012).

1.2 Ischemia Reperfusion

The restoration of blood flow is known as ischemia reperfusion (IR). Obstructing blood flow results in a depletion of oxygen to the tissue and leaves it in a state of hypoxia. The subendothelium struggles to maintain its correct metabolism because of this and impairs the function of the heart (Buja, 2005). The heart is continually stimulated, even through injury and struggles to contract. This causes acidosis and cellular swelling resulting in ischemia (Buja, 2005).

The initiation of early reperfusion can reverse this ischemic damage (Werns & Lucchesi, 1987; Verma *et al*, 2002). Ischemia can arise from spontaneous occlusion of a vessel, as seen with acute MIs, or during organ transplantation in a clinical practice and vessel clamping; all have oxygen limiting factors (Dorweiler *et al*, 2007). Reperfusion is the restoration of this blood flow to the injury site to prevent entire functional tissue loss.

1.2.1 Myocardial Ischemia Reperfusion Injury

Injury to myocardial tissue through acute MI however, is not only governed by prolonged ischemia but also reperfusion dependant damage; known as ischemia reperfusion injury (IRI) (Verma *et al*, 2002). Reperfusion is essential to salvage as much viable tissue as possible. Initially, reinstating blood flow restores regular cellular respiration and metabolism of the cells (Hausenloy & Yellon, 2013). However, extensive studies have shown that late reperfusion can result in cardiomyocyte death and increased tissue injury (Jennings *et al*, 1960; Braunwald & Kloner, 1985).

In terms of damaging steps to myocardial IRI, hypoxia initially leads to metabolic changes within the cell and causes oxidative stress (Hausenloy & Yellon, 2013). Under hypoxic conditions: synthesis of ATP is compromised leading to changes in pH, lactic acid accumulation, calcium (Ca^{2+}) overload, mitochondrial dysfunction and the accumulation of hypoxanthine. The intracellular increase of Ca^{2+} ions in turn increases the conversion of xanthine dehydrogenase to xanthine oxidase. This results in an increase of reactive oxygen species (ROS) shown in *figure 1.1*. ROS cause oxidative stress and are found to be considerably increased in myocardial tissue undergoing reperfusion; having a detrimental effect on vascular endothelial cells (Dhalla *et al*, 2000).

IR leads to the incomplete reduction of oxygen. This generates extremely reactive superoxide anion (O_2^-) (Taniyama & Griendling, 2003), hydrogen peroxide (H_2O_2) and reactive hydroxyl radical (•OH). O_2^- and •OH are free radicals that cause damage to proteins and cellular integrity. H_2O_2 is not a free radical but has been known to have a longer half-life (Slezak *et al*, 1995). It can however spontaneously react with O_2^- to form oxygen and •OH (Zweier *et al*, 2006). In times of stress, cells usually have their own line of defence with antioxidants known as free radical scavenging enzymes (Zweier *et al*, 2006); a method in which to protect themselves. In an IRI however, antioxidant levels become depleted and thus cellular damage is far greater (Dorweiler *et al*, 2007).

Cells of the microvascular region are extremely prone to IR damage where endothelial cells in particular may undergo further cellular necrosis after reperfusion. Vascular endothelial cells line the inside of coronary blood vessels and are predisposed to damage following blood flow change (Verma *et al*¸ 2002). This damage may be caused by both

hypoxic conditions and the reintroduction of oxygen through reperfusion. To further exacerbate injury, the endothelium releases a number of mediators which limit blood flow through vasoconstriction. The increase in ROS and the release of endothelin-1 by the impaired endothelial cells causes constriction of blood vessels (Verma *et al*, 2002). This was described by Kloner *et al* (1974), as the 'no-reflow' phenomenon; they discovered extensive damage to capillaries and myocardial cell swelling after ischemia reperfusion, which all contributes to the limited microvasculature blood flow at the injury site (Kroner *et al,* 1974; Ku, 1982).

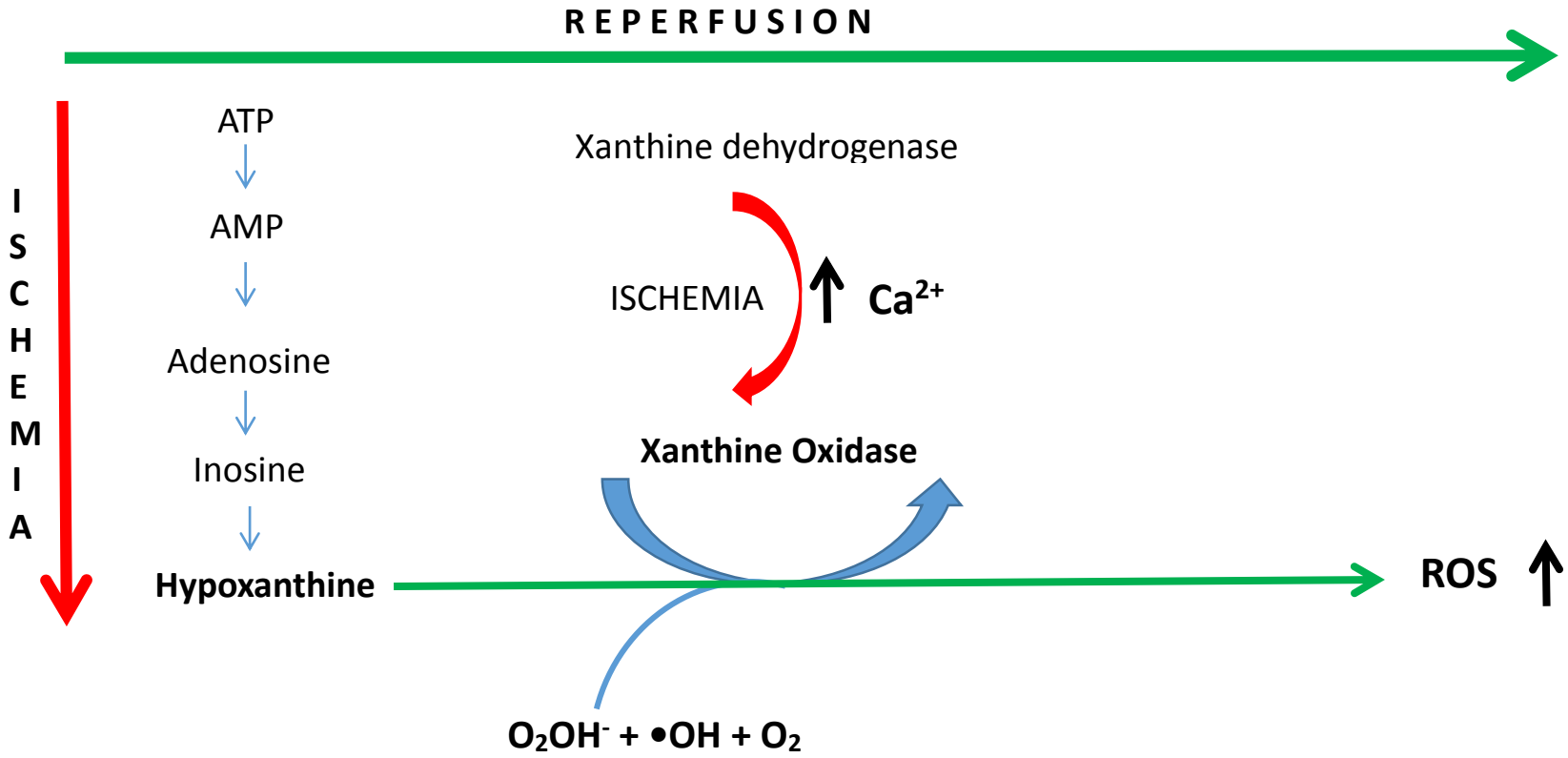

Figure 1.1 *Generation of reactive oxygen species in ischemia reperfusion injury.* During ischemia there is a depletion of ATP as well as defective ATP resynthesis. This leads to the accumulation of hypoxanthine. Xanthine oxidase becomes activated through a conformal change of xanthine dehydrogenase due to intracellular Ca^{2+} increase. All of these changes promote ROS generation (Dorweiler *et al,* 2007).

1.2.2 Myocardial Ischemia Reperfusion injury and Inflammation

Damage by IRI to endothelial cells causes a release of proinflammatory mediators such as cytokines for the initiation of inflammation. Endothelial cell adhesion molecules (CAM) also become upregulated through inflammation (Granger *et al*, 2010). Inflammation is a biological process that plays a vital role in innate immunity and arises as a result of a damaging stimulus within vascular tissue (Diegelmann & Evans, 2004).

There is a continuous - if not exaggerated - release of these mediators throughout reperfusion (Eltzschig & Collard, 2004) and reperfusion of injured tissue independently activate the innate immune response (Diegelmann & Evans, 2004). This results in the recruitment of polymorphonuclear neutrophils (PMN) to the site of injury; penetrating the already delicate endothelial lining of myocardial tissue (Taniyama & Griedling, 2003). This inflammatory response furthers the upregulation of ROS and other pro-inflammatory mediators contributing to microvascular plugging and endothelial dysfunction (Moens *et al*, 2005). Previous studies have shown that a reduction in proinflammatory mediators is correlated with a reduction in myocardial related as a result of IR injury; subsequently displaying how inflammation is a major component of IRI (Frangogiannis *et al*, 2002).

1.2.3 Pro-inflammatory Mediators

Potent proinflammatory mediators are generated throughout IR injury. The complement cascade plays a vital role within the innate immune response and becomes activated throughout reperfusion; resulting in the production of anaphylotoxins.

Specific to IR: C3a, C5a, iC3b and C5b-9 (membrane attack complex: MAC) are all up regulated (Frangogiannis *et al,* 2002; Moens *et al*, 2005). Complement contributes to injury through its chemotactic influences and also significantly increase cell permeability; inducing lysis of vulnerable cells (Moens *et al,* 2005). C5a is seen to be the most influential mediator and increases PMN recruitment. The production and activation of other proinflammatory cytokines such as TNF-α, IL-6 along with many more can be increased with C5a (Eltzschig & Collard, 2004; Frangogiannis *et al*, 2002).

1.2.4 Neutrophil Mediated Stress

PMN recruitment during reperfusion have an undesirable effect on already vulnerable endothelial tissue. Peripheral neutrophils in the blood, generally only interact with endothelium at times of inflammation. Reperfusion associated ROS increases chemokine release which acts to amplify PMN recruitment and to upregulate adhesive mediators on the endothelial cell surface. IL-8 a chemokine that is amplified within myocardial IRI plays a key role in this process (Entman *et al,* 1991; Frangogiannis *et al*, 2002).

Neutrophils once at the site of injury release degrading capable proteases such as collagenase, which will encourage the breakdown of cellular integrity with specific catalytic reactions. Local ROS generation is increased with the arrival of neutrophils, and ROS cells have a direct apoptotic effect on myocardial cells (Johansen, 2004). Autacoid release triggered by neutrophil activation are responsible for: vasoconstriction of blood vessels, activation of the endothelium and further neutrophil recruitment and activation. Above all, adhesion molecules, shown in *figure. 1.2.* are up regulated at

reperfusion to ensure specific binding of neutrophils to endothelium; a role also played by ROS (Johansen, 2004).

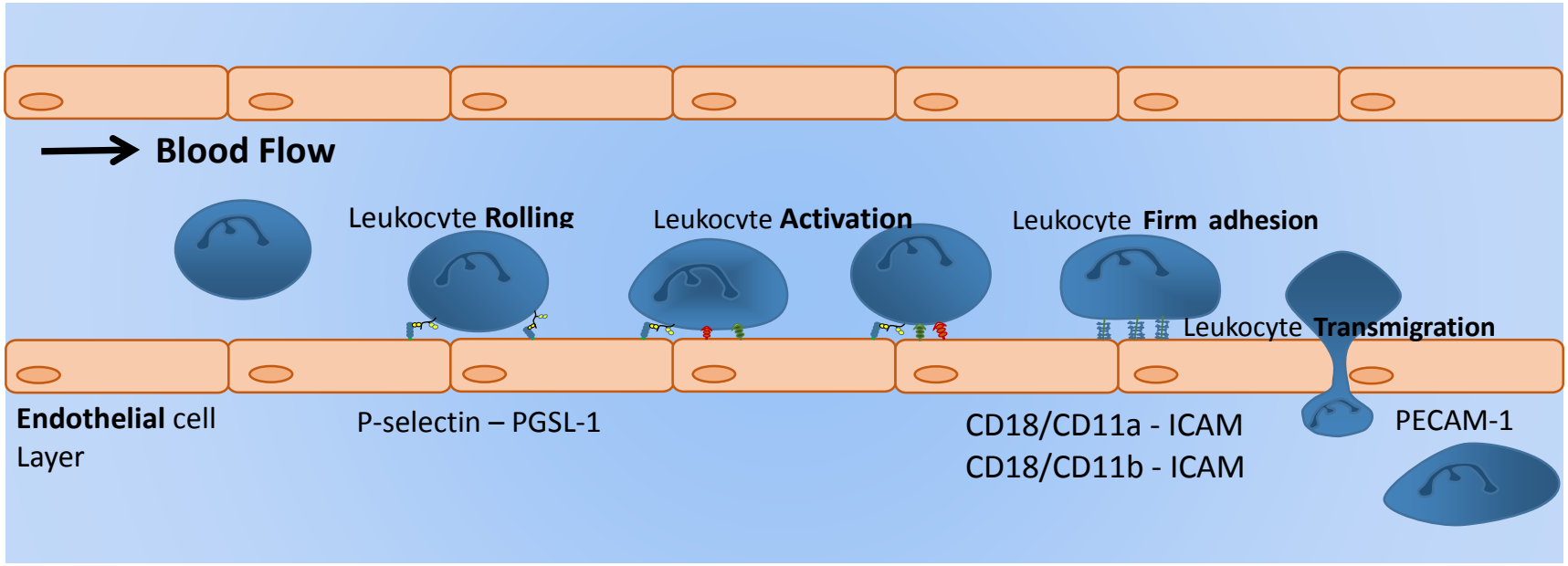

Figure. 1.2 *Neutrophil adhesion cascade*. The cascade is governed by 4 steps: rolling, activation firm adhesion and finally migration. P-selectin expressed on the endothelium and its counter receptor; P-selectin glycoprotein ligand-1 (PSGL-1) expressed on neutrophils interact at a low infinity to form a loose interaction, resulting in a 'rolling' process. Chemoattractants are increased at times of inflammation and attract leukocytes to this site. CD11a/CD18 alongside CD11b/CD18 integrins on the leukocytes form firm adhesions with intracellular adhesion molecule 1 (ICAM-1) expressed on the endothelium. PECAM-1 allows for transmigration of neutrophils across the endothelial layer (Jordan *et al*, 1999).

1.3 Clinical Manifestations

One of the first clinical problems that arise due to myocardial IRI is reperfusion-induced arrhythmias. Reperfusion-induced arrhythmias are dependent on the length of time ischemia; as little as 15-20 minutes in a clinical setting, and immediate reperfusion (Eltzschig & Collard, 2004). The heart may undergo arrhythmias; most commonly surgically related ventricular fibrillation and ventricular tachycardia (Manning & Hearse, 1984; Moens *et al,* 2005; Hausenloy & Yellon, 2013). Rapid ion concentration change has been known to play a key role in the likelihood of arrhythmias (Eltzschig & Collard, 2004) and the clinical repercussions have varied from reversible arrhythmias and potentially lethal arrhythmias (Moens *et al,* 2005; Hausenloy & Yellon, 2013).

Myocardial stunning is a very prominent clinical manifestation and dependant on reperfusion (Moens *et al*, 2005). It refers to the continuous dysfunction of intracellular metabolic reactions of myocardial tissue after reperfusion; independent of ischemic damage. This is where the heart continues to undergo anaerobic respiration; with reduction of ATP resynthesis and ROS generated cell damage through reperfusion (Eltzschig & Collard, 2004). There is reduced contractility of the heart through stunning; but again this is a reversible manifestation (Eltzschig & Collard, 2004; Moens *et al*, 2005).

