UNIVERSITY OF BIRMINGHAM

Molecular Events Governing Hematopoietic Stem Cell Recruitment *in vivo* in Murine Liver Following Ischaemia-Reperfusion Injury

by

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A thesis submitted to
The University of Birmingham
for the degree of
Doctor of Philosophy

College of Medical and Dental Sciences
School of Clinical and Experimental Medicine
The University of Birmingham
July 2009

Abstract

Evidence suggests haematopoietic stem cells (HSCs) can migrate to injured liver and influence tissue repair. However, the molecular adhesive mechanisms governing HSC recruitment to injured hepatic microcirculation are poorly understood. These mechanisms were investigated in vivo following murine hepatic ischemia-reperfusion (IR) injury. HSC adhesion was significantly enhanced in injured livers and could be reduced by blocking CD49d on HSCs or VCAM-1 in vivo. Blockade of HSC CD18, CD31 or CD44 did not alter adhesion. HSC adhesion in sham treated $CD31^{-/-}$ animals was raised compared to wild-type animals and IR injury did not further raise this adhesion. Studies in vitro demonstrated that HSC treatment with inflammatory cytokines or conditioned media/plasma did not upregulate adhesion molecule expression but CXCL12 and CXCL1 did significantly enhance HSC adhesion to endothelium. However, blockade of CXCR4 (CXCL12 receptor) failed to reduce HSC adhesion in vivo following IR injury. Furthermore, we demonstrated exogenous HSCs were identified primarily in the pulmonary circulation and intraportal injection raised recruitment within the liver irrespective of the presence of injury. This study provides novel evidence for the importance of the VLA-4/VCAM-1 pathway in HSC recruitment to IR injured liver, a pathway that may be manipulated in order to enhance hepatic engraftment of these cells clinically.

“Bernard of Chartres used to say that we are like dwarfs on the shoulders of giants, so that we can see more than they, and things at a greater distance, not by virtue of any sharpness of sight on our part, or any physical distinction, but because we are carried high and raised up by their giant size.”

John of Salisbury, Metalogicon, 1159

“The doubter is a true man of science; he doubts only himself and his interpretations, but he believes in science.”

Claude Bernard

“If The Flintstones has taught us anything, it's that pelicans can be used to mix cement.”

“I saw this movie about a bus that had to SPEED around a city, keeping its SPEED over fifty, and if its SPEED dropped, it would explode! I think it was called, ‘The Bus That couldn't Slow Down’.”

Homer Simpson

- To Caitlin and Jessica -

ACKNOWLEDGEMENTS

There are a number of people to whom I owe an enourmous debt of gratitude for their help and support during my studies. My greatest thanks must go to Dr. Neena Kalia, without whom, none of this would have been possible – I could not genuinely have asked for (or got!) a better supervisor. I must also thank Professor David Adams whose continued enthusiasm and interest for my work has proven invaluable. Thanks also to Professor Jon Frampton, whose input throughout the development of the work has proved extremely useful. I'd also like to thank the Medical Research Council for funding this work.

There are many people in the labs to whom I am grateful. My thanks to Ying Jie for chicken, Craig H for his bumpers, Craig N for his well-educated insights, Yotis for his philosophy lectures and general 'presence', Steve T for his endless supply of jokes (good and bad), Jun for the strange noises and Alice for always smiling (which is sometimes what you need first thing on a Monday morning). The Intravital microscopy laboratory can be a dark and lonely place, so my special thanks go to Ian Holyer. His help and humour over these past years have made my experience much more enjoyable. Outside of the lab, I owe a lot to my friends who have helped to keep me sane: Allan, Andy, Daniel, Gary, John G, John R, Moley, Shaun and Steve. I must also thank Lyn, who kept me supplied with Tea while I was writing.

Ultimately, my biggest thanks must go to my family. Throughout everything, good and bad, my parents have supported me and kept me going even when I thought I couldn't (not just financially!). Without them I would never have made it to this point, so for *everything*, I thank you. Also to Sarah, who has effectively shared me with my mistress (Science) for the past few years. The love, support and understanding you have given made this whole endevour possible. Obviously, I must give my greatest thanks to my children, Caitlin and Jessica. They help me more than they can ever realize. For them, this thesis is dedicated.

Publications arising from this Thesis:

Papers:

Kavanagh DPJ, Durant LE, Crosby HA, Lalor PF, Frampton J, Adams DH, Kalia N. Haematopoietic stem cell recruitment to injured murine liver sinusoids depends on α4β1 / VCAM-1 interactions. *Gut* (In Press)

Oral Presentations:

58th Meeting of the British Microcirculation Society, Kings College, London, UK
Kavanagh DPJ, Durant LE, Crosby HA, Lalor PF, Frampton J, Adams DH, Kalia N.
"Haematopoietic Stem Cell recruitment to hepatic microcirculation following ischemia-reperfusion injury is dependent on the $\alpha_4\beta_1$ integrin"

British Society for Gastroenterology Annual Meeting, SECC, Glasgow, UK
Kavanagh DPJ, Crosby HA, Lalor PF, Frampton J, Adams DH, Kalia N.
"Ischaemia-*reperfusion injury promotes hematopoietic stem cell recruitment to the murine hepatic microcirculation in vivo"*

57th Meeting of the British Microcirculation Society, Queen's University, Belfast, UK
Kavanagh DPJ, Crosby HA, Lalor PF, Frampton J, Adams DH, Kalia N.
"Ischaemia-reperfusion injury promotes hematopoietic stem cell recruitment to the murine hepatic microcirculation in vivo"

UoB Festival of Research and Enterprise, University of Birmingham, UK
Kavanagh DPJ, Durant LE, Crosby HA, Lalor PF, Frampton J, Adams DH, Kalia N.
"Haematopoietic Stem Cell recruitment to hepatic microcirculation following ischemia-reperfusion injury is dependent on the $\alpha_4\beta_1$ integrin"

Poster Presentations:

59th Meeting of the British Microcirculation Society, University of Birmingham, UK
Kavanagh DPJ, Thysse J, Adams DH, Kalia N.
"The cytokine SDF-1α promotes haematopoietic stem cell adhesion in the microcirculation"

25th Conference of the European Society for Microcirculation, Budapest, Hungary
Kavanagh DPJ, Durant LE, Crosby HA, Lalor PF, Frampton J, Adams DH, Kalia N.
"Haematopoietic Stem Cell recruitment to hepatic microcirculation following ischemia-reperfusion injury is dependent on the $\alpha_4\beta_1$ integrin"

CBCS/European Society for Cardiology Summer School on Cardiovascular Sciences, Sophia Antipolis, France

Kavanagh DPJ, Durant LE, Crosby HA, Lalor PF, Frampton J, Adams DH, Kalia N. *"Haematopoietic Stem Cell recruitment to hepatic microcirculation following ischemia-reperfusion injury is dependent on the $\alpha_4\beta_1$ integrin"*

Chapter 1

General Introduction

1. General Introduction

1.1. Hepatic disease and dysfunction

Liver disease is the UK's fifth biggest killer and is the only one of the top five on the rise (British Liver Trust, 2008). Hepatitis, or inflammation of the liver, is a feature of most acute and chronic hepatic diseases and results in the accumulation of inflammatory effector cells such as neutrophils and lymphocytes within the liver tissue. The underlying cause of hepatitis varies, including hepatotropic viruses (hepatitis virus A-E), herpes viruses (such as CMV and EBV) as well as injury induced by alcohol, autoimmune, metabolic and drug induced mechanisms. Regardless of the causative mechanism, the outcome of hepatic inflammation is broadly similar, although the severity of inflammation varies greatly. Some conditions resolve spontaneously (most cases of hepatitis A and E), while others become chronic (hepatitis C and untreated autoimmune diseases) and may progress to cirrhosis and chronic liver failure. In others, hepatocellular damage is so severe that the patient develops acute or subacute hepatic failure, potentially leading to multiple organ dysfunction syndrome and death. In both acute and chronic liver failure treatment options are limited to hepatic transplantation. However, the number of available cadaveric livers in recent years has fallen rapidly, leading surgeons to turn to marginal livers (Angelico, 2005) or, increasingly in many countries to living donor liver transplantation (Abbasoglu, 2008).

1.1.1. Cellular therapy for hepatic disease

Cellular therapies have been used for the treatment of various liver diseases. Indeed, work spanning three decades has suggested that hepatocyte transplantation may be useful in the treatment of metabolic liver disease where there is an underlying genetic defect that is either wholly or predominantly restricted to the hepatocyte. Exogenous hepatocyte administration has shown potential benefits in animal models for numerous disorders, such as Crigler-Najjar syndrome, hereditary tyrosinaemia and familial hypercholesterolemia (Fox and Chowdhury, 2004). Unfortunately, the frequency of liver repopulation from donor hepatocytes is low, although inhibition of host hepatocyte regeneration improves the seeding efficiency of exogenously introduced hepatocytes (Laconi *et al.*, 1998). Furthermore, improvements observed in human trials are dependent largely on the hepatocyte origin and route of injection (Ohashi *et al.*, 2001). In a rat model, systemic introduction of hepatocytes via the femoral artery resulted in low levels of liver sequestration (2% of injected hepatocytes) and high levels of pulmonary recruitment (30%). In the same study, injection of hepatocytes via the portal vein resulted in much greater hepatic engraftment (52%) (Picardo *et al.*, 1996).

1.1.2. Bone marrow derived cells for hepatic regeneration - role of cell differentiation

Relatively recent reports challenged the long-standing dogma that the differentiation pathways of cells are strictly defined (Slack and Tosh, 2001).This phenomena, termed transdifferentiation or metaplasia, is observed when a cell of one lineage adopts the phenotype of another lineage without first dedifferentiating into a more primitive cell or precursor. The potential for transdifferentiation has brought about much interest from the

field of tissue repair, especially for restoration of tissue damaged by degenerative diseases (Gussoni *et al.*, 1999). Bone marrow (BM) cells have received increasing clinical interest as a source for cellular therapy and more specifically bone marrow progenitor cells (BMPCs). The BM is soft tissue found within the interior of most of the major bones and is responsible for maintaining haematopoiesis. It consists of a wide mix of cell types, including immature leukocytes (termed blast cells), lymphocytes and progenitor cells, housed within a complex supporting environment termed the BM stroma (Krebsbach *et al.*, 1999).

Seminal studies by Petersen and colleagues first identified BM derived cells (BMDCs) as a potential source of hepatocytes and hepatic oval cells (Petersen *et al.*, 1999). In this study, female rats were lethally irradiated and rescued with male BM. Animals were subsequently treated with 2-AAF to block endogenous hepatocyte proliferation and with CCl_4 to induce hepatic injury. Y-chromosome positive oval cells were detected at day 9 and day 13 post transplant, while Y-chromosome positive hepatocytes could be detected only at day 13. This suggests initial repopulation of the hepatic oval cells, which then subsequently differentiate and repopulate hepatocytes by day 13 post transplant. This finding was confirmed by the studies of Theise and colleagues (2000a). Female mice were subject to irradiation prior to administration of male BMDCs. Recipients were subsequently tested for expression of the male chromosome. All mice tested at 2 months displayed some degree of Y-chromosome positivity in the liver, suggesting hepatocyte generation from donor cells. It should be noted that in this study, there was no prior injury except for irradiation (which in itself is insufficient to cause severe hepatic injury) suggesting that hepatocyte generation from BMDCs may play a role in basal tissue renewal. Subsequent work by Alison and co-

workers also implicated BMDCs in human hepatic regeneration (Alison *et al.*, 2000). Livers were examined from both female patients who had received a BM transplant from a male donor, and males who had received a liver transplant from a female donor. In both cases, donor derived cells expressing hepatic markers could be detected in the liver.

Subsequent work sought to clarify the functional relevance of this recruitment. Lagasse *et al* injected BMDCs into the *Fah*$^{-/-}$ knockout mouse which represents a model of familial tyrosinemia (Lagasse *et al.*, 2000). Lack of the enzyme fumarylacetoacetate hydrolase leads to the build up of toxic levels of tyrosine which is responsible for the injury. In this model, they found that injection of 1 x 10^6 unfractionated donor BM cells into *Fah*$^{-/-}$ mice generated large nodules of functional, donor derived hepatocytes accounting for up to 30-50% of liver mass. More importantly, this large engraftment was accompanied by an improvement in biochemical liver function.

In order to identify the cell type responsible for this improved outcome, Lagasse and colleagues purified haematopoietic stem cells (HSCs) from BM and used these for transplantation into *Fah*$^{-/-}$ mice. They found that only the HSC population led to wide repopulation in mutant mice, with other purified populations having relatively little hepatic potential. The specific importance of the HSC fraction in tissue repair was re-enforced by work carried out in other organs. It was shown that direct injection of HSCs, defined as Lin$^-$ c-kit$^+$, into the infarcted left ventricular wall resulted in widespread regeneration and improved cardiac function scores. Similar regeneration was not seen with Lin$^-$ c-kit$^-$ cells (Orlic *et al.*, 2001a).

Research from the Grompe laboratory demonstrated in the $Fah^{-/-}$ mouse that hepatic engraftment by donor cells occurred even without the presence of injury, further suggesting that BMDCs play a role in basal hepatic turnover and repair (Wang *et al.*, 2002). However, they found that transplanting enriched HSCs did not enhance hepatocyte replacement beyond that seen with whole BM transplantation. A major finding of the study was that hepatocyte replenishment did not occur without prior irradiation, suggesting that haematopoietic engraftment within the BM was an essential prerequisite for hepatocyte replenishment.

Work by the Weissman lab published in Science (Wagers *et al.*, 2002) further examined the ability of HSCs to transdifferentiate by injecting a single GFP-marked BM derived HSC into lethally irradiated recipients. Analysis of tissues at 4-9 months post-transplant showed GFP^+ cells present in both the brain and the liver, although the frequency of this transdifferentiation was very low. GFP^+ cells were shown to form viable Purkinge cells and hepatocytes, suggesting that HSCs do indeed possess some potential for transdifferentiation. However, the authors stated that HSC transdifferentiation was an 'extremely rare event if it occurs at all'.

The plastic potential of HSCs was further addressed in a paper by Jang *et al,* where HSCs were treated in a porous transwell system with humoral factors released from CCl_4 injured hepatic tissue (2004). Incubation of HSCs with injured liver tissue resulted in a phenotypic shift in the HSCs, which began expressing the hepatic marker cytokeratin-18. Another

recent study by Khurana and Mukhopadhyay also confirmed the ability of HSCs to undergo transdifferentiation *in vitro* after incubation with CCl_4 injured liver (2008). Expression of albumin, CK-18 and hepatocyte enzymes were seen in transdifferentiated cells. Importantly, both studies demonstrated hepatic engraftment of transdifferentiated cells when injected into recipients (Jang *et al.*, 2004, Khurana and Mukhopadhyay, 2008).

1.1.3. Role of Cell Fusion in Hepatic Regeneration

A number of studies have raised doubts about whether transdifferentiation actually occurs, with subsequent studies demonstrating that adult stem cells spontaneously fuse with host cells and take on their characteristics. It was suggested that this event may have been misinterpreted as transdifferentiation in previous studies. Ying and colleagues demonstrated that cells isolated from the embryonic stem cells could fuse with cells from peripheral tissues and undergo reprogramming (Ying *et al.*, 2002). Further studies implicated similar fusion mechanisms in therapeutic liver repair. In serial transplantation studies, Wang *et al* showed that BM derived hepatocytes were formed by cell fusion (2003). Animals received BM from donors which were $Fancc^{-/-}$ (Fanconi anaemia group C gene). These animals have no hepatic disorder and were solely used as they provided a genetically traceable marker for subsequent analysis. Hepatocytes isolated from tertiary recipients had lost the $Fancc^{-/-}$ phenotype, indicating that their formation did not arise from transdifferentiation alone. The authors also noted various chromosomal abnormalities suggesting male/female cell fusion, including 80-XXXY, 120-XXXXYY and 144-4X4Y. It should be noted that transdifferentiation could not be totally ruled out in this study; some 40-XX

hepatocytes were detected in male livers who had been the recipients of female BM. Again, HSC engraftment was essential before hepatocyte replenishment was seen.

A study by Newsome and colleagues examined the role of human cord-blood derived progenitor cells in hepatic regeneration (2003). In this study, human cord blood progenitor cells were shown to be rich in haematoprogenitor cells and to stably engraft NOD/SCID mice at 8 weeks post transfusion. Human hepatocytes could be detected in the liver of NOD/SCID mice at four, six and sixteen weeks post transfusion. Importantly, this study could not find any evidence for cell fusion. Detected human hepatocytes in NOD/SCID mice did not express mouse DNA suggesting fusion had not occurred. In terms of therapy, the level of engraftment observed in this study was relatively low. At most, 0.17% of hepatocytes in NOD/SCID liver were human in origin. Whether this frequency is relevant therapeutically is unclear.

1.1.4. Role of HSC Progeny in Hepatic Regeneration

The prior need for haematopoietic engraftment within the BM suggested that HSC progeny may be responsible for hepatic regeneration, rather than (or in addition to) HSCs themselves. This was further examined by Willenbring and colleagues using the $Fah^{-/-}$ animal model (Willenbring *et al.*, 2004). Transplantation studies revealed that macrophage progenitors or pure populations of macrophages were sufficient for hepatocyte replacement. Indeed macrophage fusion has been documented for some time, especially in the formation of osteoclasts (Vaananen and Laitala-Leinonen, 2008). Willenbring and colleagues further highlighted the specific importance of macrophages as lymphocytes

were not critical for hepatic replenishment. Animals, transplanted with HSCs isolated from $Rag1^{-/-}$ animals (which are unable to form lymphocytes) displayed similar levels of hepatocyte replacement to those receiving wildtype HSCs (Willenbring *et al.*, 2004).

1.1.5. Paracrine functions of stem cells

Building evidence suggests stem cells are able to contribute to tissue repair via paracrine anti-inflammatory mechanisms. In the paw odema model of murine inflammation, tail vein administration of fetal-liver derived HSCs significantly reduces paw weight, lymphatic organ weight and TNFα release following injury (Biziuleviciene *et al.*, 2007). Following transient cochlear ischemia in gerbils, intrascalar injection of HSCs significantly ameriolates hearing loss by inducing expression of glial cell line-derived neurotrophic factor (Yoshida *et al.*, 2007). Mounting evidence suggests that MSCs may possess more potent anti-inflammatory abilities than HSCs. Indeed, administration of MSCs following renal IR injury ameliorates injury through differentiation independent mechanisms (Tögel *et al.*, 2005). In the study by Togel *et al,* reduced pro- and increased anti-inflammatory cytokine expression was noted just 24 hours after injury in MSC treated animals when compared to controls.

1.1.6. Mobilisation of HSCs after Hepatic Injury

Mobilization of HSCs by granulocyte-colony-stimulating factor (G-CSF) has also been observed to ameliorate CCl_4 induced liver injury. Furthermore, these mobilized HSCs were able to promote host hepatocyte repair (Yannaki *et al.,* 2005). Such reparative functions of mobilised HSCs are not limited to the liver. Orlic *et al* demonstrated that stem cell factor

(SCF) and G-CSF led to a 250-fold increase in Lin^- c-kit^+ cells in the circulation which resulted in more circulating HSCs were available to home to infarcted heart and promote repair. Indeed, this mobilisation protocol enhanced heart function post infarct and led to a significant increase in survival rates post injury (Orlic *et al.*, 2001b). Furthermore, BM cells mobilized by G-CSF improve renal function and animal survival following acute renal injury. This effect was shown to be wholly reliant on BM mobilization as ablation of the BM by irradiation removed the protective effect of G-CSF treatment (Iwasaki *et al.*, 2005). In a model of murine renal IR injuy, mobilization of HSCs with SCF/G-CSF reduces damage by lowering granulocyte migration to the kidney (Stokman *et al.*, 2005).

1.1.7. Clinical Trials

As a result of these initial studies, clinical trials have been initiated that investigate the role of stem cells in hepatic injury. To date, there are at least 11 published studies of clinical trials using BM cells for hepatic disease, namely cirrhosis (Houlihan and Newsome, 2008). In a study by Gaia *et al*, eight patients with liver cirrhosis were treated 12 hourly for 3 days with a 5μg/kg dose of G-CSF to induce progenitor cell mobilization (2006). This dose led to robust peripheral mobilization of $CD34^+$ progenitor cells (over thirteen times that seen in untreated controls). Clinical improvement was observed in six of the eight patients at follow-up (between 5-17 months). Although the authors note peripheralisation of $CD34^+$ progenitor cells, this study does not investigate any direct effects of G-CSF on the injured liver. This is important, as it has been suggested G-CSF acts directly on vascular and tubular cells in the kidney (Nishida and Hamaoka, 2006). Furthermore, G-CSF mobilizes other cell

populations, including endothelial progenitor cells and monocytes (Capoccia *et al.*, 2006, Takahashi *et al.*, 1999).

This latter point has been addressed by a number of clinical trials which have used sorting techniques to administer specific subsets of cells to patients. In a study by Gordon and colleagues, five patients with liver insufficiency were treated with a subset of autologous $CD34^+$ cells collected by leukapheresis following G-CSF treatment (2006). Injection of between 1×10^6 and 2×10^8 $CD34^+$ cells resulted in a fall in serum bilirubin and an increase in serum albumin at follow-up after 60 days. A subsequent study of the same patients was published after 12-18 months follow-up. Four out of the five patients had maintained raised serum albumin levels for at least six months (Levicar *et al.*, 2008). It is important to consider that these studies used G-CSF to mobilize $CD34^+$ progenitor cells for leukapheresis and the authors did not take into consideration any direct effect of G-CSF on hepatic injury.

Mohamadnejad and colleagues infused $2.5 – 8.0 \times 10^6$ magnetically separated autologous $CD34^+$ cells into four patients with liver cirrhosis (2007). Cells were obtained from 200ml of BM isolated from the iliac crest and reintroduced, after CD34 sorting, via the hepatic artery. At two weeks post procedure, one patient died as a result of renal and hepatic failure. Another patient in the study had an onset of renal failure at 5 months post procedure. Although all patients displayed some improvement in serum albumin levels, they also had worsened MELD scores (model for end-stage liver disease) which is a quantitative scale of liver disease. Results from this study raise serious concerns regarding the safety of stem cell administration via the hepatic artery. The development of hepatorenal dysfunction appears

to be specific either to the volume or type of cells administered. However, a study from a Brazilian group infused 1 x 10^8 unsorted mononuclear cells via the hepatic artery with no mortality (Lyra *et al.*, 2007).

1.1.8. Mechanisms underlying stem cell therapy – an overview

The mechanisms involved in HSC mediated repair of the liver are not clear. The literature currently suggests that it is in fact HSC progeny that perform restorative functions in the liver following injury rather than HSCs *per se.* A number of groups have shown that BM-derived progeny are responsible for the therapeutic liver repair in the $Fah^{-/-}$ knockout mouse implicating a potential role for BM engraftment prior to therapeutic benefit (Wang et al., 2003, Vassilopoulos et al., 2003). In the $Fah^{-/-}$ injury model, repopulation of the liver by donor BM derived cells can not be identified without preparative whole body irradiation, further suggesting that donor cell engraftment of the BM is required for liver regeneration (Wang et al., 2002). The role of direct recruitment of HSCs to the liver is unclear.

The primary mechanism by which HSCs and BM cells provide therapeutic benefit in the liver is likely to be via cell fusion with the majority of the literature now supporting this process. The role of transdifferentiation appears minor, although many studies do confirm the existence of the phenomena – even in papers that predominantly propose cell fusion as the majoritive mechanism (Wang et al., 2003). While MSCs do hold some potential in terms of paracrine activities at sites of injury, this may not represent a major mechanism for HSC mediated benefit as the evidence for this behavior in HSCs has not been shown in major injury models such as IR injury.

1.2. Bone Marrow Stem Cells

1.2.1. Haematopoietic stem cell definition and function

HSCs are the stem cells responsible for maintenance of homeostasis through continual replenishment of all of the various cellular components of the blood such as lymphocytes, leukocytes and erthyocytes (Figure 1.1). HSCs were identified in the early 1960s by Till & McCulloch as “colony forming units (CFUs)”; defined as cells which were able to give rise to haematopoietic nodules in the spleen following transplantation of BM into irradiated animals (Till and McCulloch, 1961, Till *et al.*, 1964).

The classical definition of a stem cell infers two properties; self-renewal and potency. In terms of self-renewal, the cell must be capable of division, where at least one daughter cell retains the phenotype of the parent cell. This is critical to maintain the stem cell pool. In terms of potency, the cell must be capable of differentiation into more specialized cell types, distinct from the mother cell. The majority of stem cell populations are either totipotent (able to form all cell types) or pluripotent (form multiple tissues/organs). Some stem cells are unipotent, in that they are only able to differentiate into one cell type (hepatocytes are unipotent stem cells).

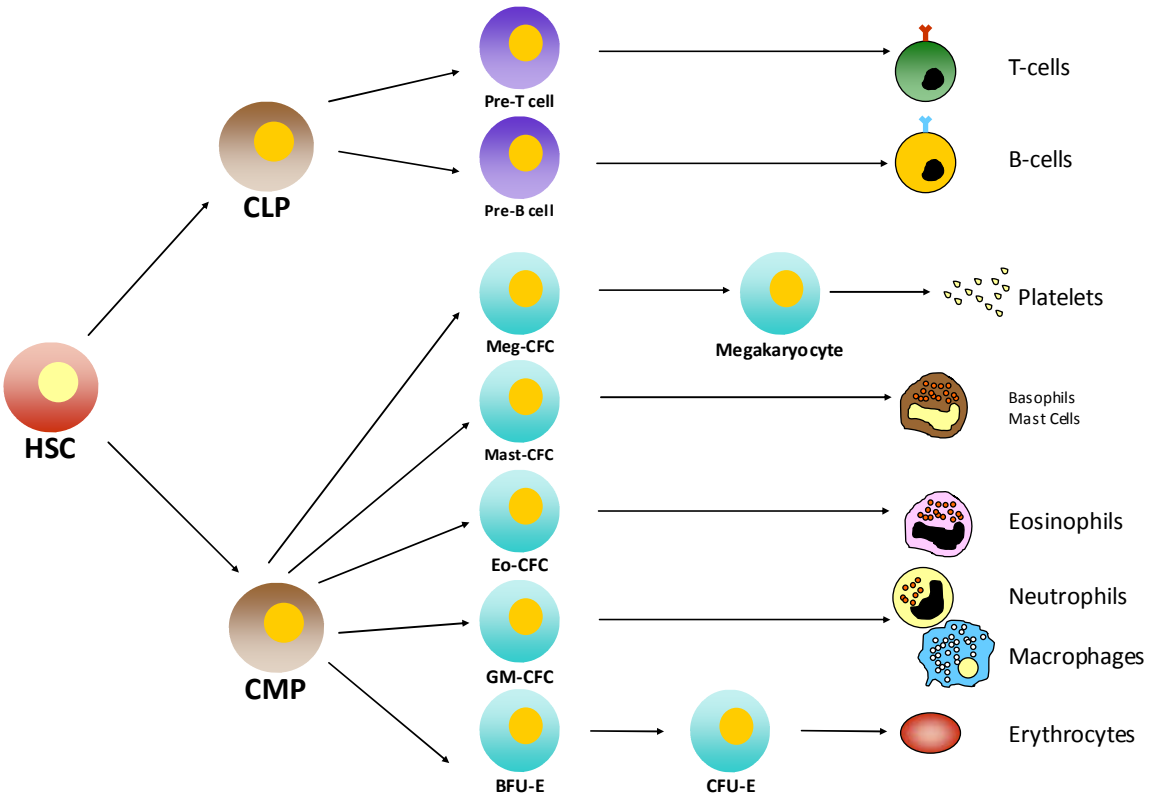


Figure 1.1. *The Haematopoietic System.* The haematopoietic system is maintained by differentiation of HSCs into more committed progenitors. HSCs primarily differentiate into Common Lymphoid Progenitors (CLP) and Common Myloid Progenitors (CMP). These progenitor cells then undergo rounds of differentiation and specialize to form the multitude of cells of the blood. Adapted from a figure from Blood Lines (Metcalf, 2005).

1.2.2. Haematopoietic stem cell identification and isolation

HSCs are found in the BM of the major bones of the body, such as the femur and the tibia. They are typically isolated based on surface marker expression, with the most common defining population being c-kit^{+} Sca-1^{+} Lin^{-} (KSL) (Wognum *et al.*, 2003). Recent work has also suggested endothelial protein C receptor (CD201) be used as an additional marker of HSCs in murine BM (Balazs *et al.*, 2006). In humans, CD34 is commonly used as a stem/progenitor cell marker (Civin *et al.*, 1984) although this is unsuitable in mice because up to 90% of adult murine HSCs do not express this marker (Osawa *et al.*, 1996). HSCs can also be isolated based on their cellular phenotypes. A small population of cells, termed the Side Population (SP), exists in BM and is found to be highly enriched for HSCs. Goodell *et al* demonstrated that a small subset of BM cells, which could be characterized by their ability to efflux Hoescht 33342, are highly enriched for repopulating HSCs (1996).

1.2.3. Other bone marrow stem cell populations

The BM is made up of a number of other stem cell populations in addition to HSCs. Mesenchymal stem cells (MSCs) give rise to the connective tissues of the body, such as osteoblasts, myocytes and adipocytes. Adipose derived adult stem cells (ASCs) possess many of the characterstics of MSCs and are commonly regarded as a population of MSCs located within a different anatomical compartment to 'traditional' MSCs. The term mesenchymal stem cell is under some debate, as they do not appear to maintain characteristics of the mesenchyme (they cannot differentiate into HSCs). Other terms given to this population include marrow stromal cells, multipotent stromal cells and mesenchymal stromal cells.

Endothelial progenitor cells (EPCs) are circulating, BM-derived cells believed to be important in vascular maintenance and vasculogenesis. These cells are thought to derive from hemangioblasts, which themselves are derived from HSCs (Grant *et al.*, 2002). Tissue committed stem cells (TCSCs) have also been found in the BM and give rise to cells that are responsible for repairing individual tissues. Cells expressing early liver markers (such as α-fetoprotein and CK19) have been shown to be located in the BM and possess the ability to mobilize into the blood upon trauma (Kucia *et al.*, 2004). It is thought these cells may act as a 'reserve pool' for tissue repair. Such cells are also resident within the tissues they replenish (Majka *et al.*, 2005). The BM also contains multipotent adult progenitor cells (MAPCs) which are able to differentiate into mesenchymal cell types, as well as endothelium, neural cells and hepatocytes (Schwartz *et al.*, 2002). Interestingly, when MAPCs are injected into early blastocysts, they contribute to almost all somatic cells (Jiang *et al.*, 2002).

1.3. Mechanisms of stem cell recruitment

The surface adhesion molecule profile of HSCs is similar to that of mature leukocytes, likely consistent with the fact that leukocytes are derived from HSCs (Becker *et al.*, 1999, Chan and Watt, 2001). Studies of HSC recruitment in the BM suggest similar adhesive events govern recruitment of HSCs and mature leukocytes and will be discussed later. Although our understanding of HSC recruitment to the BM is increasing, little is known about HSC recruitment to extra-medullary non-BM sites. An increased understanding of the molecular adhesive mechanisms which HSCs use to find their way into damaged or inflamed tissues

may give us a starting point from which to manipulate recruitment to enhance regeneration and repair.

1.3.1. Inflammation and the leukocyte adhesion cascade

The major elements of the leukocyte adhesion cascade that take place during inflammation will be discussed further as HSC recruitment appears to follow a similar pattern as identified from studies conducted within the BM. Inflammation is the localised protective reaction to irritation, injury or infection mediated by the immune system. The immune system is comprised of five classes of leukocytes (neutrophils, eosinophils, basophils, lymphocytes and monocytes), which together form the innate and the adaptive arms of the immune response. The innate response is the immediate or first line of immune defence against new pathogens. It requires no prior exposure to the pathogen and is activated in response to recognition by pattern-recognition receptors (PRRs) of broad ranges of pathogen-specific molecular structures (Akira *et al.*, 2006). Upon infection or tissue damage, resident tissue cells are activated to secrete cytokines and subsequently leukocytes (neutrophils and macrophages) from the innate immune system are recruited into the target area from the circulation. Once within the tissue, neutrophils and macrophages clear invading micro-organisms and damaged cells by engulfment / phagocytosis.

To complement this innate activity, the body has an adaptive immune response, of which the effector cells include T- and B-cells. Although the adaptive immune response occurs later (7-10 days), it has the benefit of antigenic specificity and immune memory, in that subsequent exposure to the same pathogen results in a rapid specific immune response

from memory T and B-cells. Recruitment of the cellular components of the immune system is increased in response to underlying tissue damage or infection. Recruitment of effector leukocytes into inflamed tissue from blood is mediated primarily at the level of the local vascular endothelium, which up-regulates cell adhesion molecules (CAMs) on the cell surface in response to factors released from the underlying tissue. The ability of endothelium to selectively up-regulate combinations of adhesion molecules and chemokines allows it to promote the recruitment of specific types of leukocyte to areas of inflammation.

The concept of leukocyte capture from flow has been known since the observations of Cohnheim some 130 years ago (Cohnheim, 1877). Since then, our understanding of leukocyte recruitment has increased exponentially. The leukocyte recruitment paradigm, in its current form, was initially proposed by Butcher and has tethering, rolling, firm adhesion and endothelial transmigration as its main dynamic components (1991). The adhesion cascade and the major CAMs involved are summarized in Figure 1.2.

1.3.1.1. Leukocyte Capture and Rolling

Blood flow velocity in human skin capillaries is approximately 0.5 mm/sec as detected by laser Doppler (Stucker et al., 1996). Unsuprisingly, leukocytes must be slowed and captured from the prevailing flow if they are to have stable interactions with the local vascular endothelium. This capture step usually involves a phase of rolling in which the leukocyte rolls along the vessel wall as an essential prerequisite to stable adhesion in vessels with high flow. The majority of rolling interactions are mediated by a group of adhesion

molecules called the selectins, so named for their similarity to C-type lectins (Rosen and Bertozzi, 1996). Selectins are Ca^{2+}-dependent transmembrane adhesion proteins found both in vascular endothelium and on circulating leukocytes. A total of three selectins have been identified. E-selectin is found on endothelium, P-selectin on endothelium and platelets and L-selectin on leukocytes.

1.3.1.1.1. E-selectin

Expression of E-selectin, a Type I transmembrane glycoprotein, on inactivated endothelium in most tissues is negligible but is rapidly induced following treatment with cytokines (Brewer and Dam, 2000). Hypoxia is unable to directly mediate up-regulation of E-selectin on isolated endothelial monolayers (Zund *et al.*, 1996), although up-regulation is seen *in vivo* following ischaemia-reperfusion injury (Burke *et al.*, 1998), suggesting an indirect role of hypoxia in regulating this endothelial selectin. The ligands for E-selectin are expressed by a wide variety of cell types, including neutrophils, monocytes, eosinophils, and lymphocytes (Brewer and Dam, 2000). E-selectin recognizes sialylated and fucosylated carbohydrate structures resembling the tetrasaccharide sialyl Lewis x (sLe^x) or sialyl Lewis a (sLe^a) (Zollner and Vestweber, 1996). P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes acts a ligand for E-selectin in addition to its role as a ligand for P-selectin (Katayama *et al.*, 2003). An additional molecule, E-selectin ligand-1 (ESL-1), has been identified as a major adhesive ligand for E-selectin (Steegmaier *et al.*, 1995).

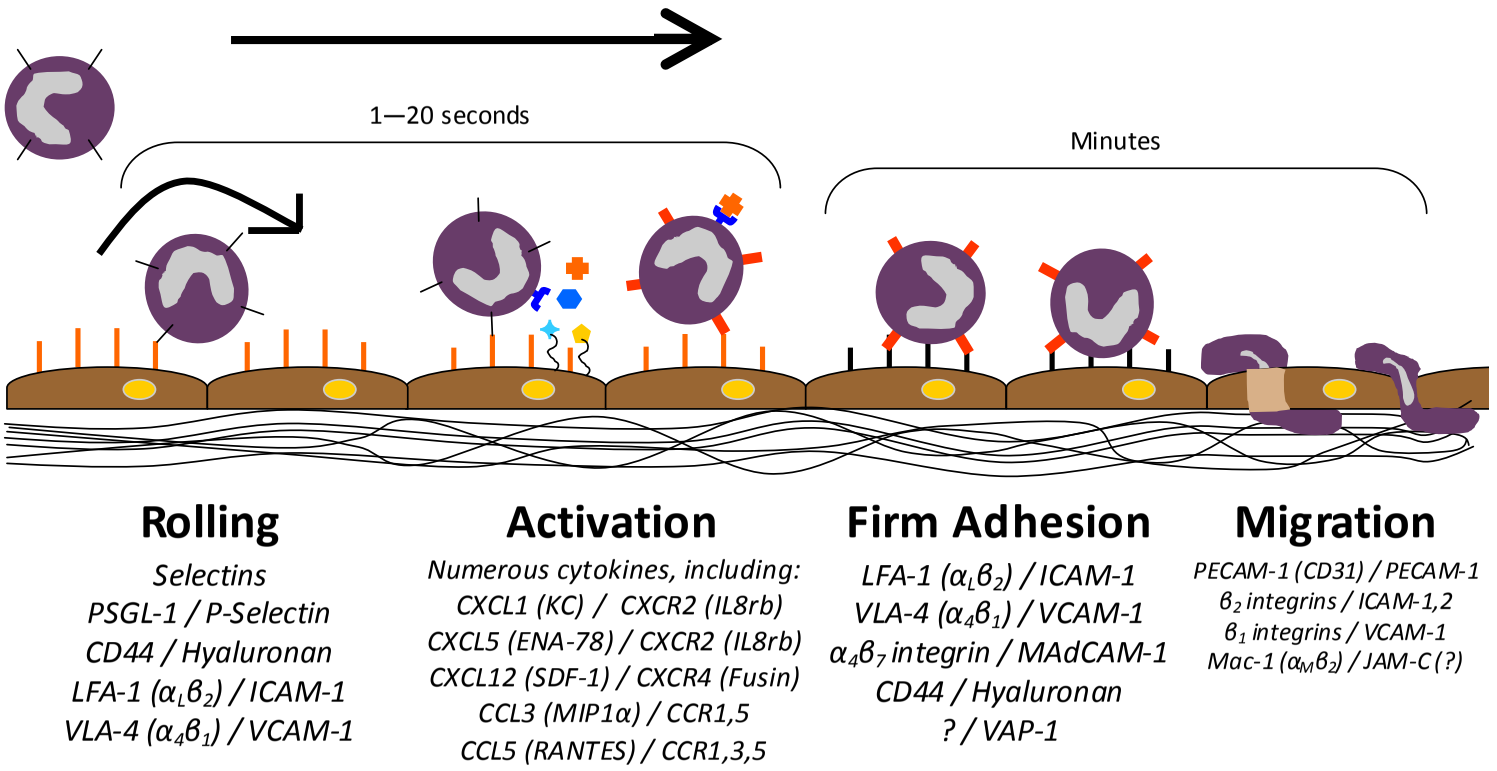


Figure 1.2. *Leukocyte adhesion cascade*. The modern form of the leukocyte adhesion cascade was proposed by Butcher in 1991. Since then, the molecules that govern the various steps of recruitment have been rigorously studied. Additional stages in the cascade have been proposed, including a slow rolling step taking place between the initial higher velocity rolling and activation. There is evidence to suggest that the final step, transmigration, can take place in one of two forms. Transcellular transmigration (A) occurs by the extravasation of leukocytes from blood, though an existing endothelial cell. Paracellular transmigration (B) occurs when leukocytes extravasate through the junctions between endothelial cells. This model is adapted from Butcher & Picker and Ley *et al* .

1.3.1.1.2. P-selectin

P-selectin, similar in structure to E-selectin, is expressed within the Weibel-Palade bodies of endothelial cells and in the α-granules of platelets (Ivetic and Ridley, 2004). Endothelial expression of P-selectin is typically short-lived and achieved by translocation of synthesized P-selectin to the cell membrane in response to inflammatory mediators such as histamine and some cytokines (Chen and Geng, 2006). Increased transcription of P-selectin occurs in response to tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and lipopolysaccaride (LPS) (Brewer and Dam, 2000). Leukocytes express just one high affinity ligand for P-selectin, namely PSGL1, although this can be differentially glycosylated to regulate ligand binding. Located principally in leukocyte microvilli, PSGL-1 binds P-selectin on endothelium and may be important in mediating the initial step of leukocyte rolling. Interestingly, there is evidence to suggest that E- and P-selectin share a degree of redundancy. E-selectin *null* mice display no obvious defect in leukocyte trafficking with a defect only uncovered when the activity of P-selectin is also blocked using a function blocking antibody (Labow *et al.*, 1994).

1.3.1.1.3. L-selectin

L-selectin is expressed on a wide range of circulating cells including monocytes, neutrophils, lymphocytes and haematopoietic progenitors (Brewer and Dam, 2000). L-selectin is structurally similar to the other selectins, but contains only two consensus repeats. There is evidence for at least three different mechanisms by which L-selectin mediates adhesive functions in the microvasculature. Firstly, it is thought that L-selectin expressed on the surface of a circulating neutrophil may display glycan ligands for E-selectin, permitting

direct selectin-selectin rolling interactions on activated endothelium (Picker *et al.*, 1991). Secondly, some evidence from biochemical assays suggests that endothelial cells express molecules which directly mediate L-selectin rolling including heparin sulfate proteoglycans although the precise nature of the interaction is not well understood (Brewer and Dam, 2000). The third mechanism suggests L-selectin can mediate rolling of leukocytes on other stationary leukocytes. Studies carried out in a flow-based adhesion assay provide evidence that stationary leukocytes constitute an L-selectin rich surface on which other leukocytes can roll. It is thought that much of the L-selectin rolling is mediated by PSGL-1 on the surface of stationary leukocytes. This type of L-selectin mediated rolling is completely absent in PSGL-1 knockout mice (Sperandio *et al.*, 2003).

1.3.1.1.4. CD44, ICAM-1 and VCAM-1

Non-selectin molecules can also mediate rolling although their contribution to rolling *in vivo* is not clear. CD44, a cell surface glycoprotein, binds the glycosaminoglycan (GAG) hyaluronan (hyaluronic acid; HA) and has been shown to partly mediate rolling of lymphocytes on tonsillar stromal cells (Clark *et al.*, 1996). Other GAGs such as chondroitin sulfate also serve as rolling ligands for CD44. Interestingly, a specific glycoform of CD44 (HCELL) can act as a ligand for L-selectin (Dimitroff *et al.*, 2000).

There is some evidence to suggest that members of the immunoglobulin superfamily intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) can mediate rolling of leukocytes on endothelium contrary to their 'traditional' role in stable adhesion. Arteriolar leukocyte rolling is significantly decreased in ICAM-1 knockout

animals (Sumagin and Sarelius, 2007), while the integrin VLA-4 (CD49d/CD29, $\alpha_4\beta_1$) is able to mediate tethering and rolling on VCAM-1 (Alon *et al.*, 1995). These findings are particularly interesting, as they suggest the existence of a large degree of redundancy across the adhesion cascade as a whole.

1.3.1.2. Leukocyte Activation

After leukocytes have been slowed from flow, factors released from the local endothelium mediate activation of the leukocyte which subsequently allows the leukocyte to progress to firm adhesion. It has been shown previously that for successful leukocyte recruitment, the most important factor is the duration for which the cell remains in contact with the local endothelium (Jung *et al.*, 1998). Therefore, for firm adhesion to occur, the leukocyte must be sufficiently activated by factors released from the local endothelium.

Cytokines are small (8-30kDa) secreted proteins, which play a central role in the maintenance and development of an immune response. In the context of inflammation, cytokines are normally secreted from activated endothelial cells where they interact with specific cytokine receptors on target cells. Typically, the target cells are inflammatory cells such as neutrophils and monocytes. Cytokines are classified according to their structure/function. Chemokines are cytokines that are able to establish a chemotactic gradient for surrounding cells **(chemo**tactic cyto**kines)**. There are four main families of chemokines whose names are based on their structure - **CC, CXC, C, and CX3C** (representing cysteine crosslinks and amino acid sequence between them). CXCL12 (CXC-Ligand 12; CXCL12) is a well established member of this family. Interleukins (IL) are cytokines that were

first identified to be released by leukocytes and which act on other leukocytes but can also be released from cells including the endothelium (Delneste *et al.*, 1994). Hence, IL-1 is a typical pro-inflammatory member of this family.

The mechanisms by which cytokines act are relatively varied. They commonly operate in a paracrine fashion, acting on cells within the local environment. Cytokines can also operate in an endocrine fashion, acting on cells distant to the local environment. This is a common cause of tissue-initiated systemic inflammation (Van der Meide and Schellekens, 1996). Cytokines are also able to act in an autocrine fashion, acting on the very cells they have been produced by.

1.3.1.2.1. Endothelial proteoglycans

Although most chemokines are secreted in soluble form from the local endothelium, a significant proportion are found bound to the luminal surface of the endothelial cell via proteoglycans such as heparin sulfate (Middleton *et al.*, 2002). This creates a localized inflammatory environment, allowing chemokines to interact with leukocytes without being removed from the local area by blood flow. Furthermore, it is suggested that chemokine binding to proteoglycans actually enhances their biological activity (Middleton *et al.*, 2002). Indeed, the binding of IL-8 to endothelial proteoglycans prolongs its biological activity by preventing degradation (Goger *et al.*, 2002).

1.3.1.2.2. Integrin priming

One of the major mechanisms by which chemokines activate leukocytes is by the priming of integrins, facilitating firm adhesion. Chemokines and cytokines can up-regulate adhesion molecule expression on the leukocyte cell surface. This process is also relatively quick as treating neutrophils or monocytes with IL-8 or GRO-α for 15 minutes induces a robust upregulation in CD11b (α_M integrin) (Conklyn *et al.*, 1996). In addition to upregulation, treatment of lymphocytes with the CC-chemokine ligand CCL25 or CCL28 activates the $\alpha_4\beta_7$ integrin, facilitating adhesion via mucosal addressin cell adhesion molecule-1 (MAdCAM-1) under conditions of shear stress (Miles *et al.*, 2008). Furthermore, treatment of immature human $CD34^+$ cells with the chemokine CXCL12 (SDF-1) activates the integrins LFA-1 ($\alpha_L\beta_2$ integrin; CD11a/CD18), VLA-4 and VLA-5 ($\alpha_5\beta_1$; CD49e/CD29), promoting ICAM-1 and VCAM-1 dependent adhesion and transmigration (Peled *et al.*, 2000). The activation of adhesion molecules is relatively rapid, with some cells needing less than five minutes treatment with chemokine to elicit activation, and some displaying adhesive characteristics after just one minute of treatment (Peled *et al.*, 1999a).

Integrin-mediated adhesion can be upregulated independent of changes in either surface expression or avidity. Treatment of lymphocytes with CXCL12 promotes rapid (sub-second) clustering of VLA-4 via G-protein coupled signaling; this permits stronger adhesive bonds between VLA-4 clusters and VCAM-1 (Grabovsky *et al.*, 2000). Interestingly, there is evidence to suggest that affinity and clustering can be stimulated independently (Abram and Lowell, 2009). An additional level of complexity exists; integrin clustering can take place in either microclusters (0.1nm – 1.0nm) or macroclusters (200+ nm) (Kim *et al.*, 2004).

Cytokines released from leukocytes can also act upon local vascular endothelium, raising the adhesive status of the bed and increasing the chance of firm leukocyte adhesion. Treatment of endothelial monolayers with IL-4 or IL-13, produced by a variety of immune cells, induces a robust increase in VCAM-1 expression and morphological changes, including the appearance of endothelial fenestrations (Kotowicz *et al.*, 1996).

1.3.1.3. Leukocyte Firm Adhesion

Following activation, leukocytes firmly adhere to endothelium. In the majority of tissue beds, firm adhesion does not occur without prior rolling. However, in low-flow tissue beds such as the liver, it has been shown that tethering and rolling on endothelium is not a prerequisite for adhesion. This may be a result of the diameter of leukocytes being similar to the diameter of the sinusoidal capillary (Kubes *et al.*, 2002).

1.3.1.3.1. Integrins and their receptors

Traditionally, the major adhesion molecule family involved in firm adhesion are the integrins and their ligands (Eniola *et al.*, 2003). The integrins are a family of adhesion molecules that regulate cell-cell and cell-matrix adhesion. Integrins exist in their functional form as a heterodimer, with larger α-subunits (120-180kDa) and smaller β-subunits (90-110kDa) (Seftor, 1998). The specific combination of α- and β-subunits defines the specificity of the integrin for its ligand (Table 1.1). There are a total of 24 heterodimers known in existence, made up from 18 α-subunits and 8 β-subunits (Takada *et al.*, 2007). There is very little homology between subunits. The sequence homology between α-subunits is 30%,

while amongst β-subunits sequence homology is 45% (Takada *et al.*, 2007). The importance of integrins becomes apparent from the human disorder of leukocyte adhesion deficiency (LAD). Three classes of LAD have been identified, with LAD I resulting from a deficiency or complete lack of β_2 integrin expression. LAD I patients tend to succumb to frequent aggressive bacterial infections and rarely survive past the first two years of life (Anderson and Springer, 1987). Failure of bacterial clearance results from severe failures in the leukocyte adhesion cascade and a marked inability of blood leukocytes to emigrate into peripheral tissues (Etzioni *et al.*, 1999).

Efficient adhesion of integrins to their ligands requires conformational changes in the molecule which facilitate binding to endothelial counter-receptors. Integrins bind to members of the Immunoglobulin superfamily (IgSF) expressed on the endothelial surface namely ICAM-1, ICAM-2, VCAM-1 and MAdCAM-1. All members of the IgSF have an immunoglobulin domain, named after the immunoglobulin molecules in which the structural domain was first identified (Barclay, 2003). Endothelial beds show tissue-specific differences in levels of the various IgSF adhesion receptors; for example, MAdCAM-1 is preferentially expressed in the intestine where it promotes gut specific recruitment of lymphocytes (Ogawa *et al.*, 2005). Even though some molecules are tissue specific in normal conditions, they can exhibit ectopic expression in some disease states. Indeed, during some inflammatory hepatic states, expression of MAdCAM-1 can be identified on sinusoidal endothelium (Adams and Eksteen, 2006).

Integrin	Synonyms	Ligands	Found on
$\alpha_L\beta_2$	LFA-1, CD11a/CD18	ICAM-1, ICAM-2, ICAM-3, ICAM-5	Lymphocytes, monocytes, macrophages, neutrophils, HSCs
$\alpha_M\beta_2$	Mac-1, CR3, CD11b/CD18	iC3b, fibrinogen, heparin, plasminogen, ICAM-1, RAGE, JAM-C	Monocytes, macrophages, neutrophils, NK cells
$\alpha_X\beta_2$	p150,95, CR4, CD11c/CD18	ICAM-1, ICAM-2, ICAM-4, VCAM-1, iC3b, fibrinogen, heparin, plasminogen,	Monocytes, macrophages, NK cells, dendritic cells, HSCs
$\alpha_D\beta_2$	-	ICAM-3, VCAM-1	Monocytes, macrophages, neutrophils, eosinophils
$\alpha_4\beta_1$	VLA-4, CD49d, CD49d/CD29	VCAM-1, fibronectin	Lymphocytes, monocytes, eosinophils, HSCs
$\alpha_4\beta_7$	LPAM-1	MAdCAM-1, fibronectin	Lymphocytes, monocytes, NK cells
$\alpha_E\beta_7$	HML-1	E-cadherin	T-lymphocytes
$\alpha_1\beta_1$	VLA-1, CD49a/CD29	Collagen	T-/B-lymphocytes, monocytes
$\alpha_2\beta_1$	VLA-2, GPIa, CD49b/CD29	Collagen	T-/B-lymphocytes, monocytes, progenitor cells(?)
$\alpha_5\beta_1$	VLA-5CD49e, CD49e/CD29	Fibronectin	T-lymphocytes, monocytes, progenitor cells(?)
$\alpha_6\beta_1$	VLA-6, GPIc, CD49f/CD29	Laminin	T-lymphocytes, monocytes

Table 1.1. *Integrin expression on leukocytes.* Numerous integrins are expressed on circulating leukocytes, with a large degree of promiscuity between integrin ligands. There is a small degree of evidence for the presence of $\alpha_2\beta_1$ and $\alpha_5\beta_1$ on $CD34^+$ progenitor cells, although this has not been confirmed on more 'pure' HSC isolates. Adapted from various sources.

1.3.1.3.2. CD44

The step of firm adhesion may also involve other non-integrin molecular mechanisms. CD44 can support firm adhesion through binding either to HA (Zhuo *et al.*, 2006) or via interactions with VLA-4 (Nandi *et al.*, 2004). Additionally, CD44-mediated activation of surface integrins has been shown to promote recruitment of cancer cell lines to endothelium (Fujisaki *et al.*, 1999).

1.3.1.4. Transmigration

Following firm adhesion, leukocytes must transmigrate through the endothelial monolayer and into the underlying tissue parenchyma/stroma. Paracellular transmigration describes the passage of a leukocyte through the endothelium, traveling between the junctions of endothelial cells. This is the more well-established method of transmigration, although evidence now also exists for transcellular transmigration in which the leukocyte migrates *through* a single endothelial cell (Feng *et al.*, 1998). However, this occurrence is much rarer. Various adhesion molecules have been implicated in the transmigration process, including the integrins, the integrin IgSF counter-receptors, platelet-endothelial cell adhesion molecule-1 (PECAM-1; CD31), CD99 and junctional adhesion molecules (JAMs) (Ley *et al.*, 2007).

In the paracellular route, leukocytes migrate along the endothelium until they reach sites between endothelial cells which are supportive of leukocyte transmigration. The process of transmigration itself is complex (Petri *et al.*, 2008). Engagement of endothelial adhesion molecules with their ligands reduces inter-endothelial contacts, thus promoting

transmigration across the endothelial layer (Ley *et al.*, 2007). Endothelial cells dynamically reorganize the localization of adhesion molecules to further promote leukocyte transmigration. Therefore, molecules which may hinder transmigration are translocated away from the inter-endothelial junction, while those which enhance transmigration are concurrently concentrated at the junctional areas (Ley *et al.*, 2007, Shaw *et al.*, 2001). Engagement of junctional adhesion molecules may cause downstream signaling that results in the loosening of adhesive contacts between endothelial cells, particularily VE-cadherin (Dejana, 2004). The adhesion molecules JAM-A and PECAM-1 are connected to the cadherin system, via intracellular binding partners (Ley *et al.*, 2007). There appears to be some stimulus specific modulation of the transmigration process; PECAM-1, ICAM-2 and JAM-A dependent venular transmigration of leukocytes is seen after treatment with IL-1β, but not after treatment with TNF-α (Nourshargh *et al.*, 2006). This may act to selectively permit transmigration of specific leukocyte subsets.

