OPTIMISING STEM CELL THERAPY FOR LIVER DISEASE THROUGH MODULATION OF SPHINGOSINE 1-PHOSPHATE SIGNALLING

by

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ABSTRACT

Liver Disease is a rapidly rising cause of mortality and morbidity in the United Kingdom. There is an urgent need for new therapies for liver disease, given that the only curative treatment is liver transplantation which is limited by organ availability. A potential role of bone marrow stem cells as a novel therapy for liver disease has been proposed although understanding of the mechanisms involved in mediating both the anti fibrotic effect and the trafficking of cells to the liver are limited. In this thesis I have investigated the beneficial effect of a purified population of haematopoietic stem cells(HSC) in liver injury and studied mechanisms by which their effect may be optimised. Firstly I confirmed the increased mobilisation and recruitment of HSC to the injured liver and demonstrated a significant anti fibrotic effect of repeated injections of HSC in a murine model of liver injury. Observations from these studies suggested that stimulation of endogenous repair is the mechanism responsible. Sphingosine 1-phosphate (S1P) has bee shown to mediate egress of HSC from peripheral tissue into draining lymphatics, I have shown increased levels of S1P in both the blood and liver during liver injury, mediated by upregulation of sphingosine kinase 1. The S1P receptor modulator FTY720 increased the hepatic accumulation of HSC during liver injury without altering HSC recruitment to the liver, suggesting reduced egress of HSC. Ultimately I demonstrated that administration of FTY720 increased the hepatic retention of injected HSC which was associated with an enhanced anti fibrotic action. These studies provide encouraging evidence for the use of HSC in clinical studies and propose a mechanism by which the effect of this rare population of cells may be optimised.

DEDICATION

To my wife Sarah and my boys Oliver and Samuel

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FTY720 increases the hepatic retention of purified haematopoietic stem cells in chronic liver injury resulting in an enhanced anti-fibrotic action

King A, Houlihan D, Kavavanagh D, Suresh S, Garg A, Sumption H, Frampton J, Adams D, Newsome P

British Association for the Study of the Liver, Annual Meeting, London, September 2013

Liver Diseases in 2013: Advances in Pathogenesis and Treatment, London, October 2013

American Association for the Study of Liver Disease, Annual Meeting , Washington DC, November 2013

Bone Marrow Stem Cell Therapy for Liver Disease

King A, Newsome P

Digestive Diseases – in press

1 INTRODUCTION

1.1 Overview

In the introduction to this thesis I will begin by describing the urgent need for new treatments for chronic liver disease given the rapidly rising mortality from end stage liver disease and the limitations of the currently available therapies. I describe the characteristics of the liver, the changes that occur in liver disease and the current evidence for the reversibility of liver scarring. I introduce the concept of using bone marrow derived stem cells to treat liver disease and discuss the studies to date that support the beneficial effect of such therapy and the proposed mechanisms underlying this. I outline the characteristics of haematopoietic stem cells and our understanding of the mechanisms of tissue homing, concluding by discussing trafficking of HSC and the effect of modulation of sphingosine 1-phosphate signalling.

1.2 The Need for new therapies for Liver Disease

The incidence of chronic liver disease is rapidly rising both in the United Kingdom and worldwide. In the United Kingdom it is the fifth most common cause of death, and whilst the mortality from the leading causes of death (such as heart disease, stroke and cancer) has continued to fall over the past forty years, deaths from liver disease continue to rise at an alarming rate (Fig 1.1). The average age of death from liver disease is 59 years compared with between 72 and 84 years for other leading causes of death. The increase in cases of chronic liver disease is driven by the sharply rising rates of obesity and diabetes contributing to non alcoholic fatty liver disease, increases in alcohol related liver disease particularly amongst younger age groups than in the past and a rise in the number of cases of chronic hepatitis C infection(Leon *et al.* 2006; UK Chief Medical Officer 2012).

At present there are no established treatments for end stage liver disease other than replacement of the damaged organ through liver transplantation. The major limitation of liver transplantation remains supply of donor organs, whilst the need for transplantation continues to increase year on year the number of suitable donor livers for transplantation has not risen to meet demand. Each year only 50-60% of patients on the liver transplant waiting list receive a transplant, with significant numbers of patients dying or deteriorating whilst on the waiting list(NHS Blood and Transplant 2012). Liver transplantation carries significant peri-operative risk, so not all patients with end stage liver disease can be considered for a transplant particularly those who are older or who have other medical problems. Post operatively transplant recipients are at increased risk of kidney disease,

heart disease and the complications of taking immunosuppressant medications (Lucey *et al.* 2013).

The current management of chronic liver disease is predominantly aimed at preventing and treating the complications, such as ascites and variceal bleeding (D'Amico *et al.* 2006). Whilst the management of the complications of liver disease has improved over time (Garcia-Pagan *et al.* 2008), it remains the case that there are no effective treatments that can reverse the underlying liver damage.

Given the rapid rise in liver disease and the lack of widespread effective treatments there is clearly an urgent need for newer therapies for liver disease, that can prevent or reverse the progression of chronic liver disease and provide an alternative to liver transplantation.

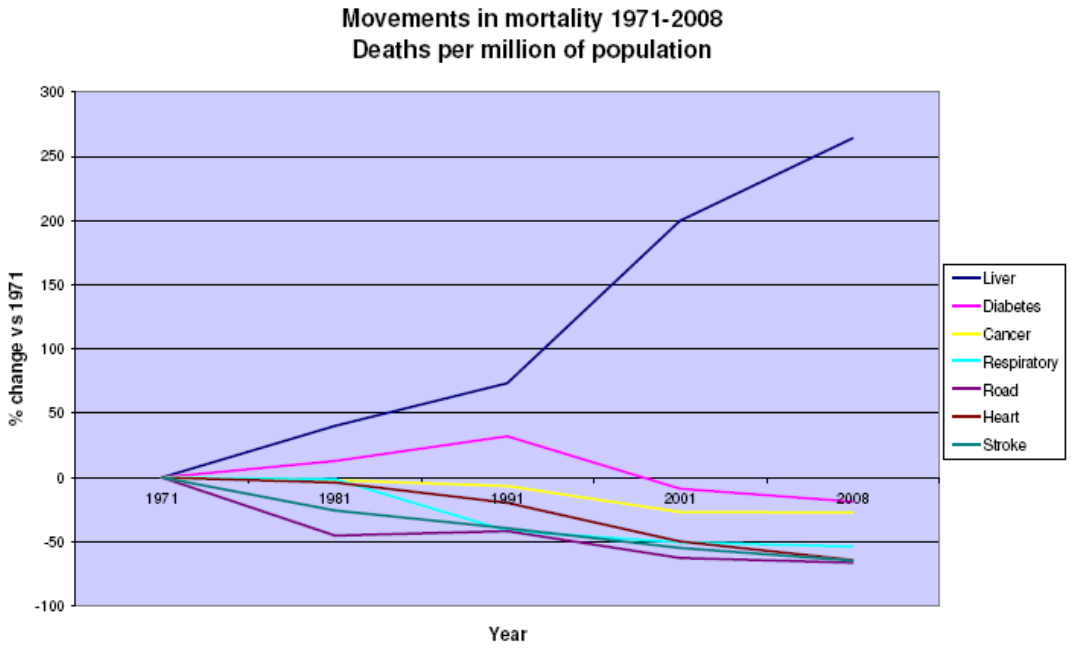


Figure 1.1 Rising Mortality from Liver Disease

Change in mortality from 1971 to 2008 in the six commonest causes of death in the United Kingdom (Reproduced from Health Service Quarterly (Office for National Statistics, Winter 2008))

1.3 Liver Disease

1.3.1 The Normal Liver

The liver is the largest solid organ in the body and is vital to both maintaining homeostasis and responding at times of stress. It is crucial to several hundred vital metabolic functions including the synthesis and excretion of bile acids, glucose metabolism through glycogenolysis and gluconeogenesis, metabolism of nitrogenous waste products, cholesterol metabolism, plasma protein synthesis and steroid hormone metabolism. Additionally the liver has vital roles in clearing toxins from the bloodstream and regulating the immune system(Dooley 2011).

The liver is closely associated with the gastrointestinal tract and possesses a unique dual blood supply whereby approximately 80% of blood enters the liver through the portal vein and 20% through the hepatic artery (Fig1.2A). The portal vein drains blood from the GI tract, spleen and pancreas and supplies low pressure venous blood which is high in nutrient and toxin content and low in oxygen content. The hepatic artery arises from the celiac axis and supplies high pressure oxygenated blood. After entering the liver, these vessels divide into smaller branches until the terminal branches come together at portal tracts where the blood is mixed and enters the hepatic sinusoids. Blood drains from the sinusoids into central venules which come together to form the hepatic veins which drain into the inferior vena cava(Fig 1.2B)(Dooley 2011).

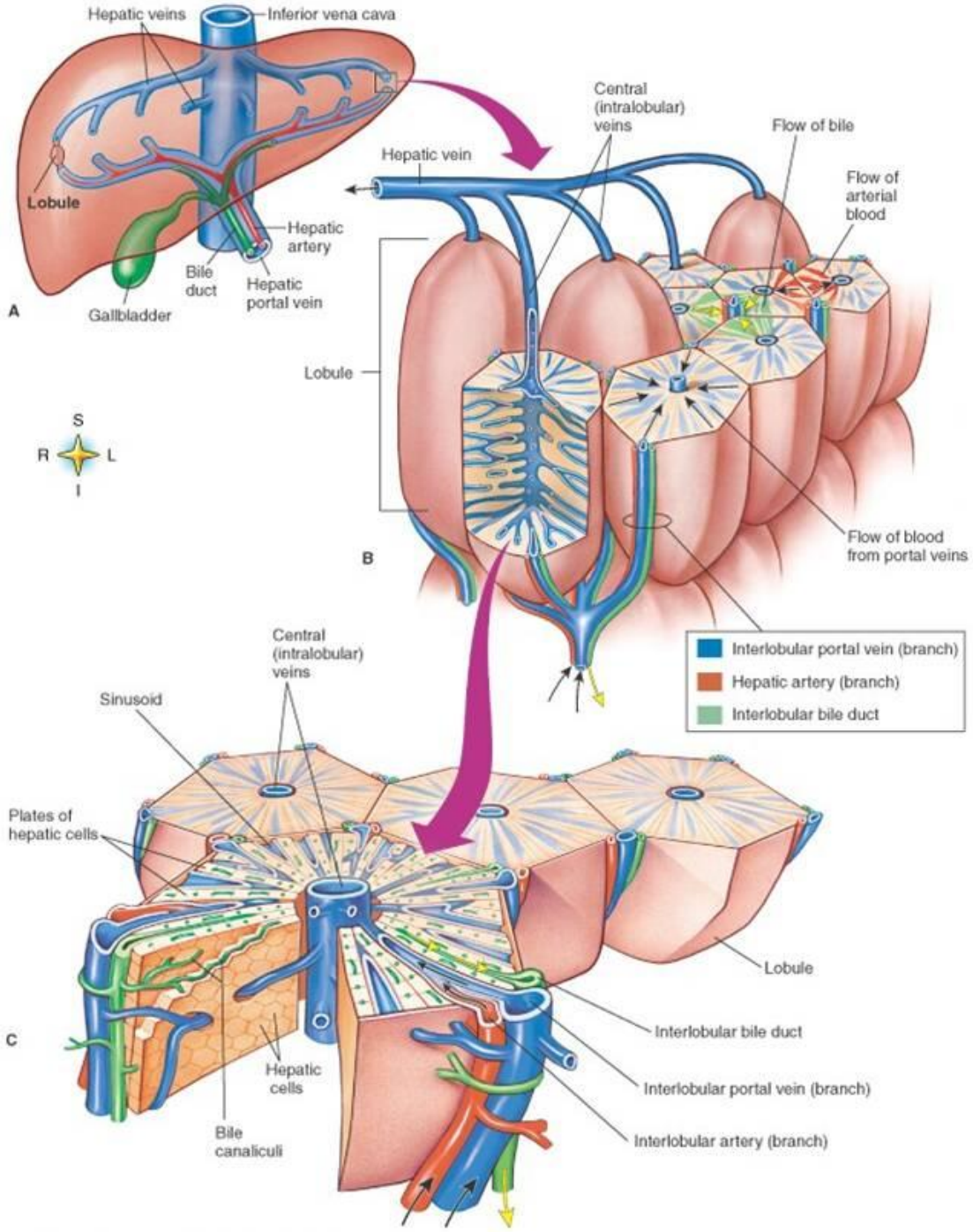


Figure 1.2 Anatomy of the Human Liver

Diagrammatic representation of the macroscopic and microscopic anatomy of the human liver, indicating (A)blood supply, (B)lobule structure and (C)sinusoidal anatomy (Reproduced from Anatomy & Physiology,OpenStax-CNX6/19/2013 http://cnx.org/content/col11496/1.6)

The liver produces approx 25-50% of the lymph flowing through the thoracic duct (Barrowman *et al.* 1984; Barrowman 1991) and in common with many other organs the lymphatic vessels are responsible for both tissue drainage and immunological control (Ohtani *et al.* 2008; Othani 2008). The hepatic sinusoids are the predominant source of hepatic lymph fluid. Fluid within the space of Disse passes through channels of interstitial space, between sinusoidal endothelial cells and hepatocytes and enters the interstitial space of either the portal tract or the sublobular veins. Fluid and migrating cells within this interstitial space enter prelymphatic and then lymphatic vessels(Ohtani *et al.* 2003; Ohtani *et al.* 2008). Increased portal lymph flow has been observed in patients with liver disease(Ludwig *et al.* 1968; Witte *et al.* 1969) and up to 30-fold increases in lymph flow have been demonstrated in cirrhotic rats, which correlated well with portal pressure (Barrowman *et al.* 1984).

The hepatic sinusoids represent the functional units of the liver and consist of interconnecting plates of hepatocytes lined on the basolateral surface by specialised fenestrated endothelial cells; this creates the space of Disse which allows direct contact between sinusoidal blood and hepatocytes. At the apical surface of the hepatocytes, bile canaliculi are formed which drain into terminal branches of the biliary tree lined by specialised epithelial cells called cholangiocytes. These bile ductules drain into larger ducts and eventually into the common hepatic duct leaving the liver and draining into the common bile duct (Fig1.2C)(Dooley 2011).

Hepatocytes make up about 80% of the total liver volume and large, polygonal cells. High numbers of mitochondria mean that they are the key metabolic cell within the liver and are

responsible for the vast majority of the functions of the liver. The fenestrations of the sinusoidal endothelium ensure that the basal surface of hepatocytes can be in direct contact with blood containing nutrients and toxins in the space of Disse, and microvilli on the surface membrane increase the surface area available for exchange of solutes(Fig 1.3A)(Dooley 2011).

Hepatic sinusoidal endothelial cells are characterised by the presence of open fenestrations and the absence of tight junctions between cells, thus allowing the entry of blood into the space of Disse and direct contact with hepatocytes (Fig1.3A). Functionally these cells possess the ability to endocytose waste products and toxins and to upregulate surface adhesion molecules at times of injury or inflammation to enhance cellular recruitment to the liver (Smedsrod *et al.* 1994; Lalor *et al.* 2002; Edwards *et al.* 2005).

Kuppfer cells are liver resident macrophage present in the space of Disse in association with the endothelial cells (Fig1.3A). Their function is to phagocytose bacteria and cellular debris from sinusoidal blood and also regulate hepatic inflammation and regeneration through production and secretion of soluble mediators such as cytokines and chemokines (Matsuoka *et al.* 1990; Duffield 2003; Fallowfield *et al.* 2007).

Hepatic stellate cells possess intracytoplasmic stores of vitamin A and retinoids, and are located within the space of Disse where they regulate turnover of the extra cellular matrix (Fig1.3A). During liver injury these cells become activated and transdifferentiate into fibrogenic cells called hepatic myofibroblasts. The specific role of these cells in the pathogenesis of liver disease will be further discussed(Matsuoka *et al.* 1990; Friedman 2008; Henderson *et al.* 2008).

The liver of a mouse is proportionally larger than that of a human, comprising approximately 6% of total body weight compared with approximately 2% in a human. It is made up of 4 anatomically distinct lobes – medial, left, caudate and right which is subdivided into cranial and caudal segments. The mouse liver possess the same dual blood supply as the human liver, together with a biliary drainage system to the intestine and a gall bladder(Hollander *et al.* 1987).

The microscopic appearances of the murine liver are similar to that of human liver. Portal areas containing a portal vein branch, hepatic artery branch and a bile ductule can be identified, with hepatocytes arranged in plates radiating from central venules towards the portal areas and separated by sinusoidal capillaries demonstrating the same lobular arrangement as seen in humans(Hollander *et al.* 1987). The cellular composition of the mouse liver is also similar to that of humans with hepatocytes comprising approximately 55% of total cell numbers, Kuppfer cells 18%, Hepatic Stellate cells 8% and liver sinusoidal endothelial cells 22% (Baratta *et al.* 2009).

1.3.2 Pathophysiology of Hepatic Fibrosis and Cirrhosis

The development of hepatic fibrosis and cirrhosis represents the common end result of chronic liver injury regardless of the underlying aetiology. Examples of injurious agents resulting in repeated hepatocyte damage include the toxic effects of excessive alcohol consumption, chronic viral infection or sustained auto immune injury. The pathophysiological process of cirrhosis is common to all causes of chronic liver disease and results in (i) a disordered scarred hepatic architecture with associated intrahepatic resistance (portal hypertension) leading to the clinical manifestations of varices, ascites and encephalopathy and (ii) loss of hepatocyte mass resulting in failure of hepatic synthetic function(D'Amico *et al.* 2006).

Hepatic injury results in the release of inflammatory mediators (such as reactive oxygen species and Transforming Growth Factor β)(Parola *et al.* 2001) from necrotic and apoptotic hepatocytes, cholangiocytes and endothelial cells (Jaeschke 2002),this results in the activation of liver resident cells of the innate immune system and recruitment of additional inflammatory cells, such as macrophages and neutrophils via upregulation of adhesion molecules on sinusoidal endothelial cells and secretion of pro inflammatory and pro fibrotic cytokines (including TGFβ, IL-1β, Platelet Derived Growth Factor, CCL2)(Friedman 2008; Ramachandran *et al.* 2012). Pro fibrogenic cytokines released by apoptotic hepatocytes, activated kupffer cells and recruited inflammatory cells promote activation of hepatic stellate cells into activated hepatic myofibroblasts which is associated with loss of vitamin A stores and expression of αSMA(Friedman 2008; Friedman 2008; Ramachandran *et al.* 2012). Activation results in the acquisition of key properties which contribute to fibrogenesis –

most importantly a direct contribution to hepatic fibrosis through increased production and secretion of collagens I,III and IV (Fig1.3B)(Schuppan 1990; Bataller *et al.* 2005). This increased collagen production is accompanied by increased expression of tissue inhibitors of metalloproteinases (TIMPs), enzymes which are responsible for the breakdown of components of the extracellular matrix (ECM) under normal circumstances, the resulting effect of this is a shift in the balance between TIMP and matrix metalloproteinases (MMPs) in favour of ECM accumulation(Bataller *et al.* 2005; Friedman 2008). Increases in ECM further promote hepatic stellate cell activation, survival and proliferation. Activated myofibroblasts secrete inflammatory and fibrogenic cytokines and chemokines (such as PDGF, MCP1, IP10 and VEGF) which further promote the proliferation and migration of myofibroblasts and other inflammatory cells which results in an amplification of the fibrogenic process (Fig 1.3B)(Bataller *et al.* 2005). The deposition of collagen and accumulation of ECM results in the development of bridging fibrosis and eventually fibrous septae and regenerative hepatic nodules which distort the hepatic parenchyma and disrupt hepatic blood flow(Friedman 2008). This results in the development of portal hypertension which underlies the clinical complications of ascites, varices and encephalopathy, and ultimately hepatic synthetic failure(D'Amico *et al.* 2006).

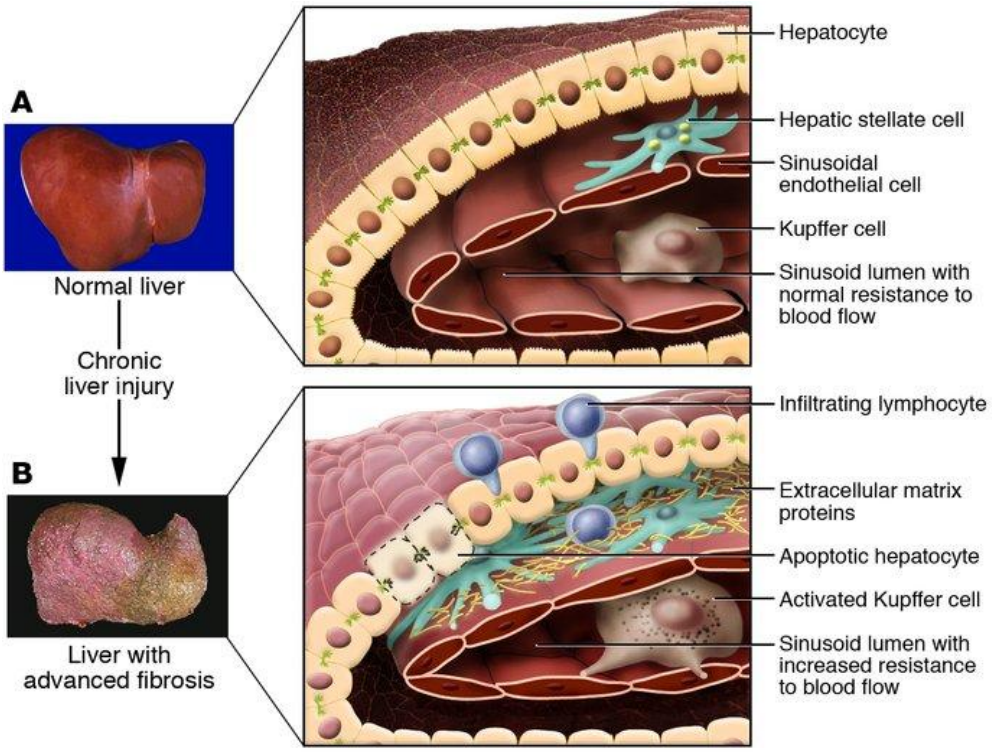


Figure 1.3 Changes in the Hepatic Architecture in Liver Fibrosis

(A) Architecture of normal liver demonstrating structural relationship between hepatocytes and sinusoidal endothelial cells creating the space of Disse containing hepatic stellate cells and normal sinusoidal lumen. (B) During liver injury inflammatory lymphocytes, apoptotic hepatocytes and activated Kupffer cells release fibrogenic mediators resulting in phenotypic activation of hepatic stellate cells to fibrogenic myofibroblasts resulting in deposition of ECM, capilliarisation of the sinusoidal lumen and increased resistance to luminal blood flow. (Reproduced from Bataller et al, J Clin Invest. 2005;115(2):209-218)

1.3.3 Reversibility of Hepatic Fibrosis and Cirrhosis

Historically the progression of hepatic fibrosis to cirrhosis was thought to be a relentless unidirectional process, however it is now recognised that this is not the case and that there is evidence from both animal models and humans that hepatic fibrosis is a reversible process with both progression and resolution possible. Clinical trials of new drugs for the treatment of chronic hepatitis B (HBV) and C (HCV) have demonstrated resolution of fibrosis in large numbers of patients in liver biopsies taken before and after treatment(Ellis *et al.* 2012). Furthermore treatment or removal of the toxic insult has been shown to improve hepatic fibrosis in alcohol related liver disease(Pares *et al.* 1986), autoimmune hepatitis(Dufour *et al.* 1997), biliary obstruction (Hammel *et al.* 2001)and non alcoholic fatty liver disease(Ellis *et al.* 2012). Reports of the reversibility of fully established cirrhosis have been less frequent, but in one series of 113 patients with cirrhosis due to either chronic HCV, chronic HBV or autoimmune hepatitis 12.4% of patients showed regression of cirrhosis after treatment with antiviral or immunosuppressive medications (Serpaggi *et al.* 2006). In an experimental rodent model using carbon tetrachloride to induce liver injury, fibrosis which had developed after 8 weeks treatment resolved almost completely after withdrawal of the injury. After 12 weeks injury cirrhosis had developed, which only partially resolved after removal of injury(Issa *et al.* 2004).

The resolution of hepatic fibrosis is dependent upon two key processes, the degradation of collagenous scar tissue and a reduction in activated hepatic myofibroblasts through apoptosis(Iredale *et al.* 1998) or reversion to a quiescent stellate cell phenotype(Kisseleva *et al.* 2012).

Macrophages have been shown to play a critical role in the progression and resolution of hepatic fibrosis, and both pro- and anti- fibrotic macrophage phenotypes have been described (Ramachandran *et al.* 2012). Duffield and colleagues used a CD11b-DTR transgenic mouse model which allowed selective depletion of macrophages at different timepoints during the progression and resolution of hepatic fibrosis. They were able to demonstrate that depletion of macrophages during the progression of fibrosis resulted in lower levels of fibrosis and reduced numbers of activated myofibroblasts, but that macrophage depletion during resolution of fibrosis impaired the recovery of fibrosis(Duffield *et al.* 2005). During the progression of hepatic fibrosis direct cellular contact between collagen I in the ECM and activated myofibroblasts promotes myofibroblast survival(Bataller *et al.* 2005). Direct contact between macrophages and myofibroblasts may result in myofibroblast apoptosis, as pro apoptotic signals (such as MMP-9, TRAIL and NGF) are also released from infiltrating macrophages or regenerating hepatocytes(Elsharkawy *et al.* 2005; Iredale 2008). During resolution of fibrosis, macrophages within the liver are derived from both liver specific Kupffer cells and circulating cells from the bone marrow (Duffield *et al.* 2005)and are localised to areas of hepatic scar(Duffield *et al.* 2005; Fallowfield *et al.* 2007). Fallowfield et al demonstrated that these scar associated macrophages express both MMP-9 and MMP-13 capable of degrading collagenous fibrotic scar tissue, and showed failure of resolution of fibrosis in MMP-13 deficient mice(Fallowfield *et al.* 2007). Tissue inhibitor of metalloproteinase-1 (TIMP-1) acts to both inhibit the collagenolytic activity of MMP (Iredale *et al.* 1992; Iredale *et al.* 1996)and to prevent myofibroblast apoptosis(Elsharkawy *et al.* 2005), which during the progression of fibrosis results in an accumulation of ECM fibrotic scar tissue. During resolution of fibrosis levels of TIMP-1 and -2 are significantly lower

resulting in a change in the balance between TIMP and MMP and a net degradation of ECM scar tissue and an increased tendency towards myofibroblast apoptosis(Iredale *et al.* 1998; Issa *et al.* 2004).

1.4 Bone Marrow Stem Cells

Adult bone marrow contains two major populations of stem cells, Haematopoietic Stem Cells and Mesenchymal Stem Cells. Haematopoietic stem cells provide continual renewal and replacement of all blood cell lineages whilst Mesenchymal stem cells differentiate into cells of three lineages constituting skeletal tissue – fat, cartilage and bone.

1.4.1 Haematopoietic Stem Cells

Haematopoietic Stem Cells (HSC) were first described over 50 years ago(Till *et al.* 1961) and since then have become the most widely studied stem cell and have also been used extensively in clinical practice as part of the treatment for haematologic malignancies (Gratwohl *et al.* 2010). HSC possess the typical properties of a stem cell – self renewal and potency. They have the ability to differentiate into mature cells committed along specific lineages and are responsible for the continual replenishment of the constituent cellular components of the blood (Fig 1.4). The ability for self renewal ensures one or both progeny retain the potential of the parent cell, thus ensuring a lifelong stem cell population(Siminovitch *et al.* 1963). HSC reside within the bone marrow niche and can be subdivided into a smaller quiescent population in the endosteal region of the bone marrow (long term repopulating HSC) and a more frequently occurring, active, cell cycling population associated with the vascular endothelium of the bone marrow (short term repopulating HSC)(Kiel *et al.* 2005). Although the bone marrow represents the primary niche for HSC, sites of other niches have been shown to include organs such as the liver, spleen and skin(O'Malley 2007; Miyata *et al.* 2008).

Haematopoietic multipotent progenitor cells represent the first descendant progeny of true HSC, and although lacking in the ability to self renew maintain the potential to differentiate into cells of all haematopoietic lineages. Studies of both the therapeutic effects of HSC and the mechanisms involved in the trafficking of HSC have tended to use populations of cells enriched for both haematopoietic stem cells and haematopoietic progenitor cells rather than studying purely HSC which are present at very low frequency within the bone marrow (Muller-Sieburg *et al.* 1986; Mazo *et al.* 2011).

HSC can be identified and isolated based upon cell surface marker expression, vital dye staining ability (eg Hoescht) and performance in functional cell assays(Purton *et al.* 2007). In humans, surface expression of CD34 or CD133 have been used to identify populations of cells enriched for HSC (Civin *et al.* 1984). In mice expression of the c-kit and sca-1 surface markers together with the absence of expression of lineage specific markers represent a population of cells enriched with both HSC and haematopoietic multipotent progenitor cells (Fig 1.4) (Wognum *et al.* 2003). Further discrimination into long term repopulating HSC, short term repopulating HSC and HPC can be made according to expression of Flt3 and CD34(Wognum *et al.* 2003).

Stem cell antigen 1 (sca-1) is a phosphatidylinositol- anchored protein, a member of the lymphocyte antigen 6 family(Lai *et al.* 1998) which identifies early haematopoietic stem and progenitor cells(Spangrude *et al.* 1988). Sca-1 is found predominantly on undifferentiated cells but also regulates the activation of T- and B-cells (Rock *et al.* 1989). c-kit, also known as Stem Cell Factor receptor or CD117, is a cytokine receptor found on haematopoietic stem cells but also on mast cells, thymocytes and common myeloid progenitor cells (Edling *et al.*

2007). The binding of SCF to the receptor results in dimerisation and activation of intrinsic tyrosine kinase activity which phosphorylates signal transduction molecules controlling cell survival, proliferation and differentiation(Yarden *et al.* 1987; Edling *et al.* 2007). Lineage specific markers used to eliminate differentiated cells include Ter119 for erythroid cells, CD5, CD8a and CD45R for lymphoid cells and, Gr-1 and CD11b for myeloid cells.

Identification using surface markers can have limitations, for example it has been shown that sca-1 can be upregulated on non-stem cells during inflammation(Malek *et al.* 1989; Purton *et al.* 2007). The test of true HSC is the ability to reconstitute haematopoiesis in serial transplantation assays, ensuring that transplanted cells possess both self renewal and full haematopoietic potential, whilst colony forming cell (CFC) assays can be used as a test of haematopoietic potential alone (Purton *et al.* 2007; Mazo *et al.* 2011).

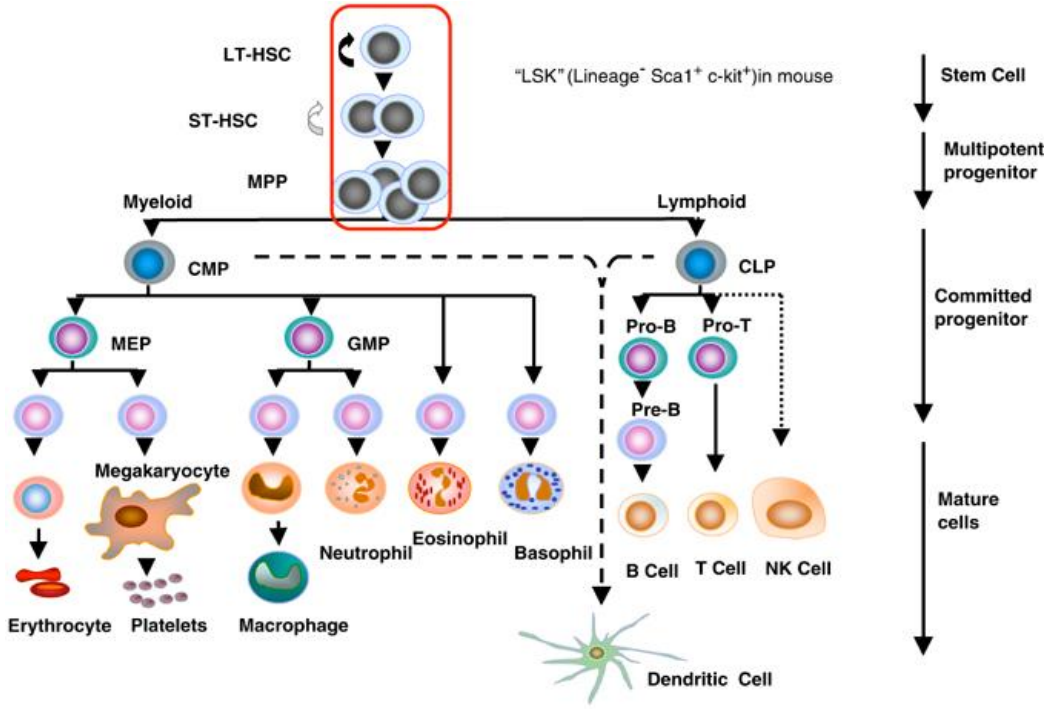


Figure 1.4 Differentiation of Haematopoietic Stem Cells

Differentiation of long term repopulating HSC (LT-HSC) to short term repopulating HSC (ST-HSC) and multipotent progenitors (MPP) is associated with progressive loss of self renewal capability, and in mice these populations of cells are defined by expression of c-kit and sca-1 and absence of lineage marker expression (red box). Mulitpotent progenitors differentiate into committed myeloid (CMP) or lymphoid progenitors (CLP) and eventually to terminally differentiated mature cells of the haematopoietic system. (Reproduced from Larsson and Karlsson, Oncogene 2005;24:5676-5692)

1.5 Bone Marrow Stem Cell Therapy for Liver Disease

1.5.1 Contribution of the Bone Marrow to Liver Repair and Regeneration

The first reports of the contribution of the bone marrow to liver regeneration came from Petersen and colleagues who performed sex mis-matched bone marrow transplants in mice undergoing liver injury(Petersen *et al.* 1999). In addition to the use of carbon tetrachloride(CCl_4) to induce liver injury, mice also received 2-Acetyl-Aminofluorene(2-AAF) to suppress endogenous hepatocyte proliferation (which would be the expected mechanism of regeneration following CCl_4 injury). Notably, they demonstrated the presence of donor (bone marrow) derived hepatocytes in the livers of transplanted animals(Petersen *et al.* 1999). This was subsequently corroborated by other groups who infused wild type bone marrow into fumarylacetoacetate hydrolase deficient mice ($FAH^{-/-}$) mice. In this model transplanted wild-type BM cells were able to generate FAH-expressing hepatocytes and improve the liver function and survival of the FAH deficient mice(Petersen *et al.* 1999; Lagasse *et al.* 2000; Theise *et al.* 2000; Wang *et al.* 2003).

In contrast to these initial reports suggesting that bone marrow cells made a significant contribution to the hepatocyte population of the liver, Wagers et al demonstrated that in irradiated mice receiving GFP tagged, single stem cell transplants, detection of donor-derived hepatocytes was an extremely rare event despite full reconstitution of the bone marrow by GFP positive cells(Wagers *et al.* 2002), whilst subsequently other studies have also shown a negligible contribution of BM cells to hepatocyte repopulation (Kanazawa *et al.* 2003; Li *et al.* 2012).

In the absence of a significant survival advantage, such as in metabolic liver disease, it is likely that the contribution of bone marrow derived cells to the hepatocyte population is limited and unlikely to be of significant clinical relevance.

The progression of liver disease not only results in liver failure due to hepatocyte death but also in the deposition of fibrous matrix leading to the development of cirrhosis resulting in portal hypertension and its clinical complications. To investigate the contribution of bone marrow derived cells to the development of hepatic fibrosis, Forbes and colleagues examined fibrotic liver tissue from a) male patients who had received a liver transplant from a female donor and b) a female patient who had received a bone marrow transplant from a male donor. Y-chromosome positive cells with a myofibroblast phenotype (αSMA^+, $vimentin^+$, $CD45^-$) were observed within the fibrotic livers suggesting that scar forming cells in liver fibrosis may originate from the bone marrow(Forbes *et al.* 2004). A subsequent animal study utilising sex mismatched bone marrow transplants also observed bone marrow derived hepatic stellate cells and myofibroblasts in the livers of mice with hepatic fibrosis(Russo *et al.* 2006).

In contrast however, Higashiyama et al transplanted bone marrow from transgenic mice with a collagen reporter (GFP or luciferase) into wild type mice and induced liver fibrosis with carbon tetrachloride or bile duct ligation and found no significant contribution of BM to myofibroblasts in the fibrotic livers of the recipients(Higashiyama *et al.* 2009). A further study also failed to find any significant number of BM derived cells amongst the myofibroblast population of fibrotic livers(Paredes *et al.* 2012).

Further evidence for the role of bone marrow cells in the response to liver injury is suggested by the significant mobilisation of cells from the bone marrow into the circulation following regenerative stimuli such as hepatic resection or transplantation(De Silvestro *et al.* 2004; Lemoli *et al.* 2006; Gehling *et al.* 2010), and during acute or chronic liver injury(Ghanem *et al.* 2012; Wan *et al.* 2013). Kollet and colleagues demonstrated mobilisation and homing of human CD34+ bone marrow cells to the liver in the NOD/SCID mouse during carbon tetrachloride induced chronic liver injury. A similar finding was observed following direct hepatic injection of the cytokine SDF-1, which is upregulated in liver injury, and which was abrogated by a CXCR4 neutralising antibody(Kollet *et al.* 2003).

1.5.2 The Anti Fibrotic potential of Bone Marrow Stem Cells in Liver Disease

The observation that bone marrow may contribute to hepatic repair and regeneration lead to further study of the therapeutic benefits of bone marrow derived cells in liver injury. Sakaida and colleagues infused GFP^+ bone marrow into mice with CCl_4 induced chronic liver injury and demonstrated a significant reduction in liver fibrosis and improvement in serum albumin levels. No clear mechanism or cell type responsible for this effect was determined but an increase in hepatic MMP9 expression was observed(Sakaida *et al.* 2004). Rather than infuse bone marrow cells into mice with liver injury, Yannaki et al administered GCSF to mobilise bone marrow cells into the circulation. Using sex mismatched bone marrow transplants and CCl_4 to induce either acute or chronic liver injury, they demonstrated that administration of GCSF reduced the severity of the liver injury and improved survival but that this was mediated through stimulation of endogenous repair mechanisms rather than engraftment and differentiation of bone marrow derived cells(Yannaki *et al.* 2005). However, the evidence for a beneficial effect of bone marrow cells in liver injury is not consistent, Quintanilha et al infused bone marrow cells into rats with chronic CCl_4 liver injury and failed to show any improvement in fibrosis either during or after the onset of injury(Quintanilha *et al.* 2008). Furthermore, in a recent study Thomas et al showed that in mice with chronic liver injury, infusions of unsorted whole bone marrow cells worsened hepatic fibrosis(Thomas *et al.* 2011). Bone marrow contains a heterogeneous population of cells, including both haematopoietic and mesenchymal stem cells, progenitor cells at varying stages of differentiation and mature, fully differentiated cells therefore the varying outcomes seen in these studies is perhaps not surprising. Despite the lack of clarity regarding the beneficial effects of bone marrow cells, and in particular the cell type

responsible for these effects, attempts have been made to optimise the performance of bone marrow cell infusion to improve hepatic injury. Bone marrow cells cultured in serum free media for 14 days reduced liver fibrosis and improved serum albumin in a CCl_4 model of liver injury, although the infused cells consisted mainly of cultured macrophages and mesenchymal stem cells(Iwamoto *et al.* 2013). A further study of cultured bone marrow cells found that after passage two, 94% of the cells were mesenchymal stem cells and infusion of these cells resulted in a significant reduction in hepatic fibrosis(Tanimoto *et al.* 2013). Splenectomy followed by bone marrow cell infusion in mice with chronic liver injury resulted in higher numbers of cells within the liver and an improved therapeutic effect(Iwamoto *et al.* 2012). Interestingly when bone marrow cells for infusion were harvested from animals with chronic liver injury, no beneficial effect on liver injury was observed(Mannheimer *et al.* 2011). This observation may be of particular relevance given that clinical studies to date have used autologous cells in patients with chronic liver disease.