Irreversible cell death will occur after 20-30 minutes occlusion of a coronary artery (Entman *et al*, 1991). There are two irreversible forms of myocardial injury through reperfusion (Hausenloy & Yellon, 2013). The first of which is microvascular

plugging of capillaries and the second; known as lethal myocardial reperfusion injury. Plugging limits reperfusion to some areas of cardiac tissue resulting in necrosis and apoptosis of these cells. The biochemical mechanisms described in *figure. 1* alongside an increased inflammatory response all contribute to an accelerated myocardial cell death (Hausenloy & Yellon, 2013).

1.4 Remote Organ Injury

Remote organ injury is a phenomena that occurs after local reperfusion injury in one organ has a knock on effect upon other vital organs of the body (Kyriakides *et al*, 2000). This can on occasions result in multiple organ dysfunction syndrome (MODS) and one major organ effected is the heart (Carden & Granger, 2000). A direct injury to myocardium can be described as remote organ injury (Carden & Granger, 2000).

During reperfusion, when leukocyte trafficking and homing is increased locally to injury site, there may be a generation of circulating chemoattractants at remote organs (Kyriakides *et al*, 2000). Chemoattractants such as proinflammatory mediators play a key role in the recruitment of neutrophils to an injury and increase injury through reperfusion (Jordan *et* al 1999; Kyriakides *et al*, 2000). Kyriakides *et al,* (2000) have shown the importance of selectins and complement components and the damage caused by their activation at remote organ sites (Kyriakides *et al*, 2000). Heart function in a previous study has been shown to be compromised following renal IRI; with cardiac cells undergoing apoptosis as a result (Kelly, 2003). TNF-α and IL-1 release at times of inflammation from renal

reperfusion can increase ICAM-1 expression in heart tissue; which eventually lead to left ventricular damage and increase vasoconstriction (Kelly, 2003).

1.5 Clinical Treatment

The most effective procedure to limit acute myocardial ischemia is through rapid reperfusion of tissue; as already described, late reperfusion can have harmful outcomes. There are some clinical strategies in place to combat particular clinical manifestations that result from a myocardial Ischemic injury (Hausenloy & Yellon, 2013).

For management of stunned myocardium, inotropic stimulation therapies help to restore sufficient contractility of the heart (Verma *et al*, 2002). Previous inotropic stimulation studies have shown that intravenously delivered inotropes such as dopamine can aid in contractility of the heart for up to 3 hours; without increasing demand for oxygen (Arnold *et al,* 1985). This can reduce chances of left ventricular failure; a clinical manifestation not uncommon through myocardial IRI. This treatment does not increase infarct size or reduce myocardial tissue function (Arnold *et al*, 1985; Verma *et al*, 2002).

Antioxidant administration methods have been used to combat the readily depleting levels of antioxidants at times of ROS generation. Endogenous antioxidants and their activation pathways have been known to diminish through reperfusion when oxidative stress is at its peak. Experimental data using antioxidants such as Vitamin E and glutathione peroxidase administration have shown that these antioxidants may have beneficial effects in particular models of myocardial IRI, thus reducing its damaging effects (Dhalla *et al,* 2000; Verma *et al*, 2002; Marczin *et al*, 2003).

Preconditioning is a relatively emerging idea; this is where hearts undertake short periods of repeated ischemia and reperfusion before a longer period of ischemia was first applied; this was first considered by Murry *et al* in 1986 (Zhao *et al*, 2003). Preconditioning has shown a reduction in infarct size in comparison to non-preconditioned tissue. The dilemma comes from the lack of opportunity for preconditioning at times of spontaneous occlusion of a heart vessel leading to acute MI. Recently postconditioning with a similar approach has shown to equally reduce infarct size with little difference between both methods (Zhao *et al*, 2003). Again, postconditioning has been shown to be most effective in the first few minutes of reperfusion; which is difficult for acute MIs and therefore clinical practice (Kin *et al*, 2004).

Many studies over the years have shown the effects of anti-neutrophil therapies in order to reduce infarct size. Non-steroid anti-inflammatory drug therapies have been used to reduce the synthesis of neutrophil adhesion molecules; but their effectiveness to reduce infarct size remains low (Johansen, 2004). To reduce neutrophil activation at injury sites, the release of proinflammatory mediators such as tumour necrosis factor alpha (TNF-α) can be blocked (Werns & Lucchesi, 1987; Eltzchig & Collard, 2004). Monoclonal antibody use against adhesion molecules such as: PSGL-1, ICAM-1, PECAM-1 and integrin CD11 have all contributed to the attenuation of activated neutrophils at myocardial IRI sites (Frangogiannis *et al,* 2002). Although these reports have always indicated that infarct size is reduced; clinically there is little evidence of these effects (Frangogiannis *et al,* 2002). Even with a reduction in neutrophil activation at injury site, clinical repercussions

of myocardial IRIs have not ceased to exist; as myocardial stunning still occurs as well as arrhythmias and endothelial dysfunction (Baxter, 2002; Jordan *et al*, 1999).

1.6 Stem Cells for Regenerative Therapy

Treatments for MIs have been limited therefore the therapeutic capabilities of stem cells (SC) have become a novel and recent strategy to tackle the burden of MI. The two vital characteristics of SCs are the ability to differentiate into other cell lineages, and to produce an identical daughter cell, thus a capacity for self-renewal (Morrison *et* al, 1997; Kondo *et al*, 2003). Depending from where they are derived determines their level of plasticity as progenitor cells. Embryonic SCs are pluripotent and give rise to all cells of the 3 germ layers: mesoderm, ectoderm and endoderm (Thomson *et al*, 1998). The ethical issues for the use of embryonic SCs requires an alternative approach in cellular therapy.

This can be provided through the use of multipotent cells present in adult tissue that have a lesser plasticity in comparison. These cells act as a reservoir for the body in times or tissue renewal or repair. In particular, bone marrow (BM) is home to primary immature cell populations that have shown their ability for regeneration and restorative capabilities of injured tissues (Yin & Li, 2006). Mesenchymal stem cells (MSC) and haematopoietic stem cells (HSC) are two well documented bone marrow derived stem cells (BMSC) and have previously been used for myocardial regeneration studies and other organ injuries (Orlic *et al*, 2002; Kavanagh *et al*, 2010). SC therapy therefore provides an exciting opportunity to find ways to treat acute MIs and myocardial IRI (Wollert & Drexler, 2005).

1.6.1 Haematopoietic Stem Cell

1.6.1.1 Haematopoiesis and HSC Source

HSCs were first identified by Till and McCulloch in 1963 and at the time identified as colony forming units (CFU). HSCs give rise to two different groups/classes of blood cells: lymphoid and myeloid cells. Once they have taken a path of differentiation they begin to express lineage commitment markers and lose their ability to self-re-renew (Cheshier *et al*, 1999).

Once matured, all blood cells deriving from the BM have a limited lifespan; therefore to maintain an adequate number of cells for haematopoiesis, it is vital that there is a naturally occurring renewal of HSCs throughout adult life (Gunsilius *et al*, 2001). Studies with irradiated mice have shown to develop haematopoietic cell colonies in the spleen, from a single progenitor cell, showing their renewal capabilities (Becker *et al*, 1963).

1.6.1.2 HSC Identification

Specific cell surface proteins known as lineage markers can be used to identify HSCs when isolated. Markers for HSCs were first identified by Spangrude *et al* (1988), where they determined the purest form of haematopoietic progenitors isolated from the BM. Thy-1^{lo}, Lin^- (Lineage-negative) and Sca-1^+ expression markers were shown to be cells that give rise to all other blood cell types (Sprangrude *et al*, 1988). Later c-kit^+ expression alongside Lin^- and Sca-1^+ (LSK) expressed markers have become the universally accepted form of identification of HSCs (Ogawa *et al*, 1991; Yokota *et al*, 2012). Human hematopoietic progenitors present $CD34^+$ antigens as a marker (Berenson *et al*, 1988)

whereas murine models vary in expression of CD34, making specific identification markers different for humans and murine models (Okuno *et al*, 2002).

1.6.1.3 HSC Mobilisation & Trafficking

HSCs require an appropriate SC niche to survive. Within the BM, the microenvironment provided for by adult stromal cells is ideal for them to self-renew, proliferate when needed and then transmigrate (Yin & Li, 2006). As BM provides a pool of renewing SCs throughout adult life, it raises the question about why HSCs are also found regularly in peripheral blood. Trafficking of HSCs out of the BM allows them to find other sites of SC niches; in order to occupy as many BM microenvironments that are available (Méndez-Ferrer & Frenette, 2007). This can be seen as a defence mechanism for if ever there has been damage or trauma to one site of SC pool; ensuring that there are adequate sites from which haematopoiesis can still occur. The circulating HSCs continuously look for empty spaces that are ideal for proliferation (Méndez-Ferrer & Frenette, 2007).

Granulocyte colony stimulating factor (G-CSF) has been used as means to traffic HSCs out of the BM in numerous studies (Molineux *et al*, 1990; Méndez-Ferrer & Frenette, 2007). Other proteolytic enzymes such as metalloproteinase-9 (MMP-9) have also been seen to promote mobilisation (Méndez-Ferrer & Frenette, 2007). Orlic *et al's* (2001) use of G-CSF to decrease myocardial infarct size has reported improved functioning of the heart after injury; indicating the importance of increased mobilisation and trafficking of HSCs from the BM (Orlic *et al*, 2001b).

1.6.1.4 HSC Homing & Recruitment

Endothelial cells in the BM express E-selectin, P-selectin and Vascular Cell Adhesion Molecule-1 (VCAM-1) and play a vital role in the homing of HSCs to BM niches. Similar to neutrophil rolling; P-selectin interacts with PSGL-1 on HSCs for loose adherence and facilitate rolling, the primary step in the leukocyte adhesion cascade (Frenette *et al*, 1998).

Areas of the endothelium in BM that are rich in CXCL12 (also known as SDF-1); a chemokine, provides an important interaction for homing of HSCS (Méndez-Ferrer & Frenette, 2007). This chemokine is upregulated in hypoxic conditions which means BM is rich in CXCL12 due to its relatively low levels of oxygen (Ceradini *et al*, 2004). CXCL12 interacts with the G-coupled protein receptor CXCR4 on HSCs (Méndez-Ferrer & Frenette, 2007).

Homing mechanisms to a number of injured sites remain unclear, although the mechanisms underlying HSC recruitment in a small number of tissues is understood (Kavanagh *et al*, 2010; Kavanagh *et al*, 2013). As seen in the BM, hypoxic conditions cause high expression of CXCL12; in injured tissue there is increased hypoxia which may contribute to the recruitment of HSCs within these regions (Hattori *et al*, 2001; Caradini *et al,* 2004; Méndez-Ferrer & Frenette, 2007). In response to liver injury, there have been reports of a CXCL12 concentration gradient development between the BM and the liver resulting in increased recruitment of HSCs to the liver (Dalakas *et al*, 2005).

1.6.1.5 HSCs & Myocardial Ischemia Repair

There has been a lot of conflicting data amongst studies using Lin^-, c-kit^+ cells from the BM and the role they have at the site of cardiac ischemia to reduce infarct size. Orlic *et*

al published work in 2001 using Lin-/c-kit+ cells in an effort to reduce infarct size after myocardial ischemia. They reported that injection of these cells (as well as a non-invasive strategy of cytokine directed release of BM cells, such as that seen following G-CSF administration) have led to myocardial regeneration (Orlic *et al*, 2001a; Olic *et al,* 2001b).

Studies using HSCs for myocardial injury have described two different methods by which they become cardiomyocytes. They must undergo either cellular fusion or transdifferentiation to form cardiomyocytes rather than forming cells of haematopoiesis lineage. As the mechanisms by which HSCs home and differentiate into injured heart tissue is still relatively unknown, clinical trials are limited as a precaution (Wollert & Drexler, 2005).

Jackson *et al*, (2001) in a recent study used BM derived HSC marker cells called side-population (SP) cells. After myocardial IRI, donor SP cells from transgenic mice expressing LacZ were used for treatment. LacZ and α-actinin expression were used as markers for detection of donor derived cells. They were found to be expressed in 0.02% of cardiomyocytes within the infarcted region; showing the plasticity of these cells (Jackson *et al*, 2001). Although the percentage of newly formed myocytes was low, the potential of HSC therapy for myocardial infarction treatment seems encouraging.

There are however many conflicting findings of HSCs and their roles in cardiac ischemia repair. Later studies have claimed that HSCs upon arrival to ischemic injury do not transdifferentiate into cardiomyocytes (Norol *et al*, 2003); increasing the scepticism around the therapeutic use of HSCs for myocardial injury. This data however, has not

ruled out a beneficial process of cellular fusion by which HSC cells may fuse with another resident cell within myocardial tissue for the production of new myocytes (Wagers *et al,* 2002). A study of apoptotic cardiomyocytes incubated with HSCs has shown both cells undergo cellular fusion and reduce apoptosis effects (Yang *et al*, 2012) *in vitro*. Studies of cytokine mobilisation treatment in nonhuman primates have shown to initiate angiogenesis of small cardiac blood capillaries after myocardial infarction injury, however these studies found no evidence of the myocyte regeneration shown in mice studies by Orlic *et al* (Norol *et al*, 2003). Studies hereafter have continued to question the fate of HSCs to improve cardiac function, as many, have failed to observe the same findings as Orlic *et al* (Murry *et al*, 2004). This discrepancy in data may be due to the scarce understanding of HSC homing to injured tissue and the lack of retention of these cells to myocardial injury (Kavanagh & Kalia, 2011). Another factor could be that not all studies use the same population of HSCs for myocardial regeneration and therefore may result in variation of results.

1.6.2 Mesenchymal Stem Cell

MSCs were first discovered by Friedenstein *et al* as a population of BM cells (Afanasyev *et al*, 2009) with a non-haematopoietic cell fate. These isolated cells were identified as having the capability to differentiate into chondrocytes, osteoblasts and adipocytes, and later studies have shown that they can have cell fates of many different lineages such as cardiomyocytes (Bobis *et al*, 2006). They act as stromal cells, vital for forming a suitable microenvironment for HSCs within the BM, and their multipotency make them an exciting new approach to cellular therapy (Bobis *et al*, 2006).

1.6.2.1 MSC Identification

MSCs must adhere to plastic in culture, they must also express (and also lack expression of) particular cell surface antigens. Human MSCs (hMSC) must be positive for CD105, CD73, CD90 and negative for the following antigens: CD45, CD34 CD11b, CD19 and HLA-DR (Dominici *et al*, 2006). When using murine MSC (mMSC) the surface antigens they express can vary from hMSCs along with inter-strain differences between mMSCs. Therefore, must be taken into account when identifying MSCs from mice (Rostovskaya & Anastassiadis, 2012; Kavanagh *et al*, 2014). Particular murine cells isolated from bone marrow such as platelet derived growth factor receptor-α (PDGFR-α) MSCs are positive in PDGFR-α and Sca-1 expression and negative for CD45 and TER119. They can be isolated and cultured without contamination of HSCs (Kavanagh *et al*, 2014).

1.6.2.2 MSCs & Myocardial IRI

Both *in vitro* and *in vivo* conditioning of MSCs can see them differentiate into cardiomyocytes. Transplantation of MSCs in previous studies improve cardiac functioning through capillary density increase and replacement of lost cardiomyocytes following myocardial IRI (Tang *et al*, 2006). In times of myocardial IRI the administration of MSCs has shown to reduce infarct size. Reports of paracrine factors released by MSCs have shown beneficial effects on injured cardiac tissue (Lai *et* al, 2010). A study by Lai *et al* (2010) suggested that exosomes released by MSCs can also have the same desired effect as paracrine factors, cytokines and chemokines in reducing infarct size. This can be seen as an important breakthrough for myocardial IRI treatment. Differentiating MSCs to replace damaged myocardial cells remains relatively unknown; but the

therapeutic benefits from factors released by MSCs have therapeutic relevance (Lai *et al*, 2010).

1.6.3 Limitations of Stem Cell Therapy

Clinically, the use of HSC therapy for myocardial ischemic injuries are limited, due to such low retention of the cells within the damaged tissue. Only 0.02% of HSCs were found to be differentiated into cardiomyocytes by the studies from Jackson *et al* (2001). Cytokine directed methods using SDF-1α have been used to increase circulating HSCs. These therapies are also restricted due to the difficulty of delivering vectors containing the cytokines. Therefore the need for non-invasive methods to increase recruitment of SCs to infarcted tissue seem like the most applicable for clinical use (Hiasa *et al*, 2004; Kavanagh *et al*, 2013).