1.3.1.5. Sub-endothelial migration

Once leukocytes have traversed the endothelial layer, they then must navigate through the layers of basement membrane and into the surrounding tissue. The basement membrane of most tissues is relatively complex and is made up of laminins, collagen IV and connecting proteins. Therefore, it constitutes a formidable barrier for a leukocyte to cross. This process appears to be mediated, at least in part, by PECAM-1 and α6 integrin family members (Wang *et al.*, 2005). Migration of leukocytes is further influenced by the presence of a pericyte layer subsequent to the basement membrane in most vessels. Currently, the

importance of interactions between leukocytes and pericytes is unclear, although pericytes do express adhesion molecules that are able to interact with passing leukocytes (Verbeek *et al.*, 1995). The liver sinusoidal bed differs from most vascular beds in that the sinusoidal endothelium lacks a classical basement membrane as it is separated from the underlying hepatocytes by the Space of Disse. How cells cross the Space of Disse is unknown but recent evidence suggests that hepatic stellate cells, which act as pericytes for sinusoidal endothelial cells, can modulate migration beyond the endothelium (Holt *et al.*, 2009).

1.3.2 HSC recruitment to Bone Marrow Microcirculation

The BM microcirculation also consists of sinusoidal endothelium, similar to that seen in the liver. Futhermore, primary bone marrow endothelial cells also express CD31, CD44, ICAM-1, and VCAM-1 (Candal *et al.*, 1996). Recent *in vivo* intravital microscopy (IVM) studies have partially elucidated adhesion pathways governing transplanted HSC and progenitor cell (HPC) homing to BM microcirculation. All the different classes of adhesion molecules appear to play a role in anchoring immature hematopoietic cells to BM microcirculation. Work by Frenette *et al* suggests that the BM homing of HSCs in normal animals is regulated primarily by the P- and E-selectins, with the role of VCAM-1 only uncovered after the selectin-mediated recruitment has been compromised (1998). Hence, there appears to be a potential redundancy for VCAM-1 in BM homing. Indeed, treatment of wildtype mice with an anti-VCAM-1 antibody did not reduce HSC homing in lethally irradiated mice (Frenette *et al.*, 1998). HSC homing to BM was significantly impaired in P- and E-selectin knockout mice, an impairment which could be further exaggerated by treatment of the mice with a VCAM-1 blocking antibody (Frenette *et al.*, 1998). The ligand expressed on HSCs for these selectins

is unclear. Although enzymatic modification of PSGL-1 on the HSC surface increases initial interactions with the BM stroma, it does not significantly improve their homing (Hidalgo and Frenette, 2005).

CD44 and HA have also been implicated in HSC homing to the BM. Enriched human $CD34^+$ cells treated with anti-CD44 mAbs display a marked defect in BM homing following intravenous injection into NOD/SCID mice (Avigdor *et al.*, 2004). This defect is at least partially mediated by HA in the BM. Treatment of animals with the enzyme hyaluronidase, which degrades HA, elicits a similar, although not as marked, decrease in BM homing (Avigdor *et al.*, 2004). It is thought that HA-mediated homing is dependent on CXCL12 activation of CXCR4, which then acts on pathways that modify the localization and activity of CD44 (Avigdor *et al.*, 2004). Interestingly, a specific glycoform of CD44 (termed HCELL) also acts as a ligand for E- and L-Selectin (Sackstein, 2004). In fact, the affinity of HCELL for L-selectin is around five times the affinity of PGSL-1 for L-selectin (Dimitroff *et al.*, 2001). Furthermore, HCELL is the most potent ligand for E-selectin expressed by any cell (Sackstein, 2004).

1.3.2.1. CXCL12/CXCR4 in BM homing

The G-protein coupled chemokine receptor CXCR4 and its accompanying ligand CXCL12 (SDF-1), are implicated heavily in the recruitment of HSCs into the BM microcirculation. Interestingly, it should be noted that CXCR4 appears to act as a universal BM homing molecule as it is also utilised by CXCR4 expressing neutrophils to home to BM prior to

apoptosis (Martin *et al.*, 2003). Furthermore, tumour micrometastases in the BM also express high levels of CXCR4 (Kaifi *et al.*, 2005). Injection of HSCs into the venous circulation results in rapid clearance, within minutes (Lapidot *et al.*, 2005). These cells home to various tissues, including the spleen, liver and lungs. From these tissues, HSCs may subsequently migrate toward and home into the BM (Lapidot *et al.*, 2005), where they are observed within CXCL12 expressing microdomains (Sipkins *et al.*, 2005). This observation further highlights the importance of the CXCL12/CXCR4 axis in HSC recruitment. Expression of CXCL12 is regulated in part by oxygen tension via the activities of the transcription factor hypoxia inducible factor-1α (HIF-1α) (Ceradini *et al.*, 2004). As the BM is itself partially hypoxic (Ceradini *et al.*, 2004, Lapidot *et al.*, 2005), CXCL12 is expressed by many of the stromal cells that line the microenvironments in which HSCs home (Kortesidis *et al.*, 2005). The CXCL12 concentration gradient is critical in retaining HSCs within the BM. Reduction of BM CXCL12 levels results in HSC mobilization into the circulation (Petit *et al.*, 2002), while increased levels of circulating CXCL12, such as that seen following an extramedullary hypoxic injury, promote emigration of HSCs from the BM following an CXCL12 concentration gradient (Hattori *et al.*, 2001). In addition to the chemotactic properties of CXCL12, it also has a well defined signaling effect on HSCs. Activation of CXCR4 by CXCL12 results in the subsequent rapid and robust activation of integrins on the HSC surface, namely LFA-1, VLA-4 and VLA-5 (Peled *et al.*, 2000). This may help facilitate the firm adhesion of these cells by anchoring them to their respective ligands.

Although the CXCL12/CXCR4 axis works to promote HSC adhesion and retention in the BM, ultimately retention is reliant on specific adhesion molecule interactions within the BM

stroma. BM endothelial cells constitutively express the IgSF molecule VCAM-1 (Jacobsen *et al.*, 1996) and interactions between this molecule and VLA-4 are important in the homing of these cells to the BM following systemic administration. Early work illustrated this importance by demostrating that pretreatment of murine haematopoietic progenitors with an $\alpha_4\beta_1$ integrin or VCAM-1 blocking antibody significantly impaired BM homing in lethally irradiated mice by around 50% (Papayannopoulou *et al.*, 1995).

1.3.3 HSC recruitment to Non-BM tissue Microcirculation

Although there is conflicting evidence whether HSCs are actually beneficial during hepatic injury and if so, the mechanism of benefit (fusion vs differentiation), it remains irrefutable that if stem cells are to be beneficial in treating human disease, a proper understanding of their recruitment to damaged tissues is crucial. Our knowledge on the molecular adhesive mechanisms that govern recruitment of HSCs to BM is increasing, but it is still unclear whether similar adhesive events govern HSC recruitment to injured non-BM tissue endothelium. Indeed, while much published work has examined the capacity of stem cells as a therapy, very little work has tried to underpin the recruitment mechanisms these cells use to engraft within non-BM sites.

A recent study by Zhang *et al* has identified the molecular mechanisms that underpin progenitor cell recruitment in experimental models of mice undergoing myocardial infarction (Zhang *et al.*, 2007). The authors report that recruitment and subsequent fusion of progenitor cells is dependant on VLA-4 interactions with VCAM-1. This currently appears

to be the only published study that identifies a specific adhesion molecule pair that regulates HSC engraftment to an inflamed non-medullary site. Recruitment mechanisms are still unclear for other injured sites, including the liver. A better understanding of such mechanisms would facilitate future manipulation of the engraftment process.

1.4. Hepatic ischemia-reperfusion injury

In order to investigate the adhesive molecular mechanisms governing HSC recruitment to the injured hepatic microcirculation, the studies within this thesis have utilised an acute model of murine hepatic injury induced by ischaemia and subsequent reperfusion. Hepatic ischemia-reperfusion (IR) injury is a clinically important problem. Poor success and morbidity associated with orthotopic cadaveric liver transplantation and major hepatic resections is commonly a result of IR injury (Carden and Granger, 2000). IR injury refers to tissue damage caused paradoxically when blood flow is returned to the tissue after a period of restricted blood flow (greek. *isch*- restriction, *haema*- blood). Lack of oxygen and nutrients creates a condition in which the restoration of blood flow results in inflammation and oxidative damage through generation of reactive oxygen species (ROS). Reperfusion therefore exacerbates the ischemic injury. Indeed, in the liver, the level of hepatic damage seen after 3 hours of ischaemia and 1 hour of reperfusion is much greater than after 4 hours of ischaemia alone (Eltzschig and Collard, 2004).

Initially, ischaemia causes metabolic disturbances within cells resulting in dysregulation of energy pathways and ROS release (Figure 1.3). At the outset, cells experience a loss of ATP,

rise of intracellular sodium and cell oedema (Fondevila *et al.*, 2003). Ischaemic metabolic failure of the mitochondrial respiratory chain leads to the generation of hydroxyl radicals during reperfusion through an increased rate of hydrogen peroxide (H_2O_2) production (Gonzalez-Flecha *et al.*, 1993). Evidence suggests that hepatocytes and Kupffer cells are also important origins of oxidative stress, although the latter appear to play a minor role as depleting Kupffer cells fails to attenuate IR injury (Jaeschke, 2003, Reinders *et al.*, 1997, Kumamoto *et al.*, 1999).

Hepatic sinusoidal perfusion failure is also common during the post-reperfusion phase, resulting in microcirculatory failure (Jaeschke, 2003). The vascular tone of the hepatic sinusoids is a result of a delicate balance between vasodilatory molecules (such as nitric oxide and prostacylin) and vasoconstrictory molecules (such as endothelin-1, ET-1). Any imbalance between these factors during IR results in dysregulation of vascular tone and sinusoidal blood flow, promoting mechanical leukostasis (Vinores *et al.*, 2007). Indeed, pharmacological restoration of vascular tone following IR improves blood flow and attenuates injury (Shiraishi *et al.*, 1997). Injury is also exacerbated by the substantial endothelial recruitment of platelets. Upto one third of circulating platelets are found in the IR injured liver during reperfusion (Sindram *et al.*, 2000). These platelets form micro-aggregates and are activated when examined by electron microscopy (Cywes *et al.*, 1993). Platelets can induce apoptosis in sinusoidal endothelial cells during tissue reperfusion which can be prevented by blocking their adhesion (Sindram *et al.*, 2000).

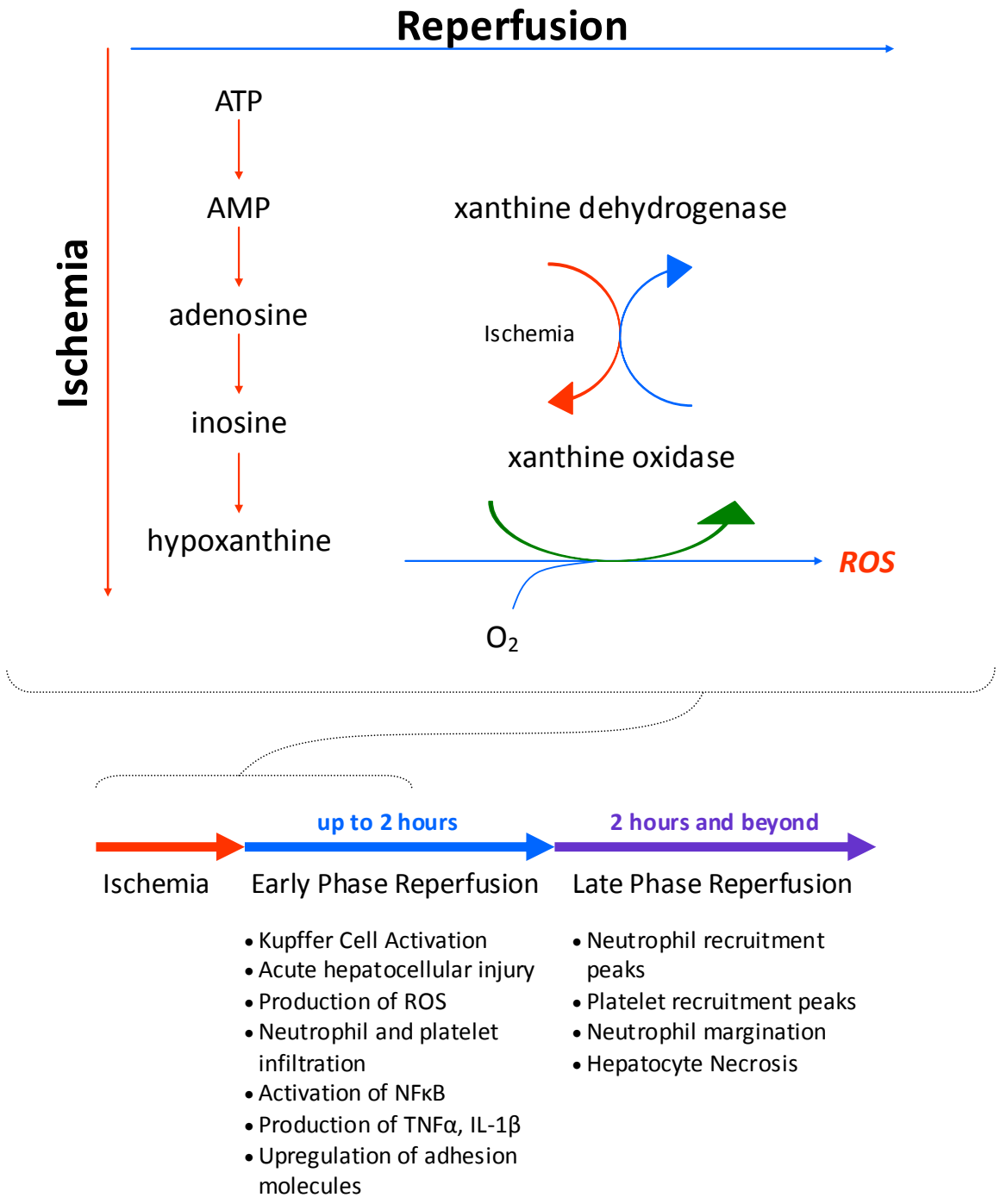


Figure 1.3. *Mechanisms of Ischemia-Reperfusion injury.* Initial injury during the ischemic phase takes place intracellularly, with dysregulation of energy metabolism. This leads on to further oxidative damage, which subsequently promotes direct damage and leukocyte recruitment during the early and late phases of reperfusion. Adapted from various sources.

As a result of both the oxidatively stressed environment and the vascular mechanical disturbances during IR, hepatic microvessels display marked neutrophil accumulation, at least in part as a consequence of increased capture by endothelial cell adhesion molecules. During the reperfusion phase, neutrophil activation occurs which also results in the generation of further damaging ROS (Marti *et al.*, 2004). Leukocyte recruitment is additionally amplified by the release of cytokines from the injured tissue, such as tumour necrosis factor-α (TNF-α) and interleukin (IL)-1. During IRI, plasma levels of TNF-α and IL-1 are raised within 5 minutes of the reperfusion phase (Suzuki and Toledo-Pereyra, 1994). Both TNF-α and IL-1 mediate activation of the pro-inflammatory transcription factor NF-κB which leads to the generation of further cytokines and chemokines, as well as synthesis of adhesion molecules (Ghosh *et al.*, 1998). Hypoxia itself activates a number of additional transcription factors, such as hypoxia-inducible-factor-1α (HIF-1α) (Lopez-Lazaro, 2006). Hypoxic induction of HIF-1α drives a robust release of CXCL12, establishing a chemokine gradient for inflammatory cell recruitment (Ceradini *et al.*, 2004).

Although neutrophil accumulation has been shown in the sinusoids *in vivo* (Vollmar *et al.*, 1994), the adhesion molecules responsible have not been fully identified (Jaeschke, 2003). It is also thought that the accumulation of neutrophils in sinusoids is a result of numerous mechanical factors, such as vasoconstriction, swelling of the vascular lining and reduced flexibility of activated neutrophils (Jaeschke, 2003). Whether these mechanical recruitment mechanisms also apply to other cell types is unclear.

1.4.1. Role of sinusoidal adhesion molecules in hepatic IR

During IR, the hepatic sinusoidal endothelium plays an active role in both establishment of injury and maintenance of an inflammatory state (Montalvo-Jave *et al.*, 2008). In the hyperacute stage of the injury, sinusoidal constriction resulting from vasomodulatory imbalance promotes leukostasis (Jaeschke *et al.*, 1990). Accumulation of neutrophils within the sinusoids perturbs flow, but is not thought to cause complete occlusion (Vollmar *et al.*, 1996).

The recruitment of leukocytes and platelets is mediated by adhesion molecules expressed on the sinusoidal endothelium. Under basal conditions, sinusoidal endothelium expresses low levels of ICAM-1 and VCAM-1, with no expression of any of the selectin family members (Lalor and Adams, 1999). Following insult, levels of ICAM-1 and VCAM-1 are raised, and although some vessels express E- and P- selectin, selectins are largely absent from the sinusoids (Wang *et al.*, 1999, Adams *et al.*, 1996, Grant *et al.*, 2001). Other endothelial adhesion molecules such as MAdCAM-1 can be detected under certain specific inflammatory conditions and could play a role in IR injury (Ando *et al.*, 2007). Additionally, vascular adhesion protein-1 (VAP-1) is constitutively expressed in the liver and is one of the few endothelial beds to exhibit constitutive expression (Lalor and Adams, 1999). VAP-1 has been shown to play an important role in lymphocyte recruitment to rejecting rat and murine liver allografts (Martelius *et al.*, 2004) and during inflammation in cerebral IR injury (Xu *et al.*, 2006), but it is unclear whether it has any role in hepatic IR.

The adhesion pathways mediating neutrophil recruitment during IR injury also include an important role identified for ICAM-1 (Montalvo-Jave *et al.*, 2008). ICAM-1 is upregulated on

sinusoidal endothelium during hepatic IR injury, due at least in part to the activity of TNF-α released from activated Kupffer Cells (Mosher *et al.*, 2001, Colletti *et al.*, 1998). Pretreatment of animals with an ICAM-1 blocking antibody, immediately following reperfusion, reduces the level of hepatic injury seen at 72 hours post-reflow (Kuzume *et al.*, 1997). Indeed, ICAM-$1^{-/-}$ animals show a higher rate of survival following total hepatic IR injury when compared with wild-type controls (Yadav *et al.*, 1998). It is thought that neutrophil adhesion via ICAM-1 is mediated through interactions with integrins LFA-1 and/or Mac-1 (Monson *et al.*, 2007).

Despite the lack of strong E and P-selectin expression on inflamed sinusoidal endothelium, functional studies suggest that selectins play a role in the pathogenesis of hepatic IR injury. Administration of β-SQDC9, an L- and P- selectin inhibitor, into IR treated animals immediately prior to reperfusion significantly reduces plasma transaminase levels, as well as reducing neutrophil infiltration (Shima *et al.*, 2005). Following hepatic IR, P-$selectin^{-/-}$ mice display a marked attenuation of neutrophil infiltration and subsequently less hepatic injury (Singh *et al.*, 1998). Recruitment may be mediated via direct interactions between P-selectin and neutrophil PSGL-1, although it should be noted there is evidence to suggest that P-selectin, in tandem with ICAM-1, may attenuate injury by modulation of inflammatory chemokine release rather than direct adhesive actions (Monson *et al.*, 2007). A similar role has been suggested for L-selectin in hepatic IR injury. L-$selectin^{-/-}$ animals display a marked decrease in neutrophil infiltration compared to wild-type animals (Yadav *et al.*, 1998). Hepatic IR injury increases E-selectin expression on sinusoidal endothelial cells *in vitro*, although whether this translates to an *in vivo* role is less clear (Burke *et al.*, 1998).

E-selectin has been shown to play a role in neutrophil recruitment during cerebral IR injury (Zhang *et al.*, 1996), but whether this finding correlates with the events during hepatic IR injury is currently unclear.

The role of VCAM-1 in hepatic IR injury is unclear. Blockade of VCAM-1 alone in other models of IR shows no benefit (Justicia *et al.*, 2006, Bowden *et al.*, 2002) but this finding has not been investigated fully in hepatic IR. It has been suggested that the α_4 integrins may act as an alternative mechanism for neutrophil recruitment into inflamed tissues (Johnston and Kubes, 1999) and this may be uncovered in the absence of β_2 integrin mediated recruitment (Monson *et al.*, 2007, Bowden *et al.*, 2002). Neutrophils isolated from arthritic mice display increased adhesion to VCAM-1 in a flow-based adhesion assay when compared to neutrophils isolated from controls (Johnston and Kubes, 1999) suggesting that neutrophils activated *in vivo* can bind to VCAM-1. Interestingly, this increased adhesion is seen without an upregulation of α_4 expression levels, suggesting a change in integrin activity or affinity (Johnston and Kubes, 1999).

1.5. Summary

BM-derived HSCs may potentially aid tissue repair and contribute to restoration of damaged hepatocytes. The precise beneficial mechanisms are poorly understood and probably include hepatocyte restoration via fusion-dependent mechanisms, a small effect due to transdifferentiation into functional hepatocytes and/or paracrine effects that promote liver regeneration and suppress local inflammation. Regardless of the beneficial

mechanisms, understanding the molecular basis of hepatic HSC recruitment and engraftment is critical for design of future therapeutic trials.

Evidence supporting a beneficial role for HSCs in liver injury includes the fact that they can differentiate into hepatocyte-like cells when co-cultured with injured hepatocytes and elevated numbers of circulating HSCs have been observed after extensive liver resection and alcoholic hepatitis. Expression of the potent HSC chemokine, CXCL12, is increased within injured liver which facilitates HSC recruitment from BM via the circulation (Hatch *et al.*, 2002). The liver acts as a HSC niche during foetal development and low numbers of c-kit^{+} Sca-1^{+} Lin^{-} (KSL) HSCs can be detected within adult mouse liver. Collectively, these studies provide evidence that BM-derived HSCs infiltrate inflamed liver and may have potential therapeutic use as tools to promote liver regeneration.

However, HSCs are rare cells constituting <0.05% of the BM (Chen *et al.*, 1997) and this scarcity has hindered their clinical use (Dahlke *et al.*, 2006). If HSC therapy is to be realized, a better understanding of the molecular adhesive factors regulating engraftment at injured sites is essential in order to develop strategies to enhance recruitment. It is possible that HSCs utilise the same classes of adhesion molecules used by mature leukocytes for recruitment to inflamed sites. However, there is an extreme lack of published literature that has addressed this and the molecular adhesive events that govern HSC recruitment within injured non-BM sites is currently unknown. If HSCs do hold promise in the context of regenerative diseases or the repair of tissue injury, what is clear is that we need to have a

better understanding of how HSCs home to tissues and what local factors influence their retention and integration.

1.6. Aims and Hypotheses

The major hypothesis of this thesis is that HSCs are recruited to the hepatic microcirculation following hepatic IR injury and that the mechanism is selective and can be experimentally manipulated. More specifically, the aims of this thesis are as follows :

1. Hepatic IR injury significantly promotes adhesion of HSCs to the sinusoidal microcirculation
2. Blockade of specific adhesion molecules on the HSCs would significantly reduce HSC adhesion post-injury *in vitro* and *in vivo*
3. Blockade of specific adhesion molecules on the endothelium would significantly reduce HSC adhesion post-injury *in vivo*
4. HSC adhesion can be modulated positively by pre-treatment with specific cytokines
5. HSCs recruited to the injured liver may have immunomodulatory effects thereby reducing subsequent mature leukocyte recruitment

Chapter 2

Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1. Antibodies

All antibodies used in this thesis were used at the concentrations detailed in **Table 2.1**, unless specifically listed elsewhere.

2.1.2 Common Materials

A number of materials were used repeatedly in this thesis and are presented in **Table 2.2** for clarity. Materials referred to in the text were obtained from the indicated suppliers unless otherwise stated.

2.2. Cell Culture, Cell Isolation and Cell Preparation

2.2.1. Cell Counting

For all cell counting, a standard Neubauer haemocytometer was used. Trypan blue (9:1) was added to cell suspensions prior to counting to ensure exclusion of dead cells from the final count. Cells were centrifuged and subsequently resuspended in 1ml and well mixed. 90μL of Trypan Blue was added to 10ul of cell suspension, and approximately 20μl of the resulting suspension was viewed using a hemocytometer under a x10 objective. The number of cells that had not taken up the blue stain (viable cells) were counted in the four

Antibody	Conj	Clone	Origin	Target	Isotype	Company	Conc^{n}
IgG2 Control	N/A	RTK4530	Rat	N/A	IgG2b	Cambridge Bioscience	80µg/ml
IgG1 Control	N/A	RTK2071	Rat	N/A	IgG1,κ	Cambridge Bioscience	80µg/ml
LE-AF anti-CD31	N/A	MEC13.3	Rat	Mouse	IgG2a	Cambridge Bioscience	80µg/ml
LE-AF anti-CD49d	N/A	R1-2	Rat	Mouse	IgG2b	Cambridge Bioscience	80µg/ml
LEAF anti-CD44	N/A	IM7	Rat	Mouse	IgG2b	Cambridge Bioscience	80µg/ml
NA-LE anti-CD18	N/A	GAME-46	Rat	Mouse	IgG1	BD Pharmingen	80µg/ml
LE-AF anti-CD62L	N/A	MEL-14	Rat	Mouse	IgG2a	Cambridge Bioscience	80µg/ml
Purified anti-CXCR4	N/A	247506	Rat	Mouse	IgG2b	R&D Systems	80µg/ml
anti-CD31	FITC	MEC13.3	Rat	Mouse	IgG2a	Cambridge Bioscience	1:50
anti-CD49d	FITC	R1-2	Rat	Mouse	IgG2b	Cambridge Bioscience	1:50
anti-CD44	PE	IM7	Rat	Mouse	IgG2b	eBioscience	1:50
anti-CD18	FITC	GAME-46	Rat	Mouse	IgG1	Santa Cruz Biotech.	1:50
anti-CD62L	FITC	MEL-14	Rat	Mouse	IgG2a	eBioscience	1:50
anti-Sca-1	PE	D7	Rat	Mouse	IgG2a	eBioscience	1:50
anti-c-kit (CD117)	Cy5	2B8	Rat	Mouse	IgG2b	eBioscience	1:50
anti-CD34	FITC	RAM34	Rat	Mouse	IgG2a	eBioscience	1:50
anti-CD11b	FITC	M1/70	Rat	Mouse	IgG2b	eBioscience	1:50
anti-CD45R	FITC	RA3-6B2	Rat	Mouse	IgG2a	eBioscience	1:50
anti-Ter-119	FITC	Ter-119	Rat	Mouse	IgG2b	eBioscience	1:50
anti-CD8	FITC	53-6.7	Rat	Mouse	IgG2a	eBioscience	1:50
anti-CD5	FITC	CD5	Rat	Mouse	IgG2a	eBioscience	1:50
anti-Gr-1	FITC	RB6-8C5	Rat	Mouse	IgG2b	eBioscience	1:50
anti-VCAM-1	N/A	MK/3	Rat	Mouse	IgG1	Abcam	1:100
CD16/32	N/A	93	Rat	Mouse	IgG2a	eBioscience	1:20
Polyclonal anti-rat IgG	TR	Polyclonal	Goat	Rat	IgG Frc.	Abcam	1:250
Rat IgG control	FITC	eB149/10H5	Rat	Mouse	IgG2b	eBioscience	1:50
Rat IgG control	PE	eB149/10H5	Rat	Mouse	IgG2b	eBioscience	1:50

Table 2.1. *Antibodies used in this thesis.* Antibodies were purchased from: Cambridge Bioscience, Cambridge, UK; BD Pharmingen, Oxford, UK; Santa Cruz Biotechnology, Santa Cruz, US; eBioscience, San Diego, US; abcam, Cambridge, UK.

Material	*Abbreviation*	*Manufacturer*	*Location*
Acetone	-	Fisher Scientific	Leicestershire, UK
3-aminopropyltriethoxysaline	APES	Sigma	Poole, UK
Ammonium Hydrochloride	NH_4Cl	Sigma	Poole, UK
Bovine Serum Albumin	BSA	Sigma	Poole, UK
5'6-carboxyfluorescein diacetate succinimidyl ester	CFSE	Molecular Probes (Invitrogen)	Paisley, UK
Dextran	-	Pharmacosmos	Holbaek, Denmark
Dimethyl Sulphoxide	DMSO	Sigma	Poole, UK
Heat inactivated Fetal Bovine Serum	FBS	Invitrogen	Paisley, UK
Gelatin (Type I from porcine skin)	-	Sigma	Poole, UK
Ketamine Hydrochloride	-	Pharmacia Animal Health	Northamptonshire, UK
L-Glucose	-	Sigma	Poole, UK
L-Glutamine (Glutamax^{TM})	-	Invitrogen/PAA Labs	Paisley/Peteboro', UK
Murine Epidermal Growth Factor	mEGF	Peprotech	London, UK
Murine Interleukin-6	mIL-6	Peprotech	London, UK
Non-essential amino acids	-	Invitrogen	Paisley, UK
Penicillin	-	Invitrogen/PAA Labs	Paisley/Peteboro', UK
Phosphate Buffered Saline	PBS	Invitrogen	Paisley, UK
Potassium Bicarbinate	$KHCO_3$	Sigma	Poole, UK
Propridium Iodide	PI	Sigma	Poole, UK
Sodium ethylene tetraacetic acid	Na.EDTA	Sigma	Poole, UK
Sodium Chloride	NaCl	Sigma	Poole, UK
Streptomycin	-	Invitrogen	Paisley, UK
TrypLE – Trypsin Like Enzyme	TrypLE	Invitrogen	Paisley, UK
Trypan Blue	-	Sigma	Poole, UK
Trypsin-EDTA	-	Invitrogen	Paisley, UK
Xylazine Hydrochloride	-	Millpledge Vetinary	Nottinghamshire, UK

Table 2.2. *Chemicals used frequently in this thesis.* Products were purchased from companies shown. Abbreviations (if applicable) are also shown.

grid 1 x 1mm^2 fields. The average count from the four grids was calculated and multiplied by 1 x 10^5 to yield the number of cells per ml.

2.2.2. HPC-5 and HPC-7 culture

HSC lines have been used for most of the experiments within this thesis. Intravital studies monitoring HSC trafficking have been limited due to cell rarity and difficulty isolating sufficient numbers for detection following systemic infusion (Chen *et al.*, 1997). Therefore, this study utilized an adult and embryonic murine hematopoietic progenitor cell line, HPC-5 and HPC-7 respectively. HPC-5 and HPC-7 cells were a generous gift from from Professor Leif Carlsson (University of Umeå, Sweden). These cell lines display characteristics of primary HSCs, such as being highly enriched for surface markers characteristic of the most immature HSCs including c-kit$^+$, Sca-1$^+$, CD34$^-$ and Lin$^-$ (Pinto do Ó *et al.*, 2002, Pinto do Ó *et al.*, 1998). Additionally, HPC-5 cells reconstitute haematopoiesis upon transplantation into a lethally irradiated host (Pinto do Ó *et al.*, 2002). The HPC-7 cell line has been used extensively for studies investigating the control of HSC growth and differentiation at a molecular and cellular level (Wilson et al., 2009, Pimanda et al., 2008). Furthermore, this cell line is considered superior to other HSC lines such as FDCP-1 mix or FDC-P1 mix, which have been routinely utilized by leading laboratories to monitor HSC trafficking with the murine BM intravitally (Mazo et al., 1998, Katayama et al., 2004). In this thesis (Chapter 4), the adhesion molecule profile of HPC-7 and HPC-5 was examined and found to be consistent with the profile expected of HSCs (although data for HPC-5 is not shown, results

are similar). Furthermore, we also show these cells express CXCR4 – the receptor for CXCL12 commonly found on HSCs (Chapter 5).

Cells were cultured in Stem Pro Serum Free Medium (SFM) 34 (Invitrogen) supplemented with 50U/ml penicillin, 50U/ml streptomycin and 2mM L-Glutamine, and conditioned medium from CHO K3 cells at a concentration of 1 in 10. Both HPC-5 and HPC-7 growth is strictly dependent upon the presence of SCF. CHO-K3 cells generate SCF as a result of a stable transfection (see below). In addition to SCF, HPC-5 cells are dependent on IL-6 and were cultured in the presence of 10ng/ml murine IL-6. Both cell types were maintained daily at a density of between 0.8 – 1.3 x 10^6 cells/ml.

2.2.3. CHO K3 cell culture

CHO K3 cells were also generously obtained from Professor Leif Carlsson (University of Umeå, Sweden). CHO K3 cells were grown to confluence in gelatin coated flasks. CHO K3 were maintained in Dulbecco's Modified Eagles Medium (DMEM, Invitrogen) containing 10% FBS, 50U/ml penicillin, 50U/ml streptomycin, and 2mM L-Glutamine. To generate SCF-containing medium, confluent CHO K3 cells were left for 48 hours without a change of media. Thereafter, the media was changed to Stem Pro 34 SFM (Invitrogen) supplemented with 0.5% FBS. Cells were cultured for 24 hours and the media replaced with fresh Stem Pro 34 SFM with 0.5% FBS. Cells were incubated for a further 48 hours and the media from these cells was collected and filtered. Conditioned media was then stored at -20^{o}C until required for supplementation of HPC media.

2.2.4. Murine Cardiac Endothelial Cell (MuCEC-1) Culture

Isolation of primary murine endothelial cells is typically very difficult and requires elaborate and time consuming purification techniques (Marelli-Berg *et al.*, 2000). Furthermore, overgrowth of contaminating cells often prevents long-term culture of these populations (Marelli-Berg *et al.*, 2000). As a result of the difficulties encountered with primary isolates, an immortalised murine cardiac endothelial cell line was used (MuCEC-1) (Bouis *et al.*, 2001). MuCEC-1 cells were obtained from Dr. Elaine Lidington (Imperial College School of Medicine, UK). Cells were grown to confluence in gelatin coated flasks. MuCEC-1 cells are isolated from H-2K^{b}-tsA58 mice, which express a thermoliable strain (tsA58) of the SV(40) large T antigen (tsA58 TAg) which allows the production of conditionally immortalised cell lines (Lidington *et al.*, 2002). Endothelial cells generated from these cell lines are characterised by their phenotypic cobblestone appearance and their ability to form tubes on an artificial extra-cellular matrix, as detailed in the paper by Lidington *et al* (2002). Furthermore, these cells were shown to be positive for ICAM-1 and VCAM-1, with selectin expression neglible (Lidington *et al.*, 2002). MuCEC-1 were maintained in DMEM containing 10% FBS, supplemented with 50U/ml penicillin, 50U/ml streptomycin and 2mM L-Glutamine and 10ng/ml mEGF. At confluence, cells were enzymatically dissociated using Trypsin-EDTA, washed in complete media and split 1 in 3. For static adhesion assays, a confluent flask of MuCEC-1 was enzymatically dissociated using Trypsin-EDTA, washed in complete media and one third resuspended in 30ml of complete medium. 1ml of the resulting cell suspension was then added to each well of a 24 well plate containing a pre-treated coverslip (please refer to section 2.4.) and allowed to grow to confluence prior to use.

2.2.5. KG1a cell culture

The KG1a cell line is a primitive human haematopoietic myeloid progenitor cell line, characterised by a robust expression of the human stem cell marker CD34 (Koeffler *et al.*, 1980). KG1a cells were a kind gift from Dr. Heather Crosby (University of Birmingham, UK). They were used in intravital experiments to compare human and murine progenitor cell trafficking within murine hepatic microcirculation *in vivo.* Cells were maintained in RPMI 1640 (PAA Laboratories), containing 10% FBS (Invitrogen), 100U/ml L-glutamine, 100ug/ml penicillin and 292ug/ml streptomycin.

2.2.6. Whole bone marrow (WBM) cell preparation

Whole bone marrow (BM) cells were isolated from the femurs and tibias of donor C57BL/6 mice (Harlan, Oxon, UK). Animals were sacrificed by cervical dislocation and the hind limbs (both fibias/tibias) were separated from the hip-bone. Muscle was carefully removed from the bones and each bone was placed on ice until bone removal was complete. Small sections were moved from the epiphysis of each bone. The bone was subsequently washed through with ice-cold DMEM (supplemented with 10% FCS, 2mM L-Glutamine, 50U/ml Streptomycin, and 50U/ml Penicillin) using a 26G needle. Cells were pelletted and subsequently treated with ACK lysis buffer to lyse erythrocytes (0.15mM NH_4Cl, 10mM $KHCO_3$, 0.1mM Na.EDTA, pH 7.2-7.4). ACK lysis buffer was incubated with cells for 3 minutes and then immediately diluted 1:1 with ice-cold PBS. Cells were pelletted and washed twice by cold centrifugation (1200rpm, 4°C, 5 mins) in PBS supplemented with 0.1% BSA.

2.2.7. Leukocyte isolation

Leukocytes were isolated from donor 8-10 week old C57Bl/6 mice. Mice were anaesthetised by isoflurane, followed by CO_2 narcosis. Following the cessation of breathing, a laporotomy was performed to enable access to the descending aorta. Blood was drawn from the descending aorta into heparin coated 1ml syringes. Blood obtained (approximately 700µl) from one animal was added to 3ml of 1% dextran (in 0.9% saline) and subsequently topped up with 1% dextran (in 0.9% saline) to a final volume of 10ml. Erythrocytes were allowed to sediment for 30 minutes at room temperature and the leukocyte rich supernatant transferred to a new tube. Cells were pelletted and washed twice by cold centrifugation (4°C) in cold PBS supplemented with 0.1% BSA. Residual erythrocytes were subjected to hypotonic lysis by suspension in 7ml 0.2% NaCl with ten gentle inversions followed by hypertonic rescue by one inversion of an additional 7ml of 0.6% NaCl supplemented with 0.1% L-Glucose. Remaining leukocytes were washed once in PBS supplemented with 0.1% BSA. Leukocytes were resuspended in 1ml PBS with 0.1% BSA and counted. Cells were washed a further time in PBS with 0.1% BSA and resuspended to 1 x 10^6 cells/100µl.

2.2.8. MACS cell isolation of primary HSCs

Some experiments were conducted using a freshly purified population of Lin^{-ve} $Sca\text{-}1^{+ve}$ BM cells. These cells were isolated using a magnetic activated cell sorting (MACS) technique. Isolations were performed as per the manufacturers protocol (Mitenyi Biotech, Surrey, UK). Whole BM cells were obtained from the BM of the tibia and fibia of donor animals. Once isolated, cells were washed in MACS buffer by centrifugation (1200rpm, 4ºC, 5 mins). MACS

buffer consisted of PBS supplemented with 0.5% BSA and 2mM EDTA at pH 7.2. Cells were counted and washed a further time in MACS buffer. Cells were then resuspended in 90µl of MACS buffer per 10^7 total cells. 10µl of primary antibody was added and cells were incubated in darkness on ice for 10 minutes. For lineage depletions, the primary antibodies were biotinylated lineage cocktail antibodies (CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7-4, and Ter-119). For Sca-1^+ isolations, the primary antibody used was anti-Sca-1-FITC. Following incubation with primary antibodies, 1ml of MACS buffer was added per 10^7 cells and cells were washed by centrifugation (1200rpm, 4°C, 5 mins). Supernatant was removed, and cells were resuspended in 80µl of MACS buffer per 10^7 total cells. To this, 20µl of secondary antibody coated microbeads were added to the cell suspension. For Sca-1^+ isolation, the secondary antibodies were anti-FITC-microbeads. For lineage depletions, the secondary antibodies were anti-Biotin microbeads. Cells were washed by adding 1ml MACS buffer per 10^7 cells and centrifugation (1200rpm, 4°C, 5 mins). Cells were resuspended in 500µl MACS buffer and run through an MS (for Sca-1) or LS (for Lineage depletions) separation column in the presence of a magnetic field, in order to retain positively labeled cells. The column was rinsed, and washed through with 3 washes of 500µl (MS) or 1.5ml (LS) MACS buffer. The column was then removed from the magnet and positively retained cells were washed from the column by flushing at high force 1ml (MS) or 3ml (LS) of MACS buffer through the column. To isolate Lin^-Sca-1^+ cells, whole BM was initially depleted for lineage positive cells, and then subsequently Sca-1 positively selected from the remaining population. Cells were collected and analysed by FACS to check purity. Isolates were typically 95% positive for the target population.

2.2.9 Fluorescent cell labelling

Cell labelling was achieved using the flourescein label, 5'6-Carboxyfluorescein Diacetate Succinimidyl Ester (CFDA-SE). CFDA-SE is a non-fluorescent, cell permeable flourescein derivative, which is modified by intracellular esterases to render the molecule fluorescent. Modification by these intracellular enzymes also renders the modified molecule cell impermeant. Cells (HPC-7 & HPC-5: 3 x 10^6 cells; whole bone marrow (WBM) ≈ 5 x 10^7 cells; KG1a: 3 x 10^6 cells; Leukocytes: ≈ 2 x 10^6) were washed with 1ml pre-warmed (37^{o}C) PBS with 0.1% albumin in PBS (0.1% PBSA). Cells were then resuspended in 2ml pre-warmed 0.1% PBSA, in preparation for staining. Stock CFDA-SE was diluted to 10mM in DMSO and aliquoted for future use. 2µl of 10mM CFDA-SE was added to 2ml of PBSA resulting in a staining solution at 10µM. 2ml of this solution was added to 2ml of cell suspension, resulting in a final staining volume of 4ml at 5µM CFDA-SE. Cells were incubated with the CFDA-SE solution for 10 minutes at room temperature in the dark. The staining reaction was quenched with 6ml of ice-cold complete Stem Pro SFM 34 medium (Invitrogen) for HPC-7 and HPC-5 cells or DMEM (Invitrogen) for WBM cells and leukocytes. Cells were then washed three times by centrifugation (1200rpm, 4°C, 5 mins) in relevant media and recounted. Cells were suspended to 1 x 10^6 per 100µl and kept on ice until 30 minutes prior to systemic injection for intravital experiments.

2.2.10. Acridine orange labelling of leukocytes

For some experiments, leukocytes were labelled *in vivo* using the cell-permeable nucleic dye, Acridine Orange (Sigma-Aldrich, Poole, UK). When bound to DNA, Acridine Orange displays a similar fluorescent spectra to FITC, with an excitiation maximum of 502nm and an

emission of 525nm (green). To visualise leukocytes, animals were injected intra-arterially with 100µl of 200µg/ml Acridine Orange. If required, an additional 50µl of 200µg/ml of Acridine Orange was injected every 45 minutes.

2.2.11. Cell adhesion molecule blocking

To identify critical surface adhesion molecules involved in recruitment, some cells were resuspended in 250µl of PBS supplemented with 0.1% BSA and containing 80µg/ml of the appropriate blocking antibody. These included either the LE-AF isotype control (RTK4530) or LE-AF anti-CD31 (MEC13.3), LE-AF anti-CD49d (R1-2), LE-AF anti-CD44 (IM7) or NA-LE anti-CD18 (GAME-46). FACS studies were performed in order to ensure 80µg/ml is an adequate dose to ensure complete blockade of the respective antigen. Cells were incubated with blocking antibody for 15 minutes prior to CFSE staining. For some experiments, animals were injected with anti-VCAM-1 blocking antibodies (clone 429). Animals were surgically prepared as described previously. Immediately following ischemia, 70µg of anti-VCAM-1 in 100µl was injected via the carotid artery. Control animals received an isotype control immediately following ischemia. A dose of 70µg is consistent with concentrations published in the literature using the same antibody (Belcher *et al.*, 2005, John and Crispe, 2004, Orito *et al.*, 2007).

2.2.12. FITC-BSA production

At the end of all intravital experiments, 200µl of FITC-conjugated bovine serum albumin (FITC-BSA) was injected intra-arterially. When introduced systemically, it is retained within the vasculature, unless the integrity of the vessel is disturbed, thereby facilitating easy

visualisation of the vasculature and confirming blood flow (Kalia *et al.*, 2002b). 10% FITC on celite was conjugated to bovine serum albumin (both Sigma, Poole, UK) by mixing the FITC, BSA and 10ml of bicarbonate buffer (pH 9.0) overnight at 4ºC . The resulting mixture was centrifuged at 10-12,000rpm for 10 minutes, and conjugated/unconjugated BSA separated by dialysis (12,000MW cut off; Spectrum Laboratories, CA, USA). Prepared FITC-BSA was frozen and stored until use in eppendorfs.

2.3 Monitoring Cell Trafficking *in vivo* using Intravital Microscopy

2.3.1. Animals

Animal experiments were completed with prior approval of the local ethics committee and the United Kingdom Home Office, in accordance with the Animals (Scientific Procedures) Act of 1986 under Project Licence 40/2749 (held by Dr. Neena Kalia). 9-11 week old male C57Bl/6 mice were obtained locally (Biomedical Services Unit, University of Birmingham, UK). Some experiments were condcuted on PECAM$^{-/-}$ mice (generous gift from Dr. Tak Mak, Amgen Institute, Toronto, Canada). PECAM$^{-/-}$ mice were bred from heterozygotes on a C57Bl/6 background and maintained locally (colony held by Professor Chris Buckley). PECAM$^{-/-}$ phenotype was confirmed by flow cytometric analysis of peripheral blood leukocytes (Duncan *et al.*, 1999). All animals had *ad libitum* access to food and water.

2.3.2. Surgical Preparation

Anaesthesia was induced by intraperitoneal administration of Ketamine (100 mg/kg Vetalar; Amersham Biosciences and Upjohn Ltd., UK) and xylazine hydrochloride (10 mg/kg;

Millpledge Pharmaceuticals, UK) delivered in 0.9% saline. Maintenance anaesthesia was delivered intra-arterially as required. The trachea and right common carotid artery was cannulated with polyethylene portex tubing (Smiths Medical, Hythe, UK) to assist breathing and to allow administration of labelled cells and additional anaesthetic. A midline laporotomy was perfomed by cutting along the linea alba, an avascular fibrous structure found between the left and right rectus abdominis muscles. To facilitate liver exteriorisation, the left and right rectus abdominis muscles were removed using a cautery pen and the facilform ligament was cut releasing the liver from the diaphragm.

2.3.3 Induction of Ischemia-Reperfusion Injury

In some animals, uni-lobular ischaemia was induced by application of an atraumatic vascular clamp to the hepatic artery and hepatic portal vein supplying the left hepatic lobe for a period of 90 minutes. Importantly, this allowed ischaemia to be induced in the left lobe alone as our preliminary studies demonstrated that even relatively short periods of total hepatic ischaemia resulted in significant mortality. The liver lobe was reperfused by removal of the clamp and the animal was immediately prepared for intravital monitoring by moving it onto a custom designed plastic board with the liver exteriorised onto a glass microscope slide. The liver was then covered with Saran wrap to reduce the effects of respiratory movements and also to prevent superfusate buffer (see below) from accumulating beneath the liver. Throughout the experiment, the preparation was continuously perfused by a bicarbonate-buffered saline solution (36ºC, pH 7.4) aerated with

5% CO_2, 95% N_2. Animals in sham procedures were exposed to the same liver manipulations as injured animals except the vascular clamp was not applied.

2.3.4. Alanine Aminotransferase (ALT) levels following IR injury

To biochemically confirm that hepatic injury had occurred, blood samples were obtained from mice subject to sham or IR procedures at different time points. Increased levels of serum ALT are directy proportional to the extent of liver injury. Samples were analysed using an Olympus Au400 analyser. The automated analyzer facilitates ALT activity and monitors light absorbance at 340nm which is relative the level of enzyme in the sample. Sampling was performed by the Department of Clinical Chemistry at the Birmingham Women's Healthcare NHS Trust (Birmingham, UK).

2.3.5. Quantification of Cell Trafficking *in vivo*

Using the x10 objective, one hepatic field of view (413µm x 308µm) was pre-selected containing 1-2 post-sinusoidal venules and sinusoidal capillaries. After capturing a 10 sec digital video, fluorescent cells were administered intra-arterially as a 100µl bolus at 30 minutes post-reperfusion. 1 minute recordings were taken from the same pre-selected field of view every five minutes for a period of 60 minutes (ie. until 90 min post-reperfusion). At the end of this monitoring period, an additional six fields of view were taken in order to ensure the events taking place in the pre-selected field of view represented those taking place in other regions of the liver. These 6 regions were not randomly selected but followed a strict pre-determined pattern. The six regions were selected relative to the position of the initial field (Figure 2.1). Events in the pre-selected

region were considered representative if the final level of cell adhesion was within two standard deviations of the mean of the six fields examined following the experiments. Two standard deviations are typically expected statistically to contain around 95% of the population. At the end of some intravital experiments, 100µl of FITC-conjugated BSA was injected to visualise the vasculature and confirm the presence of blood flow. All recordings were stored digitally using Slidebook software (versions 4.1 – 4.1.16, Intelligent Imaging Innovations, Denver, US) and analysed offline.

Quantification of the cell trafficking intravitally has been well documented in the field of microcirculation research (Vowinkel *et al.*, 2004, Anthoni *et al.*, 2006, Wallace *et al.*, 1999). Free-flowing cells were identified as cells which were visible in the field of view but were either non-static, or static for less than 30 seconds. Observation of rolling interactions within sinusoidal capillaries were extremely rare – as a result, experiments were not analysed for numbers of rolling cells. Adherent cells were identified as cells which were visible in the field and static for more than 30 seconds. Cells which entered the field of view after the mid-point of the 1 minute recording were classified as free-flowing, as it was impossible to ascertain if they remained adherent beyond 30 seconds. Adhesion of fluorescently labelled cells was extremely rare within the post-sinusoidal venules and occurred predominantly within sinusoids. Therefore, adhesion was presented as total adhesion per field of view. When quantifying levels of adhesion, only those cells observed within the focal plane were counted. Therefore, out of focus cells, identified by their inability to present as punctate ovoid elements in the field of view, were not counted.

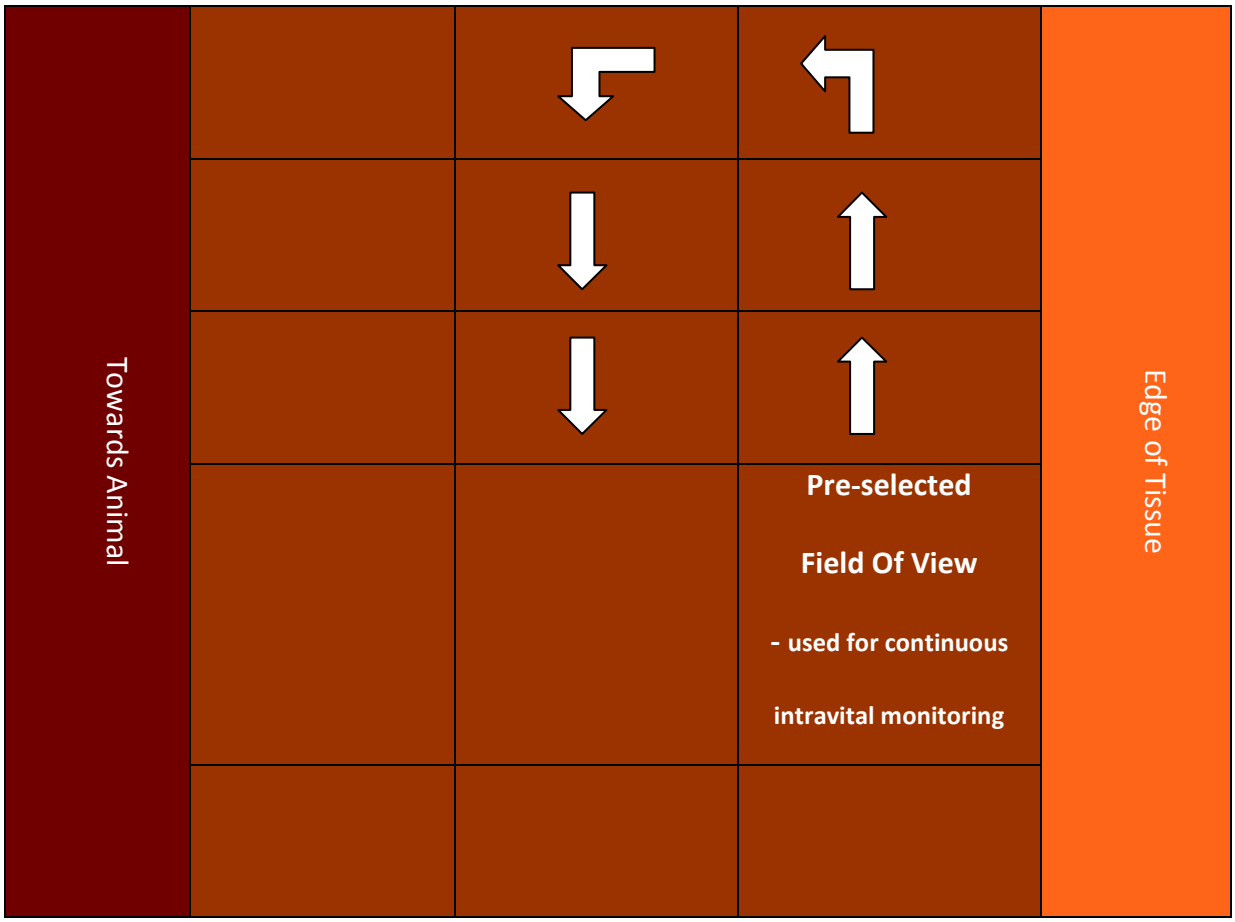


Figure 2.1. **Selection Criteria for Field of View to Monitor for Adhesive Events Intravitally.** Six fields of view were selected at the end of experiments to ensure data obtained from the initial pre-selected field of view were representative of events taking place across the whole liver. Events in the pre-selected region were considered representative if the final level of cell adhesion was within two standard deviations of the mean of the six fields examined following the experiments. Two standard deviations are typically expected to contain around 95% of the population.