Cho et al examined the beneficial effects of three different approaches, G-CSF injection alone to mobilise bone marrow cells, infusion of bone marrow derived mononuclear cells and infusion of purified haematopoietic stem cells. In this study there were significant reductions in hepatic fibrosis with similar benefits seen with both mononuclear cells and haematopoietic stem cells, whilst GCSF injections alone resulted in a significant but less marked reduction(Cho *et al.* 2011).

Mesenchymal stem cells (MSC) infused into mice with chronic liver injury resulted in a reduction in liver fibrosis although very few cells were seen to engraft the injured liver(Rabani *et al.* 2010). Zhao and colleagues examined the effects of MSC in rats with CCl_4

induced chronic liver injury and found a significant reduction in mortality and hepatic fibrosis compared with controls. Repeatedly infusing mesenchymal stem cells on 3 occasions gave rise to an improvement in survival and fibrosis when compared with a single injection of cells(Miryounesi *et al.* 2013).

The relative beneficial effects of MSC and HSC on hepatic fibrosis in a chronic carbon tetrachloride model of liver injury in mice were compared in a study by Li and colleagues. They found that both survival and fibrosis improved in the MSC and HSC groups when compared with controls but that the effect of MSC was greater than that of HSC(Li *et al.* 2013). The authors proposed a synergistic effect for the two cell types, although administration of MSC alone was significantly better than a combined infusion of HSC and MSC. This study also confirmed a previous finding that MSC engraft the injured liver with a greater frequency than either purified HSC or bone marrow mononuclear cells(Cho *et al.* 2009; Li *et al.* 2013).

1.5.3 Proposed Mechanisms responsible for the anti fibrotic effect

Despite mounting evidence for the beneficial effects of bone marrow derived stem cells, the mechanisms by which these effects are mediated remain poorly understood and have not been well addressed in studies to date. Initial reports suggesting that transdifferentiation of stem cells to hepatocytes was the predominant mechanism have largely been discounted as the frequency of hepatocyte production is extremely low and unlikely to account for the benefits observed. Reports of transdifferentiation are variable and seem more likely to occur following infusion of unsorted bone marrow cells rather than more defined populations.

The concept of bone marrow derived stem cells exerting a stimulatory effect on endogenous repair mechanisms is gaining increasing popularity as a likely mechanism. This is best described as a paracrine effect, which is defined as the ability of a cell to generate signals inducing changes in nearby cells. In chronic liver injury the endogenous repair mechanisms are impaired by the inhibitory effect of scar tissue and replicative senescence following repeated injury(Fassett *et al.* 2003; Trak-Smayra *et al.* 2004).

The anti-fibrotic effect of bone marrow derived stem cells has been reported to be accompanied by significant upregulation of matrix metalloproteinases in the injured liver, predominantly MMP-9 but also MMP-2 and MMP-13(Rabani *et al.* 2010; Roderfeld *et al.* 2012; Iwamoto *et al.* 2013). This has been observed following infusions of both HSC and MSC and reported to persist for up to 20 weeks following cell infusion(Roderfeld *et al.* 2012). Although increased MMP expression may mediate the anti fibrotic effect, the cellular source remains unclear. Some have reported that increases in MMP-9 in the liver is derived from

donor cells(Sakaida *et al.* 2004; Higashiyama *et al.* 2007), whilst others have demonstrated MMP-9 derived from increased numbers of endogenous mononuclear cells recruited into the liver following cell infusion(Thomas *et al.* 2011; de Oliveira *et al.* 2012).

The stimulatory effect of cytokines and growth factors released by infused cells may result in increased hepatocyte proliferation and stimulate hepatic progenitor cells whilst also reducing inflammatory cell infiltration. Administration of GCSF, a cytokine produced by bone marrow derived stem cells, during liver injury resulted in the proliferation of endogenous hepatocytes rather than the production of new hepatocytes from bone marrow derived cells recruited to the liver(Yannaki *et al.* 2005). Increased levels of GCSF within the liver have been demonstrated following bone marrow cell infusion(Mizunaga *et al.* 2012), and increases in HGF and VEGF following cell infusion further suggest a beneficial role in hepatocyte proliferation and angiogenesis in improving liver injury(Asano *et al.* 2007; Nakamura *et al.* 2007). Exogenously administered IL-10 has been shown to reduce fibrosis in an animal model and serum IL-10 levels have been reported to increase following cell infusion(Huang *et al.* 2006; de Freitas Souza *et al.* 2012). Alterations in the infiltration of endogenous cells in the liver following cell infusion include a reduction in inflammatory cells(de Oliveira *et al.* 2012) and increases in reparative macrophage populations(Thomas *et al.* 2011). Macrophages recruited into the liver may exert a direct anti fibrotic effect and also stimulate hepatic progenitor cell proliferation(Ramachandran *et al.* 2012; Bird *et al.* 2013).

1.5.4 Clinical Studies of Bone Marrow Stem Cells in Liver Disease

Although some considerable uncertainty remains in the pre-clinical work regarding the nature of the effect of bone marrow derived stem cell therapy, the most efficacious cell population and the exact mechanism of action, clinical trials of bone marrow derived stem cell therapy have moved forward rapidly since the first reports in 2005 (Fig 1.5). Am Esch and colleagues reported the use of autologous CD133+ HSC in patients without intrinsic liver disease undergoing hepatic resection for predominantly metastatic disease. The low residual liver volume in these patients (after a putative resection) would have precluded immediate surgery. All patients underwent selective portal vein embolisation to stimulate growth of the non occluded liver segments, followed by portal venous infusion of either autologous CD133+ HSC or saline control. Strikingly they found that liver volume increased significantly quicker in patients who had received cells compared with controls and surgery could be performed sooner in that group(am Esch *et al.* 2005; Furst *et al.* 2007). This work has subsequently been replicated in a larger study with similar findings and, more importantly, improved long term survival in patients receiving infusion of stem cells with portal vein embolisation (am Esch *et al.* 2012).

The initial reports of the use of BM derived stem cells in patients with chronic liver disease took alternative approaches to the method of cell isolation and route of delivery. One study administered GCSF to patients with cirrhosis and collected bone marrow cells mobilised into the blood, isolated CD34+ HSC and infused these cells via the hepatic artery(Gordon *et al.* 2006). Alternatively, the investigators in another study directly harvested bone marrow cells from the iliac crest under general anaesthesia, selected out mononuclear bone marrow cells

References	Types of stem cell infused	Number of patients	Improvement after cell infusion
Mesenchymal stem cells			
Zhang Z, *et al.,* J Gastroenterol Hepatol, 2012	Umbilical cord-MSC	30 treatment, 15 control	Improved liver function, MELD and reduced ascites
Shi M, *et al.,* Stem Cell Transl Med, 2012	Umbilical cord-MSC	24 treatment, 19 control	Improved liver function, MELD and reduced survival rates
MoHamadnejad M, *et al.,* Arch Iran Med, 2007	BM-MSC	3 cryptogenic, 1 AIH	Improved MELD
Kharaziha P, *et al.,* Eur J Gastroenterol Hepatol, 2009	BM-MSC	4 HBV, 1 HCV, 1 alcoholic, 2 cryptogenic	Improved MELD and liver function
Peng, *et al.,* Hepatology, 2011	BM-MSC	53 treatment, 105 control	Improved ALB, TBIL and MELD
Bone marrow-derived precursors			
Lyra AC, *et al.,* World J Gastroenterol, 2007	BMNC	3 HCV, 3 alcoholic, 4 others cryptogenic	Decreased TBIL and INR and improved ALB
Amer, *et al.,* Eur J Gastroenterol Hepatol, 2011	BMNC	10 intrasplenic, 10 intrahepatic, 20 control	Improved ascites and MELD
Terai S, *et al.,* Stem cell, 2006	BMNC	5 HCV, 3 HBV, 1 unknown	Improved ALB and CP
Kim JK, *et al.,* Cell Transpl, 2012	BMNC	10 HBV	Increased liver volume and CP score
Saito T, *et al.,* Stem Cell Dev, 2011	BMNC	5 treatment, 5 control	Improved ALB, PT and CP score
Lyra AC, *et al.,* Gut, 2007	BMNC	15 treatment, 15 control	Improved ALB and CP score
Hematopoietic stem cells			
Gordon MY, *et al.,* Stem Cells, 2006	$CD34^+$ cells	5 treatment	Improved ALB
MoHamadnejad M, *et al.,* World J Gastroenterol, 2007	$CD34^+$ cells	1 HBV, 1 AIH, 1 PBC, 1 cryptogenic	Improved MELD
Pai M, *et al.,* Am J Gastroenterol, 2008	Cultured $CD34^+$ cells	9 treatment	Improved ALB and CP score
Garg V, *et al.,* Gastroenterol, 2012	G-CSF mobilized $CD34^+$ cells	23 treatment, 24 placebo	Increased survival, reduced MELD scores
Han Y, *et al.,* Cytotherapy, 2008	PBMCs from G-CSF mobilized PB	20 treatment, 20 control	Improved ALB and CP score
Am Esch JS, *et al.,* Stem Cells, 2005	$CD133^+$ cells	3 treatment, 3 control	Improved liver volume after liver resection

MSC, mesenchymal stem cell; BMNC, bone marrow mononuclear cells; PB, peripheral blood; G-CSF, granulocyte colony-stimulating factor; CP, Child-Pugh score; MELD, model for end-stage liver disease; AIH, autoimmune hepatitis.

Fig 1.5 Clinical Studies of Bone Marrow Stem Cells for Liver Disease

Summary of published studies of mesenchymal stem cells, bone marrow derived cells and haematopoietic stem cells, indicating type of stem cell injected, number of patients included and information on outcomes following infusion. (Reproduced from Zhang and Wang, J Hepatology 2013;59(1):183-85)

and infused these cells via the portal vein(Terai *et al.* 2006). Whilst both studies involved only small numbers of patients, 5 and 9 respectively, and lacked control participants, they demonstrated that this form of therapy appeared safe and feasible. Clinical outcomes were reported in both studies and showed reduced serum bilirubin and improved serum albumin two months following cell infusion in the first study(Gordon *et al.* 2006) and improved serum albumin and Child Pugh score at six months in the second(Terai *et al.* 2006). These two initial studies highlight the significant number of variables involved in comparing these and subsequent studies. Further small scale, uncontrolled studies have included variation in the patients enrolled (aetiology and severity of liver disease), the method of cell harvest (direct bone marrow aspiration or peripheral blood leukophereis), the type and number of cells infused (various populations from unsorted mononuclear cells to purified $CD133^+$ HSC) and the route of cell infusion (hepatic artery, portal vein or peripheral vein)(Yannaki *et al.* 2006; Lyra *et al.* 2007; Mohamadnejad *et al.* 2007; Levicar *et al.* 2008; Pai *et al.* 2008; Lyra *et al.* 2010). The aim of many of these studies was to determine safety and feasibility and in that sense the outcomes were promising, however the size and nature of many of these subsequent studies meant that meaningful conclusions on clinical outcomes could not be made (Fig 1.5).

In more recent small controlled trials Lyra and colleagues demonstrated significant improvements in liver function one year following infusion of bone marrow mononuclear cells into the hepatic artery when compared with untreated control patients(Lyra *et al.* 2010). Ismail and colleagues examined the role of infusion of bone marrow mononuclear

cells prior to liver resection in cirrhotic patients with hepatocellular carcinoma and showed improved outcomes three months post operatively compared with patients undergoing resection alone(Ismail *et al.* 2010).

Human studies of BM stem cell therapy have predominantly used either unsorted mononuclear cell preparations (consisting of cells of haematopoietic lineages) or cell populations enriched for HSC (eg $CD34^+$ or $CD133^+$). The harvest and ex vivo isolation of HSC is a well established technique used extensively for the preparation of auto- or allo- grafts in patients with haematological malignancy. The techniques used are well validated and processes conform to guidelines and standards for preparation of clinical grade products(Sutherland *et al.* 1996). MSC represent a more heterogeneous population of cells than HSC and their isolation requires more intensive manipulation *ex vivo*, meaning that studies of these cells are more challenging to perform. The reported studies of MSC have included differences in the variables previously discussed but also in the techniques used to define and isolate the cells.

1.6 Sphingosine 1-phosphate

Sphingosine 1-phosphate (S1P), named in 1884 after the Greek mythological creature the Sphinx because of its enigmatic nature(Thudichum 1884) , is a bioactive sphingolipid metabolite derived from sphingomyelin via the sphingomyelinase pathway. Sphingolipids are structural components of plasma membranes designed to protect the cell surface. Initial reports described the role of S1P in regulating cell growth and suppressing apoptosis (Hannun *et al.* 2008), however further investigations have revealed its role as a intra-and extra-cellular signalling mediator involved in multiple cellular processes including cell growth and survival, regulation of cell motility and invasion, angiogenesis and vascular maturation, lymphocyte trafficking and immune regulation(Spiegel *et al.* 1993; Rivera *et al.* 2007). There is also evidence that S1P is important in the pathophysiology of diseases such as cancer, allergy, atherosclerosis and auto immune disease(Maceyka *et al.* 2012). Mechanisms by which S1P exerts its action include alterations in subcellular localisation, cellular import and export, protein binding and variation in receptor expression on target cells(Rosen *et al.* 2009) (Olivera *et al.* 2013).

1.6.1 Metabolism of S1P

The ability of S1P to act as a cell signalling molecule relies on the generation of a S1P concentration gradient across cell membranes and between body compartments. S1P has a short half life of only 15 minutes(Venkataraman *et al.* 2008) and its concentration in cells, tissues and fluids is governed by the balance between synthesis and degradation or export. Enzymes responsible for the synthesis of S1P are Sphingosine Kinase 1 and 2 and for the

reversible and irreversible degradation of S1P are Sphingosine Phophastase and Sphingosine Lyase respectively (Fig 1.6) (Takabe *et al.* 2008; Maceyka *et al.* 2012).

Sphingosine kinase 1 and 2 (SphK1,2) catalyse the ATP dependent phosphorylation of sphingosine (an 18 carbon aliphatic amino alcohol breakdown product of sphingomyelin present in cell membranes (Rivera *et al.* 2008)). SphK1 and SphK2 contain 5 conserved domains(Pyne *et al.* 2009) but differ in enzymatic activity, substrate preference, subcellular location and tissue distribution. (Liu *et al.* 2002; Pitson 2011). SphK1 preferentially phosphorylates sphingosine and SphK2 is less selective and phosphorylates a larger number of substrates, including FTY 720 (Kharel *et al.* 2005; Don *et al.* 2007). There is evidence of redundancy between the two enzymes as single SphK1 or SPhK2 knock out mice retain normal serum S1P levels and normal phenotype, whereas double knock out mice are embryonically lethal due to defects in the vascular system that are incompatible with life (Allende *et al.* 2004; Mizugishi *et al.* 2005; Takabe *et al.* 2008). It has been proposed that SphK1 predominantly synthesizes S1P destined for cellular export and SphK2 generates S1P for use as an intracellular messenger(Johnson *et al.* 2002; Sensken *et al.* 2010; Olivera *et al.* 2013) as the concentration of S1P in the blood of the SphK2 knock out mouse is higher than wild type mice due to increased cellular export after synthesis by SphK1(Olivera *et al.* 2013). S1P synthesis and secretion can be upregulated following exposure to a variety of cytokines and growth factors, including TNFα, LPS, PDGF and VEGF, resulting in translocation of SphK1 to the cell membrane and increased enzymatic activity (Pitson *et al.* 2003; Pitson *et al.* 2005; Stahelin *et al.* 2005; Takabe *et al.* 2008).

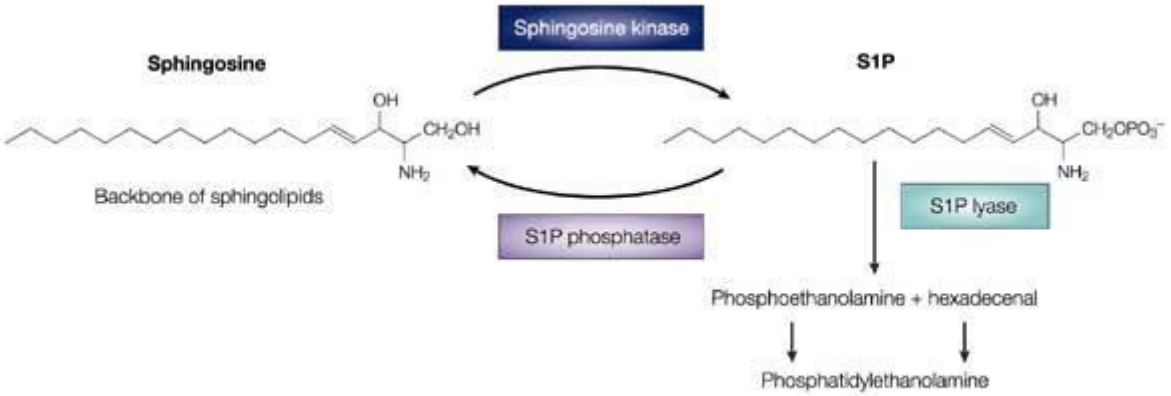


Figure 1.6 Metabolism of Sphingosine 1-phosphate

(Reproduced from Spiegel and Milsiten Nature Rev Mol Cell Biol 2003;4:397-407)

Sphingosine lyase (SphL) is a pyridoxal phosphate dependent enzyme present in the endoplasmic reticulum(Pyne *et al.* 2009; Olivera *et al.* 2013) which catalyses the irreversible degradation of S1P to hexadecanal and phosphoethanolamine (Bandhuvula *et al.* 2007). It is present in a wide variety of cells and tissues (Bektas *et al.* 2010) but is absent from platelets and erythrocytes (Yatomi *et al.* 1997; Ito *et al.* 2007). The S1P concentration in the serum and tissues of the SphL knock out mouse is up to 100 fold higher than wild type mice(Vogel *et al.* 2009; Bektas *et al.* 2010), demonstrating the importance of the enzyme in regulating S1P levels. Sphingosine phosphatase(SphP) is also present in the endoplasmic reticulum and catalyses the reversible dephosphorylation of S1P back to sphingosine(Mandala *et al.* 1998; Le Stunff *et al.* 2002).

The concentration of S1P within the blood is in the micromolar range and lower within the lymph. Tissue concentrations have been harder to accurately determine within the literature and have been estimated in the low nanomolar or picomolar range (Schwab *et al.* 2005; Pappu *et al.* 2007). These significant differences result in the formation of an S1P gradient between tissue (including lymphoid tissue) with a low S1P concentration and blood and lymph with high S1P concentrations(Pappu *et al.* 2007; Olivera *et al.* 2013). The difference is partly as a result of the differential cellular expression of sphingosine lyase, this enzyme is present in the majority of tissue resident cells and degrades tissue S1P to maintain low S1P levels (Stoffel 1970), whereas platelets and erythrocytes lack sphingosine lyase and therefore generate high S1P levels in the blood and lymph through the unopposed synthesis of S1P by sphingosine kinase (Ito *et al.* 2007). Maintaining low tissue levels of S1P means that small, acute changes in S1P levels can result in alterations to cell behaviour mediated by S1P receptor signalling – Schwab and colleagues demonstrated a pivotal role for the S1P

gradient in lymphocyte trafficking. Pharmacological inhibition of sphingosine lyase resulted in a significant elevation of the S1P concentration within lymphoid tissue which abolished the gradient between tissue and lymph and blocked the egress of lymphocytes from lymphoid tissue(Schwab *et al.* 2005).

1.6.2 Cellular Sources of S1P

Whilst synthesis and degradation of S1P occurs in most mammalian cells and S1P acts as an intracellular signalling molecule, the generation of S1P gradients relies on the cellular synthesis and export from cells that are 'net producers' of S1P.

Many of the cells within peripheral blood contribute to the production of S1P present at high levels within the blood. Erythrocytes have been shown to be the main cellular source of S1P within the vasculature(Hanel *et al.* 2007; Pappu *et al.* 2007) and generate approximately half of the total blood S1P concentration (Ito *et al.* 2007). Erythrocytes have high sphingosine kinase activity (Yang *et al.* 1999; Camerer *et al.* 2009) and lack sphingosine lyase (Ito *et al.* 2007). The release of S1P from erythrocytes occurs in a continuous, time dependent fashion and is not dependent upon cellular activation(Yang *et al.* 1999). S1P release from erythrocytes is regulated by the ABCC family of membrane transporter proteins and transported in the plasma bound to albumin or high density lipoprotein (Takabe *et al.* 2008; Kobayashi *et al.* 2009). Supporting evidence for the major role of erythrocytes in S1P production have been the observations of a correlation in humans between the red blood cell concentration and S1P concentration (Ohkawa *et al.* 2008) and of lower S1P levels in the blood of anaemic patients than normal controls (Bode *et al.* 2010).

Yatomi and colleagues demonstrated high levels of SphK activity and absence of SphL activity within mature platelets, resulting in high levels of storage of S1P(Yatomi *et al.* 1997). In contrast to erythrocytes, the release of S1P from platelets is not continual but is dependent upon activation by a variety of platelet stimuli including thrombin, TPA (a protein kinase C activator) and collagen (Yatomi *et al.* 1997; Yang *et al.* 1999; Kobayashi *et al.* 2009). The *NF-*

E2 (a transcription factor required for normal platelet formation(Shivdasani *et al.* 1995)) knockout mouse lacks circulating platelets yet maintains normal blood S1P levels (Pappu *et al.* 2007) It has been proposed that platelets do not contribute significantly to circulating levels of S1P under conditions of normal homeostasis, but that in response to tissue injury or inflammation activated platelets release stored S1P(Kim *et al.* 2009; Olivera *et al.* 2013).

Yang et al have shown that cell preparations of neutrophils and mononuclear cells (mainly lymphocytes) possess potent sphingosine kinase activity and that uptake and conversion of radiolabelled sphingosine to S1P in vitro occurred rapidly and with similar efficiency to both platelets and erythrocytes. The synthesis and release of S1P from both neutrophils and mononuclear cells was also independent of cellular stimulation or activation(Yang *et al.* 1999).

The role of endothelial cells in the maintenance of S1P gradients is vital as they are a direct barrier between varying concentrations of S1P (Olivera *et al.* 2013). Their unique structure and location means these cells can generate S1P to be exported into blood or lymph via the luminal surface or into tissue via the parenchymal surface. Endothelial cells have been shown to express all the enzymes responsible for S1P metabolism providing them with another regulatory role, for example activation of endothelial cells by exposure to shear stress resulted in upregulation of SphK and down regulation of SphL and SphP with the net effect being the increased secretion of S1P (Venkataraman *et al.* 2008). Venkataraman and colleagues showed that SphK1 knockout mice have lower blood S1P levels than wild type mice, however injection of SphK1 expressing adenoviral particles resulted in SphK1 expression in liver sinusoidal endothelium and restored blood S1P levels back to those of

wild type mice(Venkataraman *et al.* 2008). Pappu et al transplanted bone marrow cells from SphK 1/2 knock out fetal mice into irradiated wild type mice and despite the blood cells in these mice not having the ability to produce S1P, the blood concentration of S1P remained adequate(Pappu *et al.* 2007). Recently Fukuhara et al have elucidated that the membrane protein Spns2 is responsible for the extracellular secretion of S1P (Fukuhara *et al.* 2012). They showed that spns2 knock out mice have lower blood S1P concentration than wild type, and that an endothelial cell specific knock out mouse had low blood S1P concentration despite adequate production and secretion by blood cells, concluding that production and secretion of S1P by endothelial cells is responsible for a significant proportion of the blood S1P concentration(Fukuhara *et al.* 2012).

1.6.3 S1P Receptor Signalling

S1P is currently recognised to act as both an intracellular and extracellular signalling molecule, however until recently it was thought to be solely an intracellular messenger (Zhang *et al.* 1991; Olivera *et al.* 1993; Fyrst *et al.* 2010). Cell stimulation by growth factors and cytokines (Xia *et al.* 1998; Xia *et al.* 1999) increases intracellular sphingosine kinase activity resulting in increased intracellular S1P concentrations, however the intracellular signalling target for S1P remains unclear(Sanchez *et al.* 2004; Blaho *et al.* 2014).

In 1998 Lee and colleagues recognised that S1P was the ligand for the orphan receptor Endothelial Differentiation Gene 1 (EDG1) resulting in its reclassification as Sphingosine 1 Phosphate Receptor 1 (S1P1) (Lee *et al.* 1998). Further members of the EDG family were subsequently found to bind S1P with high affinity and renamed as S1P2 (EDG5), S1P3 (EDG3), S1P4 (EDG6) and S1P5 (EDG8)(Chun *et al.* 2002). All five S1P receptors structurally consist of an NH_2 terminus and seven transmembrane domains with hydrophilic intracellular and extracellular loops(Sanchez *et al.* 2004).Extracellular binding of S1P to surface receptors results in activation of intracellular signalling pathways mediated by G protein coupling(Takabe *et al.* 2008; Blaho *et al.* 2014).

The wide variety of diverse physiological functions regulated by S1P are all mediated through S1P receptor signalling(Sanchez *et al.* 2004). This is achieved by variable expression of receptor subtypes, differential levels of expression and differential G protein coupling across different cells and tissues(Sanchez *et al.* 2004). For example amongst haematopoietic cells, S1P1 is widely expressed (Rivera *et al.* 2008)whilst S1P4 is found on T lymphocytes(Wang *et al.* 2005), S1P2 on mast cells and macrophages(Kurashima *et al.* 2007), S1P5 on NK cells(Jenne *et al.* 2009) and S1P3 and S1P5 on dendritic cells (Czeloth *et al.* 2005). Whilst

S1P1 is crucial for immune cell migration(Matloubian *et al.* 2004), its role in mediating functions such as angiogenesis and vascular tone is revealed by the observation that the S1P1-/- knockout mouse is embryonically lethal due to failure of blood vessel formation (Liu *et al.* 2000).

The role of S1P receptors in regulating cell migration and trafficking has been well studied, particularly in cells of the immune system(Schwab *et al.* 2007). S1P1 signalling controls egress of T and B cells from secondary lymphoid organs and egress of thymocytes from the thymus (Matloubian *et al.* 2004; Allende *et al.* 2008). Mobilisation of both antibody secreting plasma cells from the spleen and B cells from Peyers patches to the blood is also dependent on S1P1(Gohda *et al.* 2008; Kunisawa *et al.* 2008). In contrast, NK cell migration from bone marrow into the blood is regulated by S1P5(Walzer *et al.* 2007). As previously discussed the role of S1P1 in controlling the egress of HSC from peripheral tissues into draining lymphatics has been recently described(Massberg *et al.* 2007).

Expression of S1P receptors by haematopoietic stem cells has been described as variable on human $CD34^+$ HSC, with S1P1 the only consistently expressed receptor in a study by Kimura et al (Kimura *et al.* 2004)whilst Massberg demonstrated expression of S1P1-4 on murine HSC (Massberg *et al.* 2007). Migration of HSC to S1P in vitro is similar in both human and murine cells with a dose dependent response(Kimura *et al.* 2004; Massberg *et al.* 2007; Ratajczak *et al.* 2010) however at high concentrations of S1P, migration is inhibited. The exact mechanism behind these observations is not clear but it has been postulated that exposure to S1P results in downregulation of S1P1 thus impairing migration(Rivera *et al.* 2008), alternatively at higher concentrations of S1P, receptor signalling is mediated by S1P2 which

exerts an inhibitory effect on cell migration(Ratajczak *et al.* 2010). More recently the observation that S1P is a chemokine for HSC has lead to further investigation into its role in HSC mobilisation and trafficking. There is growing evidence that S1P has a role in HSC mobilisation from the bone marrow into peripheral blood (Ratajczak *et al.* 2010; Golan *et al.* 2012; Juarez *et al.* 2012). Golan and colleagues demonstrated that the mobilising cytokine GCSF alters S1P levels within the blood thus promoting mobilisation (Golan *et al.* 2012)and Ratacjzak showed that the increase in S1P following GCSF administration was due to complement activation with membrane attack complex mediated erythrocyte lysis(Ratajczak *et al.* 2010). Juarez et al showed that S1P signalling alone did not promote mobilisation but that an S1P receptor agonist together with a CXCR4 antagonist resulted in effective HSC mobilisation without the need for GCSF(Juarez *et al.* 2012).

1.6.4 S1P Receptor Agonists/Antagonists

FTY720 was the first described modulator of S1P receptors, and was first used as an immunosuppressive agent (Brinkmann *et al.* 2002; Mandala *et al.* 2002). FTY720 is phosphorylated in vivo by sphingosine kinase 2 to form FTY720-phosphate (FTY720-P) (Billich *et al.* 2003; Don *et al.* 2007), which is structurally similar to S1P(Sanchez *et al.* 2004). FTY720 is an agonist at S1P1, 3, 4 and 5 as it binds and activates these receptors, however subsequent irreversible downregulation and ubiquitin dependent proteosomal degradation of S1P1 renders cells unresponsive to S1P (Oo *et al.* 2007). In this way FTY720 acts as a functional antagonist to S1P receptors (Fig 1.7).

Administration of FTY720 induces a rapid and reversible lymphopenia (Mandala *et al.* 2002), with lymphocytes sequestered into secondary lymphoid organs (Schwab *et al.* 2005). It does not induce any directly toxic effects on lymphocytes nor affect the total numbers of lymphocytes within the body making it an excellent potential immunosuppressive drug (Brinkmann *et al.* 2002). In vivo, FTY720 significantly improved outcomes in mice with experimental autoimmune encephalitis and has shown excellent promise (as the drug Fingolimod) in clinical trials for patients with multiple sclerosis (Brinkmann *et al.* 2010).

W146 is a competitive antagonist specific for the S1P1 receptor(Sanna *et al.* 2006). In contrast to FTY720, which induces S1P receptor internalisation and degradation rendering cells unresponsive to S1P, W146 directly blocks the effects of both S1P and FTY720 at the S1P1 receptor (Fig 1.7)(Tarrason *et al.* 2011). In vivo, W146 induces a peripheral lymphopenia in mice through sequestration of lymphocytes in lymph nodes(Tarrason *et al.*

2011) and also causes vascular capillary leakage, particularly in the lungs resulting in lung oedema (Sanna *et al.* 2006).

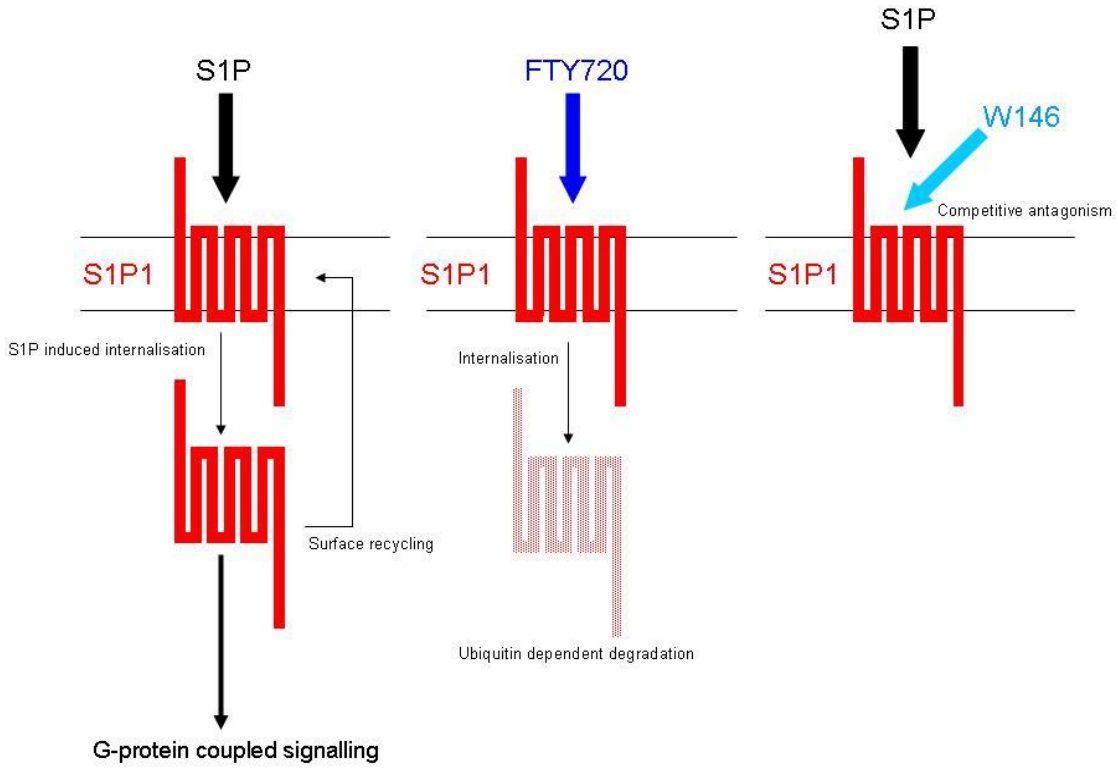


Fig 1.7 Mechanism of Action of S1P Receptor Agonists and Antagonists

(A) S1P activates S1P1 resulting in receptor internalisation, down stream G protein coupled signalling and subsequent surface recycling. (B) FTY720-P acts as a functional antagonist through internalisation and ubiquitin dependent degradation of S1P1 without downstream signalling or surface recycling, rendering cells unresponsive to S1P. (C) W146 is a competitive antagonist specific for S1P1, blocking activation of S1P1 by S1P. (Adapted from Obintata and Hla, Chemistry and Biology 2012;19:1080-81)

1.7 Haematopoietic Stem Cell Trafficking

1.7.1 HSC Mobilisation and Recruitment

The trafficking of HSC between different body compartments and organs occurs initially during the embryonic development of haematopoesis(Mazo *et al.* 2011). HSC are initially found within the fetal liver, which is the predominant site of fetal haematopoiesis, subsequently colonisation of the thymus and spleen occurs(Djaldetti *et al.* 1972; Jenkinson *et al.* 2006). The bone marrow is the primary site of adult haematopoiesis and is colonised by HSC derived from the fetal liver prior to birth, at which point fetal liver HSC become quiescent and do not significantly contribute to haematopoiesis (Christensen *et al.* 2004; Bowie *et al.* 2006).

Wright and colleagues described the continuous exchange of HSC between the bone marrow and peripheral blood and estimated that approximately 400 single HSC were present in the peripheral blood of a mouse at any one time (Wright *et al.* 2001). HSC have also been detected in various organs other than the bone marrow including the liver, lungs, gastrointestinal tract, kidney, thymus and skin(Wright *et al.* 2001; Sackstein 2004; Massberg *et al.* 2007; Zlotoff *et al.* 2011). Following systemic injection HSC were largely undetectable within the peripheral blood and were distributed widely in varying organs, with only 1-2% of the injected cells detectable in the bone marrow and 4% within the peripheral blood at 6 minutes after injection compared with 7% in the liver and 12% in the lungs (Wright *et al.* 2001).

HSC reside mainly in specialised niches within the bone marrow where cytokines, chemokines and growth factors control cell proliferation, differentiation, self renewal and

survival(Adams *et al.* 2006; Officer 2012) thus maintaining a regenerative pool of HSC in a quiescent state (Fortunel *et al.* 2003).

The presence of HSC in peripheral blood has been demonstrated and that there is a constant exchange of HSC between the BM and the peripheral circulation(Wright *et al.* 2001; Massberg *et al.* 2007) with approximately 400 HSC in the murine peripheral circulation at any one time (Wright *et al.* 2001). Circulating HSC re-engraft the BM in the absence of either myelosuppression or inflammation and is important in maintaining haematopoietic homeostasis(Wright *et al.* 2001).

The ongoing exchange of HSC between blood, bone marrow and other organs is an important homeostatic mechanism under normal physiological conditions (Mazo *et al.* 2011). Furthermore it has been shown in various animal models that tissue injury and inflammation increase trafficking of HSC to the injured tissue where differentiation into effector cells can occur(Kollet *et al.* 2003; Massberg *et al.* 2007; Si *et al.* 2010). The cellular and molecular mechanisms responsible for regulating this trafficking have provoked much investigation given the potential role of HSC as a cellular therapy for tissue injury, including liver disease.

Autologous or allogenic HSC transplantation following myeloablative therapy in patients with haematological malignancy have been in routine clinical use for some time(Gratwohl *et al.* 2010). The success of bone marrow transplantation is dependent upon the effective homing of infused HSC from the blood to the bone marrow . The mechanisms regulating the homing of cells to the bone marrow have therefore been well studied and follow a sequence

of molecular events similar to the well established model of lymphocyte recruitment(Lalor *et al.* 2002; Shetty *et al.* 2008).

Circulating HSC enter the bone marrow via a series of adhesion steps prior to entry into the bone marrow (Springer 1994; Mazo *et al.* 2011) similar to those involved in the recruitment of lymphocytes to the liver– the 'multi step adhesion cascade'(Fig 1.8)(Ley *et al.* 2007; Shetty *et al.* 2008). The first step involves the slowing and tethering of fast moving cells from the circulation to the vascular endothelium, achieved by the interaction between adhesion molecules expressed on the vascular endothelium with ligands on the cell surface. Bone marrow endothelium uniquely expresses P-selectin, E-selectin and VCAM-1, which are not found on other endothelial cells under physiological conditions and the ligands for these adhesion molecules PSGL-1 and VLA α4β1 respectively are expressed on the surface of HSC (Feuerer *et al.* 2004; Katayama *et al.* 2004). The interaction between CD44 on the cell surface of HSC and its ligand hyaluronate, a glycosaminoglycan present in the connective tissue of the bone marrow is also responsible for initiating the slowing and rolling of fast moving HSC from the circulation(Vermeulen *et al.* 1998; Avigdor *et al.* 2004). Slowly rolling cells receive an activation signal from the chemokine CXCL12, which acts via the G-protein coupled chemokine receptor CXCR4 and triggers conformational change and cytoskeletal rearrangement resulting in firm adhesion to the endothelium. (Cyster 1999; Peled *et al.* 1999). Adherent cells then transmigrate across the endothelium along a chemotactic gradient of CXCL12 which is constitutively expressed at high levels in the bone marrow (Peled *et al.* 1999; Hillyer *et al.* 2003).

The mechanisms regulating the trafficking of HSC to organs other than the bone marrow have not been as well studied and are not yet well understood. The role of the interaction between VCAM1 and α4β1 in the recruitment of HSC to the liver has been studied. Kavanagh and colleagues used an ischaemia reperfusion model of liver injury to stimulate recruitment of HSC to the liver and showed that in common with the bone marrow the interaction of VCAM with α4β1 is vital for HSC recruitment and that blockade of this signalling significantly impaired HSC recruitment, however HSC recruitment was not impeded by the blockade of CD44 or CD18(Kavanagh *et al.* 2010). The VCAM1 α4β1 interaction has also been shown to be an important mechanism for the recruitment of HSC to the heart but does not have a role in recruitment to the gastrointestinal tract or spleen (Papayannopoulou *et al.* 1995; Mazo *et al.* 2011). It is likely that recruitment of HSC to extramedullary organs is mediated by mechanisms similar to those in the bone marrow, but also involving organ specific mechanisms that are not yet completely understood.