Pre-treatment of HSCs with H_2O_2 can increase recruitment to the injured gut as previously shown. A study by Kavanagh *et al* (2013) was the first to show the use of different incubating concentrations of H_2O_2 with HSCs and its effects of the recruitment to injured tissue. Concentrations generally lower than those produced in IRI can be used to incubate HSCs to increase adhesion integrins on the surface of the cells for enhanced recruitment (Kavanagh *et al*, 2013).

The potential for SCs has become an encouraging field for repair and regeneration of ischemic and inflamed tissue and heart conditions such as MIs (Kavanagh & Kalia, 2011). This paves the way for cellular therapy; in particular the use of adult SCs to tackle such issues.

1.7 Hypothesis

The main hypothesis of this thesis is that HSC adhesion to murine heart sections is increased following myocardial IR injury and pre-treatment of HSCs enhances adhesion caused by injury.

1.7.1 Aims

The main aims of this thesis are:

- Myocardial IR injury can increase adhesion of neutrophils and HSCs significantly to heart sections *in vitro*
- Blocking specific adhesive molecules on HSCs can lessen adhesion of HSCs *in vitro* following injury to tissue
- HSC adhesion on injured heart tissue *in vitro* may be enhanced following pre-treatment of HSCs
- Other organ injuries can show an increase in adhesive properties *in vitro* of heart tissue
- Other organ injuries can cause an increase in adhesion of HSCs to heart tissue *in vitro*

CHAPTER 2:

METHODS & MATERIALS

(2) Methods & Materials

2.1 Materials

2.1.1 Antibody

	Conjugated	Clone	Origin	Target	Isotype	Company
Anti-CD31	-	MEC13.3	Rat	Mouse	IgG2a	Cambridge Bioscience
Anti-CD44	-	IM7	Rat	Mouse	IgG2b	Cambridge Bioscience
Anti-CD49	-	RI-2	Rat	Mouse	IgG2b	Cambridge Bioscience
Anti-CD54	-	MK/3	Rat	Mouse	IgG2b	Cambridge Bioscience
IgG	488	-	Rabbit	Rat	IgGb	Life technologies
LE-AF anti-CD31	-	MEC13.3	Rat	Mouse	IgG2a	Cambridge Bioscience
LE-AF anti-CD49	-	RI-2	Rat	Mouse	IgG2b	Cambridge Bioscience
LE-AF anti-CD44	-	IM7	Rat	Mouse	IgG2b	Cambridge Bioscience
IgG Control	-	cB149/10H5	Rat	Mouse	IgG2b	eBioscience
Anti-Ly6G	660	RB6-8C5	Rat	Mouse	IgG2b	eBioscience

2.1.2 Common Materials

	Abbreviation	Company	Location
Acetone	-	Fisher Scientific	Leicestershire, UK
Ketamine			
Xylazine hydrochloride	-	Millpledge Pharmaceuticals	Nottinghamshire, UK
5'6-carboxyfluorescein di-acetate succinimidyl ester	CFSE	Life Technologies	Paisley, UK
Dulbeccos modified eagle medium	DMEM	Sigma	Poole, UK
L-Glutamine	L-Glu	Invitrogen	Paisley, UK
Penicillin	Pen	Invitrogen	Paisley, UK
Streptomycin	Strep	Invitrogen	Paisley, UK
Percoll	-	Sigma-Aldrich	Poole, UK
Transforming Growth Factor-beta	TGF-β	Sigma-Aldrich	Poole, UK
Trypsin/ Ethylenediaminetetraacetic acid	Trypsin/EDTA	Invitrogen	Paisley, UK
Stempro 34 SFM	-	Life Technologies	Paisley, UK
Trypan Blue	-	Sigma-Aldrich	Poole, UK

2.2 Methods

2.2.1 Animal Maintenance

All procedures were performed with male C57BL/6 mice obtained from the Harlan group in Oxfordshire. Animal experiments were all with accordance to the United Kingdom Home Office, Animals (Scientific procedures) Act of 1986; under the project licence 40/3336. All mice had access to food and water *ad libitum.*

2.2.2 Cell Counting

Viable cells were identified using the Trypan Blue exclusion method. 10µL of cell solution was added to 90µL of Trypan blue and roughly around 20µL of this solution was added to a Neubarer Haemocytometer containing 1mm x 1mm square grids. All viable cells remained white whilst non-viable cells became blue. 4 grids were counted for viable (non-blue stained) cells of which the average was taken. Subsequent dilution calculations were used to determine the number of cells and resuspended to the required number per ml.

2.2.3 Culturing Haematopoietic Progenitor Cells - 7 (HPC-7)

A murine HSC line, haematopoietic progenitor cell-7 (HPC-7) has previously been used experimentally for HSC trafficking within injured gut in the Kalia lab (Kavanagh, 2010; Kavanagh *et al*, 2013). HPC-7 cells are an immortalised cell line that express very similar adhesive properties and markers as primary HSCs. Altering these embryonic SCs by inducing expression of the LH2; a member of the LIM-homeobox family of genes, allows the production of this immortalised cell line (Pinto do Ó *et al*, 1998). This allows vast

expansion *in vitro* for sufficient numbers of HPCs for experimental use and eliminates the use of multiple donor mice (Pinto do O *et al*, 2001).

HPC-7s were cultured and maintained in Stem-Pro 34 media with additives: L-Glutamine, Penicillin/streptomycin, Stem Pro Supplement (SPS); provided by the manufacturer and Stem cell factor (SCF). This cell line remained cultured at the desired number of around $1X10^6$ cells per ml in a $25cm^2$ or $75cm^2$ conical flask. Cells were incubated at 37°C. Media was removed and changed completely every day.

2.2.4 Culturing of Platelet Derived Growth Factor Receptor α (PDGFR-α) MSCs

Platelet derived growth factor receptor α (PDGFR-α) has been proposed as a useful selection marker for murine MSC isolation. Murine MSCs have few truly selective markers and many isolation methods rely on their ability of plastic adherence; which increases chance of contamination by other BM cells. PDGFR-α MSCs can be isolated with very little contamination from hematopoietic cell lines from within the BM which make them an advantageous MSC line for experimental use (Houlihan *et al*, 2012).

PDGFR-α MSCs were cultured for growth in Modified Eagle Medium, Alpha modification (αMEM) with the following additives: L-Glutamine, Penicillin/streptomycin, fetal bovine serum/fetal calf serum (FBS/FCS), transforming growth factor beta (TGF-β). Cells were cultured in $75cm^2$ conical flask and incubated at 37°C. They were passaged when confluency reached 90-100% and media changed every 2-3 days.

For passaging, cells were washed with PBS w/o Mg^{2+}/Ca^{2+}. 2x Trypsin/EDTA was added and incubated with the cells at 37°C for a maximum of 5 minutes. MSC media addition neutralised the trypsin and cell suspension was centrifuged at 300g for 10 minutes at

room temperature. Cells were resuspended in 3ml of media and split amongst three 75cm^2 flasks.

2.2.5 Neutrophil Preparation from bone marrow

Neutrophils were isolated from the BM as they reside there in larger densities and so fit the purpose for experimental use (Boxio *et al*, 2004). They have also been shown to have a higher lifespan than peripheral neutrophils when cultured (Boxio *et al*, 2004). An existing method with the use of a Percoll gradient was used to isolate the neutrophils from other cell populations within the BM (Boxio *et al*, 2004).

C57BL/6 anaesthetised mice were culled via a Schedule 1 method. Their rear legs were removed and all tissue and cartilage surrounding the bones was removed, and immediately stored on ice. To remove the legs, the skin is cut above the knee joint and up along the abdomen; cut so that the skin is released from around the leg and is pulled up and over the heel leaving the leg exposed.

0.01% EDTA was used to flush out the marrow, using a 25-gauge needle; approximately 5ml of 0.01% EDTA was used per bone. Using a 19-gauge needle to flush the cell mixture and clumped cells were broken down in the solution. The cell solution was then passed through a 70µm filter and centrifuged at 1500rpm at room temperature for 5 minutes.

The pellet was resuspended in 1ml of 0.01% EDTA and 4ml ice cold water were added. To restore isotonicity, 2ml of 4x PBS is added 5 seconds after the addition of the ice cold water. Cells were respun at the previously described settings and were resuspended in 2ml 0.01% EDTA.

2ml of Cell suspension was added to a Percoll gradient (2.5ml, 72% Percoll; 2.5ml, 64% Percoll; 2.5ml, 52% Percoll). The gradient along with the cell suspension were centrifuged at a speed of 1500g for 30 minutes. A 2ml band at the interaction of 72% and 64% Percoll can be identified as the neutrophil band and any surrounding Percoll was removed. The band was separated and washed with 2-3ml of PBS containing 0.1% BSA without Ca^{2+}/Mg^{2+} (PBSA-), centrifuged at 1500rpm for 5 minutes at room temperature. The supernatant was discarded and this wash was repeated once more. After removal of the supernatant the pellet was resuspended in 1ml of PBSA+. The cells were counted and suspended in media at a density of $1x10^6$ cells per ml.

2.2.6 FACS analysis of Bone Marrow Isolated Neutrophils

FACs refers to fluorescently-activated cell sorting. To check the purity of the neutrophils isolated from the BM, the cells were analysed using FACS assessed by flow cytometry. Flow cytometry was used to calculate the number of Gr-1 positive cells in each suspension. 1ml of isolated cell suspension was centrifuged and the pellet was resuspended in 100µl Flow Cytometry (FC) buffer (PBS containing 5% FCS; 0.1% sodium azide) and incubated on ice for 30 minutes. This step reduces non-specific binding. 2µl of either rat (PE) IgG control or rat (PE) anti-mouse Gr-1 was added to separate suspensions and incubated on ice for 30 minutes. Cells were washed three times with excess FC buffer and ultimately resuspended into 300µl for analysis on a flow cytometer (BD FACSCalibur). Positive regions were calculated using the negative controls by selecting the extreme right end of the peak and running this across to the far right hand side of the relevant histogram. This was then applied to the sample stained with Gr-1.

2.2.7 CFSE Labelling of cells

To label cells for both *in vitro* and *in vivo* studies cells were fluorescently labelled using 5'6-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE). The non-fluorescent CFDA-SE crosses the plasma membrane readily but is cleaved by esterases already present in cells. The following process changes CFDA-SE into the less permeable and fluorescent CFSE; which continues to reside in the cell (Quah & Parish, 2010).

Cells are spun out of any previous solution and resuspended in 2ml of PBS. Stock CFDA-SE was stored and aliquoted at a dilution of 10mM in DMSO. 2µl of this dilution of CFDA-SE was added to a separate 2ml of PBS. Both of these suspensions were pooled into one and left to incubate in the dark at 37°C for 10 minutes. 6ml of the appropriate media was added to terminate the staining and the combined 10ml solution was centrifuged at 300g for 5 minutes. The supernatant was removed and the pellet was resuspended in required volume of the correct medium to ensure the desired number of cells per ml.

2.2.8 Surgical Preparation

C57BL/6 mice were anaesthetised by intra-peritoneal (IP) injection of Ketamine (100 mg/kg Vetalar) and xylazine hydrochloride (10 mg/kg) with 0.9% saline. The volume administered was determined by the weight of the mouse. Depth of anaesthesia was monitored every 10 minutes with the use of an automated timer and tested using the pedal reflex. A tracheostomy was performed for adequate breathing and cannulation of the carotid artery with polyethylene portex tubing for the administration of anaesthesia.

A thoracotomy was performed; incision into the skin above the chest cavity was used to separate it from the pectoral muscles. Between the pectoral muscles a small hole was

made using a cautery. Once exposed these muscles were cut and further cauterised to allow access to the heart (Eckle *et al*, 2011). Pectoral muscles were removed from both left and right of the chest cavity to allow complete exposure for imaging purposes using intravital microscopy (IVM). Throughout this process the lungs dropped down and so were able to avoid any damage.

2.2.9 Induction of Myocardial Ischemia Reperfusion Injury

After full thoracotomy, the mouse was placed on a ventilator and the settings determined were dependant on the weight of the mouse. For coronary occlusion a recently designed method previously used by Eckle *et al*, (2011) allowed for a much less invasive procedure and easy reperfusion. A hanging weight system for coronary occlusion of the left anterior descending (LAD) artery was used to stop the blood flow and induce ischemia; details shown in *figure. 2.2.1.* The LAD provides the majority of the required blood supply to the interventricular septum (James & Burch, 1958) therefore would provide the largest form of ischemia from the apex of the heart ascending towards the ventricles. Occlusion lasted for 60 minutes and tissue was reperfused for 120 minutes as already demonstrated by Eckle *et al* (2011), who saw no further infarct size increase with 240 minutes reperfusion (Eckle *et al*, 2011). To prepare sham hearts; animals were subjected to the same surgical procedures recorded in section 2.2.8 but without the application of the hanging weight system. These hearts were harvested after 180 minutes; equivalent to the occlusion and reperfusion time of the IR injured hearts.

Throughout the procedure for both IR and sham hearts, 0.9% saline soaked gauze was used to maintain hydration of the tissue.

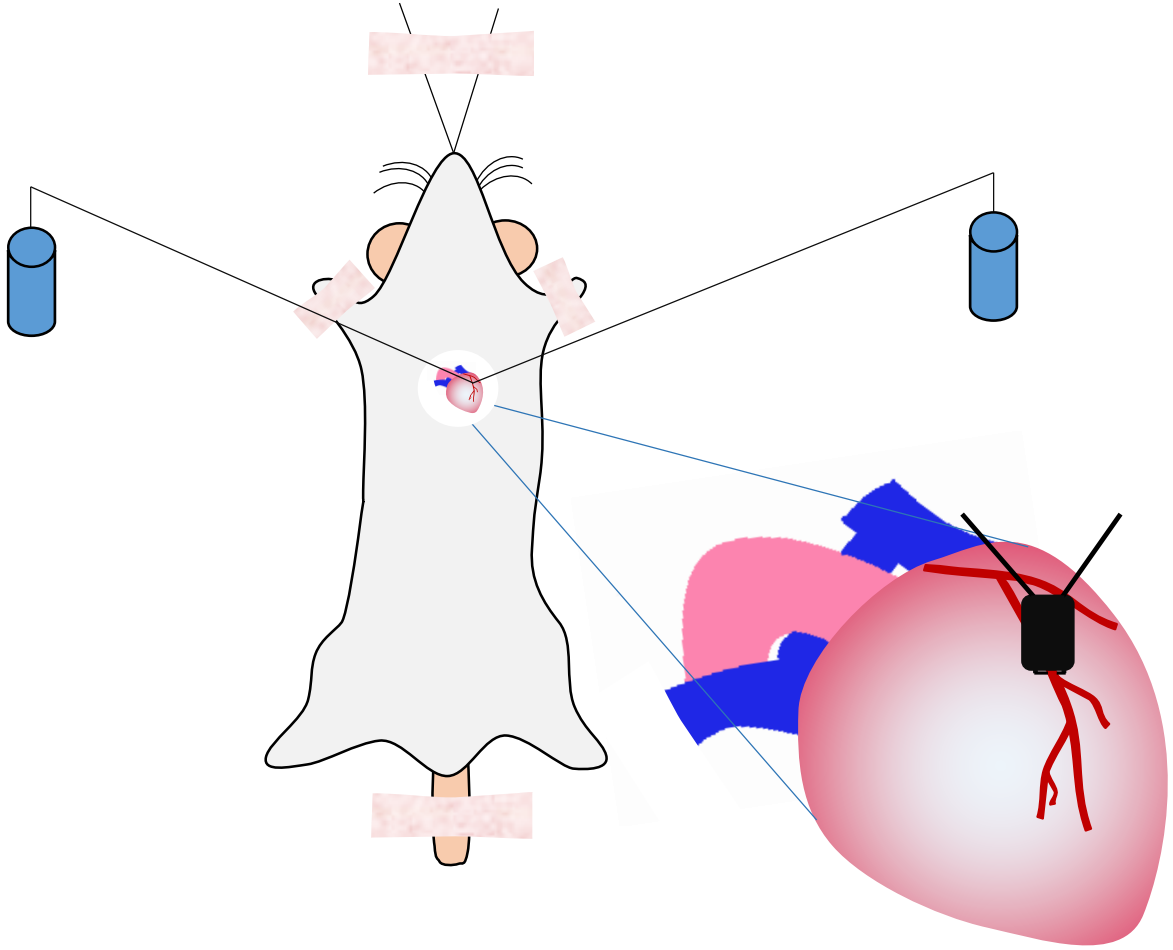

Figure 2.2.1 *The use of a hanging weight system to induce ischemia and reperfusion injury.* A 8.0 nylon suture was threaded through a piece of small piece of tubing and one end of the suture passed underneath the LAD. After being pulled through, two identical hanging weights were attached to either side of the suture; in this case Eppendorf containers with 1ml of water were used. This method lifts the LAD and obstructs the flow of blood to induce ischemia. It therefore makes it easier and less invasive for times of reperfusion, as the LAD can be lowered by the removal of the weights and return blood flow to the Interventricular tissue of the heart (Eckle *et al*, 2011).