Results are expressed as mean adherent or free flowing cells per field per minute at each time point ± SEM.

2.3.6. Anti-inflammatory effects of HPC-7 cells following IR injury

To examine the potential anti-inflammatory effects of HSCs following IR injury, experiments were performed to investigate whether pre-administration of a HPC-7 bolus could subsequently reduce leukocyte recruitment following IR injury when compared to a saline bolus. Animals were surgically prepared as described above. Ischemia was similarly induced (at a time denoted T0) and reperfusion was commenced after 90 minutes ischemia (T90). One minute was allowed for restoration of hepatic flow subsequent to which $5x10^6$ or $1x10^6$ unlabelled HPC-7 cells in 100µl PBS were injected via the carotid artery or hepatic portal vein respectively. To directly inject cells via the hepatic portal vein, a 30G (BD Microlance, Drogheda, Ireland) needle was used. Bleeding was prevented by applying gentle pressure on the vessel using a small haemostatic swab. Animals suffering excessive bleeding were sacrificed at the point of blood loss and excluded from analysis. Control animals received a 100µl bolus of saline. It should be noted that injections via the portal vein led to frequent bleeding and introducing cells via this route was not persued routinely.

To monitor exogenous leukocytes, cells were isolated from two strain and age matched donor mice, labelled with CFSE and 5×10^5 cells injected at 2.5 hours post-reperfusion via the same route as the first injection. After 15 minutes, animals were subsequently culled, and tissues examined using an Olympus IX81 inverted microscope (Olympus, UK).

2.3.7. Quantification of HSC Recruitment in Major Organs e*x vivo*

For some experiments, specific organs were examined *ex vivo* for labelled cell recruitment using an Olympus IX81 inverted microscope. Animals were culled and the injured and non-injured liver, kidney, spleen and lungs were removed after intravital experiments were completed. All organs were washed twice in 0.9% saline to remove traces of blood. The lungs and liver lobes were placed on a plastic dish and the flat vascular surface was visualised. The kidneys and spleen were sectioned coronally and sagitally respectively, further washed to remove excess blood, placed on a plastic dish and the inner surface visualised. Ten fields of view were selected in a set pattern; an initial field was selected blindly in the bottom left hand corner of the tissue, and subsequent fields were selected relative to the initial field while ensuring whole organ coverage. Fluorescent cells were counted manually using blinded counting software (see 2.4.3).

2.4. Quantification of Cell Adhesion *in vitro*

2.4.1. Frozen Tissue Static Adhesion Assay

The Stamper-Woodruff static adhesion assay was used to examine cell adhesion to snap frozen sections of liver *in vitro* (Stamper and Woodruff, 1976). C57BL/6 mice were subjected to hepatic IR injury or sham surgery. The liver lobe was removed, snap frozen in liquid nitrogen and stored at -80ºC until ready for sectioning. The livers were embedded in Cryo-M-bed (Bright Instruments, Cambridge, UK) and 10µm sections were obtained using a Bright Instruments cryostat and mounted on X-tra™ adhesive microscope slides (Surgipath, Peterborough, UK). For some experiments, sections were mounted on standard glass

microscope slides pre-coated overnight with 10% Poly-L-lysine at 4ºC (Sigma-Aldrich). Sections were stored at -20ºC until use when they were fixed in 100% acetone for 5 minutes, washed in PBS and encircled using a wax immunology pen (Immedge; Vector Laboratories, Peterborough, UK). 100µl of cell suspension (HPC-5/HPC-7/WBM/KG1a) was added to each slide and incubated in a humidified tray on a laboratory rocker for 20 minutes. Slides were then immediately fixed for five minutes in 100% acetone and sections subsequently washed in PBS. Slides were dried, coverslips mounted using Gel Mount aqueous mounting medium (Sigma) and analysed using a Zeiss Axiovert Microscope (Carl Zeiss, Hertfordshire, UK) and Zeiss Axiovision Software. Ten separate fields were selected from each tissue using a set field pattern with fields of view containing large portal areas excluded. An initial field was pre-selected in the bottom left hand corner of the tissue and subsequent fields selected relative to this to ensure full organ coverage. Fluorescent cells were counted manually using blinded counting software (see 2.4.3). Results are expressed as mean adhesion per field ± SEM.

2.4.2. Endothelial Cell Static Adhesion Assay

The endothelial static adhesion assay was used to monitor cell adhesion to endothelial cells *in vitro*. Small coverslips (13mm) were pre-treated with APES to aid endothelial cell adhesion. Coverslips were further prepared for the static adhesion assay by washing with 5M nitric acid (overnight), running water (4 hours), 25ml acetone (three washes) and 25ml 4% APES in acetone (2 washes). Coverslips were then left in a dark place overnight and washed further with acetone (2 washes) and finally with distilled water. All coverslips were

autoclaved prior to use. Endothelial cells (MuCEC-1) were cultured to confluence on the treated coverslips which were placed in the bottom of 24 well plates.

Media was removed from the endothelial monolayers which were then washed twice with 1ml warm PBS containing 0.1% BSA (0.1% PBSA). Following washing, 5 x 10^5 HPC-7 cells (in 500µl) were added to the endothelial monolayers for 5 minutes. HPC-7 cells were pre-treated with 20ng/ml TNF-α, 20ng/ml CXCL12, 20ng/ml IL-1β, or 20ng/ml CXCL1 (all made up in complete Stem Pro 34 SFM) for 4 hrs. Since cytokines were diluted in PBS, an appropriate volume of PBS alone was used as a control. HPC-7 cells were labelled with 5µM CFSE to aid visualisation. For CFSE-activation studies in Chapter 3, HPC-7 cells were visualised using phase-contrast microscopy.

Endothelial monolayers were washed with warm 0.1% PBSA and left for 10 minutes to allow transendothelial migration. Following this period, HPC-7 cells and endothelium were fixed by incubation with 2% glutaraldehyde in PBS for 15 minutes at 37ºC. The fixative was then washed away three times with 0.1% PBSA. Plates were stored at 4ºC until ready for analysis. Coverslips were removed from wells and mounted on X-tra™ adhesive microscope slides (Surgipath, Peterborough, UK) with gel mount aqueous mounting medium (Sigma). CFSE labelled HPC-7 cells were imaged using a Zeiss Axiovert Microscope (Carl Zeiss, Hertfordshire, UK) and Zeiss Axiovision Software. Non-CFSE labelled HPC-7 cells were imaged using a phase contrast microscope. Ten separate fields were selected from each endothelial monolayer using a set pattern to ensure randomness. An initial field was selected blindly in the bottom left hand area of the endothelial monolayer and subsequent

fields were selected relative to the initial field while ensuring complete coverage (pattern: select initial field, move two fields up, move two up, move two right, move two down, move two down, move two right, move two up, move two up, move two right). Slides were analysed and cells counted using the public domain ImageJ analysis software (Wayne Rasband, NIH, US).

2.4.3. Development of Software to Ensure Observer Blind Quantificataion

In order to ensure observer blind quantifictaion of tissue analysis occurred, software was developed (Figure 2.2). Image frames were saved in folders representing experiments and the details fed into the application. The program subsequently randomises the order in which the images are displayed for counting. Counting takes place manually with the operator performing the counting unaware as to which experiment the frame originates from. After all fields from all experiments have been counted, the data is collated into the respective experiments. The application can be downloaded gratis from http://www.dephilon.com/Counter.exe.

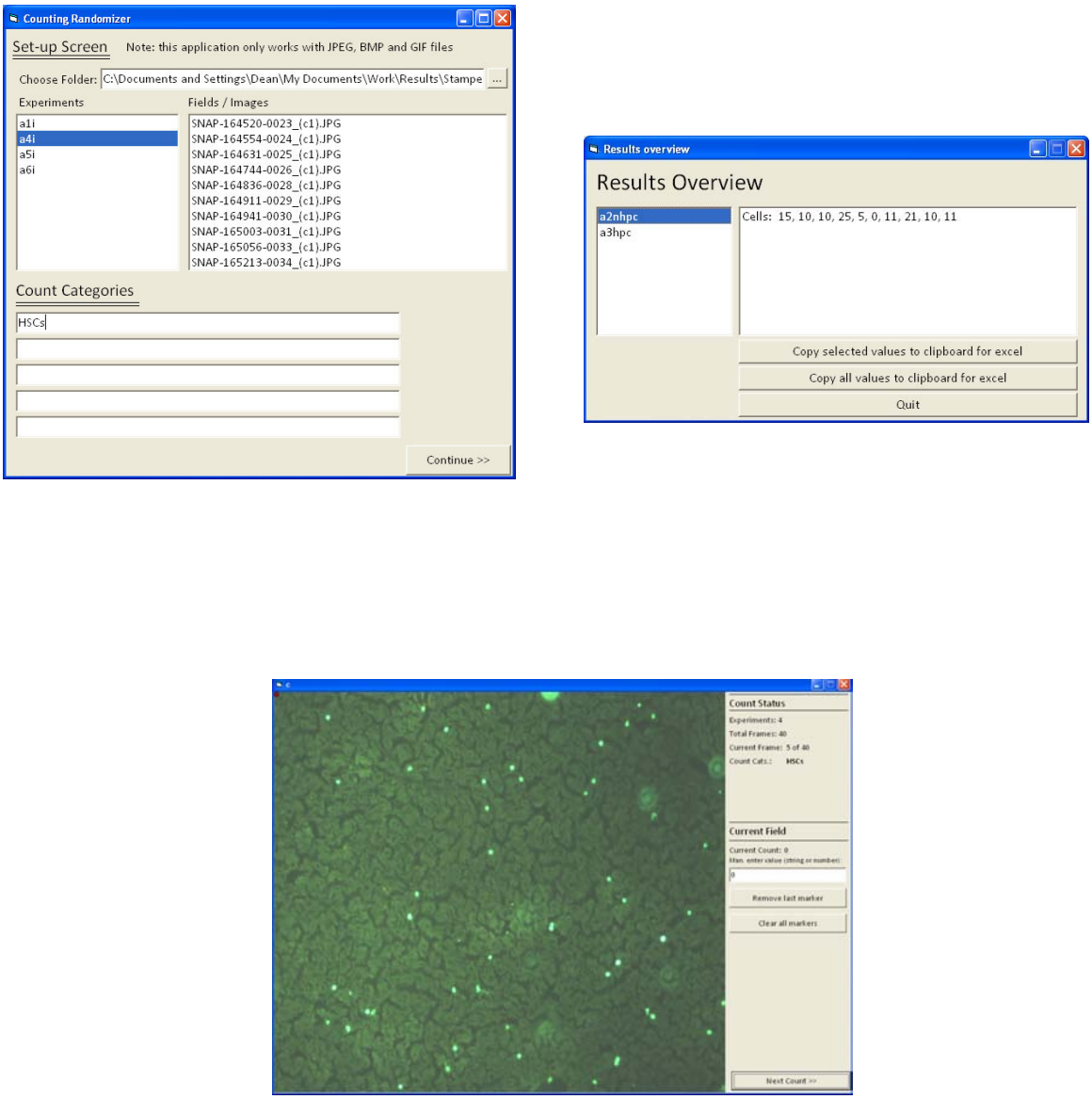


Figure 2.2. **Analysis of adhesion using custom written blind counting software.** Adhesion in frames were counted manually using custom software. The experimental set is not visible at any point during the counting procedure and thus reduces observer bias.

2.5. FACS-based studies

2.5.1. Standard FACS protocol for use on cell suspensions

Progenitor cells were removed from incubation media by cold centrifugation (1200rpm, 4ºC, 5 minutes). Cells were subsequently washed by centrifugation in cold PBS and resuspended in 100µl PBS containing 0.1% BSA and 5% FBS for 15 minutes on ice to block Fc receptors. For some experiments, Fc receptor blockade was achieved by incubation with Fc Block (CD16/32) on ice for fifteen minutes. Blocking Fc receptors reduces subsequent non-specific antibody binding. Following Fc Block, cells were incubated with primary antibody at the recommended dilution (see **Table 2.1**) for 15 minutes in darkness and on ice. If appropriate, following incubation with primary antibody, cells were incubated with secondary antibodies (again at the appropriate dilution) for a further 20 minutes in darkness and on ice. Following antibody incubation, cells were washed three times by addition of 100µl PBS with 0.1% albumin and centrifugation. Following washes, cells were resuspended in 300µl 0.1%PBSA and supplemented with 0.6µl of 0.5mg/ml propidium iodide (PI), a popular red fluorescent nuclear and chromosome counterstain. It is not permeant to live cells so enables visualisation of dead cells from a general population.

Following PI addition, suspensions were loaded onto and analysed on a BD FACSCalibur flow cytometry system. The system has two available excitation lasers (488nm and 635nm) allowing the use of multiple coloured dyes, which can be detected on four different filters (FL1-4). FL1 allows detection of dyes with an emission wavelength of around 530nm including flourescein isothiocyanate (FITC). FL2 allows detection of dyes with an emission

wavelength of around 585nm including phycoerythrin (PE). FL3 allows detection of dyes with an emission wavelength of around 670nm including propidium iodide (PI). FL4 allows detection of dyes with an emission wavelength of around 661nm including Cy5. Cell counting was terminated following the collection of 10,000 live (PI-negative) events.

2.5.2. Confirmation of adhesion molecule blockade

The role of various adhesion molecules in permitting adhesion of HPC-7 cells both *in vitro* and *in vivo* was determined using an array of specific function blocking antibodies. However, prior to antibody use for blocking experiments, FACS based studies were used to confirm complete blockade of the specific adhesion molecule. HPC-7 cells were suspended in aliquots of 1.2 x 10^7 cells in 100µl PBSA. Each aliquot was treated with increasing concentrations of the function blocking antibody (0, 10, 20, 40, 80, 160µg/ml) for 15 minutes. Cells were subsequently washed twice in 100µl of 0.1% PBSA. Following washing, cells were incubated with 2µl of a fluorescent conjugate of the same clone as the blocking antibody (see Figure 2.3) for 15 minutes in darkness. Cells were subsequently washed twice in 200µl of 0.1% PBSA and finally resuspended in 300µl of 0.1% PBSA. Suspensions were supplemented with 0.6µl of PI (1:500). Cells were analysed using standard FACS procedures. Fluorescent intensity was taken as the mean fluorescent intensity of 10,000 live (PI-negative) cells.

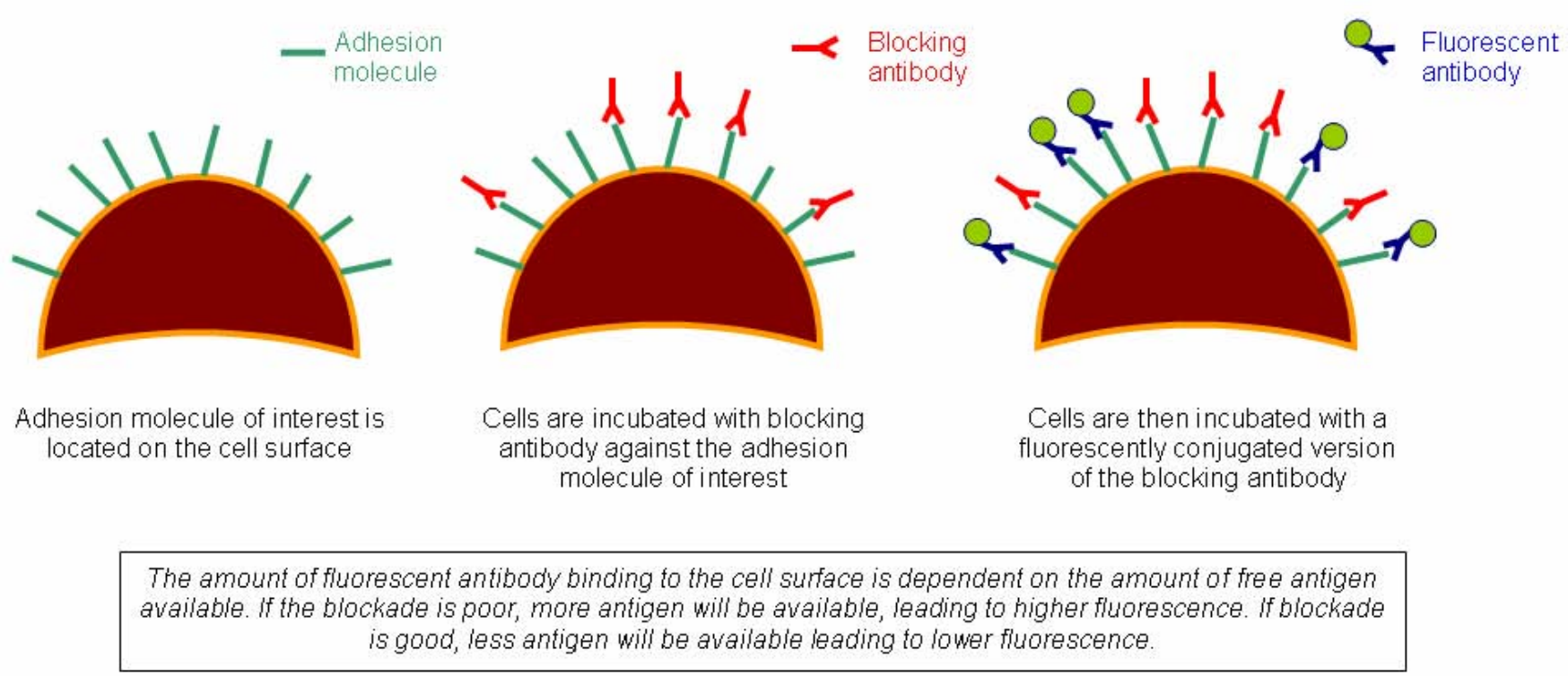


Figure 2.3. **Determination of adequate function blocking concentrations for the anti-adhesion molecule antibodies**. Cells were pre-treated with varying concentrations of blocking antibody. Blocked cells were subsequently treated with a fluorescently conjugated version of the blocking antibody. Fluorescent antibody capture is proportional to free antigen available on the cell surface.

2.5.3. Conditioned media incubations

In order to determine whether factors released from injured liver could modulate expression of adhesion molecules on HSCs, HPC-7 cells were treated with tissue conditioned media (TCM). Experiments were conducted using hepatic and intestinal tissue in order to determine whether any changes in adhesion molecule expression were induced specifically by the liver or any similarly injured organ. C57BL/6 mice were subjected to 90 minutes partial hepatic ischaemia or, for comparison purposes, 45 minutes small intestinal ischemia (clamping of the superior mesenteric artery) followed by reperfusion for one hour. Control animals underwent sham surgery.

Following reperfusion, the entire injured liver lobe or a 2cm section of jejunal gut tissue was harvested into 2ml of complete Stem Pro and disrupted using an electronic tissue homogeniser (Power Gen 125; Fisher Scientific, Leicestershire, UK). Homogenates were incubated for 24 hours and then filtered to remove cells and leave soluble mediators. For liver homogenates, 2ml of complete Stem Pro was added to make a total volume of 4ml for filtering. Suspensions were initially passed through a 70µm filter (BD Falcon, Oxford, UK) to remove larger pieces of tissue and then passed through a 0.45µm filter (Millipore, Watford, UK). Intestinal homogenates were filtered directly through a 0.45µm filter. 5×10^6 HPC-7 cells were incubated with 125µl of conditioned medium in 375µl Stem Pro. Controls were incubated in 500µl Stem Pro (conditioned medium was generated into Stem Pro). Following 24 hours incubation, cells were washed and analysed by FACS (using standard protocols) for any shifts in adhesion molecule expression (see section 2.5.1).

2.5.4 Cytokine incubations

To analyse the effect of inflammatory cytokines on HPC-7 adhesion molecule expression, cells were incubated with various cytokines. HPC-7 were incubated for 24 hours with 100ng/ml TNF-α, 100ng/ml CXCL12, 25ng/ml IL-1β, or 100ng/ml IL-6 (all made up in complete Stem Pro 34 SFM). As cytokines were diluted in PBS, negative controls consisted of Stem Pro 34 SFM with an identical volume of PBS. For some experiments, cells were treated for different lengths of time with a cocktail of all the above cytokines at 10ng/ml or 20ng/ml. Following treatment, cells were washed and analysed as per the standard FACS protocol (see section 2.5.1).

2.5.5. Plasma incubations

In order to analyse the effect of plasma derived from animals undergoing hepatic IR injury on CD49d expression, HPC-7 cells were incubated with conditioned plasma in media. Animals (C57BL/6; 25g bwt) were subjected to 90 minutes ischaemia followed by reperfusion for one hour. Control animals underwent sham surgery. Following reperfusion, Mice were anaesthetised by isoflurane, followed by CO_2 narcosis. Following the cessation of breathing, a laporotomy was performed to enable access to the descending aorta. Blood was drawn from the descending aorta into heparin coated 1ml syringes. Blood was then transferred to an eppendorf and plasma isolated by centrifugation (3000rpm, 7 mins, RT). This plasma was subsequently isolated, and added 1:4 to Stem Pro medium. 1 x 10^6 HPC-7 cells were treated with 1ml of the plasma/medium mixture overnight and CD49d expression assessed by FACS, as per standard FACS protocols (see section 2.5.1).

Chapter 3

Recruitment of haematopoietic stem cells following partial hepatic ischemia-reperfusion injury

3. Recruitment of HSCs by Ischemia-Reperfusion Injury

3.1. Introduction and hypotheses

3.1.1. Introduction

While the mechanisms by which HSCs convey benefit and induce tissue repair remain unclear, recruitment to the tissue of interest is a vital prerequisite. The experiments within this chapter investigated whether an experimental model of acute hepatic injury could be used to monitor stem cell recruitment after systemic administration. Ischemia-reperfusion (IR) injury is a serious clinical problem and has been reported to stimulate stem cell recruitment. Following renal IR injury, transplanted HSCs ($Rh^{lo}Lin^{-}Sca\text{-}1^{+}ckit^{+}$) integrate into the renal tubule and contribute to regeneration (Lin *et al.*, 2003). In mice subjected to myocardial infarction, human $CD34^{+}$ HSC fusion with host cells prompted cell-cycle re-entry of fused cardiomyocytes and thus stimulated their proliferation (Zhang et al, 2007). It was suggested that cardiomyocyte re-entry into the cell cycle was a result of reprogramming of the host cell by the HSC (Zhang *et al.*, 2007). While studies in the liver have shown that populations of progenitor cells can home to the liver post-systemic injection (Jiang *et al.*, 2002), the chronological dynamics of this adhesion have not been elucidated.

This chapter also presents data comparing the recruitment of adult HSCs with embryonic HSCs. Much ethical debate surrounds the therapeutic use of embryonic HSCs (Scott and Reijo Pera, 2008). During development, haematopoiesis begins in the dorsal aorta within the aorta–gonads–mesonephros region, shifting to the foetal liver before finally occurring

in the BM, where haematopoiesis continues during adult life (North *et al.*, 2002, Lux *et al.*, 2008). Therefore, the function of embryonic HSCs is essentially identical to adult HSCs, however, the anatomical compartment in which these cells sit may influence their behaviour and properties. While much of the work investigating tissue repair has focused on adult HSCs, the recruitment potential, and indeed the regenerative potential, of these cells has not been compared in detail to embryonic HSCs in models of the injured liver. Should embryonically derived HSCs behave in a manner similar to their adult counterparts, it may provide support for use of the more ethically acceptable adult HSCs. Furthermore, this chapter also investigated and compared the recruitment of HSCs with whole BM (WBM) cells. It has been suggested that unfractionated WBM cells may also have potential for hepatic regeneration (Grompe, 2003).

3.1.2. Hypotheses

For the work included in this chapter we hypothesized:

1. The adhesion status of HSCs is unaffected by treatment with low concentrations of the fluoresent labeling dye, CFSE
2. Short periods of total hepatic ischemia provide a reproducible, robust and severe injury model for monitoring the trafficking of administered cells
3. The adhesion of whole bone marrow (WBM) cells and HSCs to snap frozen hepatic tissue sections is significantly greater for tissue derived from IR injured animals compared to sham liver
4. The adhesion of WBM cells and HSCs to the hepatic microcirculation is significantly raised *in vivo* in animals subjected to IR injury

5. The recruitment of primary isolated HSCs ($Lin^{-}Sca\text{-}1^{+}$) is significantly raised *in vivo* in animals subjected to IR injury
6. The recruitment of a human HSC-like cell line, KG1a, is significantly raised *in vivo* in animals subjected to IR injury

3.2. Methods

The methods used in this chapter are described in detail in Chapter 2. Briefly, HPC-7 cells were incubated with varying concentrations (0µM, 5µM, 50µM) of CFSE in order to determine if the staining protocol activates cells. To examine this possibility, cells were stained and then exposed to endothelium and their adhesion quantitated using static adhesion assays. In order to establish a viable experimental injury model, C57BL/6 mice were subjected to varying periods of ischemia and reperfusion. Animals underwent either total (THI) or partial (PHI) hepatic ischemia as an insult. THI, which injured all four lobes of the liver, was achieved by clamping the extra-hepatic portal vein and hepatic artery. PHI was achieved by clamping only the left portal vein and left hepatic artery branches that feed the left lobe of the liver. All subsequent experiments within this thesis were conducted on animals undergoing 90min PHI / 30min R, as high mortality rates were observed with THI (see results section).

For immunohistochemistry, sections were isolated from IR (90min PHI / 30min R) or sham injured animals, snap frozen and hematoxylin and eosin (H&E) stained to examine the morphology of the tissues. In blood analysis experiments, samples from IR (90min PHI / 30min R) or sham animals were analysed for circulating cell fraction percentage and plasma ALT using an automated analysis system (ABX, Montpellier, France). Enzyme levels were

obtained using an automatic analysis system (Olympus Au400). Stamper-Woodruff adhesion assays were used to quantify cell adhesion to tissue sections in vitro. Tissues were isolated from animals and immediately snap frozen, maintaining their morphology and protein expression. Cells were then incubated with sections to allow them to adhere to adhesion molecules on the section. Stamper-Woodruff assays were performed on sham and IR (90m PHI / 30m R) sections, using 1 x 10^5 HPC-5, HPC-7, WBM or KG1a cells.

Fluorescent intravital microscopy was performed on male 25-30g C57Bl/6 mice. The carotid artery and trachea were cannulated and the animal subjected to either sham or IR treatment (90min PHI / 30min R). During reperfusion, the animal was moved on to the stage of an intravital microscope and a field of view for analysis was pre-selected. At 30 minutes reperfusion, 1 x 10^6 5µM CFSE labeled cells were introduced via the carotid artery and their recruitment analysed. For the investigation of primary cell adhesion, between 1.2 x 10^5 and 3.3 x 10^5 $Lin^-Sca\text{-}1^-$ cells were introduced via the carotid artery.

3.3. Results

3.3.1. CFSE labelling does not affect the adhesion of HPC-7 cells

Although HPC-7 adhesion decreased slightly following treatment with 5µM and 50µM, this was not statistically significant when compared to untreated (0µM) cells (0µM: 31.20 ± 4.87, 5µM: 24.10 ± 3.05, 50µM: 24.52 ± 2.23) *(figure 3.1.A)*. Transmigration of cells was not observed within the duration of the experiment, even with untreated cells *(figure 3.1.B-D)*.

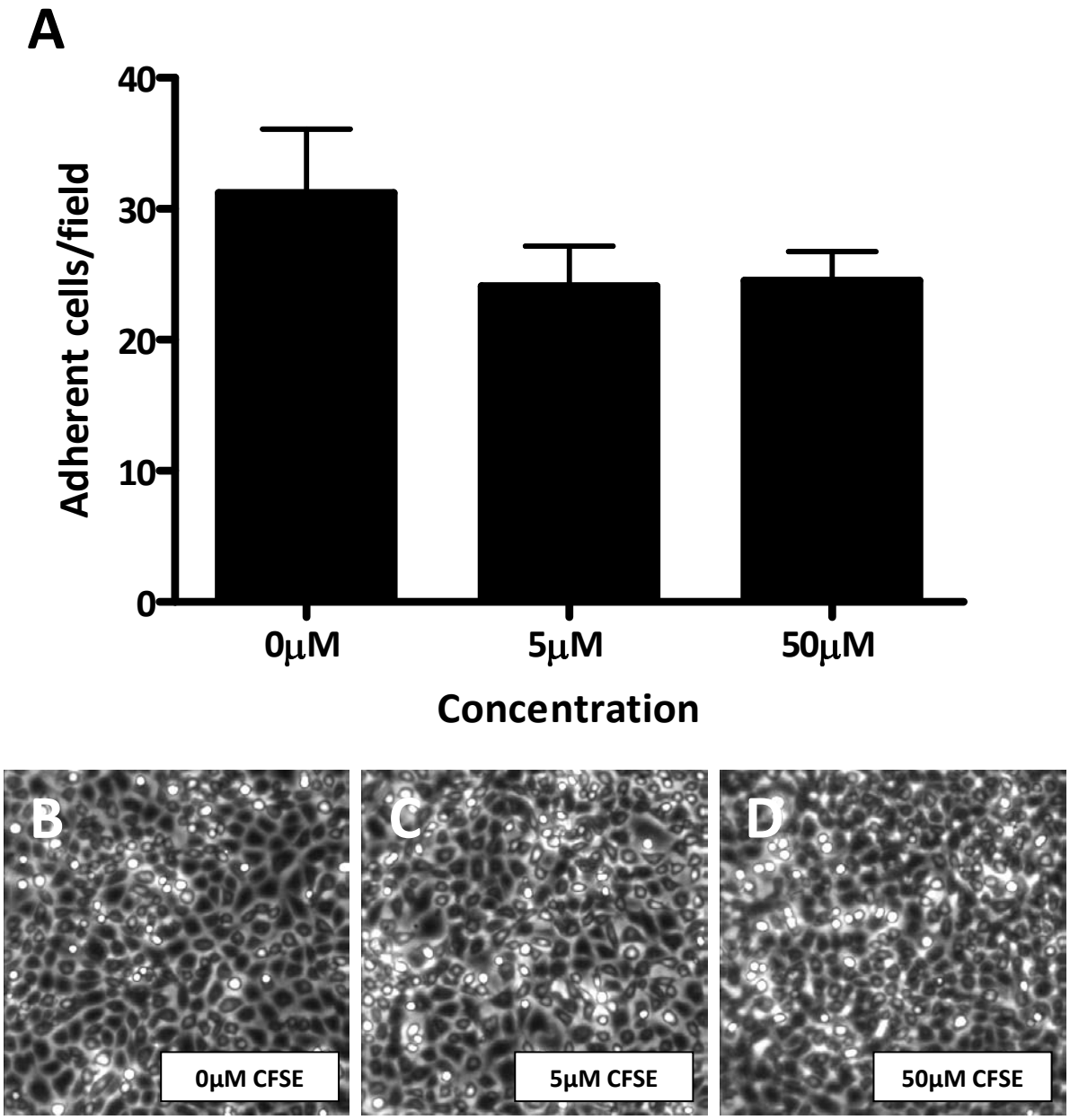


Figure 3.1. *HPC-7 cells are not activated by CFSE labeling.* Cells were treated with increasing concentrations of Carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE). No significant effect of CFDA-SE staining on the adhesion of HPC-7 cells (white cells) to MuCEC-1 (dark cells) is observed at either 5μM or 50μM compared to volume control **(panel A)**. Representative images are shown from 0μM **(panel B)**, 5μM **(panel C)** and 50μM **(panel D)** CFSE treated HPC-7. Bars represent mean adhesion ± SEM of 5 separate experiments; (Panel A: one-way ANOVA).

3.3.2. Total hepatic ischemia has a high mortality rate and is therefore unsuitable for continuous monitoring

IR injury can be fatal, especially if it involves the entire liver (THI). Congestion of blood within the feeding portal vessels subsequently leads to intestinal microcirculatory congestion which contributes significantly to the assosciated mortality. In order to identify a protocol for IR injury which did not cause mortality within the intravital observation period, we performed experiments with varying different ischemic protocols. 100% mortality was observed in animals subjected to 60m THI or 45m THI, with a slight improvement survival in the 30m THI and 20m THI groups (33%) *(figure 3.2.)*. All animals in all groups survived their respective ischemic phase, with mortality occurring during the reperfusion phase (data not shown). Almost all mortality during reperfusion occurred between 1-20 minutes reperfusion (data not shown). As THI was incompatible with animal survival, another group was subjected to 90m PHI followed by 90 minutes reperfusion. All animals survived this procedure, with survival at 100%. Hereafter IR injury refers to partial IR injury of the left hepatic lobe (90 minutes ischemia).

3.3.3. Histological damage is not present on sections from IR injured animals

Liver sections were taken from animals subjected to hepatic IR and sham procedures and stained with H&E (sections were taken at 30 minutes reperfusion). Sections were examined for signs of acute morphological damage. Microscopic examination revealed no obvious morphological damage on sections from IR treated when compared to sham treated animals *(figure 3.3.A-B).* Anatomically, tissues appeared undamaged and displayed good sinusoidal structure.

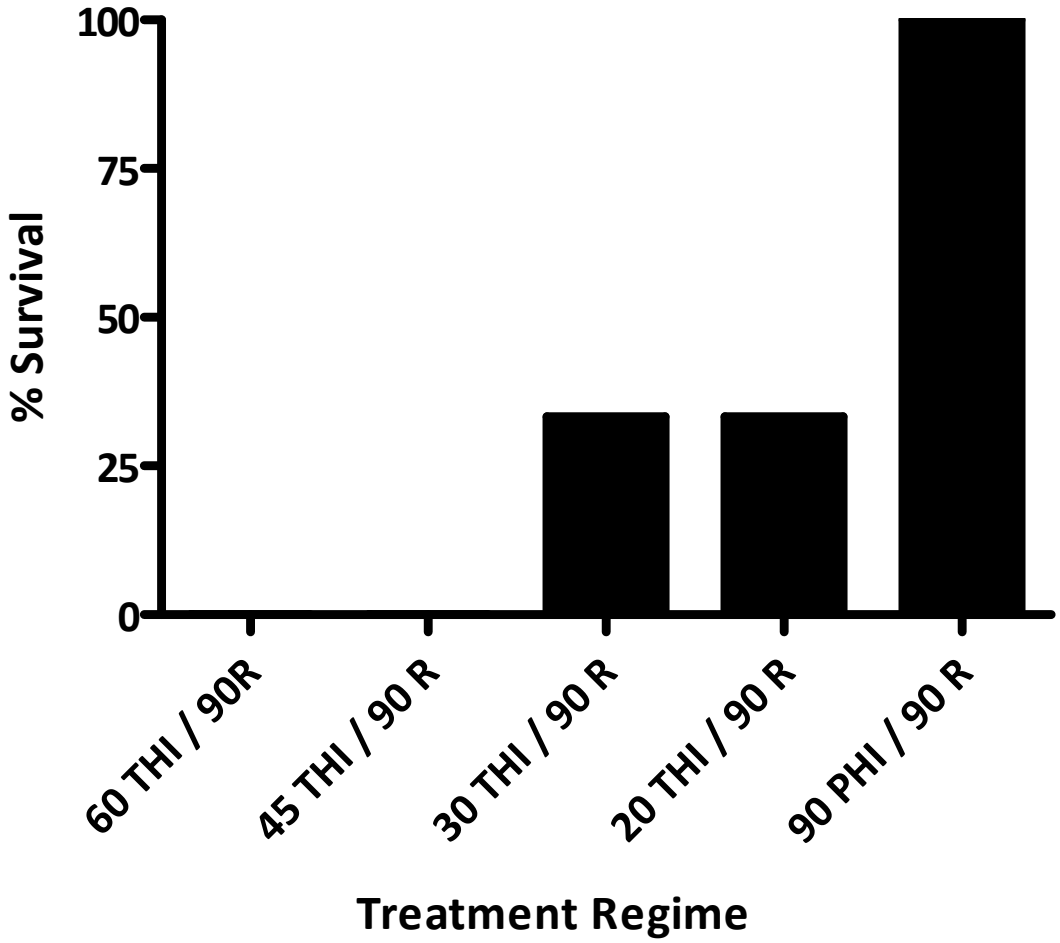


Figure 3.2. *Periods of total hepatic ischemia cause significant mortality in treated animals.* Animals were subjected to varying periods of total hepatic ischemia (THI) followed by 90 minutes reperfusion. Mortality within the 90 minute reperfusion duration was monitored. Even relatively short periods of THI were associated with significant mortality in mice. In contrast, all animals tolerated 90 minutes of partial hepatic ischemia (PHI) to the left hepatic lobe followed by 90 minutes reperfusion. The data is presented as percentage survival of at least 2 animals (60min THI / 90min R n=2; 45min THI / 90min R n=2; 30min THI / 90min R n=3; 20min THI / 90min R n=3; 90min PHI/90min R n=3)

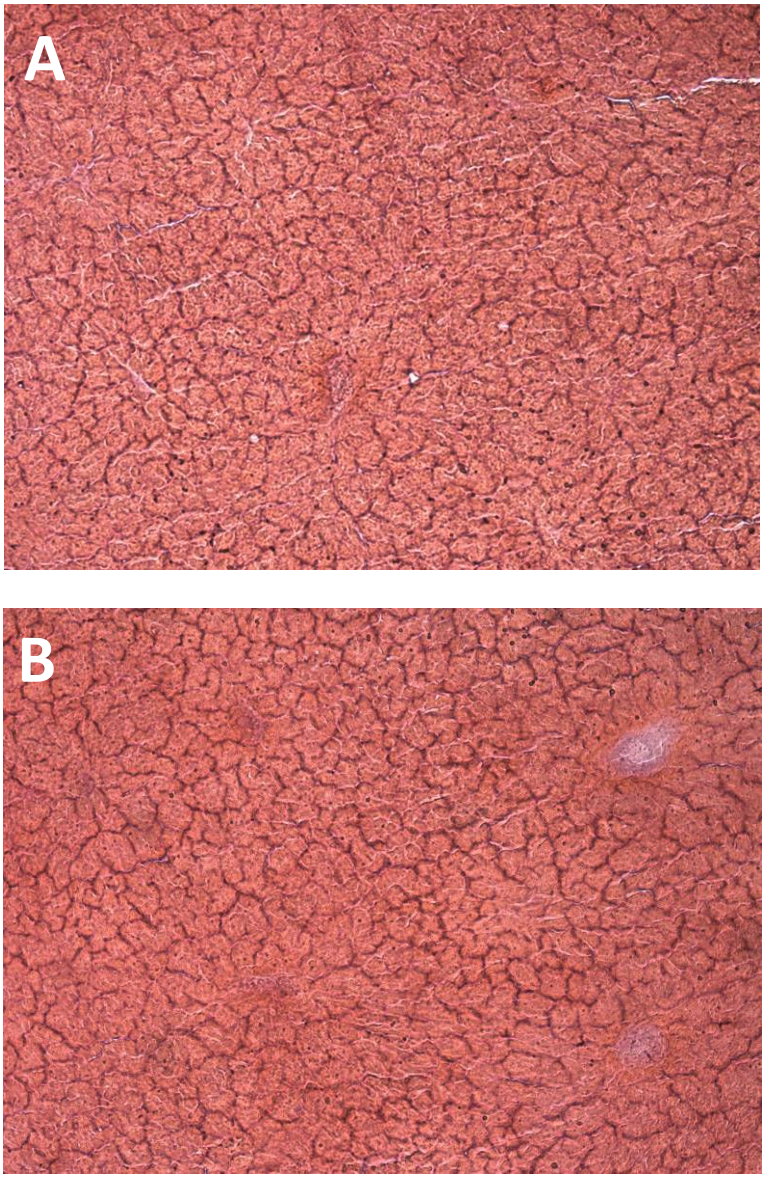


Figure 3.3. *Acute hepatic injury is not evident histologically on sections from IR Treated animals when compared to Sham Treated animals*. Livers were isolated from Sham **(panel A)** and IR (90min / 30min R) treated **(panel B)** animals (at 30 minutes reperfusion) and stained with hematoxylin and eosin (H&E) to examine for acute damage to the hepatic architecture. No disruption of the hepatic architecture is visible on sections from either Sham or IR treated animals. Images are representative of sections seen in 3 separate sham or IR treated animals.

3.3.4. ALT levels and circulating neutrophil counts are raised in IR injury

In order to confirm the presence of injury, blood samples were examined following 90min PHI / 30min R. Levels of ALT were slightly, but insignificantly, raised as a result of sham treatment compared to an untreated control (sham: 53.67 ± 7.27 U/L vs untreated: 34 ± 3 U/L; $p > 0.05$) *(figure 3.4.A)*. Levels of ALT in both untreated and sham treated animals are within normal ranges reported in the literature for mice (Car *et al.*, 1994). However, IR injury significantly increased circulating plasma ALT over 30-fold when compared to sham controls (sham treated: 53.67 ± 7.27 U/L vs IR treated: 1422 ± 637.80 U/L; $P < 0.05$) *(figure 3.4.A)*.

A slight decrease in circulating white blood cells was observed following IR injury but this decrease was not significant (sham treatment: 1.80 ± 0.20 x $10^3/mm^3$ vs IR injury: 1.02 ± 0.75 x $10^3/mm^3$; $P > 0.05$) (figure not shown). PHI injury significantly reduced the circulating lymphocyte percentage (IR injury: 96.28% ± 0.54% vs sham treated: 83.83% ± 5.51%; $P < 0.05$) *(figure 3.4.B).* A concomitant significant increase in circulating neutrophil percentages was observed (sham treated: 2.28% ± 0.46% vs IR treated: 12.39% ± 2.87%; $P < 0.01$) *(figure 3.4.B)*.

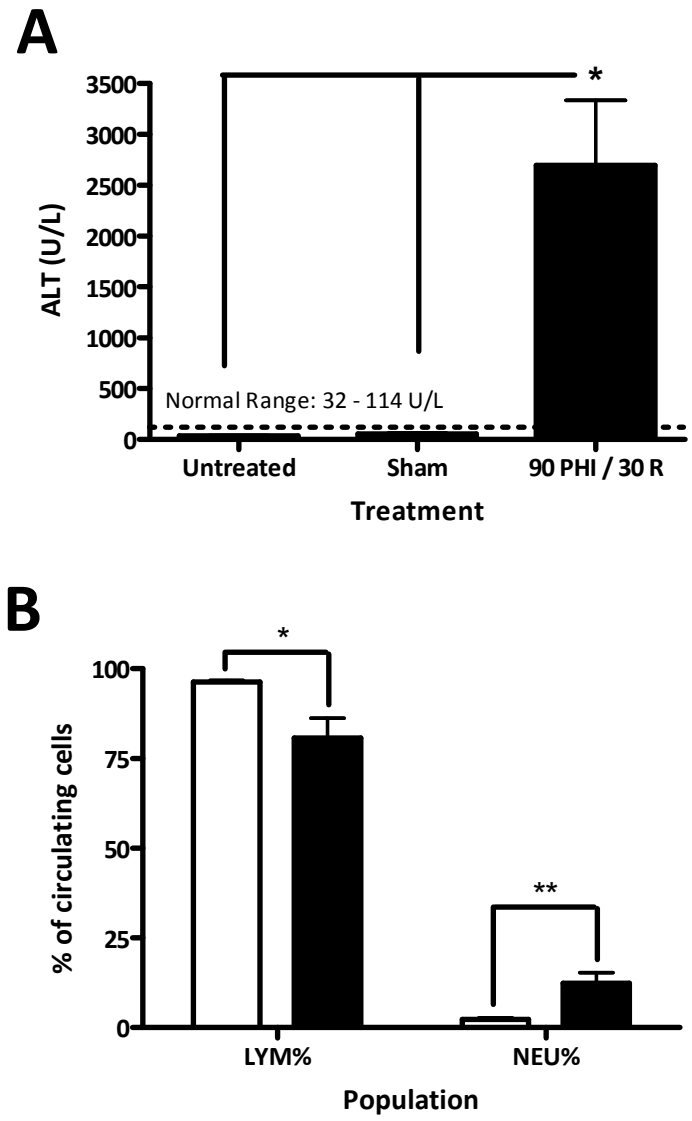


Figure 3.4. Plasma Alanine Aminotransferase (ALT) levels and circulating neutrophil levels are significantly increased acutely as a result of Partial Hepatic Ischemiai. Blood samples were analysed from Sham and IR treated animals (90 minutes partial ischemia, 30 minutes reperfusion) for plasma levels of ALT (a commonly used marker of liver injury) and circulating white blood cell counts. Sham treatment did not significantly raise plasma ALT when compared to untreated animals **(panel A)**. PHI significantly raised plasma ALT levels when compared to Sham treated animals **(panel A)**. Normal range for mice is identified by a dashed bar, and obtained from Car et al (1994). PHI significantly increased circulating neutrophil levels as a proportion of whole blood **(panel B**; clear bar: sham injury, filled bar: IR injury**)**. Bars represent mean ± SEM of at least 3 experiments; * $p < 0.05$, ** $p < 0.01$ (Panel A: One-way ANOVA with Bonferroni Multiple Comparison Test. Panel B: Unpaired t-test). Normal range from Car et al (1994).

3.3.5. Whole bone marrow cell adhesion is raised *in vitro* on IR liver sections

Significantly more WBM cells bound to sections isolated from IR injured animals when compared to sections from sham treated controls (sham control: 31.57 ± 10.56 vs IR treated: 63.43 ± 6.45; $P < 0.05$) *(figure 3.5.A).* Adhesion was predominantly to the sinusoidal capillaries and rarely in the larger portal vessels, therefore portal adhesion was not assessed separately *(figures 3.5.B-C).*

3.3.6. HPC-7 adhesion is raised *in vitro* on IR liver sections

HPC-7 cells are an immortalised embryonic stem cell line, generated by transfection with the LIM-Homeobox gene, Lhx2 (Pinto do Ó *et al.*, 2001) and HPC-5s are an immortalised adult stem cell line, generated from adult BM by genetic modification (Pinto do Ó *et al.*, 1998). We used these cell lines as model stem cells to provide a reliable and pure source of cells for functional studies.

Significantly more HPC-7 bound to frozen sections of IR tissue compared to sections isolated from sham controls (sham sections: 5.13 ± 0.27 vs IR sections: 15.00 ± 3.31; $P < 0.05$) *(figure 3.6.A)*. Adhesion of HPC-7 cells was also predominantly sinusoidal *(figures 3.6.B-C)*. However, the overall adhesion of HPC-7 cells to frozen liver sections was significantly less when compared to WBM cells (Sham: WBM: 31.57 ± 10.56 vs HPC-7: 4.75 ± 0.43; $p < 0.05$; PHI: WBM: 63.43 ± 6.45 vs HPC-7: 15.00 ± 3.31; $P < 0.01$) *(WBM: Figure 3.5.A; HPC-7: Figure 3.6.A)*

3.3.7. HPC-5 cell adhesion is not raised *in vitro* on IR liver sections

Adhesion of HPC-5 cells did not increase on IR sections compared to tissue from sham controls (sham controls: 2.03 ± 0.34 vs IR treated sections: 2.17 ± 0.83; $P > 0.05$) *(figure 3.7.A)*. Again, adhesion was predominantly sinusoidal with very little portal adhesion *(figures 3.7.B-C)*. The overall adhesion of HPC-5 cells to frozen liver sections was significantly less when compared to HPC-7 cells (Sham: HPC-5: 2.03 ± 0.34 vs HPC-7: 4.75 ± 0.43, $P < 0.01$; PHI: HPC-5: 2.17 ± 0.83 vs HPC-7: 15 ± 3.31, $P < 0.05$) *(HPC-7: Figure 3.6.A; HPC-5: Figure 3.7.A)*.

3.3.8. Whole bone marrow adhesion is significantly raised *in vivo* by IR injury

Significantly more WBM cells adhered to the hepatic vascular bed in IR animals compared with sham controls (e.g. at 55 minutes, sham control: 6.20 ± 1.68 vs IR injury: 16.63 ± 2.28; $P < 0.01$) *(figure 3.8.A)*. WBM adhesion in IR animals increased steadily throughout the duration of the experiment with adhesion at 60 minutes post-reperfusion significantly different from 30 minutes (30 minutes post reperfusion: 9.56 ± 1.71 vs 60 minutes post reperfusion: 17.88 ± 2.64; $P < 0.05$) *(figure 3.8.A)*. In comparison, adhesion in sham treated animals remained unchanged throughout the time course of the experiment (eg. 30 minutes: 2.86 ± 0.88 vs 60 minutes: 6.50 ± 2.47; $P > 0.05$) *(figure 3.8.A)*. Consistent with the *in vitro* experiments, WBM adhered to sinusoids and not portal areas *(figures 3.8.C-D)*. No significant difference was observed between the number of free flowing cells in IR animals compared to controls at any time point (e.g. at 55 minutes: sham control: 2.60 ± 1.4 vs IR injured: 3.00 ± 0.96; $P > 0.05$) *(figure 3.8.A)*. Unsurprisingly, there was a tendency for more

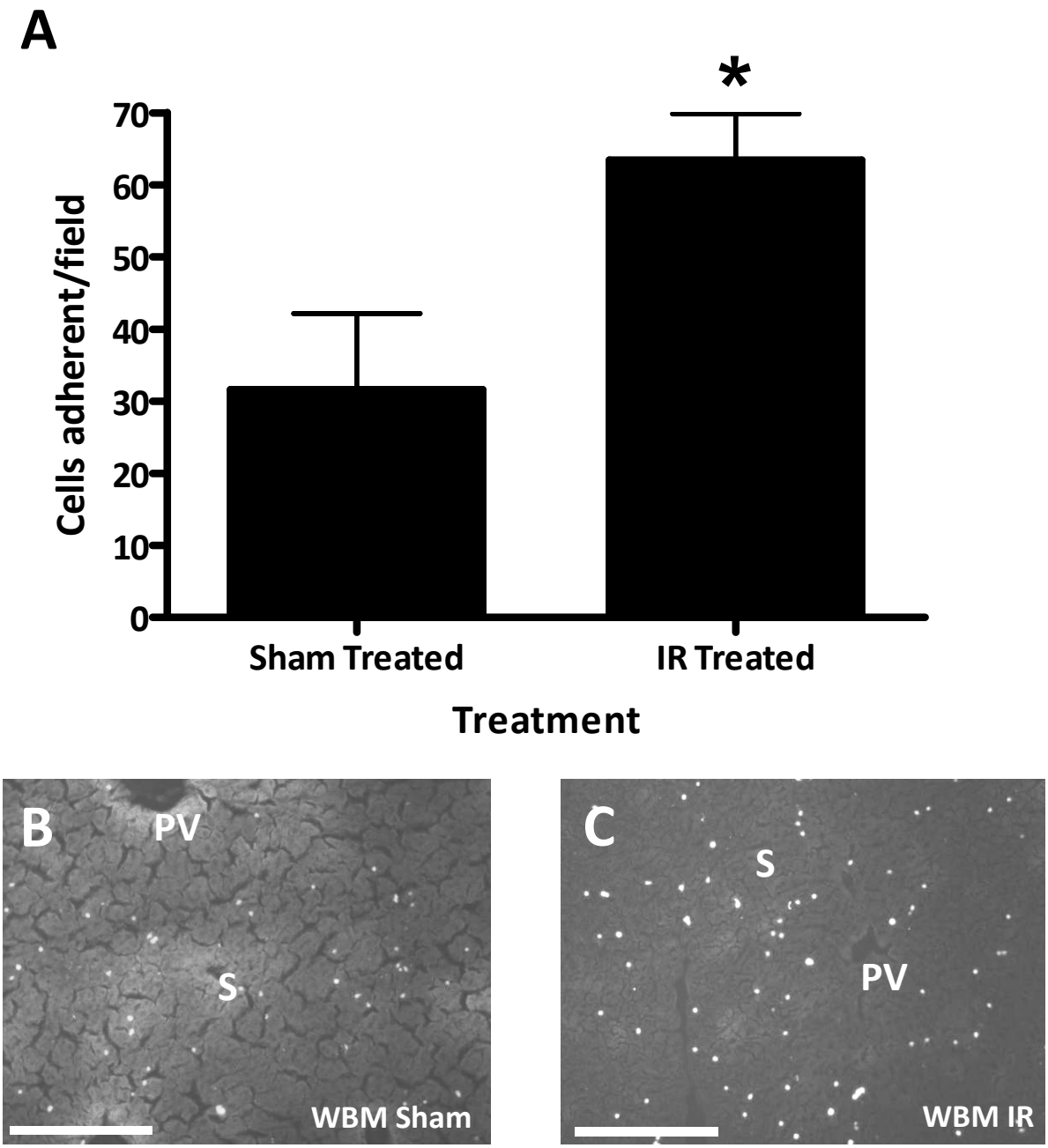


Figure 3.5. *WBM cell adhesion is significantly raised on sections isolated from IR treated animals when compared to sham controls.* WBM cell adhesion was examined on hepatic sections using the Stamper-Woodruff assay. Sections were isolated from sham and IR treated (90 PHI / 30 R) animals and 1 x 10^5 CFSE-labelled WBM cells were exposed to sections for 20 minutes. Adhesion was counted in 10 fields on each section, and an average for each section obtained. Significantly raised adhesion was observed on sections isolated from IR treated animals when compared to sham sections. Results are shown in **panel A.** Representative images are shown of Sham **(panel B)** and IR treated **(panel C)** sections. White bars represent 100µm. Bars represent mean adhesion ± SEM of at least 3 separate experiments; * $p < 0.05$ (unpaired t-test).