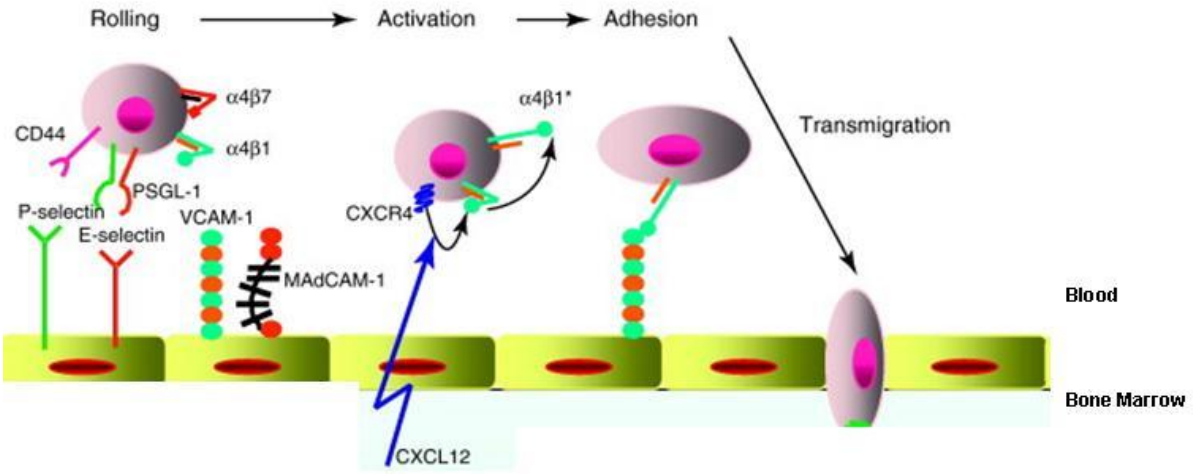


Fig 1.8 Recruitment of HSC from Blood into Bone Marrow

Fast moving circulating HSC are tethered to the endothelium by P- and E- selectins binding to ligands expressed on HSC (PSGL-1 and CD44). Tethered, slowly rolling HSC are activated by CXCL12 thorugh CXCR4 signalling, inducing a conformational change in á4â1resulting in increased affinity for VCAM-1 and mediating firm adhesion to the endothelium. Adherent HSC then transmigrate across the vessel wall along a chemotactic gradient of CXCL12. (Reproduced from Mazo et al Trends in Immunology (2011) 32;10:493-503)

1.7.2 Chemokine Receptor Signalling

Chemokines are a 8-12kDa heparin-binding cytokines, all with the ability to induce directed chemotaxis to specific sites and all possessing amino acid residues in conserved locations that are vital to maintaining their tertiary structure. The Chemokine family can be classified into four groups based upon the spacing of their first two cysteine residues – in CC chemokines the cysteine residues are adjacent, in CXC chemokines the residues are separated by a single amino acid residue, CX3CL1 is the only chemokine with three amino acids separating the cysteine residues and XCL1 and XCL2 lack two adjacent cysteine residues.(Wright *et al.* 2002; Oo *et al.* 2010; Bachelerie *et al.* 2014).

Chemokines may also be described as either 'inflammatory' or 'homeostatic/constitutive' depending on whether they are induced by inflammation (eg CXCL6,7,8 :neutrophil migration) or constitutively expressed and responsible for homeostatic immune regulation (eg CXCL12 :HSC homing to BM)(Bachelerie *et al.* 2014).

Chemokines are crucial for lymphocyte recruitment to the liver and patterns of chemokine expression within the liver have been well studied. CCL3-5 are strongly expressed in vascular endothelium within portal tracts particularly in immune mediated liver disease but also at low levels in normal liver(Goddard *et al.* 2001; Oo *et al.* 2010). CXCL9, CXCL10 and CXCL11 are present at high levels on sinusoidal endothelium in the injured liver (viral, autoimmune disease) and at low levels in normal liver (Shields *et al.* 1999; Harvey *et al.* 2003). These chemokines are also secreted by other liver cells including hepatocytes, cholangiocytes and hepatic stellate cells, and are presented on sinusoidal endothelium(Oo *et al.* 2010). Transcytosis allows chemokines produced by underlying hepatocytes or stellate cells to be

transported from the basolateral to the luminal surface of the endothelium, and chemokines secreted by cholangiocytes can be retained in the proteoglycan rich endothelial glycocalyx(Curbishley *et al.* 2005). The differences in injury induced chemokine expression may reflect a mechanism by which localised lymphocyte recruitment occurs(Oo *et al.* 2010).

Kollet and colleagues examined the role of CXCR4 in the recruitment of HSC to the liver, using NOD/SCID mice with acute and chronic carbon tetrachloride liver injury they demonstrated that blocking CXCR4 reduced homing and engraftment of human $CD34^{+}$ HSC. Localised hepatic injections of CXCL12 acted to increase homing, mediated via CXCR4(Kollet *et al.* 2003). Liver injury was associated with increase hepatic CXCL12 levels and MMP-9 expression. MMP-9 has been shown to upregulate CXCR4 expression on bone marrow cells(Kollet *et al.* 2003; Kawai *et al.* 2012) and in MMP-9 knock out mice limited recruitment of HSC to the liver was observed (Kawai *et al.* 2012). MMP-9 degraded bone marrow CXCL12, promoting HSC mobilisation and synergistically enhanced CXCL12 directed migration in vitro(Jin *et al.* 2008). CXCR4 has been shown to present, albeit at low levels on the surface of murine HSC (Sasaki *et al.* 2009).

Injury or inflammation stimulates recruitment of HSC to the site of injury(Dalakas *et al.* 2005; Badami *et al.* 2007), which corresponds with upregulation of CXCL12)(Stumm *et al.* 2002; Kollet *et al.* 2003). CXCL12 is the predominant chemoattractant for HSC mediated via CXCR4 expresssion (Wright *et al.* 2001; Wright *et al.* 2002), and is widely expressed in many tissues including the liver(Shirozu *et al.* 1995; Imai *et al.* 1999; Goddard *et al.* 2001). It has been shown that irradiation or chemotherapy results in a localised increase in bone marrow CXCL12 expression (Ponomaryov *et al.* 2000) and Kollet and colleagues investigated its

potential role as a stress signal for HSC mediating recruitment to injured or inflamed tissues (Kollet *et al.* 2003). They found an increase in membrane bound CXCL12 in carbon tetrachloride liver injury and that the increase in CXCL12 promoted homing of HSC to the liver, furthermore blocking CXCL12/CXCR4 interactions abolished recruitment of HSC to the liver. HSC recruited to the liver were localised to areas rich in CXCL12 in a similar fashion to that seen in the CXCL12 rich endosteal region of bone marrow(Weiss *et al.* 1991; Kollet *et al.* 2003). They also demonstrated the role of interactions between matrix metalloproteinases 2 and 9, and the cytokines Stem Cell Factor (SCF) and Hepatocyte Growth Factor (HGF) in HSC recruitment during liver injury. Increased MMP2 and 9 expression resulted in shedding of membrane bound SCF which in turn increased CXCR4 expression on HSC, whilst HGF expression induced cytoskeletal rearrangement and increased motility of HSC(Kollet *et al.* 2003).

Si and colleagues demonstrated the expression of CCR2 on HSC and confirmed their migration to the ligand CCL2 *in vitro*. Following APAP induced acute liver injury, significant numbers of HSC were recruited into the livers of wild type mice, however this effect was not seen in CCR2 knockout mice. Infusion of HSC derived from the bone marrow of wild type mice significantly improved hepatic injury, but no improvement was seen following infusion of HSC from CCR2 knockout mice(Si *et al.* 2010). CCR2 knockout mice display higher levels of hepatic necrosis following APAP induced injury (Hogaboam *et al.* 2000). Macrophages with the reparative M2 phenotype were absent from CCR2 knockout mice with liver injury (Holt *et al.* 2008)and in contrast to wild type mice, HSC from CCR2 knock out mice did not differentiate into macrophages of a reparative phenotype(Si *et al.* 2010).

1.7.3 HSC Recirculation

The continual exchange of HSC between the peripheral blood and bone marrow has been long recognised (Goodman *et al.* 1962) and more recently it has been shown that bone marrow derived HSC are present in organs other than the bone marrow(Wright *et al.* 2001). Until recently the mechanisms by which HSC recirculate between bone marrow and other tissues was not well understood. Massberg and colleagues investigated whether HSC undergo a similar recirculation to that of lymphocytes, whereby lymphocytes egress from organs via draining lymphatics and return to the peripheral blood circulation via the thoracic duct(von Andrian *et al.* 2000; Massberg *et al.* 2007). They were able to demonstrate the presence of bone marrow derived HSC within the thoracic duct lymph but that in contrast to lymphocytes, egress from tissues was directly into the lymph and did not require homing to lymph nodes to enter the lymphatic circulation(von Andrian *et al.* 2000; Massberg *et al.* 2007). Through the use of pairs of parabiotic mice, including a GFP expressing mouse, HSC chimerism was detected within organs outside the bone marrow and within the thoracic duct lymph, demonstrating continual recirculation. After separation of the pararbiotic pairs, HSC chimerism within the blood reduced immediately whilst a similar reduction was observed within the lymphatic fluid 36 hours later, inferring from this that the dwell time of HSC within organs is about 36 hours or more(Massberg *et al.* 2007).

Sphingosine 1-phosphate is a bioactive lipid signalling molecule that has been shown to regulate the egress of lymphocytes into efferent lymphatics, mediated by S1P receptor 1 signalling (Rosen *et al.* 2005; Schwab *et al.* 2005; Massberg *et al.* 2006). Massberg and colleagues blocked the action of S1P receptors by the administration of FTY720 which

resulted in a dramatic reduction in HSC within the lymphatic fluid, a similar effect was seen with S1P1 specific blockade using SEW2871. The reduction on HSC was seen within 6 hours of FTY720 administration and was accompanied by a reduction in the numbers of HSC within the peripheral blood, however the authors concluded that as they had shown the dwell time of HSC within tissues to be 36 hours or more that the reduction in HSC in the lymph was as a consequence of reduced tissue egress rather than reduced tissue entry (Fig 1.9). This finding was supported by the observation that after 7 days of FTY720 administration increased numbers of HSC could be found within various organs other than the bone marrow and numbers of HSC within the bone marrow remained constant(Massberg *et al.* 2007).

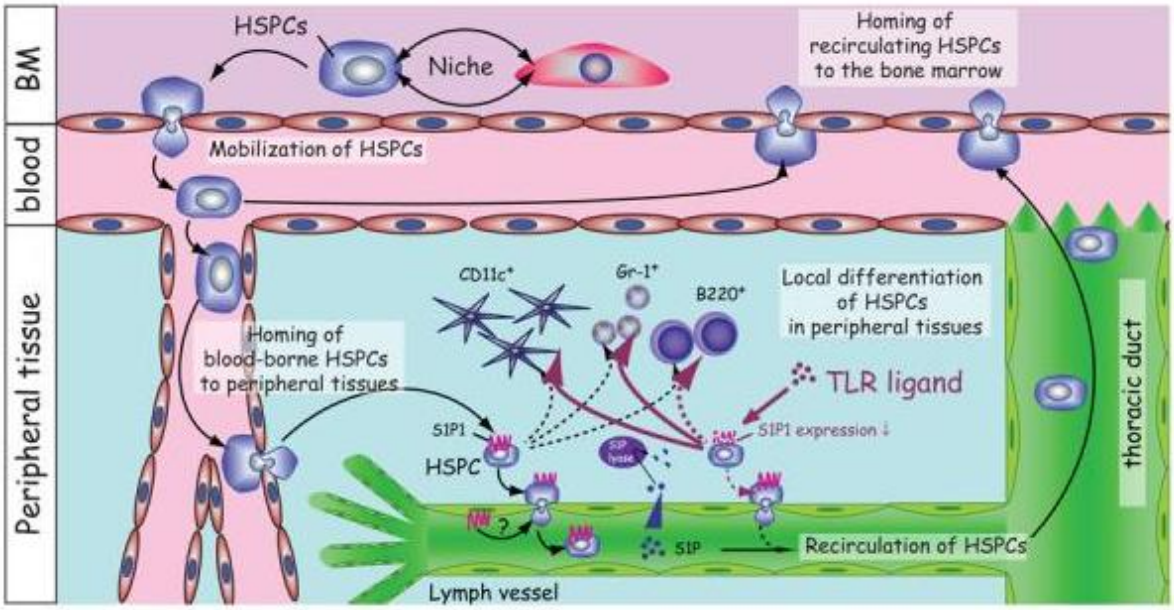


Figure 1.9 Recirculation of Haematopoietic Stem Cells

Schematic model illustrating the trafficking of migratory HSPCs under physiological conditions and during inflammation (Reproduced from Massberg et al Cell 2007;131(5):994-1008)

1.8 Aims of this project

There is a clear need for new therapies for liver disease, particularly those which may halt or reverse the progressive nature of hepatic fibrosis and cirrhosis. Adult bone marrow stem cells represent a promising therapeutic option but clarity on the specific cell types that may be of benefit is lacking and information on the mechanisms mediating these beneficial effects remains sparse. The mechanisms controlling the trafficking of stem cells to peripheral tissues such as the liver are not completely understood with emerging evidence that the bioactive lipid Sphingosine 1-phosphate is a key regulator. A greater understanding of the trafficking of stem cells may help optimise and improve their beneficial effect.

Therefore, the aims of this project are:

i) To determine whether repeated peripheral injections of a purified population of haematopoietic stem cells (c-kit+, sca-1+ lineage -) improve hepatic fibrosis in a carbon tetrachloride model of chronic liver injury, and through the use CD45 congenic mice determine the fate of injected cells

ii) To determine the alterations that occur in S1P concentrations in blood, lymph, liver and bone marrow in a murine model of chronic liver injury and determine changes in expression of the enzymes responsible for S1P synthesis and degradation that occur in both murine and human chronic liver injury

iii) To determine whether increased numbers of HSC are mobilised from the bone marrow and recruited to the liver during chronic liver injury using bone

marrow transplantation between CD45 congenic mice to identify bone marrow derived cells

iv) To determine whether modulation of S1P receptor signalling by FTY720, a functional S1P receptor antagonist, increases the accumulation of HSC within the injured liver and, using intravital microscopy investigate the effects of FTY720 on HSC recruitment to the injured liver

v) To determine whether administration of FTY720 leads to an increase in the number of injected HSC within the livers of mice with chronic carbon tetrachloride induced liver injury, and whether administration of FTY720 in conjunction with repeated peripheral injections of purified HSC augments their anti fibrotic effect

2 EXPERIMENTAL METHODS

2.1 Animal Experiments

All animal experiments performed were conducted in accordance with the Animals(Scientific Procedures) Act 1986 under a Project Licence held by Dr. Phil Newsome (PPL 40/3201). Some animals were transferred to a Project Licence held by Dr. Neena Kalia (PPL40/2749) for Intravital Microscopy. Approval of the University of Birmingham Ethics Committee was also obtained.

All animals were housed in an approved animal facility (Biomedical Services Unit, University of Birmingham) under conditions of 12 hour light/dark cycles and with unlimited access to food and water.

Wild type, inbred C57/BL6 mice were supplied directly to the Biomedical Services Unit by Harlan UK and used for cell isolation at 6-8 weeks of age and for experimental procedures at 8 weeks of age.

The BoyJ mouse(B6.SJL-*Ptprc*^{a} *Pep3*^{b}/BoyJ) is a congenic C57/BL6 mice expressing the differential pan leukocyte marker Ptprc^{a} (CD45.1). Wild type inbred C57/BL6 express the Ptprc^{b} (CD45.2) allele. BoyJ mice were supplied from a colony held by Prof Jon Frampton maintained in the Biomedical Services Unit and used for experimental procedures at 8 weeks of age.

At the completion of experimental procedures mice were euthanased under terminal inhalational anaesthesia (3% Isoflurane) allowing blood sampling via cardiac puncture to be

performed prior to death. Mice used for cell isolation were euthanased using cervical dislocation as approved under Schedule1 of the Animals(Scientific Procedures) Act.

2.2 Human Tissue

Human liver tissue was obtained from patients of the Liver Transplant Unit at the Queen Elizabeth Hospital Birmingham in accordance with approval granted by the Local Research Ethics Committee and as regulated by the Human Tissue Act. Diseased liver tissue samples were obtained from explanted livers removed from patients undergoing liver transplantation and normal liver samples were obtained from donor liver tissue not required for transplantation.

2.3 HPC7 Cell Culture

The HPC-7 cell line was obtained from Dr Neena Kalia (University of Birmingham) having originally been a gift from Prof Leif Carlsson (University of Umea, Sweden). HPC-7 is a haematopoietic progenitor cell line derived from murine embryoinic stem cells transduced with LHX2 (Pinto *et al.* 1998, Pinto *et al*. 2002). LHX2 is a LIM homeodomain protein regulating cell division and differentiation. HPC-7 express transcription factors and surface markers characteristic of haematopoietic progenitor cells. Multilineage differentiation capacity is maintained and culture and expansion of HPC-7 cells has been shown not to affect progenitor properties (Pinto *et al*. 1998, Dasse *et al*. 2012).

HPC-7 cells were cultured in StemPro Serum Free Media 34 (SFM-34) (Invitrogen) supplemented with 50U/ml Penicillin (Gibco), 50U/ml Streptomycin (Gibco), 2mM Glutamine (Gibco) and 100ng/ml recombinant murine Stem Cell Factor (SCF)(Invitrogen). HPC-7 cell proliferation is dependent upon SCF and upon maintenance in culture ($37^{o}C$, 5%CO_2) at a density of between 0.8 and 1.5 $x10^6$ cells /ml.

Cell counts were performed by staining with 0.4% Trypan blue solution (in a 4:1 ratio) to exclude dead cells and viable cells counted using a standard Neubauer haemocytometer.

2.4 Isolation of Cells from Bone Marrow

8-12 week old male C57/BL6 mice were euthanased by an approved Home Office Schedule 1 method and both femurs and both tibias removed. After removal of muscle and other soft tissue, the epiphyses of each bone were gently removed and the bone flushed through with cold Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS), 50U/ml Penicillin, 50U/ml Streptomycin, and 2mM Glutamine using a 26-gauge needle and 1ml syringe. The extracted bone marrow cells were filtered through a 70um cell strainer (BD Biosciences) followed by wash in Phosphate Buffered Saline (PBS) + 1% FCS and centrifugation at 2000rpm for 5 minutes, cells were resuspended in ACK Red Cell Lysis Buffer (Sigma) for 10 minutes at room temperature followed by a further wash in PBS + 1% FCS and centrifugation at 2000rpm for 5 minutes.

2.5 Isolation of Cells from Tissue Samples

After euthanasia organs were removed and washed thoroughly in PBS. Livers, Kidneys and Lungs were directly injected throughout with 2mg/ml Collagenase IV in DMEM/10% FCS followed by mincing using a scalpel. The tissue suspension was then incubated at 37^{o}C for 40 minutes with continuous gentle agitation. The suspension was then washed through a coarse (250µm) mesh and a fine (50µm) mesh using Roswell Park Memorial Institute (RPMI) media to obtain a cell suspension, this was centrifuged at 2000rpm for 5 minutes and the pellet resuspended in RPMI (Liver 20ml, Kidney/Lung 10mls). A working dilution of Optiprep (Sigma) at 1.09g/ml was obtained by mixing neat Optiprep with PBS in a 4:11 ratio. In a 15ml conical tube 5ml of cell suspension was layered carefully over 5ml of Optiprep solution, this was centrifuged at 1050g for 25 minutes at 4^{o}C with no brake applied to the centrifuge. Mononuclear cells were carefully aspirated from the interface between Optiprep and media, washed in PBS, centrifuged at 2000rpm for 5 minutes and the resulting cells used in further experiments.

2.6 Isolation of Cells from Peripheral Blood

Mice were anaesthetised using 3% Isoflurane and venesected via cardiac puncture to obtain 0.5-1.0 ml peripheral blood, followed by euthanasia by an approved Home Office Schedule 1 method. The volume of blood obtained was recorded and collected into tubes containing ethylenediaminetetraacetic acid (EDTA, BD Biosciences). ACK Red Cell Lysis Buffer was added to the sample at 10 times the volume of blood, vortexed and incubated at room temperature until the resulting suspension became transparent indicating lysis of

erythrocytes. The suspension was then three times washed in PBS and centrifuged at 2000rpm for 5 minutes. The resulting leukocyte pellet was then used for further experiments.

2.7 Flow Cytometry and Cell Sorting

Cells isolated using the described techniques were resuspended in PBS + 1%FCS and purified anti-mouse CD16/32 antibody (ebioscience) added at 1:25 dilution to block non specific Fc-receptor binding and incubated on ice for 20 minutes. After a further wash in PBS + 1%FCS and centrifugation, cells were resuspended in PBS+1%FCS for antibody staining. Antibodies used are listed in Table 2.1. Tubes were incubated on ice for 30 minutes then washed and centrifuged. Cells were resuspended in PBS + 1%FCS and passed through a 50µm filter immediately prior to analysis or cell sorting.

Analysis of extracellular surface markers using directly conjugated antibodies was performed as described above. Analysis of intracellular targets using unconjugated antibodies was performed with the following procedure. After completion of labelling with extracellular antibodies cells were resuspended in CytoFix solution (BD Biosciences) for 20 minutes at room temperature followed by washing in PermWash solution (BD Biosciences) and centrifugation at 2000rpm for 5 minutes. Cells were resuspended in PermWash solution and staining with the relevant intracellular antibodies (Table 2.1) was performed for 30 minutes on ice, followed by wash in PermWash and centrifugation. After resuspending the cells in PermWash solution, Goat Serum (Sigma) at a dilution of 1:20 was added to the staining tubes for 20 minutes on ice to prevent non specific binding of the secondary fluorochrome

conjugated antibody. Secondary antibody (Table 2.1) was added for 30 minutes on ice followed by washing in PermWash solution, centrifugation and resuspension in PBS+1%FCS for analysis by flow cytometry.

Cells sorting was performed using a MoFloCell Sorter(Dako Cytomation) running Summit 5.2 software. The gating strategy used and a representative analysis of the sort purity is outlined in figure 2.1. Cells to be used for PCR analysis were sorted directly into 350ul RLT lysis buffer (Qiagen) ready for RNA extraction and cells to be used for experimental injections were sorted into PBS and used immediately.

Figure 2.1 Isolation of c-kit+ sca1+ lineage- cells

Bone marrow cells were obtained from donor mice following dissection of the tibia and femurs by flushing the marrow cavity with DMEM + 10%FCS. Cells were treated with red cell lysis buffer and filtered prior to labelling with fluorescently conjugated antibodies to lineage specific markers (CD11b, CD8a, CD5, Ter119, Gr-1, B220), c-kit and sca-1. Cells were isolated by initially excluding dead cells and debris on a forward scatter – side scatter plot (A) and excluding doublets of cells on a forward scatter – pulse width plot (B). The lineage negative population was determined (C) and plotted according to expression of c-kit and sca-1 surface markers (D). HSC were defined as c-kit+, sca-1+ and lineage marker negative ('KSL') and represented 0.05% of the total bone marrow cell population. The purity of the isolated cells was then checked by determining lineage marker, c-kit and sca-1 expression on a sample of isolated cells (E).

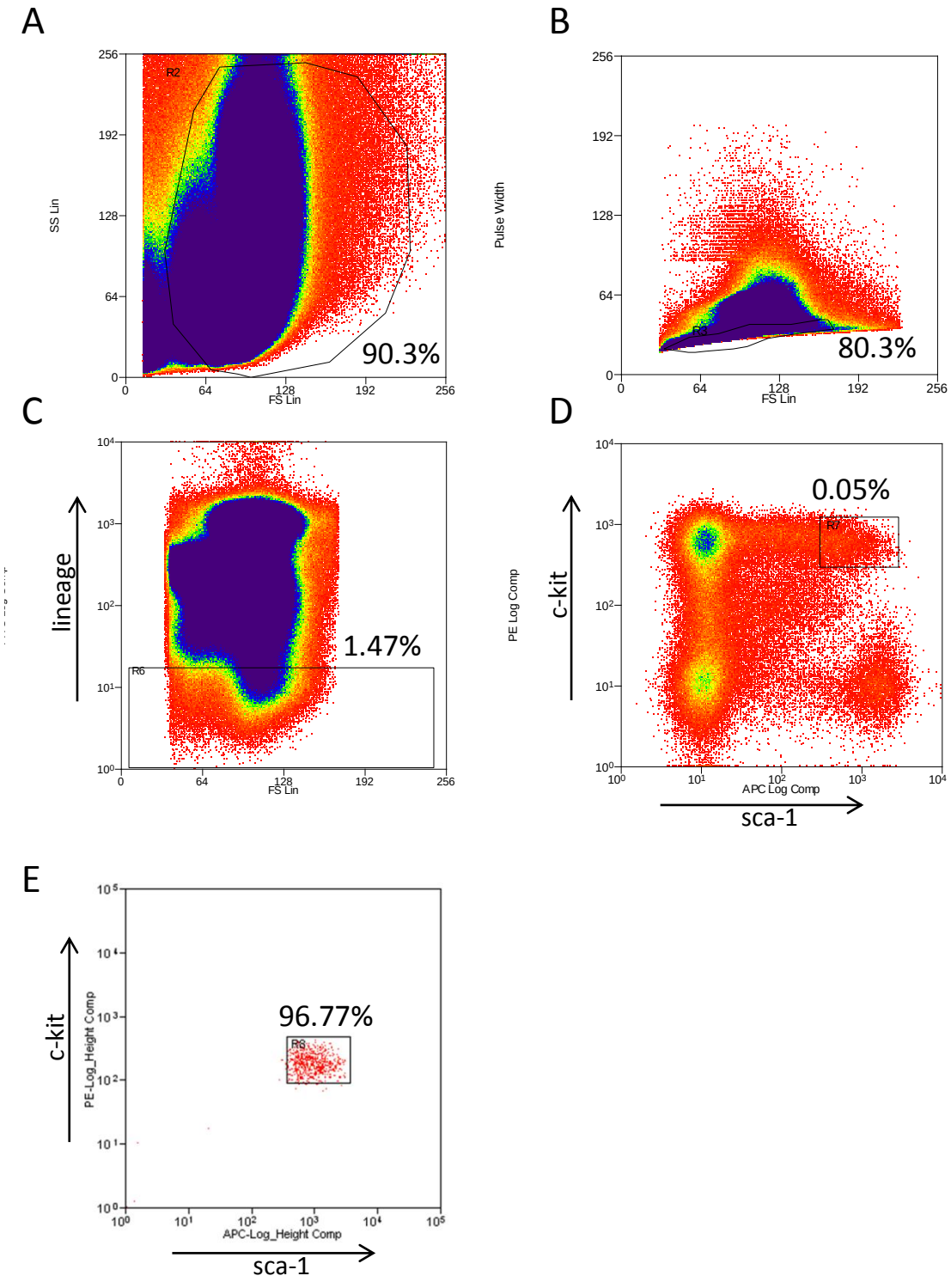


Figure 2.1
Isolation of c-kit+ sca1+ lineage- cells

Table 2.1 Antibodies used for Flow Cytometry

Antibody	Conjugate	Origin	Target	Isotype	Catalogue number	Manufacturer	Concentration Used
Isotype Control	FITC	Rat	Mouse	IgG2a	11-4321-82	eBioscience	1:50
Isotype Control	FITC	Rat	Mouse	IgG2b	11-4031-82	eBioscience	1:50
Isotype Control	APC	Rat	Mouse	IgG2a	17-4210-82	eBioscience	1:50
Isotype Control	Pacific Blue	Rat	Mouse	IgG2b	400627	BioLegend	1:50
Isotype Control	PE	Rat	Mouse	IgG2a	12-4210-82	eBioscience	1:50
Isotype Control	PE	Rat	Mouse	IgG2b	12-4211-82	eBioscience	1:50
Isotype Control	PE	Hamster	Mouse	IgG	12-4112-82	eBioscience	1:50
Isotype Control	PE	Rat	Mouse	IgG2a	IC006P	RnD Biosytems	1:20
Isotype Control	APC	Rat	Mouse	IgG2	17-4321-81	eBioscience	1:50
Isotype Control	PE	Rat	Mouse	IgG1	12-4031-82	eBioscience	1:50
Isotype Control	Alexa 647	Rat	Mouse	IgG	400526	Biolegend	1:50
Isotype Control	-	Rabbit	Mouse	IgG	sc-3888	Santa Cruz Biotech	1:100
Anti-CD11b	FITC	Rat	Mouse	IgG2b	11-6112-82	eBioscience	1:50
Anti-CD5	FITC	Rat	Mouse	IgG2a	11-6051-82	eBioscience	1:50
Anti-CD8a	FITC	Rat	Mouse	IgG2a	11-0081-82	eBioscience	1:50
Anti-Ter119	FITC	Rat	Mouse	IgG2b	11-5921-82	eBioscience	1:50
Anti-CD45R	FITC	Rat	Mouse	IgG2a	11-0452-82	eBioscience	1:50
Anti-Gr-1	FITC	Rat	Mouse	IgG2b	11-5931-82	eBioscience	1:50
Anti-CD117 (c-kit)	PE	Rat	Mouse	IgG2b	15-1171-82	eBioscience	1:50
Anti-CD117 (c-kit)	Pacific Blue	Rat	Mouse	IgG2b	105820	BioLegend	1:50
Anti-CD117 (c-kit)	APC-Cy7	Rat	Mouse	IgG2b	25-1171-82	eBioscience	1:50
Anti-Sca1	APC	Rat	Mouse	IgG2a	17-5981-82	eBioscience	1:50
Anti-CD127 (IL-7Ra)	PE	Rat	Mouse	IgG2b	12-1271-81	eBioscience	1:50
Anti-EDG1 (S1P1)	PE	Rat	Mouse	IgG2a	FAB7089T	RnD Biosytems	1:20
Anti-EDG1(S1P1)	-	Rabbit	Mouse	IgG	sc-25489	Santa Cruz Biotech	1:100
Anti-CD45	PE	Rat	Mouse	IgG2b	12-0451-81	eBioscience	1:50
Anti-CD45.1	PE	Mouse	Mouse	IgG2b	12-0453-81	eBioscience	1:50
Anti-CD45.2	APC	Mouse	Mouse	IgG2	17-0454-81	eBioscience	1:50
Anti-CD146	PE	Rat	Mouse	IgG1	12-1469-81	eBioscience	1:50
Anti-CCR2	PE	Rat	Mouse	IgG2b	FAB5538P	RnD Systems	1:10
Anti-CCR4	PE	Rat	Mouse	IgG2a	12-1949-80	eBioscience	1:50
Anti-CCR7	PE	Rat	Mouse	IgG2a	12-1971-80	eBioscience	1:50
Anti-CCR9	PE	Rat	Mouse	IgG2b	12-1991-80	eBioscience	1:50
Anti-CXCR2	Alexa 647	Rat	Mouse	IgG	129101	Biolegend	1:50
Anti-CXCR3	PE	Hamster	Mouse	IgG	12-1831-80	eBioscience	1:50
Anti-CXCR4	PE	Rat	Mouse	IgG2b	12-9991-80	eBioscience	1:50
Anti-CD16/32 Purified	-	Rat	Mouse		14-0161-82	eBioscience	1:50
Secondary	PE	Donkey	Rabbit	IgG	12-4739-81	eBioscience	1:200
LIVE/DEAD Cell Stain	Yellow	-	-	-	L34959	Invitrogen	1:200

2.8 Isolation of Liver Cells

Single cell suspensions of digested murine livers were prepared as previously described using collagenase digestion (Section 2.5). The resulting cell suspension was centrifuged slowly at 30g for 3 minutes to obtain a hepatocyte pellet which was resuspended in 350µl RLT buffer for RNA extraction. The remaining supernatant was used for isolation of liver sinusoidal endothelial cells.

To isolate liver sinusoidal endothelial cells the supernatant was centrifuged at 2000rpm for 5 minutes and resuspended in 20ml PBS + 1% FCS. In a 50ml tube 20ml of 23% Percoll was layered carefully onto 20ml of 50% Percoll to maintain a distinct interface between the two concentrations, on to this 10ml of the cell suspension was carefully added and the tubes centrifuged at 1300g for 10 minutes with no brake applied. The cells suspended at the interface between the Percoll concentrations were removed by manual pipetting and resuspended in PBS + 1% FCS, centrifuged at 2000rpm for 5 minutes and resuspended in 270µl PBS + 1% FCS and 30µl of anti-CD146 magnetic microbeads (Miltenyi Biotec). This was left to incubate for 20 minutes on ice followed by centrifugation at 2000rpm for 5 minutes and resuspended in 1ml PBS. Magnetically activated cell sorting (MACS, Miltenyi Biotec) MS columns were placed into MACS magnets and primed with 500µl PBS, 500µl aliquots of the magnetically labelled cell suspension were added to each column and washed through three times with 500µl PBS. The MACS MS columns were then removed from the magnets, and flushed through with 1ml PBS. The resulting cell suspension was collected, centrifuged at 2000rpm for 5 minutes ensuring all supernatant was removed and resuspended in RPMI media for culture or 350µl RLT lysis buffer for RNA extraction.

2.9 Isolation of Peripheral Blood Mononuclear Cells

A working dilution of Optiprep (Sigma) at 1.09g/ml was obtained by mixing neat Optiprep with PBS in a 4:11 ratio. In a 15ml conical tube 5ml of peripheral blood cell suspension was layered carefully over 5ml of Optiprep solution, this was centrifuged at 1050g for 25 minutes at 4oC with no brake applied to the centrifuge. Mononuclear cells were carefully aspirated from the interface between Optiprep and media, washed in PBS and centrifuged at 2000rpm for 5 minutes and the resulting cells resuspended in 350µl RLT buffer for RNA extraction.

2.10 Colony Forming Unit Assay

Quantification of the number of colony forming cells in cell populations was performed by culture of cells in semi solid methylcellulose based media. The media used was Methocult GF (StemCell Technologies, M3434) containing cytokines stimulating growth of myeloid cell colonies (CFU-GM, CFU-G, CFU-M, CFU-GEMM). The Methocult GF media contains 1% methylcellulose in IMDM, 15% fetal bovine serum, 1% bovine serum albumin, 10µg/ml recombinant human Insulin, 200µg/ml human Transferrin, 0.1mM 2-mercaptoethanol, 50ng/ml recombinant murine Stem Cell Factor, 10ng/ml recombinant murine IL-3, 10ng/ml recombinant human IL-6, 3U/ml recombinant human Erythropoietin and 2mM Glutamine.

Cell populations under investigation were prepared as previously described and resuspended in IMDM at a concentration ten fold higher than required in the assay. 0.3ml of cell suspension was added to 3mls of thawed Methocult GF media, vortexed well and left to stand for 5 minutes to allow air bubbles to dissipate. Using a blunt ended needle attached

to a 2ml syringe, 1.1ml of the cell containing media was added to a 35mm sterile petri dish and distributed evenly by gentle rotation. Assays were incubated at 37^{o}C 5%CO_2 inside 100mm petri dishes together with a separate 35mm petri dish containing 3ml sterile water to prevent dessication of the media. Incubation proceeded for 10-12 days followed by enumeration of individual colonies within each assay. Colonies were counted using an inverted microscope at low power magnification and to enable accuracy a square grid was printed onto transparent film and placed underneath the 35mm petri dish.

2.11 Pharmacological Agents

S1P and FTY720-phosphate

Sphingosine 1-phosphate and FTY720-phosphate (Cayman Chemicals) were dissolved in 95% Dimethyl sulphoxide / 5% 1N Hydrochloric Acid (Sigma) to form a 20mM stock solution, aliquoted and stored at -20^{o}C. Prior to use further dilutions were made in PBS containing 3% fatty acid free bovine serum albumin (Sigma) to form a 1mM solution which was then diluted to the appropriate concentration in cell culture media for use in experiments.

FTY720 – In vivo

FTY720 (Cayman Chemicals) was dissolved in sterile Dimethyl sulphoxide (Sigma) to form a 10mg/ml stock solution which was aliquoted and stored at -20oC. Immediately prior to use aliquots were thawed and diluted in phosphate buffered saline to a concentration of 0.2µg/µl ready for injection.

W146

W146 hydrate (Sigma Aldrich) was dissolved in Methanol (Sigma) containing 0.05% Acetic Acid (Sigma) to form a 3mM stock solution and stored at -20oC. Stock solution was diluted in cell culture media for use in experiments.

2.12 Transwell Migration Assays

A Transwell system (Corning Costar)was used to measure cell migration in response to chemoattractants. In this assay transwell supports are placed into standard cell culture plates thus separating the well into two, an upper compartment and a lower compartment with a permeable membrane between the two compartments. The membrane contains pores of the appropriate size for the cell type being studied. Cells are placed in the upper compartment and media with or without the chemoattractant to be studied is place in the lower compartment, the wells are incubated and at the end of the designated time period the number of cells that have migrated to the lower chamber is enumerated.

For migration assays using both primary KSL cells and HPC7 cells, 6.5mm 12 well plate Transwell inserts with a 5µm pore diameter were used (Corning Costar). 0.6ml of serum free media (StemPro 34 for HPC7 and DMEM + 0.1%BSA for primary KSL cells) containing the relevant concentration of chemoattractant was placed in individual wells of a 12 well plate, media alone was used as the negative control and in some experiments 100ng/ml SDF-1 was used as a positive control. The transwell inserts were then carefully placed into each well containing media to avoid the formation of air bubbles beneath the membrane. Cells were washed and resuspended at $1x10^6$ cells/ml in the relevant serum free media and 100µl of

cell suspension added to each transwell insert, with 100µl of cell suspension retained in order to calculate numbers of input cells. In primary KSL cell assays, lin- bone marrow cells were isolated from bone marrow using techniques described (Section 2.4) and used as the input cell population. The lin- fraction is 10-fold more enriched for KSL cells than whole bone marrow. The wells were incubated at 37^{o}C 5%CO_2 for 3 hours allowing migration to proceed. The inserts were then carefully removed from the 12 well plate and the contents of each individual well transferred to a round bottomed tube. In HPC7 assays 20µl of Trucount counting beads (Invitrogen) was added to each tube and the numbers of migrated cells in each well together with the number of input cells was determined and migration as a percentage of input cells was calculated. The contents of each well was centrifuged and resuspended in 100µl PBS + 1%FCS and labelled with anti-sca1 and anti-c-kit antibodies (Table 2.1) using the flow cytometry protocol (Section 2.7) with the addition of 20µl Trucount counting beads and the numbers of c-kit+ sca1+ cells migrating was determined and migration as a percentage of input cells was determined.