2.2.10 Liver IR Injury, ConA treated & Kidney IR Injury

Hearts were harvested of animals that had under gone other organ injuries; Liver and Kidney injuries. For liver IRI the blood flow to the left and median lobes of the liver were clamped for 90 minutes. After 90 minutes the clamp was removed and the tissue was reperfused. After 2 hours reperfusion; the hearts were harvested. Concanavalin A (ConA) liver injury model was first used by Tiegs *et* al (1992) and was used as an alternative; as another liver injury model in this study. ConA treated mice were dosed with an injection of 20mg/kg concanavalin A (Sigma, UK) via the carotid artery and hearts harvested after 4 hours of ConA administration.

(All liver surgery was carried out by Joe Robinson in the Kalia Microcirculation research lab; and hearts were harvested after procedures for the purpose of this study).

For kidney IRI, a midline laparotomy was performed and an atraumatic vascular clamp was used to obstruct blood flow to the left renal pedicle. It was clamped for 45 minutes and after the clamp was removed; the tissue was reperfused for 60 minutes (White, 2014) before hearts were harvested.

The sham hearts used for comparison against liver and kidney injured heart models were those obtained from the previously described shams in *section 2.2.9.* Sham liver and kidney surgery was not performed for the basis of this study, therefore heart surgery shams were used as the control groups.

2.2.11 Sectioning of Heart

IR injured and sham hearts were snap frozen in liquid nitrogen after imaging, and stored at -80°C for sectioning. A cryostat (Bright instruments) was used for sectioning of heart tissue. The hearts were mounted on a disc using optimum cutting temperature (OCT) embedding compound to hold the tissue in place. The aortic end of the heart was embedded into the OCT, leaving the apex facing upwards as shown in *figure 2.2.2*. Sections were taken from the base/apex of the heart. 10µm sections were cut and mounted on poly L-lysine coated glass slides, with either 2 or 3 sections per slide. The slides were fixed in 100% acetone for 15 minutes and stored at -20°C.

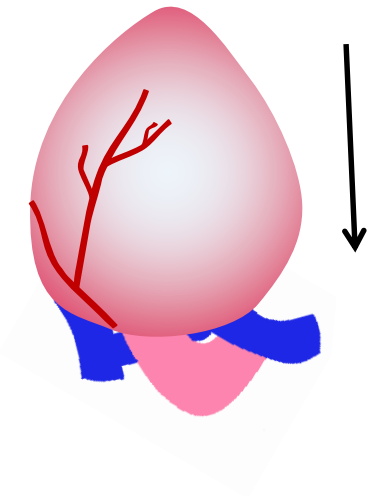

Figure 2.2.2. *Orientation of the hearts when embedded for sectioning.* The hearts were embedded into OCT on the aortic side. The apex faces upwards and the arrow represents the direction in which the hearts were sectioned.

2.2.12 Immunohistochemistry

Immunohistochemistry experiments were used to give an indication of the different surface adhesion molecules present on murine myocardial tissue. It would also provide scope to compare sham heart sections against IR treated heart sections.

Sectioned heart slides of both sham and IR tissue were warmed at room temperature for 5 minutes, and fixed in 100% acetone for 5 minutes. All sections were primarily incubated for 30 minutes with 100µl of PBS containing 0.1% BSA and 5% FCS for blocking

non-specific binding. The primary antibody was diluted 1:100 with PBS containing 0.1% BSA and 5% FCS and the antibodies used were: anti-Rat IgG as a control, anti-CD31, anti-CD44, anti-CD106 and anti-CD54. The sections were then incubated overnight at 4°C in darkness. The primary antibody was washed off using a small amount of PBS and washed thoroughly in a PBS bath, on a laboratory stirrer at room temperature for 30 minutes. Throughout this wash the slides remained in the dark. After being removed from the bath the slides were dried, taking care not to touch parts of the tissue section.

The secondary antibody; fluorescently labelled Alexa 488, was added for 30 minutes at room temperature on a laboratory rocker. The concentration of the secondary antibody was at 1:200 with PBS containing 0.1% BSA and 5% FCS. Following staining, the excess antibody was removed and washed again for 30 minutes. Slides were dried and fixed in a fluorescent mounting medium. All slides were left overnight in the dark at room temperature after fixing.

An antibody marker for endogenous neutrophils was also used to compare sham and IR heart treated sections. Ly-6G (Gr-1) antibody (eFluor 660) was incubated with the fixed sections for 90 minutes at a concentration of 1:100, washed and mounted as previously described in this section.

2.2.13 Frozen Tissue Static Adhesion Assay

The Stamper-Woodruff static adhesion assay was carried out to identify *in vitro* adhesion of neutrophils, PDGFR-α MSCs and HPC-7. Slides of heart sections previously stored at -20°C, were thawed at room temperature and fixed in 100% acetone for 5 minutes. Before acetone fixation the slides were labelled clearly with what cell types were added

to each section. The sections were washed with PBS and encircled using a wax immunology pen (Immedge; Vector Laboratories, Peterborough, UK). Cells were prepared by CFSE fluorescent labelling. For each cell type the assay was initially optimized, to determine the number of cells used per section and the most ideal incubation period.

Neutrophils were suspended at $1x10^6$ cells per ml, and 100µl of this cell suspension was added to the individual heart sections; providing $1x10^5$ cells on each section. These were then incubated in the dark on a laboratory rocker at room temperature for 20 minutes. The slides had one wash to remove the remaining unbound cells, and were again fixed in 100% acetone for 5 minutes. All sections were then washed with PBS three times, dried and mounted using Gel Mount aqueous mounting medium (Sigma).

HPC-7 cells were suspended at $1x10^5$ cells per section and incubated for 20 minutes. The protocol was then reworked, and cell suspension changed from $1x10^5$ cells per section to $5x10^5$ cells and time increased to 40 minutes. Later the most optimum number of cells per section was decided as $2.5x10^5$ with 40 minutes as the ideal incubation period.

PDGFR-α MSCs were trypsinised and incubated in media at 37°C for 1 hour. They were then stained with CFSE with the same method described in *section 2.2.6.* The incubation period was added to allow the MSCs to recover any lost adhesion molecule expression following trypsinisation. As the staining process takes an additional hour, MSCs were used at approximately 2 hours post dissociation. PDGFR-α MSCs were used at $1x10^5$ cells per section and incubated at both 30 minutes and 45 minutes to see the effects of different incubation periods had on adhesion.

To image all sections, five already determined fields of view were selected as shown in *figure 2.2.3.,* using EVOS Digital microscope. Fluorescently labelled cells were manually counted using software which blinds the observer to the source of each field (developed by Dean Kavanagh in the Kalia microcirculation research lab). This software presented all the images in a randomised order for manual counting without the knowledge of which respective group each image belongs to. All data was then presented in a table format after completing counts of all images. Results were all expressed as a mean per field ± SEM.

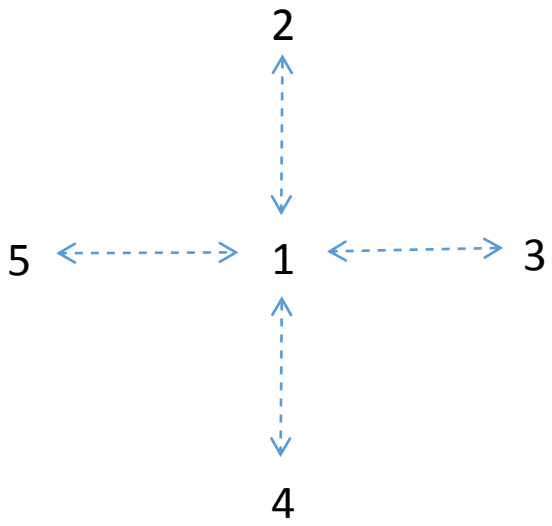

Figure. 2.2.3. *Predetermined pattern for counting fields of view of frozen tissue static adhesion assay.* This predetermined pattern allows adequate tissue section coverage, of which a mean was calculated. The first image was taken at the centre of the tissue (1) the subsequent 4 images that followed were: above, below, left and right of this starting point. Each time an image was taken, the microscope was returned to the central zone and moved onto the next field of the tissue.

2.2.14 Blocking of Cell Adhesion Molecules

In order to identify the cell adhesion molecules present on HPC-7 involved in the adhesion to heart sections *in vitro*, cells were incubated with blocking antibodies. HPC-7 were suspended in 0.1% PBSA with the blocking antibody at a dose of 80µg/ml. This concentration was determined by previously performed FACs studies in the Kalia microcirculation lab (Kavanagh, 2010) and have been sufficient for complete blockade of the antibodies used. Low endotoxin azide-free (LE-AF) IgG2, anti-CD31, anti-CD49 and anti-CD44 antibodies were incubated with HPC-7 separately (at 80µg/ml in 0.1% PBSA) for 15 minutes at room temperature. Cells were then CFSE labelled as described in section 2.5. and used for the Stamper-Woodruff static adhesion assay details of which are included in section 2.11.

2.2.15 H_2O_2 Pre-treatment of HPC-7

HPC-7 cells were pre-treated with two different concentrations of H_2O_2: 10µM and 100µM. Concentrations were based on previous work by Kavanagh *et al* (2013) where this treatment was used to enhance recruitment of HPC-7 cells to the murine gut; higher concentrations were shown to induce apoptosis in HPC-7. Cells were labelled with CFSE using the previously stated protocol and then suspended in PBS (with Ca^{2+}/Mg^{2+}) at $2.5x10^6$ cells per ml. The cells were aliquoted into three separate groups; two for the separate concentrations of H_2O_2 and the third serving as a control. For every ml of PBS cell suspension, 1µl of H_2O_2 solution was added of each concentration in subsequent aliquots and PBS added to the vehicle group. The cells were incubated for an hour in the dark at 37°C; although the cells were given a quick vortex every 15 minutes, to resuspend

the settled cells amongst the cells in solution. After the hour incubation cells were used immediately for stamper-woodruff static adhesion assay.

2.2.16 Statistics

Unpaired Students t-test was used to compare two experimental groups. For more than two experimental groups one-way ANOVA was followed by Dunnett's post-test for the use of the Stamper-Woodruff static adhesion assay. Values were considered as significant when $p<0.05$.

CHAPTER 3:

RESULTS

(3) Results

3. 1. Immunohistochemistry and Myocardial IRI

3.1.1 Expression of CD31 is not enhanced as a result of acute myocardial IRI

Heart sections taken from sham control and myocardial IR procedures were stained with fluorescently labelled antibody CD31 (*Figure 3.1;* panels: *B-C, E-F*). Sections were imaged to reveal abundant expression of CD31 amongst the tissue section; there was no observed difference of expression when comparing sham to IR (panels B-C; E-F). Histologically CD31 staining can be observed on both small and large vessels of both sham and IRI tissue (B-C; E-F).

3.1.2 CD106, CD44, CD54 expression is not enhanced as a result of acute myocardial IRI

There was no visible upregulation of CD106 (*Figure. 3.2*; A-B), CD44 (*Figure. 3.2;* C-D) and CD54 (*Figure 3.2;* E-F) observed by immunohistochemistry when comparing sham (panels; A, C, E) to IR tissue (panels; B, D, F). CD106 expression was lacking on smaller vessels (panels; A-B) and majority of the staining was localised at the larger blood vessels. CD44 (panels; C-D) staining was only observed on the smaller vessels and no detection of staining was observed on the larger vessels. CD54 (panels; E-F) staining was most abundant on larger blood vessels.

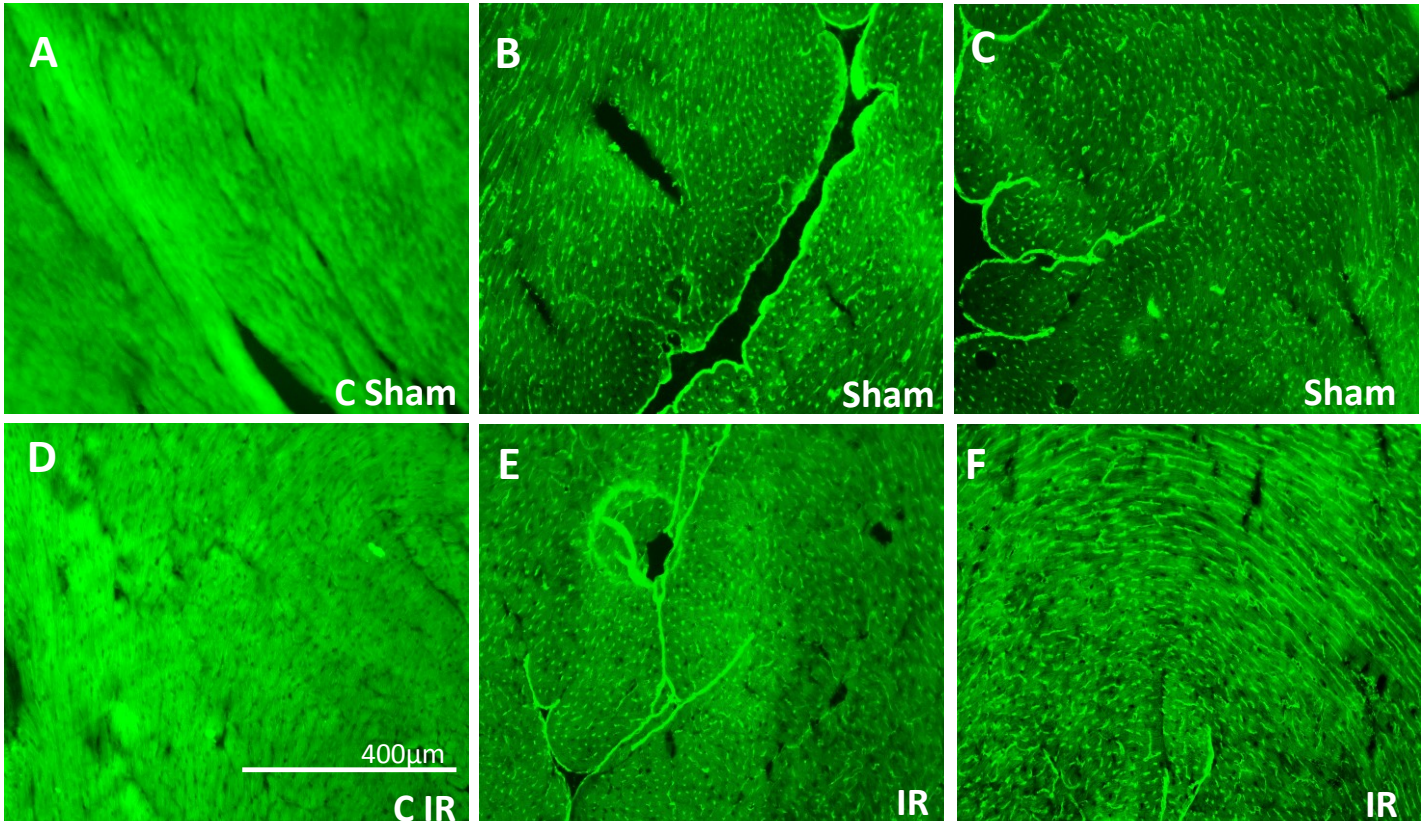

Figure 3.1. *Immunohistochemistry shows no upregulation of CD31 when comparing myocardial sham against IR tissue.* Anti-IgG immunohistochemistry was performed (panels A & D) and used as a running control throughout. Sections taken from sham and IR injured tissue visibly express CD31 and was detected across the entire tissue. There was no difference in expression detected when comparing sham and IR tissue (Sham: panel B, C; IR: panel E, F).