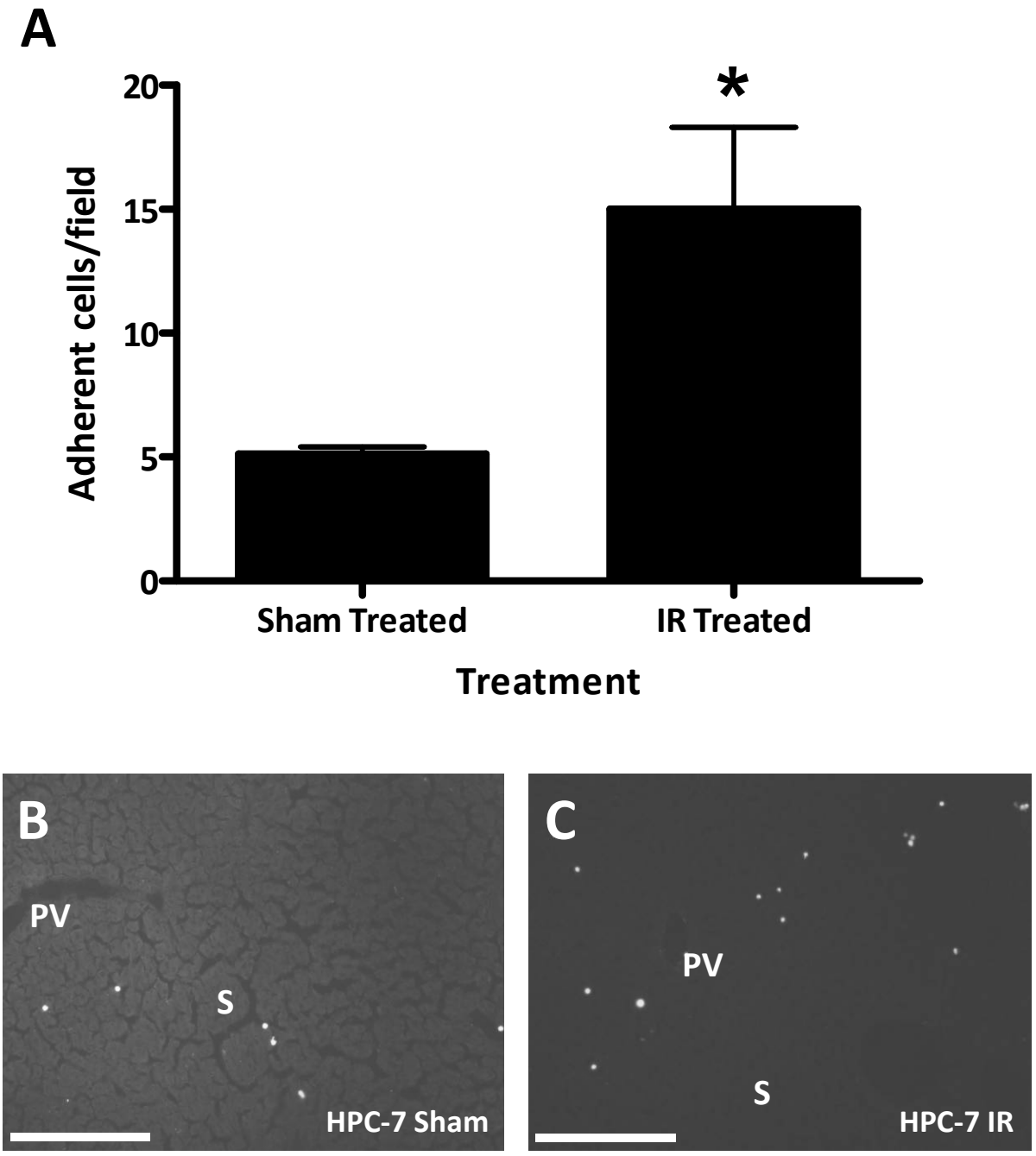


Figure 3.6. *HPC-7 cell adhesion is significantly raised on sections isolated from IR treated animals when compared to sham controls.* HPC-7 cell adhesion was examined on hepatic sections using the Stamper-Woodruff assay. Sections were isolated from sham and IR treated (90 PHI / 30 R) animals and 1 x 10^5 CFSE-labelled HPC-7 cells were exposed to sections for 20 minutes. Adhesion was counted in 10 fields on each section, and an average for each section obtained. Significantly raised adhesion was observed on sections isolated from IR treated animals when compared to sham sections. Results are shown in **panel A.** Representative images are shown of Sham **(panel B)** and IR treated **(panel C)** sections. White bars represent 100µm. Bars represent mean adhesion ± SEM of 3 separate experiments; * $p < 0.05$ (unpaired t-test).

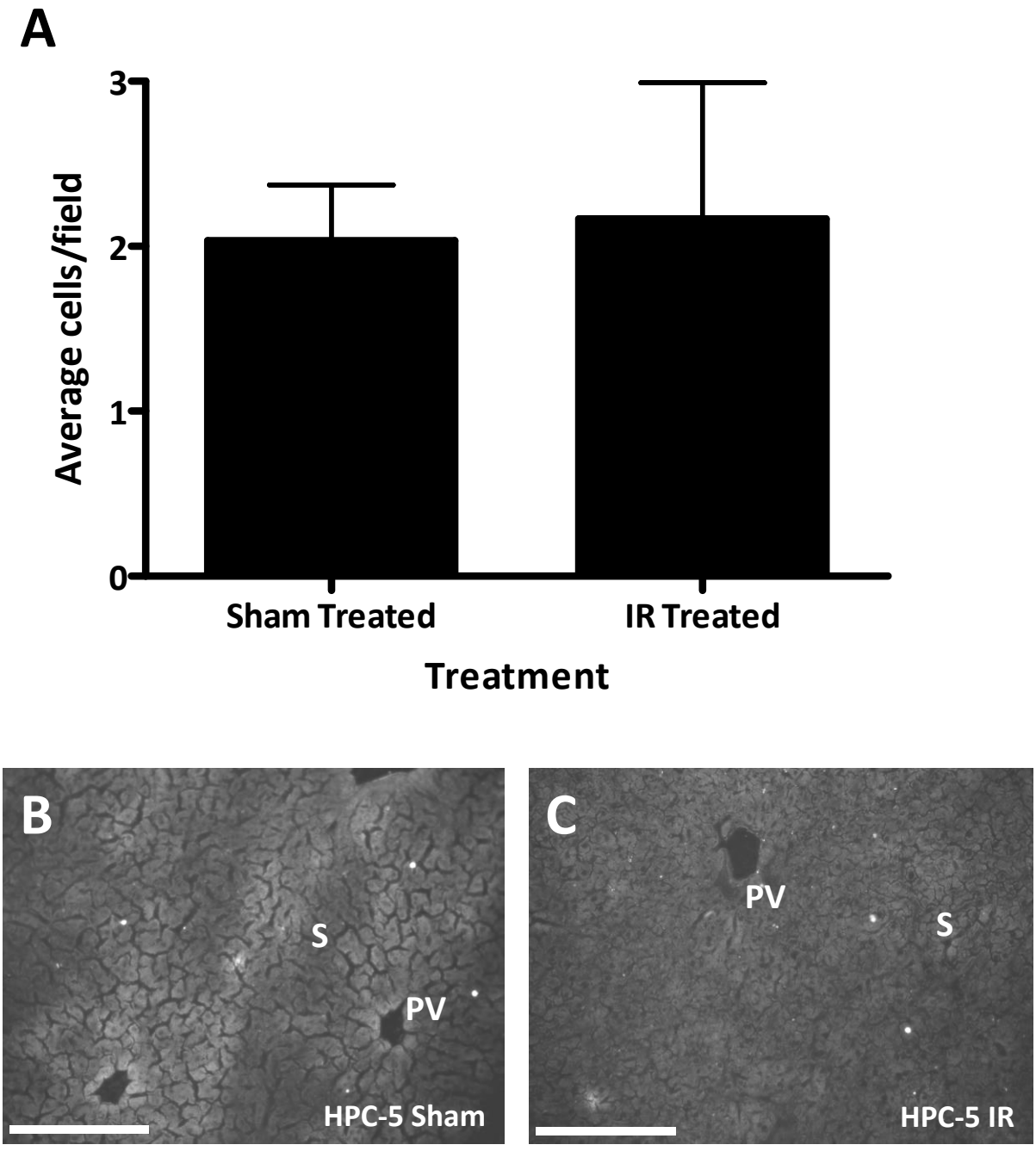


Figure 3.7. *HPC-5 cell adhesion is not raised on sections isolated from IR treated animals when compared to sham controls.* HPC-5 cell adhesion was examined on hepatic sections using the Stamper-Woodruff assay. Sections were isolated from sham and IR treated (90 PHI / 30 R) animals and 1 x 10^5 CFSE-labelled HPC-5 cells were exposed to sections for 20 minutes. Adhesion was counted in 10 fields on each section, and an average for each section obtained. Adhesion was not raised on sections isolated from IR treated animals when compared to sham sections. Results are shown in **panel A**. Representative images are shown of Sham **(panel B)** and IR treated **(panel C)** sections. White bars represent 100µm. Bars represent mean adhesion ± SEM of 4 separate experiments (unpaired t-test).

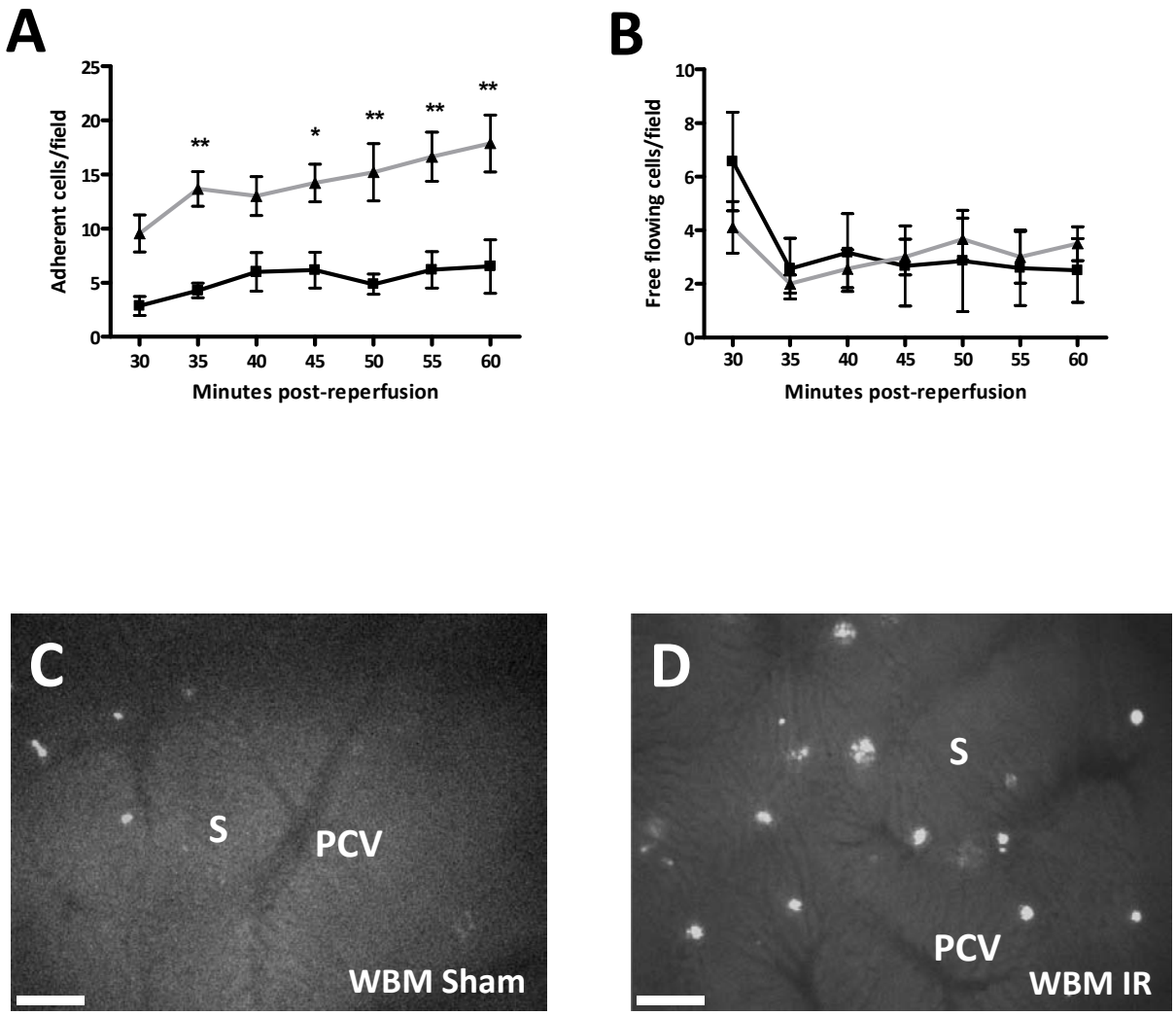


Figure 3.8. *WBM adhesion is significantly increased in animals subjected to IR injury.* Adhesion of WBM cells to the hepatic microcirculation *in vivo* was examined using intravital microscopy. Animals were subjected to either sham procedure or IR injury (90min PHI / 30min R) prior to the introduction of labelled WBM cells (1 x 10^6 cells, 5µM CFSE, i.a.). One field of view was selected prior to introduction of cells and continuously monitored every 5 minutes to analyse WBM adhesion dynamics. WBM cell adhesion was significantly raised in animals subjected to IR injury when compared to sham treated animals **(panel A;** black line: sham treated animals; grey line: IR treated animals). No significant difference was seen in free flowing cells between treatments **(panel B;** black line: sham treated animals; grey line: IR treated animals). Representative images are shown of WBM cell adhesion at 50 minutes post reperfusion in sham **(panel C)** and IR treated **(panel D)** microvasculature. White bars represent 100µm. Plots in **(A)** represent mean adhesion ± SEM of at least 5 separate experiments; * $p < 0.05$, * $p < 0.01$ (two-way ANOVA with Bonferroni Post Test).

free flowing cells to be observed immediately after introduction (ie. at 30 minutes post reperfusion).

3.3.9. HPC-7 adhesion is significantly raised *in vivo* by IR injury

HPC-7 adhesion was significantly raised in IR animals compared to sham controls (e.g. at 45 minutes, sham control: 5.60 ± 1.08 vs IR injury: 13.82 ± 1.95; $P < 0.05$) *(figure 3.9.A)*. In contrast to WBM cells, adhesion of HPC-7 in IR animals remained relatively constant throughout the time-course of the experiments, and there was no significant difference in the number of cells adhering at 30 minutes and 60 minutes post reperfusion (30 minutes: 12.60 ± 1.67 vs 60 minutes: 12.27 ± 1.31; $P > 0.05$) *(figure 3.9.A)*. HPC-7 adhesion in sham controls also increased gradually throughout the time course of the experiment with significantly more cells adhering at 60 compared with 30 minutes (30 minutes: 2.75 ± 1.18 vs 60 minutes: 7.20 ± 1.2; $P < 0.05$). In line with *in vitro* observations, HPC-7 adhesion was predominantly sinusoidal *(figures 3.9.C-D)*. No significant difference in free-flowing cells is seen between sham and IR treatment *(figure 3.9.B)*. Again, maximal levels of observed free-flowing cells were seen at 30 minutes post reperfusion *(figure 3.9.B)*.

3.3.10. HPC-5 adhesion is significantly raised *in vivo* by IR injury

Although adhesion of HPC-5s was not increased on frozen injured liver PHI sections, HPC-5 adhesion was significantly raised in IR animals compared to sham controls (e.g. at 45 minutes: sham control: 5.00 ± 0.70 vs IR injury: 17.17 ± 1.90; $P < 0.001$) *(figure 3.10.A)*. Adhesion remained relatively constant during the time-course of the experiment, with adhesion at 30 minutes not significantly different from adhesion at 60 minutes (30 minutes:

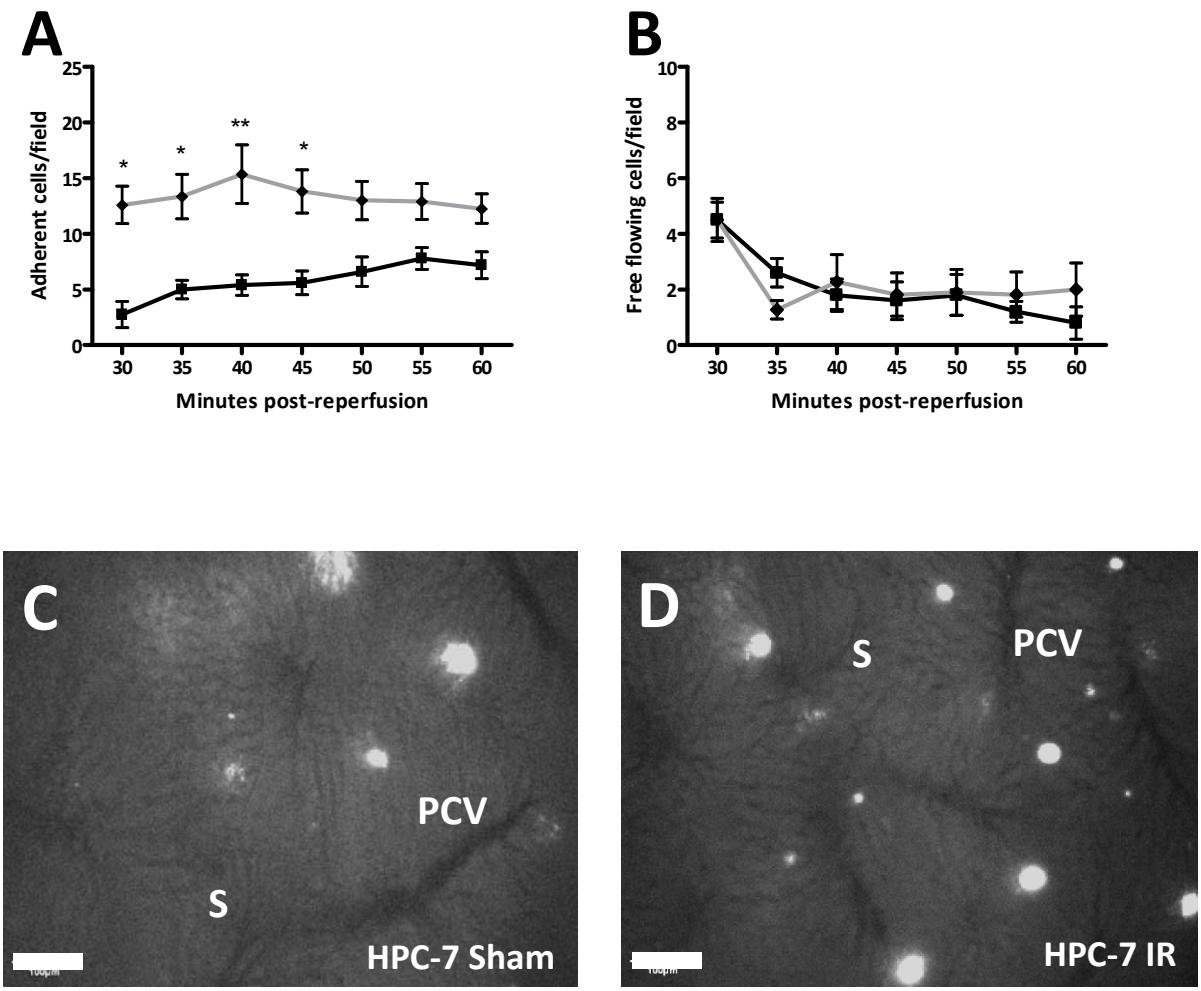


Figure 3.9. *HPC-7 adhesion is significantly increased in animals subjected to IR injury.* Adhesion of HPC-7 cells to the hepatic microcirculation was examined following IR using intravital microscopy. Animals were subjected to either sham procedure or IR injury (90m PHI/30 R) prior to the introduction of labelled HPC-7 cells (1 x 10^6 cells, 5µM CFSE, I.A.). One field of view was selected prior to introduction of cells and monitored to analyse HPC-7 adhesion dynamics. HPC-7 cell adhesion was significantly raised in animals subjected to IR injury when compared to sham treated animals **(panel A;** black line: sham treated animals; grey line: IR treated animals**)**. No significant difference was seen in free flowing cells between treatments **(panel B**; black line: sham treated animals; grey line: IR treated animals**)**. Representative images are shown of HPC-7 cell adhesion at 50 minutes post reperfusion in sham **(panel C)** and IR **(panel D)** treated microvasculature. The appearance of cells of various sizes is a result of cells occupying differing focal planes. White bars represent 100µm. Plots in **panel A** represent mean adhesion ± SEM of at least 5 separate experiments; * $p < 0.05$, ** $p < 0.01$ (two-way ANOVA with Bonferroni Post Test).

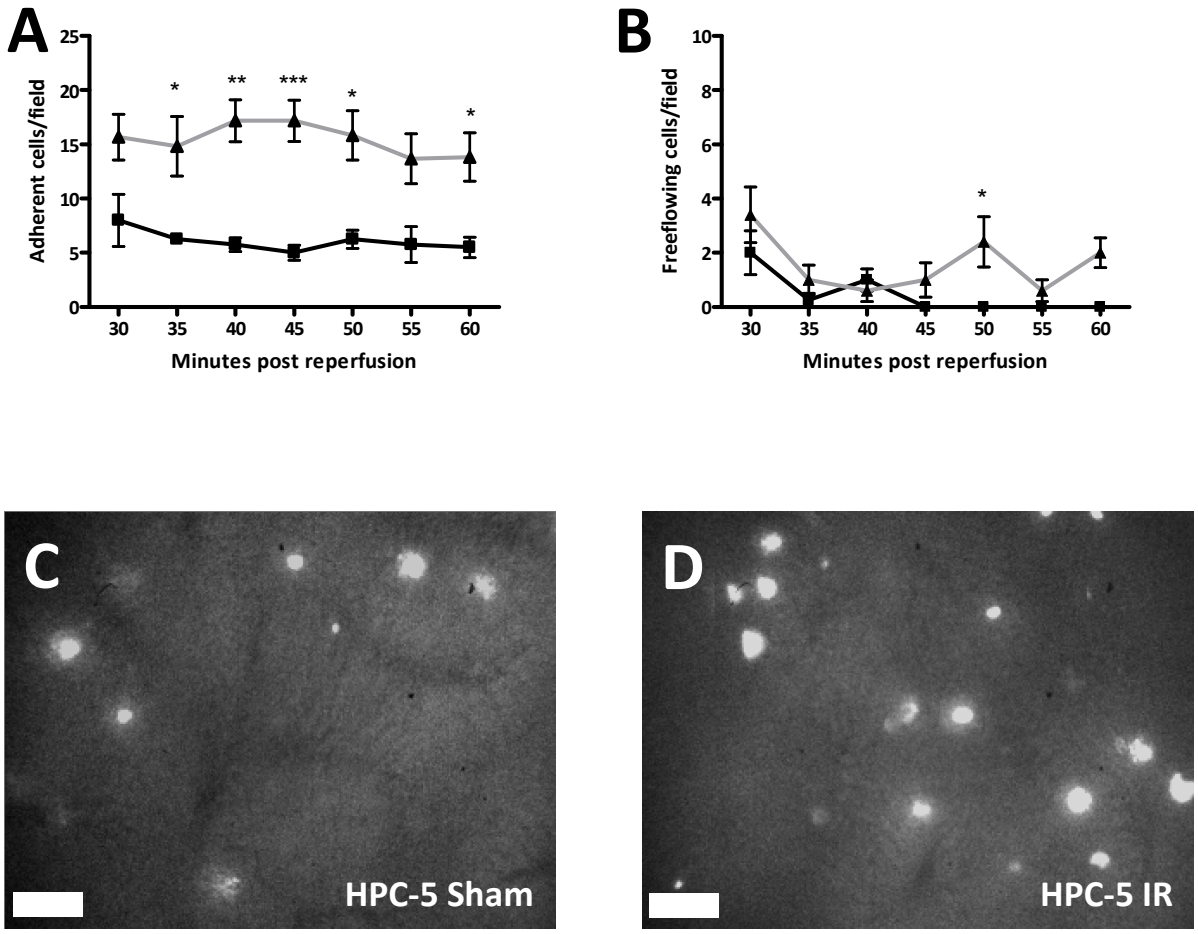


Figure 3.10. *HPC-5 adhesion is significantly increased in animals subjected to IR injury.* Adhesion of HPC-5 cells to the hepatic microcirculation was examined following IR using intravital microscopy. Animals were subjected to either sham procedure or IR injury (90m PHI/30 R) prior to the introduction of labelled HPC-5 cells (1 x 10^6 cells, 5µM CFSE, I.A.). One field of view was selected prior to introduction of cells and monitored to analyse HPC-5 adhesion dynamics. HPC-5 cell adhesion was significantly raised in animals subjected to IR injury when compared to sham treated animals **(panel A;** black line: sham treated animals; grey line: IR treated animals**)**. No significant difference was seen in free flowing cells between treatments **(panel B;** black line: sham treated animals; grey line: IR treated animals). Representative images are shown of HPC-5 cell adhesion at 50 minutes post reperfusion in sham **(panel C)** and IR treated **(panel D)** microvasculature. White bars represent 100µm. Plots in **panel A** represent mean adhesion ± SEM of at least 5 separate experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-way ANOVA with Bonferroni Post Test)

15.67 ± 2.11 vs 60 minutes: 13.83 ± 2.21; P > 0.05) *(figure 3.10.A)*. Similarly, HPC-5 adhesion in sham controls remains constant, with adhesion at 30 minutes not significantly differing from 60 minutes (30 minutes: 8.00 ± 2.42 vs 60 minutes: 5.50 ± 0.96; P > 0.05).

Adhesion of HPC-5 cells was predominantly sinusoidal *(figures 3.10.C-D)*. The observed number of free-flowing cells in IR animals did not generally differ from that seen in sham controls; an exception being at 50 minutes post reperfusion where a significant increase in free flowing cells was seen in IR animals (sham control: 0.00 ± 0.00 vs 2.40 ± 0.92; P < 0.05) *(figure 3.10.B)*.

3.3.11. Introducing HPC-7 or WBM cells at 5 minutes reperfusion does not significantly alter adhesion at 60 minutes post-reperfusion

In the above intravital experiments, exogenous cells were introduced at 30 miniutes post-reperfusion. However, these exogenously administered cells may compete with endogenous cells (eg. inflammatory neutrophils) for sites of adhesion (Rey-Ladino *et al.*, 1998). In order to examine this possibility, WBM and HPC-7 cells were introduced into IR treated animals at 5 minutes post-reperfusion, when endogenous leukocyte recruitment was likely to be less than at later time points post-reperfusion (Fox-Robichaud *et al.*, 1998). No difference in adhesion was seen with WBM cells introduced at 5 or 30 minutes post-reperfusion (e.g. WBM adhesion at 60 minutes post reperfusion: cells introduced at 5 minutes: 19.00 ± 4.34 vs cells introduced at 30 minutes: 17.88 ± 2.64; P > 0.05) *(figure 3.11.A)*. No significant difference was observed in free-flowing WBM cells whether introduced at 5 or 30 minutes reperfusion (e.g. WBM free-flowing at 60 minutes post

reperfusion: cells introduced at 5 minutes: 4.50 ± 1.94 vs cells introduced at 30 minutes: 3.50 ± 0.63; P > 0.05) *(figure 3.11.B)*.

Although fewer HPC-7 cells adhere at the earlier time points when cells were introduced at 5 minutes post reperfusion rather than 30 minutes, this reduction was not significant at any time point (e.g. HPC-7 adhesion at 40 minutes post reperfusion: cells introduced at 5 minutes: 9.60 ± 0.81 vs cells introduced at 30 minutes: 15.36 ± 2.62; P > 0.05) *(figure 3.11.C)*. At 60 minutes reperfusion, adhesion was comparable between both populations of cells (e.g. HPC-7 adhesion at 60 minutes reperfusion: cells introduced at 5 minutes: 12.40 ± 1.36 vs cells introduced at 30 minutes: 12.27 ± 1.31; P > 0.05) *(figure 3.11.C)*. Perhaps unsurprisingly, significantly more HPC-7 cells were seen free-flowing at the earliest time-point (30 minutes reperfusion) when introduced at 30 minutes reperfusion (free-flowing cells at 30 minutes: introduced at 5 minutes: 0.60 ± 0.24 vs introduced at 30 minutes: 4.50 ± 0.64; P < 0.05) *(figure 3.11.D)*. No significant differences in free-flowing HPC-7 cells was observed at any other time-points *(figure 3.11.D)*.

3.3.12. $Lin^-Sca\text{-}1^+$ cell adhesion is not enhanced by IR injury

To examine whether primary freshly isolated immature cells share adhesion characteristics with the HPC-7 cell line, $Lin^-Sca\text{-}1^+$ cells were injected into hepatic IR treated animals and adhesion assessed using intravital microscopy. Interestingly, no significant differences were seen in the levels of recruitment of $Lin^-Sca\text{-}1^+$ cells in IR injured animals compared to sham controls (e.g. adhesion at 55 minutes: sham: 9.35 ± 1.81 vs. IR injury: 9.26 ± 2.32; P > 0.05) *(figure 3.12.A)*. Levels of free-flowing $Lin^-Sca\text{-}1^+$ are not significantly different between sham

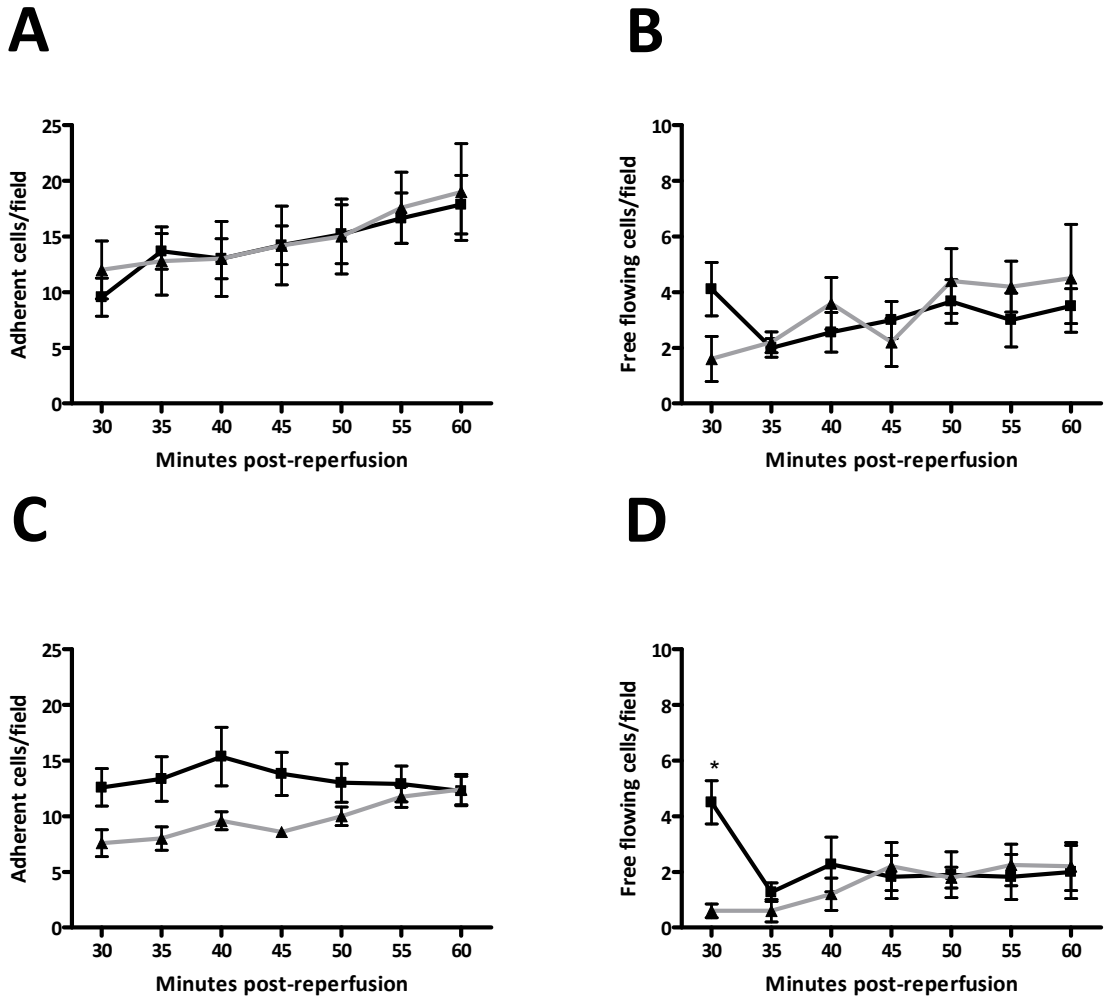


Figure 3.11. *Introducing WBM or HPC-7 cells at 5 minutes post-reperfusion does not significantly alter their adhesion compared to cells introduced at 30 minutes reperfusion.* Animals were subjected to 90 minutes partial hepatic ischemia, with $1x10^6$ cells introduced at either 30 minutes post reperfusion (black line) or 5 minutes post reperfusion (grey line). The time point at which WBM cells are introduced does not significantly alter adhesion at any point during reperfusion (panel A), nor does it significantly effect free flowing WBM cells (panel B). Similarly, the time point at which HPC-7 cells are introduced does not significantly alter adhesion at any point during reperfusion (panel C), although significantly more cells are seen free flowing at 30 minutes when introduced at 30 minutes reperfusion rather than 5 minutes (panel D). Figures represent mean adhesion (or free flowing cells) ± SEM of at least 5 separate experiments; * $p < 0.05$ (two-way ANOVA with Bonferroni Post Test).

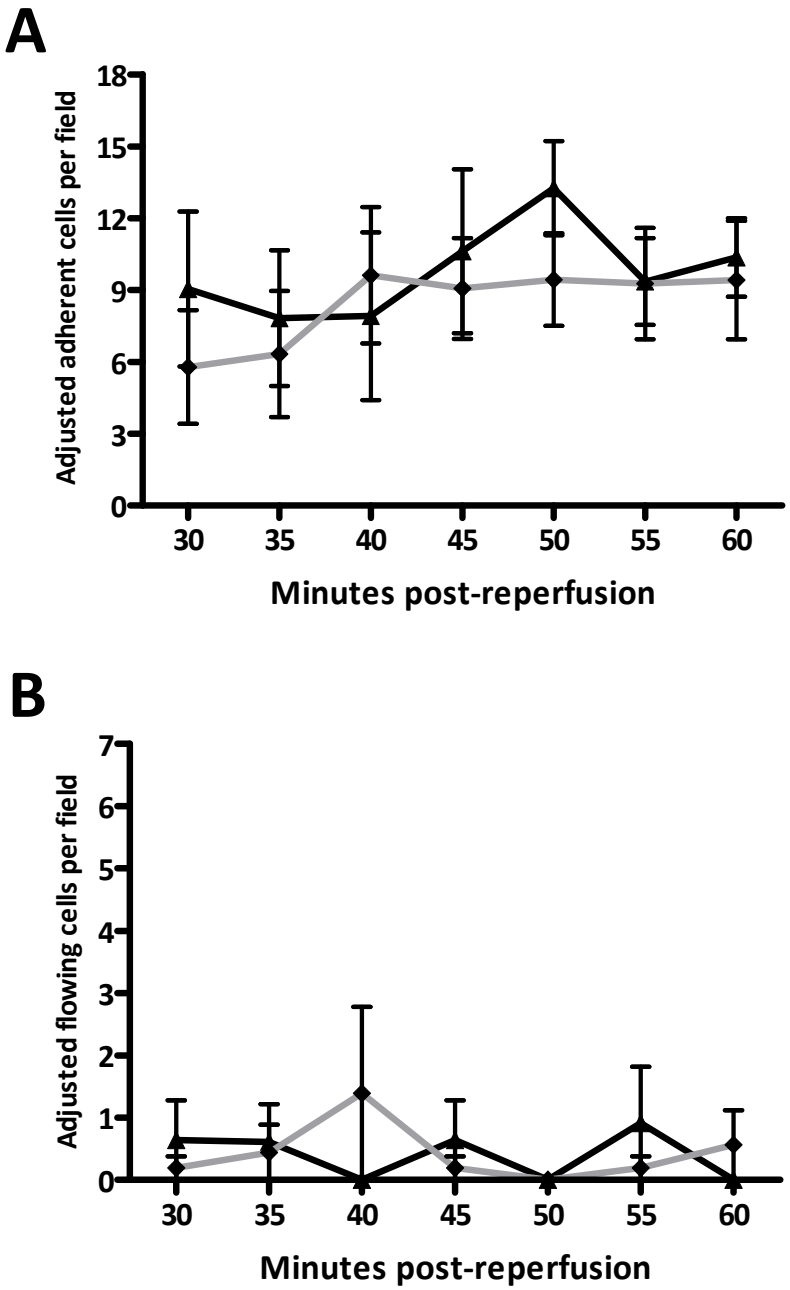


Figure 3.12. *Recruitment of Lin$^-$Sca-1$^+$ cells to the liver is not enhanced by IR injury*. Adhesion of Lin$^-$Sca-1$^+$ cells to the hepatic microcirculation was examined following IR using intravital microscopy. Animals were subjected to either sham procedure (black line) or IR injury (90m PHI/30 R; grey line) prior to the introduction of labelled Lin$^-$Sca-1$^+$ cells (between 1.2 x 10^5 and 3.3 x 10^5, 5µM CFSE, I.A.; data is adjusted to 1 x 10^6 cells – see methods). One field of view was selected prior to introduction of cells and monitored to analyse adhesion dynamics. No difference in adhesion is noted between sham and IR treated animals **(panel A)**. No difference in free-flowing cells is seen between control and IR animals **(panel B)**. Figures represent adjusted adhesive/flowing cells ± SEM from six separate experiments (multiple comparison test shows no points of significance).

and IR treated animals (e.g. free-flowing cells at 55 minutes: sham: 1.52 ± 1.52 vs. IR: 0.28 ± 0.27; P > 0.05) *(figure 3.12.B)*. The adhesion of $Lin^{-}Sca\text{-}1^{+}$ cells was again predominantly sinusoidal.

3.3.13. Adhesion of the human progenitor cell line, KG1a, is not raised by IR injury

The human myelomonocytic cell line, KG1a, has been frequently used as a surrogate for human haematopoietic progenitor cells (Wagner *et al.*, 2008, Dimitroff *et al.*, 2005). KG1a recruitment was assessed in both the *in vitro* Stamper-Woodruff and the *in vivo* intravital IR injury model. *In vitro*, KG1a adhesion was not significantly raised on sections isolated from IR animals compared to sections from sham controls (sham sections: 2.27 ± 0.87 vs IR sections: 2.80 ± 1.25; P > 0.05) *(figure 3.13.C)*. Levels of KG1a adhesion observed on hepatic sections were comparable to those seen with HPC-5 *(figure 3.7.A)* and HPC-7 *(figure 3.6.A)* but not as high as WBM adhesion *(figure 3.5.A)*. In the intravital model, KG1a cells were introduced into C57Bl/6 mice at 30 minutes reperfusion. Immune rejection was deemed unlikely to occur within such a short time-scale removing the need to use immunodeficient mice. No significant difference in KG1a adhesion was observed between sham and IR treated animals (e.g. at 50 minutes reperfusion: sham control: 43.67 ± 13.35 vs IR treated: 34.40 ± 10.48; P > 0.05) *(figure 3.13.A)*. Much higher numbers of KG1a cells were recruited *in vivo* to the hepatic microvasculature when compared with WBM *(figure 3.8.A)*, HPC-7 *(figure 3.9.A)* and HPC-5 cells *(figure 3.10.A)*. Numbers of free flowing cells were not affected by IR injury when compared to sham control (e.g. free flowing cells at 45 minutes reperfusion: sham control: 2.67 ± 1.45 vs IR treated: 2.20 ± 0.66) *(figure 3.13.B)*.

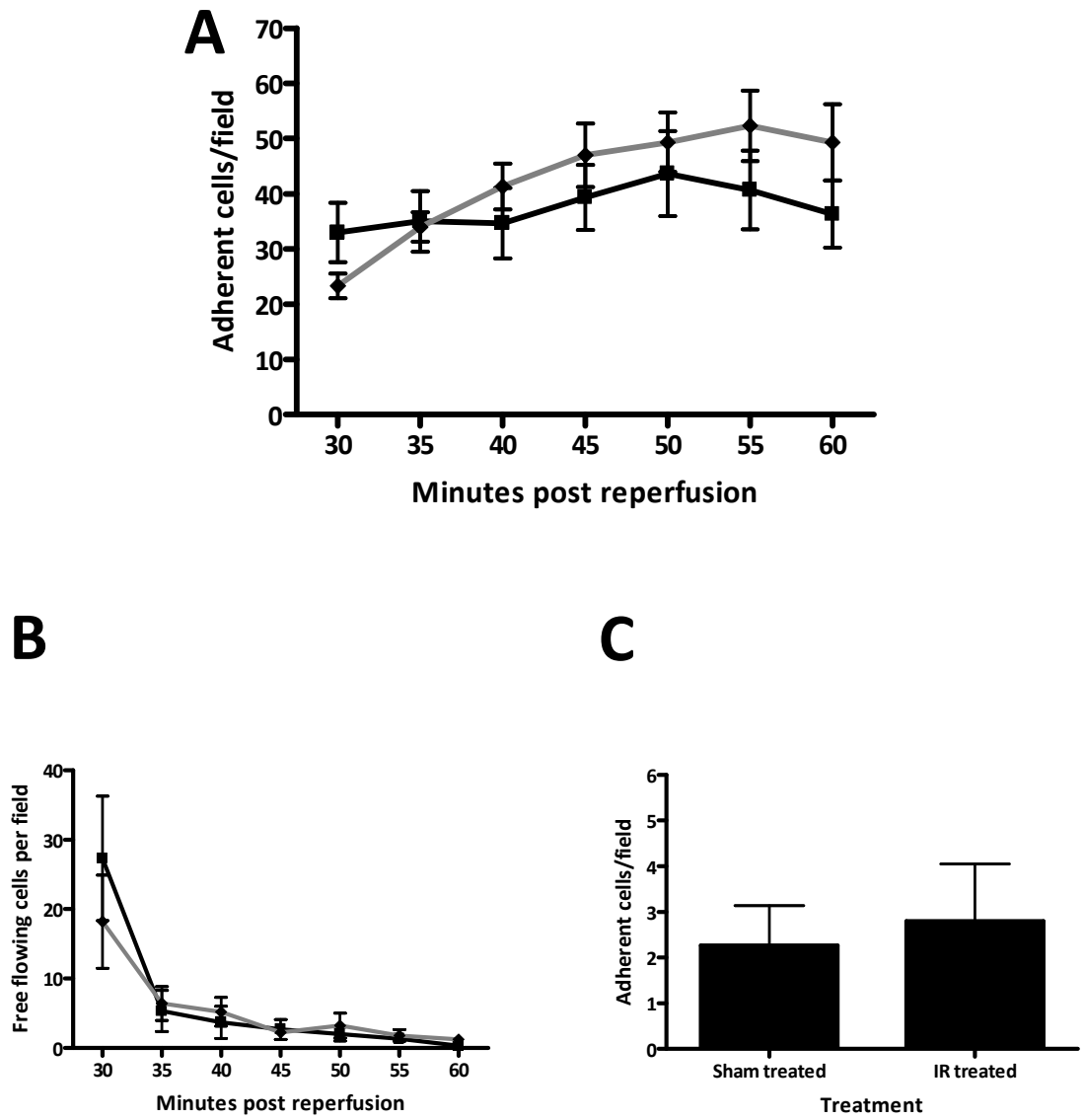


Figure 3.13. *IR injury does not promote hepatic recruitment of the human progenitor cell line, KG1a.* Intravital microscopy was used to monitor the recruitment of the human myeloid progenitor cell line, KG1a, following hepatic IR injury. Animals were subjected to IR injury (90min PHI / 30min R) prior to the introduction of labelled KG1a cells (1 x 10^6 cells; 5µM CFSE). Levels of KG1a adhesion and free flowing cells are similar in both sham and IR treated animals **(panels A and B;** black line: sham treated, grey line: IR treated**)**. Additionally, KG1a adhesion is not raised on sections isolated from IR treated animals **(panel C)**. Panels A and B represent mean adhesion/free-flowing cells ± SEM of at least 3 separate experiments. Panel C represents mean adhesion ± SEM of 3 separate experiments (panels A and B: two-way ANOVA; panel C: unpaired t-test).

3.4. Discussion

This study provides novel *in vitro* and *in vivo* evidence that hepatic HSC recruitment is increased in response to murine IR injury. This response was observed for three different cell populations; WBM cells and both adult and embryonic HSCs. Interestingly, hepatic IR injury does not significantly alter adhesion of primary isolates of progenitor cells ($Lin^{-}Sca\text{-}1^{+}$ cells) or human progenitor cells (KG1a). However, this study does demonstrate for the first time that primary HSCs can be indentified in the liver (injured or not) immediately after exogenous administration and without previous BM engraftment. Although previous studies of injury mediated HSC recruitment to the liver used chronic injury models (Alison *et al.*, 2000, Theise *et al.*, 2000b, Petersen *et al.*, 1999) the current data provides the first evidence that HSCs are also recruited rapidly to the hepatic microvasculature following an acute hepatic injury.

The increased adhesion of WBM, HPC-5 and HPC-7 cells to hepatic sinusoids after IR injury is consistent with studies reporting recruitment of HSCs to hypoxic organs along cytokine concentration gradients (Ceradini *et al.*, 2004). The role of these cells following recruitment is unclear and it is not possible to determine whether transmigration and thus tissue engraftment into the surrounding interstitium occured as the the resolution of images obtained from these intravital experiments is too low. Interestingly, similar levels of adhesion were observed with HPC-5, HPC-7 and WBM cells which suggests the presence of a potential upper limit for progenitor cell adhesion. Alternatively, the comparable levels of adhesion may represent consistent levels of rapid cell clearance. In a similar study, labeled MSCs are cleared from the rat circulation within 15 minutes (Yocum *et al.*, 2005). Work

from our lab has suggested that HSCs are rapidly cleared from the circulation. It was demonstrated that high levels of adhesion were only seen within the murine cremaster muscle microcirculation after four bolus injections of HPC-7 cells were administered systemically every 30 minutes (Y Zhao, unpublished observations, 2007).

A potential concern with introducing exogenous cells and monitoring their adhesion in vivo is the fact that they may have to compete with endogenous circulating cells. Exogenously introduced stem cells injected into early embryos display competition with endogenous cells for engraftment space (Harder *et al.*, 2002). To account for this, we also introduced cells at 5 minutes reperfusion as well as at 30 minutes as endogenous cell adhesion early on in reperfusion is likely to be lower than at later time points (Fox-Robichaud *et al.*, 1998). Introducing WBM cells at 5 minutes rather than 30 minutes had no significant effect on adhesion suggesting that recruitment is dependent upon the stage of injury rather than the length of time the cells are in the circulation. Similar results were seen with HPC-7 cells, although if introduced at 5 minutes reperfusion, recruitment is initially lower than those injected at 30 minutes reperfusion (albeit not significantly). This may represent a low initial recruitment during first pass due to a lower severity of injury at that time or poor availability of cells as a result of rapid their clearance. Such rapid clearance of progenitor cells has been shown previously; very few cells with CFU capability can be retrieved from the circulation just 6 minutes following an intravenous injection of donor Lin^- cells into recipient mice (Wright *et al.*, 2001).

Adhesion of HSCs in the hepatic microcirculation occurs primarily within the hepatic sinusoidal capillaries. The hepatic sinusoids represent a unique low flow vascular bed, with three different points of entry for leukocytes. Blood enters the liver either via the portal tract or the hepatic artery and then enters the sinusoids either by small communicating vessels in or close to the portal tracts or more distally by vessels that drain into the distal sinusoids or terminal hepatic veins. The majority of blood flow, however, is distributed through the hepatic sinusoids before returning to the systemic circulation via the hepatic veins (Lalor *et al.*, 2007). Blood flows through the sinusoids at relatively low levels of shear stress, facilitating mature leukocyte adhesion in the sinusoids. The data within this chapter also suggests the same applies for recruitment of HSCs. Interestingly, HSC adhesion remains predominantly sinusoidal on static adhesion assays where flow is not an influencing factor. As the sinusoids play an important role in IR injury, the delivery of HSCs directly to the sinusoids may be an important prerequisite for repair.

Interestingly, IR injury does not enhance the recruitment of $Lin^{-}Sca\text{-}1^{+}$ progenitor cells. HSCs constitute a rare compartment within the BM, with at most 0.05% of murine BM cells thought to be primitive HSCs (Chen *et al.*, 1997). The most basic accepted definition of HSCs is defined by expression of Sca-1 and c-kit and a lack of markers of maturation and commitment (Lineage markers), termed KSL cells (Chen *et al.*, 1997). In a typical sort, approximately 3000 cells can be retrieved from a single wild-type murine donor (data not shown). Such small yields are too small for physiological studies and many donor mice would be required to yield enough cells for a single intravital experiment. In a study by Massberg and colleagues, 1.5×10^5 KSL cells were introduced intravenously for monitoring

of adhesion in the carotid artery (Massberg *et al.*, 2006). To isolate this number of KSL cells, approximately fifty donor mice would be required. As a result, our study uses a less restrictive isolation profile. Primary cells used for comparison in this study were lineage negative and stem cell antigen-1 positive ($Lin^-Sca\text{-}1^+$). Approximately 0.3% of the adult bone murine BM is $Lin^-Sca\text{-}1^+$ (data not shown). The $Lin^-Sca\text{-}1^+$ fraction contains cells possessing long term haematopoietic reconstituting potential, although it should be noted that other cell populations exist within this fraction (Welner *et al.*, 2008). Therefore, the different responses *in vivo* may represent the different make up of these populations.

It is plausible that the HSC lines used within the study are not truly representative of 'true' primary HSCs which is why their pattern of recruitment is different. However, the HPC-7 cell line has been used extensively both locally and in international studies for investigating the control of HSC growth and differentiation at a molecular and cellular level (Wilson *et al.*, 2009, Pimanda *et al.*, 2008). Thus, it is well accepted as being representative of pure HSCs. Furthermore, this cell line can reconstitute the hematopoietic compartment in lethally irradiated mice, a property of a true stem cell (Pinto do Ó *et al.*, 2002).

This cell line is also considered superior to other HSC lines such as FDCP-1 mix or FDC-P1 mix, which have been routinely utilised by leading international laboratories to monitor HSC trafficking within murine BM intravitally (Katayama et al., 2004, Mazo et al., 1998). It is well accepted that *in vivo* trafficking studies cannot be easily and routinely performed using primary cells because of the difficulty in isolating sufficient numbers of primary cells for intravital studies. However, trafficking studies conducted using the FDCP mix cell line have

increased our understanding of HSC trafficking within BM and our results provide important insights into the molecular events governing HSC homing to non-BM sites – this is currently unknown.

However, the fact remains that only the cell lines show increased recruitment to the injured liver compared with the non-injured liver. There may be other explanations for this difference; the methods used to purify Lin^- $Sca\text{-}1^+$ cells most likely leads to the isolation of a non-heterogeneous non-identical population of BM cells, which may differ substantially in their biological properties. Furthermore, Lin^- $Sca\text{-}1^+$ cells do not represent a pure HSC population and these cells were used because they can be isolated in sufficient numbers for *in vivo* trafficking studies. The differences between the homogeneous cell line and the less well-defined primary cells emphasise the need for further purification to define a more uniform population of cells.

This study demonstrates that an acute liver injury is able to attract the HPC-7 cell line to a greater degree than non-injured livers. This in itself is a novel finding since most studies investigating stem (HSC or MSC) or progenitor cell recruitment use more severe and chronic models of injury. It is therefore possible that Lin^- $Sca\text{-}1^+$ cells would be more favourably recruited above and beyond controls in more chronic severe models of hepatic injury. This is certainly worthy of future investigations and is something our lab are currently pursuing in collaborative studies with Dr Phil Newsome (University of Birmingham) using the carbon tetrachloride model of chronic liver injury.

In vitro, HPC-7 and WBM cell adhesion was raised significantly on hepatic sections. Interestingly, much higher levels of WBM cell adhesion were seen compared to HPC-7 cells. This may again be a result of the relative heterogeneity of the WBM cell preparation. Studies by Takeuchi and colleagues suggest that approximately 50% of the BM of mice is composed of neutrophils (Takeuchi *et al.*, 1987). The higher levels of adhesion observed with WBM cells compared to HPC-7 cells may be explained by the relative abilities of neutrophils and HSCs to engraft in inflamed tissues. Alternatively, the heterogeneity of WBM cells could facilitate adhesion of various leukocyte subsets through various adhesion pathways. On the other hand, a homogenous population of HPC-7 cells are likely to utilize a narrower range of adhesion pathways.

Somewhat surprisingly, adhesion of HPC-5 cells to frozen hepatic sections is not enhanced after partial IR injury. Furthermore, the levels of HPC-5 baseline adhesion seen *in vitro* are much lower than baseline adhesion of any other cell populations. This may reflect differential responses to chemokines present immobilised on the surface of the section or differences in adhesion molecule expression. For instance, murine embryonic and adult HSCs express different levels of Mac-1 (α_M integrin) (Ivanova *et al.*, 2002). Interestingly, HPC-5 adhesion is raised *in vivo* following partial hepatic IR injury suggesting distinct pathways of adhesion *in vitro* and *in vivo*. This may highlight a requirement for flow for adhesion of HPC-5 cells. Alternatively, HPC-5 adhesion *in vivo* may be enhanced by factors that are not present on the *in vitro* Stamper-Woodruff assay. Interestingly, the innate homing mechanisms of adult and embryonic HSCs do appear to have some differences with

embryonic HSCs homing to the BM less efficiently in irradiated adult hosts compared to adult HSCs (Perlingeiro *et al.*, 2001).

KG1a cells were originally isolated in 1980, as a subtype of the human myeloid cell line, KG1 (Koeffler *et al.*, 1980). KG1a cells are frequently used as a model of human hematopoietic progenitor cells (Morimoto *et al.*, 1994, Wagner *et al.*, 2008, Dimitroff *et al.*, 2000). We found that KG1a adhesion is not increased in IR treated C57Bl/6 mice when compared to sham controls. This may be a result of species differences or more subtle differences in phenotype between HPC-5/HPC-7 cells and KG1a cells. For example, KG1a cells do not express surface CXCR4 unlike primary HSCs and thus their response to the chemokine CXCL12 may be limited (Tavor *et al.*, 2004, Lapidot *et al.*, 2005). Furthermore, KG1a cells display different levels of response to CD44 blocking antibody when compared to primary HSCs (Wagner *et al.*, 2008). These differences may explain the inability of IR injury to promote KG1a adhesion to the hepatic microcirculation.