2.13 Protein Extraction and Assay

Samples of frozen tissue (approx 80mg) were weighed, placed in a GentleMACS M tube (Miltenyi Biotec) with 20µl/mg ice cold Cell Lytic MT buffer (Sigma) containing 1% protease inhibitor cocktail (Sigma) and 5U/ml DNAse (Qiagen) and processed on programme *Protein-01*. The suspension was then centrifuged at 3000g for 3 minutes and the supernatant agitated on a Vibraplatform for 1 hour at 4^{o}C and centrifuged at >8000g for 30 minutes. The supernatant was then aliquoted and protein concentration determined using the Bradford

protein assay. The Bradford reagent (Sigma) contains Brilliant Blue G dye which forms complexes with proteins in solution resulting in a shift in light absorbance proportional to the concentration of protein. A series of protein standards were prepared by dilution of bovine serum albumin (Sigma) and 5µl of either standard or sample were added to individual wells of a 96 well plate, followed by the addition of 250µl Bradford reagent. The plate was agitated and allowed to incubate for 5 minutes, absorbance at 595nm was then determined using a spectrophotometer. Protein concentration in individual samples was determined from the standard curve (Figure 2.2).

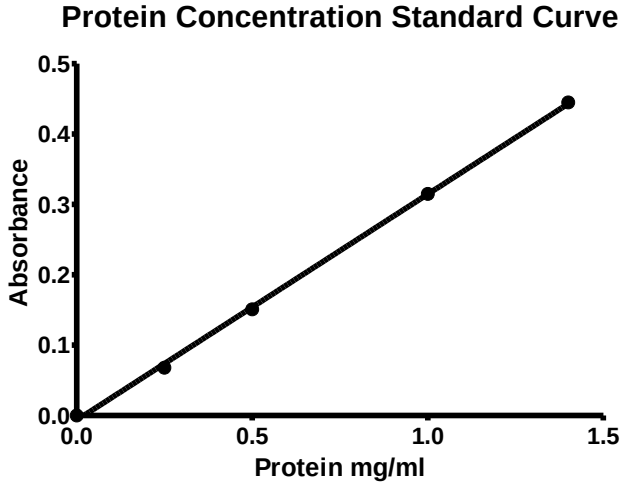


Figure 2.2 Standard Curve for determination of protein concentration using the Bradford Assay

2.14 Western Blotting

The protein samples were first separated by SDS polyacrylamide gel electrophoresis, stacking and resolving gels (Table 2.2) were placed between glass plates (BioRad) to construct a 1.5mm thick gel, inserted into the plate holder and placed in the electrophoresis tank. Electrophoresis running buffer was poured into the tank to eliminate air bubbles from the top of the gel and to cover the electrodes. Protein samples were diluted to 5µg/µl and 10µl (50µg) added to 40µl SDS-PAGE loading buffer and heated for 5 minutes at 100^{o}C. 20µl of the sample was added to each well of the stacking gel and 10µl of Precision Plus Protein molecular weight marker (BioRad) was added to the end well, electrophoresis at 200V continued for 0.5 -1 hour allowing the protein to reach the bottom of the resolving gel. The protein was transferred to Hybond ECL nitrocellulose membrane (Amersham) by placing the gel and membrane between filter paper and sponges inside a transfer cassette (BioRad). The cassette was placed in an electrophoresis transfer tank filled with transfer buffer and containing an ice pack and electrophoresis at 100V proceeded for 1 hour. The membranes were then blocked to avoid non specific antibody binding by soaking in a 5% non fat milk solution (10g milk powder in 20mls PBS + 0.02% Tween) for 1 hour at room temperature followed by the addition of the primary antibodies (Table 2.3) and incubated overnight at 4^{o}C. The membrane was washed three times in PBS-Tween and the secondary antibody diluted in PBS-Tween added, incubated for 1 hour at 4^{o}C and washed for 30 minutes in PBS-Tween. Pierce ECL Western Blotting Substrate (Thermo Scientific) was added to the membrane for 2 minutes following which the membrane was blotted with tissue and placed in a film cassette. Hyperfilm ECL (Amersham) was placed in the film cassette and after exposure developed in an X-Omat automated processor.

The membranes were stripped for reanalysis by incubating in stripping buffer for 45 minutes at 50^{o}C followed by 2 hours of continuous washing under running water. The stripped membrane was then probed using the same protocol commencing with blocking with 5% milk solution.

Table 2.2 Reagents used for Western Blotting

Reagent	Components
Electrophoresis Buffer	Per litre - 30.3g Trizma base, 144g Glycine, 10g Sodium Dodecyl Sulphate (all Sigma Aldrich)
Transfer Buffer	Per litre – 14.4g Glycine, 3g Trizma base, 200ml Methanol (Fisher Scientific), 0.5g Sodium Dodecyl Sulphate (otherwise Sigma Aldrich)
Stripping Buffer	Per 100ml – 67.5ml distilled water, 20ml 10% Sodium Dodecyl Sulphate, 12.5ml 0.5M Trizma base, 0.8ml 2-mercaptoethanol (all Sigma Aldrich)
Loading Buffer	200mM Trizma Base pH 6.8, 20% Glycerol, 10% Sodium Dodecyl Sulphate, 0.05% bromophenol blue, 10µM B-mercaptoethanol
4% Stacking Gel	Per gel – 6.1ml distilled water, 1.3ml 30% Degassed Acrylamide/Bis, 2.5ml 0.5M Tris-HCl pH 6.8, 0.1ml 10% Sodium Dodecyl Sulphate THEN ADDED 40ul 10% Ammonium Persulphate and 20µl N,N,N',N'-Tetramethylethylenediamine
15% Resolving Gel	Per gel – 2.4ml distilled water, 5.0ml 30% Degassed Acrylamide/Bis, 2.5ml 1.5M Tris-HCl pH 8.8, 0.1ml 10% Sodium Dodecyl Sulphate THEN ADDED 40ul 10% Ammonium Persulphate and 20µl N,N,N',N'-Tetramethylethylenediamine

Table 2.3 Antibodies used for Western Blotting

Antibody	Origin	Target	Isotype	Catalogue no.	Supplier	Dilution
Anti- SphK1	Rabbit	Mouse/Human	IgG	ab16491	Abcam	1:500
Anti-GAPDH	Rabbit	Mouse/Human	IgG	G8795	Sigma Aldrich	1:2000
Anti-Rabbit IgG HRP conjugated	Goat	Rabbit	IgG	A6154	Sigma Aldrich	1:10000

2.15 RNA Extraction

RNA Isolation was performed using the RNeasy Mini Kit (Qiagen) for all samples other than primary KSL cells, when the RNeasy Micro Kit (Qiagen) was used.

For RNA isolation from tissue, approximately 30mg of the relevant tissue was placed in a GentleMACS M tube (Miltenyi Biotec) together with 600µl of RLT buffer supplemented with 10µl/ml 2-mercaptoethanol (Sigma). Samples were processed on the Miltenyi GentleMACS processor using program *RNA.01_01* producing a tissue homogenate ready for RNA extraction.

For RNA isolation from cell suspensions, cells were harvested by centrifugation for 5 minutes at 2000rpm and complete aspiration of the cell media. The resulting cell pellet was resuspended in 600µl RLT buffer supplemented with 10µl/ml 2-mercaptoethanol. For RNA isolation from primary KSL cells, cells were sorted directly into 350µl RLT buffer. This cell suspension was passed through a 21-gauge needle attached to a 1ml syringe multiple times to ensure complete cell lysis and membrane disruption.

For all samples one volume of 70% Ethanol was added and immediately mixed by pipetting and this mixture transferred to an RNeasy Spin Column and centrifuged at >10,000 rpm for 15 seconds. The column flow through was discarded and 350µl RW1 buffer was added to the spin column and centrifuged at >10,000 rpm for 15 seconds. 80µl of RNase free DNase solution was then added directly to the spin column membrane and left to incubate for 15 minutes at room temperature. A further 350µl RW1 buffer was added to wash the spin column and centrifuged at >10,000 rpm for 15 seconds. 500µl RPE buffer was twice added to the spin column and centrifuged at >10,000 rpm firstly for 15 seconds then for 2 minutes.

The columns were transferred to a new collection tube and centrifuged for 1 minute to allow solvent to evaporate from the membrane, after which 40µl of RNase free water was added to the membrane and centrifuged at >10,000 rpm for 1 minute to elute the RNA.

All RNA samples were assessed for quantity and purity of RNA using a Nanodrop spectrophotometer. Samples were used for cDNA synthesis if the ratio of absorbance at 260nm (A260) to absorbance at 280nm (A280) was close to 2.0, indicating low levels of genomic DNA contamination. RNA samples were aliquoted and stored at -80^{o}C pending use in further applications.

2.16 cDNA Synthesis

Each cDNA synthesis reaction was run in a thin walled PCR tube and consisted of 1µg RNA template added to 4µl iScript Reaction Mix and 1µl iScript Reverse Transcriptase (Bio Rad) and made up to a total volume of 20µl with RNase DNase free water (Qiagen). The reaction mix was incubated in a thermal cycler at 25^{o}C for 5 minutes followed by 30 minutes at 42^{o}C and finally the RT was inactivated by incubation at 85^{o}C for 5 minutes. The reaction was then cooled to 10^{o}C and either used immediately for further applications or stored at -80^{o}C.

2.17 Quantitative PCR

Polymerase chain reaction is a process by which samples of DNA undergo geometric amplification, yielding many copies of the original DNA sequence. The reaction is catalysed by a heat stable DNA polymerase (Taq polymerase). Taqman gene expression assays were used in this work. Taqman fluorescent probes have the reporter dye FAM at the 5' end and the quencher MGB at the 3' end. The intact probes do not fluoresce as the reporter and quencher are in close proximity. As the reaction mixture cools during each cycle after the denaturation step the probe is able to hybridise with its target sequence. During the elongation step which follows the polymerase encounters the bound probe and the 5' nuclease activity of the enzyme separates the reporter from the quencher, thus rendering the reporter fluorescent. The free reporter molecules are excited by the detection instrument which detects emission of fluorescence. The amount of fluorescence is proportional to the number of copies of the target sequence.

Individual Taqman Gene Expression Assays used in this work are listed in Table 2.4. Each of these assays were inventoried and validated by the manufacturer. Where possible exon spanning assays were used to avoid interference from contaminating genomic DNA.

In order to assess gene expression of multiple chemokine receptors, a custom 96 well plate was designed and ordered (Chemokine AK). This comprised gene expression assays for the chemokine receptors together with endogenous control genes and allowed rapid analysis of multiple genes in small numbers of samples.

Each individual PCR reaction was performed in a total volume of 20µl comprising 4µl cDNA sample (containing 20ng cDNA), 1µl target Taqman Gene Expression Assay, 10µl Universal

Master Mix (Applied Biosystems) and 5µl RNase DNase free water. Reactions were performed on a 96 well PCR plate (ABI). Each reaction on the Custom Taqman 96 Well Plate (Chemokine AK) consisted of 5µl cDNA sample (containing 10ng cDNA), 10µl Universal Master Mix and 5µl RNase DNase free water added to each well of the plate.

PCR using Individual Assays was performed on a Stratagene MX3000p instrument and on an ABI Prism 7900HT instrument for the custom plate. The programme used consisted of polymerase activation at 95^{o}C for 10 minutes followed by 40 cycles of denaturing (95^{o}C for 15 seconds) and annealing (60^{o}C for 1 minute). The fluorescence of each reaction was recorded at the end of each cycle. Each reaction was performed in triplicate and control reactions which did not contain any cDNA template were also included. Cycle threshold (cT) values were obtained and the difference between the reference and target gene calculated (ΔcT = cT target – cT reference). Relative differences between samples was expressed using the $2^{-\Delta\Delta CT}$, where ΔΔcT = ΔcT sample1 – ΔcT sample2. The use of the ΔΔcT method to determine relative differences between samples under varying conditions is reliant upon there being no differences in the amplification efficiencies of the target and reference genes. Validation experiments were performed to examine whether ΔcT values differed across a range of cDNA template concentrations from the same sample. ΔcT was plotted against the log of cDNA amount in each reaction and the value of the gradient of the resulting semi-logarithmic regression line was obtained. It is generally accepted that a slope value <0.1 signifies a valid experiment with no significant difference in amplification efficiency. All validation experiments performed using the Taqman gene expression assays met this criterion.

Table 2.4 Gene Expression Assays used for PCR

Target (Murine)	Gene Expression Assay
Sphingosine Kinase 1	Mm00448841_g1
Sphingosine Kinase 2	Mm00445021_m1
Sphingosine Lyase	Mm00486079_m1
Sphingosine Phosphatase	Mm00473016_m1
Sphingosine 1-phosphate Receptor 1	Mm02619656_s1
Sphingosine 1-phosphate Receptor 2	Mm03039030_m1
Sphingosine 1-phosphate Receptor 3	Mm02620181_m1
Sphingosine 1-phosphate Receptor 4	Mm00468695_s1
Sphingosine 1-phosphate Receptor 5	Mm02620565_s1
Alpha Smooth Muscle Actin	Mm00725412_s1
Collagen 1 alpha 1	Mm00483387_m1
Glyceraldehyde 3-phosphate Dehydrogenase	Mm99999915_g1

Target (Human)	Gene Expression Assay
Sphingosine Kinase 1	Hs01116530_g1
Sphingosine Kinase 2	Hs01016543_g1
Sphingosine Lyase	Hs00187407_m1
Sphingosine Phosphatase	Hs00229266_m1
Glyceraldehyde 3-phosphate Dehydrogenase	Hs02758991_g1

All gene expression assays were supplied from Applied Biosystems.

Table 2.4 contd. Gene Expression Assays used for PCR (continued)

Target	Gene Expression Assay
CCR1	Mm00438260_m1
CCR2	Mm99999051_gH
CCR3	Mm00515543_s1
CCR4	Mm99999052_s1
CCR5	Mm01963251_s1
CCR6	Mm99999114_s1
CCR7	Mm01301785_m1
CCR8	Mm99999115_s1
CCR9	Mm02620030_s1
CCR10	Mm01292449_m1
CXCR1	Mm00731329_s1
CXCR2	Mm99999117_s1
CXCR3	Mm99999054_s1
CXCR4	Mm99999055_m1
CXCR5	Mm00432086_m1
CXCR6	Mm02620517_s1
CXCR7	Mm00432610_m1
CX3CR1	Mm02620111_s1
GAPDH	Mm99999915_g1
B-Actin	Mm00607939_s1
18S	Hs99999901_s1

All gene expression assays were supplied from Applied Biosystems.

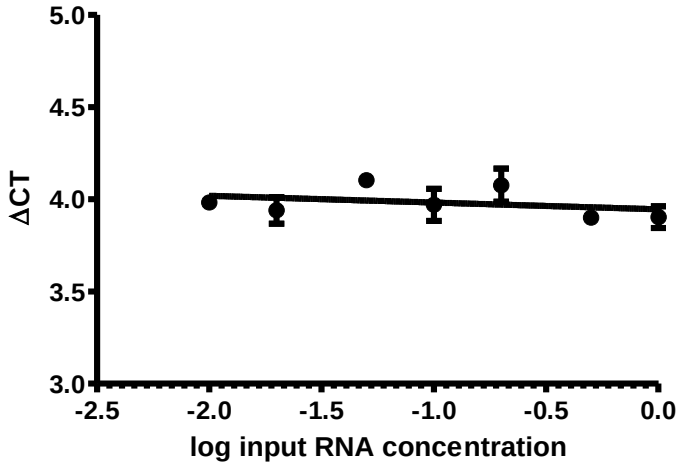


Figure 2.3 Validation plot of gene assay amplification efficiency

RNA was isolated from liver tissue of mice with chronic carbon tetrachloride liver injury and cT values for SphK1 (target) and GAPDH (control) de termined across a range of RNA concentrations. ΔcT was calculated as cT_{target} - $cT_{control}$ and plotted against RNA concentration. The gradient of the resulting regression line was calculated as -0.036 ,demonstrating no variation in amplification efficiency.

2.18 Immunohistochemistry

Frozen tissue was embedded in OCT mountant (Tissue Tek) and 5µm thick sections cut on a cryostat (by Mrs.Janine Youster). Sections were mounted on glass microscope slides coated with 0.01% Poly-L-Lysine (Sigma), fixed by immersing in Acetone (Sigma) for 5 minutes and stored at -20^{o}C until further use.

Formalin fixed tissue was placed in tissue cassettes and embedded in paraffin wax to construct tissue blocks stored at room temperature. 4µm sections were cut from paraffin embedded tissue blocks (by Mrs. Jean Shaw) and mounted using a heated water bath onto glass microscope slides and stored at room temperature for subsequent use. Staining of

formalin fixed paraffin embedded sections started with dewaxing the sections through a series of graded alcohols and washing in deionised water. Sections were then placed in Methanol (Analat) containing 0.3% hydrogen peroxide (Sigma) to quench endogenous peroxidise activity followed by washing three times in PBS Tween. Antigen retrieval was then performed with a citrate buffer, prepared by adding 1.92g anhydrous Citric Acid (Sigma) to 1000ml distilled water and adjusted to pH 6.0 using 1M sodium hydroxide solution, this was then heated to 95-100^{o}C using a microwave oven, the sections were immersed and heated for 20 minutes. The sections were allowed to cool slowly to room temperature and then washed three times in PBS Tween. Non specific binding of the antibodies to the sections was blocked by incubating the sections for 30 minutes with 10x Casein solution (Vector) diluted in PBS. The casein solution was tipped off the slide and the primary antibody was added diluted in PBS to the appropriate concentration (Table 2.5) and incubated for 1 hour at room temperature in a humidified chamber on a rocker. The sections were then washed three times in PBS Tween followed by addition of the relevant horse radish peroxidise conjugated secondary antibody (ImmPRESS Peroxidase Anti-Rabbit IgG or ImmPRESS Peroxidase Anti-Rat (Mouse Adsorbed) IgG, Vector Labs) for 30 minutes. Diaminobenzidine (DAB) was used as the peroxidase substrate and sections were incubated with ImmPACT DAB reagent (VectorLabs). Sections were then washed once in water and counterstained with Mayer's haematoxylin for 1 minute, washed again in water, dehydrated through graded alcohols and coverslips mounted using DPX mountant.

Frozen sections were fixed by immersion in Acetone for 20 minutes followed by blocking of endogenous peroxidase activity with Methanol containing 0.3% hydrogen peroxide for 20 minutes. The sections were washed three times in Tris buffered Saline pH 7.6. Non specific

binding was blocked by incubating the sections with 10x Casein solution diluted in PBS for 30 minutes and then the relevant primary antibody (Table 2.5) was added and incubated for 1 hour at room temperature in a humidified chamber on a rocker. The sections were then washed three times in PBS followed by addition of the relevant horse radish peroxidise conjugated secondary antibody (ImmPRESS Peroxidase Anti-Rabbit IgG or ImmPRESS Peroxidase Anti-Rat (Mouse Adsorbed) IgG, Vector Labs) for 30 minutes. Diaminobenzidine (DAB) was used as the peroxidase substrate and sections were incubated with ImmPACT DAB reagent (VectorLabs). Sections were washed in distilled water for 5 minutes and counterstained with Mayer's Haematoxylin for 1 minute, washed with water and coverslips mounted

Table 2.5 Antibodies used for Immunohistochemistry

Antibody	Origin	Target	Isotype	Catalogue	Supplier	Dilution	Antigen Retrieval
Anti - aSMA	Rabbit	Mouse	IgG	ab5694	Abcam	1:400	Citrate, pH6.0
Anti- F4/80	Rat	Mouse	IgG2a	MCA497R	AbD Setrotec	1:200	Citrate, pH6.0
Anti-Ly6G	Rat	Mouse	IgG2a	MCA2387T	AbD Serotec	1:500	N/A (Frozen sections)
Anti-CD45.2	Mouse	Mouse	IgG	14-0454-82	eBioscience	1:200	N/A (Frozen sections)

2.19 S1P Quantification

A competitive S1P ELISA assay (Echelon Bioscience) was used for quantification of S1P concentrations in tissue and serum samples. The ELISA assay performs well when compared with high pressure liquid chromatography giving consistently similar results (Kirby *et al.* 2009) (personal communication with Prof L. Arend). Liver and Bone Marrow samples were homogenised in a buffer containing 50mM Trizma-Hydrochloric Acid buffer pH 7.4, 1mM EDTA, 0.1mM potassium chloride, 10% Glycerol and 1% protease inhibitor. The protein content of each homogenate was determined by Bradford assay and stored at -20^{o}C. Serum and lymph samples were diluted 1:10 with delipidised human serum (Echelon).

150µl of block solution (K-1903, Echelon) was added to each well of the 96 well plate, sealed and incubated at room temperature for 1 hour. The plate was then washed 4 times with phosphate buffered saline.

Dilutions of S1P standards (0, 0.06, 0.13, 0.25, 0.5, 1.0, 2.0 µM) were prepared by diluting 100µM S1P stock solution in delipidised human serum. 63µl of anti-S1P antibody (K-1901, Echelon) was added to each well of a mixing plate together with 188µl of either sample or S1P standard, the plate was mixed on a plate shaker for 1 minute.

100µl of the mixture from each well of the mixing plate was placed in duplicate in the coated and washed plate, sealed and incubated at room temperature for 1 hour. The plate was then washed 4 times with PBS and 100µl of streptavidin-HRP was added to each well, sealed and incubated for 1 hour. After again washing 4 times with PBS, 100µl TMB substrate was added to each well and the plate incubated in the dark for 30 minutes, 50µl of H_2SO_4 stop solution was added to each well and light absorbance at 450nm recorded. A standard curve

was constructed using the absorbance readings of the S1P standards (Fig 2.4). S1P concentrations in serum and lymph were expressed as molar quantities and in liver and bone marrow were normalised to total protein content and expressed as pmol of S1P per mg of protein.

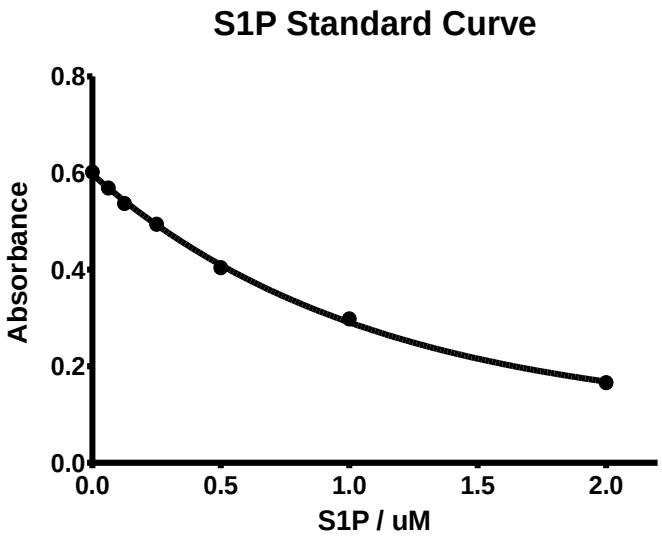


Figure 2.4 Standard curve for S1P ELISA

2.20 Sphingosine Kinase Activity Assay

Sphingosine Kinase activity was quantified using an adenosine triphosphate (ATP) depletion assay. The reaction consists of liver homogenate (prepared and protein content quantified as above) as the source of SphK, sphingosine added as the substrate and initiated by the addition of ATP. The reaction is stopped by the addition of ATP detector which quantifies the remaining ATP levels in solution, the resulting luminescent signal is inversely correlated with SphK activity.

Each sample reaction was performed in a 40µl volume, consisting of 10µl of tissue sample homogenised in S1P assay buffer (total protein content approx 30mg), 10µl of 100µM sphingosine (K-4401, Echelon) and 20µl 10µM ATP (K-ATP1, Echelon). Each ATP standard contained 10µl of tissue homogenisation buffer, 10µl of 100µM sphingosine and 20µl of each ATP dilution (0, 0.15625, 0.3125, 0.625, 1.25, 2.5, 5, 10 µM). The reaction was started by the addition of the ATP to the sample wells and the plate sealed, mixed and incubated at room temperature for 2 hours. 40µl of luminescent ATP detector (K-LUMa, Echelon) was added to each well, mixed and incubated for a further 10 minutes in the dark. The total luminescent signal from each well was recorded. A standard curve using the ATP standards was constructed and used to determine the quantity of S1P produced from the residual amount of ATP in each reaction (Fig 2.5). The results from each reaction were normalised to total protein content and time and expressed as pmol of S1P produced per mg of protein per minute.

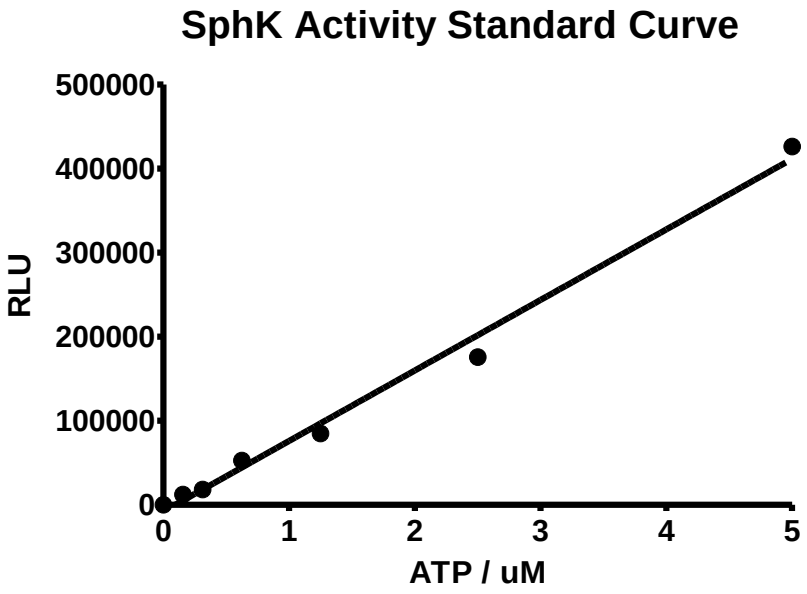


Figure 2.5 Sphingosine Kinase Activity Assay Standard Curve

2.21 Intravital Microscopy

In these experiments anaesthesia and operative surgery was performed by Dr .Dean Kavanagh (Centre for Cardiovascular Sciences, University of Birmingham), who also provided guidance on cell counting techniques.

Intravital Microscopy was performed on C57/BL6 mice which had undergone 6-8 weeks of carbon tetrachloride administration to induce chronic liver inury. Mice were anaesthetised by intraperitoneal administration of Ketamine (Vetalar, 100mg/kg) and Xylazine hydrochloride (10mg/kg) in 0.9% Saline. Further administration was delivered as required. Cannulation of the right common carotid artery using polyethylene portex tubing was performed to enable cell delivery. Laparotomy was performed followed by removal of the rectus abdominis muscles and transection of the falciform ligament allowing exteriorisation of the liver. The mouse was placed on a transparent dish and placed onto the stage of the inverted microscope. One x10 power magnification field of view (approx 400 x 300 µm) of the exteriorised liver was selected for imaging, containing at least one post sinusoidal venule and sinusoidal capillaries. The same field of view was used for all recordings.

HPC-7 cells were counted and $1x10^6$ cells were labelled with the fluorescent dye concentration 5µM CFSE. $1x10^6$ cells were centrifuged at 2000rpm for 5 minutes and reuspended in 2ml prewarmed PBS + 0.1% BSA and 2ml 10µM CFSE solution was added giving a final staining concentration of 5µM. After incubating at room temperature in the dark for 10 minutes, 6ml Stem Pro 34 SFM media was added and centrifuged at 2000rpm for 5 minutes. Cells were resuspended in Stem Pro 34 media until immediately prior to infusion

when they were centrifuged at 2000rpm for 5 minutes and resuspended in 100µl PBS for injection. Labelled cells were injected intra arterially in a volume of 100µl.

Video recordings lasting one minute were taken every five minutes from the time of injection until 60 minutes after the injection of cells. At the conclusion of monitoring, six further fields of view were imaged to ensure that findings observed in the selected field of view were representative of other areas of the liver. Video recordings were stored and analysed using Slidebook software (Intelligent Imaging Innovations).

Quantification of the recruitment and adhesion of labelled cells to the liver was determined by counting the numbers of labelled cells within the monitored hepatic field of view. Cells that remained static within the field of view for more than 30 seconds were considered adherent and those static for less than 30 seconds were considered free flowing. Recruitment of cells to the liver was quantified as the number of adherent labelled cells per field of view.

2.22 Hydroxyproline Assay

A representative tissue sample of the liver (50-100mg) was used to determine the hydroxyproline content of the liver. The liver sample was accurately weighed and placed in a 1.5ml centrifuge tube (Eppendorf), 1ml of ice cold distilled water was added and the tissue was homogenised with a hand held Polytron homogeniser (Sigma Aldrich). 125µl 50% Trichloroacetic Acid :50% Water (Sigma Aldrich) was added and the sample incubated on ice for 30 minutes. The protein was then precipitated by centrifugation in a bench top microcentrifuge for 5 minutes at 13,000rpm. The resultant pellet was resuspended in 500µl

6N Hydrochloric Acid (Sigma Aldrich) and transferred to a 12x75mm borosilicate glass tube tightly sealed with a screw top lid containing a rubber insert (Sigma Aldrich). The samples were placed in an oven at 120^{o}C for 16 hours, the lids of the tubes were then carefully removed and the samples placed back in the oven at 80^{o}C until completely dessicated (usually 48 hours). The dessicated precipitate was resuspended in 500µl distilled water and vortexed vigorously, this was then filtered by adding to the top chamber of a centrifuge filter tube (Costar, 0.45µm nylon membrane, polypropylene tube). In a clean borosilicate tube 50µl of either the filtered solution or of hydroxyproline standard (Sigma Aldrich, diluted in 0.001N hydrochloric acid) was added to 950µl of distilled water. Chloramine-T solution was made by dissolving 1.75g chloramine-T (Sodium N-chloro-p-toluenesulfonamide, Sigma Aldrich) in 10ml distilled water, 10ml 2-propanol and 80ml buffer (25g citric acid monohydrate, 6ml glacial acetic acid, 60g sodium acetate trihydrate, 17g sodium chloride dissolved in 500ml water, adjusted to pH 6.0 with the addition of sodium hydroxide pellets). 500µl of Chloramine-T solution was added to each sample, vortexed and incubated at room temperature for 20 minutes. Ehrlich's solution (p-dimethylaminobenzaldehyde perchloric acid) was made by adding 30ml 2-propanol to 7.5g p-dimethylaminobenzaldehyde, slowly mixing in 13ml 60% Perchloric Acid and bringing the total volume to 50ml with 2-propanol. 500µl Ehrlich's solution was added to each sample, vortexed and incubated in a 65^{o}C heated water bath for 15 minutes. The samples were then cooled to room temperature, 200µl of each sample and standard was placed in triplicate in a 96 well plate and a reading of light absorbance at 561nm taken. A standard curve (Fig 2.6) was created using absorbance readings of the standards (0, 0.125, 0.25, 0.5, 1.0, 2.0, 5.0 mcg hydroxyproline). Using the

previously determined weights of the samples the hydroxyproline content was corrected and expressed as micrograms of hydroxyproline per gram of liver tissue.

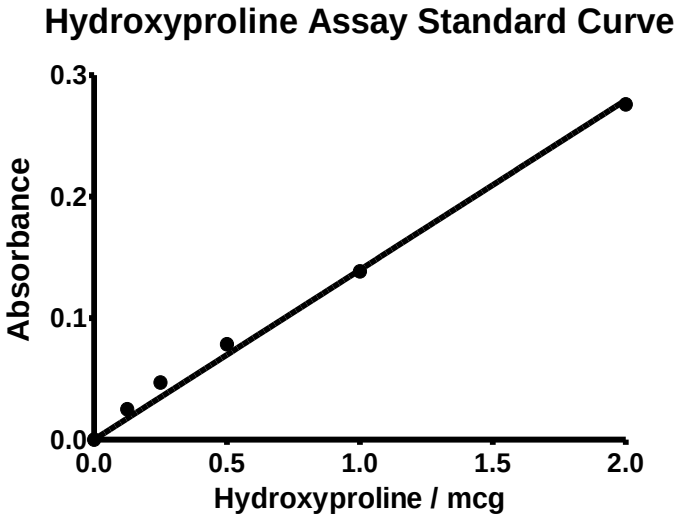


Figure 2.6 Hydroxyproline Standard Curve

2.23 Picrosirius Red Staining of Liver Sections

Formalin fixed paraffin embedded sections of liver tissue cut at 5µm thickness were used for Picrosirius Red staining. Tissue sections were dewaxed through a series of graded alcohols and washed in water. The sections were incubated with 0.5% polymobidic acid (Sigma) for 5 minutes and then immediately incubated with Picrosirius Red solution (100mg Direct Red 80 (Sigma) in 100ml saturated Picric Acid) for 3 hours. The sections were washed three times in 100% Ethanol and coverslips mounted using non aqeuos DPX mountant.

2.24 Morphometric Quantification of Liver Tissue Sections

For quantification of Picrosirius Red and αSMA staining a series of 10 random, non overlapping fields of view from each section were imaged at x20 magnification using Axiovision software. Using ImageJ software (NIH) the area of positive staining was determined and expressed as a percentage of the total area of the field of view.

For quantification of specific cell numbers positive for either F4/80 or Gr-1, the number of positively stained cells in each field of view were manually counted. 6 random, non overlapping fields of view from each section were counted and quantified as the numbers of positively stained cells per field of view.

2.25 Albumin assay

Serum albumin concentrations were measured using the Quantichrom BCG Albumin assay kit (Bioassay Systems). Bromcresol green (BCG) forms a coloured complex with albumin with the intensity being proportional to the concentration of albumin. Albumin standards were made by diluting BSA with distilled water and 5µl of each standard or 5µl of each sampled was added to the individual wells of a 96 well plate (Corning Costar). 200µl of supplied BCG solution was added to each well and incubated for 5 minutes at room temperature. The optical density of each well of the plate was measured at 620nm absorbance. A standard curve was constructed for each individual experiment and the concentration of albumin in each serum sample was determined.

2.26 DiR labelling

DiR (DilC18(7), 1,1'-dioctadecyltetramethyl indotricarbocyanine Iodide, MW 1013.4)(Invitrogen) was used to fluorescently label cells prior to use in cell tracking experiments. DiR is a near infra red fluorescent cyanine dye with lipophilic properties whose fluorescence is enhanced by incorporation into cell membranes or binding to lipophilic molecules but remains weakly fluorescent in water. When applied to cells the dye diffuses laterally within the cell membrane allowing even staining of the entire cell. The excitation wavelength of DiR is 748nm and emission wavelength 780nm which means it is particularly suited to tissue and in vivo imaging as near infrared light transmits well through tissues and less tissue autofluorescence is observed at higher light wavelengths.

Stock solution was prepared by dissolving 10mg DiR in 10ml Methanol and stored in the dark at room temperature. Cells were resuspended in complete Stem Pro media at $1x10^6$ cells/ml and 5µl/ml of DiR stock solution added to create a staining solution of 5µM DiR. The cell suspension was incubated for 30 minutes in the dark at 37^oC 5%CO_2. Cells were then washed in sterile PBS and centrifuged at 2000rpm for 5 minutes three times, and resuspended in sterile PBS for injection (volume 100-200µl).

2.27 Near Infra Red Fluorescent Imaging

Fluorescence is the result of a process whereby susceptible molecules (such as polyaromatic hydrocarbons or heterocycles) known as fluorophores or fluorescent dyes absorb excitation photons from light at a specific wavelength from an external source (such a lamp or a laser) raising their energy levels to an excited, unstable state. As these fluorophores decay from the excited state they emit fluorescent light at a characteristic wavelength determined by the difference in energy levels between the resting and excited state. The emission wavelength is always longer than the excitation wavelength due to loss of energy as heat during the excited state prior to emission of fluorescent light.

Fluorescent imaging of animal and tissue samples requires excitation photons to travel through tissue to reach the fluorescent dye. Tissue that absorbs light may also emit fluorescent light in the absence of a fluorescent dye – known as autofluorescence. Fluorescent dyes which absorb and emit light of lower wavelengths (400-600nm) are limited by poor tissue penetration of excitation photons due to absorbance by pigmented substances such as haemoglobin and deoxyhaemoglobin and by water and lipids(Sutton *et*

al. 2008). Tissue autofluorescence, particularly from skin, small intestine, gall bladder and bladder are particularly high at these wavelengths and limit the ability to use these fluorescent dyes in tissue imaging(Frangioni 2003).

The use of longer wavelength (700-1000nm) in the near infra red (NIR) spectrum is particularly suited to tissue imaging. NIR imaging benefits from vastly superior tissue penetration and almost eliminates tissue autofluorescence (Frangioni 2003; Sutton *et al.* 2008). NIR fluorescent dyes are commercially available .

Fluorescent imaging of tissue samples was undertaken using the IVIS Spectrum Imaging System (Perkin Elmer). The IVIS Spectrum comprises a 150 watt quartz tungsten halogen lamp with a dichroic reflector providing a light source for fluorescent excitation across wavelengths from 400-950nm (Fig 2.7). Light output from the lamp is provided to the excitation fliter wheel assembly where it passes through a collimating lens followed by an excitation filter. After passing through the excitation filter a second collimating lens focuses the light into a fused silica fibre optic bundle which then splits into four separate bundles that deliver filtered light to four reflectors in the ceiling of the imaging chamber, providing a relatively uniform illumination of the sample stage. An emission filter located at the top of the imaging chamber collects emission from the target fluorophore and focuses it onto the CCD camera. The IVIS Spectrum has a total of 10 narrow band excitation filters (ranging from 415 to 760nm, 30nm bandwidth) and 18 narrow band emission filters (ranging from 490 to 850nm, 20nm bandwidth).

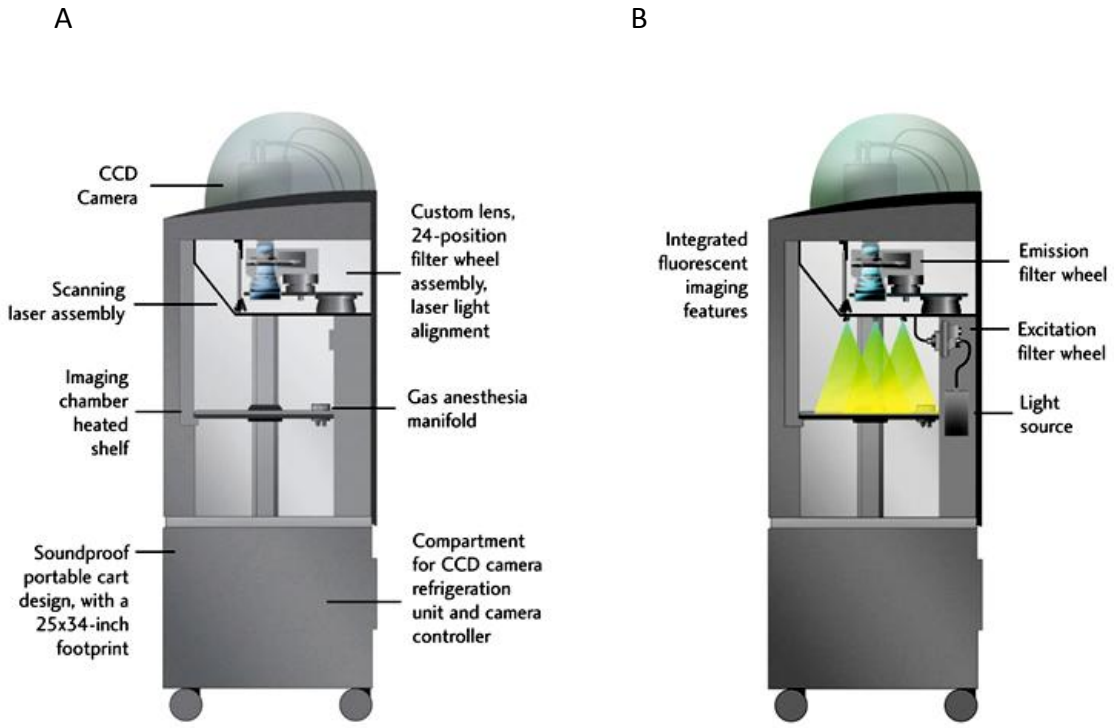


Figure 2.7 Schematic Diagram of the IVIS Spectrum Imaging System

(A) A pictoral representation of the key components of the IVIS Sectrum. (B) Generated light passes through the selected excitation filter and is reflected into the imaging chamber. Emitted fluoresnce is filtered by the selected emission filter wheel and directed onto the CCD camera generating a fluorescent image.