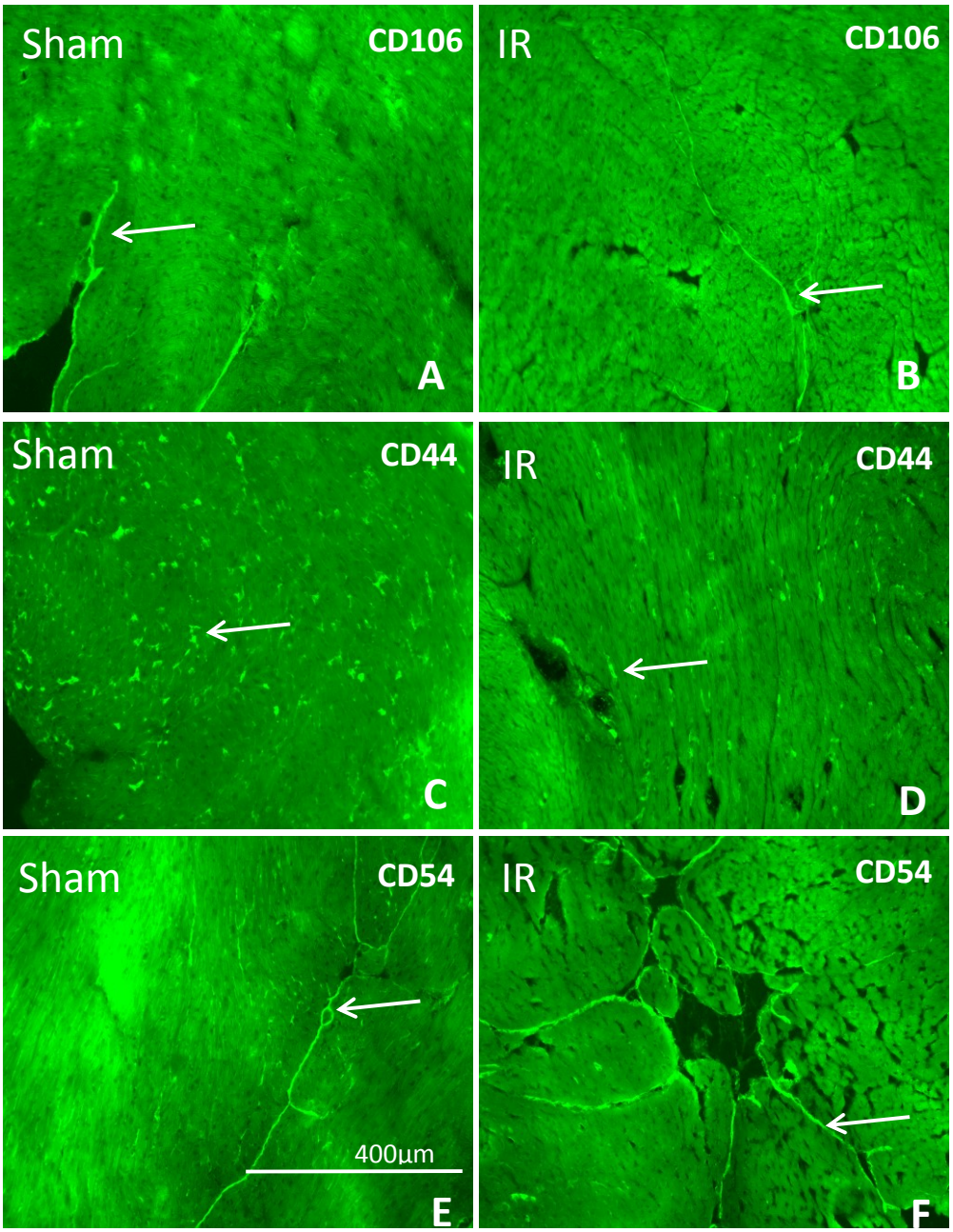

Figure 3. 2. *Immunohistochemistry shows no upregulation of CD106, CD44 and CD54 when comparing sham and IR heart sections.* Immunohistochemistry was performed on sections of both sham and IR heart tissue. CD106 and CD54 were only visible on large vessels on both sham (panel A & E) and IR (panel B & F) respectively. CD44 expression was detected on smaller vessels across the tissue (panels C-D). There was no visible difference in the upregulation of these surface molecules when comparing sham and IR sections.

3.2 Neutrophils and Myocardial IRI

3.2.1 Immunohistochemistry: There was no enhanced expression of endogenous neutrophils after acute myocardial IRI

Heart sections taken from both sham control and myocardial IR procedures were fluorescently stained for Gr-1 expression (*Figure 3.3; panels A-B).* Sections were imaged and there was no visible difference in the expression of Gr-1 when comparing sham (panel A) and IR (panel B).

3.2.2 Neutrophil adhesion is significantly increased *in vitro* by myocardial IRI.

Stamper-woodruff static adhesion assays showed significantly more neutrophils bound to frozen sections of IR tissue when compared to sham tissue (sham: 8.23 ± 1.165; IR: 18.83 ± 4.394; $p<0.05$). Representative images are shown (*Figure 3.4;* panels: B-C). FACS analysis was conducted to determine the percentage of neutrophils present in the cells isolated from the BM used for this assay (panel: D; 79.54%).

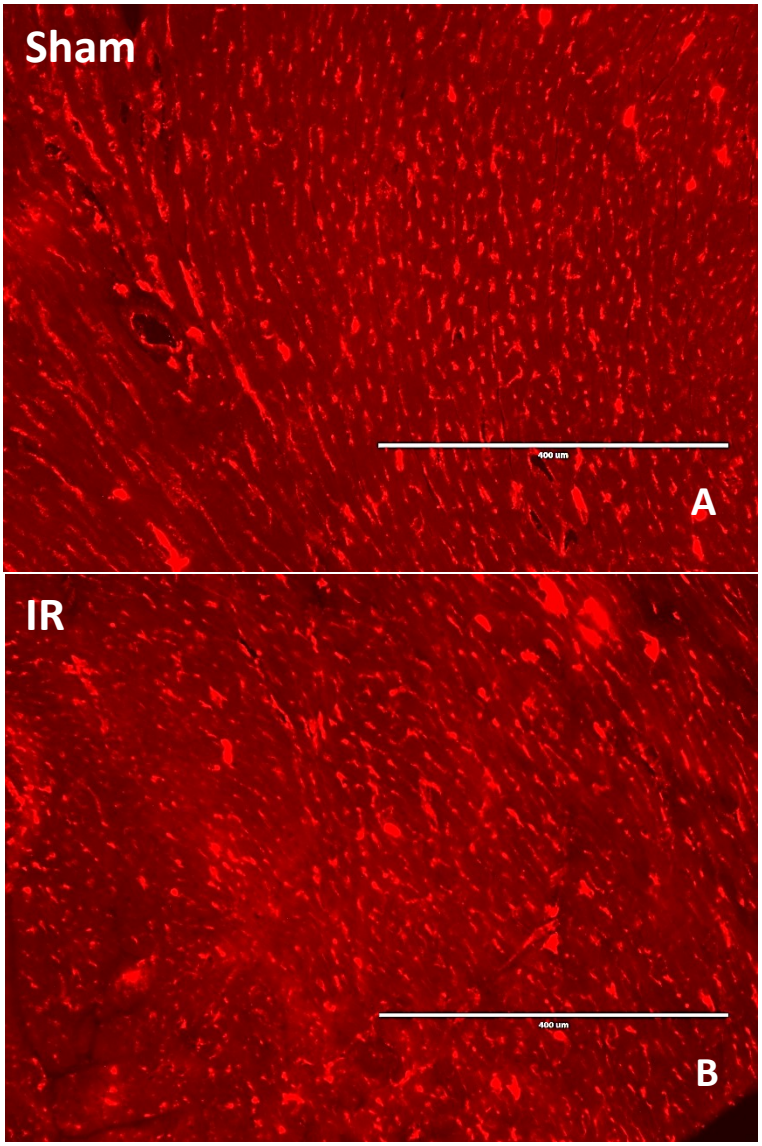

Figure 3.3. *Immunohistochemistry shows no enhancement of Gr-1 neutrophil marker when comparing myocardial sham against IR tissue.* Anti-Gr-1 immunohistochemistry was performed to compare endogenous neutrophils in sections from heart sham (panel A) and IR treated tissue (panel B). There was no visible difference in the expression when comparing the two.

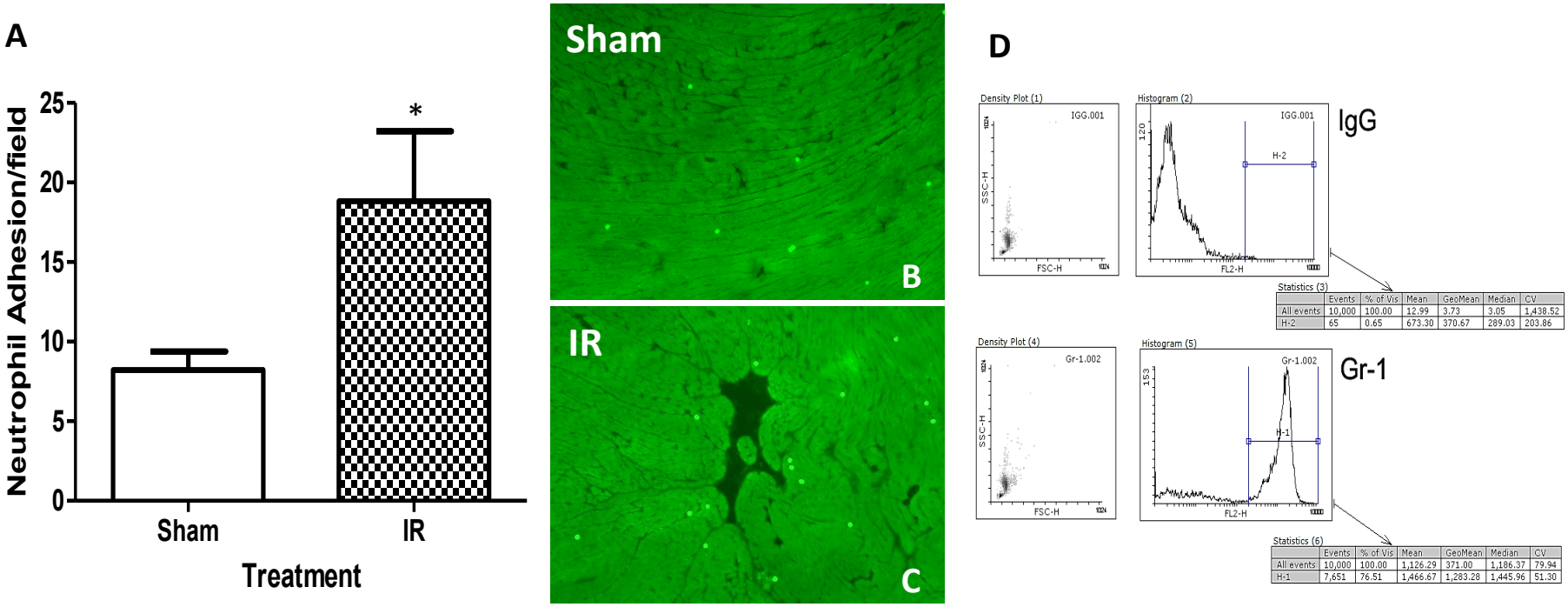

Figure 3.4. *Neutrophil adhesion is significantly increased on IR sections when compared to sham controls.* Sections taken from sham and IR treated hearts were examined for neutrophil adhesion using the Stamper-Woodruff static adhesion assay. Two of each Sham and IR treated (60/120min; isch/rep) heart sections were incubated with $1x10^5$ CFSE labelled neutrophils isolated from BM of C57/BL6 mice for 20 minutes. Adhesion was counted as 5 fields per section and an average value was obtained from all 10 fields of view of each tissue. Adhesion was significantly increased; comparing sham (panel B) and IR sections (panel C). FACS plot represents the percentage of neutrophils isolated from the BM and used in this assay (panel: D; 79.54%).Results are represented in panel A and bars represent mean adhesion ± SEM of 4 separate experiments. (*$p<0.05$; using unpaired t-test).

3.3 HPC-7 adhesion and Myocardial IRI

3.3.1 Method for HPC-7 adhesion *in vitro* on heart tissue sections

A method development step was required to optimize the Stamper-Woodruff static adhesion assay for adhesion of CFSE labelled HPC-7 to IR injured heart sections (*Figure 3.5.).* The method initially used $1x10^5$ cells per section with a 20 minute adhesion period (panel A). The incubation period was doubled to 40 minutes and the number of cells per section remained the same at $1x10^5$ cells (panel B). The number of cells were then increased to $2.5x10^5$ (panel C) and $5x10^5$ (panel D) cells per section; incubated for 40 minutes. The use of $1x10^5$ cells per section for 20 and 40 minutes incubation period saw very little adherence. $5x10^5$ cells per section showed increased clustering of HPC-7 to each other. For sufficient adherence and to avoid clustering of cells the ideal incubation period was defined at: 40 minutes and the number of cells to be used were $2.5x10^5$.

3.3.2 HPC-7 adhesion is significantly increased *in vitro* after myocardial IRI.

The immortalised cell line HPC-7 have very similar characteristics of primary HSC lines and are able to fully reconstitute haematopoiesis in lethally irradiated mice (Pinto do Ó *et al*., 2002). HPC-7 were therefore used as part of the Stamper-woodruff static adhesion assay to observe adhesion to IR and sham tissue sections. Significantly more HPC-7 adhesion was observed to IR tissue when compared with sham (sham: 9.30 ± 2.56; IR: 38.72 ± 10.19; $p<0.05$). Representative images are shown (*Figure 3.6;* panels: B-C).

3.3.3 HPC-7 adhesion was not significantly increased after pre-treatment with H_2O_2; for both sham myocardial IRI tissue *in vitro.*

HPC-7 adhesion was not significantly increased or either sham or IR treated sections; after pre-treatment with H_2O_2 using the Stamper-Woodruff static adhesion assay (*Figure 3.7)*. Different concentrations of H_2O_2 were used ranging from: non/treated (n/t), 10µM and 100µM. Although not significant, a general increase of HPC-7 adhesion can be seen for both sham sections (panel: A; Sham: n/t; 25.13 ± 7.55, 10µM; 115.80 ± 29.39, 100µM; 148.00 ± 64.52; $p<0.05$) and IR sections (panel: B; IR: n/t; 42.88 ± 6.36, 10µM; 290.10 ± 123.80, 100µM; 170.30 ± 70.76; $p<0.05$). Images are a representation of adhesion of n/t, 10µM and 100µM H_2O_2 pre-treatment of HPC-7 (Sham: panels: C, E & G; IR: panels: D, F & H).

3.3.4 HPC-7 adhesion is not significantly reduced *in vitro* after blockade of CD31, CD44 and CD49d

To identify the roles CD31, CD44 and CD49d play in the adhesion of HPC-7 *in vitro* with the use of the Stamper-Woodruff static adhesion assay. HPC-7s were pre-treated with: anti-CD31, anti-CD44 and anti-CD49d antibodies and their adhesion to heart IR sections were quantified (*Figure 3.8.)*. Interestingly, the adhesion of HPC-7 showed a decrease after CD31 and CD49d blockade, although this was not statistically significant. CD44 block showed little or no change from control (IgG control: 15.98 ± 6.04; CD31: 6.03 ± 1.51; CD44: 19.05 ± 6.03; CD49d: 9.60 ± 1.82; $p<0.05$).