Total hepatic ischemia (THI) in mice is fatal (Matsumoto *et al.*, 2005) and we demonstrated that even short periods of ischemia (20 minutes total hepatic ischemia) result in high mortality and thus THI was not a viable option for our experimental model. Alternatively, 90 minutes partial hepatic ischemia (PHI) was well tolerated by all mice for up to 90 minutes post-reperfusion. Although other murine models do exist to replicate THI, they require relatively complex surgery and long recovery times (~ 3 weeks) prior to the initiation of THI (Matsumoto *et al.*, 2005). We found that our model of hepatic IR injury resulted in a consistent and significant 50 fold increase in circulating ALT comparable to those seen in

other published studies using PHI (Llacuna *et al.*, 2006). Furthermore, previous studies confirm that PHI periods below 50 minutes are insufficient to produce robust injury, confirming the suitability of the 90 minute partial ischemic period used in the current studies (Devey *et al.*, 2008). Although no histological damage was visualised on hepatic sections stained with H&E, it has been shown previously that apoptotic liver damage occurs within 6 hours post IR injury (Schlossberg *et al.*, 1996). It should be noted that male mice were used throughout these studies. It has been shown that there are gender differences in the cytokine response to hepatic IR injury; TNF-α release and hepatocellular damage is 'moderately' raised in male mice compared to females (Crockett *et al.*, 2006).

Blood sampling post IR injury revealed a significant shift in the mature leukocyte profile within the systemic circulation. IR injury significantly increased the level of circulating neutrophils while reducing the level of circulating lymphocytes. It is unclear from these results whether this represents a recruitment of lymphocytes in the liver, which subsequently reduces their numbers in the circulation, or a BM release of mature/immature neutrophils into the circulation of IR injured animals. The shifts in leukocyte compartmentalisation are likely to be a collective result of these factors. In other models of acute injury, significant mobilisation of neutrophils takes place some hours following insult (Sato *et al.*, 1998). It has been shown previously that metamyelocytes (immature granulocyte precursors) can be detected in the pulmonary microcirculation following intestinal IR injury (Kalia *et al.*, 2002a) and acute recruitment of $CD4^+$ lymphocytes can occur within one hour of hepatic reperfusion (Caldwell *et al.*, 2005). This recruitment may be important for initiating the neutrophil-dependent phase of IR injury; $CD4^+$

lymphocytes release IL-17 upon activation, a pro-inflammatory cytokine that stimulates recruitment of neutrophils (Yu and Gaffen, 2008). Whether this behaviour is important for HSC recruitment is unclear, and not deducible from this study.

To summarise, we have demonstrated using both *in vitro* and *in vivo* techniques that HSC recruitment to the hepatic microcirculation is significantly raised as a result of IR injury. Interestingly, IR injury promotes the recruitment of both adult and embryonic derived HSCs to similar levels, suggesting potential similarities in their recruitment mechanisms. *In vitro*, HSCs appeared to adhere to the vessel linings of the sinusoids on hepatic sections, and *in vivo,* recruitment of HSCs took place exclusively within the sinusoidal vessels. Interestingly, the recruitment of primary $Lin^{-}Sca\text{-}1^{+}$ cells was not raised as a result of IR injury, nor was the recruitment of human progenitor cell line, KG1a. The molecular basis for this recruitment will be addressed in the following chapter.

Chapter 4

Mechanisms of haematopoietic stem cell recruitment during murine ischemia-reperfusion injury – role of specific adhesion molecules

4. Mechanisms of haematopoietic stem cell recruitment during murine IR injury

4.1. Introduction and Hypotheses

4.1.1. Introduction

The adhesion mechanisms involved in HSC recruitment to medullary sites are relatively well defined in comparison to extra-medullary sites (Mazo et al., 1998, Frenette et al., 1998). The liver has been at the centre of experimental and clinical studies investigating injury mediated HSC recruitment, yet it is still not clearly demonstrated whether exogenously administered HSCs home to ischemic liver or sites of inflammation from peripheral blood. The efficiency and molecular basis of this process and the vascular adhesion molecules involved are not known.

The previous chapter provides evidence for the recruitment of HSCs during acute IR injury in both *in vitro* and *in vivo* adhesion assays. Furthermore, both adult (HPC-5) and embryonic (HPC-7) HSCs are recruited to the liver in this model of injury. Should HSCs prove beneficial in models of hepatic injury, one of the major problems in translating the studies to therapy is the sub-therapeutic recruitment of transplanted cells. Methods that allow an improvement in the engraftment of HSCs (and indeed other adult stem cells) are a high priority for cellular therapies. A better understanding of the adhesion mechanisms that modulate recruitment, and more specifically the adhesion molecules involved, may allow us to modulate recruitment and enhance engraftment.

The adhesive mechanisms governing HSC recruitment to BM microcirculation are relatively well understood with contributions of various selectins and integrins well described (Lapidot et al., 2005, Mazo et al., 1998). Very few studies have examined the adhesion pathways utilised by HSCs to home to non-BM tissues. Recently, studies by Zheng and colleagues suggested that fusion of HSCs and cardiomyocytes *in vivo* is dependant on the $\alpha_4\beta_1$ integrin/VCAM-1 pathway (Zhang *et al.*, 2007).

HSCs do express adhesion molecules also utilised by mature leukocytes for recruitment into inflamed liver and $Lin^-Sca\text{-}1^+$ HSC express α_L, α_1, α_3, α_4, α_5, α_6, β_1 integrins, L-selectin, CD44 and PECAM-1 (Becker *et al.*, 1999). Hepatic sinusoidal endothelial cells express various counter-receptors, including VAP1, ICAM-1 and VCAM-1 (Adams and Eksteen, 2006). It is therefore plausible that interactions between these molecules may play important roles in HSC recruitment.

4.1.2. Hypotheses

For the work in this chapter, we hypothesised:

1. Individual blockade of HSC adhesion molecules (CD18, CD31, CD44, CD49d) significantly reduces HSC adhesion post IR injury
2. HSC recruitment seen following IR injury is reduced in $CD31^{-/-}$ animals
3. Adhesion molecule upregulation is detectable on samples of hepatic IR tissue when compared to sham treated controls

4.2. Methods

The methods used in this chapter are described in detail in Chapter 2. Briefly, HPC-7 cells were phenotyped for expression of the adhesion molecules of interest after incubation with fluorescently conjugated antibodies against CD49d (FITC), CD49e (PE), CD44 (PE), CD31 (FITC), CD18 (FITC) and CD11b (FITC). The effective function blocking concentration was also determined for these antibodies allowing us to use them in Stamper-Woodruff *in vitro* adhesion assays and intravital microscopy studies. CD31 deficient mice were used to determine the role of endothelial CD31. Immunohistochemistry was performed on sham and IR treated animals (90 PHI / 30 R) treated with primary antibodies against murine CD31, CD44 and VCAM-1 conjugated to a secondary Texas Red antibody.

4.3. Results

4.3.1. HPC-7 cells express a panel of common adhesion molecules

In order to investigate adhesion mechanisms of HPC-7, a prior knowledge of adhesion molecule expression on these cells is essential. HPC-7 cells were analysed by flow cytometry for surface expression of the adhesion molecules CD49d, CD49e, CD44, CD31, CD18 and CD11b. HPC-7 cells showed a robust expression of CD49d *(figure 4.1.A)*, CD49e *(figure 4.1.B)*, CD44 *(figure 4.1.C)*, CD31 *(figure 4.1.D)***,** CD18 *(figure 4.1.E)* and L-Selectin/CD62L *(figure 4.1.F)*. However, HPC-7 cells did not express the α_M integrin subunit, CD11b (data not shown)**.** The adult HSC line, HPC-9 (an equivalent of the line adult HPC-5) expressed a similar repertoire of adhesion molecules CD49d, CD49e, CD44 and CD31 (figure not shown).

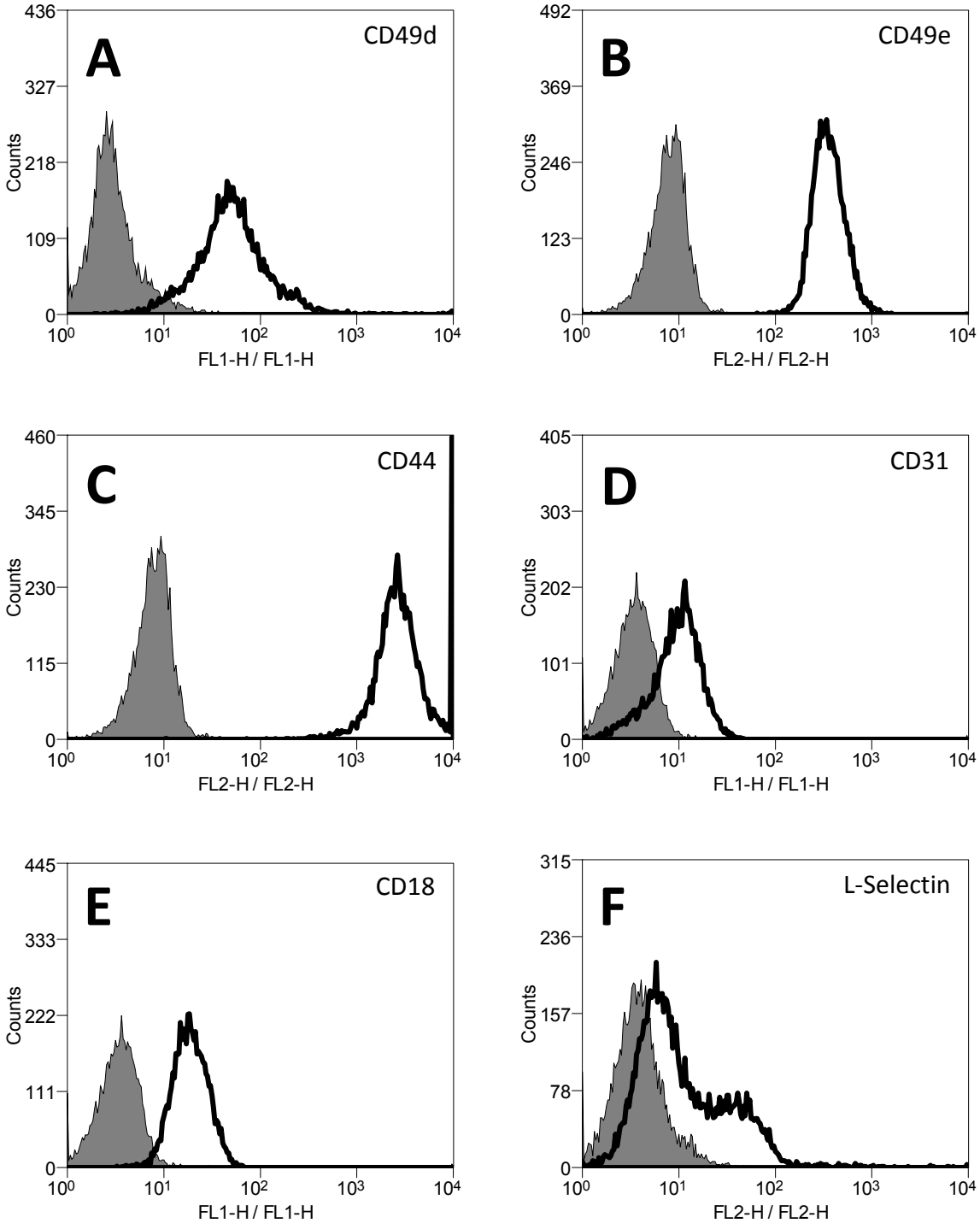


Figure 4.1. *HPC-7 cells express a repertoire of common adhesion molecules*. HPC-7 cells were examined using FACS for expression of common adhesion molecules involved in homing. HPC-7 cells express CD49d **(panel A)**, CD49e **(panel B)**, CD44 **(panel C)**, CD31 **(panel D)**, CD18 **(panel E)** and L-Selectin **(panel F)** (filled panel: negative control, solid line: relevant adhesion molecule antibody). FACS histograms are representative of 3 separate experiments.

4.3.2. Adhesion molecules can be blocked by pretreatment with function blocking antibodies

To ensure that the function blocking antibodies used in the molecular studies were able to block at given concentrations, a FACS based assay was used. For all antibodies tested, 80μg/ml of blocking antibody was an effective concentration to reduce fluorescence intensity down to control levels, suggesting blockade of surface antigen (CD49d: 0μg/ml: 8.44 ± 0.27 vs 80μg/ml: 2.82 ± 0.19; $P < 0.001$; CD44: 0μg/ml: 393.2 ± 31.72 vs 80μg/ml: 5.5 ± 0.74; $P < 0.001$; CD31: 0μg/ml: 8.09 ± 0.15 vs 80μg/ml: 2.280 ± 0.07; $P < 0.001$; CD18: 0μg/ml: 19.83 ± 0.39 vs 80μg/ml: 2.59 ± 0.04; $P < 0.001$) *(figures 4.2.A-H)*. No difference was seen between 80μg/ml and 160μg/ml blocking antibody treated cells. Conversely, 40μg/ml blocking antibody was not sufficient to elicit complete blockade in CD49d and CD44 treated cells *(figures 4.2.A-D)*. Interestingly, we were unable to block L-selectin with blocking antibodies at any concentration up to 160μg/ml (data not shown).

4.3.3. HPC-7 adhesion is not significantly reduced *in vitro* by blockade of CD49d

The role of CD49d was investigated *in vitro* by monitoring HPC-7 adhesion to frozen liver sections using the Stamper-Woodruff assay, HPC-7 cells were pre-treated with an anti-CD49d blocking antibody and their adhesion to IR sections quantified. Although treatment with an anti-CD49d blocking antibody resulted in a minor reduction in adhesion this was not statistically significant (IgG treated cells: 4.63 ± 2.10 vs anti-CD49d treated cells: 2.47 ± 1.14; $P > 0.05$). Treatment of cells with an anti-CD49d blocking antibody did not affect the anatomical localisation of recruitment as cell adhesion still remained exclusively along sinusoidal linings *(figures 4.3.B-C)*.

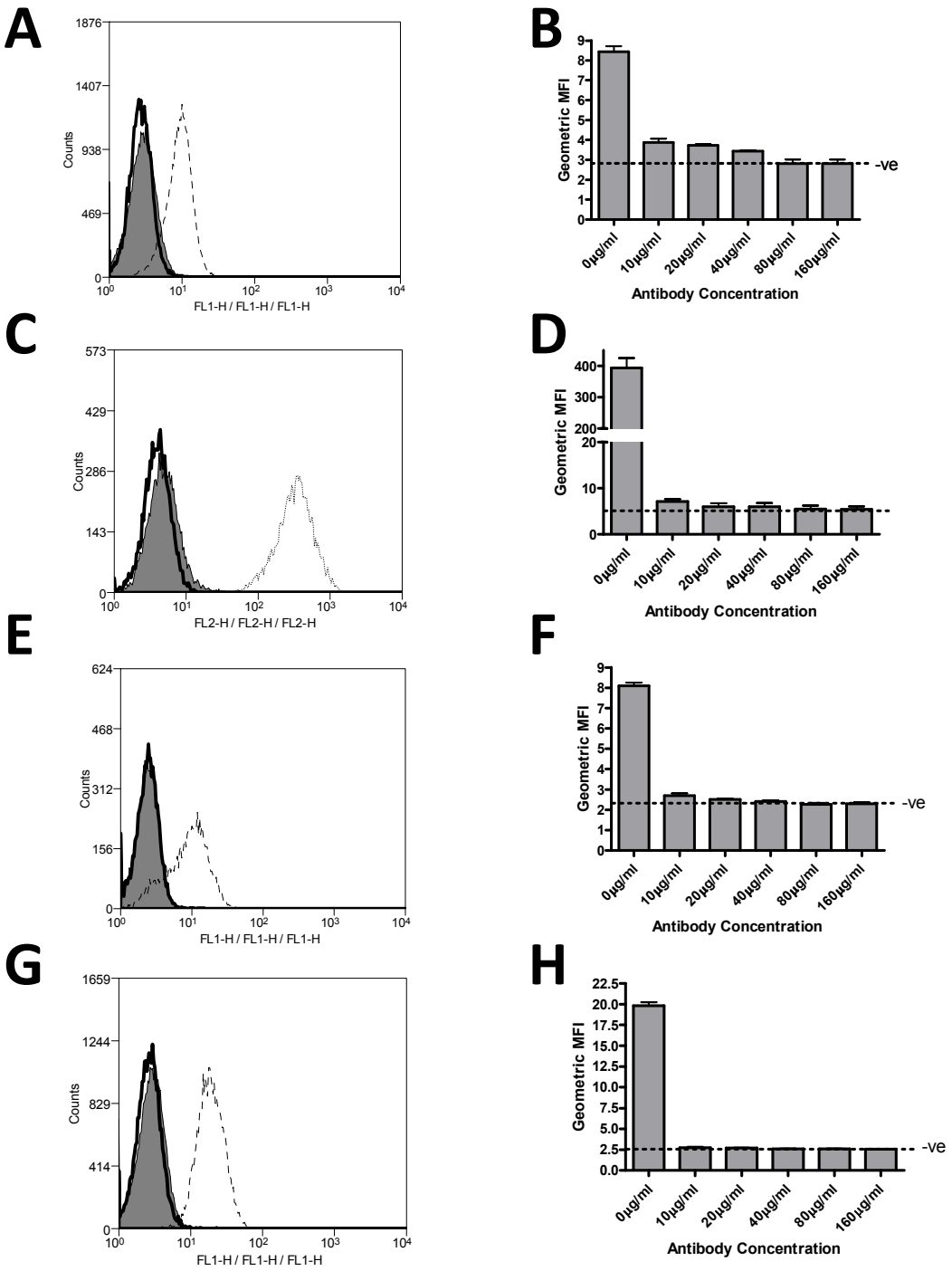


Figure 4.2. *CD49d, CD44, CD31 and CD18 can all be blocked with treatment of 80µg/ml of relevant blocking antibody.* HPC-7 cells were treated with specified concentrations of blocking antibody, then analysed by FACS for free antigen (see Figure 2.3). In all cases, 80µg/ml of blocking antibody is enough to reduce fluorescence to control levels. Plots and figures are shown for CD49d (panels A and B), CD44 (panels C and D), CD31 (panels E and F) and CD18 (panels G and H) (solid fill: negative control, solid line: 80µg/ml treated, dashed line: 0µg/ml treated/volume control). FACS plots are representative of an individual experiment, while bar graphs represent geometric mean MFI ± SEM of 3 experiments.

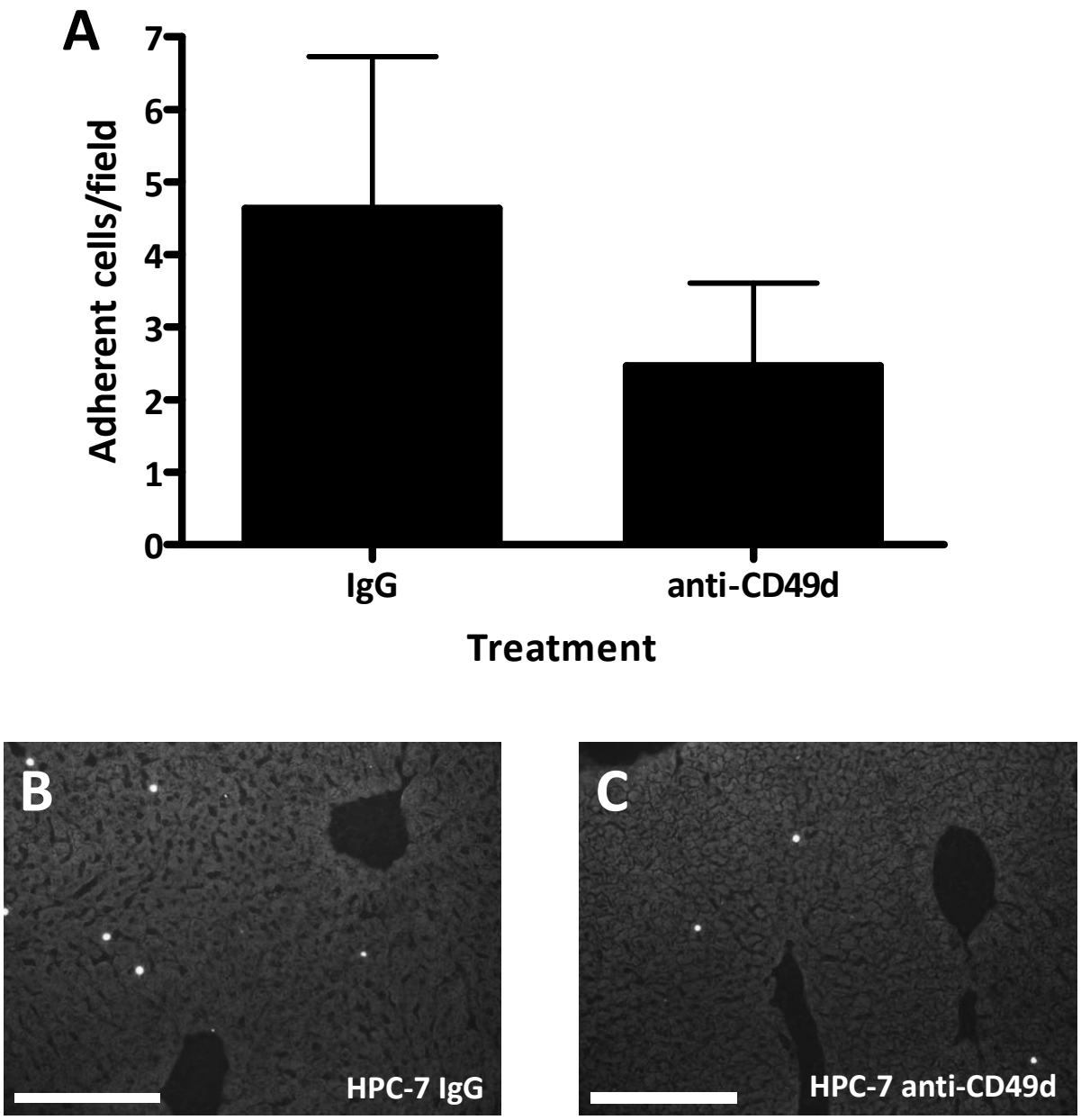


Figure 4.3. *Blocking CD49d on HPC-7 does not significantly reduce adhesion in vitro on sections isolated from IR treated animals.* The effect of CD49d blockade on HPC-7 adhesion was examined using the Stamper-Woodruff static adhesion assay. Treatment of cells with 80μg/ml anti-CD49d mAb fails to reduce adhesion on IR sections when compared to cells treated with IgG control (80μg/ml). Although blockade of CD49d appears to cause a general decrease in adhesion, this drop is not statistically significant. Representative images of IR sections with IgG treated **(panel B)** and anti-CD49d mAb treated **(panel C)** HPC-7 cells are shown. White bars represent 100μm. Data represents mean adhesion ± SEM of 6 separate experiments (unpaired t-test).

4.3.4. HPC-7 adhesion is significantly reduced *in vivo* by blockade of CD49d

The role of CD49d was also investigated *in vivo*. Anti-CD49d pre-treated cells (1×10^6) were introduced into the intravital model of partial hepatic IR injury via the carotid artery. HPC-7 adhesion was significantly reduced by pretreatment with an anti-CD49d antibody (e.g. at 55 minutes, isotype control: 15.5 ± 1.89 vs anti-CD49d treated: 6.33 ± 1.61; $P < 0.01$) *(figure 4.4.A)*. Levels of adhesion remain constant for both groups throughout the monitoring period *(figure 4.4.A)*, with levels of adhesion at 60 minutes not being significantly different from adhesion at 30 minutes (eg. isotype control, 30 minutes post reperfusion: 10.5 ± 1.52 vs 60 minutes reperfusion: 14.17 ± 2.02; $P > 0.05$; anti-CD49d treated, 30 minutes post reperfusion: 6.33 ± 1.43 vs 60 minutes post reperfusion: 6.17 ± 1.77; $P > 0.05$). Treatment of cells with an anti-CD49d antibody did not significantly alter the number of free-flowing cells observed during monitoring (e.g. free-flowing at 55 minutes: isotype control: 1.50 ± 0.56 vs anti-CD49d treated: 0.50 ± 0.37; $P > 0.05$) *(figure 4.4.B)*. Significantly more HPC-7 cells were observed free-flowing at 30 minutes reperfusion in both groups, this being the time point closest to injection *(figure 4.4.B).* Treatment of cells with an anti-CD49d blocking antibody did not alter the anatomical localisation of recruitment as cell adhesion still remained exclusively sinusoidal *(figures 4.4.C-D)*.

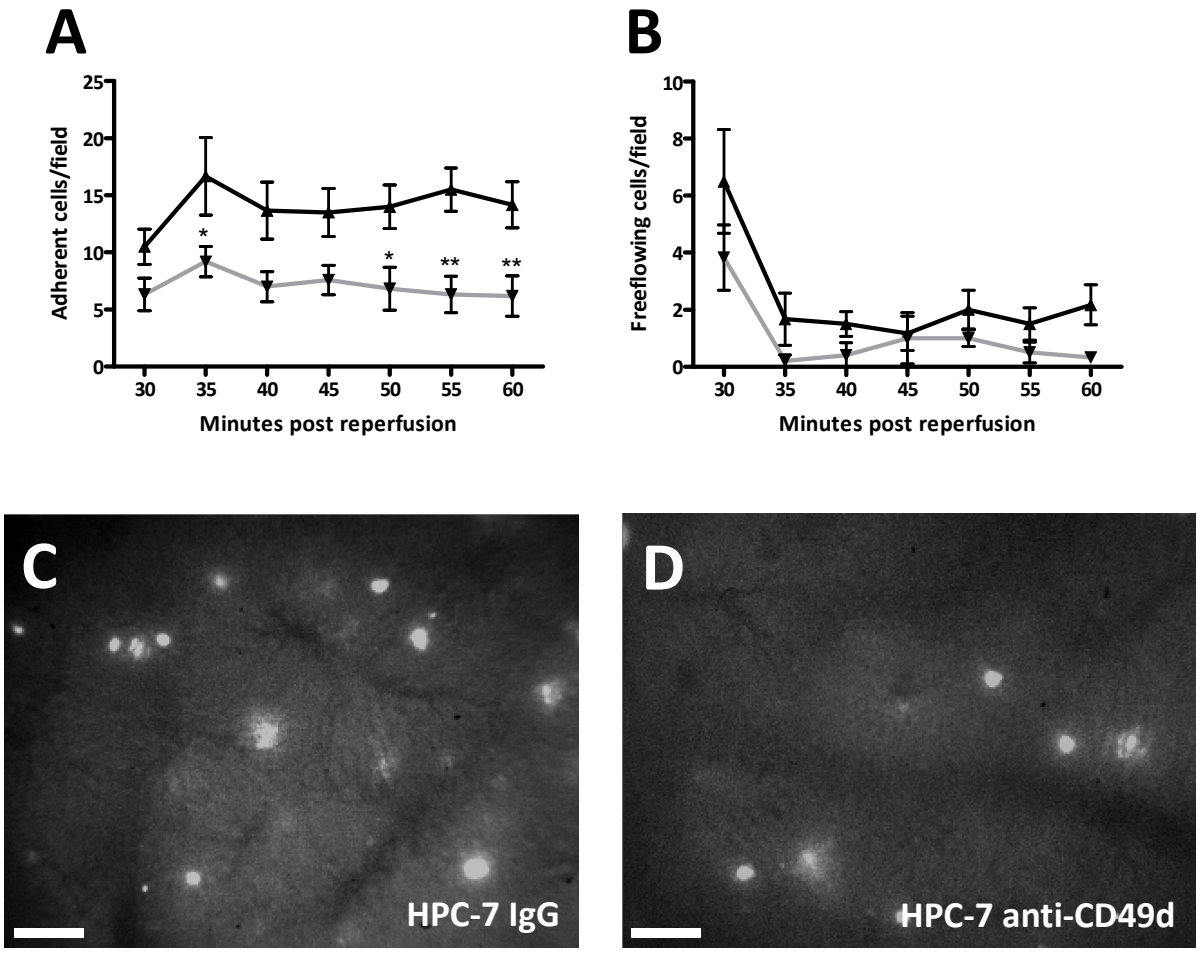


Figure 4.4. *HPC-7 adhesion is significantly reduced in vivo by treating HPC-7 cells with a CD49d blocking antibody.* Adhesion of HPC-7 cells to the hepatic microcirculation was examined using intravital microscopy following treatment with blocking antibodies. Animals were subjected to IR injury (90m PHI/30 R) prior to the introduction of either CD49d blocked or IgG treated HPC-7 cells (1 x 10^6 cells, 5μM CFSE, i.a.). HPC-7 cell adhesion was significantly reduced by pre-treatment with a CD49d blocking antibody **(panel A;** black line: IR + IgG treated cells; grey line: IR + CD49d treated cells). No significant difference was seen in free flowing cells between treatments (**panel B;** black line: IR + IgG treated cells; grey line: IR + CD49d treated cells). Representative images are shown of HPC-7 cell adhesion at 50 minutes post reperfusion with IgG (**panel C**) and CD49d (**panel D**) treated cells. White bars represent 100μm. Plots in **panel A** and **panel B** represent mean ± SEM of at least 5 separate experiments; * $p < 0.05$, ** $p < 0.01$ (two-way ANOVA followed by Bonferroni Post Test for points of significance).

4.3.5. HPC-7 adhesion is significantly reduced by *in vivo* blockade of VCAM-1

To determine whether VCAM-1, one of the counter-receptors for VLA-4, was involved in recruitment, VCAM-1, was blocked by intra-arterial administration of a blocking antibody *in vivo*. Animals were subjected to partial hepatic IR injury and treated with VCAM-1 blocking antibody at 1 minute post-reperfusion. Control animals received an isotype matched control antibody. HPC-7 adhesion is significantly reduced by treating animals with anti-VCAM-1 (e.g. at 55 minutes post reperfusion, isotype control: 12.8 ± 1.77 vs. anti-VCAM-1 treated: 5.25 ± 1.03; $P < 0.01$) *(figure 4.5.A)*. Levels of adhesion remain constant throughout the monitoring period *figure 4.5.A)*, with adhesion for both groups at 60 minutes not significantly different from adhesion at 30 minutes (e.g. isotype control treated, 30 minutes post reperfusion: 12.20 ± 1.53 vs. 60 minutes reperfusion: 11.60 ± 0.68; $P > 0.05$; anti-VCAM-1 treated, 30 minutes post reperfusion: 5.75 ± 1.44 vs. 60 minutes post reperfusion: 6.75 ± 1.44; $P > 0.05$) *(figure 4.5.A)*. Intraarterial treatment with an anti-VCAM-1 antibody did not significantly alter the number of free-flowing cells observed during monitoring (e.g. free-flowing at 55 minutes: isotype control: 0.00 ± 0.00 vs. anti-VCAM-1 treated: 0.00 ± 0.00; $P > 0.05$) *(figure 4.5.B)*. Intraarterial treatment with an anti-VCAM-1 blocking antibody did not alter the anatomical localisation of recruitment as cell adhesion still remained exclusively sinusoidal *(figures 4.5.C-D)*.

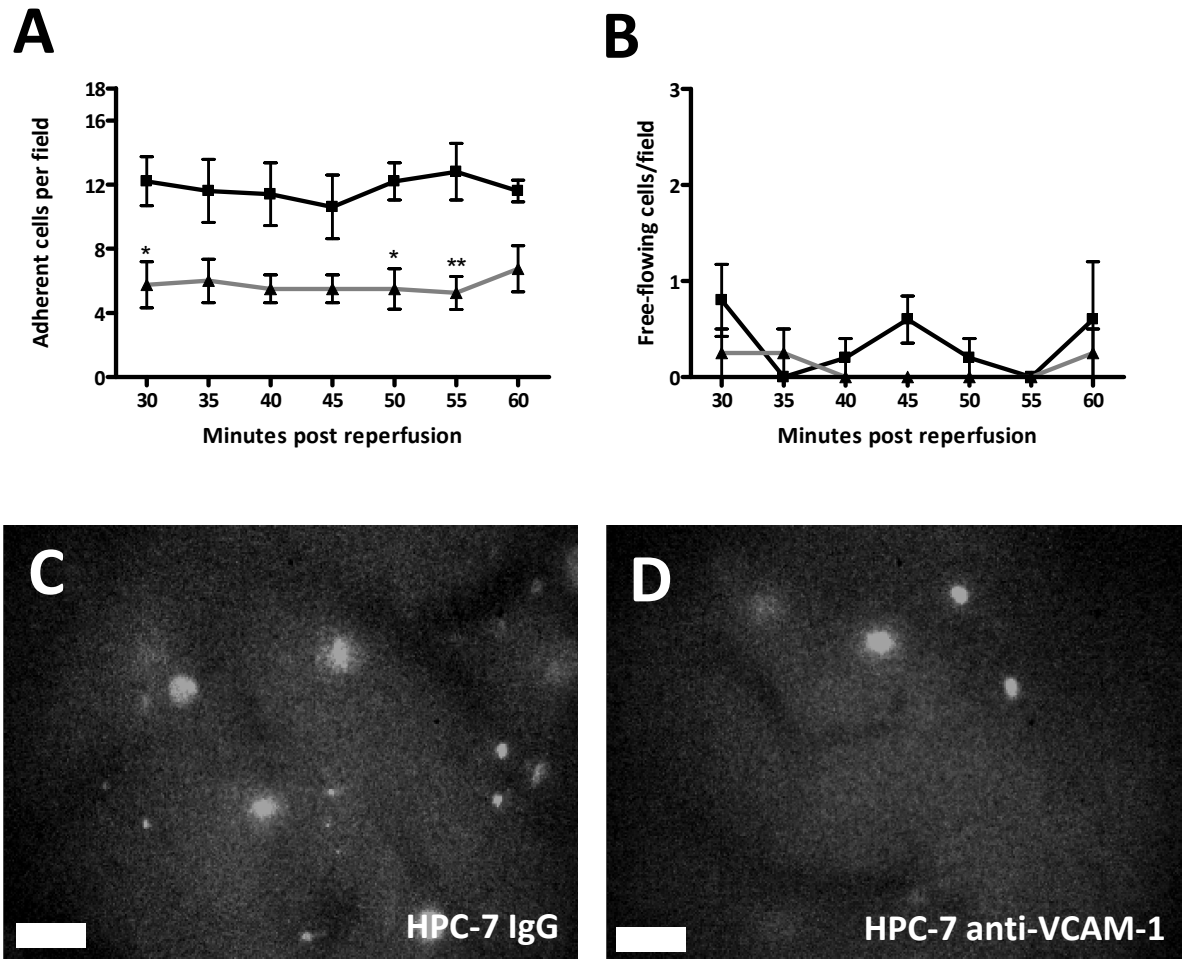


Figure 4.5. *HPC-7 adhesion is significantly reduced in vivo by intra-arterial administration of a VCAM-1 blocking antibody.* Adhesion of HPC-7 cells to the hepatic microcirculation was examined using intravital microscopy following intra-arterial treatment with a VCAM-1 blocking antibody. Animals were subjected to IR injury (90m PHI/30 R), with intra-arterial administration of 70µg (in 100µl saline) of VCAM-1 or IgG control at 1 minute reperfusion. At 30 minutes reperfusion, HPC-7 cells were introduced (1 x 10^6 cells, 5µM CFSE, I.A.). HPC-7 cell adhesion was significantly reduced by pre-treatment with a VCAM-1 blocking antibody **(panel A;** black line: IR + IgG administered; grey line: IR + anti-VCAM-1 administered). No significant difference was seen in free flowing cells between treatments (**panel B;** black line: IR + IgG administered; grey line: IR + anti-VCAM-1 administered). Representative images are shown of HPC-7 cell adhesion at 50 minutes post reperfusion with IgG (**panel C**) and VCAM-1 (**panel D**) treated animals. White bars represent 100µm. Plots in **panel A** and **panel B** represent mean ± SEM of at least 5 separate experiments; * $p < 0.05$, ** $p < 0.01$ (two-way ANOVA followed by Bonferroni Post Test for points of significance)

4.3.6. HPC-7 adhesion to IR injured hepatic microvasculature *in vivo* is independent of CD44

HPC-7 cells were pre-treated with a CD44 blocking antibody prior to injection at 30 minutes post-reperfusion. Control cells were treated with an isotype control antibody. Pre-treatment of HPC-7 cells with a CD44 blocking antibody did not significantly reduce adhesion post-IR injury when compared to cells treated with an isotype control (e.g. adhesion at 55 minutes: isotype control: 15.50 ± 1.89 vs anti-CD44 treated: 17.00 ± 3.86; $P > 0.05$) *(figure 4.6.A)*. Levels of adhesion remain constant throughout the monitoring period *(figure 4.6.A)*, with adhesion in both groups at 60 minutes not significantly different from adhesion at 30 minutes (e.g. isotype control treated, 30 minutes post reperfusion: 10.50 ± 1.52 vs. 60 minutes reperfusion: 14.17 ± 2.02; $P > 0.05$; anti-CD44 treated, 30 minutes post reperfusion: 13.40 ± 2.94 vs. 60 minutes post reperfusion: 17.80 ± 3.60; $P > 0.05$) *(figure 4.6.A).* Significantly fewer free-flowing HPC-7 cells were observed when cells were pre-treated with a CD44 blocking antibody (e.g. free-flowing cells at 50 minutes: isotype control: 2.00 ± 0.28 vs. anti-CD44 treated: 0.20 ± 0.09; $P < 0.01$) *(figure 4.6.B)*. Again, the maximum number of free-flowing cells was seen at 30 minutes post-reperfusion *(figure 4.6.B)*. Treatment of cells with an anti-CD44 blocking antibody did not alter the anatomical localisation of HPC-7 recruitment as cell adhesion still remained exclusively sinusoidal *(figures 4.6.C-D).*

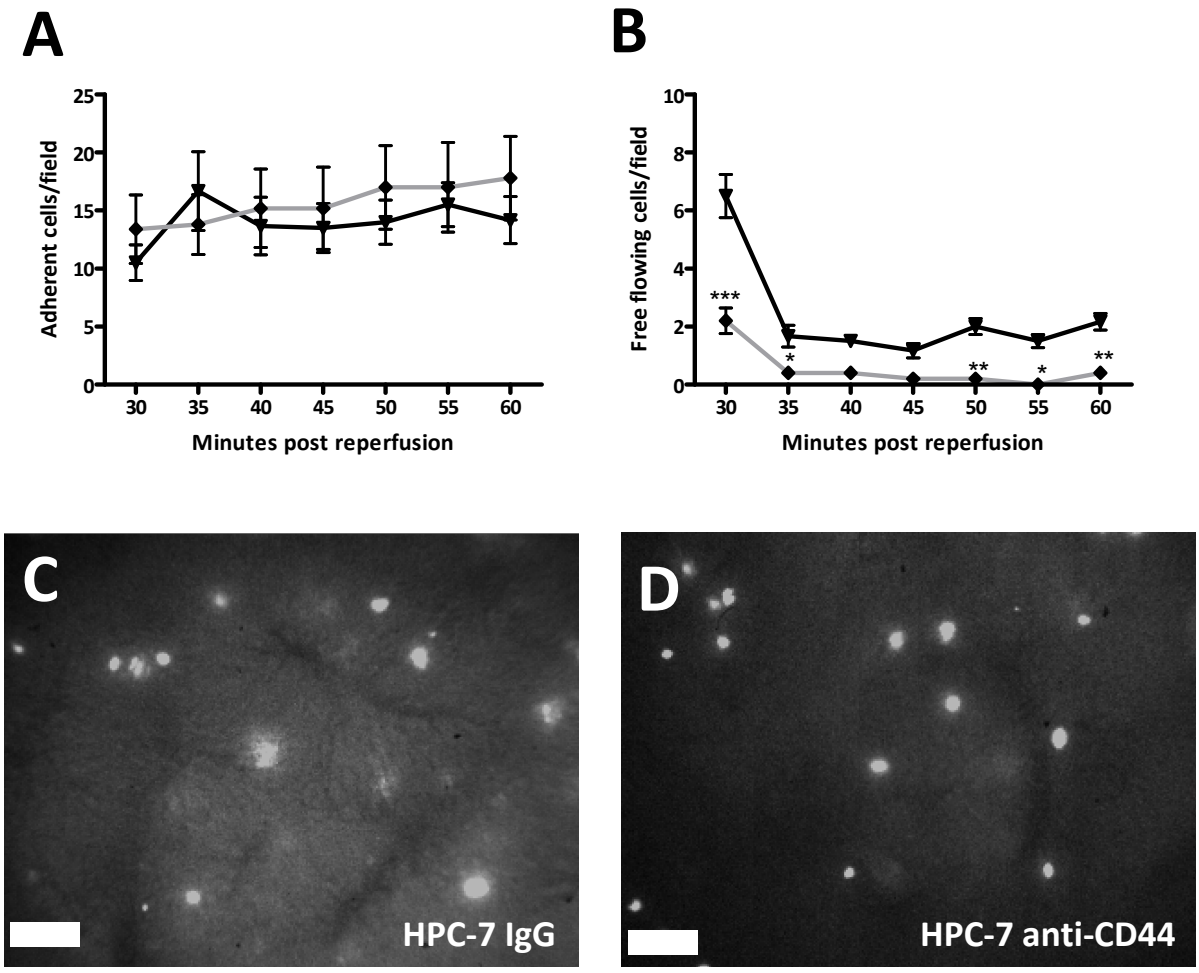


Figure 4.6. *HPC-7 adhesion is not affected in vivo by pre-treating HPC-7 cells with a CD44 blocking antibody.* Adhesion of HPC-7 cells to the hepatic microcirculation was examined using intravital microscopy following treatment with blocking antibodies. Animals were subjected to IR injury (90m PHI/30 R) prior to the introduction of either CD44 blocked or IgG treated HPC-7 cells (1 x 10^6 cells, 5µM CFSE, I.A.). HPC-7 cell adhesion was not significantly reduced by pre-treatment with a CD44 blocking antibody **(panel A;** black line: IR + IgG treated cells; grey line: IR + CD44 treated cells). Significantly fewer free flowing cells were observed when they had been pre-treated with the CD44 blocking antibody (**panel B;** black line: IR + IgG treated cells; grey line: IR + CD44 treated cells). Representative images are shown of HPC-7 cell adhesion at 50 minutes post reperfusion with IgG (**panel C**) and CD44 (**panel D**) treated cells. White bars represent 100µm. Plots in **panel A** and **panel B** represent mean ± SEM of at least 5 separate experiments; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-way ANOVA followed by Bonferroni Post Tests)

4.3.7. HPC-7 adhesion to IR injured hepatic microvasculature *in vivo* is independent of CD18

HPC-7 cells were pre-treated with a CD18 blocking antibody prior to injection at 30 minutes reperfusion. Control cells were treated with an isotype control antibody. Pre-treatment of HPC-7 cells with anti CD18 did not significantly reduce adhesion seen post-IR injury when compared to cells treated with an isotype control (e.g. adhesion at 55 minutes: isotype control: 15.5 ± 1.89 vs. anti-CD18 treated: 13.17 ± 1.66; $P > 0.05$) *(figure 4.7.A)*. Levels of adhesion remained constant throughout the monitoring period *(figure 4.7.A)*, with adhesion in both groups at 60 minutes not significantly different from adhesion at 30 minutes (e.g. isotype control treated, 30 minutes post reperfusion: 10.50 ± 1.52 vs. 60 minutes reperfusion: 14.17 ± 2.02; $P > 0.05$; anti-CD18 treated, 30 minutes post reperfusion: 11.17 ± 1.89 vs. 60 minutes post reperfusion: 14.00 ± 1.26; $P > 0.05$) *(figure 4.7.A)*. Blocking CD18 did not significantly alter free-flowing cells observed when compared to IgG control (e.g. free-flowing at 55 minutes: isotype control: 1.50 ± 0.23 vs. anti-CD18 treated: 1.33 ± 0.88; $P > 0.05$*) (figure 4.7.B)*. The majority of free-flowing cells were observed at 30 minutes reperfusion, the earliest time point after injection *(figure 4.7.B)*. Treatment of cells with an anti-CD18 blocking antibody did not alter the localisation of HPC-7 recruitment and adhesion remained exclusively sinusoidal *(figures 4.7.C-D)*.

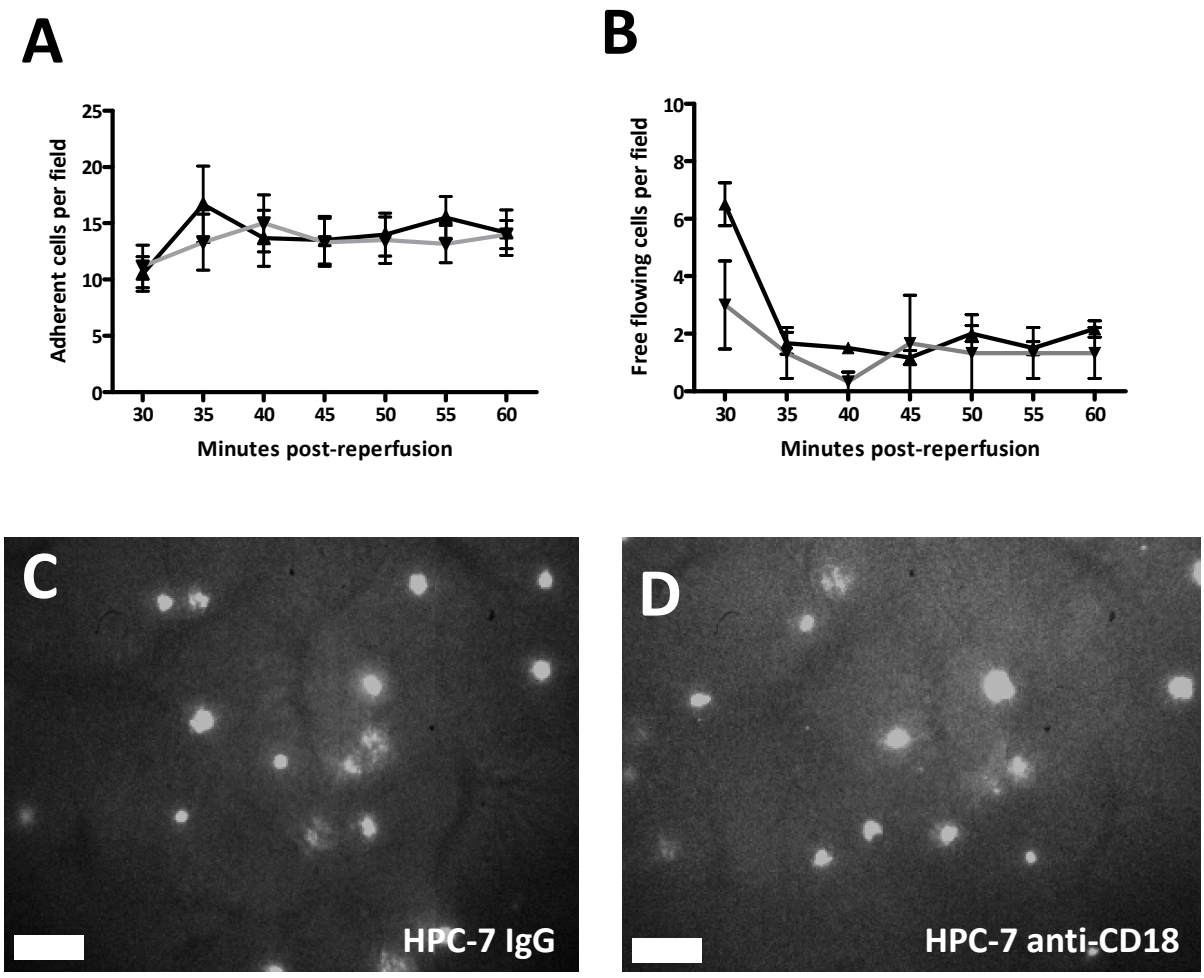


Figure 4.7. *HPC-7 adhesion is not affected in vivo by pre-treating HPC-7 cells with a CD18 blocking antibody.* Adhesion of HPC-7 cells to the hepatic microcirculation was examined using intravital microscopy following treatment with blocking antibodies. Animals were subjected to IR injury (90m PHI/30 R) prior to the introduction of either CD18 blocked or IgG treated HPC-7 cells (1 x 10^6 cells, 5µM CFSE, I.A.). HPC-7 cell adhesion was not significantly reduced by pre-treatment with a CD18 blocking antibody **(panel A;** black line: IR + IgG treated cells; grey line: IR + CD18 treated cells). No differences were observed in flowing cells regardless of treatment (**panel B;** black line: IR + IgG treated cells; grey line: IR + CD18 treated cells). Representative images are shown of HPC-7 cell adhesion at 50 minutes post reperfusion with IgG (**panel C**) and CD18 (**panel D**) treated cells. White bars represent 100µm. Plots in **panel A** and **panel B** represent mean ± SEM of at least 5 separate experiments (two-way ANOVA).

4.3.8. HSC adhesion to IR injured hepatic microvasculature *in vivo* is independent of CD31 expressed on the HSC

HPC-7 cells were treated with a CD31 blocking antibody prior to injection at 30 minutes reperfusion. Control cells were treated with an isotype control antibody. Pre-treatment of HPC-7 cells with a CD31 blocking antibody did not significantly reduce adhesion seen post-IR injury when compared to cells treated with an isotype control (e.g. adhesion at 55 minutes: isotype control: 15.50 ± 1.89 vs. CD31 treated: 13.43 ± 3.23; $P > 0.05$) *(figure 4.8.A)*. Levels of adhesion remain constant throughout the monitoring period *(figure 4.8.A)*, with adhesion in both groups at 60 minutes not significantly different from adhesion at 30 minutes (e.g. isotype control treated, 30 minutes post reperfusion: 10.50 ± 1.52 vs. 60 minutes reperfusion: 14.17 ± 2.02; $P > 0.05$; anti-CD31 treated, 30 minutes post reperfusion: 12.29 ± 3.01 vs. 60 minutes post reperfusion: 15.14 ± 3.94; $P > 0.05$) *(figure 4.8.A)*. Blocking CD31 on HPC-7 cells did not significantly alter free-flowing cells observed when compared to IgG control treated cells (e.g. free-flowing cells at 55 minutes: isotype control: 1.50 ± 0.23 vs. CD31 treated: 1.50 ± 0.67 $P > 0.05$) *(figure 4.8.B)*. The majority of free-flowing cells were observed at 30 minutes reperfusion, the earliest time point after injection *(figure 4.8.B)*. Treatment of cells with an anti-CD31 blocking antibody did not alter the anatomical localisation of HPC-7 recruitment as cell adhesion still remained exclusively sinusoidal *(figures 4.8.C-D)*.

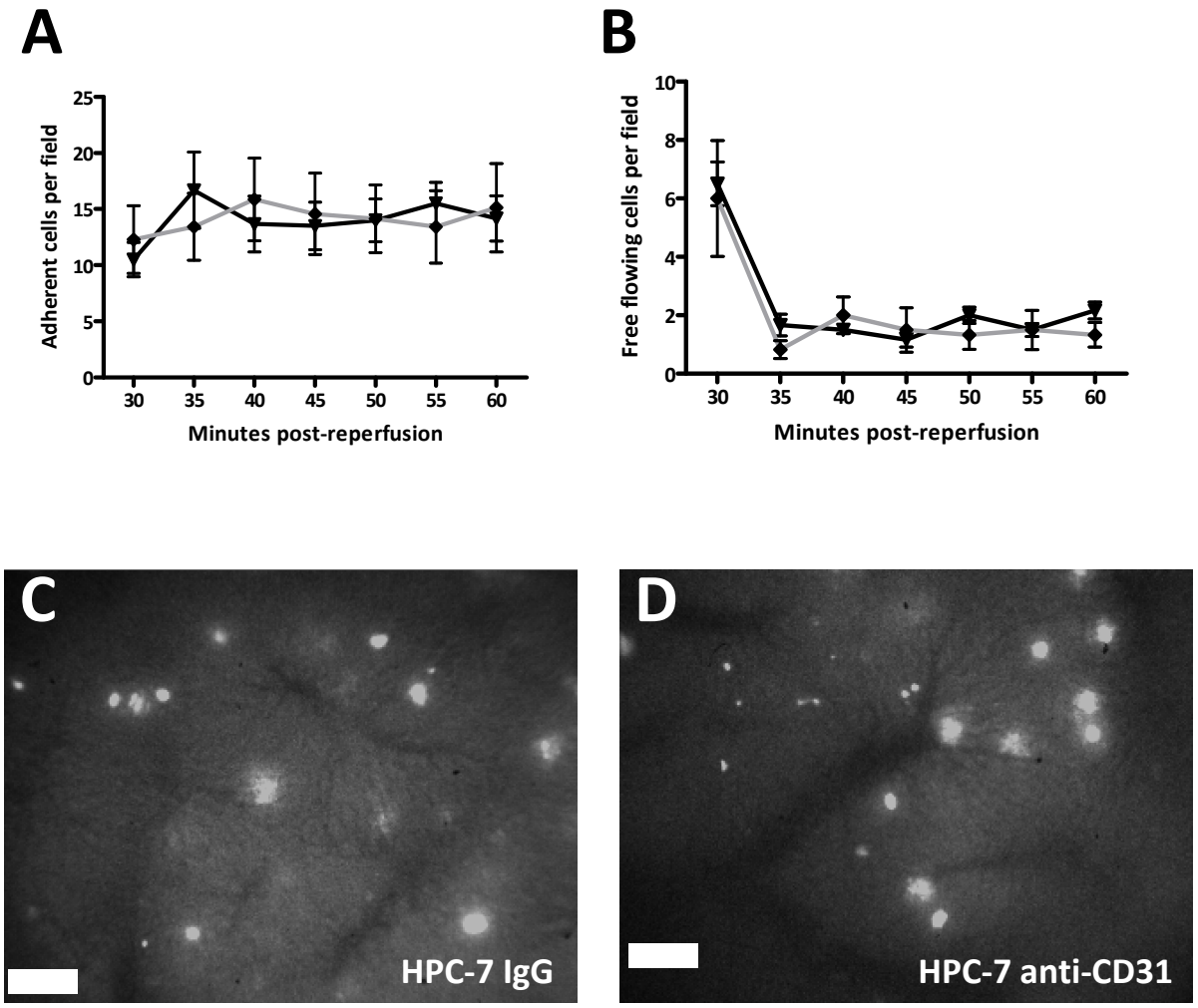


Figure 4.8. *HPC-7 adhesion is not affected in vivo by pre-treating HPC-7 cells with a CD31 blocking antibody.* Adhesion of HPC-7 cells to the hepatic microcirculation was examined using intravital microscopy following treatment with blocking antibodies. Animals were subjected to IR injury (90m PHI/30 R) prior to the introduction of either CD31 blocked or IgG treated HPC-7 cells (1 x 10^6 cells, 5µM CFSE, I.A.). HPC-7 cell adhesion was not significantly reduced by pre-treatment with a CD31 blocking antibody **(panel A;** black line: IR + IgG treated cells; grey line: IR + CD31 treated cells). No differences were observed in flowing cells regardless of treatment (**panel B;** black line: IR + IgG treated cells; grey line: IR + CD31 treated cells). Representative images are shown of HPC-7 cell adhesion at 50 minutes post reperfusion with IgG (**panel C**) and CD31 (**panel D**) treated cells. White bars represent 100µm. Plots in **panel A** and **panel B** represent mean ± SEM of at least 5 separate experiments (two-way ANOVA)

4.3.9. $CD31^{-/-}$ animals display raised and maximal adhesion for HPC-7 without a prior need for IR injury

There is evidence to suggest there is a low basal expression of CD31 in the hepatic sinusoids (Neubauer *et al.*, 2000, Neubauer *et al.*, 2008). Therefore, the role of endothelial CD31 in HSC recruitment was also investigated using $CD31^{-/-}$ mice. HPC-7 cells (expressing CD31) were injected into $CD31^{-/-}$ mice at 30 minutes reperfusion and adhesion monitored. HPC-7 cells were also injected into sham treated $CD31^{-/-}$ animals. Following sham treatment, adhesion of HPC-7 cells was significantly raised in $CD31^{-/-}$ animals when compared to strain and age matched wildtype sham controls, although adhesion falls to control levels at 60 minutes reperfusion (e.g. sham treatment, adhesion at 30 minutes reperfusion: wild-type animals: 2.75 ± 1.18 vs. $CD31^{-/-}$ animals: 15.17 ± 2.93; $P < 0.001$; sham treatment, adhesion at 60 minutes reperfusion: wild-type animals: 7.20 ± 1.20 vs. $CD31^{-/-}$ animals: 9.67 ± 1.33; $P > 0.05$) *(figure 4.9.A)*. Significantly fewer free-flowing cells are observed in $CD31^{-/-}$ animals at early time points when compared to wild-type animals (e.g. sham treatment, free-flowing cells at 30 minutes: wild-type animals: 4.50 ± 0.65 vs. $CD31^{-/-}$ animals: 1.67 ± 0.92; $P < 0.01$) *(figure 4.9.B)*. Interestingly, following IR injury in $CD31^{-/-}$ animals, HPC-7 adhesion is not significantly different from that seen in sham controls (e.g. adhesion at 30 minutes: sham treated $CD31^{-/-}$: 15.17 ± 2.93 vs IR treated $CD31^{-/-}$: 15.67 ± 5.02; $P > 0.05$) *(figure 4.9.C)*. Significantly fewer free-flowing cells were observed at the earliest time point following reperfusion in sham $CD31^{-/-}$ animals compared to IR $CD31^{-/-}$ animals (e.g. free-flowing cells at 30 minutes: sham treated $CD31^{-/-}$: 1.67 ± 0.92 vs. IR treated $CD31^{-/-}$: 5.67 ± 1.67; $P < 0.001$) *(figure 4.9.D)*. Very few free-flowing cells are observed in the later periods of reperfusion and there is no significant difference between sham and IR treated animals

(e.g. free-flowing cells at 60 minutes: sham treated $CD31^{-/-}$: 0.60 ± 0.40 vs. IR treated $CD31^{-/-}$: 0.33 ± 0.21; P > 0.05) *(figure 4.9.D)*. Lack of CD31 did not alter the localisation of HPC-7 recruitment as cell adhesion again remained exclusively sinusoidal similar to wild-type controls *(figures 4.9.E-F)*.