Fluorescent image data can be quantified by measuring the photon emission from the subject, known as radiance, it is the number of photons per second that leave a square centimetre of tissue and radiate into a solid angle of one steradian. A steradian is a three dimensional cone of light emitted from the surface and in the same way that a radian is a unit of arc length for circle a steradian is a unit of solid angle for a sphere, an entire sphere having 4π steradians. Measurements of radiance are unaffected by camera settings and image data can be quantitatively compared.

The fluorescent signal detected from a sample is dependent on both the amount of fluorescent dye present in the sample and the intensity of the incident excitation light. The excitation light incident on the sample stage is not uniform over the entire field of view, the peak intensity is in the centre of the stage but can drop by up to 30% across the stage. To eliminate the variability in the excitation light and to allow quantitative comparisions, fluorescent image data is converted into efficiency where Efficiency = Radiance of the subject / Illumnation intensity. Illumination intensity is specific to each imaging device and is provided by the manufacturer. A reference image of excitation light intensity incident on a highly reflective white plate is stored in the data analysis software for each excitation filter at every field of view and lamp power. Radiance efficiency represents a ratio of the number of fluorescent photons relative to each incident excitation photon in each pixel and does not have units.

Fluorescent and photographic images of individual organs were analysed using Living Image software. Regions of interest (ROI) were defined around each organ and the radiance efficiency after subtraction of background were calculated by the software (Fig 2.8).

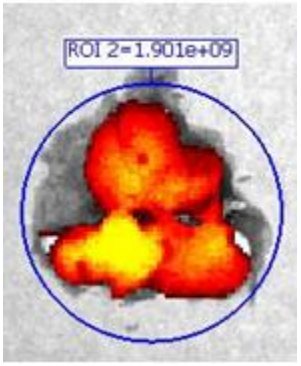


Figure 2.8 Combined photograph and fluorescence image of murine liver demonstrating ROI gating and quantification of radiance efficiency

Individual organs were photographed and fluorescently imaged using the IVIS Spectrum, the Living Image software generated a photographic image of the organ overlaid with a representation of fluorescence intensity. Region of interest (ROI) are selected around individual organs and total fluorescence intensity within the ROI is calculated by the software. Background fluoresecne intensity is measured and subtracted meaning that only fluorescence generated by the imaged organ is measured. This representative image taken of a liver one day following the injection of $1x10^6$ DiR labelled HPC-7 cells demonstrates fluoresecne intensity within the ROI of $1.901x10^9$.

2.28 Identification of Injected Cells from Tissue Samples

Single cell suspensions of livers, spleens, kidneys and lungs from each mouse were prepared and enriched for mononuclear cells by centrifugation on Optiprep as previously described. The resulting cell suspensions were centrifuged and resuspended in PBS + 1% FCS. PE conjugated anti-CD45 antibody was added at a concentration of 1:100 and incubated on ice for 30 minutes. The stained cells were centrifuged at 2000rpm for 5 minutes and resuspended in 1ml PBS + 1% FCS for analysis by flow cytometry. Prior to analysis 20µl of Trucount beads were added to the sample to enable an accurate determination of total cell numbers. The excitation and emission wavelengths of the DiR dye (748nm, 780nm) best correspond to those of the APC-Cy7 channel of the flow cytometer (650nm, 774nm). Infused cells were identified by positive fluorescent staining in the APC-Cy7 channel and positive PE staining indicating cells which are CD45 positive and therefore haematopoietic lineages rather than as a result of dye contamination of other cells within the tissues.

3 THE ANTI-FIBROTIC EFFECT OF HAEMATOPOIETIC STEM CELLS

3.1 Introduction

There is a clear need for new therapies for liver disease, particularly with the increasing mismatch between supply and demand for liver transplantation. There is considerable interest in the use of adult bone marrow stem cells as a new therapeutic option, intriguingly there have been a significant number of clinical studies performed despite a lack of convincing evidence from pre-clinical studies. In particular questions remain unanswered as to the most effective cell population to use and the mechanisms by which theses cells exert a beneficial effect.

Both animal and human studies to date have examined the beneficial effect of a variety different bone marrow cell populations, ranging from fresh unsorted bone marrow preparations to specific differentiated populations of bone marrow derived cells, for example macrophages (Forbes *et al.* 2012). Stem and progenitor cells comprise a very small proportion of the total bone marrow cell population, which is an extremely heterogeneous population of cells containing cells at differing stages of differentiation and maturity. This means that in many of the animal and human studies it is not clear whether there is a specific cell type within the bone marrow exerting a beneficial effect. The heterogeneous nature of the studies to date make cross comparison of efficacy and pooling of data virtually impossible, however a common message from the human studies to date is that bone marrow stem cell therapy in patients with liver disease is safe and feasible (Houlihan *et al.* 2008). The initial human studies have suggested a beneficial effect but none have been designed, controlled or powered to robustly detect a significant improvement in liver

function. Larger, randomised controlled trials of stem cell therapy for liver disease are currently underway which will hopefully help determine the efficacy of specific cell types but are unlikely to elucidate the mechanisms of benefit.

Haematopoietic stem cells can be readily defined by their surface marker phenotype in mice and in humans, isolation from donor mouse bone marrow is straightforward and in humans routine protocols are in place for obtaining autologous stem cells (Gratwohl *et al.* 2010). The isolation of clinical grade HSC from human donors is now a routine procedure and means that use of HSC in clinical studies can be facilitated.

There have only been two studies reporting the used of purified (ie KSL) HSC in animal models of liver injury. Both studies reported a significant improvement in hepatic fibrosis following infusion of HSC. Li and colleagues demonstrated a reduction in hepatic fibrosis and improved hepatocyte regeneration in a carbon tetrachloride model of liver injury, and whilst they showed that some infused cells adopted a hepatocyte phenotype this in itself did not explain the beneficial effect. Significant alterations in serum concentrations of inflammatory and regenerative cytokines and growth factors were seen after cell infusion(Li *et al.* 2013). Cho and co-workers also showed a reduction in hepatic fibrosis following infusion of HSC.(Cho *et al.* 2011). In both studies $1x10^6$ cells were infused and in one study cells were isolated on KSL and then cultured in vitro with no reassessment of cell phenotype or function following culture. This number of cells corresponds to approximately 40-50 million HSC per kilogram of bodyweight which is vastly higher than the number of cells used in any previous animal or human study.

The cellular and molecular mechanisms underlying any potential beneficial effect of HSC cell therapy have yet to be determined. It is now apparent that the concept of transdifferentiation or cell fusion of bone marrow stem cells to hepatocytes is now an extremely rare event and would not be of functional significance(Aurich *et al.* 2007). It is likely when this phenomenon is observed that it is due to differentiation of pluripotent very small embryonic like stem cells contaminating preparations of purified stem cells, rather than transdifferentiation of more committed stem and progenitor cells(Kucia *et al.* 2006).

Observations from more recent studies have given greater weight to the concept that infused cells provoke alterations in the surrounding tissue environment – the so-called 'paracrine effect' – which result in fibrosis regression and hepatocyte regeneration. Increased expression of matrix metalloproteinases by either host derived cells(Thomas *et al.* 2011) or infused cells(Sakaida *et al.* 2004) drive the regression of fibrosis, and recruitment of host derived cells crucial to the regression of fibrosis such as macrophages and neutrophils may be as a result of increased chemokine production from infused cells(Thomas *et al.* 2011). Infused cells expressing growth factors such as HGF and VEGF may stimulate hepatocyte proliferation and progenitor cell responses(Majka *et al.* 2001; Houlihan *et al.* 2008).

The majority of chronic liver injury is due to repeated exposure to the injurious agent and it is often not possible to treat or remove the injury. No previous studies have examined the benefits of repeated treatment with stem cell therapy in liver disease but instead have focussed on a one off treatment.

In this first section of work I will determine the effect of repeated injections of purified haematopoietic stem cells on chronic liver injury. I will attempt to elucidate further the mechanisms by which such benefits may be mediated with particular interest in the fate of the infused cells.

3.2 Repeated carbon tetrachloride administration induces chronic liver injury in mice

Carbon tetrachloride was used at a dose of 1mg/kg body weight (diluted 1:4 in mineral oil vehicle) and administered to mice by intra peritoneal injection. After 8 weeks of treatment a significant chronic liver injury had developed.

Overall survival of all animals with chronic liver injury was greater than 95%.

On macroscopic examination of the animals the livers of the injured mice appeared pale, hard with irregular edges. There was evidence of splenomegaly and ascites was present within the abdominal cavity.

Histological examination of the livers using haematoxyin and eosin staining demonstrated architectural distortion with a marked inflammatory cell infiltrate (Fig 3.1A,B). Picrosirius red staining demonstrated deposition of collagen with bridging fibrosis between portal tracts (Fig 3.1C,D).

A deterioration in hepatic synthetic function was also observed with a marked reduction in serum albumin (Control 4.22+/- 0.37 g/dl vs Injury 3.85+/-0.22 g/dl, $p<0.05$).

Figure 3.1

Carbon Tetrachloride Liver Injury

Chronic liver injury was induced in age and sex matched C57/BL6 mice by twice weekly intraperitoneal administration of carbon tetrachloride (1mg/kg) in mineral oil for 8 weeks. Control mice received twice weekly intraperitoneal injections of mineral oil. Tissue sections from both carbon tetrachloride treated mice and control mice were stained with haematoxylin and eosin (A,B) and with picrosirius red (C,D). Mice treated with carbon tetrachloride developed significant liver injury including hepatocyte necrosis, inflammatory cell infiltrates, steatosis and deposition of collagenous fibrous tissue bridging between portal tracts.

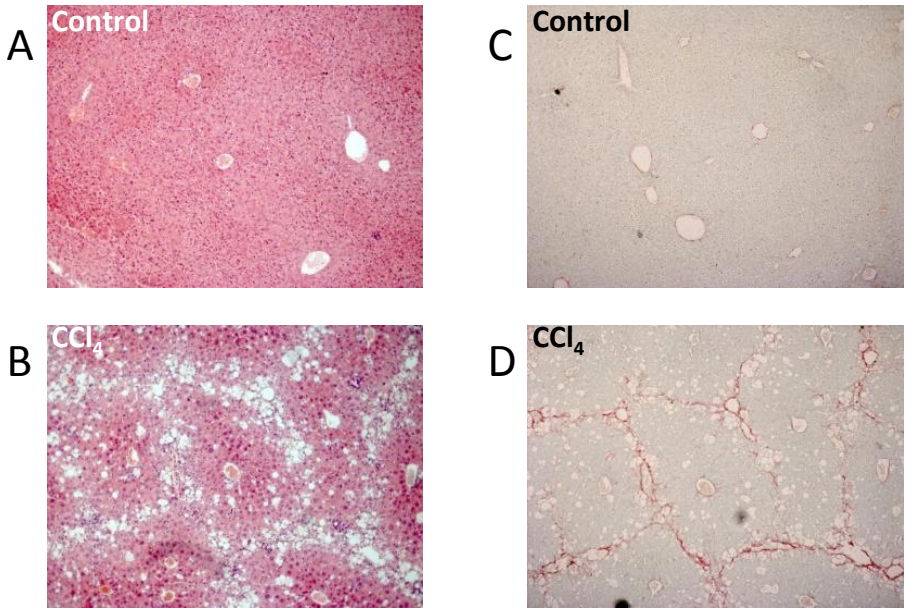


Figure 3.1 Carbon Tetrachloride Liver Injury

3.3 Increased numbers of bone marrow derived HSC are found in the liver and blood during chronic liver injury

It has been previously shown that haematopoietic stem cells can be found within the liver under physiological conditions and that following liver injury are mobilised from the bone marrow into the circulation(Wright *et al.* 2001; Lemoli *et al.* 2006; Gehling *et al.* 2010). I used the carbon tetrachloride model of liver injury to investigate in more detail the effect of chronic liver inury on c-kit+ sca1+ lin- (KSL) haematopoietic stem cell mobilisation and recruitment to the liver. Chronic liver injury was induced in mice by twice weekly administration of carbon tetrachloride for 8 weeks, mice were then sacrificed and bone marrow, peripheral blood and liver obtained. KSL cells were quantified by flow cytometry as shown in Figure 3.2. Colony forming unit assays were used as a functional assessment of stem cell number.

Significantly higher numbers of HSC determined by c-kit+ sca1+ lin- (KSL) surface phenotype were found in the livers (Injury 1163 +/- 173 KSL cells per liver vs Control 258.5+/-22.2 KSL cells per liver, $p<0.01$) and peripheral blood (Injury 0.397+/-0.055 KSL cells per µl blood vs Control 0.0674+/-0.011 KSL cells per µl blood, $p<0.001$) of mice with liver injury compared with control mice (Fig 3.3A,B). The numbers of HSC within the bone marrow remained constant (Injury 2323+/-48.9 KSL cells per femur vs Control 2327+/-64.3 KSL cells per femur, p=ns) (Fig 3.3C). A similar increase in the numbers of colony forming cells (CFC) within the liver (Injury 178.8+/-21.9 CFC per liver vs Control 38.0+/-6.15 CFC per liver, $p<0.001$)(Fig 3.4B) and peripheral blood (Injury 83.42+/-13.6 CFC per ml blood vs Control 42.29 CFC per ml blood, $p<0.05$)(Fig 3.4A), and no change

Figure 3.2

Identification of c-kit+ sca1+ lineage- cells within liver and peripheral blood

Chronic liver injury was induced in age and sex matched C57/BL6 mice by twice weekly intraperitoneal administration of carbon tetrachloride (1mg/kg) in mineral oil for 8 weeks. Control mice received twice weekly intraperitoneal injections of mineral oil. Peripheral blood was obtained by cardiac puncture under terminal anaesthesia and livers were dissected out and flushed to remove circulating blood. Following collagenase digestion and enrichment for mononuclear cells using a density centrifugation gradient a single cell suspension was obtained from liver tissue, which was labelled with fluorescently conjugated antibodies to c-kit, sca-1 and lineage markers. (A) Dead cells and cellular debris was excluded on a forward scatter – side scatter plot, lineage negative cells identified and expression of c-kit and sca-1 determined in these cells. (B) Representative plots from control and carbon tetrachloride treated mouse livers are displayed. (C) Peripheral blood samples were treated with red cell lysis buffer, labelled with fluorescent antibodies in a similar manner to liver samples and a similar flow cytometry gating strategy used. (D) Representative samples of peripheral blood from control and carbon tetrachloride treated mice are displayed.

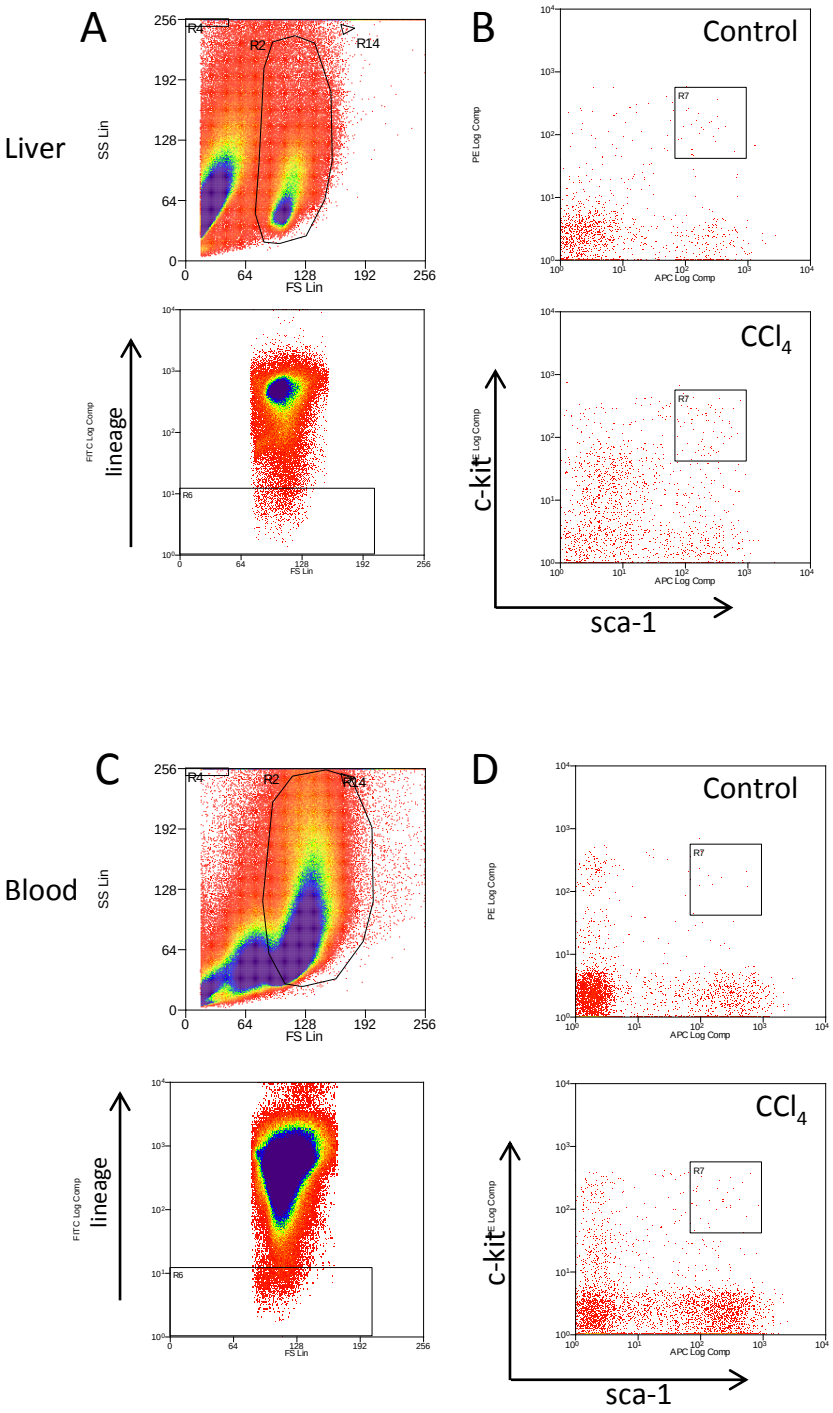


Figure 3.2

Identification of c-kit+ sca1+ lineage- cells within liver and peripheral blood

Figure 3.3

Increased numbers of c-kit+ sca1+ lineage- cells are found in the liver and peripheral blood of mice with chronic liver injury

Chronic liver injury was induced in age and sex matched C57/BL6 mice by twice weekly intraperitoneal administration of carbon tetrachloride (1mg/kg) in mineral oil for 8 weeks. Control mice received twice weekly intraperitoneal injections of mineral oil. HSC were defined as c-kit+ sca1+ lineage negative (KSL) and were identified in tissue and blood samples as previously described. Absolute cell numbers were quantified using counting beads and samples were run in duplicate. (A,B) Significantly higher numbers of KSL cells were detected in the peripheral blood and livers of carbon tetrachloride treated mice than controls. (C) No difference was seen in the bone marrow. Data from individual mice is displayed (circles : control, squares : carbon tetrachloride treated) and means indicated by horizontal line. **p<0.01 ***p<0.001(unpaired student's t-test)

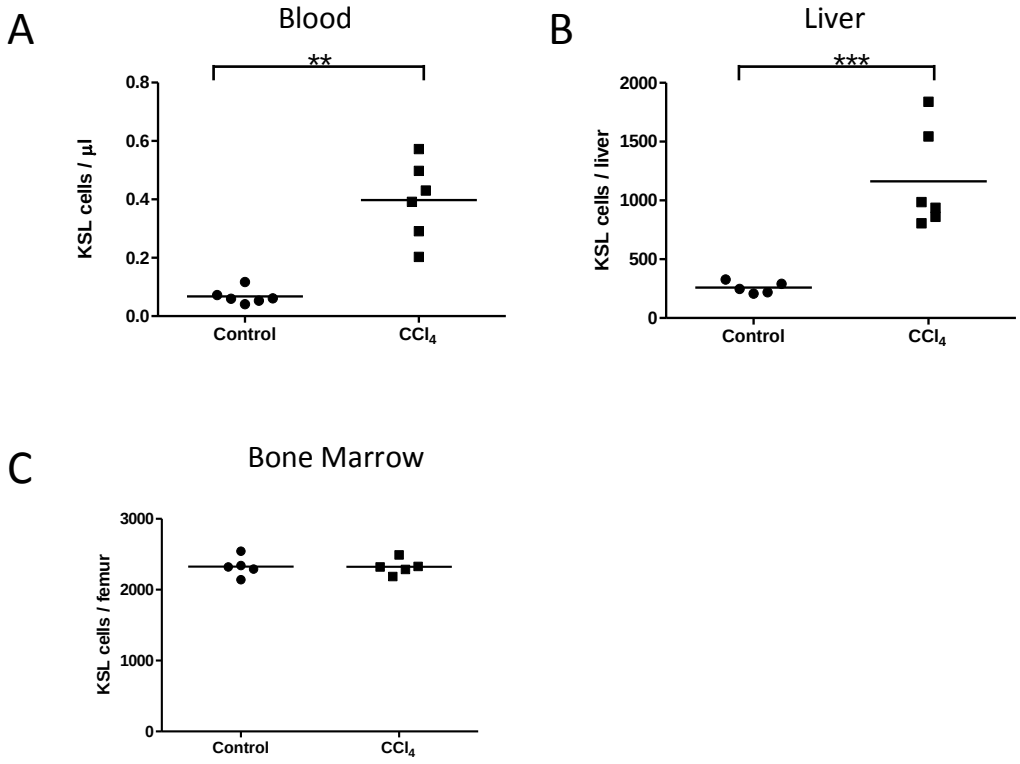


Figure 3.3
Increased numbers of c-kit+ sca1+ lineage- cells are found in the liver and peripheral blood of mice with chronic liver injury

Figure 3.4

Increased numbers of colony forming cells are found in the liver and peripheral blood of mice with chronic liver injury

Chronic liver injury was induced in age and sex matched C57/BL6 mice by twice weekly intraperitoneal administration of carbon tetrachloride (1mg/kg) in mineral oil for 8 weeks. Control mice received twice weekly intraperitoneal injections of mineral oil. Colony forming cells (CFC) within the liver, blood and bone marrow were quantified in myeloid CFU assays. (A,B) Significantly higher numbers of CFC were detected in the peripheral blood and livers of carbon tetrachloride treated mice than controls. (C) No difference was seen in the bone marrow. Data from individual mice is displayed (circles : control, squares : carbon tetrachloride treated) and means indicated by horizontal line. * $p<0.05$ ***$p<0.001$ (unpaired student's t-test)

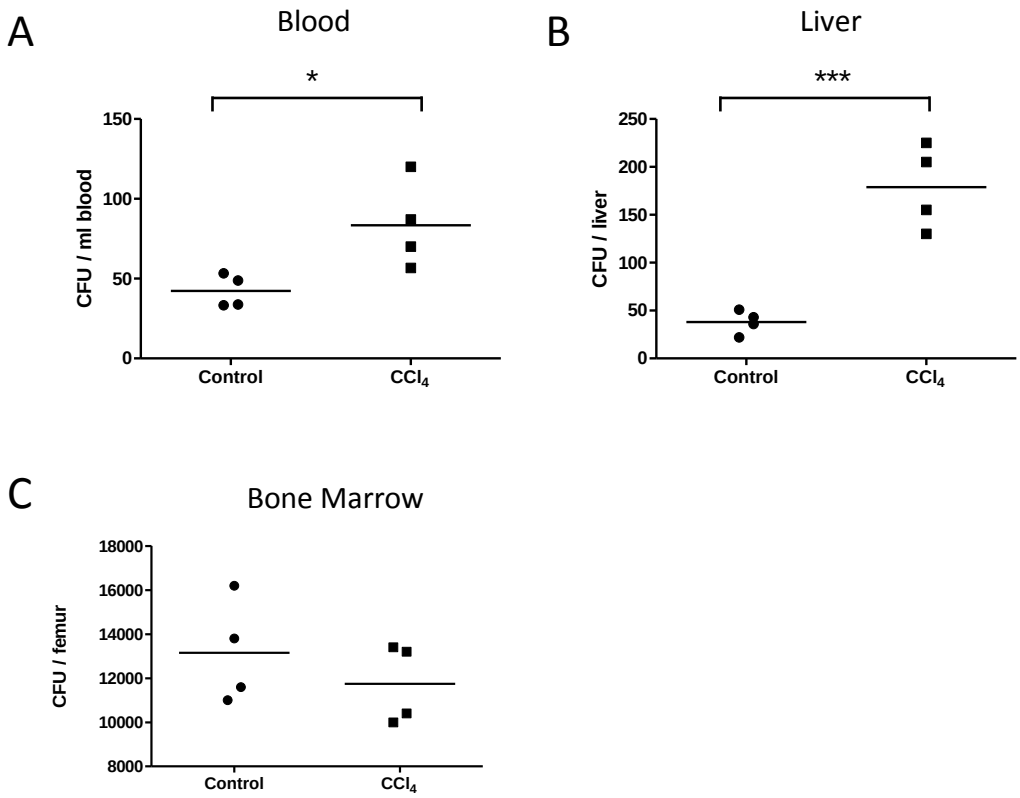


Figure 3.4
Increased numbers of colony forming cells are found in the liver and peripheral blood of mice with chronic liver injury

was observed within the bone marrow (Injury 11750+/-899 CFC per femur vs Control 131500+/-1181 CFC per femur, p=ns) (Fig 3.4C).

The liver is a site of fetal haematopoiesis and populations of liver resident HSC have been described(Taniguchi *et al.* 1996). In order to test my hypothesis that the increased numbers of HSC within the liver and blood are bone marrow derived I devised a bone marrow transplantation protocol that would enable tracking of bone marrow derived cells. BoyJ and C57BL6 mice differ in CD45 antigen expression, designated as CD45.1 ($Ptprc^{a}$ BoyJ) and CD45.2 ($Ptprc^{b}$ C57BL6). Ptprc is a protein tyrosine phosphatase, homologous to the CD45 protein (Charbonneau *et al.* 1988), the a allele is carried by BoyJ (CD45.1+) mice and the b allele by C57BL6 (CD45.2+) mice. This phenotypic difference has been widely used in competitive bone marrow repopulation experiments to track donor cells.

BoyJ (CD45.1) mice received sub lethal doses of irradiation followed by transplantation of bone marrow cells from donor C57BL6 (CD45.2) mice (Fig 3.5A). Analysis of peripheral blood and bone marrow from transplanted mice showed almost universal bone marrow reconstitution with CD45.2 (ie donor derived) cells after 4 weeks (Fig 3.5B,C). Subsequently transplanted mice underwent 8 weeks carbon tetrachloride administration, were sacrificed and bone marrow derived HSC quantified by flow cytometry, defined as CD45.2+ lin- sca1+ ckit+.

The findings were in keeping with those seen in the initial experiments, with an approximately five fold increase in the numbers of bone marrow derived KSL cells in the peripheral blood (Injury 0.333+/-0.064 CD45.2 KSL cells per µl blood vs Control 0.077+/-0.009 CD45.2 KSL cells per µl blood, $p<0.01$) (Figure 3.6A) and a three fold increase in the

Figure 3.5

Generation of Bone Marrow Chimersim and Subsequent Chronic Liver Injury

(A) Recipient BoyJ (CD45.1) mice received Baytril antibiotic in drinking water one week prior and one week following irradiation with 9Gy in two divided doses on the same day. Bone marrow cells were obtained from donor C57BL6 (CD45.2) mice by flushing of the tibia and femurs. Six hours following irradiation donor bone marrow cells were infused into recipient mice by tail vein injection. After 4 weeks recovery, transplanted mice were divided into two groups at random with one group receiving twice weekly intraperitoneal administration of carbon tetrachloride (1mg/kg) in mineral oil and the other group receiving intraperiotneal injections of mineral oil alone for a further 8 weeks. (B,C) Samples of bone marrow and peripheral blood were treated with red cell lysis buffer and labelled with fluorescent antibodies to CD45.1 and CD45.2. Analysis of CD45 expression by flow cytometry revealed almost complete CD45.2 expression following transplantation compared with CD45.1 expression prior to transplantation. Representative flow cytometry plots of multiple experiments are shown.

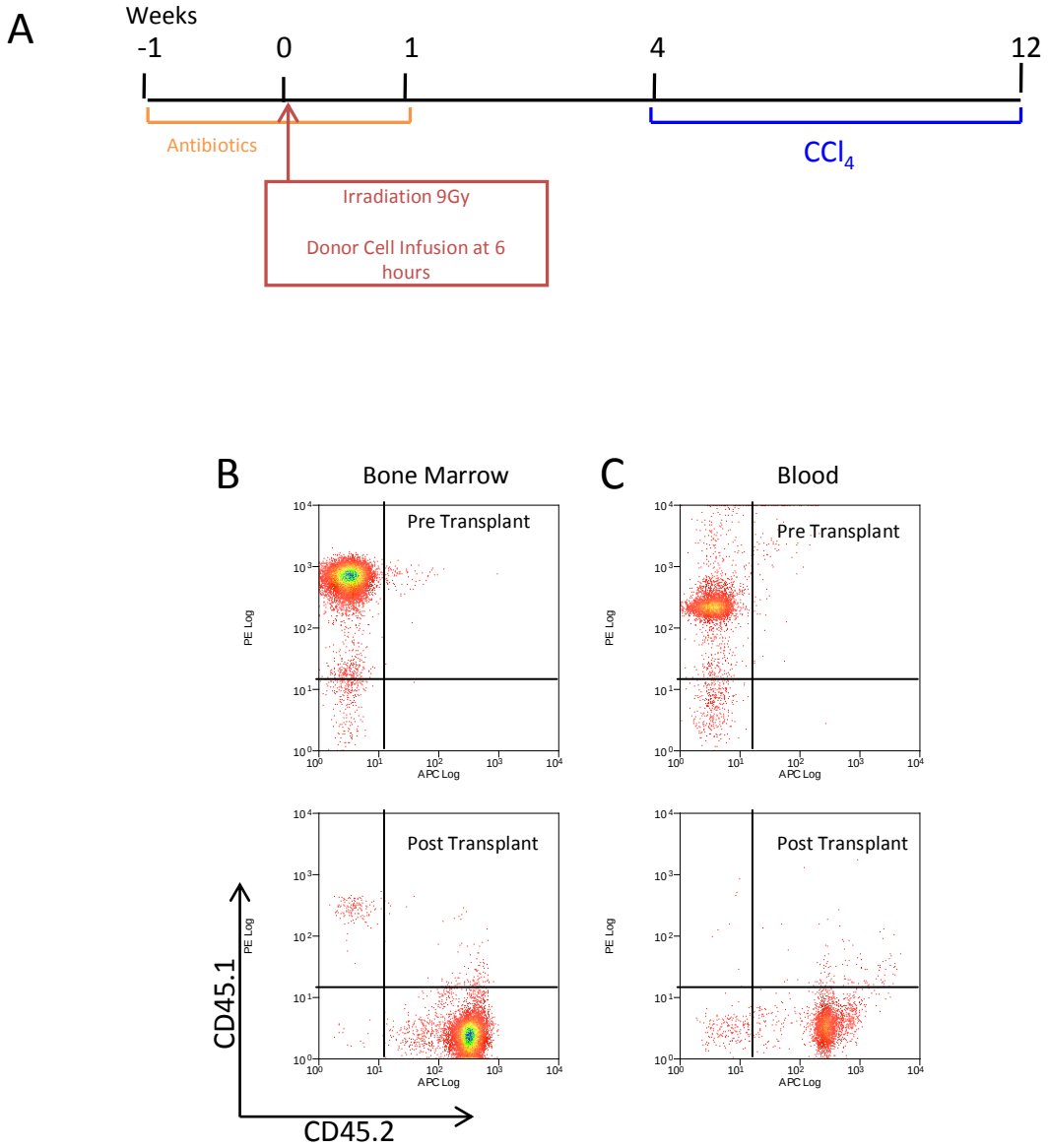


Figure 3.5
Generation of Bone Marrow Chimersim and Subsequent Chronic Liver Injury

Figure 3.6

Increased numbers of bone marrow derived c-kit+ sca1+ lineage- cells in the liver and peripheral blood during liver injury

Recipient BoyJ (CD45.1) mice received Baytril antibiotic in drinking water one week prior and one week following irradiation with 9Gy in two divided doses on the same day. Bone marrow cells were obtained from donor C57BL6 (CD45.2) mice by flushing of the tibia and femurs. Six hours following irradiation donor bone marrow cells were infused into recipient mice by tail vein injection. After 4 weeks recovery, transplanted mice were divided into two groups at random with one group receiving twice weekly intraperitoneal administration of carbon tetrachloride (1mg/kg) in mineral oil and the other group receiving intraperitoneal injections of mineral oil alone for a further 8 weeks. Bone marrow derived HSC were defined as CD45.2+ c-kit+ sca1+ lineage negative (CD45.2+ KSL) and were identified in liver and peripheral blood samples as previously described. Absolute cell numbers were quantified using counting beads and samples were run in duplicate. (A,B) Significantly higher numbers of bone marrow derived CD45.2+ KSL cells were detected in the peripheral blood and livers of carbon tetrachloride treated mice than controls. Data from individual mice is displayed (circles : control, squares : carbon tetrachloride treated) and means indicated by horizontal line. ** $p<0.01$ ***$p<0.001$ (unpaired student's t-test)

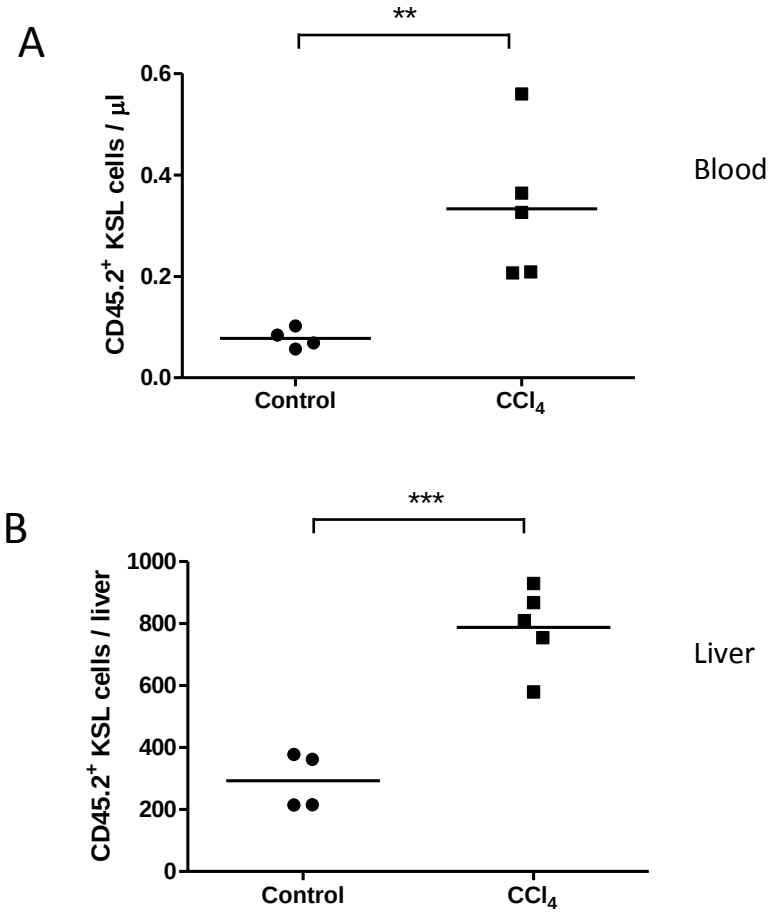


Figure 3.6
Increased numbers of bone marrow derived c-kit+ sca1+ lineage- cells in the liver and peripheral blood during liver injury

liver (Injury 788.2+/-59.79 CD45.2 KSL cells per liver vs Control 292.6+/-44.78 CD45.2 KSL cells per liver, $p<0.001$) (Fig 3.6B) of mice with liver injury when compared with control mice, whilst KSL cells within the bone marrow remained unchanged (Injury 3162+/-94 CD45.2 KSL cells per femur vs Control 2954+/-87 CD45.2 KSL cells per femur, p=ns).

3.4 Repeated infusions of HSC results in significant improvement in hepatic fibrosis

Having demonstrated that there is mobilisation and recruitment of bone marrow derived HSC in liver injury, I sought to determine whether repeated injection of purified HSC would result in a reduction in hepatic fibrosis.

BoyJ mice were randomly allocated to either treatment (n=8) or control (n=8) groups and all mice received 6 weeks of twice weekly carbon tetrachloride injections. C57BL6 mice were used as donor mice for the KSL cells to be infused, $5x10^4$ freshly isolated KSL cells were injected via the tail vein to the mice in the treatment group at the start of weeks 7,8 and 9. Carbon tetrachloride continued to be administered to both groups until seven days after the final cell injection and mice were sacrificed at 72 hours after then final carbon tetrachloride injection and 7 days after the final cell injection (Fig 3.7).

Quantitative morphometric analysis of fibrosis in the liver using picrosirius red staining revealed significantly lower levels of collagen deposition within the liver with a 49.7% reduction in staining in the KSL cell treatment group compared with controls (KSL 2.207+/-0.119 % staining area vs Control 4.388+/-0.27 %staining area, $p<0.0001$)(Fig 3.8A,B). Hepatic hydroxyproline content was also significantly lower in the KSL cell treatment group (KSL 328.5+/-30.4 mg Hyp per g liver vs Control 428.4+/-31.9 mg Hyp per g liver, $p<0.05$) (Fig 3.8C).

Serum albumin levels were measured as a marker of liver synthetic function and was previously shown to be lower in mice with chronic liver injury. In the treatment group the serum albumin levels were significantly higher than those in the control group (KSL 4.06 +/-

0.37 vs Control 3.14+/-0.39, $p<0.01$)(Fig 3.8D), demonstrating an improvement in liver function.

Figure 3.7

Investigation of the effects of repeated infusions of purified c-kit+ sca1+ lin- in a murine model of chronic liver injury - Experimental timeline

Chronic liver injury was induced in age and sex matched C57/BL6 mice by twice weekly intraperitoneal administration of carbon tetrachloride (1mg/kg) in mineral oil for 6 weeks, mice were then randomly allocated to either control or cell treatment group. (A) Mice in the control group continued to receive twice weekly injections of intraperitoneal carbon tetrachloride until week 10 and were sacrificed 72 hours following the final injection of carbon tetrachloride. (B) Mice in the cell treatment group received twice weekly carbon tetrachloride in the same way as control mice but in addition received a tail vein injection of $5x10^4$ purified KSL cells isolated from C57/BL6 donor mice on the first day of weeks 7,8 and 9, mice were sacrificed 72 hours after the final injection of carbon tetrachloride which was 7 days following the final cell injection.