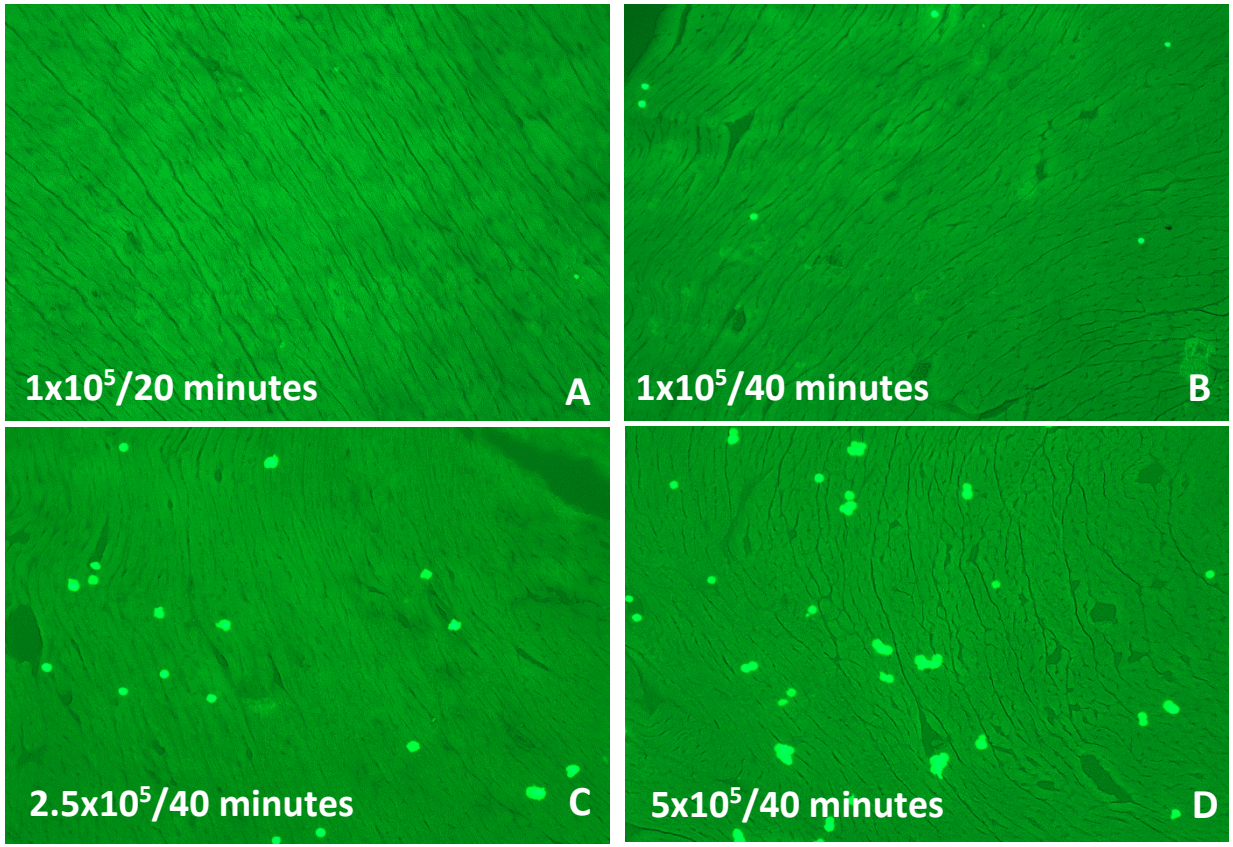

Figure 3.5. *Adhesion of HPC-7 using the Stamper-Woodruff static adhesion assay was modified to using 2.5x10^5 cells per section with an incubation period of 40 minutes.* 1x10^5 cells per section for 20 minutes (panel A) showed little or no adhesion of HPC-7. Increased adhesion time to 40 minutes (panel B) increases number of HPC-7 adhered to sections. The use of 2.5x10^5 HPC-7 for 40 minute incubation period (panel C) further increases adhesion. Using 5x10^5 HPC-7 per section with an incubation period of 40 minutes (panel D) caused cells to cluster and adhere to each other. The resulting model protocol was to use 2.5x10^5 cells per section with 40 minutes incubation.

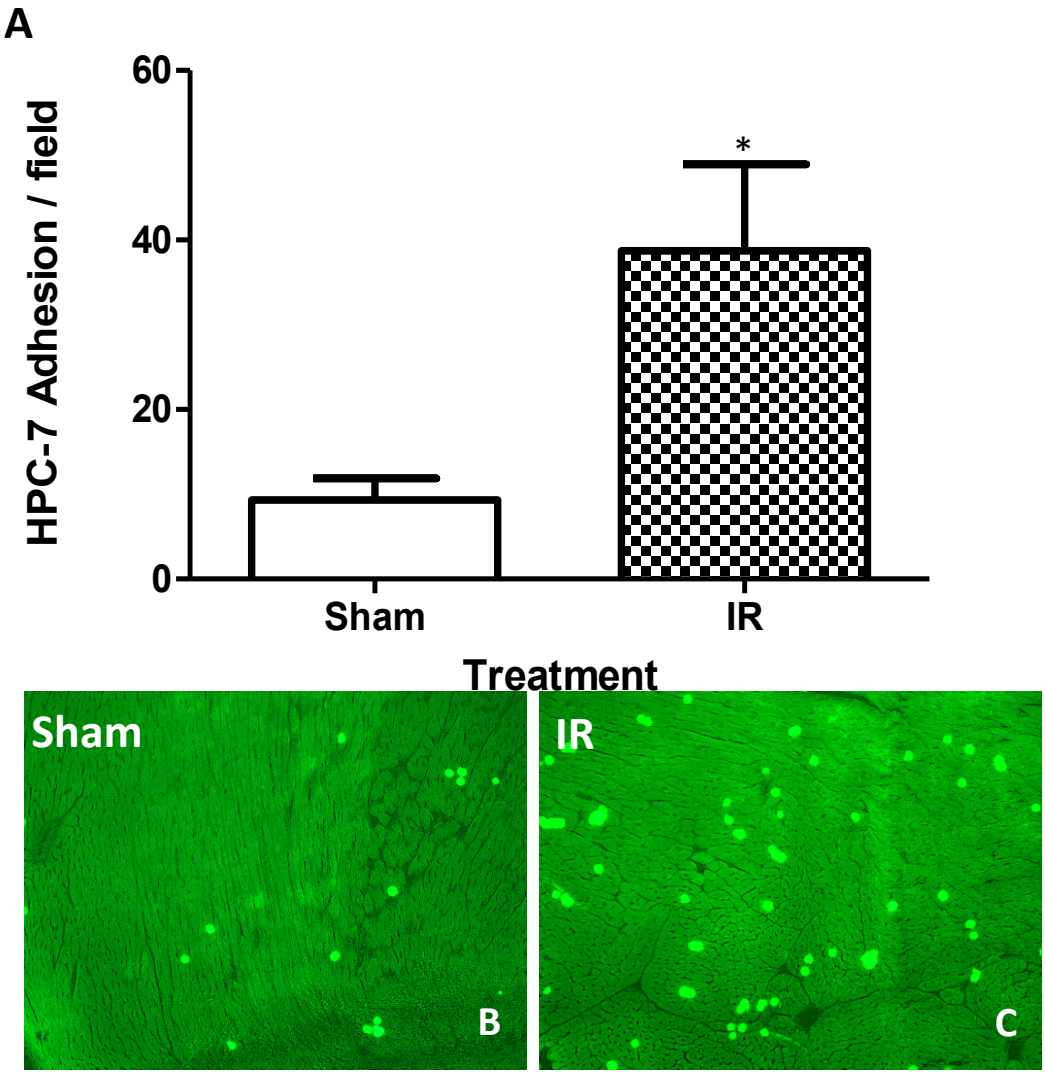

Figure 3.6. *HPC-7 adhesion is significantly increased on IR sections when compared to sham controls.* Sections taken from sham and IR treated hearts were examined for HPC-7 adhesion using the Stamper-Woodruff static adhesion assay. Two/three of each Sham and IR treated (60/120min; isch/rep) heart sections were incubated with $2.5x10^5$ CFSE labelled HPC-7s for 40 minutes. Adhesion was counted as 5 fields per section and an average value was obtained from all 10/15 fields of view of each tissue. Adhesion was significantly increased; comparing sham (panel B) and IR sections (panel C). Results are represented in panel A and bars represent mean adhesion ± SEM of 4 separate experiments. (*$p<0.05$; using unpaired t-test).

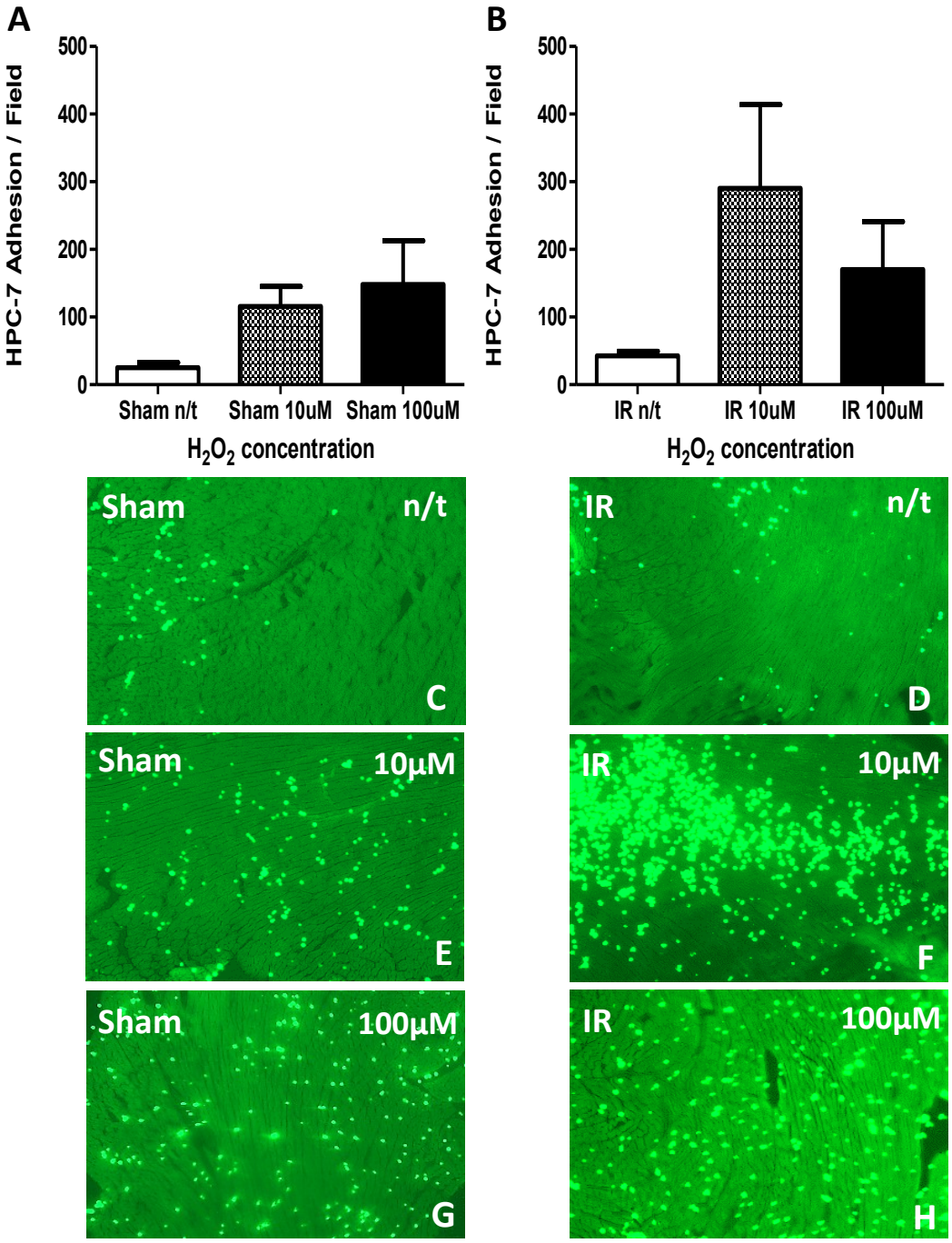

Figure. 3.7. *HPC-7 adhesion is not significantly increased with H_2O_2 pre-treatment of sham and IR sections.* Stamper-Woodruff static adhesion assay was performed with $2.5x10^5$ cells per section for 40 minutes using treated HPC-7s on both sham (panel: A) and IR treated (panel: B; 60/120; isch/rep) heart sections. CFSE labelled HPC-7s were incubated at 37°C for 1 hour with H_2O_2 prior to use for the adhesion assay. Concentrations used were: 10µM (Sham: panel E; IR: panel F), 100µM (Sham: panel G; IR: panel H) and n/t cells were used as a control (Sham: panel C; IR: panel F). Adhesion was counted as 5 fields per section and an average was obtained for each tissue. Results are represented in panel A and bars express mean adhesion ± SEM of 4 separate experiments. (*$p<0.05$; using one-way ANOVA and Dunnett's post-test).

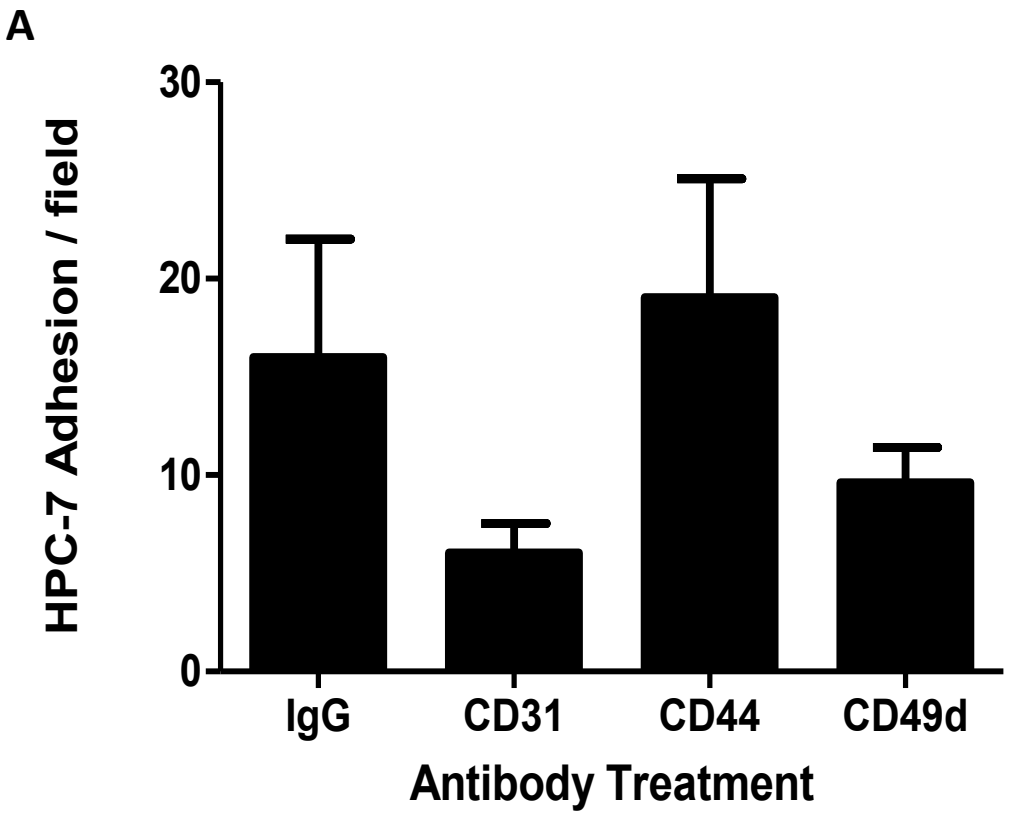

Figure 3.8. *Adhesion is not significantly reduced after blocking CD31, CD44 and CD49d on HPC-7 when tested on IR sections in vitro.* CD31, CD44 and CD49d blockade on HPC-7 adhesion was quantified using the Stamper-Woodruff static adhesion assay. Cells were treated with 80µg/ml of each mAb and when compared with IgG control statistically there was no significant reduction in adhesion. Although a general reduction was observed with blockade CD31 and CD49d, statistically, there was no significance. Results are represented in panel A and bars express mean adhesion ± SEM of 4 separate experiments. (*$p<0.05$; using one way ANOVA and Dunnetts post-test).