4.3.10. Tissue VCAM-1, CD31 and CD44 are not upregulated as a result of an acute IR injury when assessed by immunohistochemistry

No detectable upregulation in VCAM-1 *(figures 4.10.A-B)*, CD31 *(figures 4.10.C-D)* or CD44 *(figures 4.10.E-F)* was detectable by immunohistochemistry on IR injured hepatic sections when compared to sham sections. No VCAM-1 staining of large vessels or sinusoidal endothelium was visibly detectable on any hepatic sections *(figures 4.10.A-B).* CD31 staining was primarily localised to large vessels *(figures 4.10.C-D)*, while CD44 primarily stained sinusoidal vessels *(figures 4.10.E-F)*.

4.4. Discussion

Although knowledge of mechanisms governing HSC homing to BM is increasing, very little is known about the molecular basis of HSC recruitment to extramedullary sites following injury. Our novel data demonstrate that systemically administered HSCs migrate to the injured murine liver and adhere in sinusoidal capillaries with recruitment *in vivo* being dependent on CD49d ($\alpha_4\beta_1$ integrin) and endothelial VCAM-1 interactions. CD49d had a dominant role in our studies, evidenced by failure of CD18, CD44 or CD31 blockade to reduce adhesion.

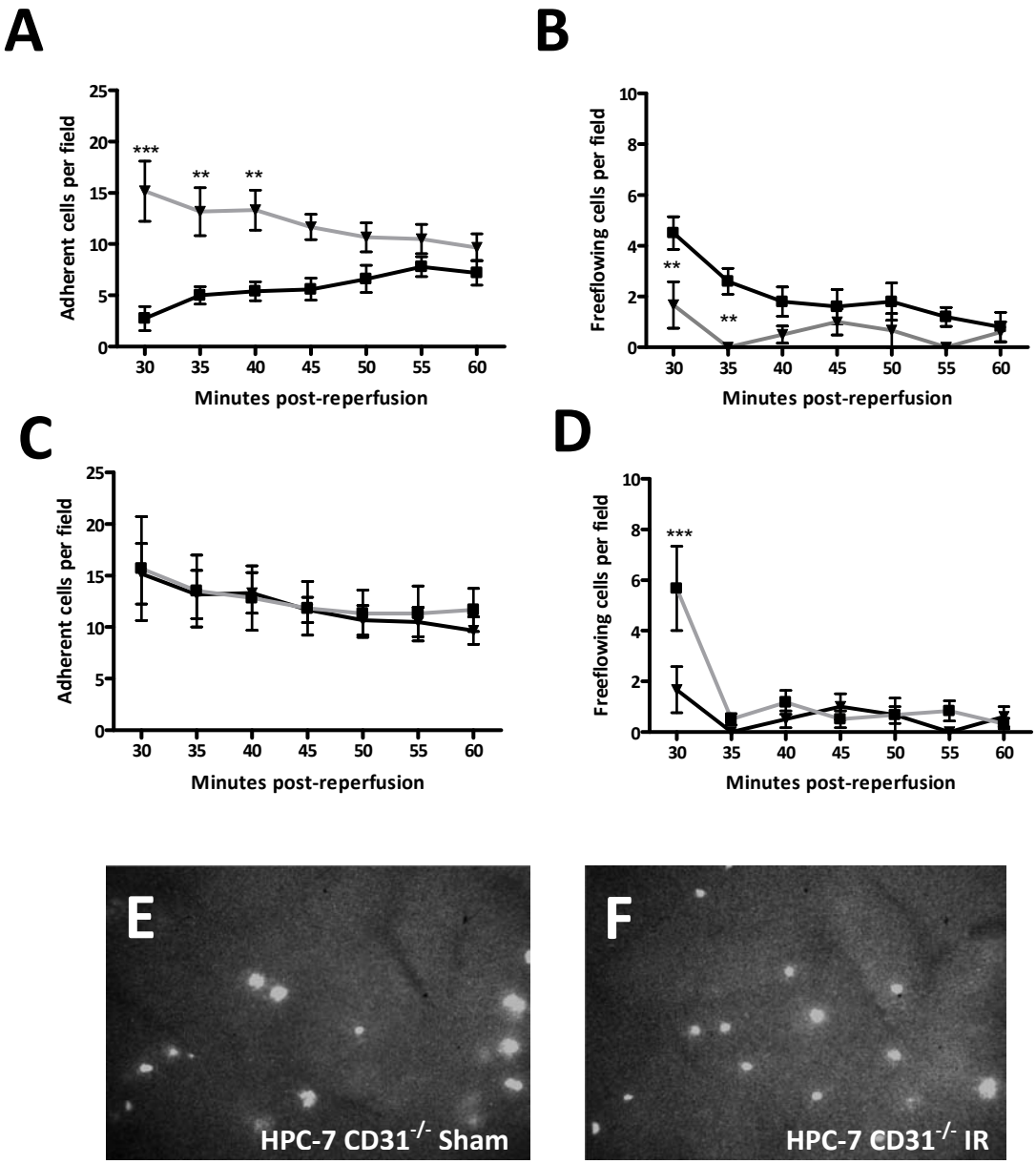


Figure 4.9. *CD31 knockout animals display raised and maximal adhesion for HPC-7 in vivo without a prior need for IR injury.* Adhesion of HPC-7 cells to the hepatic microcirculation was examined using intravital microscopy. $CD31^{-/-}$ animals were subjected to sham or IR treatment (90 PHI/30 R) and HPC-7 cells (expressing CD31) administered following treatment. HPC-7 adhesion is significantly raised in $CD31^{-/-}$ sham animals when compared to WT sham controls (**panel A;** black line: WT Sham, grey line: $CD31^{-/-}$ Sham). Significantly fewer cells are observed free-flowing in $CD31^{-/-}$ sham animals during early reperfusion compared to WT sham controls (**panel B;** black line: WT Sham, grey line: $CD31^{-/-}$ Sham). IR injury does not raise adhesion any further in $CD31^{-/-}$ animals (**panel C;** black line: $CD31^{-/-}$ Sham, grey line: $CD31^{-/-}$ IR). Significantly more free flowing cells are visualised in $CD31^{-/-}$ IR animals compared to $CD31^{-/-}$ sham (**panel D;** black line: $CD31^{-/-}$ Sham, grey line: $CD31^{-/-}$ IR). Representative images of $CD31^{-/-}$ sham **(panel E)** and $CD31^{-/-}$ IR are shown **(panel F)**. **Panels A-D** represent mean ± SEM of at least 5 separate experiments; ** $p < 0.01$, *** $p < 0.001$ (two-way ANOVA followed by Bonferroni Post Test for points of significance).

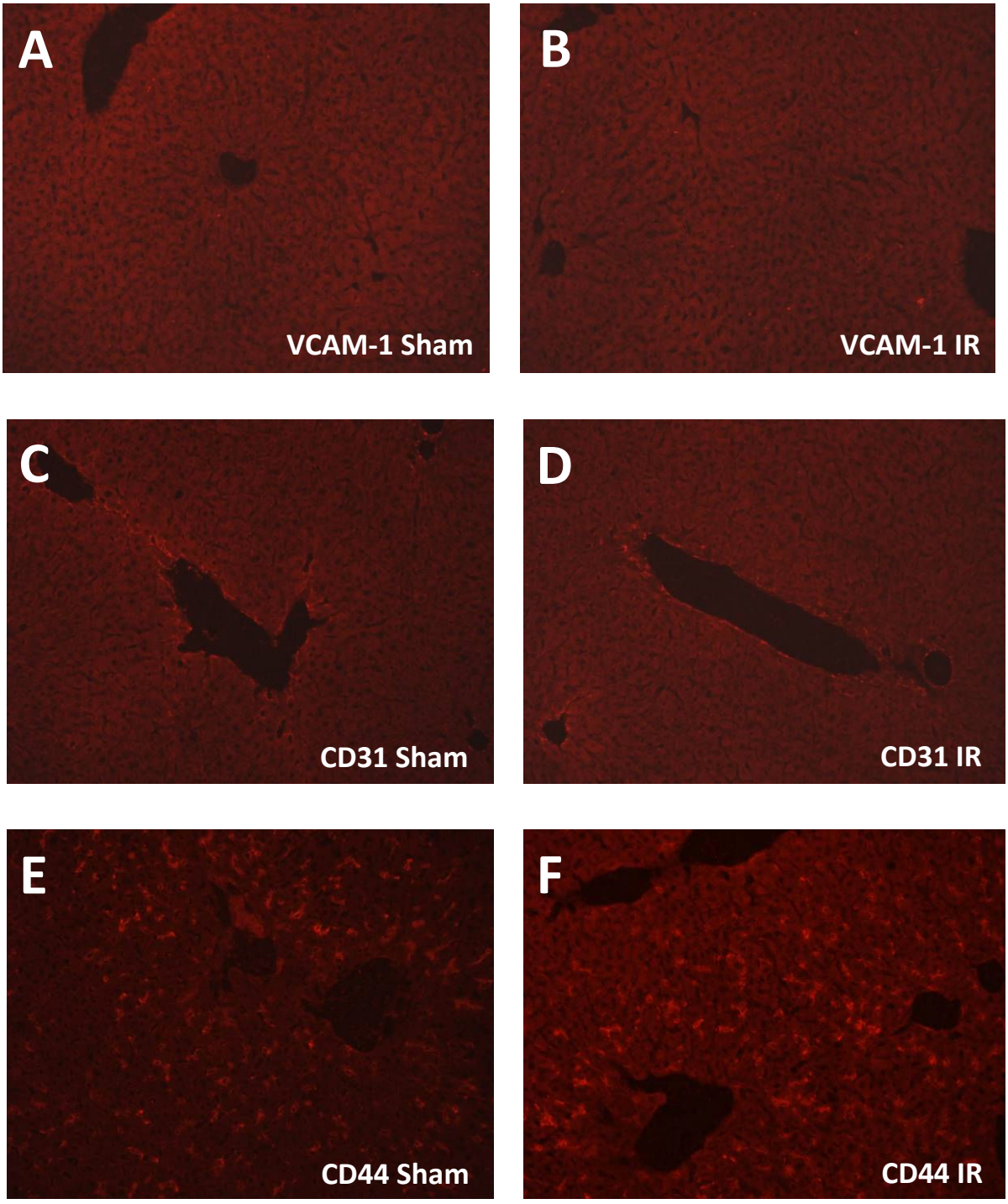


Figure 4.10. *No upregulation of VCAM-1, CD31 or CD44 is detectable by immunohistochemistry of sham and IR liver sections*. Sections were isolated from sham and IR animals, and immunohistochemistry (IHC) performed. VCAM-1 was visibly undetectable on both sham (panel A) and IR (panel B) sections. CD31 and CD44 were detectable on the larger portal vessels and sinusoidal endothelium respectively. However, there was no discernable difference in expression between sham and IR sections (CD31: Sham, panel C; IR, panel D. CD44: Sham, panel E; IR, panel F).

VLA-4 also mediates adhesion of mature leukocytes to endothelium via interactions with endothelial VCAM-1 or matrix fibronectin (Elices *et al.*, 1990). The latter interaction has been implicated in inflammatory cell recruitment following hepatic IR injury although a similar role for the VLA-4/VCAM-1 pathway has not been reported in the murine hepatic IR inury model (Moore *et al.*, 2007). Furthermore, the VLA-4/VCAM-1 pathway has been implicated heavily in homing of HSCs to the BM following exogenous stem cell transplantation in irradiated hosts (Papayannopoulou *et al.*, 1995). Blockade of VLA-4 on progenitor cells significantly reduces homing of HSCs to the BM and concomitantly increases circulating progenitor cell numbers (Papayannopoulou *et al.*, 1995). Recent work by Ceradini and colleagues (Ceradini *et al.*, 2004) has suggested that HSC trafficking to sites of hypoxic injury is a result of local increases in CXCL12. Hepatic injury also induces release of CXCL12 (Kollet *et al.*, 2003) which can subsequently rapidly activate HSC integrins including VLA-4, promoting recruitment (Peled *et al.*, 2000).

Previous work from our lab has shown that pre-treatment of HPC-7 cells with an anti-CD49d blocking antibody inhibits adhesion *in vitro* to murine cardiac endothelium under flow conditions *(figure 4.11 at the end of this chapter)*. Interestingly, while pre-treatment of HPC-7 cells with an anti-CD49d blocking antibody attenuates HPC-7 recruitment *in vivo* and *in vitro* using flow based adhesion assays, it does not modify adhesion *in vitro* using static tissue adhesion assays. This suggests that other adhesion molecules may be important in the recruitment of HSCs to the hepatic microvasculature or flow is an important component for VLA-4-dependent adhesion. Interestingly, VLA-4 supports firm adhesion of T-lymphocytes and α4 integrin transfected cells on VCAM-1 *in vitro* when treated with

integrin activating factors Mn^{2+}, phorbol ester and mAb TS2/16 (Alon *et al.*, 1995). It is possible that in our model of injury, activating factors are present in the local and systemic circulation *in vivo*, but would be absent in the Stamper-Woodruff assay explaining the lack of a role for VLA-4 in this assay.

In the current study, functional inhibition of VCAM-1 with an intra-arterial injection of blocking antibody significantly attenuated adhesion of HPC-7 cells following IR injury. In a rat model of hepatic IR injury, transcriptional upregulation of VCAM-1 can be detected within one hour of reperfusion (Bell *et al.*, 1997). Lymphocyte recruitment following IR injury is also largely reliant on VCAM-1 (Lalor and Adams, 1999). It is therefore not surprising that the VLA-4/VCAM-1 pathway mediates HSC recruitment to the liver. Indeed, the VLA-4/VCAM-1 pathway mediates recruitment of other cell types to the liver inlcuding melanoma cells (Anasagasti *et al.*, 1997). Interestingly, whether VCAM-1 plays a similar role in the recruitment of neutrophils following hepatic IR injury is currently not known. Neutrophil recruitment during liver reperfusion appears to be predominantly dependent largely on the α_M integrin Mac-1 (CD11b/CD18) interactions with endothelial ICAM-1 (Jaeschke, 2003).

Homophilic interactions of the IgSF adhesion molecule CD31 (PECAM-1) play a critical role in mature leukocyte adhesion and transendothelial migration in various organs (Rijcken *et al.*, 2007, Nolte *et al.*, 2004, Murohara *et al.*, 1996). It is also expressed on HSCs and has been shown to play a role in BM homing (Ross *et al.*, 2008). Although evidence suggests a role for CD31 generally in IR injuries (Nolte *et al.*, 2004, Nourshargh *et al.*, 2006), very little

evidence implicates the molecule specifically in hepatic IR injury. However, basal expression of CD31 has been observed immunohistochemically on human hepatic sinusoidal endothelial cells (Pusztaszeri *et al.*, 2006). In the current study, pre-treatment of HPC-7 cells with an anti-CD31 antibody did not reduce adhesion post-reperfusion suggesting HPC-7 recruitment is independent of CD31.

As CD31 also has a significant endothelial component, we investigated HSC adhesion in $CD31^{-/-}$ animals. Surprisingly, we demonstrated that HPC-7 adhesion following sham treatment was raised in $CD31^{-/-}$ animals when compared to sham treated WT controls. Interestingly, work from our lab has recently demonstrated increased whole BM cell adhesion to murine skull BM microcirculation in $CD31^{-/-}$ mice (Ross *et al.*, 2008). This may be a result of $CD31^{-/-}$ animals exhibiting a basal or constitutive pro-inflammatory state. Goel and colleagues (Goel *et al.*, 2007) investigated the role of CD31 in an endotoxin-induced model of liver inflammation and demonstrated that CD31 deficiency was associated with heightened levels of pro-inflammatory cytokines and reactive oxygen species (Goel *et al.*, 2007). CD31 engagement has also been shown to inhibit the production of pro-inflammatory cytokines *in vivo* (Carrithers *et al.*, 2005, Maas *et al.*, 2005). However, HPC-7 adhesion was not further raised in $CD31^{-/-}$ IR treated animals compared to sham treated $CD31^{-/-}$ animals. It is possible that maximal adhesion of HPC-7s is attained in the sham treated $CD31^{-/-}$ animals which cannot be further enhanced even in the presence of injury. Alternatively, it is possible that HSC recruitment to the injured liver is actually dependent on endothelial CD31 which is why recruitment within $CD31^{-/-}$ IR treated animals is similar to the appropriate control sham treated $CD31^{-/-}$ animals.

We demonstrated the abundant expresssion of CD44 on the surface of HPC-7 cells but it is also expressed on hepatic endothelium during inflammation (McDonald *et al.*, 2008). Even at basal levels, hepatic expression of CD44 is around two orders of magnitude higher than any other organ (McDonald *et al.*, 2008). Furthermore, CD44 is implicated in CXCL12 mediated trafficking of $CD34^+$ progenitor cells to the BM (Avigdor *et al.*, 2004). MSC migration on HA is also significantly mediated by CD44 (Zhu *et al.*, 2006). Collectively, these studies suggest that CD44 appears to be a good candidate to mediate HSC adhesion to the hepatic microvasculature post IR injury. However, pre-treatment of HPC-7 cells with a CD44 blocking antibody did not significantly reduce HPC-7 adhesion during IR injury in the current study. Some controversy exists regarding the role of CD44 in homing to the BM. Although studies using anti-CD44 antibodies reveal impaired homing (Avigdor *et al.*, 2004), CD44 knock-out mice demonstrate no defects in this process (Oostendorp *et al.*, 2000). In renal IR injury, CD44 blockade significantly reduces neutrophil adhesion to the injured tissue (Rouschop *et al.*, 2005). On the other hand, intestinal IR injury leads to a significant reduction in CD44 expression in jejunal mucosal endothelium (Heel *et al.*, 1998). If the hepatic response to IR injury is similar to that in the jejunum, then the result would be lower hepatic endothelial expression of CD44. As a result, blockade of CD44 would have no effect on adhesion of HPC-7 cells.

We did not demonstrate a significant role for the β_2 integrin, CD18, which is surprising given the importance of CD18/ICAM-1 interactions for IR mediated neutrophil recruitment in several organs including the liver (Jaeschke *et al.*, 1993). It is suggested that the major role

of the β_2 integrins in hepatic IR injury becomes apparent later during reperfusion, when the endothelial monolayer is disrupted and leukocyte β_2 integrins bind directly to receptors on hepatocytes (Kobayashi *et al.*, 2001). Hence the previously described benefits of anti-CD18 therapy in the liver may be due to it preventing neutrophil interactions with hepatocytes rather than sinusoids. This may explain the ineffectiveness of CD18 blockade in the current study since at the times studied, the endothelial monolayer is likely to be relatively intact, masking hepatocytes and the adhesion molecules to which HSCs would bind using β_2 integrins.

Recent data suggest that the VLA-4/VCAM-1 pathway alone is capable of facilitating capture of progenitor cells within BM, but if its function is compromised a synergistic contribution of β_2 integrins and selectins is uncovered (Papayannopoulou *et al.*, 2001). Furthermore, it is possible that VLA-4 integrin plays a site specific role in liver since studies from our laboratory demonstrate HSC recruitment to IR injured cremaster muscle venules is CD18-dependent (Zhao *et al.*, 2008). In addition, it has been shown that HSCs can be activated such that the adhesion molecules expressed on the cell surface become 'primed' (Peled *et al.*, 2000). It is conceivable that the signals released from the endothelium during hepatic IR injury are insufficient for β_2 integrin activation on HSCs.

During the reperfusion phase of hepatic IR injury, activation of the transcription factor NF-κB occurs within 30 minutes and peaks at one hour (Yoshidome *et al.*, 1999). As early as 90 minutes post-reperfusion, mRNA for ICAM-1 and VCAM-1 are significantly upregulated on hepatic endothelial cells (Essani *et al.*, 1997, Yoshidome *et al.*, 1999). The current study

used immunohistochemistry to investigate the expression of CD44, CD31 and VCAM-1 during IR injury. However, no detectable upregulation of CD44, CD31 or VCAM-1 on hepatic sections from IR treated animals was observed when compared to sham treated animals. In contrast it should be noted we were unable to detect CD31 on the hepatic sinusoids using immunohistochemistry. This may be a result of low CD31 expression which is below the sensitivity of the assay. It is well documented that immunohistochemistry is sometimes unsuitable for detecting analytes at low levels (Gusev *et al.*, 2001).

Interestingly, L-selectin could not be blocked, even with monoclonal antibody concentrations as high as 160μg/ml of antibody. Whether this represents cycling of blocked L-selectin with fresh L-selectin is unclear. It is unlikely to be a result of inappropriate antibody; a fluorescent antibody of the same clone identifies clearly identifies L-selectin expression. As a result of the relative selectin independence of the hepatic sinusoidal vascular bed, this adhesion molecule was not investigated in any detail in our studies.

In conclusion, we report for the first time that VLA-4/VCAM-1 interactions play an important role in recruitment of exogenous HSCs to inflamed liver. Modulation of this homing mechanism may be used as a therapeutic strategy to improve the efficacy of potential stem cell therapy. This is essential considering the rarity of HSCs, which currently limit the ultimate magnitude of therapeutic cellular regeneration based on administering these cells exogenously. In a study by Kumar and Ponnazhagan, ectopic expression of the α4 integrin on the surface of MSCs significantly promotes recruitment to the bone marrow. To achieve this, MSCs were transfected with the α4 integrin subunit using an adenovirus

vector. Cells transfected with the α4 integrin displayed a 10-fold enhancement in homing when compared to GFP (control) transduced cells (Kumar and Ponnazhagan, 2007). Furthermore, in a recent study by Sackstein and colleagues, chemical engineering of MSCs enhanced recruitment of these cells to the bone marrow. While MSCs natively do not express E-selectin ligands, Sackstein *et al.* enzymatically converted the native MSC CD44 isoform into HCELL, a potent E-selectin ligand (Sackstein *et al.*, 2008). Understanding the adhesion pathways in HSCs will act a starting point for similar studies aimed to enhance recruitment of HSCs to target tissues.

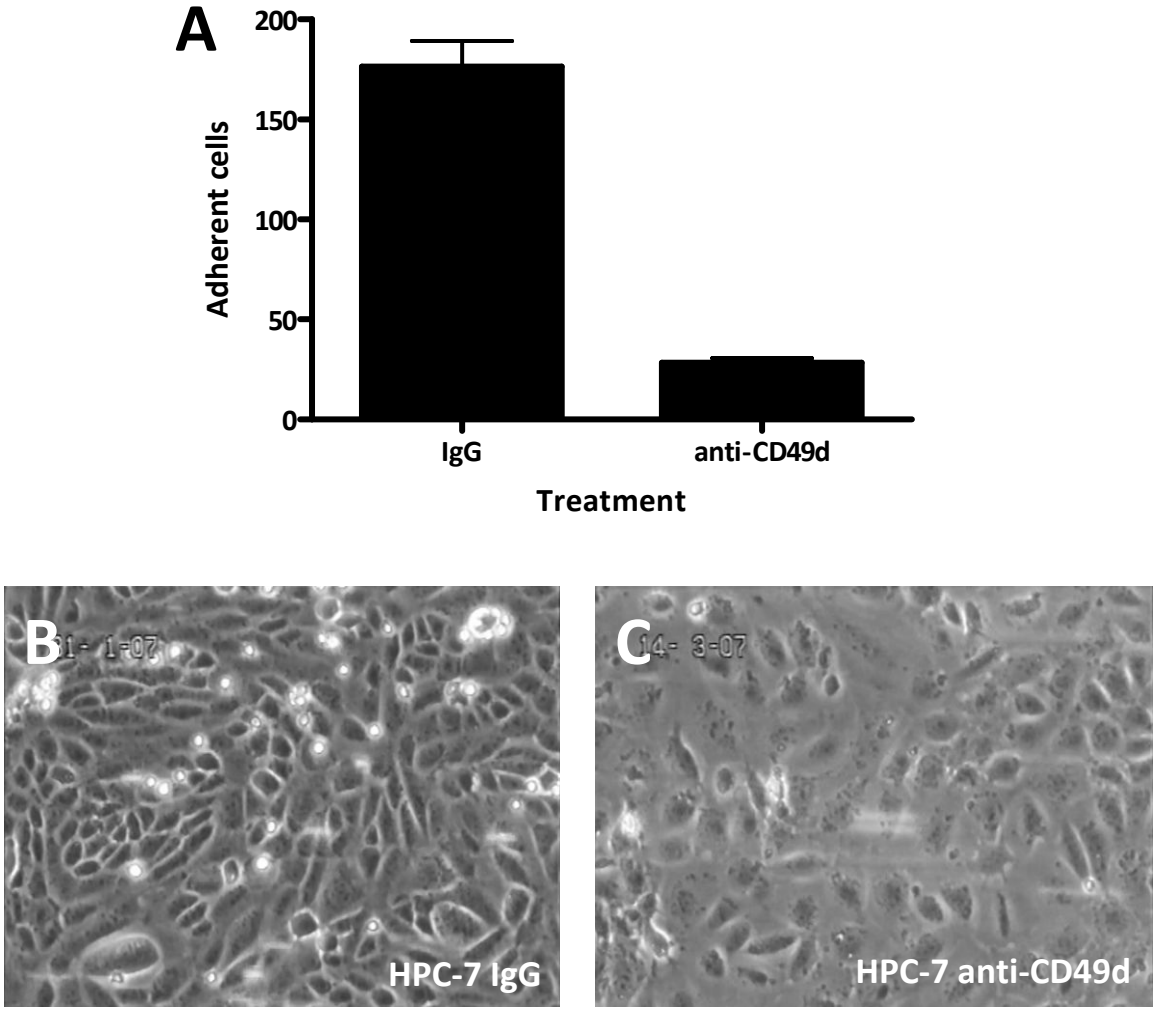


Figure 4.11. *Blocking HPC-7 cells with an anti-CD49d antibody significantly reduces adhesion to MuCEC-1 in a flow based adhesion assay.* In a flow based adhesion assay, HPC-7 cells (pre-treated with an anti-CD49d mAb) were flown across MuCEC-1. Blockade of CD49d significantly reduces adhesion **(panel A)**. Example images of the assay are presented **(panels B and C).** Work was performed by Mr Luke E Durant in our lab (unpublished results, 2006).

Chapter 5

Mechanisms of haematopoietic stem cell recruitment during murine ischemia-reperfusion injury – role of cytokines

5. Mechanisms of haematopoietic stem cell recruitment during murine ischemia-reperfusion injury – role of cytokines

5.1. Introduction and hypotheses

5.1.1. Introduction

The cytokine response to IR injury is robust (Luster, 1998), with production of a variety of cytokines providing a strong stimulus for neutrophil recruitment to the injured organ (Lentsch *et al.*, 1998, Burne *et al.*, 2001). Cytokines released during hepatic injury include TNFα, IL-1, IL-8, macrophage inflammatory protein-2 (MIP-2) but also CXCL12 and MMP-9 (Kollet *et al.*, 2003). Matrix metalloproteinases, such as MMP-9, are able to modify the composition of basement membrane, facilitating HSC emigration into tissues (Dalakas *et al.*, 2005).

Although the role of inflammatory cytokines/chemokines in mature leukocyte recruitment is well established, their role in HSC recruitment to injured tissue is not clear. Currently the role of CXCL12 in stem cell homing has been most investigated. It plays a critical role in HSC recruitment to the BM (Kopp *et al.*, 2005) and antibody mediated blockade of the CXCL12 receptor, CXCR4, prevents BM engraftment of human $CD34^+$ cells in NOD/SCID mice (Peled *et al.*, 1999b). Furthermore, CXCL12 is also implicated in the recruitment of HSCs to extra-medullary sites following hypoxic injury as its release from endothelium is mediated by activation of the transcription factor, hypoxia-inducible factor-1 (HIF-1) during periods of hypoxia (Ceradini *et al.*, 2004). CXCL12 production by cardiac tissue recruits human

$CD34^+$/c-kit^+ cells and this has been shown to subsequently confer a vasculogenic effect (Askari *et al.*, 2003). In rats, direct injection of a protease resistant form of CXCL12 (S-CXCL12(S4V)) into infarcted myocardium significantly promotes progenitor cell ($CXCR4^+$c-kit^+) recruitment and improves cardiac ejection fraction (Segers *et al.*, 2007). However, the direct role played by CXCL12 and other major inflammatory cytokines in recruiting HSCs to injured liver is not well understood.

CXCL12 mediated recruitment is likely to involve integrin activation on the HSC surface as well as the induction of cytoskeletal reorganization and a motile phenotype. CXCL12 activates integrins VLA-4, VLA-5 and LFA-1 as evidenced by increased adhesiveness to ICAM-1 and VCAM-1 *in vitro* (Peled *et al.*, 2000). During liver rejection infiltrating lymphocytes express raised levels of functional CXCR4, suggesting the CXCL12/CXCR4 pathway may be physiologically relevant for leukocyte homing during liver injury (Goddard *et al.*, 2001). In addition to inducing activation by a conformational change that switches an inactive integrin into an active one, cytokines can also upregulate integrin *expression* on the cell surface. Treatment of various subsets of mature leukocytes with MIP1α or MIP2α significantly increases surface expression of the CD11b subunit of the integrin Mac-1 (Conklyn *et al.*, 1996). Treatment of neutrophils with 200U/ml TNFα for 30 minutes significantly upregulates both CD11b and CD18 expression and increases migration responses to the chemokine, CCL3 (Montecucco *et al.*, 2008). Upregulation of CD18 on neutrophils has also been observed following a 15 minute exposure to RANTES, MIP-1 and IL-8 (Conklyn *et al.*, 1996). There is limited data on whether inflammatory cytokines can also increase adhesion molecule expression on HSCs. However, Zhang *et al* did demonstrate

that treatment of human $CD34^+$ cells with IL-6 and TNFα upregulates expression of CD49d (Zhang *et al.*, 2007).

The previous chapter demonstrated that HSC recruitment during liver injury is primarily dependent upon the VLA-4/VCAM-1 pathway. However, it is not clear whether increased expression or conformational activation of VLA-4 on HSCs is responsible for their recruitment nor what the activating factors are. Therefore, this chapter will investigate whether soluble factors released from IR injured liver alter the expression of not only VLA-4, but also CD18 and CD44 on the HSC surface. Circulating HSCs would be exposed to these soluble inflammatory factors not only within the local microcirculation at the site of injury, but also possibly within the systemic circulation. In order to determine whether CXCL12 is responsible for any changes in adhesion molecule expression, the effect of CXCL12 on HSCs will be tested. However, within an inflamed environment, cell recruitment is likely mediated by a combination of cytokines acting in synergy and we will also study the effect of proinflammatory cytokines including TNFα, IL-6, CXCL1 (CXCL1; keratinocyte derived chemokine, a functional murine homologue of human IL-8) and TGFβ. Furthermore, the direct role of CXCL12 in mediating HSC recruitment to IR injured liver *in vivo* will be investigated. Understanding these effects could facilitate modulation of HSC recruitment *ex vivo* prior to cell transfer in order to enhance recruitment.

5.1.2. Hypotheses

Our primary hypothesis is that IR injury induces the expression of integrin-activating factors in tissue which include CXCL12. More specifically, we hypothesised that :

1. Treatment of HPC-7 cells with a) media conditioned by homogenised tissue supernatant from animals subjected to IR injury b) plasma from animals subjected to IR injury would upregulate adhesion molecule expression
2. Treatment of HPC-7 cells with CXCL12, TNFα, IL-6, CXCL1 or TGFβ either alone or as a cocktail would upregulate adhesion molecule expression
3. Treatment of HPC-7 with inflammatory cytokines, including CXCL12, would enhance their adhesion to endothelium in static adhesion assays
4. Blockade of the CXCL12 receptor CXCR4 on HPC-7 cells would significantly reduce injured liver recruitment *in vivo*

5.2. Methods

The methods used in this chapter are described in detail in Chapter 2. Briefly, HPC-7 cells were initially examined by FACS for expression of the CXCL12 receptor using fluorescently conjugated antibody against CXCR4 (FITC). Expression of the TNFα receptors, TNFR1 (PE) and TNFR2 (PE) was also determined. HPC-7s were then analysed for changes in expression of adhesion molecules following treatment with media conditioned by homogenised hepatic IR tissue supernatant or plasma derived from IR animals for 24 hrs. To determine whether any effects observed were tissue specific, cells were also treated with media conditioned by homogenised IR treated gut tissue supernatant.

In order to determine which specific cytokines could induce adhesion molecule expression changes, HPC-7s were also analysed for changes in expression of adhesion molecules following treatment with 100ng/ml of CXCL12, CXCL1, IL-6 or TNF-α, 10ng/ml TGFβ or PBSA for between 30 minutes and 24 hours. FACS analysis was conducted using fluorescently conjugated antibodies against CD49d (FITC), CD44 (PE) and CD18 (FITC). The effects of a

cocktail of cytokines on CD49d expression were also determined. HPC-7 cells were treated with a cocktail of 20ng/ml IL-1β, 20ng/ml CXCL12, 20ng/ml TNF-α and 20ng/ml CXCL1 for 0.5, 1, 3, 6 and 24 hours. Data are presented as a percent of control expression for samples with an untreated control, or mean fluorescent intensity for groups lacking an untreated control (such as sham and IR treated samples).

To investigate the ability of cytokines to regulate adhesion of HPC-7 cells to endothelial monolayers, static adhesion assays were conducted using MuCEC-1 seeded on coverslips. HPC-7 cells were incubated with cytokines for four hours prior to exposure to endothelium and adhesion subsequently monitored. Data represent mean adhesion ± SEM from four experiments. Furthermore, intravital microscopy was conducted using CFSE labeled HPC-7 cells pre-treated with a CXCR4 receptor function blocking mAb. Data represent the mean adhesion or free flowing cells ± SEM of at least 4 animals per group.

5.3. Results

5.3.1. HPC-7 cells express the cytokine receptors CXCR4, TNFR1 and TNFR2

The cytokine receptor profile of HPC-7 cells is currently not known, so flow cytometry was used to determine whether these cells express CXCR4, TNFR1 and/or TNFR2. HPC-7 express CXCR4 *(figure 5.1.A)*, and both TNFR1 and TNFR2 with higher levels of the former *(figure 5.1.B) (figures 5.1.B & 5.1C)*.

5.3.2. CD44 or CD18 expression is not changed by treatment of HPC-7 cells with conditioned media from gut or hepatic IR tissues

No significant upregulation of CD18 (sham liver conditioned: 36.94 ± 5.41 vs. IR liver conditioned: 38.02 ± 1.22; $P > 0.05$; sham gut conditioned: 39.86 ± 4.51 vs. IR gut conditioned: 35.49 ± 8.27; $P > 0.05$) or CD44 (sham hepatic conditioned: 2081.00 ± 120.70 vs. IR hepatic conditioned: 2110.00 ± 164.50; $P > 0.05$; sham gut conditioned: 4105.00 ± 236.70 vs. IR gut conditioned: 3881.00 ± 961.50; $P > 0.05$) expression was observed following treatment with hepatic IR or gut IR tissue conditioned media compared with sham control *(figures 5.2.A-D)*.

5.3.3. CD49d expression is upregulated by treatment of HPC-7 with hepatic IR and gut IR conditioned media

Treatment of HPC-7 cells with hepatic IR conditioned media increased CD49d expression compared to media conditioned with a sham liver sample, but this did not attain statistical significance (MFI: sham hepatic conditioned: 31.09 ± 1.21 vs. IR hepatic conditioned: 38.57 ± 5.74; $P > 0.05$) *(figure 5.3.A)*. No change was seen after treatment with gut IR conditioned media (MFI: sham gut conditioned: 29.06 ± 1.62 vs. IR gut conditioned: 23.13 ± 7.00; $P > 0.05$) *(figure 5.3.B)*.

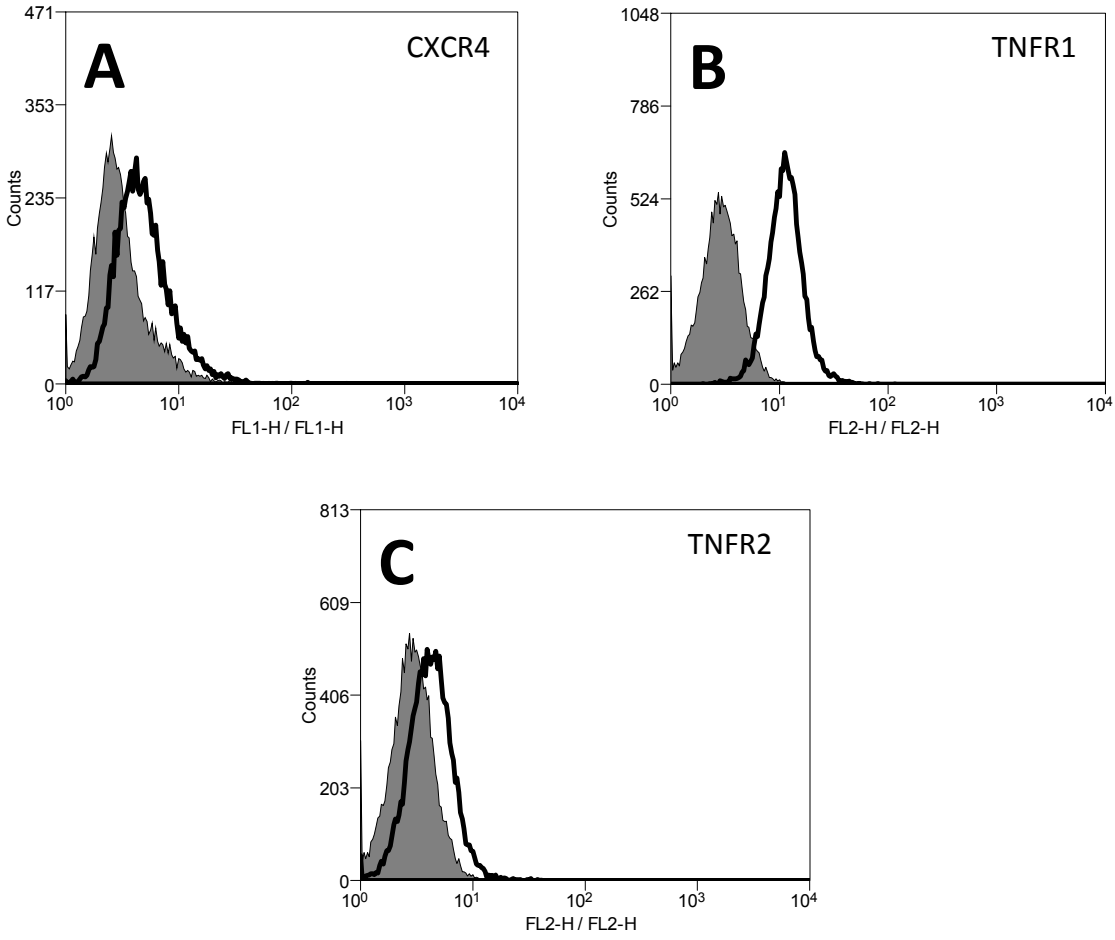


Figure 5.1. *HPC-7 cells express the cytokine receptors CXCR4, TNFR1 and TNFR2 as determined by FACS.* HPC-7 cells express CXCR4 (panel A), TNFR1 (panel B), and TNFR2 (panel C) (filled area: negative control antibody, black line: receptor antibody). Figures are typical FACS plots representative of three separate experiments.

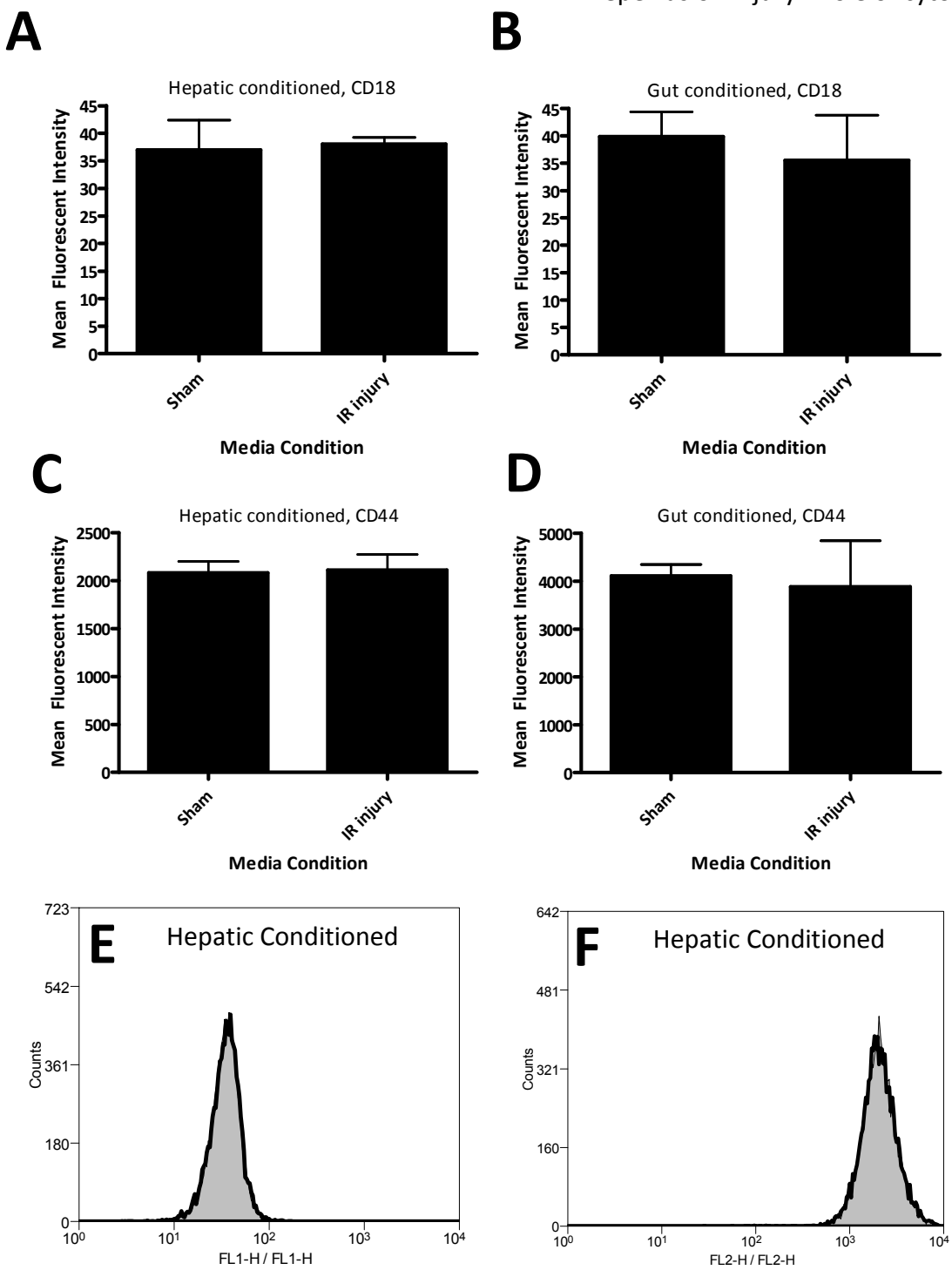


Figure 5.2. *Treatment of HPC-7 cells with media conditioned from hepatic and intestinal IR tissue does not up-regulate CD44 or CD18.* Hepatic and gut IR tissue was prepared, homogenised and incubated in media for 24 hours (sham tissue was also obtained). Treatment of HPC-7 cells with IR liver tissue media does not up-regulate CD18 **(panel A)** or CD44 **(panel C)** compared to sham gut tissue. Similarly, IR gut tissue media does not up-regulate CD18 **(panel B)** or CD44 **(panel D).** Representative FACS plots of hepato-treated HPC-7 CD18 **(panel E)** and CD44 **(panel F)** expressions are shown (filled area: sham conditioned, black line: IR conditioned). Figures represent mean ± SEM of at least three separate experiments.

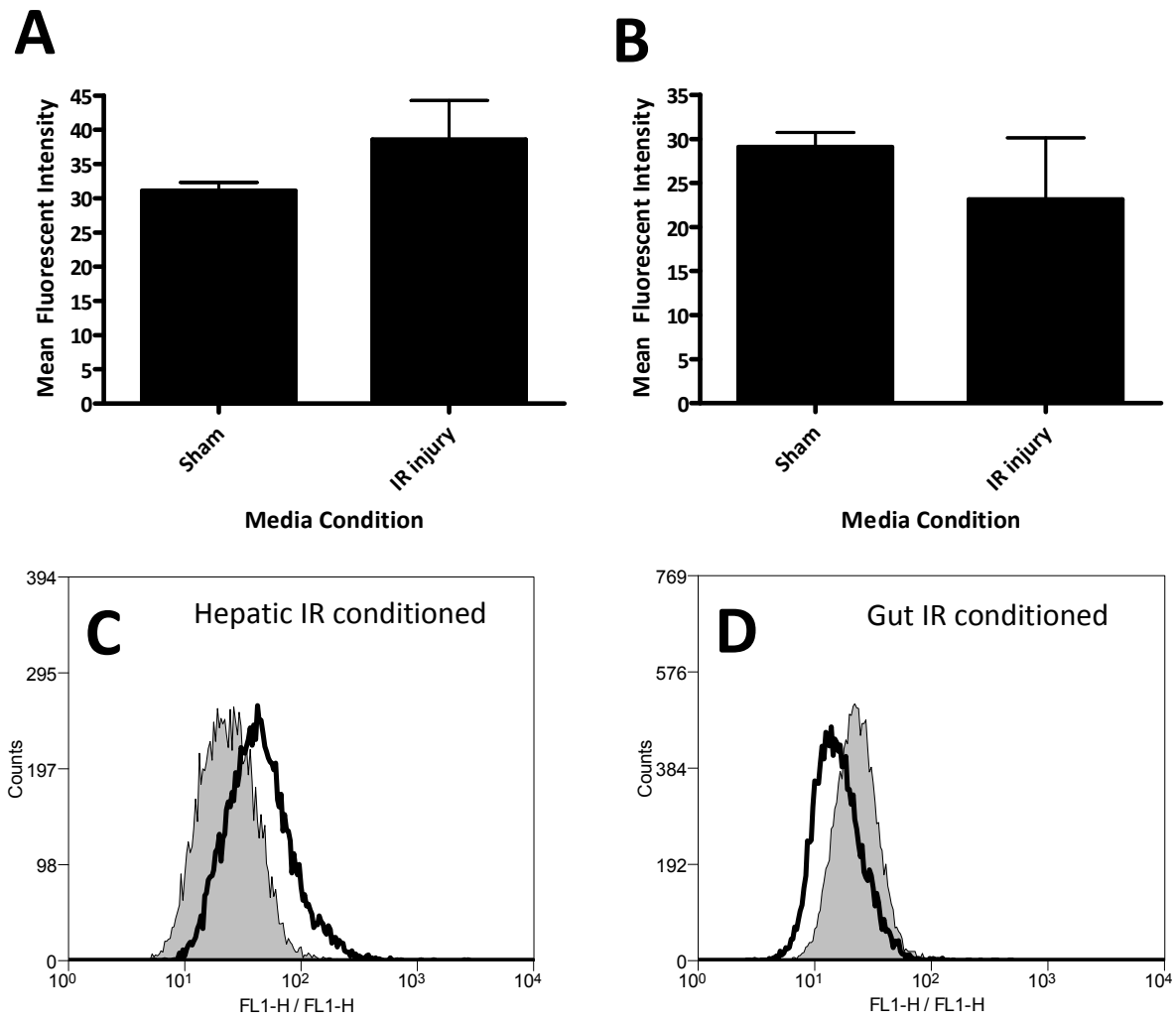


Figure 5.3. *Hepatic IR conditioned medium, but not gut IR conditioned medium, modestly (but insignificantly) up-regulates CD49d on HPC-7 cells.* Hepatic and gut IR tissue was prepared, homogenised and incubated in media overnight (sham tissue was also obtained). HPC-7 cells were incubated in the relevant media for 24 hours. Hepatic IR conditioned media modestly, but insignificantly, up-regulates HPC-7 expression of CD49d **(panels A and C)**. Gut IR conditioned medium mediates a general down-regulation of CD49d **(panels B and D)**. Panels A and B are representative of mean ± SEM of three separate experiments. Panels C and D are representative FACS images.

5.3.4. CD49d expression after treatment of HPC-7 with conditioned plasma from hepatic IR

To identify if the factors mediating the minor CD49d shift were circulating factors, HPC-7 cells were treated for 24 hours with plasma from hepatic IR treated animals or sham treated animals. No change in CD49d expression was seen following treatment with plasma from IR animals when compared to plasma from sham controls (MFI: hepatic sham – plasma: 15.99 ± 3.89 vs. hepatic IR – plasma: 15.20 ± 3.23; $P > 0.05$) *(Figure 5.4.A)*.

5.3.5. CD49d, CD44 and CD18 expression is not significantly changed by treatment with individual cytokines

CD49d expression increased slightly after treatment with CXCL12 (% of control expression: 107.74% ± 5.17%) *(figures 5.5.A & 5.5.D)* but not after other cytokine treatments. Interestingly, decreased expression was seen following treatment with 100ng/ml IL-6 (% of control expression: 74.1% ± 14.94%) *(figure 5.5.A)*, 100ng/ml TNF-α (% of control expression: 75.96% ± 2.74%) *(figure 5.5.A)* and 10ng/ml TGFβ (% of control expression: 59.22% ± 0.51%) *(figure 5.5.A)*. CD44 expression was not upregulated by cytokine treatment, although a drop in expression was observed following treatment with 100ng/ml TNF-α (% of control expression: 91.17% ± 0.36%) *(figure 5.5.B)*. CD18 expression was also not changed by any of the cytokine treatments *(figure 5.5.C)*.

5.3.6. CD49d expression is downregulated by a cocktail of cytokines in a time-dependent manner

CD49d upregulation was not observed at any time point. However, a time-dependent down-regulation in CD49d expression was observed *(figure 5.6.A)* in response to a cocktail of 20ng/ml IL-1β, 20ng/ml CXCL12, 20ng/ml TNF-α and 20ng/ml CXCL1. Compared to PBSA controls, cell surface CD49d decreased at 30 minutes (% control expression: 96.27% ± 0.73%), 1 hour (% control expression: 96.85% ± 0.21%), 3 hours (% control expression: 96.29% ± 0.31%), 6 hours (% control expression: 93.26% ± 0.57%) with maximal reduction at 24 hours (% control expression: 82.46% ± 2.68%) *(figure 5.6.A)*.