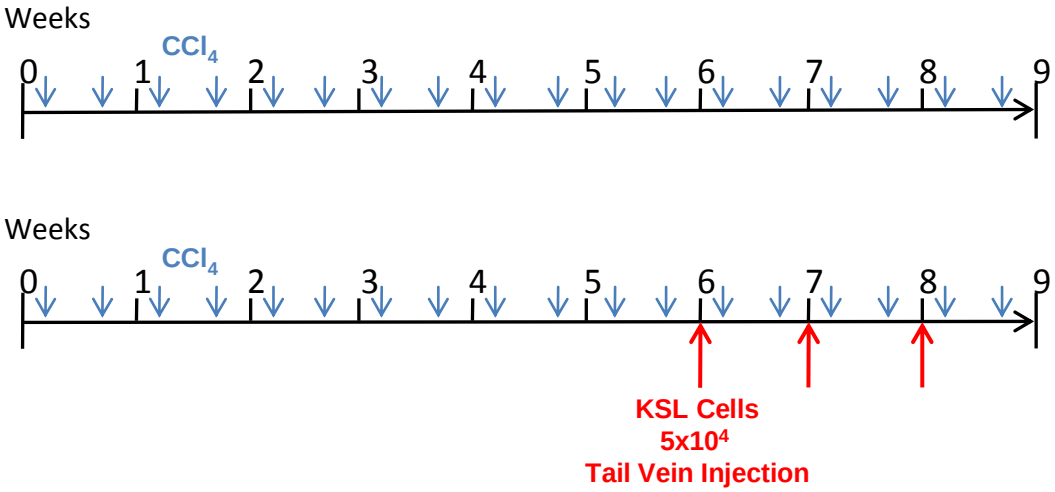


Figure 3.7
Investigation of the effects of repeated infusions of purified c-kit+ sca1+ lin- in a murine model of chronic liver injury - Experimental timeline

Figure 3.8

Significant improvement in hepatic fibrosis following repeated injection of purified KSL cells

(A) Formalin fixed paraffin embedded liver tissue sections from mice treated with KSL cell infusions (white bars) and control mice (black bars) were stained for picrosirius red. 6 random, non-overlapping images were obtained from each section and staining was quantified as a percentage of the image positive for picrosirius red using ImageJ software. Significantly lower levels of picrosirius red staining was detected in mice treated with KSL cell infusions than in control mice. n=8 each group, mean +/-SD % area stained shown, *p<0.05 (two tailed, unpaired students t-test). (B) Representative photomicrograph images (x20 magnification) of picrosirius red staining (red, no counterstain) from control and KSL treated mice. (C) Hydroxyproline content in samples of liver tissue from KSL cell treated mice (white bars) and control mice (black bars) was determined and expressed as mg of Hydroxyproline per gram of liver tissue. Significantly lower amounts of hydroxyproline were found in livers from mice receiving repeated KSL cell injections compared with livers from control mice. n=8 each group, mean+/-SD hydroxyproline content shown, *p<0.05 (two tailed, unpaired students t-test). (D) Serum samples were obtained by centrifugation of whole blood from control mice (black bars) and mice treated with KSL cell injections (white bars) and serum albumin concentration was measured by bromocresol green colorimetric assay. Serum albumin concentration was higher in mice treated with KSL cell injections than control mice. n=8 each group, mean+/-SD serum albumin concentration shown, *p<0.05 **p<0.01 ***p<0.001(two tailed, unpaired students t-test).

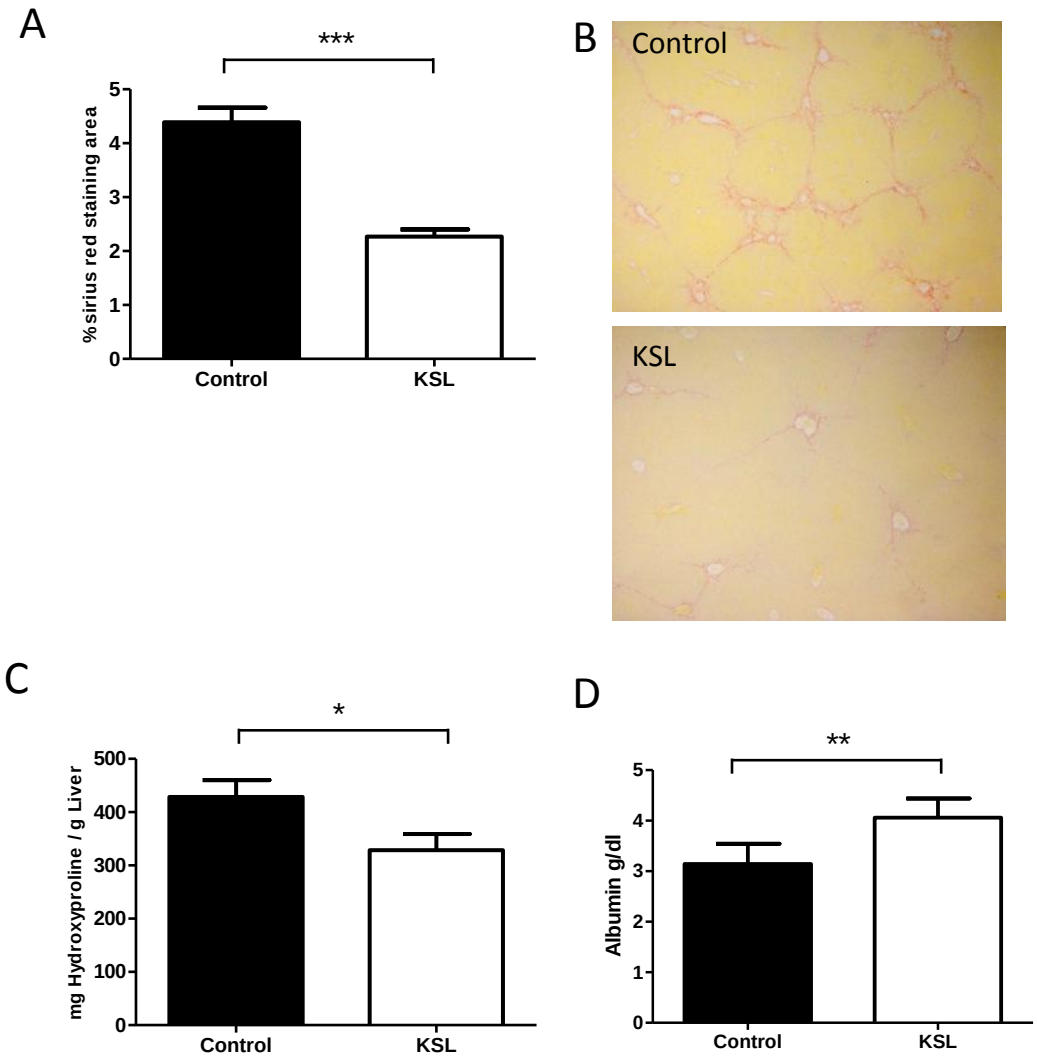


Figure 3.8
Significant improvement in hepatic fibrosis following repeated injection of purified KSL cells

A key process in the development of chronic liver injury is activation of hepatic stellate cells to αSMA expressing activated myofibroblasts. Morphometric analysis of liver sections stained for αSMA by immunohistochemistry showed a marked three fold reduction in αSMA positive staining area in the KSL cell treatment group (KSL 2.422+/-0.20 % staining area vs Control 7.055+/-0.334 % staining area, $p<0.0001$)(Fig 3.9A,B). Quantitative analysis of genes expressed by activated myofibroblasts demonstrated a 0.19+/-0.07 fold reduction in hepatic αSMA expression ($p<0.0001$) and a 0.29+/-0.14 fold reduction in hepatic col1α1 expression ($p<0.0001$) in mice treated with KSL cells (Fig 3.9 C,D).

Figure 3.9

Significant reduction in hepatic activated myofibroblasts following repeated injections of purified KSL cells

(A) Formalin fixed paraffin embedded liver tissue sections from mice treated with KSL cell infusions (white bars) and control mice (black bars) were stained for aSMA. 6 random, non-overlapping images were obtained from each section and staining was quantified as a percentage of the image positive for aSMA using ImageJ software. Significantly lower levels of aSMA staining was detected in mice treated with KSL cell infusions than in control mice. n=8 each group, mean +/-SD % area stained shown, ***p<0.001 (two tailed, unpaired students t-test). (B) Representative photomicrograph images (x20 magnification) of aSMA staining (brown, DAB substrate, haematoxylin counterstain) from control and KSL treated mice. (C,D) RNA was extracted from liver tissue from mice treated with KSL cell infusions (white bars) and control mice (black bars) and using quantitative RT-PCR gene expression of (C)col1a1 and (D) aSMA were determined, normalised to GAPDH expression and relative to control mice using the 2^{-ddCT} method. Hepatic gene expression of both col1a1 and aSMA was significantly lower in KSL cell treated mice than control mice. n=8 each group, mean +/-SD fold change shown, ***p<0.001 (wilcoxon signed rank test).

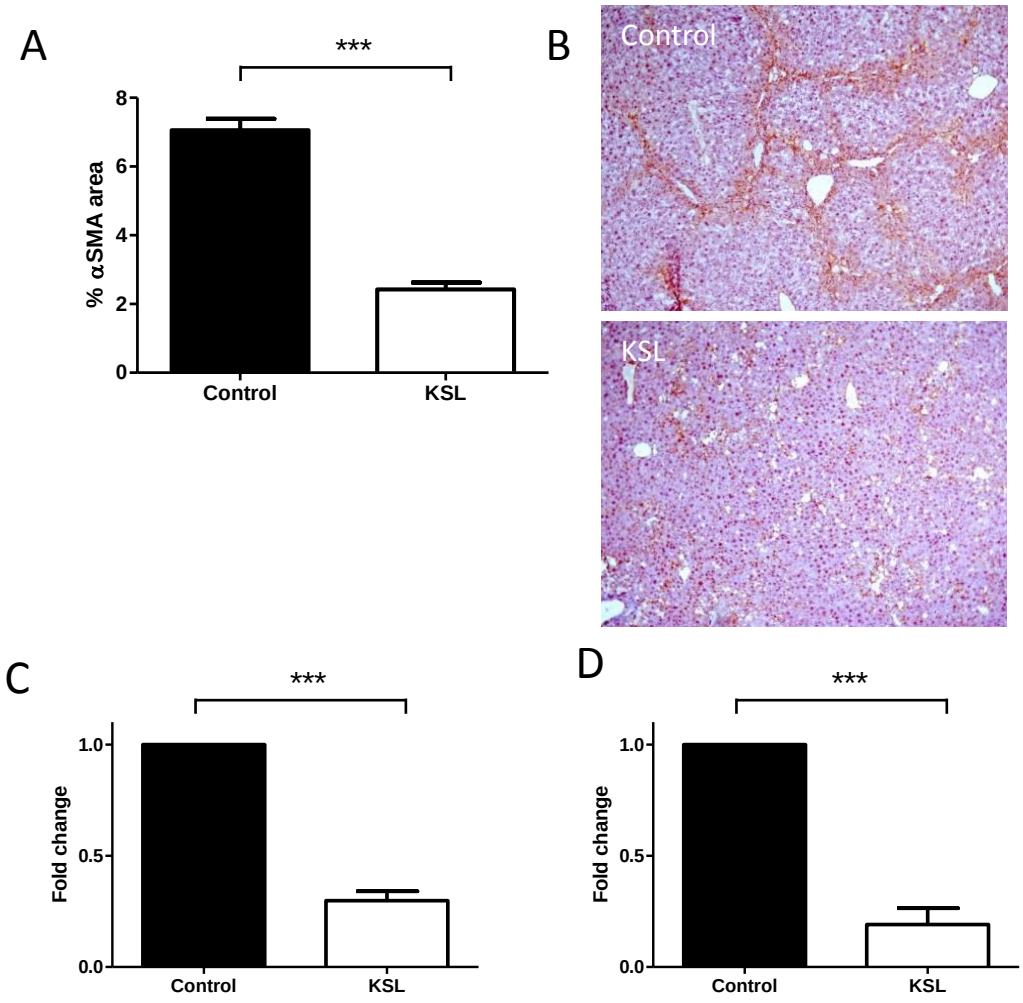


Figure 3.9
Significant reduction in hepatic activated myofibroblasts following repeated injections of purified KSL cells

3.5 Increased numbers of recipient derived rather than donor derived cells

HSC infused in this series of experiments were derived from C57BL6 mice and were CD45.2+ and the recipient mice with chronic liver injury were BoyJ CD45.1+ mice. Sections from livers of the treated mice were analysed for the presence of infused cells by immunohistochemical staining for CD45.2. Despite analysing multiple serial and non-serial tissue sections, CD45.2+ cells were largely not detected and were found in isolation only rarely (Fig 3.10). Due to the scarcity of these cells within the liver sections at seven days after infusion it was not possible to analyse further for any lineage differentiation or transdifferentiation that may have occurred to be responsible for the beneficial effect observed. It was clear, however, that there are not significant numbers of infused cells localised to areas of fibrosis or inflammation at this timepoint.

The effect of HSC infusions on endogenous repair mechanisms via a 'paracrine effect' has been postulated as the most likely mechanism by which infused mononuclear cells exert their effect (Houlihan *et al.* 2008; Forbes *et al.* 2012). The effect of repeated HSC infusions on endogenous cell populations postulated to be involved in the resolution of fibrosis was analysed by immunohistochemical analysis of liver sections for F4/80 as a marker of macrophages(Austyn *et al.* 1981) and Ly6G as a marker of neutrophils(Fleming *et al.* 1993). Liver sections from mice treated with KSL cells contained higher numbers of both F4/80+ (KSL 44.2+/-11.8 cells per fov vs Control 24.99+/-7.5 cells per fov, $p<0.0001$)(Fig 3.11C,D) and Ly6G+ (KSL 12.42+/-2.74 cells per fov vs Control 5.646+/-2.514 cells per fov, $p<0.0001$) (Fig 3.11A,B) cells compared with controls.

Figure 3.10

Absence of significant numbers of injected cells in the liver seven days after injection

Formalin fixed paraffin embedded liver tissue sections were taken from mice seven days after the final injection of KSL cells and from control mice and stained for CD45.2 expression, indicating the presence of injected cells or progeny of infused cells. The detection of CD45.2 positive cells was an extremely rare event. (A,B) Representative photomicrograph images (x20 magnification) of CD45.2 staining of liver sections from (A) control and (B) KSL cell treated mice, one image demonstrated the presence of a CD45.2 positive cell with higher magnification (x100) image shown (inset box).

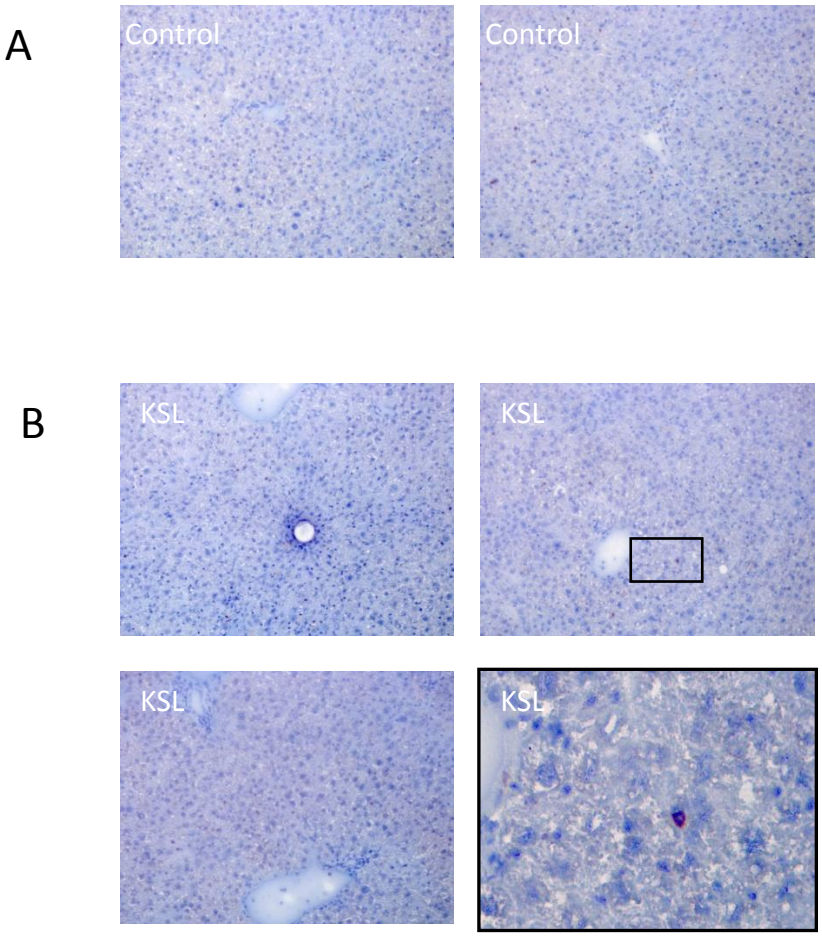


Figure 3.10
Absence of significant numbers of injected cells in the liver seven days after injection

Figure 3.11

Increased numbers of endogenous cell populations within the liver following repeated injection of purified KSL cells

Formalin fixed paraffin embedded liver tissue sections from mice treated with KSL cell infusions (white bars) and control mice (black bars) were stained for F4/80 and Ly6G. (A,C) Representative photomicrograph images of liver sections from control and KSL treated mice stained for (A) Ly6G (brown, DAB substrate, haematoxylin counterstain, x20 magnification) and (C) F4/80 (brown, DAB substrate, haematoxylin counterstain, x100 magnification). (B,D) The number of individual stained cells were manually counted in 6 random, non-overlapping fields of view (x100 for F4/80, x20 for Ly6G). Significantly higher numbers of Ly6G+ and F4/80+ cells were seen within the livers of mice treated with KSL cells compared with controls. n=8 each group, mean+/-SD number of cells per field of view shown, ***$p<0.001$ (two tailed, unpaired students t-test).

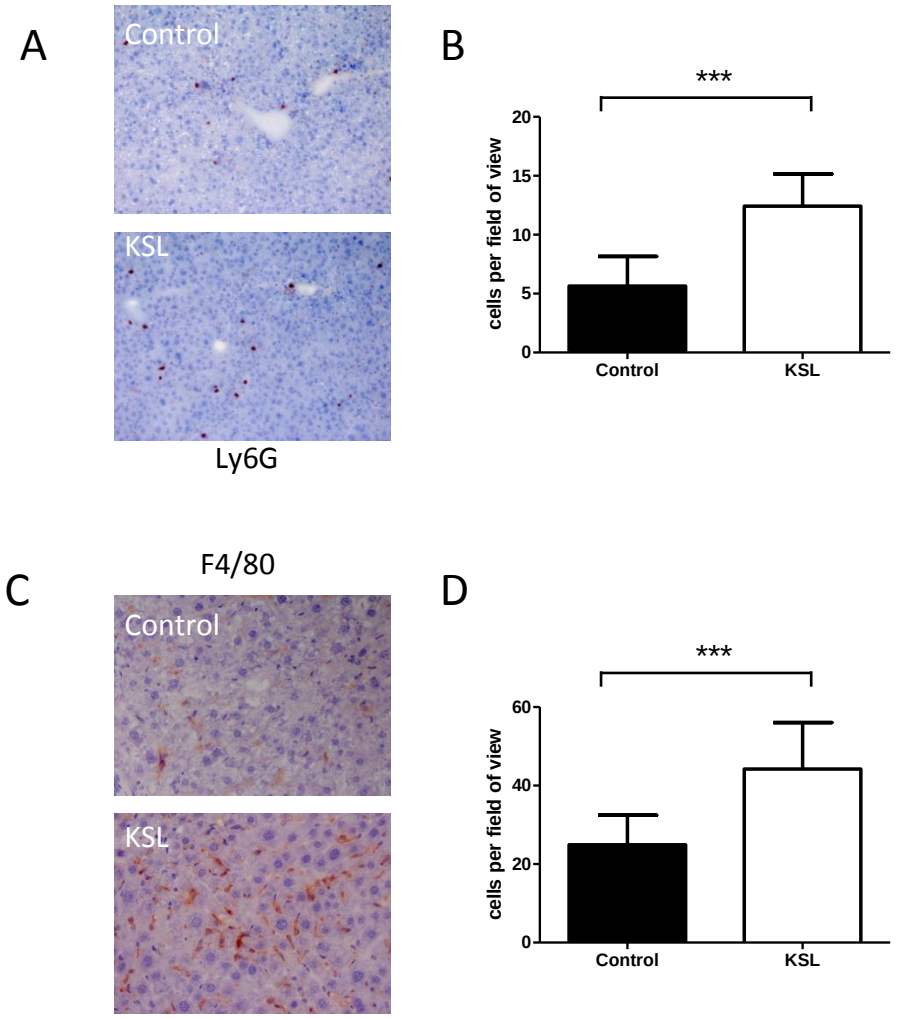


Figure 3.11
Increased numbers of endogenous cell populations within the liver following repeated injection of purified KSL cells

I concluded that these cells represented endogenous, recipient derived cells as they were present in significant numbers with a complete lack of cells staining positive for CD45.2 (Fig 3.10), the phenotype of the donor cells.

3.6 Myeloid and lymphoid progenitor cells exert a similar anti fibrotic effect as HSC

There has been particular interest in the beneficial effect of cells of the monocyte-macrophage lineage in liver injury(Thomas *et al.* 2011; Bird *et al.* 2013). One of the earliest stages in HSC differentiation is that of commitment to a common myeloid progenitor (CMP) or a common lymphoid progenitor (CLP). I investigated whether differentiation into cells of a myeloid lineage is a potential mechanism by which HSC exert their beneficial effect by examining the effect of myeloid and lymphoid progenitors on hepatic fibrosis.

CMP were defined as c-kit+ sca1- lineage- IL7Rα- and CLP as ckit lo sca1 lo lineage- IL7Rα+ (Kondo *et al.* 1997) and were freshly isolated from C57BL6 donor mice (Fig 3.12A). Colony forming unit assays were performed to determine myeloid colony forming ability. Large numbers of myeloid colonies were grown from freshly isolated HSC (73.5+/-6.4 CFC per 500 cells), whilst colonies from CMP were less plentiful (17.5+/-8.3 CFC per 500 cells). Only rarely could myeloid colonies be identified from freshly isolated CLP (1.1 +/- 0.202 CFC per 500 cells), demonstrating their inability to differentiate into cells of myeloid lineage (Fig 3.12B).

Further experiments were performed according to the previous protocol, however repeated infusions of either CMP or CLP were administered in place of KSL cells.

Assessment of hepatic fibrosis demonstrated a significant reduction in picrosirius red staining in mice receiving either CMP or CLP to levels comparable with HSC treatment (KSL 2.207+/-0.119 % staining area, CMP 1.937+/-0.129 %staining area, CLP 2.269+/-0.133 %staining area vs Control 4.38+/-0.27 %staining area, all $p<0.01$, one way anova with Bonferroni post test correction) (Fig 3.13A). Hepatic hydroxyproline content was also

significantly lower in both the CMP and CLP groups compared with control mice (KSL 328.5+/-30.3 mg Hyp per g liver, CMP 331.5+/-22.5 mg Hyp per g liver, CLP 360.8+/-27.1 mg Hyp per g liver vs Control 428.4+/-31.9 mg Hyp per g liver, all groups: $p<0.05$, one way anova with Bonferroni post test correction)(Fig 3.13B) Analysis of αSMA staining showed a significant reduction after both CMP and CLP infusions (KSL2.42+/-0.200 %staining area, CMP 2.792+/-0.255 % staining area, CLP 3.694+/-0.325 %staining area vs Control 7.055+/-0.334 % staining area, $p<0.001$ all groups, one way anova with Bonferroni post test correction)(Fig 3.13D) and αSMA and col1α1 gene expression was significantly lower in both groups at levels similar to those seen after HSC infusions (Fig 3.13E,F). Serum albumin levels were significantly higher than control in both the CMP and CLP treated mice, although the improvement was not as marked as seen in KSL treated mice (KSL 4.06+/-0.37 g/dl, CMP 3.513+/-0.28 g/dl, CLP 3.517+/-0.35 g/dl vs Control 3.146+/-0.39, $p<0.05$ all groups, one way anova with Bonferroni post test correction)(Fig 3.13C).

Overall the observed pattern of results demonstrated a similar beneficial effect of both myeloid and lymphoid progenitors suggesting that myeloid differentiation is not a key mechanism involved in the beneficial action of HSC and that early progenitor cells appear equally as efficacious as more primitive stem cells.

Figure 3.12

Isolation of common myeloid and common lymphoid progenitor cells

Bone marrow cells were obtained from donor mice following dissection of the tibia and femurs by flushing the marrow cavity with DMEM + 10%FCS. Cells were treated with red cell lysis buffer and filtered prior to labelling with fluorescently conjugated antibodies to lineage specific markers (CD11b, CD8a, CD5, Ter119, Gr-1, B220), c-kit, sca-1 and IL-7Ra. (A) After exclusion of cell debris, dead cells and cell doublets, a further division of the lineage negative fraction was made on Il7Ra expression, followed by c-kit and sca-1 expression. Common myeloid progenitors (CMP) were defined as lineage negative, IL7Rα-, c-kit+, sca1- and common lymphoid progenitors (CLP) as lineage negative, IL7Rα +, c-kit lo , sca-1 lo. (B) The ability of each stem and progenitor cell population to form myeloid colonies was determined by myeloid CFU assay. KSL (white bars) and CMP (grey bars) cells formed colonies of myeloid cells but CLP (black bars) did not. Mean +/- SD number of colonies formed per 500 plated cells displayed. Assays performed in duplicate from 3 separate isolations.

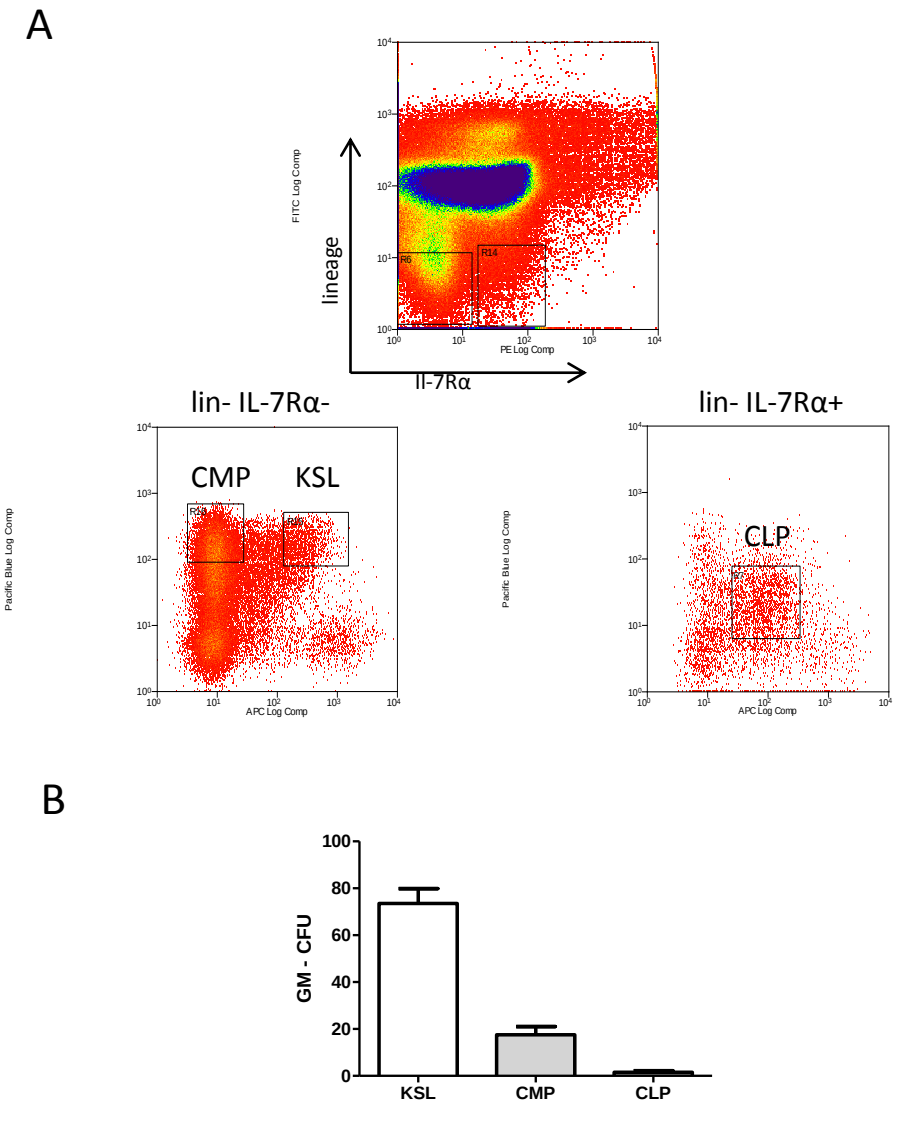


Figure 3.12
Isolation of common myeloid and common lymphoid progenitor cells

Figure 3.13

Repeated injection of either purified CMP or CLP results in a similar anti fibrotic effect to purified KSL cells

(A) Formalin fixed paraffin embedded liver tissue sections from mice treated with KSL (white bars), CMP (light grey bars) and CLP (dark grey bars) cell infusions and control mice (black bars) were stained for picrosirius red. 6 random, non-overlapping images were obtained from each section and staining was quantified as a percentage of the image positive for picorsirius red using ImageJ software. A similar reduction in picorsirius red staining was seen in all three treatment groups (KSL,CMP and CLP) compared with control mice. n=8, mean +/- SD % area stained shown, *p<0.05 vs control (one way anova with bonferroni multiple comparison test). (B) Hydroxyproline content in samples of liver tissue from KSL (white bars), CMP (light grey bars) and CLP (dark grey bars) cell treated mice and control mice (black bars) was determined. Significantly lower amounts of hydroxyproline were found in livers from mice receiving repeated KSL, CMP or CLP cell injections compared with livers from control mice. n=8, mean+/-SD hydroxyproline content shown, *p<0.05 (one way anova with bonferroni multiple comparison test). (C) Serum samples were obtained by centrifugation of whole blood from control mice (black bars) and mice treated with KSL (white bars), CMP (light grey bars) and CLP (dark grey bars) cell injections (white bars) and serum albumin concentration was measured by bromocresol green colorimetric assay. Serum albumin concentration was higher in mice treated with KSL , CMP and CLP cell injections than control mice. n=8, mean+/-SD serum albumin concentration shown, *p<0.05 (one way anova with bonferroni multiple comparison test). (D) Formalin fixed paraffin embedded liver tissue sections from mice treated with KSL (white bars), CMP (light grey bars) and CLP (dark grey bars) cell infusions and control mice (black bars) were stained for aSMA. 6 random, non-overlapping images were obtained from each section and staining was quantified as a percentage of the image positive for aSMA using ImageJ software. A similar reduction in aSMA staining was seen in all three treatment groups (KSL,CMP and CLP) compared with control mice. n=8, mean +/-SD % area stained shown, *p<0.05 vs control (one way anova with bonferroni multiple comparison test). (E,F) RNA was extracted from liver tissue from mice treated with KSL (white bars), CMP (light grey bars) and CLP (dark grey bars) cell infusions and control mice (black bars) and using quantitative RT-PCR gene expression of (E)col1a1 and (F) aSMA were determined, normalised to GAPDH expression and relative to control mice using the 2^{-ddCT} method. Hepatic gene expression of both col1a1 and aSMA was significantly lower in KSL, CMP and CLP cell treated mice than control mice. n=8 each group, mean +/-SD fold change shown, *p<0.05 (wilcoxon signed rank test).

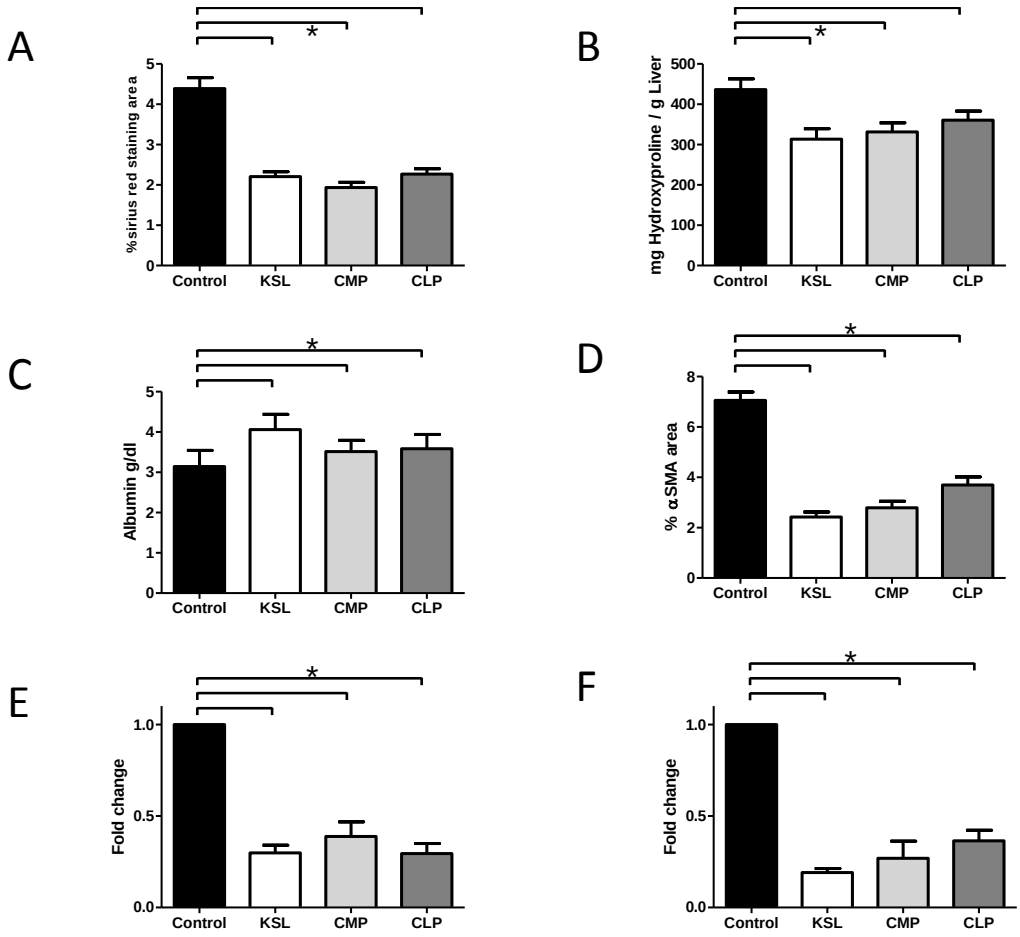


Figure 3.13
Repeated injection of either purified CMP or CLP results in a similar anti fibrotic effect to purified KSL cells

3.7 Conclusions

There has been considerable interest in the therapeutic benefits of bone marrow derived stem cells for patients with liver disease. There have however been mixed reports of the effects of injecting unselected bone marrow cells which comprise a heterogeneous population of cells, and the specific cell type or types responsible for either an anti- or pro-fibrotic effect is not clear(Sakaida *et al.* 2004; Cho *et al.* 2011; Thomas *et al.* 2011). In this first part of work I sought to clarify the effect of a specific, purified population of bone marrow cells – the c-kit+ sca1+ lineage – fraction – which represents primitive haematopoietic stem cells and early mulitpotent progenitor cells.

I have shown that increased numbers of haematopoietic stem cells are mobilised into the bloodstream and recruited into the liver during chronic liver injury and importantly, have confirmed through the generation of bone marrow chimerism that these HSC are derived from the bone marrow rather than an expansion of liver resident HSC, a site of fetal haematopoiesis (Taniguchi *et al.* 1996). This is in keeping with previous observations that HSC are present at higher numbers in the blood of patients following liver resection or transplantation (Lemoli *et al.* 2006; Gehling *et al.* 2010). This may represent a mechanism of repair and regeneration when hepatocyte replication and hepatic progenitor cell responses are overwhelmed, and it has been shown that liver regeneration is significantly improved in patients receiving CD133+ cells prior to liver resection(am Esch *et al.* 2012).

This work examined the effects on hepatic fibrosis of repeated injections of a purified population of haematopoietic stem and progenitor cells defined by the expression of c-kit

and sca-1 and lack of lineage markers. Freshly isolated cells were administered through the tail vein into the systemic circulation at weekly intervals on 3 separate occasions.

The findings of these studies demonstrate a marked reduction in hepatic fibrosis in mice treated with HSC compared with control mice receiving no treatment. The beneficial effect of repeated injections of a relatively small number of highly purified HSC has not previously been shown.

Significant reductions in extracellular matrix (ECM) deposition as measured by picrosirius red staining and hepatic hydroxyproline content were noted. This was accompanied by a 66% reduction in αSMA+ activated hepatic myofibroblasts. These observations are consistent with a reversal of the positive feedback that occurs during fibrogenesis whereby quiescent hepatic stellate cells undego activation to pro fibrogenic activated hepatic myofibroblasts with associated deposition of ECM, which in turn acts as a pro-survival signal for myofibroblasts. These data suggest that injection of KSL cells interrupts this process and promotes breakdown of ECM and inactivation or apoptosis of myofibroblasts. These studies were not designed to determine the precise mechanisms of action of KSL cell injection but rather to confirm their therapeutic effect in promoting resolution of fibrosis and to provide potential insights into the mechanisms involved which I will discuss further.

There was associated with this an improvement in serum albumin concentration which confirms that there was an improvement in liver function alongside the reduction in hepatic fibrosis.

The experimental design of these studies was deliberately chosen with translation to human applications in mind, particularly as clinical trials of HSC in liver disease are

underway(www.clinicaltrials.gov). The number of cells to be infused and the route of delivery of the cells were two key variables considered.

Two previous studies have shown the beneficial effect of a single injection of KSL cells on liver fibrosis, however in both cases $1x10^6$ cells were injected – twenty fold the number of cells per injection in this work. In one study KSL cells were cultured in vitro and injected prior to further phenotyping or assessment of colony forming ability. KSL cells represent a rare population of cells, comprising 0.05% of bone marrow cells and the yield of KSL cells from one donor mouse was in the order of 5-10,000 KSL cells per mouse. An injection of $1x10^6$ represents the administration of approximately $40x10^6$ cells /kg to a C57BL6 mouse, the equivalent number of cells in a 70kg human would be $2.8x10^9$ cells. Lorenzini et al studied GCSF induced stem cell mobilisation and harvesting in patients with liver cirrhosis and showed that the mean number of HSC isolated was $1.2x10^6$/kg(Lorenzini *et al.* 2008). The choice of number of cells to inject was made based in practical terms on the numbers of donor mice required but also for applicability of results to represent more closely the numbers of cells that could be isolated and used in patients with cirrhosis.

Previous studies in both humans and animal models have used a variety of approaches to cell injection, including peripheral vein injection, portal vein injection and hepatic artery injection (Houlihan *et al.* 2008). Access to the hepatic vessels requires specialised procedures involving significant risk, particularly if repeated injections are to be considered, and data from human studies have shown rare but catastrophic complications of hepatic vessel injection (Mohamadnejad *et al.* 2013). These studies used a peripheral vein injection to administer the cells and no complications related to cell injection in these studies were

noted, although in the bone marrow transplantation studies one mouse died immediately after injection presumably due to massive embolus of either air or cells as not other signs of overwhelming injury or infection could be found. It would be expected that a greater number of cells would engraft the liver if injected via the portal vein than if injected via a peripheral vein, however these findings suggest that the therapeutic effect is not completely lost. The trafficking of injected cells and subsequent tissue distribution has not been well studied and will be addressed in subsequent parts of this work.