3.4 PDGFR-α MSC and Myocardial IRI

3.4.1 Method for PDGFR-α MSC adhesion *in vitro*

Figure 3.9. represents the method development for the use of the Stamper-Woodruff static adhesion assay with PDGFR-α MSCs; using sham and IR sections. PDGFR-α MSCs were fluorescently labelled with CFSE and sections were incubated with $1x10^5$ cells per section. Panel A: incubated with $1x10^5$ PDGFR-α MSCs for 30minutes (sham: 0.15 ± 0.05; IR: 0.85 ± 0.17); panel B: incubated with $1x10^5$ PDGFR-α MSCs 45minutes (sham: 0.95 ± 0.24; IR 2.65 ±0.53).

3.4.2 PDGFR-α MSC adhesion is significantly increased *in vitro* by myocardial IRI.

PDGFR-α MSCs adhesion was significantly increased when comparing IR tissue to sham (*Figure 3.9.)* panel A - sham: 0.15 ± 0.05; IR: 0.85 ± 0.17; $p<0.05$) with both incubation periods (panel B – sham: 0.95 ± 0.24; IR 2.65 ±0.53; $p<0.05$). Although statistically there was an increase, the overall number of PDGFR-α MSCs adhered to the sections remained very low with both methods used. Images (*Figure 3.9*; panels: C-F) are a representation of the number of adhered PDGFR-α MSCs per field of view.

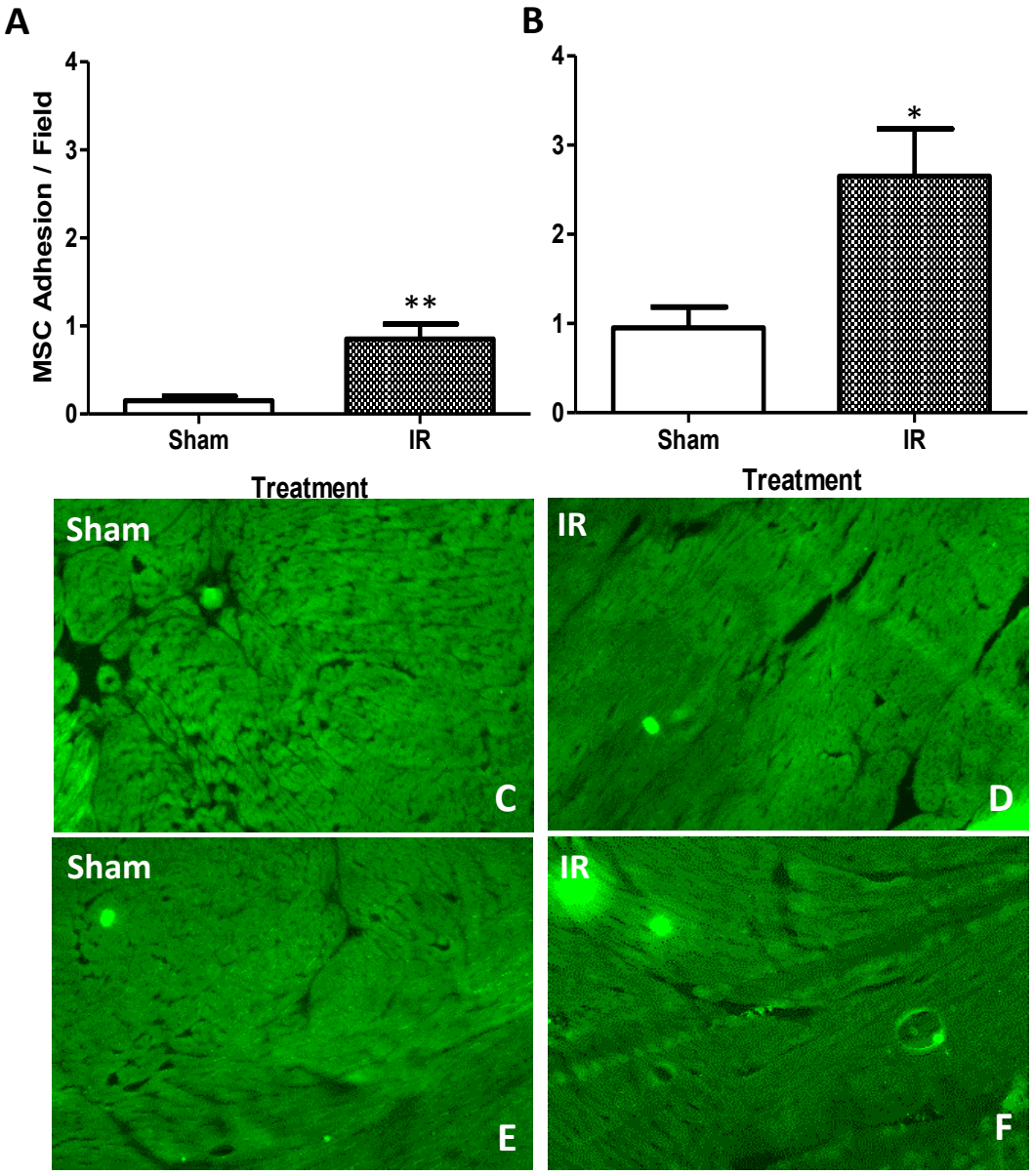

Figure 3.9. *PDGFR-α MSC adhesion is significantly increased on IR sections when compared to sham controls.* Sections taken from sham and IR treated hearts were examined for PDGFR-α MSC adhesion using the Stamper-Woodruff static adhesion assay. Sham and IR treated (60/120min; isch/rep) heart sections were incubated with $1x10^5$ CFSE labelled PDGR-α MSC for 30 minutes (panels: A, C & D); and for 45 minutes (panels: B, E & F). Adhesion was significantly increased when comparing sham and IR sections for both experiments (panels A-B). Results are represented in panel A for 30 minutes incubation and panel B for 45 minutes. Bars represent mean adhesion ± SEM of 4 separate experiments (*p<0.05 **p<0.01; using unpaired t-test).

3.5 Other Organ Injuries and Myocardial Consequences

3.5.1 Neutrophil adhesion is significantly increased *in vitro* to heart sections of ConA treated animals.

Stamper-Woodruff static adhesion assays were performed on heart sections taken from liver IR (LIR), ConA and kidney IR (KIR) treated animals. Hearts were harvested of all these treated animals for the purpose of this experiment. When comparing neutrophil adhesion; although not significant, a general increase can be observed following LIR and KIR injury compared to sham controls (sham: 8.23 ± 1.17; L IR: 22.35 ± 7.059; KIR: 15.47 ± 1.770; $p<0.05$). The control group used here was the sham group previously used to compare non-injured heart tissue against myocardial IR injured tissue. Neutrophil adhesion to heart sections isolated from ConA treated animals was significantly increased when compared to the myocardial IR sham group (sham: 8.23 ± 1.17; ConA: 28.60 ± 7.40; $p<0.05$). Images (*Figure 3.10;* panels: B-E) are a representation of the number of adhered neutrophils to the sham and LIR (panel C), ConA (panel D) and KIR (panel E) treated heart tissue sections.

3.5.2 HPC-7 adhesion does not increase *in vitro* to heart sections of liver IR, ConA and Kidney IR treated animals.

Stamper-Woodruff static adhesion assays were performed using HPC-7 on previously mentioned heart sections (see *Section **3.5.1***). Adhesion did not significantly increase on

any of the heart sections of the different injury treated animals. Interestingly, there was a general increase when comparing LIR, ConA and KIR adhesion to sham heart sections (*figure 3.12*: sham: 9.30 ± 2.56; LIR: 42.71 ± 18.30; ConA IR: 67.10 ± 37.50; KIR: 28.80 ± 4.12; $p<0.05$) although not significant. Again, the sham heart sections were from myocardial IR injury sham surgery. Images (*figure 3.11;* panels B-E) are a representation of the number of adhered HPC-7 per field.

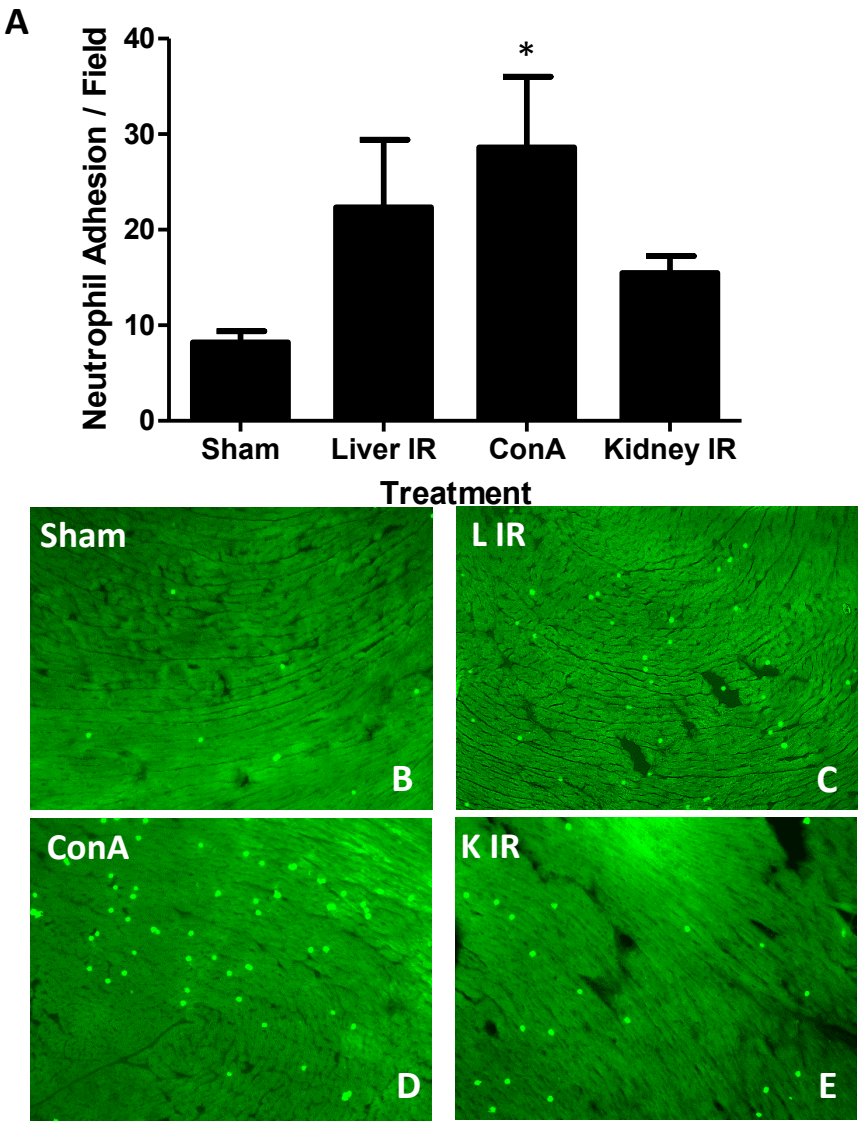

Figure 3.10. *Neutrophil adhesion is significantly increased on ConA treated heart sections compared to sham controls.* Heart sections taken from: Sham (panel B), LIR (panel C: 90/120min; isch/rep), ConA (panel D: 20mg/kg), KIR (panel E: 45/60min; isch/rep) treated animals were examined for neutrophil adhesion using Stamper-Woodruff static adhesion assay. Two sections of each tissue were incubated with $1x10^5$ CFSE labelled neutrophils isolated from BM for 20 minutes. Adhesion was counted as 5 fields per section and an average was obtained from all 10 fields of view for each tissue. Adhesion was significantly increased in ConA LIR heart sections (panel D) when compared to sham (panel B). Sham sections used were from myocardial sham tissue described in *section 2.2.9.* Adhesion of neutrophils to heart sections of LIR and KIR treated animals showed a general increase although not significant. Results are represented in panel A and bars represent mean adhesion ± SEM of 4 sham, 3 LIR, 2 ConA & KIR separate experiments. (*$p<0.05$; using one-way ANOVA and Dunnetts post-test).

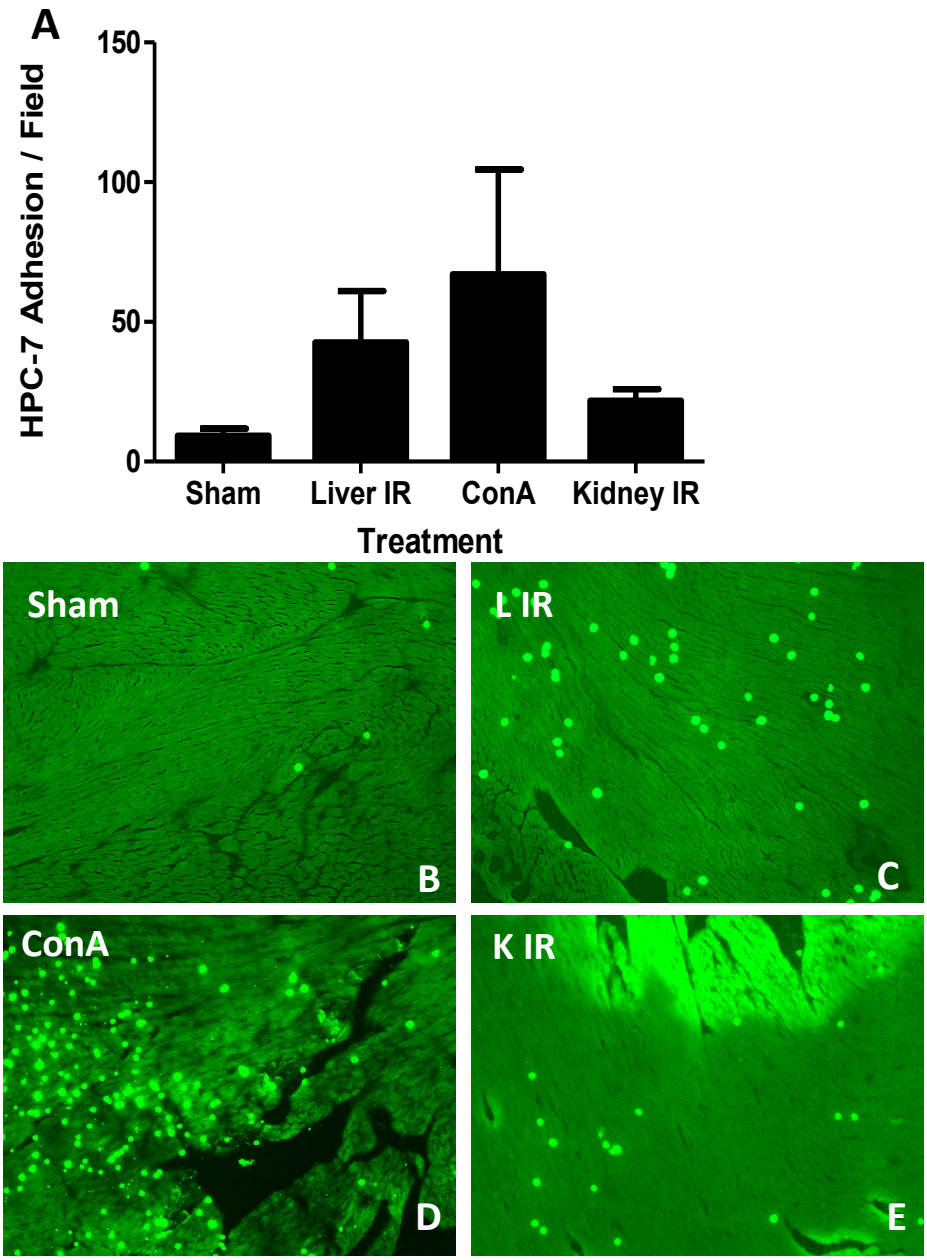

Figure 3.11. *HPC-7 adhesion is not significantly increased on heart sections from LIR, ConA or KIR treated animals.* Heart sections taken from: Sham (panel B), LIR (panel C: 90/120min; isch/rep), ConA (panel D: 20mg/kg), KIR (panel E: 45/60; isch/rep) treated animals were examined for HPC-7 adhesion using Stamper-Woodruff static adhesion assay. Two sections of each tissue were incubated with $2.5x10^5$ CFSE labelled HPC-7 for 40 minutes. Adhesion of 2 sections was counted as 5 fields per section and an average was obtained from all 10 fields of view for each tissue. Adhesion of HPC-7 to heart sections of LIR, ConA and KIR treated animals showed a general increase although not significant when compared to sham. Sham sections used were from myocardial sham tissue described in *section 2.2.9.* Results are represented in panel A and bars represent mean adhesion ± SEM of 4 sham, 3 LIR, 2 ConA & 3 KIR separate experiments. (*$p<0.05$; using one-way ANOVA and Dunnetts post-test).