5.3.7. Treatment of HPC-7 cells with CXCL12 and CXCL1 enhance adhesion to MuCEC-1

The role of various cytokines in directly mediating HSC adhesion was determined *in vitro* by pre-treating HPC-7s for 4 hours with 20ng/ml of CXCL12, CXCL1, TNF-α or IL-1β. Control HPC-7 cells were treated with PBSA. HPC-7 cells were then CFSE labelled. Adhesion of HPC-7 to MuCEC-1 was determined in a static endothelial cell adhesion assay. Treatment of HPC-7 cells with 20ng/ml TNF-α did not significantly increase adhesion to murine endothelium (cells adherent: PBSA: 21.15 ± 5.42 vs. TNF-α: 28.50 ± 2.50; $P > 0.05$) *(figure 5.7.A)*. Although there was a slight increase, IL-1β did not significantly raise adhesion either (cells adherent: PBSA: 21.15 ± 5.42 vs. IL-1β: 44.78 ± 10.01; $P > 0.05$) *(figure 5.7.A)*. However, treatment of HPC-7 cells with CXCL12 significantly enhanced adhesion on murine endothelium when compared to control (cells adherent: PBSA: 21.15 ± 5.42 vs. CXCL12: 59.33 ± 10.50; $P < 0.05$) *(figure 5.7.A)*. Similarly, treatment of HPC-7 cells with CXCL1 also

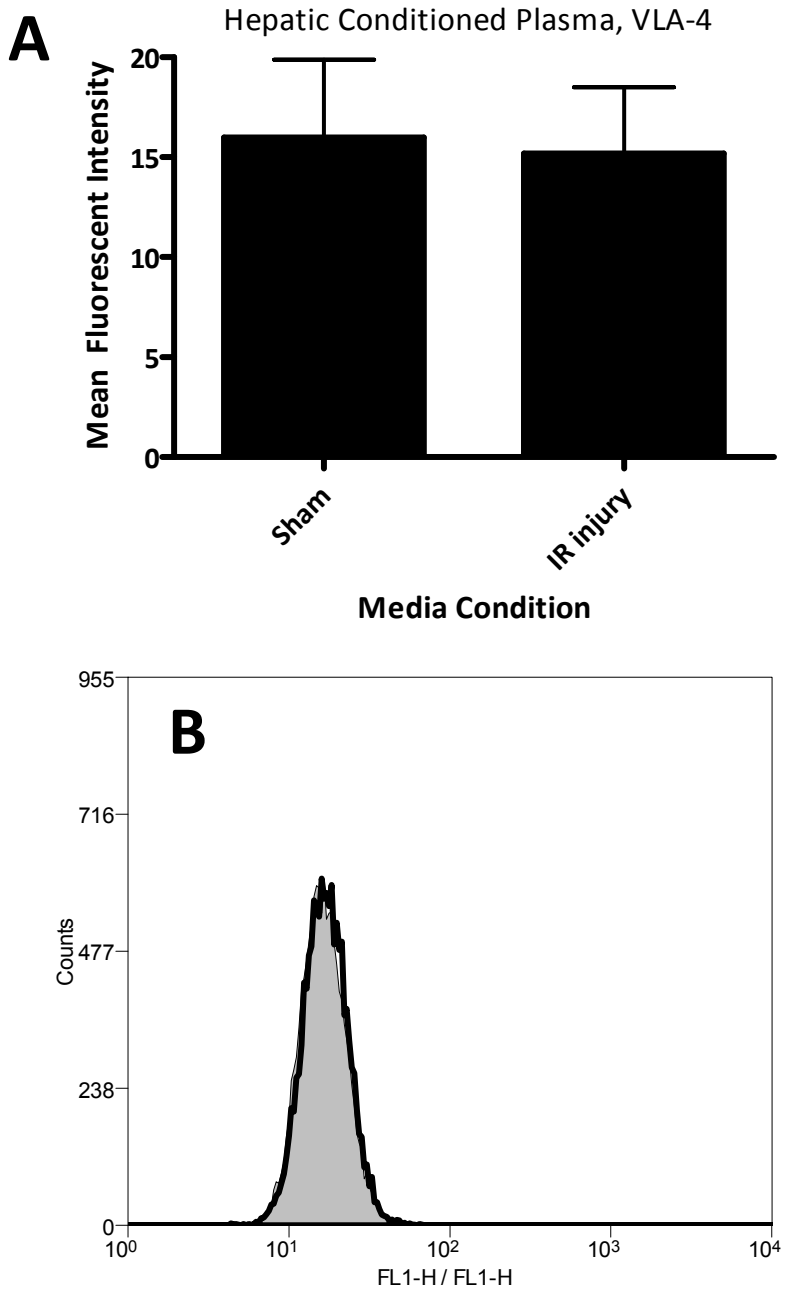


Figure 5.4. *CD49d is not upregulated on HPC-7 cells as a result of overnight incubation with plasma from IR treated animals.* HPC-7 cells were incubated for 24 hours in plasma isolated from sham and IR treated animals. Treatment of cells with plasma isolated from IR animals does not up-regulate CD49d when compared to plasma from sham animals **(panel A).** A representative FACS plot is shown **(panel B).** Panel A represents mean ± SEM of three separate experiments.

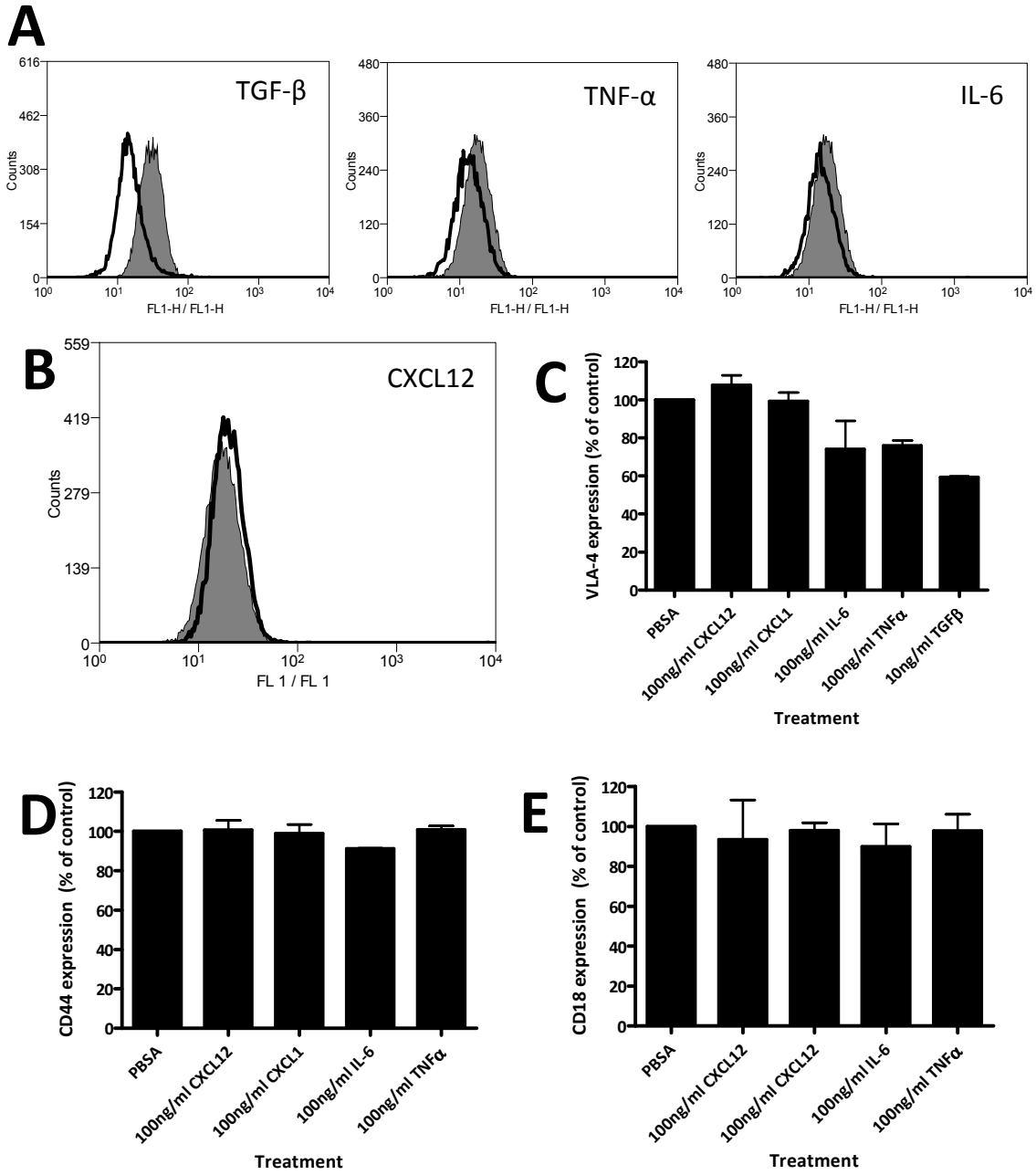
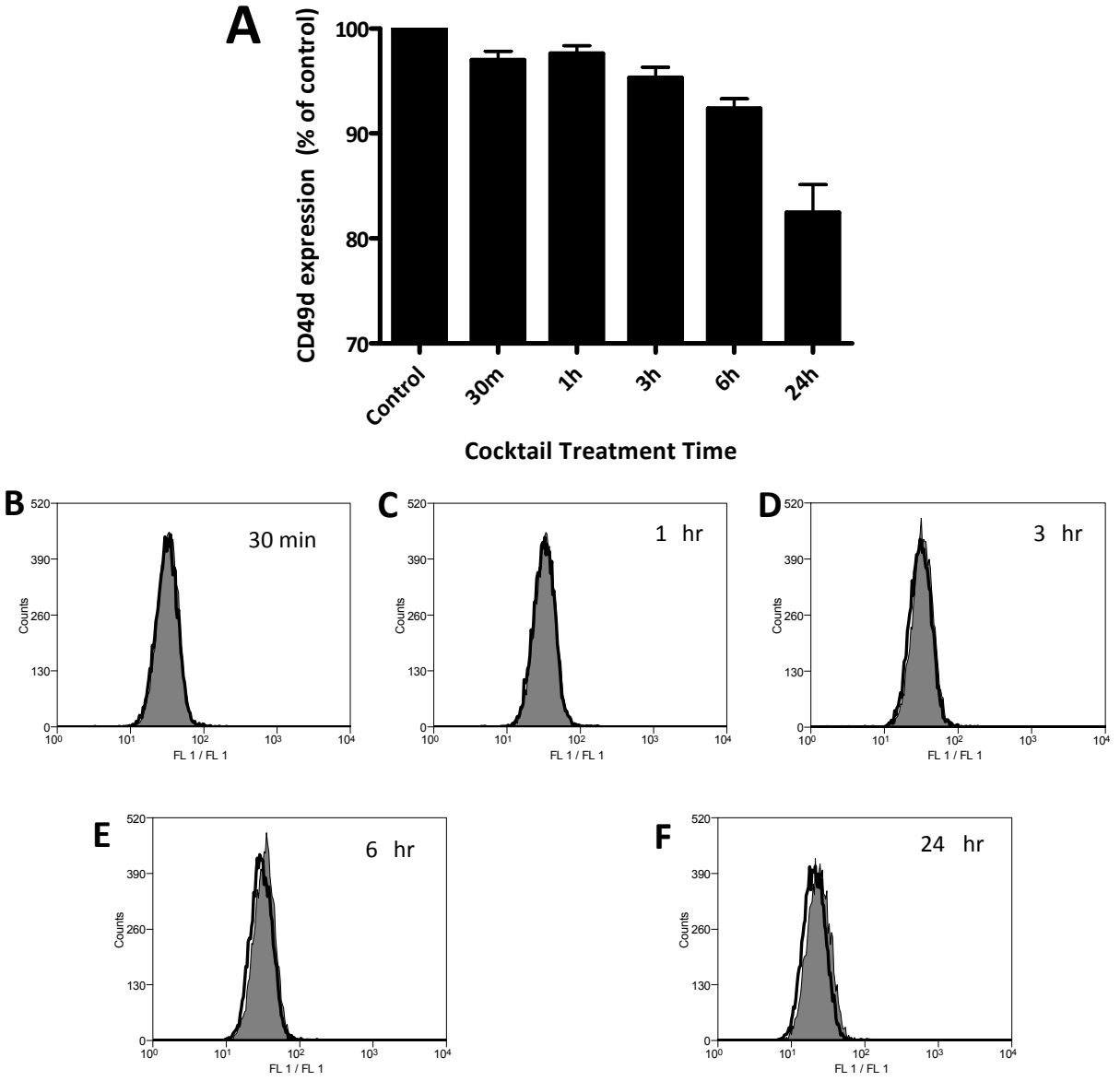


Figure 5.5. *Treatment of HPC-7 cells with various cytokines does not up-regulate expression of selected adhesion molecules.* HPC-7 cells were treated for 24 hours with various cytokines to investigate any effect on adhesion molecule expression. Expression of CD49d appears to be decreased by treatment with 100ng/ml IL-6, 100ng/ml TNF-α and 10ng/ml TGF-β. **(panel A)**. Almost all cytokines were unable to elicit an upregulation of CD49d **(panel C)**, CD44 **(panel D)** or CD18 **(panel E)**. Representative FACS plot displaying minor increase in CD49d expression after CXCL12 treatment is shown **(panel C)**. All panels represent expression as a percentage of control ± SEM from three experiments.



	Control	30m	1h	3h	6h	24h
% control expression	100.00	96.99	97.61	95.31	92.39	82.46
± SEM	0.00	0.83	0.77	1.00	0.92	2.68

Figure 5.6. *Treatment of HPC-7 cells with a cocktail of comparatively low-dose cytokines results in a time dependent down-regulation of CD49d.* HPC-7 cells were treated for various time periods with a cytokine cocktail containing 20ng/ml CXCL12, 20ng/ml IL-1β, 20ng/ml TNF-α and 20ng/ml CXCL1. A down-regulation of CD49d was visible at all time points, increasing incrementally until 24 hours **(panel A)**. Representative FACS plots are shown from 30 minutes **(panel B)**, 1 hour **(panel C)**, 3 hours **(panel D)**, 6 hours **(panel E)** and 24 hours **(panel F).** Bars represent CD49d expression as percent of control ± SEM from 3 individual experiments.

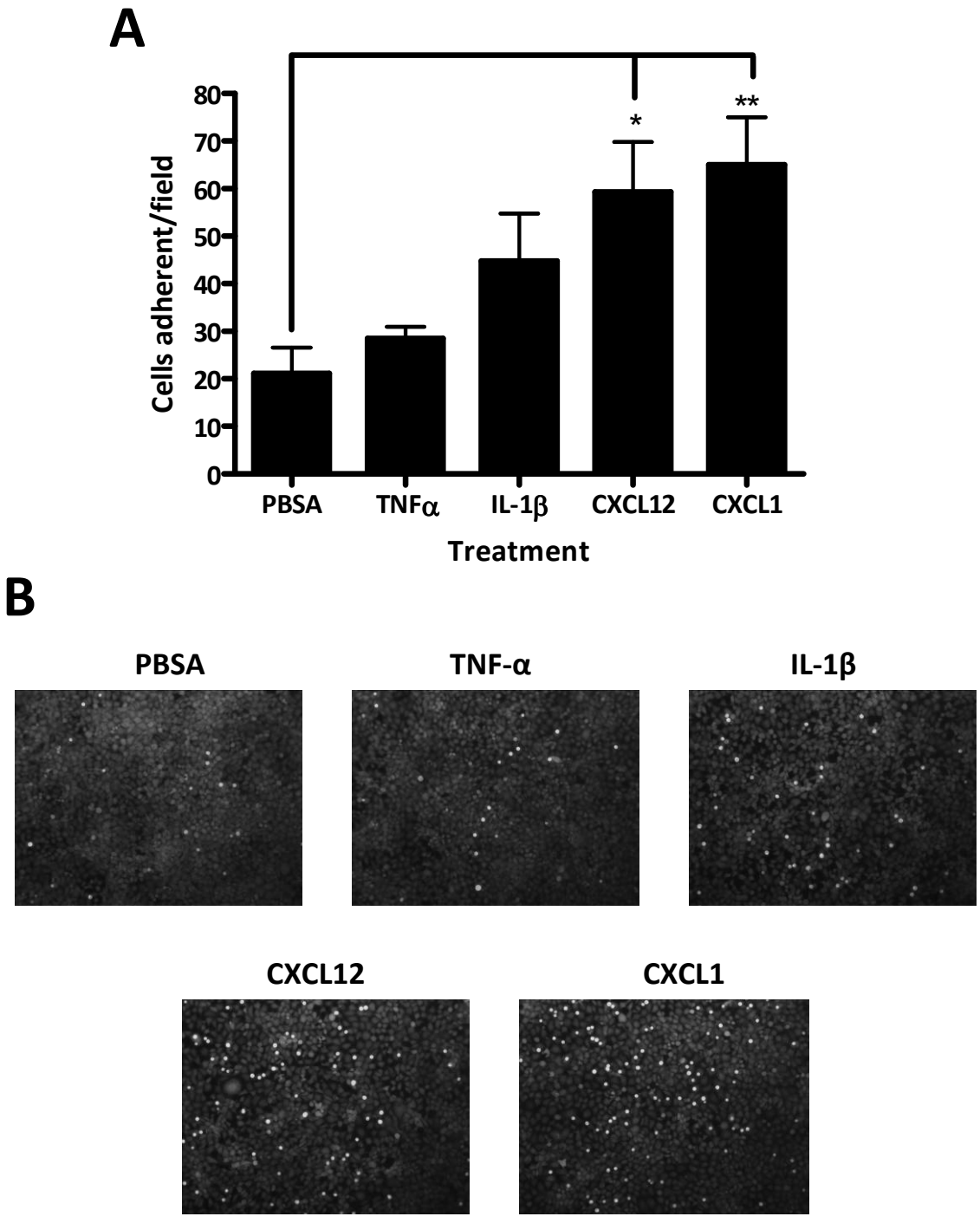


Figure 5.7. *CXCL12 and CXCL1, but not TNF-α or IL-1β, enhance HPC-7 adhesion to MuCEC-1.* HPC-7 cells were pre-treated with 20ng/ml of the relevant cytokine for four hours, and subsequently labeled with CFSE (5μM). Cells were seeded on MuCEC-1 for five minutes then unbound cells washed away. Treatment with CXCL12 and CXCL1 significantly enhance HPC-7 recruitment on MuCEC-1, a phenomenon not observed for TNF-α or IL-1β **(panel A).** Representative images are given **(panel B).** Bars represent mean ± SEM of four separate experiments; * $p < 0.05$, ** $p < 0.01$ (one-way ANOVA followed by a Bonferroni Multiple Comparison Test)

significantly augmented adhesion on murine endothelium (cells adherent: PBSA: 21.15 ± 5.42 vs. CXCL1: 65.03 ± 10.04; $P < 0.01$) *(figure 5.7.A)*.

5.3.8. CXCR4 blockade does not significantly reduce hepatic HSC adhesion post IR injury

As CXCL12 treatment significantly increased HPC-7 adhesion to endothelium *in vitro*, the counter-receptor CXCR4 was blocked on HPC-7 cells in the *in vivo* intravital model using 80μg/ml anti-CXCR4. Control cells were treated with an isotype control. However, blockade of CXCR4 did not significantly reduce the adhesion of HPC-7 to the hepatic microvasculature post IR injury (e.g. adhesion at 45 minutes: IgG treated cells: 13.50 ± 2.11 vs. CXCR4 treated cells: 12.00 ± 3.43; $P > 0.05$) *(figure 5.8.A)*. Blockade of CXCR4 did not significantly alter the number of free-flowing cells observed compared to IgG treated cells (e.g. free-flowing cells at 45 minutes: IgG treated cells: 1.17 ± 0.61 vs. CXCR4 treated cells: 0.50 ± 0.29; $P > 0.05$) *(figure 5.8.B)*. The vast majority of free-flowing cells were observed at 30 minutes post-reperfusion, the nearest time point to injection (e.g. CXCR4 treated: free-flowing cells at 30 minutes: 4.25 ± 0.63 vs. free-flowing cells at 60 minutes: 0.75 ± 0.48; $P < 0.01$) *(figure 5.8.B)*. Recruitment of cells remained exclusively sinusoidal *(figure 5.8.C-D)*.

5.4. Discussion

Despite previously demonstrating a crucial role for VLA-4/VCAM-1 interactions in mediating hepatic HSC recruitment, no significant increases in CD49d expression were observed following co-incubation of HPC-7s with injured liver conditioned media.

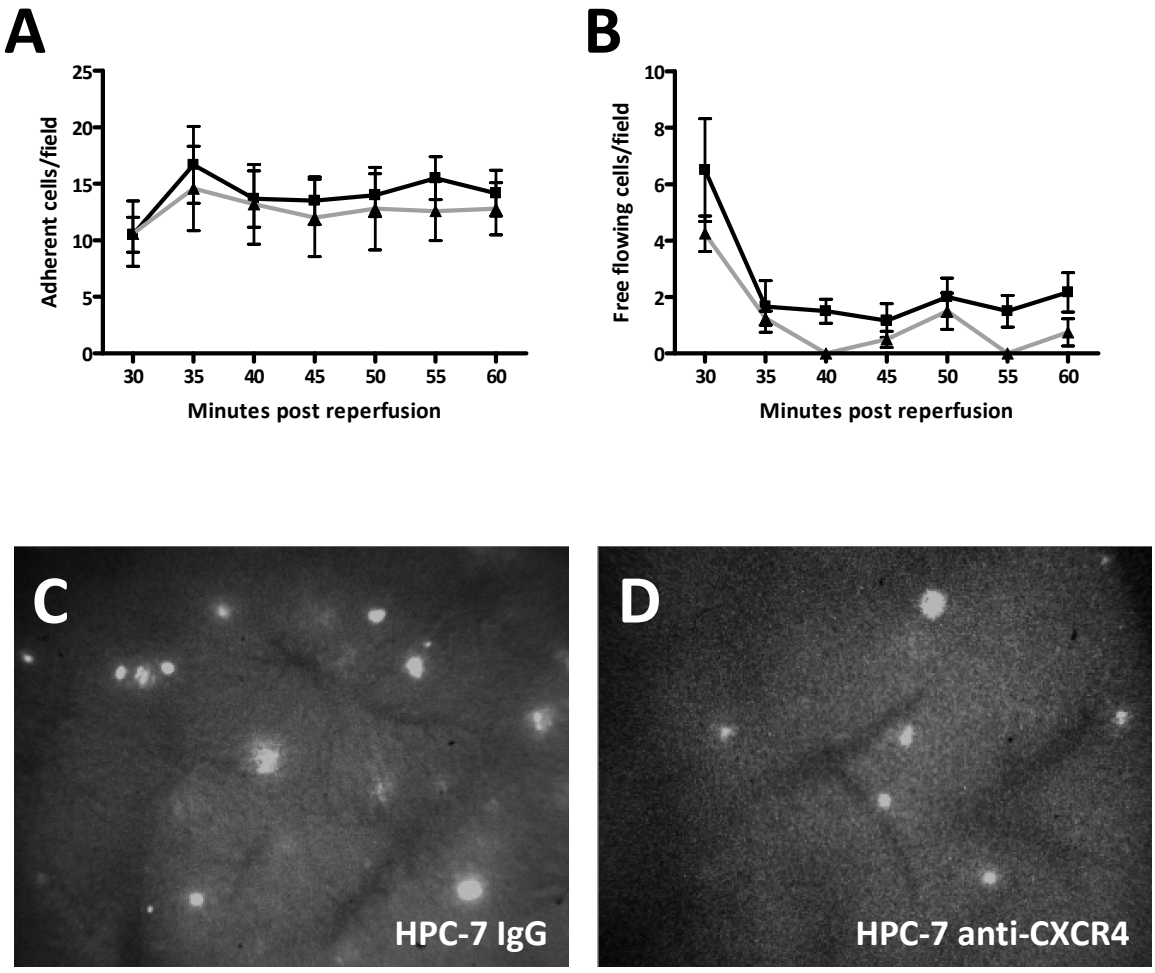


Figure 5.8. *CXCR4 blockade does not significantly reduce hepatic HPC-7 recruitment post IR injury.* Recruitment of HPC-7 cells to the hepatic microcirculation was examined using intravital microscopy following treatment with a specific blocking antibody. Animals were subjected to IR injury (90m PHI/30 R) prior to the introduction of either CXCR4 blocked or IgG treated HPC-7 cells (1 x 10^6 cells, 5µM CFSE, I.A.). Blockade of CXCR4 on HPC-7 failed to reduce the adhesion seen post IR injury **(panel A**; black line: IgG treated, grey line: anti-CXCR4 treated**).** No significant difference was seen in free flowing cells between treatments **(panel B**; black line: IgG treated, grey line: anti-CXCR4 treated**).** Representative images are shown of IgG **(panel C)** and CXCR4 **(panel D)** treated cells. Figures are representative of mean ± SEM of five separate experiments.

Furthermore, after testing a panel of cytokines known to play a role in hepatic IR injury (Serracino-Inglott *et al.,* 2001), we were also unable to identify cytokines that could significantly modulate the cell surface expression of CD49d. Both CXCL12 and murine CXCL1 significantly increased adhesion of HPC-7 cells to endothelial cells *in vitro* and given their lack of effect on integrin expression this is likely to be the results of conformational activation. However, blocking CXCR4 failed to reduce adhesion *in vivo*.

The modest increase in CD49d expression after 24 hour incubation with homogenised tissue supernatant may reflect a mechanism by which HPC-7 cells are retained within the injured microcirculation. However, the changes were not statistically significant although this might reflect a type 2 statistical error and with increased power, significance could have been reached. If a statistical significance could be demonstrated this would suggest that the local inflammatory environment can release factor(s) that activate CD49d.

Indeed, our in vitro studies demonstrated that both CXCL12 and CXCL1 were able to increase adhesion of HPC-7s to murine endothelial cells. Murine CXCL1 has been implicated in inflammatory cell recruitment following hepatic IR injury (Lentsch *et al.,* 1998) while the human functional homologue, IL-8, has been implicated partially in HSC mobilisation from the BM (van Pel *et al.*, 2006). However, while a critical role for the CXCL12/CXCR4 pathway has been demonstrated in HSC recruitment, a similar role for CXCL1 had not previously been identified.

As CXCL12 is implicated in BM homing and HSC recruitment to extra-medullary tissues, we sought to investigate whether blockade of CXCR4 would reduce adhesion post IR injury. Pre-treating HPC-7 cells with CXCR4 blocking antibodies failed to reduce HSC adhesion following IR injury. Other cytokines may compensate for the activities of CXCL12, including IL-8, which may able to promote HSC adhesion to endothelium. Although blocking CXCR4 robustly inhibits the BM homing of HSCs (Lapidot and Kollet, 2002), this may reflect more tightly defined adhesive mechanisms in the regulation of homing to the BM.

Interestingly, some of the cytokines induced downregulation of CD49d. IL-6 is released during acute hepatic IR injury (Tsuyama *et al.*, 2000) and has anti-inflammatory and hepato-protective functions (Matsumoto *et al.*, 2006). IL-6 caused a reproducible downregulation of CD49d expression and to a lesser extent CD44 and CD18. TGFβ and TNFα also decreased CD49d expression. TGFβ has been shown to play important roles in the regulation of the adhesive state of the BM compartment; treatment of BM stroma with TGFβ significantly reduces progenitor cell recruitment via CD49d (Robledo *et al.*, 1998). CD49d is crucial in the retention of HSCs in the BM (Papayannopoulou, 2004) thus it is possible that release of cytokines from the injured liver into the systemic circulation could reduce CD49d on HSCs and thereby inhibit BM engraftment. This would increase the availability of peripheral circulating HSCs for subsequent recruitment to injured sites. Indeed, cytokines such as G-CSF (granulocyte colony-stimulating factor), used clinically to mobilise stem cells from the BM, also work in a similar manner by manipulating numerous retention mechanisms. Treatment of progenitor cells with G-CSF significantly reduces their expression of CD49d and promotes egress into the circulation (Chen and Xie, 2003).

Treatment of HPC-7s with a cocktail of cytokines also reduced CD49d to a similar degree with maximal reduction after 24 hours co-incubation. It is possible that certain inflammatory environments may actively discourage HSC retention and CD49d downregulation could represent a mechanism by which HSCs can be removed from, or are prevented from being retained within, injured sites. This is consistent with the findings of other groups suggesting that BM-derived stem cells do not actually need to engraft the injured tissue to exert benefit, but rather do so by a paracrine, anti-inflammatory mechanism. Indeed, following renal IR injury, exogenously administered MSC are only present in the kidney for 2 hours post reperfusion and not observed after 24 hours (Tögel *et al.*, 2005).

Although a paracrine role has been predominantly ascribed to MSCs, HSCs can also exert benefit via a similar mechanism. Yoshida and colleagues demonstrated that exogenously administered HSCs could prevent the damage associated with cochlear IR injury. However, this benefit was not dependent on subsequent HSC differentiation or fusion, but rather paracrine effects (Yoshida *et al.*, 2006). Furthermore, HSCs have been reported to secrete growth factors with trophic properties, such as Ang1 (Takakura *et al.*, 2000). Interestingly, HSCs have been shown to ameliorate hearing loss following cochlear ischemia via upregulation of the protein Glial cell derived neurotrophic factor (GDNF) independent of fusion or transdifferentiation (Yoshida *et al.*, 2007).

Unlike treatment with the individual cytokines, no change in CD49d expression was observed when cells were treated with plasma derived from IR injured animals. During tissue damage, cytokines released from the injured endothelium establish a local inflammatory microenvironment that facilitates leukocyte recruitment. Cytokines remain within the local vasculature by binding to proteoglycans on the luminal surface of the endothelial glycoclayx. For instance, it has been shown that IL-8 and MIP-1β are immobilised on the local endothelium, which prevents their washout by flowing blood (Rot, 1992, Tanaka *et al.*, 1993). Thus CD49d activating factors may have been present only locally at the site of injury but not in the circulation. Furthermore, cytokines are rapidly cleared from the circulation by both the liver and the kidney which might have left low or undetectable levels in plasma (Wollenberg *et al.*, 1993).

TNFα is also a key mediator of neutrophil recruitment in inflammation in part by enhancing Mac-1 (CD11b/CD18) up-regulation. Mac-1 is important for leukocyte migration into inflamed tissues. Montecucco and colleagues observed that treatment of neutrophils for 30 minutes with TNFα induced significant increases in CD11b and CD18 subunits (Montecucco *et al.*, 2008). However, in the current study treatment of HSCs with TNFα does not lead to an upregulation in surface expression of any of the adhesion molecules investigated and *in vitro* only CXCL12 or CXCL1 increased adhesion of HPC-7s to murine cardiac endothelial cells. Thus, TNFα appears to play a role in the recruitment of mature leukocytes rather than stem/progenitor cells. Indeed, recent studies from our group have demonstrated that recruitment of HPC-7 cells *in vivo* to the murine cremaster muscle can

only be mediated by an intrascrotal injection of IL-1β but not TNFα (Zhao *et al.*, 2008). It is also likely that TNFα acts primarily on the endothelium rather than on HSCs.

The lack of a direct role for TNFα in mediating HPC-7 recruitment *in vitro* and *in vivo* (Zhao *et al.*, 2008) is somewhat surprising given the observation that HPC-7 cells express abundant levels of the TNF-α receptors TNFR1 and TNFR2. Meldrum *et al* also identified TNFR1 on $CD34^+$ murine BM cells (Meldrum *et al.,* 2006). However, they demonstrated that TNFα served as a signal to progenitor cells to release vascular endothelial growth factor (VEGF). Furthermore, by using $CD34^+$ cells derived from $TNFR1^{-/-}$ mice, they identified this receptor as being responsible for mediating maximum VEGF production. VEGF is released in large quantities by stem cells and decreases inflammation and apoptosis during ischemia. It is therefore possible that the role of TNF receptors on HPC-7 cells is not to mediate stem cell recruitment, but to allow the cells to mediate beneficial paracrine effects.

Pretreatment of HSCs with cytokines may be an important prerequisitie to their use in clinical therapies. Indeed, *ex vivo* treatment of BM HSCs with SCF, TPO, IGF-2 and FGF-1 enhances their *in vivo* repopulating ability when introduced into lethally irradiated recipients (Zhang and Lodish, 2005). In the context of extra-medullary recruitment, pretreatment of HSCs with cytokines may be beneficial in enhancing recruitment at sites of injury via either adhesion molecule upregulation on the HSC surface or activation of pre-existing adhesion molecules. While we were unable to show evidence for the upregulation of adhesion molecules after cytokine treatment, we have shown that particular cytokines

do enhance recruitment *in vitro*. Further work would examine whether pretreating HSCs prior to injection would enhance recruitment to injured organs.

Chapter 6

Investigating the localization of injected HSCs, their potential role in regulating inflammation and their interactions with platelets

6. Mechanisms by which HSCs may exert beneficial effects

6.1. Introduction and hypotheses

6.1.1. Introduction

The adhesion pathways by which HSCs are recruited to the IR injured liver are at least partially elucidated in the previous chapters. HSC recruitment to injured extra-medullary, non-BM sites from the peripheral circulation may prove to be benefical in certain disorders, including the treatment of tissue damage post myocardial infarction (Zhang *et al.*, 2007). However, the actual mechanisms by which HSCs may exert their benefit once recruited to damaged tissue are unclear. In the liver, evidence exists that HSCs can replace lost hepatocytes by both cell transdifferentiation and cell fusion although this is a rare event (Dalakas *et al.*, 2005, Thorgeirsson and Grisham, 2006). Alternatively, HSCs and other stem cell populations, particularly MSCs, may promote tissue repair through paracrine mechanisms. Intramyocardial injection of MSCs following infarction improves cardiac function and prevents ventricular remodelling via a secreted biological factor (Gnecchi *et al.*, 2005). Furthermore, recruitment of MSCs to the renal microcirculation protects against ischemic acute renal failure (ARF), via mechanisms which are independent of transdifferentiation or fusion. The presence of HSCs during ARF results in reduced inflammatory gene expression (IL-1β, TNF-α, IFN-γ and iNOS) and increased anti-inflammatory gene expression (IL-10, bFGF, TGF-α and Bcl-2) (Tögel *et al.*, 2005). Whether a similar mechanism occurs in the liver is unclear. Indeed, it is even unclear whether HSCs share similar paracrine functions as their mesenchymal counterparts.

Numerous studies have provided evidence that MSCs promote tissue repair through the secretion of paracrine cytoprotective factors. MSC conditioned media improved cardiac function in rat model of myocardial infarction, although the secreted beneficial factor(s) was not identified (Gnecchi *et al.*, 2005). MSCs also protect against acute renal ischemic injury by reducing pro-inflammatory gene expression (IL-1β, TNF-α, IFN-γ and iNOS) and increasing anti-inflammatory gene expression (IL-10, bFGF, TGF-α and Bcl-2) (Tögel *et al.*, 2005). Similar effects have been observed in chemically damaged corneas with infiltration of inflammatory $CD4^+$ cells reduced 3 weeks post-injury (Oh *et al.*, 2008). Whether HSCs impact the degree of inflammatory cell recruitment within the liver and share similar paracrine functions as their mesenchymal counterparts is not known.

It is likely that any anti-inflammatory effects would be proportional to the degree of HSC recruitment. Therefore, an important consideration when designing clinical trials is the route of delivery of these rare cells. Exogenous myoblasts administered following myocardial infarct show differential effects dependant upon the route of injection (Fukushima *et al.*, 2008). Injection of isolated liver sinusoidal endothelial cells (LSEC) via the portal vein increases their homing to the liver when compared to intrasplenic and intravenous introduction (Benten *et al.*, 2005). Given the overall rarity of HSCs, it is crucial that these cells are introduced into the circulation by routes which optimise their hepatic recruitment. Indeed, in this thesis, despite one million HPC-7 cells being administered systemically via the carotid artery, only small proportions were actually identified within the liver. Extra-hepatic homing of injected HSCs is certainly a significant barrier to

successful cellular therapy. Therefore, this chapter will also identify these extra-hepatic sites of recruitment.

The use of exogenously administered adult stem cells has gained clinical and experimental interest. However, endogenous HSC mobilisation from the BM and subsequent recruitment to sites of injury may also confer benefit. Many studies have shown elevated HSCs in the peripheral circulation during various models of human disease (Kollet *et al.*, 2003, Massa *et al.,* 2005, Lemoli *et al.*, 2006). Whether this has a role to play in liver regeneration is unclear. However, some studies have found that the contribution of mobilized progenitor cells to liver regeneration is negligible (Liu *et al.*, 2006). In our IR injury model, it is not known whether liver damage mobilises stem cells into the peripheral circulation and this will therefore be investigated.

Circulating neutrophils are recruited to sites of inflammation by adhesion to platelet thrombi or platelet microparticles via P-selectin (Palabrica *et al.*, 1992, Kirchhofer *et al.*, 1997, Forlow *et al.*, 2000). Recent studies have suggested that stem cells are also guided to sites of injury by platelets that release cytokines and also provide a surface for adhesion (Massberg *et al.*, 2006). Endothelial progenitor cells (EPCs) adhere to immobilized platelets *in vitro* via PSGL-1 and CD49d dependent mechanisms and subsequently drive differentiation of EPCs and $CD34^+$ cells into endothelial cells (Langer *et al.*, 2006, Stellos *et al.*, 2008). Whether the HPC-7s used in this thesis can bind to platelets adherent on endothelial cells will also be investigated *in vitro.*

6.1.2. Hypotheses

For the work contained in this chapter, we hypothesized:

1. Introducing HPC-7 cells via the hepatic portal vein would increase their recruitment
2. Hepatic recruitment of HPC-7s would reduce leukocyte recruitment and this effect would be greater when cells were introduced via the portal vein
3. Significant numbers of HPC-7 cells home to sites other than the liver
4. HSCs are mobilised into the peripheral circulation as a result of hepatic IR injury
5. Platelets can promote HPC-7 adhesion to TNFα activated endothelium *in vitro*

6.2. Methods

Methods used in this chapter are described in detail in Chapter 2. Briefly, cell recruitment following 1 x 10^6 CFSE-labelled HPC-7 administration via the carotid artery and hepatic portal vein was compared intravitally. To determine any anti-inflammatory effects, 1 x 10^6 or 5 x 10^6 unlabelled HPC-7 cells were introduced in IR injured animals via the portal vein or carotid artery respectively at 1 minute reperfusion. Reperfusion was continued for 2.5 hours at which point 5 x 10^5 CFSE-labelled donor leukocytes were administered via the same entry route as the HPC-7 cells. After a further 15 minutes, the injured (left) and uninjured (right) liver, kidney, spleen and lungs were isolated and examined for labelled exogenous leukocyte recruitment microscopically (see Figure 6.1). Extra-hepatic homing of HPC-5 and HPC-7 cells into these organs was also determined by examining 5 surface fields of view microscopically. Data represents mean ± SEM of three separate experiments. To

investigate whether ischemia promotes progenitor cell mobilisation, animals were subjected to IR injury and peripheral blood analysed by FACS for circulating progenitor cells defined as $Lin^{-}Sca1^{+}$.

A static adhesion assay was used to investigate platelet-HPC-7 interactions. MuCEC-1s, cultured to confluence on APES-coated coverslips, were treated with 10ng/ml recombinant murine TNFα for 4 hours. Although TNFα alone does not promote human platelet adhesion to HUVEC (Tull *et al.*, 2006), work in our lab (Holyer, I*.,* unpublished observations) and others (Lou *et al.*, 1997) shows treatment of MuCEC-1 with murine TNFα does support platelet adhesion. Endothelium was incubated with 2 x 10^8 platelets or PBS vehicle for 45 minutes. Unbound platelets were washed away and 5 x 10^5 CFSE-labelled HPC-7 cells were incubated with endothelium for 5 minutes. Unbound HPC-7 cells were washed away, coverslips fixed with 2% glutaraldehyde and ten fields counted for adhesive cells. Data represents mean adherent cells per field ± SEM of 5 separate experiments.

6.3. Results

6.3.1. HPC-5 and HPC-7 home primarily to the lungs following systemic injection

Similar to the intravital results presented in Chapter 3, HPC-7 and HPC-5 recruitment was enhanced in the injured liver lobe subjected to IR injury compared to sham (injured liver lobe, HPC-7: sham: 1.35 ± 0.13 vs. IR: 7.87 ± 1.57; $P < 0.05$; HPC-5: sham: 2.40 ± 0.64 vs. IR: 8.20 ± 1.40; $P < 0.05$) *(figure 6.2.A-B)*.

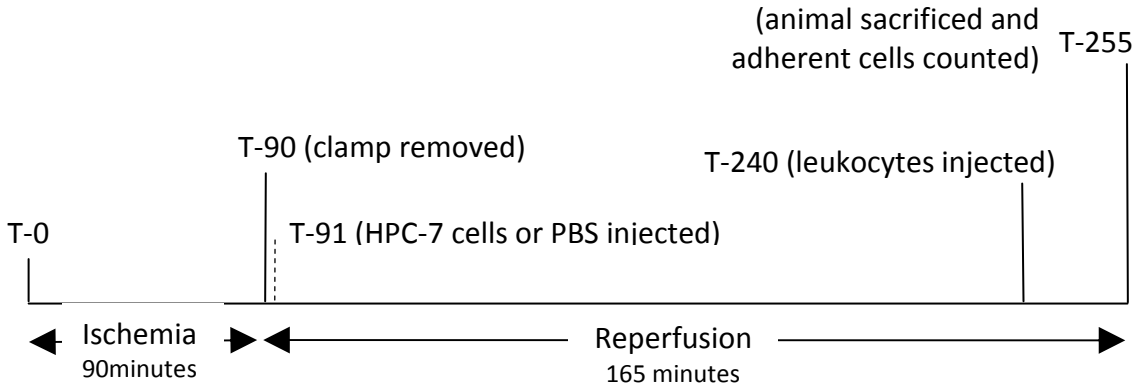


Figure 6.1. *Protocol used for anti-inflammatory studies.* HPC-7 cells were introduced following hepatic IR and their effect on the adhesion of exogenously administered leukocytes examined. T-0 represents 0 minutes post initiation of ischemia, while T-90 represents 90 minutes post initiation of ischemia. At T-91 either 1 x 10^6 or 5 x 10^6 HPC-7 cells were administered intra-portally or intra-arterially, respectively. CFSE labelled leukocytes were administered at T-240 and tissues isolated 15 minutes later for quantitation of leukocyte adhesion.

Although there was also an increase in HPC-7 and HPC-5 recruitment in the non-injured hepatic lobe, this difference was only significant for HPC-5 cells (non-injured liver lobe, HPC-7: sham: 2.00 ± 0.20 vs. IR: 6.27 ± 2.89; $P > 0.05$; HPC-5: sham: 3.66 ± 0.24 vs IR. 7.53 ± 0.71; $P < 0.01$) *(figure 6.2.A-B)*. HPC-5 and HPC-7 recruitment to the renal microvasculature was negligible and the low levels observed were not affected by injury (kidney: HPC-7: sham: 0.20 ± 0.20 vs. IR: 1.73 ± 0.81; $P > 0.05$; HPC-5: sham: 1.2 ± 0.2 vs. IR: 1.46 ± 0.27; $P > 0.05$) *(figure 6.2.A-B)*. Significant adhesion of HPC-5 and HPC-7 cells were observed in the spleen, although recruitment in this organ was not modified by hepatic injury (spleen: HPC-7: sham: 10.93 ± 1.27 vs. IR: 14.4 ± 7.67; $P > 0.05$; HPC-5: sham: 21.47 ± 6.29 vs. IR: 25.67 ± 12.88; $P > 0.05$) *(figure 6.2.A-B)*. The majority of adherent cells were visible in the pulmonary circulation, with a tendency for increased HPC-7 adhesion in IR treated animals (lungs: HPC-7: sham: 35.87 ± 8.41 vs. IR: 59.47 ± 6.35; $P > 0.05$; HPC-5: sham: 63.40 ± 12.42 vs. IR: 69.33 ± 18.10; $P > 0.05$) *(figure 6.2.A-B).*

6.3.2. Administration of HPC-7 cells via the carotid artery following ischemia does not reduce subsequent leukocyte recruitment

Intra-arterial administration of 5 x 10^6 HPC-7 during early reperfusion did not significantly reduce exogenous leukocyte recruitment to the injured liver lobe compared to controls (saline: 2.50 ± 1.30 vs. HPC-7: 3.00 ± 1.00; $P > 0.05$) *(figure 6.3A & figure 6.3B)*. HPC-7 cells also failed to reduce leukocyte adhesion in the non-injured liver lobe (saline: 3.70 ± 0.00 vs. HPC-7: 3.85 ± 0.85; $P > 0.05$), kidney (saline: 1.35 ± 0.45 vs. HPC-7: 1.85 ± 0.25; $P > 0.05$) or spleen (saline: 12.15 ± 1.45 vs. HPC-7: 12.35 ± 1.95; $P > 0.05$). In fact, pre-HPC-7

administration significantly enhanced recruitment of leukocytes to the lung (saline: 63.65 ± 6.35 vs. HPC-7: 77.5 ± 2.20; P < 0.05) *(figure 6.3A & figure 6.3B)*

6.3.3. Administration of HPC-7 cells via the portal vein enhances recruitment, but injury specific recruitment is lost

Introduction of HPC-7 cells via the hepatic portal vein significantly enhances recruitment seen intravitally at 75 minutes reperfusion by over five-fold (adherent cells at 75 minutes reperfusion: carotid artery: 10.50 ± 1.09 vs. portal vein: 51.33 ± 15.72; P<0.001) *(figure 6.4.A)*. However, no difference in recruitment is seen between sham and IR animals if cells are introduced through the portal vein at 75 minutes reperfusion and the equivalent sham time point (adherent cells, portal vein injection: 165 minutes sham: 54.67 ± 22.18 vs. 75 minutes reperfusion: 51.33 ± 15.72; P > 0.05) *(figure 6.4.B)*.

6.3.4. Administration of HPC-7 cells via the portal vein following ischemia does not reduce subsequent leukocyte recruitment

Intra-portal administration of 1 x 10^6 HPC-7 during early reperfusion did not significantly reduce exogenous leukocyte recruitment in the injured liver lobe compared to controls (saline: 5.23 ± 0.24 vs. HPC-7: 6.53 ± 2.78; P > 0.05) *(figure 6.5.A & figure 6.5.B).* HPC-7 cells also failed to reduce leukocyte adhesion in the non-injured liver lobe (saline: 6.27 ± 1.59 vs. HPC-7: 7.00 ± 3.08; P > 0.05), kidney (saline: 0.63 ± 0.35 vs. HPC-7: 0.67 ± 0.15; P > 0.05), spleen (saline: 4.70 ± 1.38 vs. HPC-7: 5.47 ± 2.77; P > 0.05) and lung (saline: 33.00 ± 6.22 vs. HPC-7: 24.77 ± 3.23; P > 0.05) *(figure 6.5.A & figure 6.5.B).*

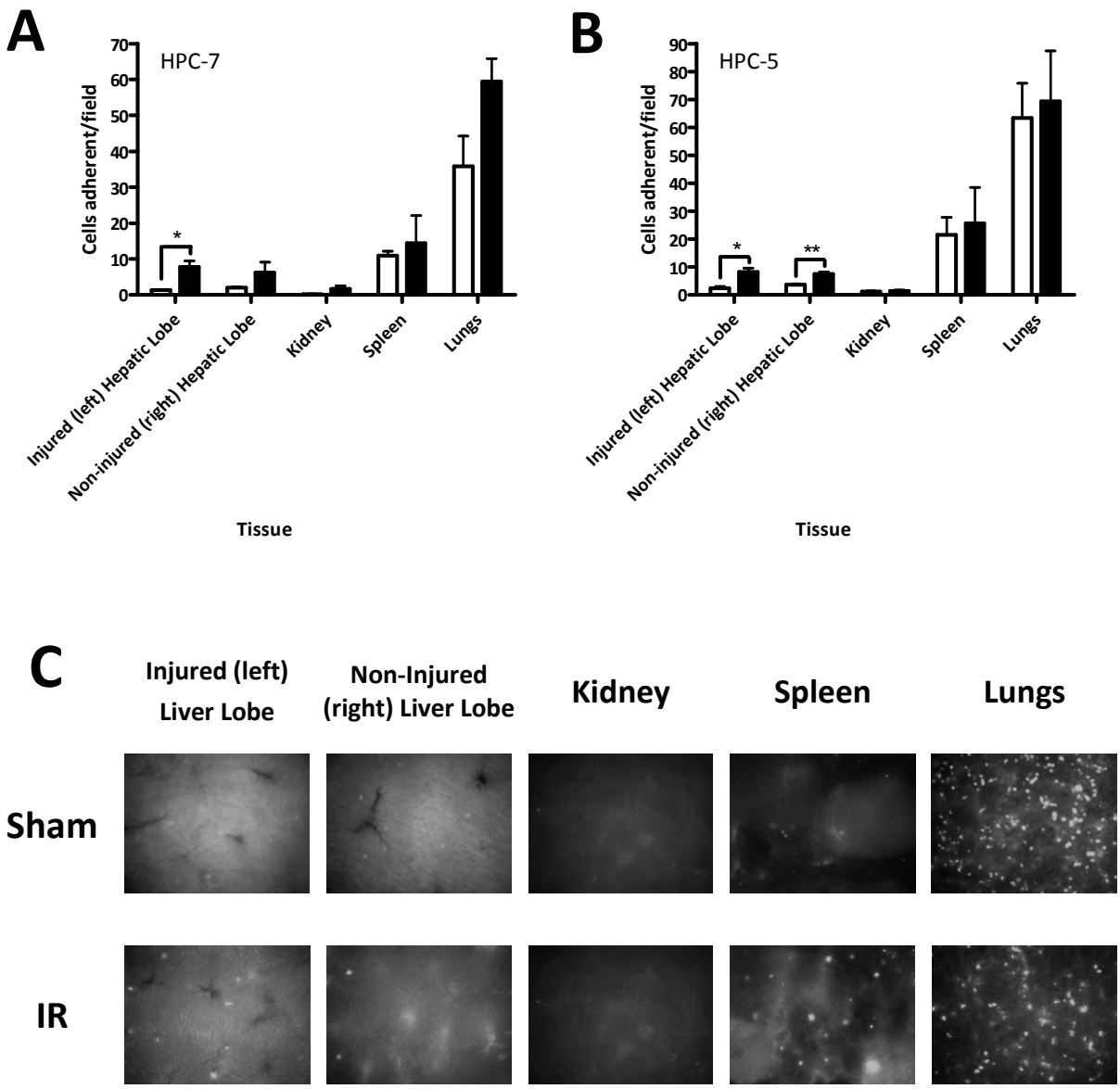


Figure 6.2. *HPC-7 and HPC-5 cells home primarily to the lungs and spleen.* Animals were analysed for the tissue homing characteristics of HPC-5/-7 following sham and IR injury. Following administration of cells (1 x 10^6 HPC-5/-7, 5µM CFSE, I.A.) into sham and IR treated animals (90 PHI/30), cells were allowed to circulate for a further 1 hour. Subsequently, tissues were harvested and analysed by fluorescence microscopy. HPC-7 **(panel A;** clear: sham, fill: IR**)** and HPC-5 **(panel B;** clear: sham, fill: IR**)** cells home primarily to the lungs and spleen. No renal homing of HPC-7 or HPC-5 was observed. Significantly raised HPC-7 adhesion was seen in the Left Hepatic Lobe following IR injury **(panel A)**. Significantly raised HPC-5 adhesion was seen in the Left and Right Hepatic Lobes following IR injury **(panel B).** Representative images of HPC-7 adhesion in the various tissues is shown **(panel C)**. Panels A and B are representative of mean adhesion ± SEM of 3 separate experiments. * $p < 0.05$, ** $p < 0.01$ (unpaired t-test).

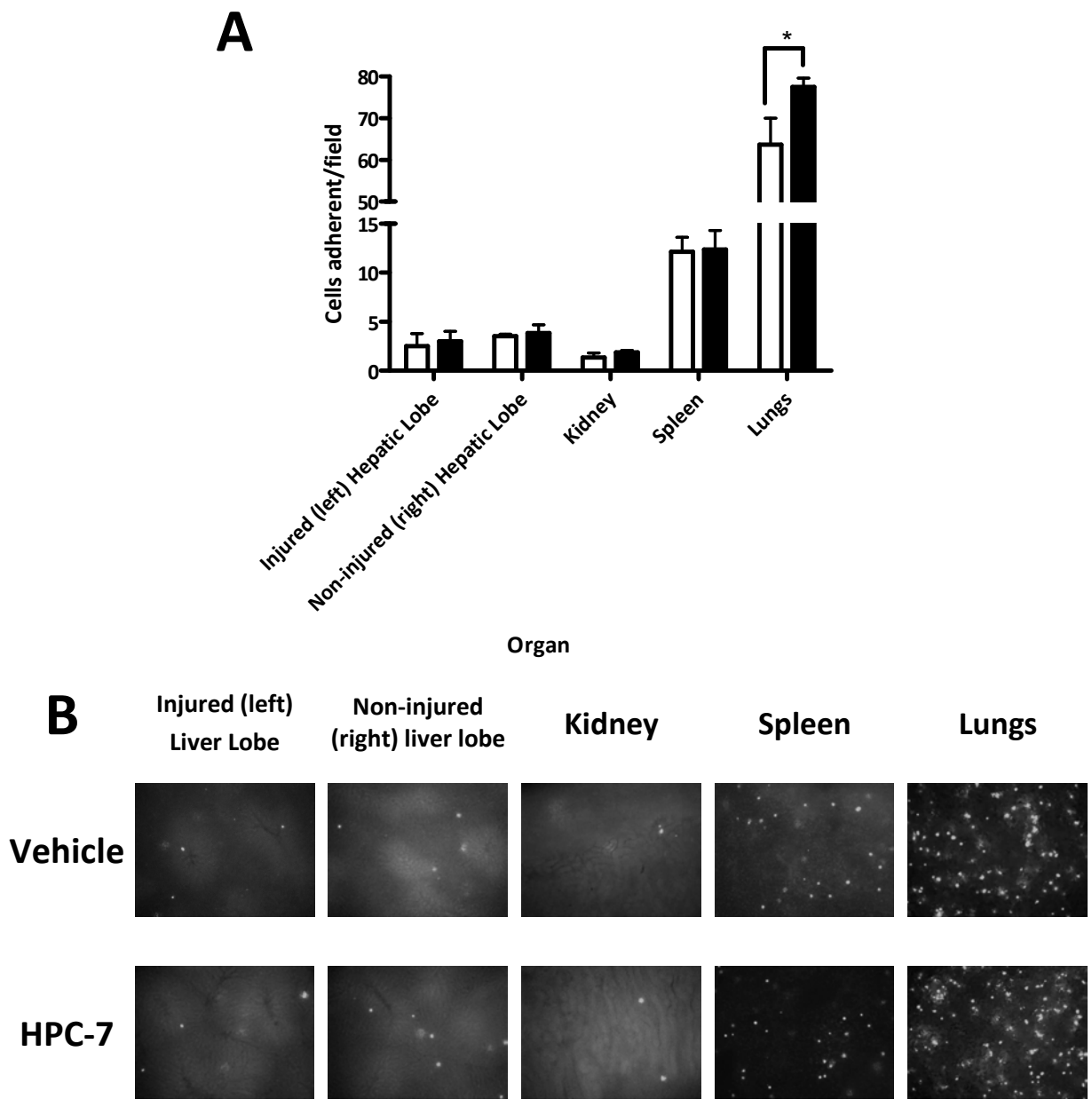


Figure 6.3. *Administration of HPC-7 cells via the carotid artery following ischemia does not reduce exogenous leukocyte recruitment during reperfusion.* WT animals were subjected to IR injury (90 minutes), and either 5 x 10^6 HPC-7 (in 100µl saline) or saline alone were introduced to the circulation through the carotid artery at 1 minute reperfusion. At 2.5 hours reperfusion, 5 x 10^5 labeled leukocytes (5µM CFSE) were introduced through the carotid artery. Cells were allowed to circulate for 15 minutes, and tissues were isolated. Administration of HPC-7 cells does not reduce leukocyte recruitment in any organs, actually resulting in an increase in leukocyte recruitment to the lungs **(panel A**; clear: saline, filled: HPC-7**)**. Example images are shown **(panel B).** Results are representative of mean ± SEM of 2 separate experiments; * $p < 0.05$ (two-way ANOVA followed by Bonferroni posttests).