I had hoped that the fate of the injected KSL cells may provide some insights into the potential mechanisms of effect and in these experiments injected donor cells could be identified by expression of CD45.2, given that recipient mice expressed CD45.1. However, the identification of injected cells within recipient liver at seven days after injection was an extremely rare event and no meaningful conclusions could be obtained with regard to the fate of injected cells, either in location within the liver or subsequent phenotypic fate. It raises the question whether repeated injection of cells is necessary to maintain the therapeutic effect or whether cell injection acts as a stimulus for endogenous repair which continues in the absence of the injected cells.

I examined the effect of KSL cell injection on endogenous cell populations through immunostaining for the macrophage marker F4/80 and the neutrophil marker Ly6G. Higher quantities of both cell populations were noted in the livers of mice which had received KSL cell injections. In previous studies there have been mixed reports, where some authors have suggested that the anti fibrotic effect is mediated through recruitment of endogenous cells producing matrix metalloproteinases (Thomas *et al.* 2011) , others have suggested that

differentiation into cells of macrophage/monocyte lineages is the mechanism involved(Sakaida *et al.* 2004). The lack of staining for the donor cell marker CD45.2 confirmed that these were recipient derived cells, implying stimulation of endogenous repair mechanisms rather than differentiation of injected pluripotent KSL cells. Further work to confirm upregulation of MMPs following KSL cell injection and to clarify the cellular source of MMPs (ie donor vs recipient) is required.

The final studies in this part of work were designed to confirm whether differentiation of injected KSL cells into cells of a myeloid lineage, particularly those of macrophage-monocyte, given the reports of the anti fibrotic effect of bone marrow derived macrophages (Thomas *et al.* 2011). I was able to isolate both common myeloid progenitors (CMP) and common lymphoid progenitors (CLP) through surface phenotyping(Kondo *et al.* 1997). An assessment of colony forming cells confirmed that CMP possessed myeloid colony forming ability whereas CLP did not.

The experimental design replicated that of the KSL studies and demonstrated that injection of either CMP or CLP resulted in an anti fibrotic effect remarkably similar to that seen with KSL cells. Injection of both cell types compared favourably with KSL cells, with significant reduction in hepatic collagen and numbers of αMSA+ hepatic myofibroblasts. Theses studies conclude that differentiation to myeloid lineages is not required for stem or progenitor cells to generate an anti fibrotic effect and is not the key mechanism of action for these cells. These findings suggest that stimulation of endogenous repair mechanisms, mediated through production and secretion of molecular signalling mediators– the so-called 'paracrine effect'- is the mechanism involved. Primitive, undifferentiated stem and progenitor cells

may function as effector cells in the immune system and may regulate immune cell trafficking through their ability to produce and secrete cytokines and chemokines at higher concentrations than more mature, differentiated cells(Granick *et al.* 2012).

In summary, I have shown for the first time the anti fibrotic effect of repeated infusions of purified HSC. This has been achieved with smaller numbers of cells than used in previous studies which is encouraging as this represents cell numbers relevant to human studies. The injected cells could not be identified in the recipient livers although significant increases in endogenous cell populations were observed.

4 SPHINGOSINE 1-PHOSPHATE METABOLISM IN LIVER INJURY

4.1 Introduction

S1P is produced by the phosphorylation of sphingosine by sphingosine kinase 1 and 2 (SPhK1,2) and degraded irreversibly for sphingosine lyase (SPhL)and reversibly by sphingosine phosphatise(SphP) (Olivera *et al.* 2013). S1P has a short half life (Venkataraman *et al.* 2008)() and therefore S1P levels are controlled by the balance of activity between synthesising and degrading enzymes(Venkataraman *et al.* 2008). Sphingosine lyase is a predominantly tissue resident enzyme vital for maintaining low tissue concentrations of S1P, and is absent from platelets and erythrocytes which contain large intracellular stores of S1P(Ito *et al.* 2007). Vascular endothelial cells, including hepatic sinusoidal endothelial cells contribute to the synthesis of S1P in the blood through expression of sphingosine kinase 1(Venkataraman *et al.* 2008). A role for the liver in S1P production is further suggested by the observation by Kurano et al that plasma S1P levels are lower in mice following hepatectomy (Kurano *et al.* 2013). Furthermore, activation of SPhK has been demonstrated following stimulation by various growth factors and cytokines induced during liver injury (Xia *et al.* 1999; Rivera *et al.* 2008). Venkataraman and colleagues demonstrated that lymphatic endothelial cells are responsible for the synthesis of lymphatic S1P(Venkataraman *et al.* 2008). Intracellular signalling of S1P regulates many cellular processes(Rosen *et al.* 2009) and the balance between intracellular S1P and its metabolic precursor ceramide determines cell fate(Takabe *et al.* 2008). S1P favours cell survival(Zhang *et al.* 1991; Olivera *et al.* 1993) whilst ceramide is pro-apoptotic(Cuvillier *et al.* 1996), controlled by the balance between synthesizing (sphingosine kinase) and degrading (sphingosine phosphatase and lyase)

enzymes. Studies on the role of S1P in the constituent cells of the liver are limited. In hepatocytes, Ikeda and colleagues demonstrated that S1P exerted an antiproliferative effect in vitro which was mediated through increased Rho activation(Ikeda *et al.* 2003). Bile acid induced hepatocyte apoptosis was accompanied by an increase in S1P levels and inhibition of sphingosine kinase 1 reduced apoptosis through suppression of intracellular calcium signalling (Karimian *et al.* 2013), whilst inhibition of sphingosine kinase 2 in hepatocytes subjected to anoxia-reoxygenation reduced both S1P production and hepatocyte death(Shi *et al.* 2012). In contrast to the effects seen in hepatocytes, Zheng et al showed that exposure to S1P reduced ethanol induced apoptosis of sinusoidal endothelial cells by increasing intracellular calcium and endothelial nitric oxide synthetase activity(Zheng *et al.* 2006). The pathological hallmark of hepatic fibrosis is the activation of hepatic stellate cells to form fibrogenic myofibroblasts(Friedman 2008), stimulated by cytokines such as TGFβ(Henderson *et al.* 2008). Yang et al observed a correlation between the hepatic expression of SphK1 and that of TGFβ (Yang *et al.* 2013), whilst TGFβ is also a potent stimulator of SphK activity(Rivera *et al.* 2008). S1P has been shown to stimulate hepatic stellate cell proliferation and migration (Ikeda et al. 2000), mediated through the S1P2 receptor as in S1P2 knock out mice accumulation of hepatic myofibroblasts was reduced following liver injury resulting in lower levels of hepatic fibrosis (Ikeda et al. 2009). Liu and colleagues demonstrated migration of human hepatic stellate cell line to S1P and stimulation of fibrogenesis by stellate cells upon exposure to s1P(Liu *et al.* 2011). The contractility of hepatic stellate cells can be increased by S1P in vitro (Yang et al. 2013) and S1P also increased portal pressure in rats with chronic liver injury (Ikeda et al. 2004). It has been suggested that there is 'crosstalk' between S1P and TGFβ during liver injury promoting the

development of hepatic fibrosis, although the exact cellular and molecular mechanisms involved are yet to be discovered (Takuwa *et al.* 2012). There is current interest in the contribution of bone marrow derived mesenchymal stem cells (MSC) to the hepatic myofibroblast population and it has been reported that homing of MSC to the injured liver is mediated by S1P signalling via the S1P3 receptor. In murine models of liver injury administration of an S1P3 antagonist resulted in reduced MSC migration to the liver and a reduction in hepatic fibrosis(Li *et al.* 2009; Li *et al.* 2009).

Alterations to S1P metabolism have been shown to occur under a variety of inflammatory and injurious conditions (Takuwa *et al.* 2012). Li and colleagues have shown significant increases in liver and serum S1P levels in both carbon tetrachloride and bile duct ligation murine models of liver injury(Li *et al.* 2009; Li *et al.* 2009). The increase in hepatic S1P was associated with upregulation of hepatic SphK1 expression during liver injury with no alternations in SphL or SphP. Hepatic SphK activity was correlated with serum S1P concentration, and as no corresponding increase in SphK in blood cells was observed lead the authors to surmise that the rise in serum S1P was derived from the liver (Li *et al.* 2009). Li also reported that in samples of human fibrotic liver, S1P levels were higher than normal liver accompanied by increased sphingosine kinase expression (Li *et al.* 2011). However, contrary to these findings, Ikeda measured plasma S1P levels in patients with chronic hepatitis C virus infection and found levels were significantly lower than in matched controls and that plasma S1P levels were inversely correlated with the degree of hepatic fibrosis (Ikeda *et al.* 2010).

S1P regulates migration of a variety of different cell types, including lymphocytes and other immune cell populations, mediated through maintenance of concentration gradients between different body fluids and tissues. In particular, Massberg and colleagues have shown that S1P regulates the egress of HSC from peripheral tissues (low S1P concentration) into draining lymphatics (high S1P concentration)(Massberg *et al.* 2007). There is also emerging evidence of the role of S1P in mobilisation of HSC from the bone marrow into the circulation (Ratajczak *et al.* 2010; Juarez *et al.* 2012)

It is clear that alterations to S1P metabolism occur during liver injury which may directly (through autocrine or paracrine mechanisms) or indirectly through altered cellular migration affect the progression of liver injury. The key to altered cell migration is changes in S1P gradients between body compartments. A greater understanding of the changes that occur to S1P metabolism during liver injury will provide further insights into the mechanisms of HSC trafficking. In this section of work my aim is to determine the changes that occur to S1P concentrations in liver injury and the mechanisms responsible for these changes.

4.2 S1P levels in liver and serum are increased during chronic liver injury

I used the carbon tetrachloride model of liver injury to investigate the differences in concentrations of S1P in different body compartments during chronic liver injury. The areas of interest were the key tissues or fluids involved in the trafficking and recirculation of HSC as previously described (Massberg *et al.* 2007), namely the bone marrow, peripheral blood, liver and lymph fluid.

Carbon tetrachloride was administered twice weekly for 8 weeks, which as previously shown induces a significant chronic liver injury. Control mice received twice weekly injections of mineral oil, the diluting vehicle for carbon tetrachloride. S1P levels were measured using a proprietary ELISA which has been shown to correlate with results obtained from high performance liquid chromatography(Kirby *et al.* 2009) (Prof L. Arend, personal communication).

Higher concentrations of S1P were found in both the livers (Injury 68.79+/-7.42 pmol per mg protein vs Control 39.45+/-7.06 pmol per mg protein, $p<0.05$)(Fig 4.1B)and serum (Injury 1.71+/-0.13 µM vs Control 1.15+/-0.18 µM, $p=0.0025$) (Fig 4.1A)of mice with chronic injury compared with control mice. Although no significant difference was seen in S1P concentration in the bone marrow or lymph, there was a suggestion that levels in the bone marrow were lower (Injury 312.7+/-74.5 pmol per mg protein vs Control 378.5+/-86.1 pmol per mg protein, p=ns) (Fig 4.1C) and in the lymph were higher (Injury 0.104+/-0.015 µM vs Control 0.084+/-0.012 µM, p=ns) during liver injury (Fig 4.1D).

Previous studies have demonstrated the importance of a gradient in S1P levels between different body compartments as being crucial for cellular migration. The differences in the

Figure 4.1

S1P Levels in Liver Tissue and Serum are elevated in Chronic Liver Injury induced by Carbon Tetrachloride

Chronic liver injury was induced in age and sex matched C57/BL6 mice by twice weekly intraperitoneal administration of carbon tetrachloride (1mg/kg) in mineral oil for 8 weeks. Control mice received twice weekly intraperitoneal injections of mineral oil. Mice were sacrificed and levels of S1P in serum and lymph were measured directly by ELISA and concentration quantified. Samples of bone marrow and liver were homogenised in buffer as described (Section 2.18) and protein concentration determined by Bradford assay. Concentration of S1P within the homogenate was determined by ELISA and quantified relative to protein concentration in each sample. Bars represent mean +/- SEM. N=5 mice per group, *$p<0.05$,** $p<0.01$ (two tailed, unpaired students t-test).

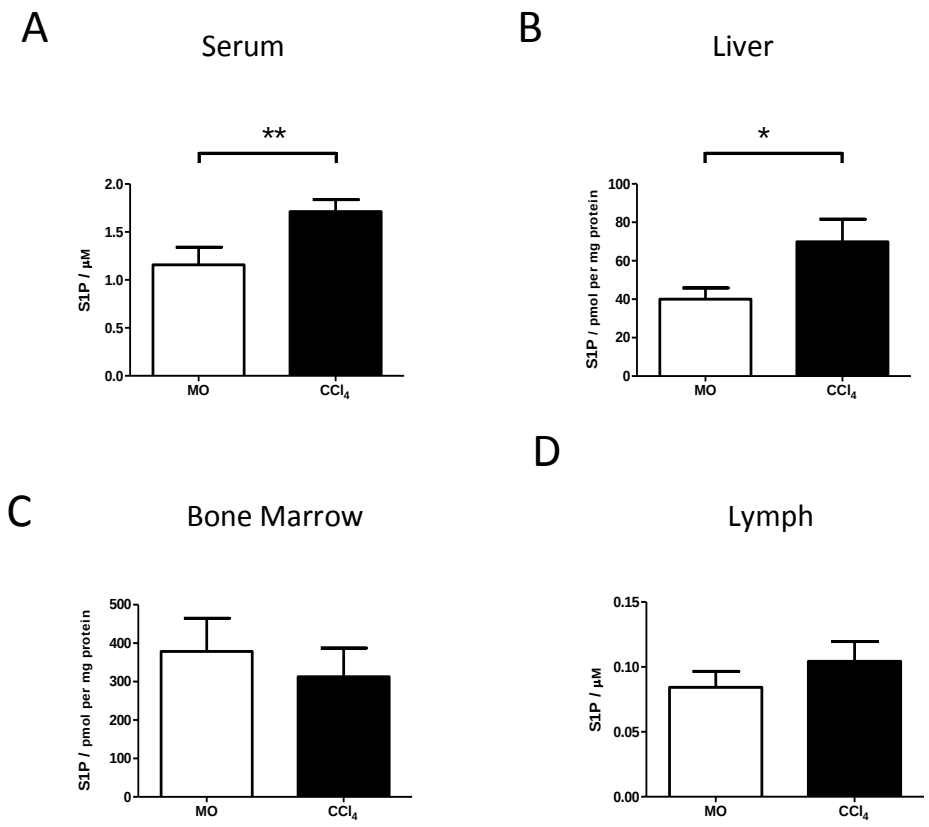


Figure 4.1
S1P Levels in Liver Tissue and Serum are elevated in Chronic Liver Injury induced by Carbon Tetrachloride

units of measurement used and the methods of normalisation do not necessarily permit a direct comparison between the values for different body compartments and direct measurement of concentrations in the tissue interstitial fluid have not been possible. However when the relative changes in levels between control and injury are examined this suggests that the gradient between the bone marrow and peripheral blood increases to favour the peripheral blood due to the 1.5 fold increase in serum S1P concentration, and the gradient between the liver and draining lymph changes to favour the liver due to the 1.7 fold increase in the liver concentration.

4.3 Sphingosine Kinase 1 expression and activity is increased in the liver during chronic liver injury

S1P has a short half life and concentrations are determined according to the balance between its synthesizing enzymes (Sphingosine Kinase 1 and 2) and degrading enzymes (Sphingosine Lyase and Phosphatase). Changes in these enzymes during liver injury were investigated further by firstly examining relative changes in liver gene expression of the four enzymes by qPCR.

The striking finding was that of a 5.5 fold upregulation of SphK1 hepatic gene expression during liver injury (CCl4 Injury 5.57+/-0.65 fold increase vs control, $p<0.001$), whilst expression of the other enzymes remained unchanged (SphK2 1.027+/-0.709, SphL 1.131+/-1.013, SphP 0.817+/-0.683 fold increase vs control, p=ns)(Fig 4.2).

To determine whether this upregulation of SphK1 during chronic liver injury was specific to the liver or represented a systemic response to toxic injury, gene expression in three other major organs – spleen, lung and kidney - was also determined. No significant changes in gene expression were seen in other organs examined (Fig 4.3).

An alternative model of liver injury was also used to confirm the findings in the carbon tetrachloride model and ensure that the observations were not restricted a one particular aetiology of liver injury. The Methionine Choline Deficient Diet was fed to C57BL6 mice for 6 weeks, resulting in a 40% loss of body weight (the maximum permitted by Home Office Project Licence for humane reasons). This resulted in an established injury with steatosis, hepatocyte ballooning, inflammatory infiltrates and fibrosis.

Figure 4.2

Hepatic Sphingosine Kinase 1 gene expression is upregulated in Chronic Liver Injury

Chronic liver injury was induced in age and sex matched C57/BL6 mice by twice weekly intraperitoneal administration of carbon tetrachloride (1mg/kg) in mineral oil for 8 weeks. Control mice received twice weekly intraperitoneal injections of mineral oil. Mice were sacrificed and hepatic gene expression of sphingosine kinase 1, sphingosine kinase 2, sphingosine lyase and sphingosine phosphatase relative to GAPDH was determined. Bars represent mean +/- SEM fold change relative to control mice. N=5 mice per group, *** $p<0.0001$

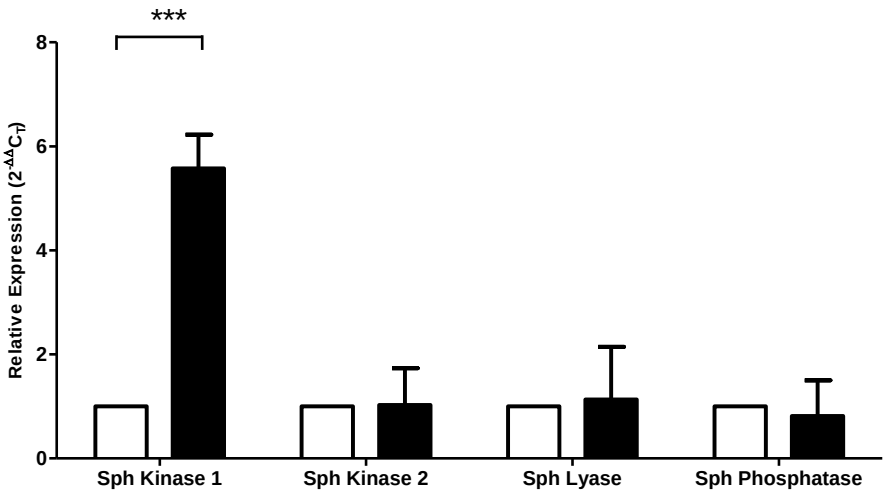


Figure 4.2
Hepatic Sphingosine Kinase 1 gene expression is upregulated in Chronic Liver Injury

Figure 4.3

Upregulation of Sphingosine Kinase 1 gene expression in Chronic Liver Injury is restricted to the liver

Chronic liver injury was induced in age and sex matched C57/BL6 mice by twice weekly intraperitoneal administration of carbon tetrachloride (1mg/kg) in mineral oil for 8 weeks. Control mice received twice weekly intraperitoneal injections of mineral oil. Mice were sacrificed and gene expression of sphingosine kinase 1, sphingosine kinase 2, sphingosine lyase and sphingosine phosphatase relative to GAPDH was determined in (A)spleen, (B) kidney and (C) lung. Bars represent mean +/- SEM fold change relative to control mice. N=5 mice per group,.

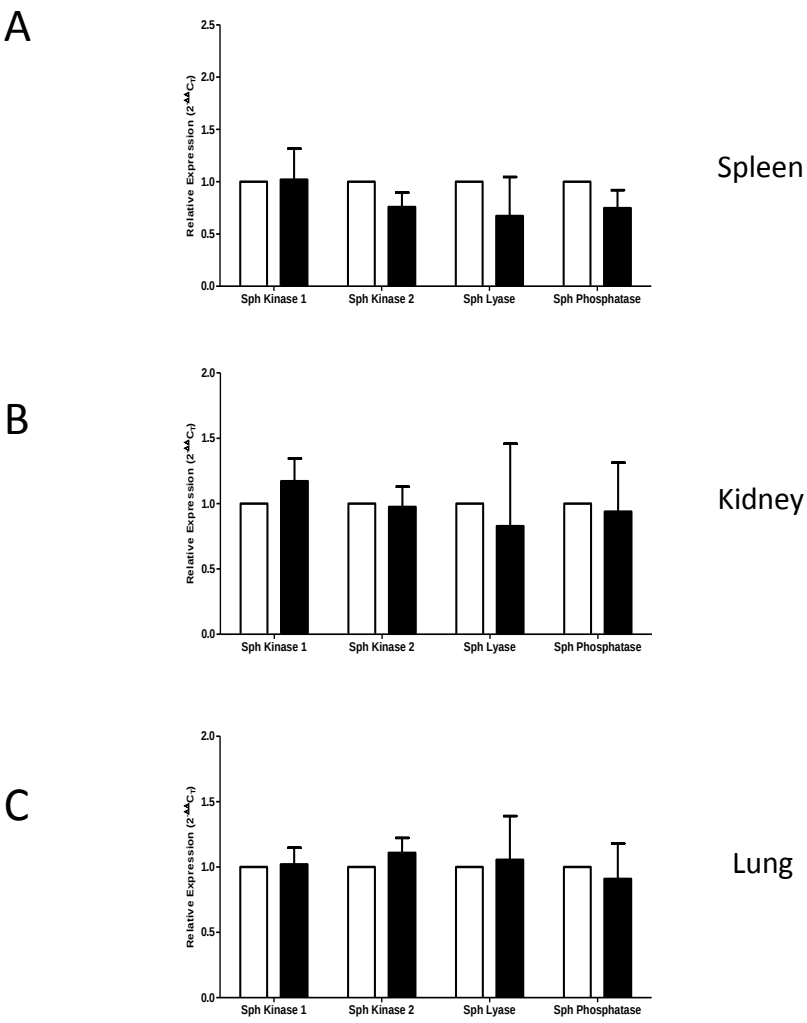


Figure 4.3
Upregulation of Sphingosine Kinase 1 gene expression in Chronic Liver Injury is restricted to the liver

A similar pattern of changes in relative gene expression was seen with 4.4 fold upregulation of hepatic SphK1 gene expression in the liver of injured mice (MCD Injury SphK1 4.408+/-1.464 fold increase vs control, $p<0.001$) (Fig 4.4). Gene expression of the other enzymes was unaltered (SphK2 0.805+/-0.221, SphL 0.774+/-0.474, SphP 0.935+/-0.065 fold increase vs control, p=ns)(Fig 4.4).

These findings suggested an important role for SphK1 in the liver during chronic liver injury, therefore further studies of this enzyme were performed by examining hepatic protein expression and tissue enzymatic activity.

Semi quantitative determination of changes in hepatic SphK1 protein levels was performed using western blotting. Clear differences in the intensities of the protein bands at the expected molecular weight (37kDa) for SphK1 were observed (Fig 4.5A).

Figure 4.4

Hepatic Sphingosine Kinase 1 gene expression is upregulated in an alternative model of Chronic Liver Injury

Age and sex matched C57/BL6 mice were fed a Methionine Choline Deficient Diet for 6 weeks to induce chronic liver injury, control mice were fed normal chow. Mice were sacrificed and hepatic gene expression of sphingosine kinase 1, sphingosine kinase 2, sphingosine lyase and sphingosine phosphatase relative to GAPDH was determined. Bars represent mean +/- SEM fold change relative to control mice. N=5 mice per group, *** $p<0.0001$

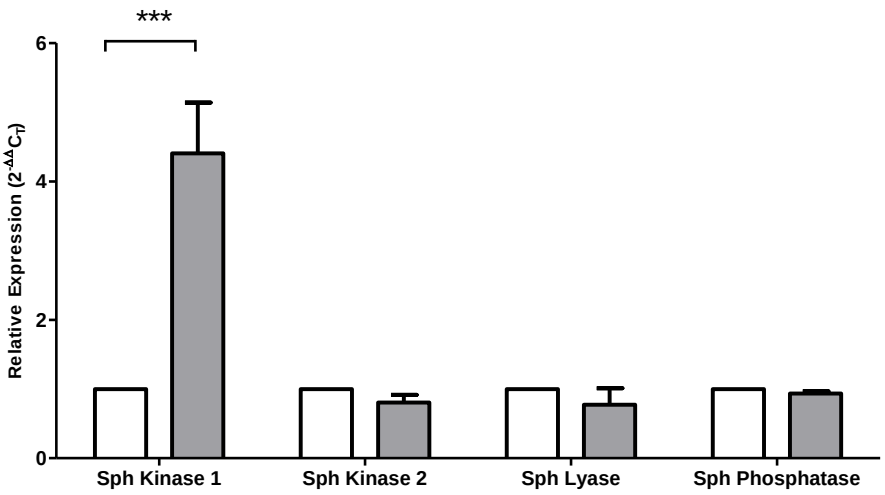


Figure 4.4
Hepatic Sphingosine Kinase 1 gene expression is upregulated in an alternative model of Chronic Liver Injury

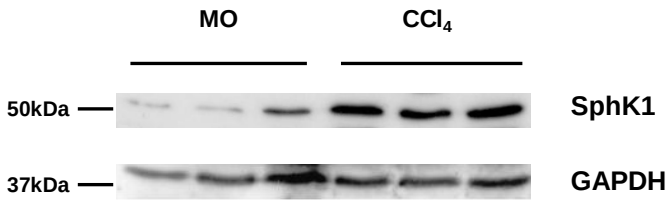
Analysis of the enzymatic activity of SphK1 within the liver was performed as previously described using a commercially available assay. Significantly higher levels of SphK activity were demonstrated in injured livers compared with controls (CCl4 Injury 5.76+/- 0.95 nmol S1P/min/mg protein vs 2.03+/-0.25 nmol S1P/min/mg protein, $p<0.01$)(Fig 4.5B).

Figure 4.5

Sphingosine Kinase Activity in Murine Liver Injury

Chronic liver injury was induced in age and sex matched C57/BL6 mice by twice weekly intraperitoneal administration of carbon tetrachloride (1mg/kg) in mineral oil for 8 weeks. Control mice received twice weekly intraperitoneal injections of mineral oil. (A)Samples of liver tissue from mice in each group were lysed for protein extraction and expression of sphingosine kinase 1 (SphK1) and GAPDH in each sample determined by western blotting. A blot demonstrating SphK1 and GAPDH expression in livers from carbon tetrachloride treated mice and mineral oil treated mice is shown and is representative of 3 separate experiments. (B) Samples of liver tissue were homogenised in buffer solution and used in an ATP depletion assay to measure the enzymatic activity of SphK within the livers of carbon tetrachloride treated (black bars) and mineral oil treated (white bars) mice. The rate of phosphorylation of sphingosine to S1P was determined from the rate of depletion of ATP and expressed as nmol of S1P produced per minute per mg of liver protein. n=5, mean+/-SD rate of S1P formation shown, **<0.01 (two tailed unpaired students t-test).

A



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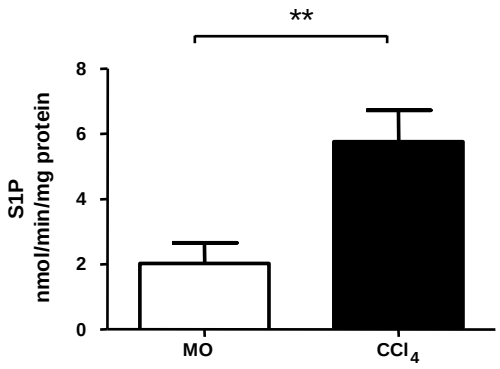


Figure 4.5
Sphingosine Kinase Activity in Murine Liver Injury

4.4 Similar changes in Sphingosine Kinase 1 expression are seen in humans with chronic liver disease

Changes in relative gene expression of the enzymes responsible for S1P metabolism were determined in samples of human liver from normal donors and explanted livers of patients undergoing liver transplantation for alcohol related liver disease (ALD). A similar pattern to that seen in murine livers was observed in the human liver samples. A significant 12.5 fold upregulation of hepatic SphK1 gene expression was noted (ALD Injury 12.55+/-6.1 fold increase vs normal liver, $p<0.001$) with no significant changes in the other enzymes (Fig 4.6).

Samples were also obtained from diseased livers with a variety of differing aetiologies of chronic liver disease (alcoholic liver disease, primary biliary cirrhosis, autoimmune hepatitis, non alcoholic fatty liver disease, chronic hepatitis C) and acute liver failure (paracetamol overdose). The same pattern of changes in enzyme gene expression was seen regardless of the underlying aetiology, however the sample sizes for each individual condition prevent a thorough statistical analysis given the amount of individual variation (Fig 4.7).

Further investigation of SphK1 protein expression by western blotting in human liver samples demonstrated increased SphK 1 expression in a variety of liver diseases (Fig 4.8A). Samples of human liver from patients with alcohol related liver disease were used to determine SPhk enzymatic activity which was 4-fold higher than in normal human liver samples (ALD Injury 4.38+/-1.11 nmol S1P/min/mg protein vs Normal 1.04+/-0.21 nmol S1P/min/mg protein, $p<0.05$)(Fig 4.8B).

Figure 4.6

Upregulation of SphK1 in Human Alcohol Related Chronic Liver Disease

Samples of frozen liver tissue were obtained from either normal donor liver or explanted livers from patients with alcohol related chronic liver disease. Gene expression of sphingosine kinase 1, sphingosine kinase 2, sphingosine lyase and sphingosine phosphatase relative to GAPDH was determined. Bars represent mean +/- SEM fold change relative to normal liver. N=6 per group, *** $p<0.0001$

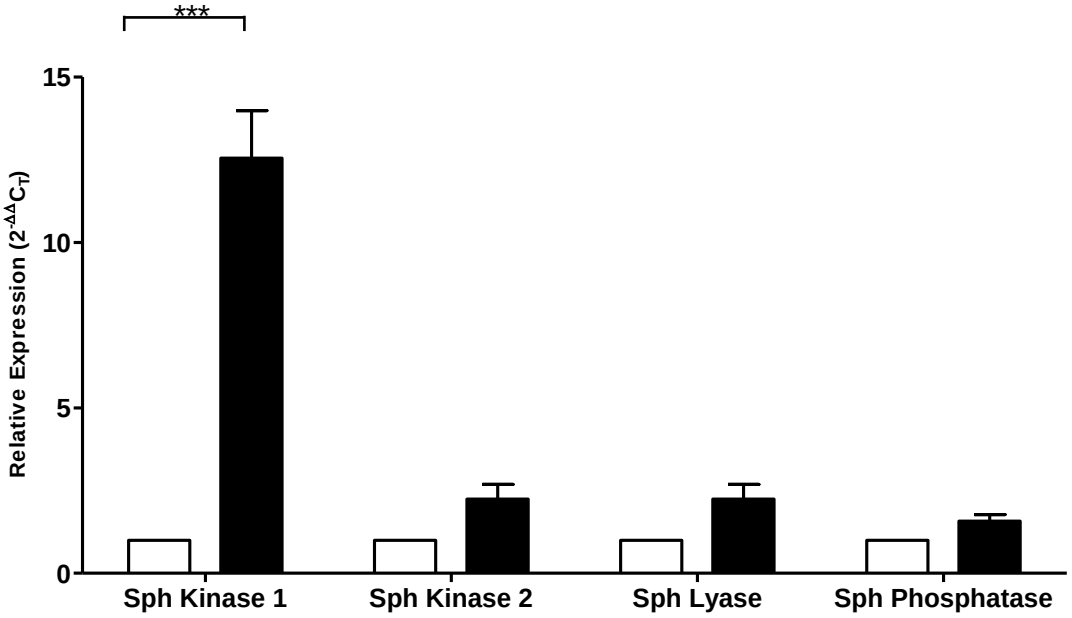


Figure 4.6
Upregulation of SphK1 in Human Alcohol Related Chronic Liver Disease

Figure 4.7

Upregulation of SphK1 in Human Liver Disease of Differing Aetiologies

Samples of frozen liver tissue were obtained from either normal donor liver or explanted livers from patients with liver disease (ALD Alcoholic liver disease, PBC primary biliary cirrhosis, AIH Autoimmune hepatitis, NAFLD Non alcoholic fatty liver disease, HCV Hepatitis C virus, POD Paracetamol ovedose). Gene expression of sphingosine kinase 1, sphingosine kinase 2, sphingosine lyase and sphingosine phosphatase relative to GAPDH was determined. Bars represent mean +/- SEM fold change relative to normal liver. N=3 per group.

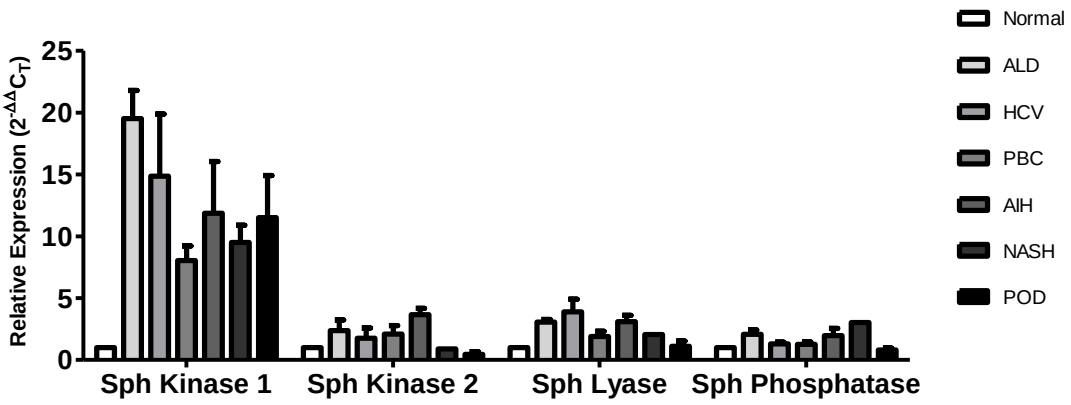


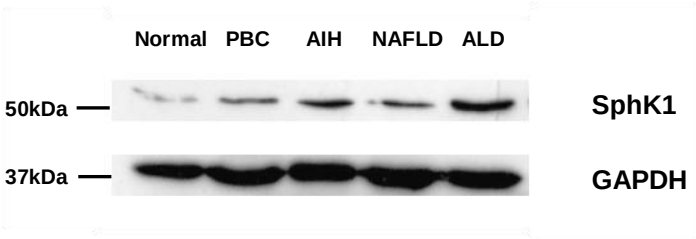
Figure 4.7
Upregulation of SphK1 in Human Liver Disease of Differing Aetiologies

Figure 4.8

Sphingosine Kinase Activity in Human Liver Disease

Samples of frozen liver tissue were obtained from either normal donor liver or explanted livers from patients with liver disease (ALD Alcoholic liver disease, PBC primary biliary cirrhosis, AIH Autoimmune hepatitis, NAFLD Non alcoholic fatty liver disease, HCV Hepatitis C virus). (A)Samples of human liver tissue were lysed for protein extraction and expression of sphingosine kinase 1 (SphK1) and GAPDH in each sample determined by western blotting. A blot demonstrating SphK1 and GAPDH expression in normal and diseased livers is shown and is representative of 3 separate experiments. (B) Samples of liver tissue were homogenised in buffer solution and used in an ATP depletion assay to measure the enzymatic activity of SphK in diseased (black bars) and normal (white bars) liver tissue. The rate of phosphorylation of sphingosine to S1P was determined from the rate of depletion of ATP and expressed as nmol of S1P produced per minute per mg of liver protein. n=5, mean+/-SD rate of S1P formation shown, *$p<0.05$, (two tailed unpaired students t-test).

A



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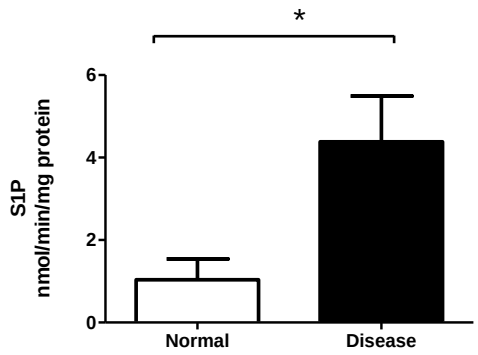


Figure 4.8
Sphingosine Kinase Activity in Human Liver Disease

4.5 Upregulation of Sphingosine Kinase predominantly occurs in liver sinusoidal endothelial cells

In order to investigate the cellular source of the increased hepatic expression and activity of Sphk1 in liver injury I isolated two main cell types from the liver. The isolation of liver sinusoidal endothelial cells from murine liver by immunomagnetic selection using CD146 as a marker of LSEC had recently been optimised in our laboratories and I was able to use this technique to isolate LSEC. I used slow centrifugation as a method of isolating hepatocytes from a single cell suspension and density gradient separation to isolate peripheral blood mononuclear cells.

Analysis of gene expression revealed differences in the relative expression of enzymes between the cell types within normal liver. Sphk1 was the most abundantly expressed enzyme in LSEC with much lower levels of SphL and SphP. In contrast hepatocytes expressed predominantly degrading enzymes SphP and SphL with less SphK1 expression (Fig 4.9).

Having previously shown significant upregulation of SphK1 during liver injury, I investigated changes that occurred to SpqK1 expression in individual cell types during liver injury. Freshly isolated LSEC, hepatocytes and peripheral blood mononuclear cells from both control mice and mice with carbon tetrachloride induced chronic liver injury were analysed for expression of SphK. During liver injury expression of SphK 1 on LSEC was markedly higher than in control mice (Injury 5.26+/-1.26 fold increase vs control, $p<0.001$.)(Fig 4.10A), although expression was unchanged in other cell types (Hepatocytes 1.70+/-0.86 fold increase vs control, p=ns, PBMC 1.24+/-0.51 fold increase vs control, p=ns) (Fig 4.10B,C).

Figure 4.9

Expression of Sphingosine Kinase, Lyase and Phosphatase in individual cell populations

Liver tissue and blood was obtained from C57BL6 mice. Liver sinusoidal endothelial cells (LSEC) were isolated by density gradient centrifugation of non parencyhmal cells and immunomagentic isolation of CD146+ cells. Hepatocytes were isolated by slow speed centrifugation of liver cell suspension. Peripheral blood mononuclear cells (PBMC) were isolated by red cell lysis and density gradient centrifugation. Gene expression of sphingosine kinase 1, sphingosine kinase 2, sphingosine lyase and sphingosine phosphatase relative to GPADH was determined in each cell type by the 2^{-dCT} method. Bars represent mean +/- SEM expression relative to GAPDH. N=3 per group.