CHAPTER 4:

DISCUSSION

(4) DISCUSSION

Embryonic SCs have shown beneficial attributes in the treatment of cardiovascular disease; for instance, their ability to form cardiomyocytes *in vitro*; but these cells pose ethical problems; which is absent in studies with adult SCs (Balsam & Robbins, 2005). The beneficial attributes and safety of the use of HSC transplantation for: blood, immunological disorders and the treatment for some types of cancer, have been demonstrated for over 50 years and abundantly studied (Copelan, 2006; Farsi, 2010). The use of HSCs for cardiac functional repair has been limited due to a number of factors, which include restricted knowledge of governing mechanisms for their recruitment within cardiac tissue. The low levels of cardiac regeneration and conflicting studies regarding HSCs plasticity and mediator release have all contributed to the hesitation of HSC clinical trials for cardiac disease (Orlic *et al,* 2001b; Wagers *et* al, 2002).

The body of the work in this thesis was dedicated to the development of myocardial IRI model and the production of a model which would allow the isolation of myocardial IR tissue following intravital microscopy of the beating heart. The initial findings have highlighted increased adhesive properties for both neutrophils and more interestingly, HPC-7 to injured heart tissue.

During *in vivo* experimentation, surgical success rate is an important factor which may influence the number of animals used experimentally and required to obtain the most useful data. The use of the previously published hanging weight system (Eckle *et al*, 2011) seemed advantageous to previously used methods of ligation of the LAD; this method has been suggested to avoid surgical trauma to the coronary vessel.

Furthermore, if the artery is not sufficiently ligated and the knot becomes loose; which is a possibility due to the movement of the heart, reperfusion can occur throughout the procedure. Thus altering the results of an IRI study (Eckle *et al*, 2011). This method was therefore used as an alternative to invoke a myocardial IRI (Eckle *et al*, 2011) for the purpose of this study.

4.1 Neutrophil adhesion *in vitro*

To confirm reperfusion injury, neutrophil adhesion *in vitro* was an initial target for this study. This was important to validate the model, as there are no previously published studies using Stamper-Woodruff type assays on isolated cardiac tissue from animal models. Although, the Stamper-Woodruff static adhesion assay has previously been used for adhesion of granulocytes on human cardiac tissue (Jaakkola *et al*, 2000). Neutrophil recruitment and adhesion to IR tissue has been well documented in the past (Hernandez *et al*, 1987; Kakker & Lefer, 2004). Myocardial IRI results in an inflammatory response, in which expression of adhesive molecules on both neutrophils and endothelium are upregulated (Jordan *et* al, 1999; Kakker & Lefer, 2004). Therefore as a method to validate the use of the Stamper-Woodruff static adhesion assay for cardiac tissue, analysis of the number of adhesive neutrophils was compared between injured and sham heart sections shown in *Figure. 3.4.* A significant increase in adhesion of neutrophils supports the use of this assay on cardiac tissue and verifies the presence of an injury.

Peripheral neutrophils arrive to the site of injury *in vivo* and their adhesion molecules are upregulated by various cytokines and proinflammatory mediators (Jordan *et al*,

1999). In this study, no attempt was made to increase adhesion molecules on the neutrophils taken from the BM; these neutrophils have been described as functionally immature (Boxio *et al,* 2004), yet adhesion was still increased (*Figure 3.4.)*. This leads to an interesting finding; damaged cardiac tissue is able to support the adhesion of neutrophils even without needing a prior stimulatory agent.

4.2 HPC-7 adhesion *in vitro*

This is the first study to demonstrate the adhesive properties of HPC-7 on murine heart sections *in vitro*, for both sham and injured tissue. HPC-7 have been used both *in vitro* and *in vivo* as a HSC line, particularly in analysing the recruitment of HSCs in IRI (Kavanagh *et al*, 2010; White *et al*, 2014). Results from this study reveal that HPC-7 adhesion is significantly increased on injured heart tissue *in vitro*. The use of the Stamper-Woodruff assay relies solely on adhesive mechanisms between the tissues and cells they are incubated with. These results show that microvascular properties must have been altered through injury and that this can facilitate amplified adhesion when comparing to non-injured tissue.

The enhancement of HPC-7 recruitment in gut injury has been shown *in vivo* using pre-treatment methods with H_2O_2 (Kavanagh *et al*, 2013). To increase this adhesion *in vitro* on heart sections, attempts were made to pre-treat the cells with similar concentrations used by Kavanagh *et al* (2013). Interestingly, HPC-7 incubation with H_2O_2 showed, for the first time, a general increase in adhesion to heart sections *in vitro*. Although not significant, it can be seen from *Figure 3.7* that overall adhesion was enhanced. Significant data may be achievable through increasing the number of animals used in

the study to provide evidence of the ability of H_2O_2 to enhance the adhesive capabilities of HPC-7 in respect to cardiac tissue.

A study by Yang *et al*, (2012) has used H_2O_2 to induce apoptosis in cardiomyocytes *in vitro*, and culture them with HSCs. They described the importance of cellular fusion of cardiomyocytes and HSCs to reduce apoptosis; in these H_2O_2 treated cardiomyocytes. They found a significant increase of cellular fusion after the induction of apoptosis in cardiomyocytes. The results from this study (*Figure 3.7)* have clearly shown an increase of adherence with H_2O_2 pre-treatment of HPC-7 to cardiac tissue. Yang *et al* suggest that inducing apoptosis is the reason for increased cellular fusion; it may well be that the presence of H_2O_2 played a role in increasing adherence and perhaps enhanced fusion; rather than increased fusion from just the injury itself.

Cardiomyocyte formation from HSCs has been reported to be low as 0.02% by Jackson *et al* (2001). Due to the unknown homing mechanisms of HSCs to injured heart tissue, groups have expanded the attempts to amplify cardiomyocyte formation by increasing mobilisation of HSCs from BM (Orlic *et* al, 2001b). The mobilisation of more HSCs from the BM were to increase the "probability" of HSC homing to injury (Orlic *et* al, 2001b). Mobilisation attempts in primates by Norol *et al* (2003) using the same mechanisms have failed at observing the same cardiomyocyte regeneration capabilities as Orlic *et al*. H_2O_2 pre-treatment however has shown that adhesion of HPC-7 can be increased to cardiac tissue *in vitro* and if shown *in vivo* to have the same effect, the process of homing and recruitment may be optimised to increase HSC recruitment within the injured heart.

Vector delivery methods using SDF1-α in the past have failed to increase homing of HSCs in sham heart tissue (Abbott *et al*, 2004). Although not significant, H_2O_2 pre-treatment of HPC-7 did show a general increase in adhesion on sham heart sections as well as injured heart sections. The homing of HPC-7 may therefore be observed using intravital microscopy in sham cardiac tissue as well as injured cardiac tissue with this pre-treatment method; if found to have the same adhesive increase *in vivo* as seen *in vitro*.

4.3 Immunohistochemistry

Although there was no evident upregulation of endothelial markers shown in *Figure 3.1 and 3.2,* previous studies have demonstrated using immunohistochemistry and static adhesion assays,the roles ICAM-1 (CD54), P-selectin and VAP-1 in the adhesion of neutrophils (Jaakkola *et al*, 2000). Therefore, it was important to identify specific adhesion molecules that were present on the murine heart sections collected for this study. Immunohistochemistry for surface markers was performed for the basis of presence rather than for quantification in this thesis. Further experimentation would be required to quantify any difference between sham and IR as the differences cannot be identified from staining and visual inspection alone; methods such as flow cytometry would be advantageous as these provide quantitative data that can be assessed easily.

4.4 Adhesive Blocking

HPC-7 adhesive blocking provided no significant differences, but interestingly, a general decrease in adhesion could be observed with both CD31 and CD49d block. Flow cytometry results of the adhesive properties of HPC-7 in the Kalia lab have previously shown the abundant expression of CD31 and CD49d on the surface of these cells

(Kavanagh, 2010). This decrease observed with the Stamper-Woodruff assay following blockade of CD31 and CD49d could suggest that these may be the mediators involved in the adhesive mechanisms of HPC-7 in murine heart tissue. Previous data in flow experiments have shown that anti-CD49d block lessens the adhesion of HPC-7 to cardiac endothelium *in vitro* (Kavanagh, 2010).

4.5 PDGFR-α MSC adhesion

Homing mechanisms of MSCs still remain fairly unknown but there is known increase of MSCs in peripheral circulation at times of MI (Wang *et al*, 2006). MSC studies following MI injuries have shown that less than 1% of systemically delivered MSCs reside in the injured heart with many more residing in undamaged lung tissue (Barbash *et al*, 2003). The static adhesion assay results have shown that there is increased adhesion of MSC on the injured heart sections, but the overall number of adhesive cells were extremely low. This poses the question of whether MSCs possess the appropriate adhesive mediators for recruitment to cardiac injury. In addition, when using MSCs for static adhesion assay use, the disassociation of the cells through trypsinisation may reduce their adhesive capabilities. Although there was a resting period before using them for the Stamper-Woodruff assay, this may not have been sufficient enough for the cells to recover and maybe the reason for the lack of adhesion to the heart sections.

4.6 Remote Organ Injury

More interestingly, the adhesion of neutrophils was increased on heart sections isolated from mice who had injuries in the liver and kidney. Kelly (2003) has shown leukocyte infiltration increases in the heart following renal IR. The results from this *in vitro* study

have shown an increase in adhesion of neutrophils following liver injuries as well, in particular with ConA treated liver injured mice. Tiegs *et al,* (1992) first used Con A as a liver disease model and their results showed no signs of damage to any major organs other than the liver itself. Other studies have shown that the toxicity effects of ConA is highly dependent on dosage and the route in which it is administered, but again there was no direct toxicity reported of the heart (Ballerstadt *et al*, 2006). Clinically, small doses of ConA administration has been suggested as a potential method to monitor glucose levels in diabetic patients, but the safety of ConA use is still questionable (Ballerstadt *et al*, 2006). The results from this study mirror this scepticism to use ConA clinically until more animal trials have taken place. This is because ConA administration has clearly shown to have affected the adhesive properties of the heart *in vitro* shown in section *3.5.1;* whether that is through direct toxicity of the heart or through the liver injury it causes.

An interesting find from this study is HPC-7 adhesion also showed a general increase; similarly to the pattern observed with neutrophils. Perhaps with increasing the number of animals in the study who were ConA treated, this could have shown a more significant result. Eventually, with further studies, if homing and recruitment of HPC-7s can be monitored and also enhanced in the heart *in vivo*; cardiac damage caused by other organ injuries such as the kidney and liver may be attenuated.

4.7 Future work

The *in vitro* findings in this study have provided scope for further investigation of mechanisms governing HPC-7 homing and recruitment to injured microvascular tissue

in murine hearts *in vivo*. Adhesive properties are enhanced by injury alone *in vitro*. The next step would be to compare how HPC-7s interact with injured and sham hearts *in vivo* using a real time visual method of intravital microscopy. To see any beneficial attributes of HPC-7s to reduce injury, it is important that they are able to home into injured tissue *in vivo*. The data from this study has provided a stepping stone and purpose for expansion into *in vivo* studies.

This entire study relies on the presence of an injury by reperfusion and using a relatively new model of invoking myocardial IRI with the hanging weight system. This model clearly enables the heart to have increased adhesive properties after injury as shown by the data collected. A comparison between ligation of the LAD and the hanging weight system however seems logical before *in vivo* research is carried out. This would eliminate any uncertainty behind the use of this particular model as comparison between the two has not previously been carried out. Eckle *et al,* (2011) have stated the disadvantages of using ligated methods against the hanging weight system; also using the same method for renal ischemia reperfusion injury (Grenz *et al*, 2011). But there is no published data to compare the two.

A method to determine the size of injury on the heart after ligation of the LAD or using the hanging weight system is to measure infarct size. This would indicate the extent of the injury on heart tissue. For the basis of the *in vitro* tests in this thesis, measuring infarct size produced by the injury was not essential. For further research *in vivo,* to test the efficiency of the hanging weight system and any change to injury with HSC recruitment, measurement of infarct size will be an essential requirement. The Evans

blue dye method has been vastly used for testing of viable tissue and infarct size measurement (Eckles *et al*, 2011). A double staining technique has been known as the gold standard method to use for determining the area at risk (AAR) within cardiac tissue (Bohl *et al*, 2009). This involves reocclusion of the LAD after IR injury and perfusion of Evans blue dye into the tissue; which results in the occluded section of the heart to remain unstained. The nonviable tissue is then determined by the use of triphenyltetrazolium chloride (TTC) for staining (Bohl *et al,* 2009; Eckles *et al*, 2011). The use of this method is important for further studies into the benefits of HPC-7s to cardiac injury, as they can help determine whether any benefit to infarct size can be seen after treatment.

Cardiac troponin level assays would also determine the presence of myocardial necrosis and is known as a biomarker for acute MIs (Thygesen *et al,* 2012). Although it seems adhesive properties of the murine heart change after other organ injuries *in vitro*, there is no indication of whether these injuries affect infarct size. As further study, after other organ injuries troponin levels may be measured to determine cardiac injury.

One of the most interesting finds in this study was the effects of other organ injury on cardiac tissue. Future study towards these effects would require true liver and kidney sham surgical procedures for comparison with liver injury and kidney injury. In this study due to time restrictions the control group used for comparison were myocardial surgery sham hearts. For more reliable comparisons for further study, true shams will be required.

Orlic *et al* (2001a; 2001b) has shown in their studies that HSCs can form new myocardium. This study has shown that adhesive properties are increased in cardiac tissue after injury, to allow an increase of HPC-7 adhesion *in vitro*. If HSC administration can show a decrease in infarct size by forming new cardiomyocytes in animal models; this technique to limit cardiac injury after myocardial infarction serves as a future purpose in clinical practise. If homing through pre-treatment of cells can increase their adhesion it may lead to an increased formation of new cardiomyocytes as a replacement (Orlic *et al*, 2001b). Many studies have scepticism about the ability of HSCs to form new cardiomyocytes (Norol *et al*, 2003) and has become a hindrance for them to be moved towards clinical trials to treat forms of heart disease. Kavanagh *et al*, (2013) showed that pre-treatment of HPC-7s can increase their recruitment to the gut, and this study has shown positive indications that this same pre-treatment method may be used to increase recruitment to cardiac tissue. If with future work this can be shown to be significant *in vitro* and *in vivo*, the potential for using HSCs to treat myocardial infarctions looks increasingly promising.

The initial step from this data obtained will be to see if adhesive properties *in vivo* mimic those seen *in vitro* throughout this study; where many other mechanisms may having an influencing factor. Further work through intravital microscopy should identify the mechanisms governing adhesion of HSCs to injured cardiac tissue, and eventually this form of treatment can be used to see if infarct size can be reduced.

4.8 Conclusion

Although mechanisms governing myocardial injury through reperfusion are well known; the techniques used to treat cardiac failure remain ineffective. Stem cell therapy for cardiac regeneration has been abundantly studied but the understanding of the homing and recruitment mechanisms of these cells remain fairly primitive (Orlic *et al*, 2001a; Orlic *et al*, 2001b). The plasticity of HSCs in the heart for one, has currently hindered its progression towards clinical trials (Wagers *et al*, 2002).

This study has presented novel data showing how changes in the adhesive properties of injured cardiac tissue can increase adhesion of beneficial cells such as HSC, and to an extent, MSCs. These initial findings have opened the doors for enhancing the adhesive potential of these cells to cardiac tissue and perhaps aid in the identification of adhesive molecules that may play a crucial role in HSC interaction with cardiac endothelium. Furthermore, this study has – for the first time – validated the use of a Stamper-Woodruff based assay using isolated murine cardiac tissue. All of these findings, taken together, can be used as a stepping stone for *in vivo* research with the ultimate aim to use stem cells to reduce infarct size.

CHAPTER 5:

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