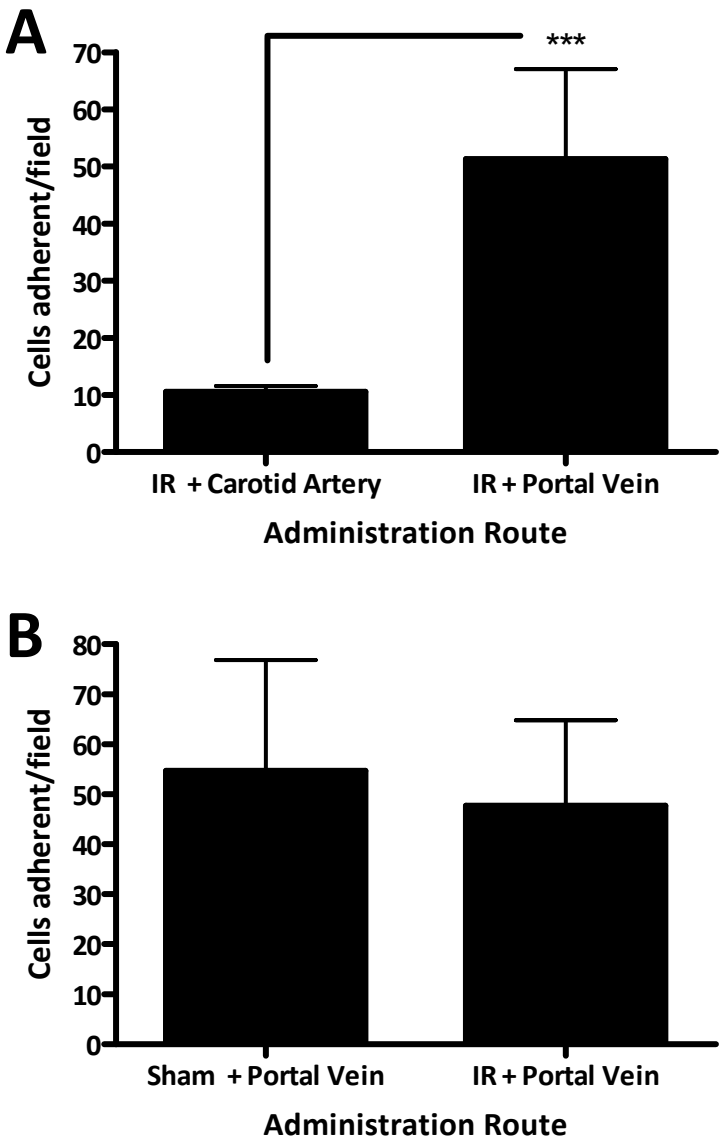


Figure 6.4. *Administration of HPC-7 cells through the hepatic portal vein significantly increases their recruitment to the liver, but abolishes the specific injury mediated recruitment.* HPC-7 cells were introduced into the circulation via the portal vein and recruitment at 75 minutes was compared to cells introduced through the carotid artery. Administration of HPC-7 cells through the portal vein significantly enhances their recruitment during IR injury compared to carotid artery introduction **(panel A).** However, no difference in recruitment is seen between sham and IR animals if cells are introduced through the portal vein **(panel B)**. Figures are representative of mean ± SEM of at least 3 separate experiments (n = 3-10); *** $p < 0.001$ (unpaired t-test)

6.3.5. HSCs are not mobilized acutely as a result of IR injury

Previous evidence suggests that HSC can be mobilized into the peripheral circulation following hepatic IR injury (Liu et al., 2006). To investigate this, peripheral blood was anlaysed by FACS following either partial hepatic IR or sham surgery. Cells were identified based on the lack or presence of lineage markers (Lin) and the lack or presence of Sca-1. Although there is a general decrease in circulating $Lin^-Sca\text{-}1^+$ cells (HSCs), the differences are not significant ($Lin^-Sca\text{-}1^+$: sham: 2.11% ± 1.35% vs. 0.59% ± 0.22; $P > 0.05$) *(Figure 6.6A)*. Similarily, although there is a general decrease in circulating cells negative for both lineage markers and Sca-1, this decrease is not significant ($Lin^-Sca\text{-}1^-$: sham: 2.39% ± 0.35% vs. IR: 1.56% ± 0.06%; $P > 0.05$) *(Figure 6.6A)*. On the other hand, IR significantly raises the levels of circulating lineage positive/Sca-1 negative cells, a population which includes neutrophils ($Lin^+Sca\text{-}1^-$ cells: sham: 35.78% ± 4.17% vs IR: 67.03% ± 2.44%; $P < 0.001$) *(Figure 6.6A)*. Consistent with FACS results, blood testing of IR animals revealed an increase in circulating neutrophils when compared to sham treated animals (data not shown). The increase in circulating $Lin^+Sca\text{-}1^-$ cells is accompanied by a concomitant decrease in cells positive for both lineage markers and Sca-1, a population which includes lymphocytes ($Lin^+Sca\text{-}1^+$: sham: 59.72% ± 5.57% vs. IR: 30.82% ± 2.43%; $P < 0.001$) *(figure 6.6.A)*.

6.3.6. Adherent platelets do not promote HSC adhesion *in vitro*

Adherence of platelets to endothelium was confirmed visually (data not shown). The presence of platelets on endothelial cells did not significantly alter HPC-7 adhesion compared to endothelial cells without platelets (HPC-7 adhesion: without platelets: 131.80 ± 16.44 vs. with platelets: 149.40 ± 10.43; $P > 0.05$) *(figure 6.7.A).*

6.4. Discussion

Recruitment of exogenously administered labelled donor leukocytes were used as a surrogate marker to monitor anti-inflammatory effects of HPC-7s. However, no differences in leukocyte recruitment were observed at the time points investigated. This was despite attempts to enhance hepatic HPC-7 recruitment by introducing high numbers of HPC-7 cells via the carotid artery or portal vein.

Furthermore, no mobilisation of $Lin^{-}Sca\text{-}1^{+}$ cells was identified within the peripheral blood in this acute injury model. Data from the *in vitro* adhesion assays also suggests that HPC-7s may not be recruited to sites of injury via interactions with platelets.

Recent studies suggest HSCs may exert their beneficial effects through paracrine anti-inflammatory mechanisms rather than direct cellular replenishment/repair (Humphreys and Bonventre, 2008, Duffield *et al.*, 2005, Tögel *et al.*, 2005). Fetal liver derived HSCs have been shown to reduce inflammatory injury observed during a paw edema model (Biziuleviciene *et al.*, 2007). However, in the current study, injection of HPC-7 s did not reduce leukocyte recruitment post IR injury. HPC-7s were administered as soon as possible after reperfusion. Thus in our systems HSCs do not inhibit leukocyte recruitment although they could mediate other anti-inflammatory processes. Inflammation of the liver causes injury and an elevation in circulating ALT from necrotic and injured hepatocytes. Even if HSCs are anti-inflammatory, it is unlikely that detection of circulating ALT levels would be

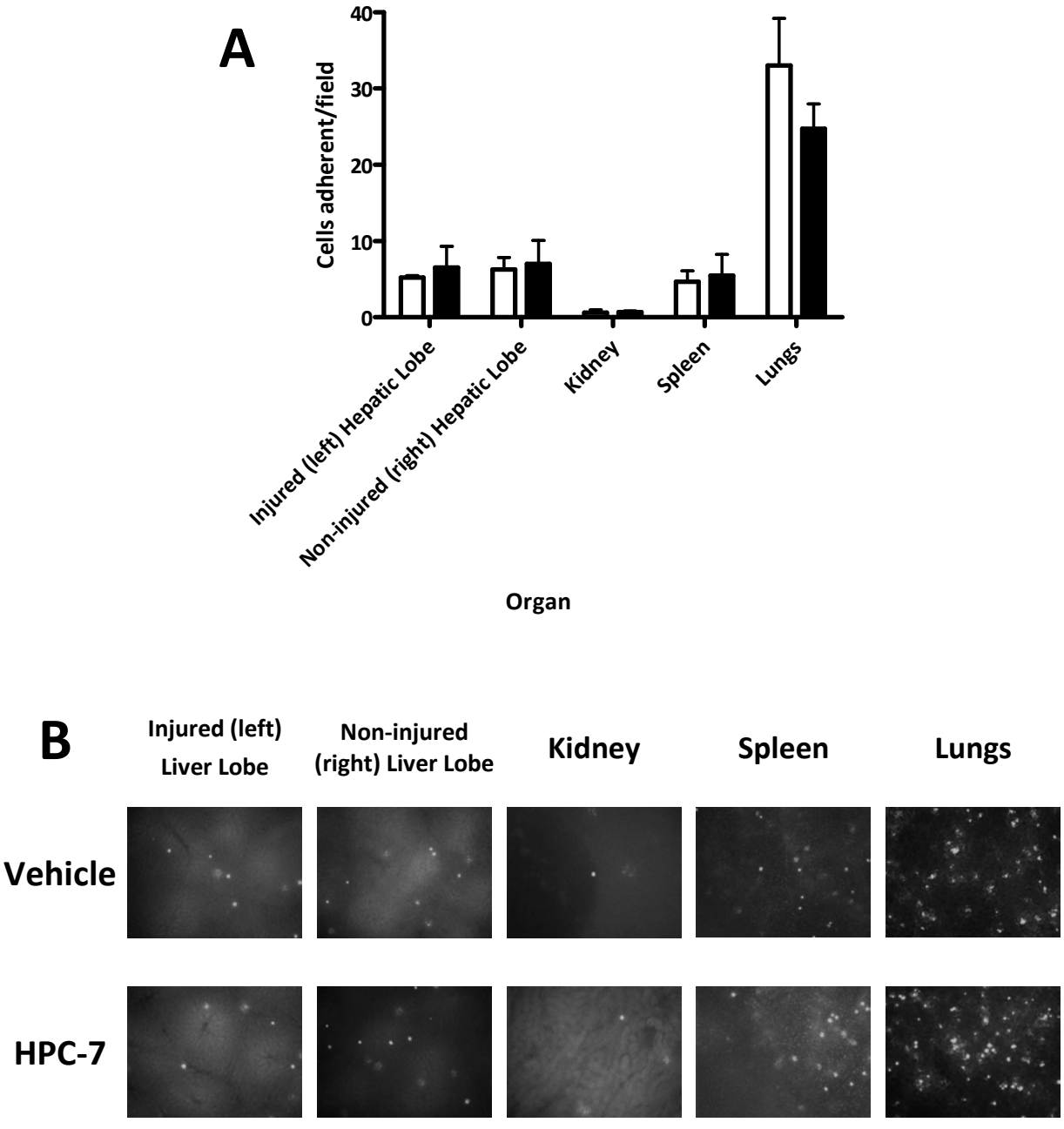


Figure 6.5. *Administration of HPC-7 cells via the portal vein following ischemia does not reduce leukocyte recruitment during reperfusion.* WT animals were subjected to IR injury (90 minutes) and either 1 x 10^6 HPC-7 (in 100µl saline) or saline alone were introduced to the circulation through the portal vein at 1 minute reperfusion. At 2.5 hours reperfusion, 5 x 10^5 labeled leukocytes (5µM CFSE) were also introduced through the portal vein. Cells were allowed to circulate for 15 minutes, and tissues were isolated. Administration of HPC-7 cells does not reduce leukocyte recruitment during IR in any organs when compared to saline vehicle alone **(panel A;** clear: saline, filled: HPC-7**)**. Example images are shown **(panel B).** Results are representative of mean ± SEM of 3 separate experiments; (two-way ANOVA).

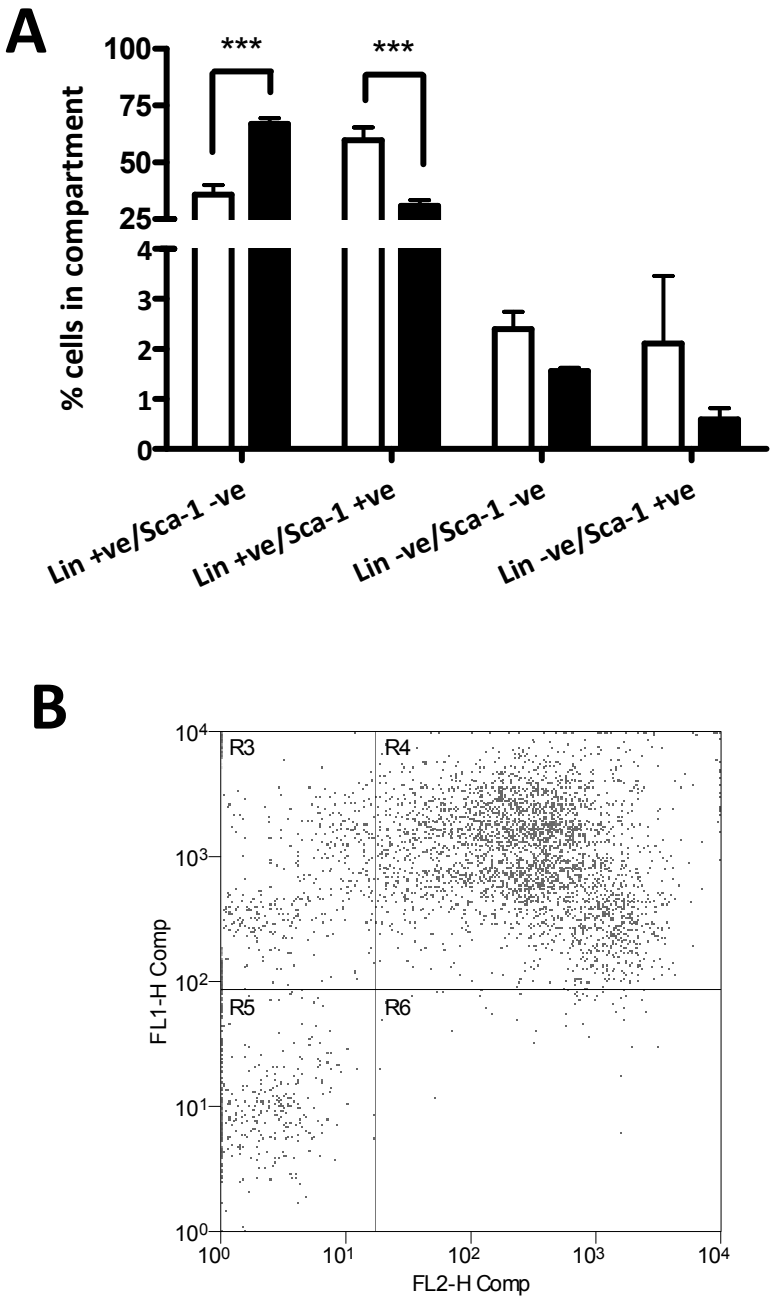


Figure 6.6. *HSCs, defined as Lin$^-$/Sca-1$^+$, are not mobilized acutely as a result of partial hepatic ischemia reperfusion injury.* Animals were subjected to partial hepatic IR injury (90 PHI/180 R) or sham treatment, and circulating peripheral blood cells analysed. As a result of injury, circulating Lin$^+$/Sca-1$^-$ are increased, while circulating Lin$^+$/Sca-1$^+$ cells decrease **(panel A;** clear: sham, fill: IR**).** Representative FACS plot is shown, R6 representing Lin$^-$/Sca-1$^+$ cells **(panel B**; FL1: Lin, FL2: Sca-1**).** Figures represent mean ± SEM of three separate experiments; * $p < 0.05$ (two-way ANOVA, followed by Bonferroni posttests).

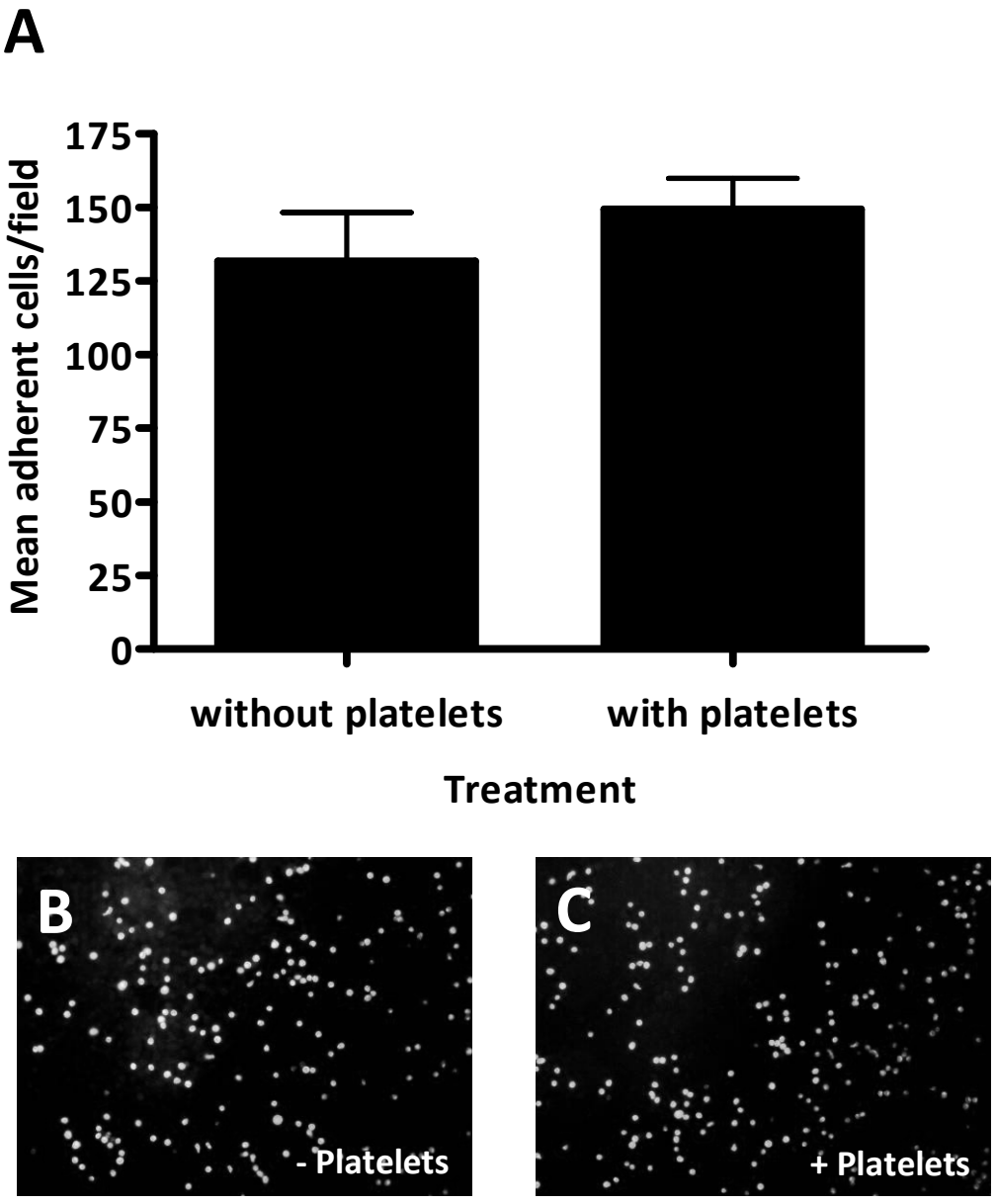


Figure 6.7. *Presence of adherent platelets does not promote HSC adhesion in vitro using a static adhesion assay.* Endothelial monolayers were treated with TNFα and platelets or PBS was subsequently added. HPC-7 were subsequently exposed to treated endothelium and adhesion analyzed. No significant difference in HPC-7 adhesion is seen between endothelium exposed to platelets and endothelium exposed to PBS **(panel A)**. Representative images are shown of HPC-7 adhesion after endothelium has been treated with PBS **(panel B)** or platelets **(panel C).** Bars represent mean adhesion per field ± SEM of five separate experiments.

sensitive enough to detect any HSC anti-inflammatory activity. In order to monitor subsequent leukocyte infiltration, labelled leukocytes derived from donor mice were introduced and their recruitment quantified. This approach of administering exogenous labelled leukocytes was used instead of injecting the fluorescent label (CFSE) systemically as this would have indiscriminately labelled both HPC-7's and endogenous mature leukocytes. Therefore, it is possible that recruitment of *endogenous* inflammatory cells was inhibited but our methodological approach did not allow us to quantitate this.

It is also possible that HSCs need a much longer duration to confer an anti-inflammatory effect and this warrants further investigation. Interestingly, MSCs can downregulate inflammatory gene expression, upregulate pro-inflammatory gene expression and subsequently protect against ischemic injury within 2 hours (Togel *et al.*, 2005). Whether HSCs can regulate protective or inflammatory gene expression is not known. Overall, our data appears to be consistent with the general finding that the stem cell compartment responsible for paracrine anti-inflammatory actions are most likely the MSCs (Gnecchi *et al.*, 2005, Humphreys and Bonventre, 2008, Tögel *et al.*, 2005).

The inability of HSCs to reduce leukocyte recruitment could be a result of sub-therapeutic HSC recruitment. In order to achieve optimal anti-inflammatory potential, cells were introduced into the liver via the hepatic portal vein which resulted in significantly more hepatic recruitment. Since cells introduced via the carotid artery pass through the lungs, the evasion of the pulmonary circulation may explain the increased adhesion seen with intraportal injection. However, selective recruitment of HSCs is lost when high cell doses

are introduced intraportally and may be due to masking of the selective recruitment component seen following carotid injection. Alternatively, the levels of adhesion observed following portal vein injection may represent the upper limit of hepatic HSC recruitment which cannot be exceeded even in the presence of injury.

Intra-arterial injection of stem cells is one of the common routes used in several clinical settings. However, this route of HSC delivery to mice after hepatic IR injury is limited by entrapment of donor cells predominantly in the lungs. Indeed, adhesion of both HPC-5 and HPC-7 in the pulmonary circulation is over five times that seen in the liver. This phenomenon has also been observed for activated peripheral blood neutrophils and monocytes with recruitment mediated by adhesion molecules (Aoki *et al.*, 1997, Aldonyte *et al.*, 2003). It is therefore possible that HSCs are also activated in the peripheral circulation which would then promote their recruitment within the pulmonary vasculature. In addition, recruitment of mature leukocytes into the pulmonary vasculature has been attributed to their reduced deformability. The capillaries of the pulmonary circulation are relatively small in diameter (approximately 5µm) and circulating cells are initially trapped due to their size. For instance, the average leukocyte diameter is 8.5µm (Ting-Beall et al., 1993). Interestingly, HSCs are similar in size to leukocytes with diameters between 6-10µm (Zuba-Surma et al., 2008). Subsequent to this initial entrapment, blood flow causes entrapped leukocytes to change shape which facilitates their further transit through the pulmonary vasculature (Gebb *et al.*, 1995). Indeed, stiffening due to the intracellular formation of filamentous actin has been implicated in leukocyte recruitment to the lung (Doerschuk, 2001). Very little work has carefully examined the physical characteristics of

HSCs and so currently the mechanism underlying pulmonary recruitment is unclear and certainly warranting of further studies.

During embryonic and fetal development, haematopoiesis occurs in a number of different anatomical sites including the fetal liver, yolk sac and spleen. It is therefore unsurprising that a significant number of HSCs are also found adherent in the spleen following systemic injection. The spleen has been shown to contain a reserve of HSCs (Kodama *et al.*, 2005), so recruitment of exogenously administered HSCs may represent a physiological replenishment mechanism.

In contrast, relatively little HSC recruitment is seen in the renal microcirculation in either basal or hepatic IR treated animals. Renal recruitment has been observed following BM transplant both in the absence (Forbes *et al.*, 2002) and presence (Lin *et al.*, 2003) of injury, although others have shown no renal engraftment of purified HSCs and no subsequent contribution to regeneration (Krause *et al.*, 2001). Whether HSCs are recruited to the renal microvasculature remains unclear, but the results from this study suggest they are not.

Interestingly, adhesion in the non-ischemic right hepatic lobe was also raised and significantly so for HPC-5s. It is well documented that during various unilobular hepatic insults, the non-ischemic lobes also suffer some degree of injury (Singh *et al.*, 1999, Nakamitsu *et al.*, 2001, Yoshida *et al.*, 2003). For instance, following partial hepatic IR, P-selectin is upregulated to the same extent in both the ischemic and non-ischemic lobes

(Singh *et al.,* 1999). This may explain the similarly raised HSC adhesion in the both the ischemic and non-ischemic liver lobes.

HSC mobilization frequently occurs as a result of peripheral injury, driven by factors such as CXCL12, MMP-9 and GM-CSF (Dalakas *et al.,* 2005, Frossard *et al.*, 2002). For example, during renal IR injury, GM-CSF is released from cells in the outer medulla of the kidney (Lentsch *et al.*, 1998, Zhang *et al.,* 2004). Following hepatic resection, HSCs in the peripheral circulation are raised (De Silvestro *et al.*, 2004). Furthermore, HSC mobilization occurs as a result of reperfusion injury during orthotopic liver transplantation (Lemoli *et al.*, 2006). Therapeutic interventions could therefore focus around augmenting this physiological response to injury.

However, in this study, HSC mobilization did not occur as a result of partial hepatic IR injury. In fact, the levels of circulating $Lin^{-}Sca\text{-}1^{+}$ cells appear to fall as a result of IR injury which could represent recruitment of these cells to the injured organ. If this is the case, then HSC mobilization could potentially be masked by the recruitment of these cells to the injured organ. Following IR injury, raised levels of $Lin^{+}Sca\text{-}1^{-}$ cells are seen in the circulation. This may represent raised circulating granulocytes, as these cells have been shown to be Sca-1 negative (Vasconcelos *et al.*, 2006). This is accompanied by a significant decrease in circulating $Lin^{+}Sca\text{-}1^{+}$ cells. These cells may represent circulating lymphocytes (Spangrude *et al.*, 1988, Stanford *et al.*, 1997). These results are in line with blood sampling results from Chapter 3 where increased circulating neutrophils are accompanied by a decrease in circulating lymphocytes.

Recent evidence suggests HSC adhesion to injured endothelium is mediated via platelets, with platelets providing a bridge between the HSC and the endothelium (Massberg *et al.*, 2006). It has additionally been shown that activated platelets, along with fibrin, guide $CD34^+$ cells to sites of vascular injury and promote their differentiation into endothelial cells (de Boer *et al.*, 2006). Interestingly, platelets express and secrete CXCL12 (Stellos *et al.*, 2008), a cytokine shown to promote adhesion of HPC-7 cells to endothelium. However, incubation of endothelium with platelets prior to HPC-7 exposure did not increase HPC-7 adhesion. The model used in this study is devoid of flow, which may partly explain the inability of platelets to promote HSC adhesion. Shear stress activates numerous intracellular pathways within platelets including Protein Kinase C and those downstream of platelet integrins (Feng *et al.*, 2006, Kroll *et al.*, 1993). The absence of shear stress in our model may lead to inadequate platelet activation and subsequent HSC recruitment. Furthermore, it is well established that activated platelets adherent to endothelium are able to promote cell recruitment by providing a P-Selectin rich rolling substrate (de Boer *et al.*, 2006). In the studies by de Boer *et al,* human $CD34^+$ cells initially roll on platelet thrombi and subsequently adhere to endothelium downstream of the original thrombus (de Boer *et al.*, 2006). It is likely that the absence of flow and the subsequent lack of platelet thrombi may prevent HSC recruitment by adherent platelets in the static assays used in these studies.

That said, studies from our lab have demonstrated that HPC-7s can indeed interact with platelets *in vivo*. Laser induced injury was utilised to denude endothelium in a spatially and temporally localised area within a cremaster muscle arteriole (Kalia *et al.*, 2008). This led to

the formation of a thrombus which regressed with time to leave a mural thrombus on the surface of the endothelium. HPC-7 cells subsequently injected into the animal, formed firm adhesive binds within the area proximal to the mural platelet thrombus (*figure 6.7* – courtesy of Dr Kalia).

To summarise, we have demonstrated that HPC-7 cells do not prevent exogenous leukocyte recruitment within the time frame of our experimental protocol despite intraportal delivery of HSCs which enhances their recruitment. Perhaps more cells could be recruited if protocols could be identified that overcome the barrier of cells becoming entrapped within the lungs. Therefore, it is possible that HSCs confer benefit primarily via fusion or differentiation dependent mechanisms (or some as yet unidentified mechanism). No mobilisation of endogenous HSCs was observed but may be due to the fact that an acute model of injury is utilised. This is in contrast to the mobilisation observed when hepatic injury is chronic or more severe. Whether the recruitment of HSCs is dependent upon interactions with platelets certainly warrants further investigation using different assays, particularly in light of increasing recent evidence that platelets can play a major role in regeneration of injured liver (Myronovych *et al.*, 2008, Nocito *et al.*, 2007, Lesurtel *et al.*, 2006).

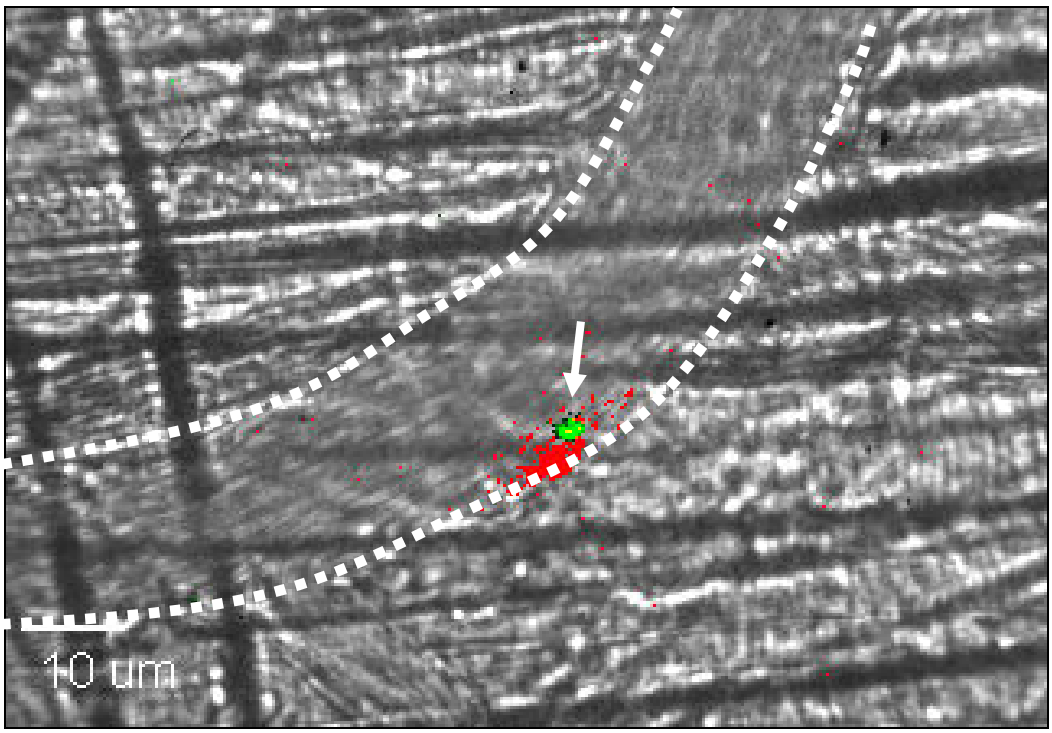


Figure 6.7. *HPC-7 cell firmly adherent to mural platelet thrombus*. In a laser model of endothelial injury in the mouse cremaster, HPC-7 cells adhere to residual mural thrombi. Endogenous platelets were labeled with Alexa 594 (red) and exogenous HPC-7 cells were labeled with CFSE (green). Photo courtesy of Dr Kalia, magnification x40.

Chapter 7

General Discussion

7. General Discussion

7.1. Summary of main findings and general discussion

HSC transplantation has been used routinely over the past 50 years for the treatment of various diseases such as cancers, numerous anemias and various inborn errors of metabolism (Copelan, 2006). The safety and efficacy of this procedure is well documented in both autologous and allogenic transplantation (Gatti *et al.*, 1968, Copelan, 2006). When the first studies suggested BM-derived cells could reconstitute injured hepatic tissue (Petersen *et al.*, 1999, Theise *et al.*, 2000b), the relatively routine nature of the technique fueled much hope in the field of regenerative medicine. Lagasse and colleagues then further demonstrated that HSCs were the major BM-derived stem cell to confer benefit within the damaged liver (Lagasse *et al.*, 2000). Furthermore, purified HSCs have also been demonstrated to be beneficial in a number of other injured organs including the regeneration of chemically injured skeletal muscle (Abedi *et al.*, 2007) and IR injured renal tubules (Lin *et al.*, 2003).

HSCs are rare cells constituting < 0.05% of the BM and this scarcity has hindered their clinical use. If HSCs do prove beneficial in the clinic then a better understanding of the molecular adhesive factors regulating engraftment at injured sites is essential in order to develop strategies to enhance recruitment. Therefore, the primary aim of this thesis was to elucidate the adhesion mechanisms involved in the recruitment of HSCs to the hepatic microvasculature. The major novel findings from this study include the following;

- Both adult and embryonic derived HSCs are recruited to the liver as a result of hepatic IR injury
- Primary isolates of Lin^- / $Sca\text{-}1^+$ cells are recruited to the liver both in the absence and presence of injury, but not enhanced during the latter
- The β_1 integrin VLA-4 appears to be the major contributor in securing the firm adhesion of HSCs to sinusoidal endothelium within the IR injured murine liver
- VCAM-1 is the counter-receptor in the liver that mediates HSC recruitment via VLA-4
- The β_2 integrin CD18, while important for neutrophil recruitment, plays no role in HSC recruitment to the IR injured murine liver
- The adhesion molecules CD31 and CD44 on HSCs play no role in HSC recruitment to the IR injured murine liver
- Knockout mice deficient in CD31 present with higher HSC recruitment than wild-types even without injury
- Treatment of HSCs with media conditioned by IR injured liver does not significantly raise adhesion molecule expression on HSCs, nor does treatment with IR conditioned plasma or individual cytokines – conversely, treatment with some cytokines reduces adhesion molecule expression
- Treatment of HSCs with CXCL12 and CXCL1 promotes their adhesion to endothelium *in vitro*, while TNFα and IL-1β do not. Blocking the CXCL12 receptor (CXCR4) on HSCs does not reduce *in vivo* IR mediated HSC recruitment
- Following system injection, significant numbers of HSCs traffic to the lungs and spleen as well as the liver

- Significantly more HSCs are recruited if they are delivered via the hepatic portal vein, but the specific injury mediated recruitment is lost

The studies contained within this thesis were performed using HPC-7 and HPC-5 cells. Both cell lines are derived from primary stem cells. HPC-5 and HPC-7 cells have been generated from murine adult BM HSCs and murine embryonic stem cells respectively (Pinto do Ó *et al.*, 1998, Pinto do Ó *et al.*, 2002, Pinto do Ó *et al.*, 2001). The cell lines have been generated by transfection with the LIM-homeobox gene Lhx2 which generates immortalized, SCF-dependent hematopoietic progenitor cell lines. Both these cell lines are similar to primary HSCs on the cellular, biochemical and molecular levels and are therefore excellent HSC surrogates (Richter *et al.*, 2006). Unfortunately, primary HSCs are rare, so isolation of a sufficient number for *in vivo* studies is difficult. Using these cells, this study has shown that both embryonic and adult HSC recruitment to the hepatic microvasculature is promoted by IR injury. The similar levels of recruitment seen between adult and embryonic HSCs may potentially negate the need to use the latter in clinical studies and thus avoid the ethical controversies surrounding their use (Towns and Jones, 2004).

In this study, flow cytometry revealed that both HPC-5 and HPC-7 cells express a large repertoire of adhesion molecules. There is much evidence corroborating the adhesion molecule expression profile detected on HPC-7 and HPC-5 with primary HSCs (Chan and Watt, 2001). Unfortunately we were unable to assess whether the expression levels of these adhesion molecules were comparable with those of primary HSCs. As isolation of primary HSCs requires multi-colour FACS techniques, it is difficult to then subsequently

re-probe the same cells for other markers. Antibody-mediated adhesion molecule blockade revealed that the recruitment of HSCs to the hepatic microvasculature following IR injury was dependent on VLA-4. Furthermore, intravital studies also revealed that blocking VCAM-1, the endothelial counter-receptor for VLA-4, significantly reduced HSC adhesion post IR injury. The VLA-4/VCAM-1 pathway is implicated in the homing of HSCs to the BM (Papayannopoulou *et al.*, 1995, Frenette *et al.*, 1998), and in leukocyte homing to the inflamed liver (Fogler *et al.*, 1996). It is therefore somewhat unsurprising that the VLA-4/VCAM-1 pathway plays such an important role in HSC recruitment to injured liver. VCAM-1 is expressed at low levels in the non-inflamed liver sinusoids, with upregulation following hepatic IR injury driven by NF-κB activation (Bell *et al.*, 1997). However, we were unable to detect any upregulation in VCAM-1 expression immunohistochemically following IR injury. Although this may be a genuine result, published literature suggests that the relatively low sensitivity of the immunostaining protocol most likely renders it unable to detect any upregulation that has occurred, especially if such upregulation is small (van Maanen *et al.*, 2004)

Alternatively, adhesion could be manipulated independent of VCAM-1 upregulation. Factors released from the injured hepatocytes or sinusoidal endothelial cells could themselves modulate integrin expression on the HSC (Montecucco *et al.*, 2008). This idea was further reinforced by the observation that media conditioned by IR injured hepatic tissue was able to elicit a small (but insignificant) upregulation of CD49d expression on HSCs, while not influencing expression of CD18 or CD44. Previous studies have shown that injured tissue may drive HSC to express hepatocyte differentiation markers such as CK18

and albumin rapidly, within 7 hours (Jang *et al.*, 2004). Interestingly, treatment of HSCs with cytokines or conditioned plasma failed to reproduce the mild upregulation seen with hepatic conditioned media. In fact, with certain cytokines CD49d expression actually decreased, suggesting a potential mechanism for removal of these cells from the site of injury (or even mobilisation from the BM). Furthermore, cytokine mediated CD49d downregulation is time dependent suggesting that downregulation of CD49d is progressive from the initial point of adhesion.

Alternatively, factors released from the injured organ itself could modulate integrin activation status (Peled *et al.*, 2000). Treatment of HPC-7 cells with CXCL12 or CXCL1 (murine IL-8 homologue) raised adhesion of HSCs on endothelium *in vitro*, independent of a significant effect on CD49d expression. However, blockade of CXCR4 on HPC-7 cells failed to significantly reduce adhesion *in vivo*, suggesting the presence of alternative compensatory pathways to offset the blockade of the CXCL12/CXCR4 pathway. The release of CXCL1 has been documented during IR injury (Lentsch *et al.*, 1998) and so makes this a potential factor that may modulate stem cell recruitment to injured sites in addition to CXCL12.

While both HPC-7 and HPC-5 express high levels of CD18 and CD44, neither play a role in HSC recruitment to the hepatic microvasculature *in vivo*. During hepatic IR injury, initial recruitment of inflammatory neutrophils to the injured organ is independent of CD18, which facilitates neutrophil adhesion to hepatocytes after endothelial integrity is lost (Kobayashi *et al.*, 2001). Although CD18 has been shown to be important in endothelial progenitor cell recruitment to the heart (Wu *et al.*, 2006), no studies have implicated CD18

in HSC recruitment. It is possible that during hepatic IR injury, CD18 is important in recruitment during the latter stages of injury rather than during the acute phase. Blockade of CD44 on HSC in the current study also had no effect on recruitment to IR injured liver. Contradictory data exists in the literature regarding the role of CD44 in HSC recruitment. Studies using anti-CD44 antibodies reveal impaired BM homing (Avigdor *et al.,* 2004) while studies using CD44 knock-out mice demonstrate no defects in recruitment (Oostendorp *et al.*, 2000).

The choice of injection route is likely to be critical to the efficacy of stem cell treatment. Clinically, BM derived cells for treatment of liver disease have been administered via the portal vein, hepatic artery and peripheral veins *(*Gordon *et al.*, 2006, Terai *et al.*, 2006). The results in this thesis suggest that the route of injection plays a crucial role in determining the level of hepatic HSC recruitment with more cells recruited to the sinusoids after administration via the portal vein compared to the carotid artery. Whether the level of recruitment is important in cellular therapy is unclear. In one study where patients showed some degree of functional hepatic improvement, between 1 x 10^6 and 2 x 10^8 cells were introduced suggesting, surprisingly, that the restoration in function is potentially unrelated to the magnitude of the cell dose (Gordon *et al.*, 2006). If the magnitude of recruitment is not important, then administration of cells via non-direct routes would potentially be sufficient for therapy. However, HSCs may play a protective role by releasing factors that promote hepatic regeneration though signals such as growth factors. In such circumstances, it is plausible to assume that increased recruitment may present greater benefit. However, progenitor cells are intricately linked to the formation of hepatic

tumours (Tang *et al.*, 2008) and therefore careful consideration must be taken to outweigh the potential negatives with clinical benefit. Unfortunately, results from this thesis were unable to display any anti-inflammatory effects of HSCs

The understanding gained on the adhesion pathways involved in HSC homing to sites of injury should prove invaluable in enhancing their recruitment. Indeed, methods for improving the trafficking and subsequent engraftment of HSCs, including other stem cells such as MSCs, are a high priority for cellular therapies (Karp and Leng Teo, 2009). Retrovirus vectors encoding homing receptors such as CXCR4 have been recently used to enhance homing of HSCs and MSCs through increasing their response to CXCL12 (Cheng *et al.*, 2008, Porecha *et al.*, 2006). Furthermore increasing the expression of the α4 subunit of VLA-4 on MSCs using adenovirus vectors led to a 10 fold increase in their homing to BM (Kumar and Ponnazhagan, 2007). An alternative approach would be to pre-treat cells prior to exogenous administration. Indeed, *ex vivo* treatment of HSCs with growth factors or cytokines significantly enhances their engraftment in their hematopoietic niches (Zanjani *et al.*, 1992, Chavakis *et al.*, In Press). Results from this thesis suggest that pre-stimulation of HSCs with CXCL12 or CXCL1 could promote recruitment of cells to injured organs and this will be investigated *in vivo* in ongoing studies within the Kalia lab. Given the degree of pulmonary HSC recruitment, it is critical to ensure that any *ex vivo* manipulations do not significantly promote pulmonary engraftment. Excessive pulmonary engraftment of HSCs following transplantation can cause respiratory failure and alveolar hemorrhage (Ho *et al.*, 2001).

The results of this thesis suggest a potential biphasic mechanism of HSC recruitment following injury (see figure 7.1). Initially, recruitment and activation of HSCs to the injured microvasculature is mediated by cytokines. CXCL12 is released during hepatic injury (Kollet *et al.*, 2003) and is likely to be an important player in this initial response. Other cytokines are likely to play a role in this initial step since blockade of CXCR4 does not prevent recruitment *in vivo* post IR injury. CXCL1 in particular may play a role in this step. Thereafter, integrins on the HSC surface are activated (Peled *et al.*, 2000). This activation of integrins, and more specifically VLA-4, promotes adhesion of HSCs to the hepatic endothelium via VCAM-1. Although changes in CD49d expression were observed, it is possible that integrin avidity for endothelium is increased without a change in expression levels. The adhesion molecule CD31 also appeared to play a role in HSC recruitment, as $CD31^{-/-}$ mice display raised post-sham adhesiveness for HSCs when compared to strain/age matched controls. This effect is more likely to occur as a result of CD31-mediated maintenance of the inflammatory status of the organ, rather than a direct adhesive mechanism.

Following firm adhesion of HSCs to the microvasculature, cells are exposed to significant levels of cytokines released from the local endothelium. Excessive treatment of HSCs with specific cytokines reduced levels of CD49d expression, suggesting that the local inflammatory environment may actually promote the egress of cells from the injured tissue. This has been demonstrated previously in the kidney whereby stem cells recruited to the IR injured renal microcirculation are visible following 2 hours reperfusion but not 24 hours reperfusion, suggesting they have migrated out of the injured tissue (Tögel *et al.*, 2005).

Cells leaving the tissue could home to a number of anatomical locations. It is possible that HSCs 'released' from the liver return to their more traditional niche, the BM. Although HSCs may be deficient in CD49d expression following this egress, the β_2 integrin and selectin pathways have been shown to compensate for VLA-4 in BM homing and may still be able to facilitate this engraftment (Papayannopoulou *et al.*, 2001). Alternatively, HSCs may egress from the liver and engraft within the lung. Pulmonary recruitment may be dependent on adhesion molecules. However, activated leukocytes become more rigid due to reorganisation of their actin skeleton and it is likely that this promotes mechanical leukostasis in the lungs. The actual true destination of these cells once they have left the liver is unclear and warranting of further study.

7.2. Future Work

Even though the body of literature on progenitor cell recruitment injured non-BM sites is expanding, there are still questions which remain to be answered. As a result, the work detailed in this thesis could be expanded in a number of ways. Recent studies have used retroviral vectors to over-express the α_4 subunit of VLA-4 in both HSCs and MSCs (Cheng *et al.*, 2008, Kumar and Ponnazhagan, 2007, Porecha *et al.*, 2006). While these studies provide excellent proof-of-principle, over-expression of this integrin has not been investigated in the hepatic IR injury model. One would hypothesize that over-expression of the α4 integrin (and subsequently VLA-4) would increase hepatic HSC recruitment following IR.

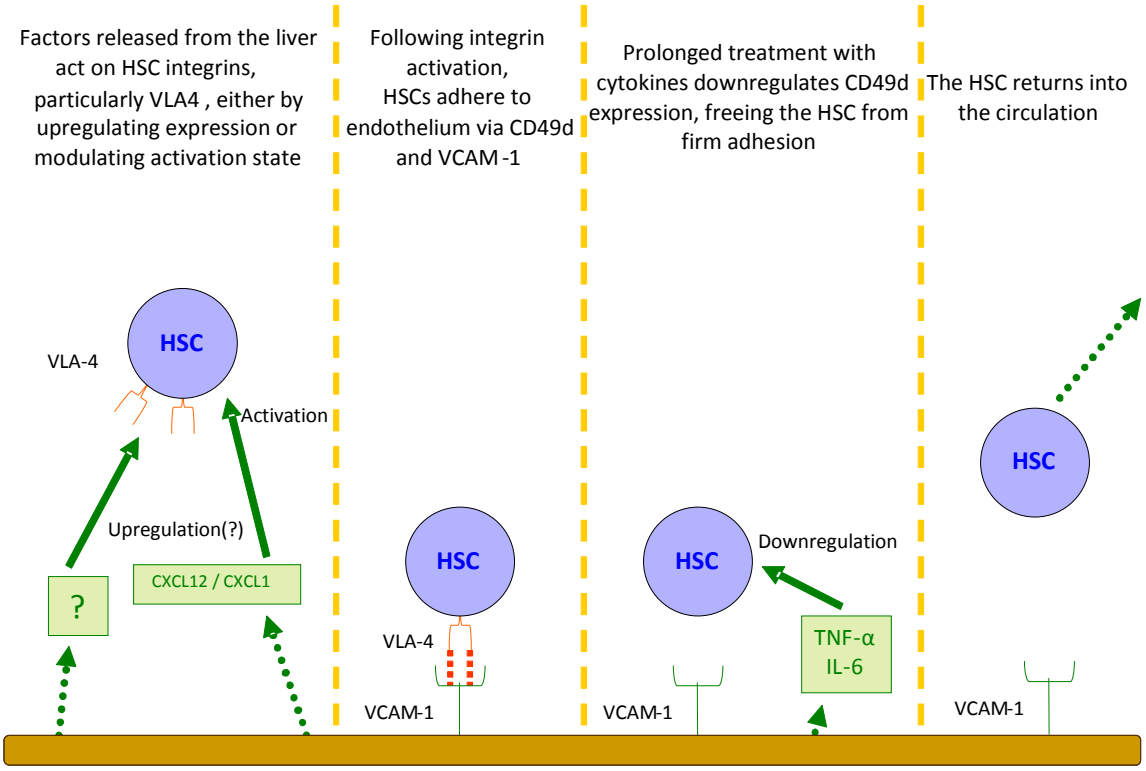


Figure 7.1. *Proposed mechanism of HSC recruitment following IR injury.* HSC recruitment is initially promoted by cytokine release which subsequently promotes firm adhesion via VLA-4/VCAM-1. Prolonged treatment with cytokines reduces adhesion molecule expression on the cell surface and promotes egress of cells from the inflammatory microvasculature.

In addition to HSCs, evidence now suggests that MSCs may be protective in animal models of hepatic IR (Baijun *et al.*, 2004). However, the molecular mechanisms that govern the recruitment of these cells to the liver post IR injury are also unknown. The similarities between HSC and MSC recruitment to the hepatic microvasculature could be investigated. Based on the results within this thesis, a role for VLA-4 could be speculated.

In our studies, we have been unable to show any anti-inflammatory effects of HSCs, although this may be a result of our chosen biological marker or sub-optimal recruitment. It has been shown in primates that co-administration of MSCs and HSCs enhances the latter's engraftment of the BM (Masuda *et al.*, 2008). Whether administration of MSCs alongside (or prior) to HSCs would promote their recruitment is unclear and warranting further study.

Treatment of HSCs with particular cytokines significantly increases their adhesion to endothelium via activation of surface adhesion molecules (Peled *et al.,* 2000). For future work, it may be useful to investigate the priming of HSCs with cytokines such as CXCL12 and CXCL1 to increase their recruitment in the tissue of interest. Alternatively, priming of the liver could be examined by injection of cytokines directly into the hepatic parenchyma. It can be appreciated that by making cells (or tissues) more adhesive and delivering them systemically, the chances of engraftment in other (non-targeted) tissues is increased. The future work in this area would need to carefully consider the site of injection in order to maximise delivery to the tissue of interest. Results in thesis show hepatic engraftment massively increased when cells are introduced via the portal vein.

When using any form of cellular therapy, it is important to consider any detrimental effects that ectopic positioning of the introduced cells may have. Indeed, evidence suggests that cells of BM origin may contribute to the formation of liver fibrosis highlighting the importance of isolation and identification (Russo *et al.*, 2006). Human hepatocellular cancers have been shown to express stem cell markers (Oct4, Nanog, Stat3, TBRII and ELF) and are thought to be progenitor cells which have adopted aberrant cell signalling (Tang *et al.*, 2008). Hence, it is clear that over-engraftment of the liver by HSCs may not be beneficial. This is something which needs to be considered carefully prior to entry of any HSC therapies to the clinic.

Resting platelets express CD31 on their cell surface, with expression raised following thrombin treatment (Metzelaar *et al.*, 1991). Recent evidence suggests that the role of CD31 is inhibitory (Falati *et al.*, 2006). Using IVM and laser-induced injury to cremaster muscle arterioles, it was demonstarted that the thrombi formed in PECAM-1 deficient mice were larger, formed more rapidly and were more stable (Falati *et al.*, 2006). Therefore in the $CD31^{-/-}$ animals used in the current study, it is possible that decreased platelet inhibition resulted in increased platelet adhesion even in the absence of injury or another activating factor. As HSCs can be recruited via platelets (Massberg *et al.*, 2006), the level of HSC adhesion seen following sham treatment may be a result of HSC recruitment to platelets. It would be interesting to replace the platelet compartment in $CD31^{-/-}$ animals with platelets from a WT mouse and look for changes in HSC adhesion in sham treated animals. The half-life of platelets is just over 100 hours (Fritz *et al.*, 1986), so it could be feasible to inject

wild-type platelets into thrombocytopenic mice and examine acutely whether CD31 mediates the raised sham adhesion via endothelial CD31 or via CD31 effects on platelets.

7.3. Concluding Remarks

Stem cell therapy for tissue disease is still very much in its infancy, with many arguing that the step from the bench to the bedside has been taken too soon (Melton, 2006, Leone and Crea, 2006). Whether this turns out to be the case has yet to be seen, and should become apparent as results from the ongoing clinical trials begin to unfold. However, there is a general consensus that many clinical trials have been undertaken without a basic knowledge of the biology of these cells. For instance, while there are numerous clinical studies examining the potential role of HSCs in liver injury, this thesis is the first to elucidate some of the molecular adhesive events that HSCs utilize to home to the injured liver. Similarly, in the field of cardiac regeneration, clinical studies have taken place before the mechanisms underlying repair have begun to be unraveled (Abdel-Latif *et al.*, 2007, Segers and Lee, 2008).

A better understanding of the basic biology underlying stem cell functions is needed if stem cell therapy is to be utilized to its optimum. Such studies need to focus on the pathways of retention, incorporation and differentiation within the injured tissue as well as continuing to focus on the molecular basis for recruitment. Despite the general excitement about the clinical trials, a major lack of understanding of how stem cells target specific tissues quite likely hinders their overall success. As this data is obtained, it should hopefully provide the scientific community with the information required to manipulate the behavior of stem

cells prior to their administration into the systemic circulation. The more tools for HSC manipulation we have, the better we can focus HSCs to where they are needed.

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