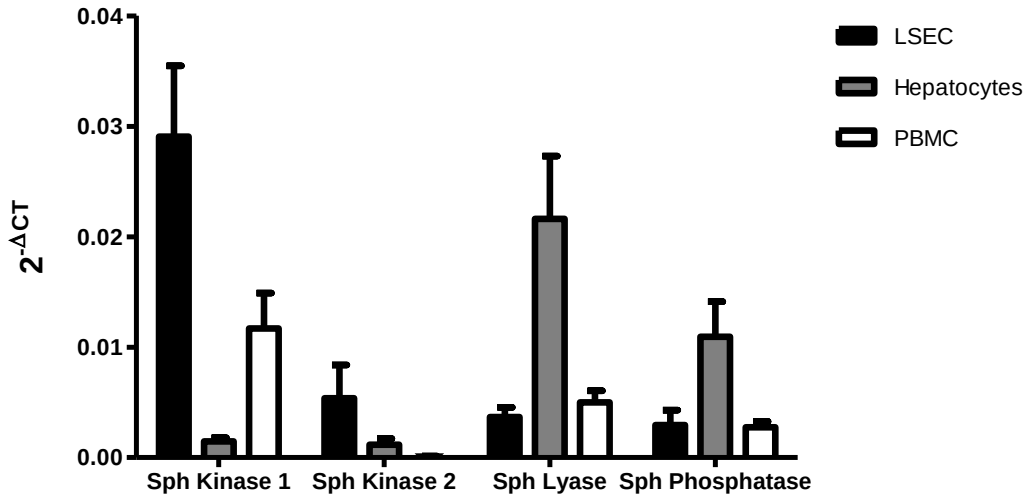


Figure 4.9
Expression of SphK, SphL and SphP in individual cell populations

Figure 4.10

Alterations in SphK1 expression during liver injury in individual cell populations

Chronic liver injury was induced in age and sex matched C57/BL6 mice by twice weekly intraperitoneal administration of carbon tetrachloride (1mg/kg) in mineral oil for 8 weeks. Control mice received twice weekly intraperitoneal injections of mineral oil. Liver sinusoidal endothelial cells (LSEC) were isolated by density gradient centrifugation of non parencyhmal cells and immunomagentic isolation of CD146+ cells. Hepatocytes were isolated by slow speed centrifugation of liver cell suspension. Peripheral blood mononuclear cells (PBMC) were isolated by red cell lysis and density gradient centrifugation. Gene expression of sphingosine kinase 1 normalised to GAPDH and fold change in cells from injured mice relative to control mice was determined. Bars represent mean +/- SEM fold change relative to control mice. N=3 per group, ***$p<0.001$, two tailed unpaired students t-test).

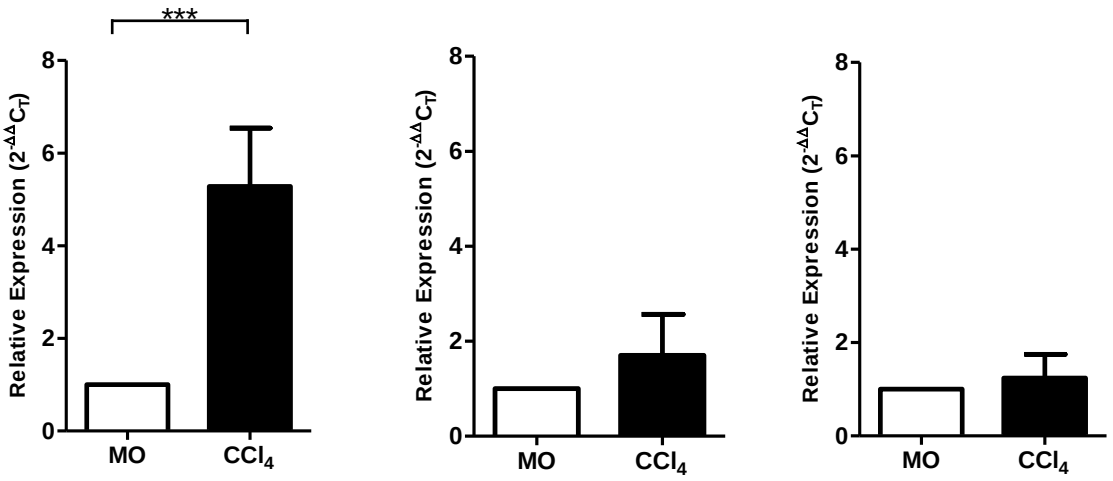


Figure 4.10
Alterations in SphK1 expression during liver injury in individual cell populations

4.6 Conclusions

Sphingosine 1-phosphate is a key regulator of many cellular processes, acting as both an intracellular and extracellular signalling molecule(Takabe *et al.* 2008; Olivera *et al.* 2013). The role of S1P during liver injury has not yet been fully elucidated but important functions are likely to include control of the balance between cell survival and apoptosis through intracellular signalling and crosstalk between S1P and TGFβ cell signalling in the development of hepatic fibrosis (Yang *et al.* 2013). S1P functions as a regulator of haematopoietic stem cell trafficking, with specific roles demonstrated in controlling the egress of HSC from peripheral tissues into draining lymphatics (Massberg *et al.* 2007)and in promoting the mobilisation of HSC from the bone marrow into the peripheral blood (Ratajczak *et al.* 2010). In both cases it is the generation of a concentration gradient of S1P between different body compartments that stimulates cell migration (Schwab *et al.* 2005). In the previous section of work I had demonstrated increased mobilisation of bone marrow derived HSC into the blood during liver injury, and increased numbers of bone marrow derived HSC within the liver during liver injury. It was therefore of great interest to determine whether changes in S1P concentrations, and in particular changes in the S1P gradients, occurred during liver injury.

I have shown that during chronic liver injury significant increases in S1P within the serum and liver are generated, whilst the bone marrow and lymph concentrations remain unchanged. It is very difficult to make direct comparisons between tissue concentrations, predominantly because it is not possible to directly measure S1P within the interistial fluid

but within the tissue as a whole. However, this limitation aside, it is clear from examining the relative changes that occur during liver injury that :

(i)an increase in serum S1P whilst bone marrow S1P remains unchanged would provide a greater stimulus to mobilisation of HSC and,

(ii) an increase in hepatic S1P without a change in lymph S1P would result in a reduction in the S1P gradient between liver and lymph.

There was a significant upregulation of sphingosine kinase 1 during liver injury in both the carbon tetrachloride and MCD models of liver injury, this is in keeping with previous observations from Li and colleagues who have also demonstrated this change in the bile duct ligation model of cholestatic liver injury(Li *et al.* 2009; Li *et al.* 2009). S1P has a short half life and concentrations of S1P are tightly regulated by the balance between the synthesizing and degrading enzymes, therefore the observed increase in hepatic sphingosine kinase 1 would explain the increased S1P concentration. The changes seen in sphingosine kinase were restricted to the liver and were not as a result of a multi system inflammatory or toxic response. Many cytokines and growth factors have been shown to stimulate sphingosine kinase 1 activity, and in particular those that are upregulated during liver injury such as TNFα(Xia *et al.* 1999).

I was also able to study the changes that occur to enzymes regulating S1P concentrations in human liver as this has not been well described previously. I have shown that in keeping with the changes seen in the animal models there is significant upregulation of sphingosine kinase 1 which is replicated across a variety of human liver diseases including those of viral, immune, cholestatic and acute aetiologies. These data provide reassurance that the animal

model is an acceptable method for further study. It was not possible for me to examine changes in serum levels of S1P from human subjects particularly as one report suggests that serum S1P levels were lower in patients with hepatitis C related cirrhosis(Ikeda *et al.* 2010), as opposed to the higher levels seen in the animal model. The significance of this is not clear and requires further investigation but it is possible that serum S1P levels may alter across the spectrum of fibrosis, with levels initially rising and then falling as cirrhosis and liver failure become established.

Having shown that there is upregulation of sphingosine kinase 1 within the liver during liver injury I investigated the cellular source of this increase. Endothelial cells of both the vascular and lymphatic vessels have been shown to be sources of S1P within the blood and lymph(Venkataraman *et al.* 2008; Fukuhara *et al.* 2012), whilst sphingosine kinase expression within the liver sinusoidal endothelial cells contributes to the serum S1P concentration(Venkataraman *et al.* 2008). Using cell isolation techniques previously optimised in our laboratories I was able to isolate populations of hepatocytes, liver sinusoidal endothelial cells and peripheral blood mononuclear cells for further analysis. Hepatocytes predominantly expressed SphP and SphL, whilst endothelial cells and mononuclear cells expressed sphk. This would suggest that hepatocytes are net 'degraders' of S1P and endothelial and mononuclear cells net 'producers' of S1P. It has been recognised that tissues are abundant in SphL, required for low S1P levels to maintain the S1P tissue gradient, whilst endothelial cells have a secretory function and contribute to serum S1P concentrations(Connolly *et al.* 2010; Fukuhara *et al.* 2012). Cells freshly isolated from mice with liver injury and control mice were also analysed for sphingosine kinase expression and I noted that there was significant upregulation of sphingosine kinase within the liver

sinusoidal endothelial cells during liver injury whilst expression in hepatocytes and mononuclear cells remained unaltered. I propose that during liver injury stimulation of liver sinusoidal endothelial cells results in upregulation of SphK1 which contributes to the increases in both hepatic and serum S1P concentrations.

Platelets and mononuclear cells have previously been shown to be important sources of S1P, with platelets in particular containing high levels of intracellular S1P, which can be secreted upon activation (Yatomi *et al.* 1997; Yang *et al.* 1999). During liver injury platelets and mononuclear cells are important in the development of liver injury and fibrosis and are recruited to the liver in significant numbers (Iannacone *et al.* 2005)and this may represent an important alternative source for the increased levels of S1P seen within the liver. Future experiments to investigate this further could include the adoptive transfer of bone marrow from SphK1 KO mice into WT mice followed by the induction of liver injury and subsequent analysis of S1P enzymes to determine the contribution SphK deficient haematopoietic cells.

In this section of work my interest has predominantly been in the effect of liver inury upon S1P concentration gradients given the emerging importance of S1P as a regulator of HSC trafficking. I have not, however, made attempts to further elucidate the role of increased S1P levels in the progression of liver injury and the development of fibrosis.

5 SPHINGOSINE 1-POSPHATE AND HAEMATOPOIEITC STEM CELL TRAFFICKING IN LIVER INJURY

5.1 Introduction

It has been previously shown that both human and murine haematopoietic stem cells express S1P receptors(Kimura *et al.* 2004; Seitz *et al.* 2005) and migrate in vitro to S1P(Seitz *et al.* 2005) , however it is only recently that the role of S1P in HSC homing and trafficking is becoming clearer(Massberg *et al.* 2007). This is an area of considerable interest, not only for the potential role of HSC in tissue regeneration, but also because a greater understanding of the mechanisms regulating mobilisation of HSC from the bone marrow into the circulation and homing from the circulation to the bone marrow will help optimise and refine bone marrow transplantation techniques for haematological disease.

Ratacjzak and colleagues have investigated the role of S1P in the mobilisation of HSC from the bone marrow following administration of the stimulatory cytokine GCSF. This resulted in activation of the complement cascade resulting in lysis of red blood cells by the membrane attack complex (Ratajczak *et al.* 2010), and as red blood cells contain large reserves of S1P (Bode *et al.* 2010)this results in increased plasma S1P levels. The increased plasma S1P promotes chemotaxis of HSC from the bone marrow into the circulation (Ratajczak *et al.* 2010). In a separate study by Golan and colleagues, administration of GCSF increased serum S1P concentration but also appeared to make bone marrow cells more responsive to S1P by upregualting the surface expression of the S1P1 receptor on bone marrow cells(Golan *et al.* 2012). The CXCR4 antagonist can also induce mobilisation of HSC by interfering with the SDF-1 retention signal within the bone marrow, a recent study by Juarez et al has shown

that this process is also dependent upon an adequate S!P gradient and S1P receptor signalling(Juarez *et al.* 2012). SphK1 knock out mice have lower plasma S1P concentrations than control mice, this results in a lower S1P gradient between the blood and bone marrow, and consequently lower CXCR4 antagonist induced mobilisation was seen in these mice than in control mice(Juarez *et al.* 2012). Similarly administration of the S1P receptor antagonist FTY720 downregulated S1P1 receptor expression on HSC and resulted in reduced mobilisation of HSC (Juarez *et al.* 2012).

The homing of HSC to the bone marrow and extramedullary tissues has been shown to be mediated by chemokine receptor signalling, and in particular CXCR4 (Kollet *et al.* 2003), CCR2 (Si *et al.* 2010)) regulate homing of HSC to the liver. Cell surface chemokine receptors recognise membrane bound ligand resulting in cell activation and transendothelial migration along chemokine gradients. To date there has yet to be demonstrated a role for S1P receptor signalling in HSC recruitment to bone marrow or other tissues. There is however, evidence of considerable crossover between S1P receptor and chemokine receptor signalling. Both S1P and chemokine receptors are G protein coupled receptors sharing signalling pathways, including the coupling of both S1P1 and CXCR4 to the Gai subunit with subsequent PI3K activation (Cyster 2005), and cross talk between CCR7 and S1P1 regulates the egress of T lymphocytes from lymph nodes (Pham *et al.* 2008). In human $CD34^+$ HSC, S1P receptor signalling has been shown to increase their CXCR4 mediated migration towards SDF-1(Whetton *et al.* 2003). However overexpression of S1P1 on human $CD34^+$ HSC resulted in reduced cell surface CXCR4 expression and a significant reduction in migration to SDF-1 but increased S1P induced migration(Ryser *et al.* 2008). Treatment of human $CD34^+$ HSC with FTY720 which downregulates S1P1 expression(Oo *et al.* 2007) resulted in the opposite effect,

with increased SDF-1 induced migration but with no alteration in surface CXCR4 expression (Kimura *et al.* 2004).

HSC circulate in peripheral blood at low frequency (Wright *et al.* 2001) and following administration of FTY720 in mice the numbers of circulating HSC are reduced, however this effect is variable with some reports suggesting a much greater reduction (Massberg *et al.* 2007) than others(Juarez *et al.* 2012). The effect of FTY720 on HSC numbers within the bone marrow is either constant (Juarez *et al.* 2012) or increased(Golan *et al.* 2012) in similar experimental models. The administration of FTY720 following injection of human $CD34^+$ HSC into NOD/SCID mice resulted in significantly higher rates of homing to the bone marrow than in control mice(Kimura *et al.* 2004). Significantly higher numbers of colony forming cells could be obtained from the livers of mice receiving FTY720 over a period of seven days than untreated mice(Massberg *et al.* 2007), this was explained to be due to reduced egress of HSC from the liver rather than increased recruitment.

Liver injury results in the mobilisation of HSC and accumulation within the liver, it is now apparent that S1P receptor signalling has a crucial role in both the mobilisation of bone marrow cells and in their trafficking to organs. It is not clear whether the recruitment of HSC to extra medullary tissues involves a direct role for S1P receptor signalling or an indirect role through modulation of chemokine receptor signalling. In this section of work I will characterise in detail the repertoire of chemokine receptors expressed by HSC and the effect of S1P signalling on chemokine receptor expression. I will determine whether FTY720 increases the number HSC within the liver during liver injury and whether HSC recruitment to the injured liver is dependent upon S1P receptor signalling.

5.2 Characterisation of HPC-7 cells

The relative scarcity of KSL cells within the bone marrow (approx 0.05% of bone marrow cells) means that studies requiring large numbers of cells are virtually impossible given the huge numbers of donor mice required for cell isolation.

The HPC-7 cell line has been widely used both within our instution and more widely as a model to study HSC at both molecular and cellular levels, particularly in studies of HSC trafficking and tissue recruitment(Kavanagh *et al.* 2010; Volpe *et al.* 2013; White *et al.* 2013).

HPC-7 cells were readily cultured in serum free media supplemented with Stem Cell Factor and antibiotics. Cell proliferation was sensitive to cell density in culture with optimum cell concentrations of 0.8-2.0 $x10^6$ cell per ml.

I confirmed the surface phenotype of HPC-7 cells by flow cytometry and demonstrated expression of c-kit and sca-1 and the absence of markers of lineage differentiation (Fig 5.1B-E). I also confirmed colony forming ability in vitro (Fig 5.1A).

Figure 5.1

Expression of Haematopoietc Stem Cell Markers on HPC-7 Cells

(A)HPC-7 cells were cultured in Methocult GF media for 7 days and significant numbers of myeloid colony forming cell clusters were identified. Representative image of CFU-GM colony shown. Cultured HPC-7 cells were labelled with fluorescently conjugated antibodies to lineage markers (CD11b, CD5, CD8a, Ter119, Gr-1, B220), c-kit and sca-1 and analysed by flow cytometry. (B) Dead cells and debris were excluded by gating on a plot of forward scatter – side scatter and surface expression of (C) lineage markers, (D) c-kit and (E) sca-1 measured. Representative histograms displayed (isotype control: grey, antibody: red line).

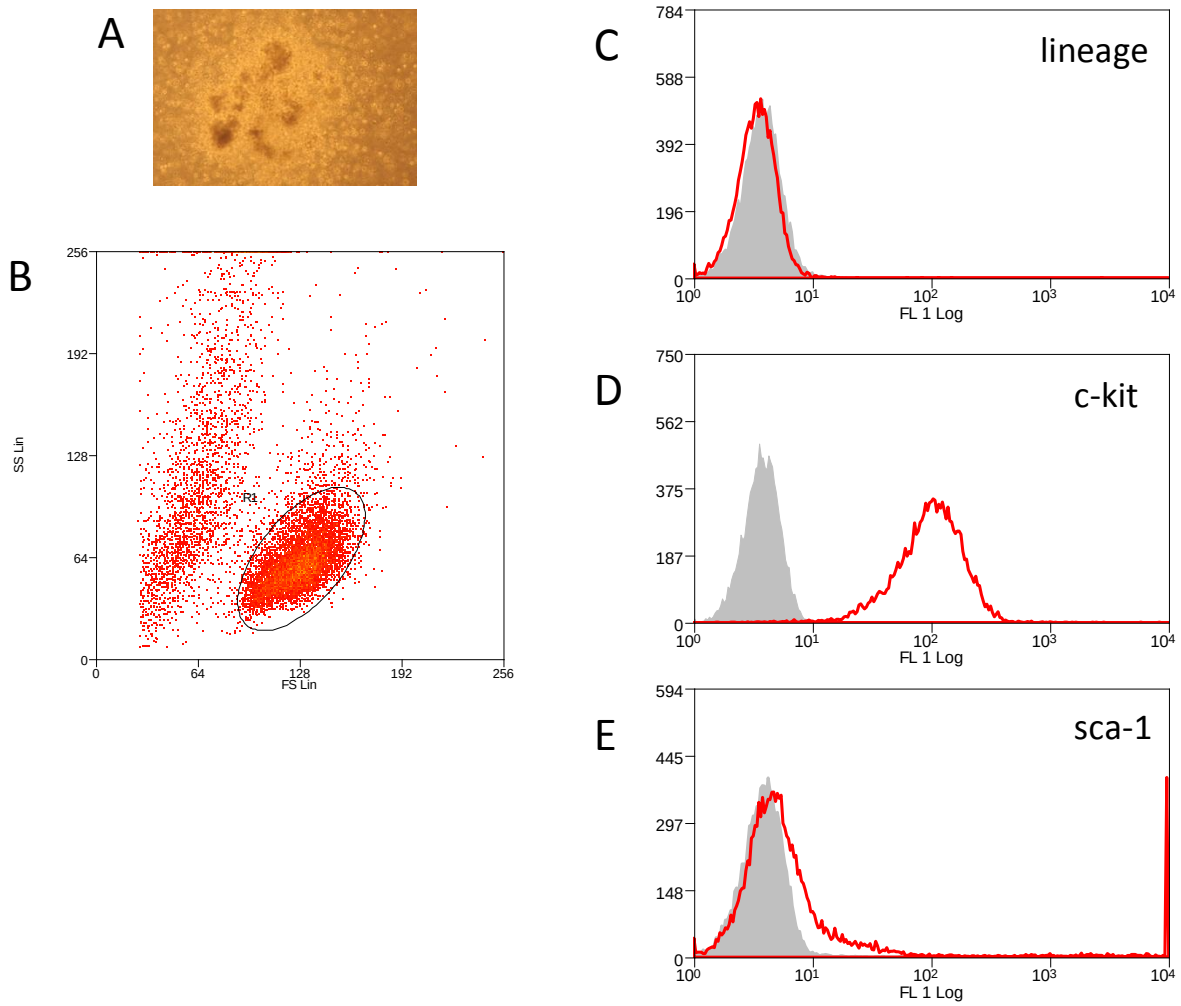


Figure 5.1
Expression of Haematopoietc Stem Cell Markers on HPC-7 Cells

5.3 HSC express S1P receptors and migrate to S1P

The relative gene expression of the S1P receptors were determined in both freshly isolated KSL cells and cultured HPC7 cells. Quantitative PCR was used and expression relative to GAPDH was determined. In both cell types S1P1 receptor was the most abundantly expressed and whilst S1P2 and S1P4 receptors were expressed in both cell types to a lesser degree, neither cell type expressed S1P5 receptor. The striking difference between the cell types was that of S1P3 receptor expression, as although strong expression was detected in KSL cells this was absent in HPC7 cells (Fig 5.2A,B).

Given the high levels of S1P1 gene expression and knowledge of its role in cellular migration, further analysis of the cellular expression of S1P1 was performed. Flow cytometry was used to determine cell surface expression of S1P1 on both freshly isolated KSL cells and cultured HPC7 cells, and in separate experiments both cell types were also permeabilised to detect both intracellular and extracellular S1P1 receptor expression. Both KSL and HPC7 cells displayed marginal cell surface expression of S1P1 receptor. However, analysis of permeabilised cells demonstrated abundant intracellular stores of S1P1 (Fig 5.2C,D).

The functional role of the S1P receptors expressed by HSCs was assessed by their migration in vitro to S1P. Using a transwell assay the migration of both KSL and HPC7 cells to varying concentration of S1P was determined. Dose dependent migration was observed in both KSL and HPC7 cells with maximal levels of migration at 1μM S1P for both cell types (KSL 12.69+/-0.99 % input cells migrated, HPC-7 14.55+/-1/776 % input cells migrated)(Fig 5.3A,B).

Figure 5.2

Primary murine haematopoietic stem cells and HPC-7 cells express Sphingosine 1-phosphate receptors

(A,B) Gene expression of S1P receptors 1-5 in freshly isolated KSL cells and cultured HPC-7 cells relative to GAPDH by the $2^{-\Delta CT}$ method was determined. Bars represent mean +/- SEM expression in three independent experiments.

(C,D)Surface $S1P_1$ receptor expression on freshly isolated KSL cells and cultured HPC-7 cells was analysed by flow cytometry. After fixation and permeabilisation of the cells, total intracellular S1P1 receptor expression was also assessed. Representative histograms of 3 independent experiments are displayed (grey area: isotype control, red line: S1P1 antibody).

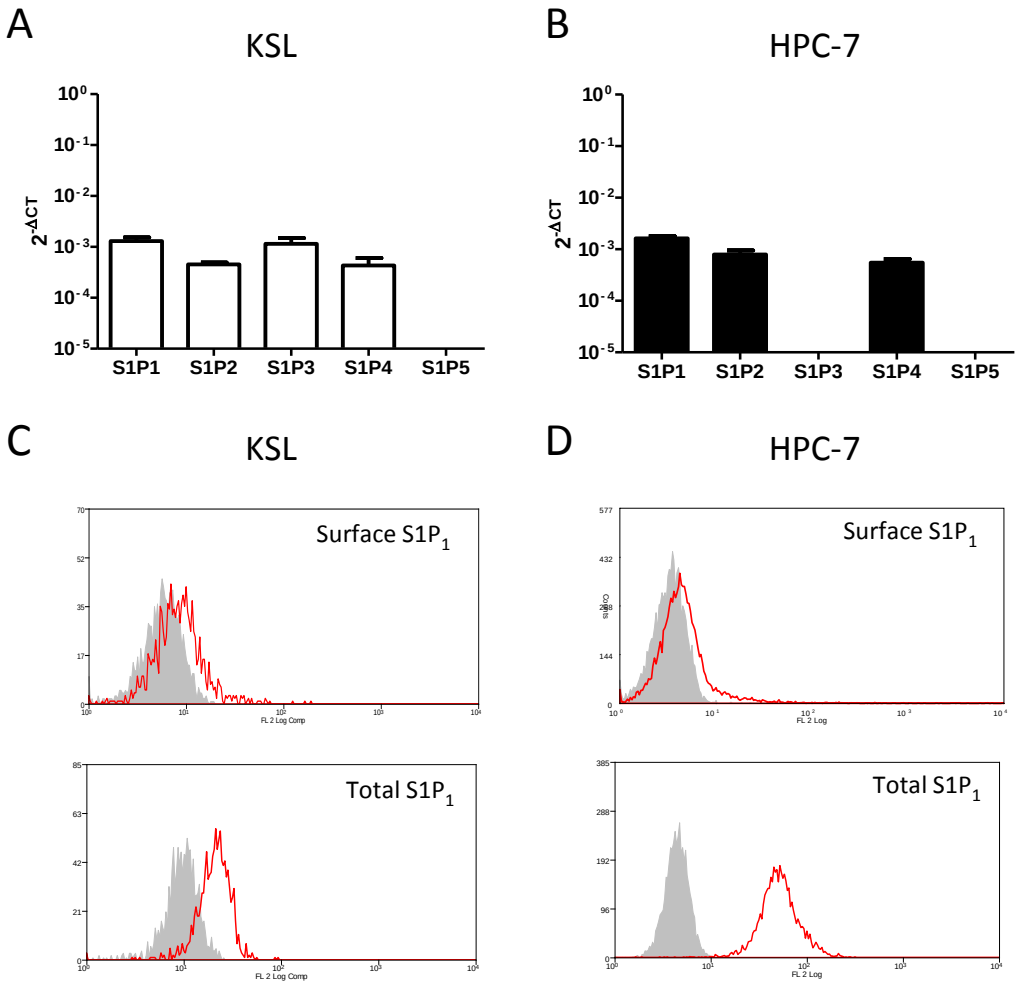


Figure 5.2
Primary murine haematopoietic stem cells and HPC-7 cells express Sphingosine 1-phosphate receptors

Figure 5.3

Primary murine haematopoietic stem cells and HPC-7 cells migrate n vitro to Sphingosine 1-phosphate

(A)Bone marrow cells negative for lineage marker expression were isolated by FACS sorting and used in a transwell migration assay. $1x10^5$ lineage negative cells were added to the top well of the transwell and migration to varying concentrations of S1P proceeded for 4 hours. Cells migrating into the bottom well were analysed for expression of c-kit and sca-1 and quantified by flow cytometry. KSL cells migrating to the lower well were expressed as a percentage of the total number of KSL cells added to the top well. Bars represent the mean +/- SEM of 3 separate experiments. (B) In similar experiments using HPC-7 cells, cells were counted using flow cytometry and expressed as a percentage of the total number of HPC-7 cells added to the top well. Bars represent the mean +/- SEM of 3 separate experiments

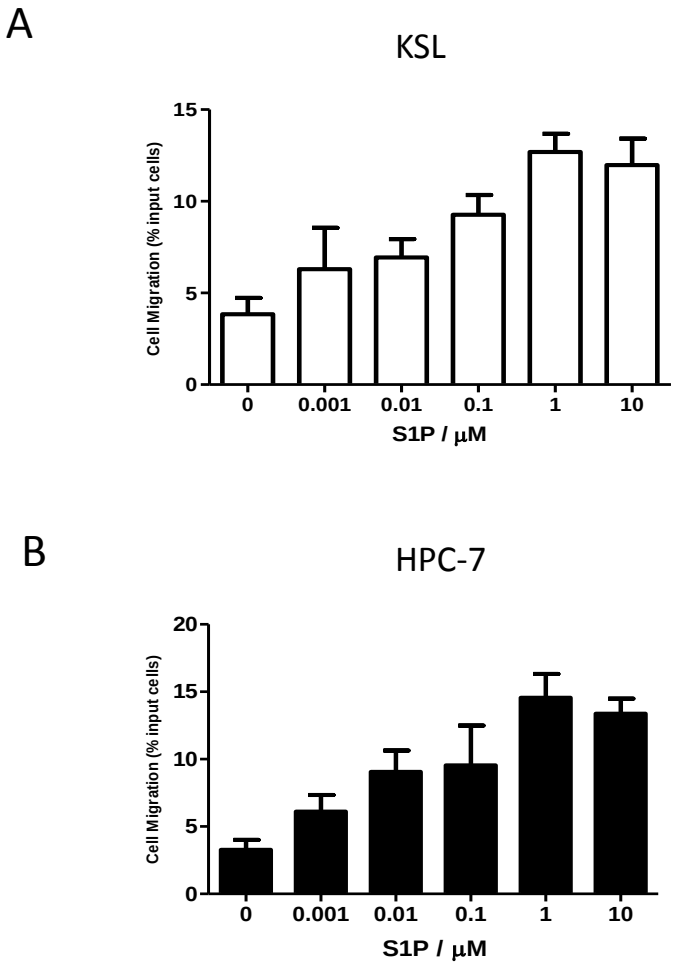


Figure 5.3
Primary murine haematopoietic stem cells and HPC-7 cells migrate n vitro to Sphingosine 1-phosphate

5.4 FTY720 inhibits migration of HSC to S1P

The partial S1P receptor agonist/ competitive antagonist FTY720 was used to study the effects of S1P receptor modulation on HSC migration using the transwell assay system. FTY720 was used at a concentration of 1μM and when incubated with both KSL and HPC7 cells did not have any adverse effect on cell viability (Fig 5.4A). Flow cytometry demonstrated that incubation with FTY720 resulted in a marked reduction in cell surface S1P1 receptor expression (Fig 5.4B).

In both KSL and HPC-7 cells, FTY720 did not affect baseline levels of migration to control media (KSL: FTY720 4.34+/-0.95 % migration vs Control 3.84+/-0.88 % migration, p=ns; HPC-7: FTY720 3.12+/-1.01 vs Control 3.11+/-1.41% migration, p=ns) (Fig5.5A,B), however significantly less migration to S1P was observed in cells treated with FTY720 than in cells treated with control media alone (KSL: FTY720 7.19+/-1.77 % migration vs Control 11.61+/-2.08 % migration, $p<0.01$; HPC-7 FTY720 5.62+/-0.89 % migration vs Control 13.03+/-1.06 % migration, $p<0.01$)(Fig 5.5A,B).

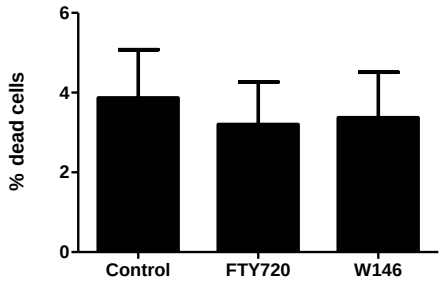
FTY720 acts at multiple S1P receptor subtypes (Brinkmann *et al.* 2002)and to examine in more detail the contribution of S1P1 receptor to HSC migration, a direct S1P1 receptor antagonist, W146, was used in repeat experiments to determine the effects of S1P1 receptor blockade alone on HSC migration. W146 had no effect on cellular viability and incubation of the cells with W146 did not alter cell surface S1P1 receptor expression using flow cytometry (Fig 5.4).

Figure 5.4

Effect of S1P receptor modulators on cell viability and cell surface S1P1 expression

(A)Whole bone marrow was incubated in media containing either dilutant control, FTY720-phosphate (FTY720-P) or W146 for 3 hours. Cells were then labelled with fluorescently conjugated antibodies to lineage markers, c-kit and sca-1. Prior to analysis by flow cytometry, a viability dye was added and the number of dead KSL cells were determined, expressed as a percentage of the total cell number of KSL cells. No significant difference was seen in cell viability following treatment with FTY720-P or W146. Bars represent the mean+/-SD of 3 separate experiments. (B)Following treatment with FTY720-P or W146, cells were also labelled with S1P1 antibody and cell surface expression of S1P1 measured by flow cytometry. FTY720-P redcued the surface of expression of S1P1 whereas W146 had no effect when compared with control media alone. Representative histogram of three separate experiments shown (grey area: isotype control, red line: control media alone, light blue line :W146, dark blue line : FTY720-P).

A



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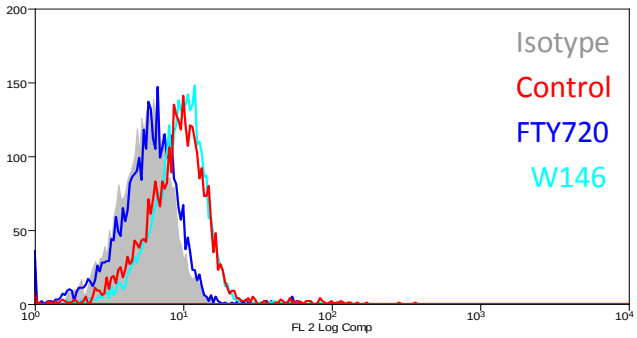


Figure 5.4
Effect of S1P receptor modulators on cell viability and cell surface S1P1 expression

W146 treatment did not alter migration of KSL or HPC-7 cells to control media (KSL: W146 4.65+/-0.70 %migration vs Control 3.84+/-0.88 % migration, p=ns; HPC-7: W146 3.25+/-1.19 vs Control 3.10+/-1.41 % migration, p=ns)(Fig 5.5A,B). Treatment with W146 resulted in significantly impaired migration to S1P in both KSL and HPC7 cells, similar to the effect of FTY720 (KSL: W146 5.96+/-1.31 % migration vs Control 11.61+/-2.08 % migration, $p<0.01$; HPC-7 W146 6.89+/-1.65 % migration vs Control 13.03+/-1.06, $p<0.01$)(Fig 5.5A,B). These observations suggest that the effect of FTY720 on HSC migration to S1P is mediated through the S1P1 receptor.

Figure 5.5

FTY720 and W146 inhibit migration of primary murine haematopoietic stem cells and HPC-7 cells to Sphingosine 1-phosphate in vitro

Bone marrow cells negative for lineage marker expression were isolated by FACS sorting and used in a transwell migration assay. $1x10^5$ lineage negative cells were added to the top well of the transwell and migration proceeded for 4 hours. Top and bottom wells of the transwell assay contained either 1µM S1P, 1µM FTY720-P, 1µM W146 or dilutant control media as shown. Transwells were incubated for 4 hours at 37^oC, 5%CO_2 and cells migrating into the bottom wells were analysed by flow cytometry for expression of c-kit and sca-1 and quantified using counting beads. Migration was quantified by calculating the numbers of c-kit+ sca1+ cells in the bottom well as a percentage of the total number added to the top well. Exposure of freshly isolated KSL cells to FTY720-P and W146 significantly inhibited in vitro migration to S1P. Results of three separate experiments performed in duplicate. Mean +/- SD % input cells shown, * $p<0.05$ (one way anova with bonferroni multiple comparison test) (B) Similar experiments using cultured HPC-7 cells were performed and cells migrating into the bottom well quantified by flow cytometry using counting beads. Migration was quantified by calculating the numbers of cells in the bottom well as percentage of the total number added to the top well. Exposure of cultured HPC-7 cells to FTY720 and W146 significantly inhibited in vitro migration to S1P. Results of three separate experiments performed in duplicate. Mean +/- SD % input cells shown, * $p<0.05$ (one way anova with bonferroni multiple comparison test)

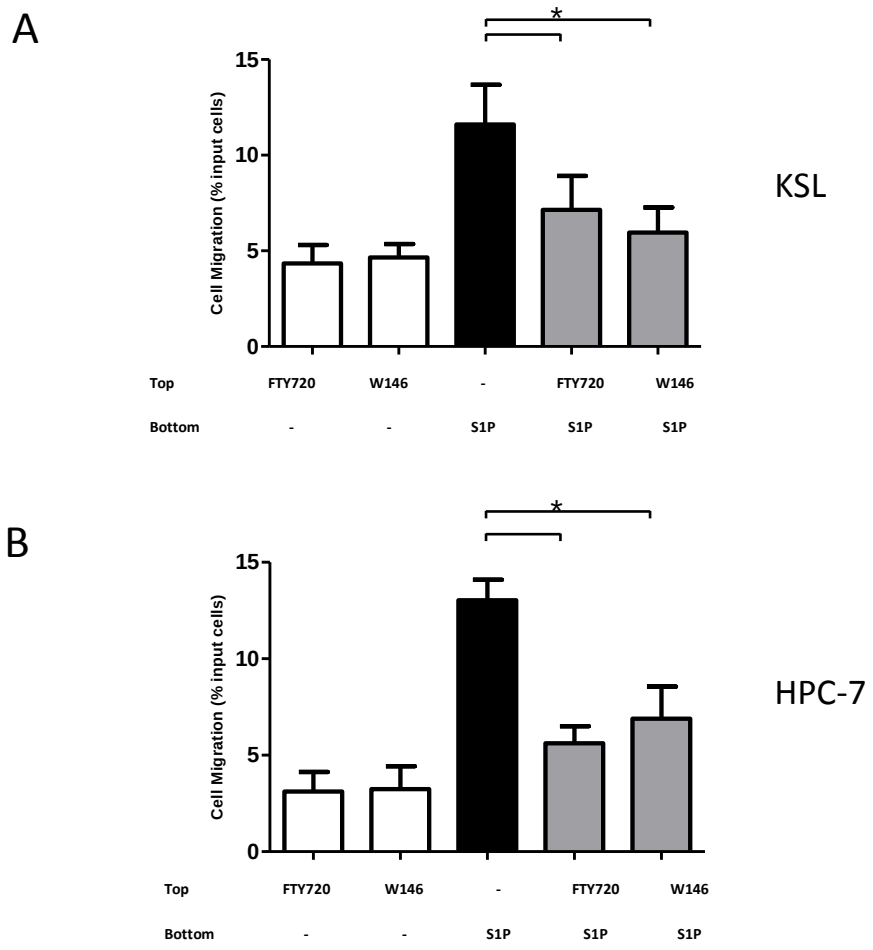


Figure 5.5
FTY720 and W146 inhibit migration of primary murine haematopoietic stem cells and HPC-7 cells to Sphingosine 1-phosphate in vitro

5.5 Chemokine receptor expression on HSC is unaltered by S1P

The chemokine system is vital for cell migration and trafficking and the full repertoire of chemokine receptors expressed by murine HSC has not previously been described(Wright *et al*. 2002). An analysis of gene expression of the chemokine receptors by both freshly isolated KSL and cultured HPC7 cells was performed using quantitative PCR with normalisation to GAPDH expression using the ΔΔCT method. Genes were considered not expressed if a signal was not detected or detected after more than 35 cycles of annealing.

CCR9 was the most abundantly expressed receptor in the KSL cells, with high levels of CCR2, CXCR2 and CXCR4 also present. CCR1,CCR4,CCR7, CXCR1 and CXCR3 were also expressed at lower levels. A similar pattern of expression was seen in HPC-7 cells with the same receptors also being expressed, however there was a striking difference in CCR9 expression with levels in HPC7 being almost undetectable compared with the abundant expression in KSL cells (Fig 5.6A). CXCR4 was most abundantly expressed in HPC7 cells at levels markedly higher than CCR2 and CXCR3 which were the next most highly expressed (Fig 5.6B).

Flow cytometry was used to analyse further the cell surface expression of the chemokine receptors for which gene expression had been detected in HSC. The cell surface expression of chemokine receptors was determined on both freshly isolated KSL (Fig 5.7A)and cultured HPC7 cells (Fig 5.8A), chemokine receptors identified by gene expression were chosen for further study. On KSL cells, CCR2, CCR9 and CXCR4 were present (6.37+/-3.14, 22.2+/-4.19, 14.84+/-2.95 % expression respectively), but there was no appreciable (less than 2%) expression of the remaining receptors (Fig 5.7B). In contrast on cultured HPC7 cells CXCR4 was almost

Figure 5.6

Chemokine Receptor Expression on primary KSL cells and HPC-7 cells

Gene expression of chemokine receptors in (A) freshly isolated KSL cells and (B) cultured HPC-7 cells was determined by quantitative PCR. Expression relative to GAPDH by the $2^{-\Delta CT}$ method was determined. Bars represent mean +/- SEM expression in three independent experiments.

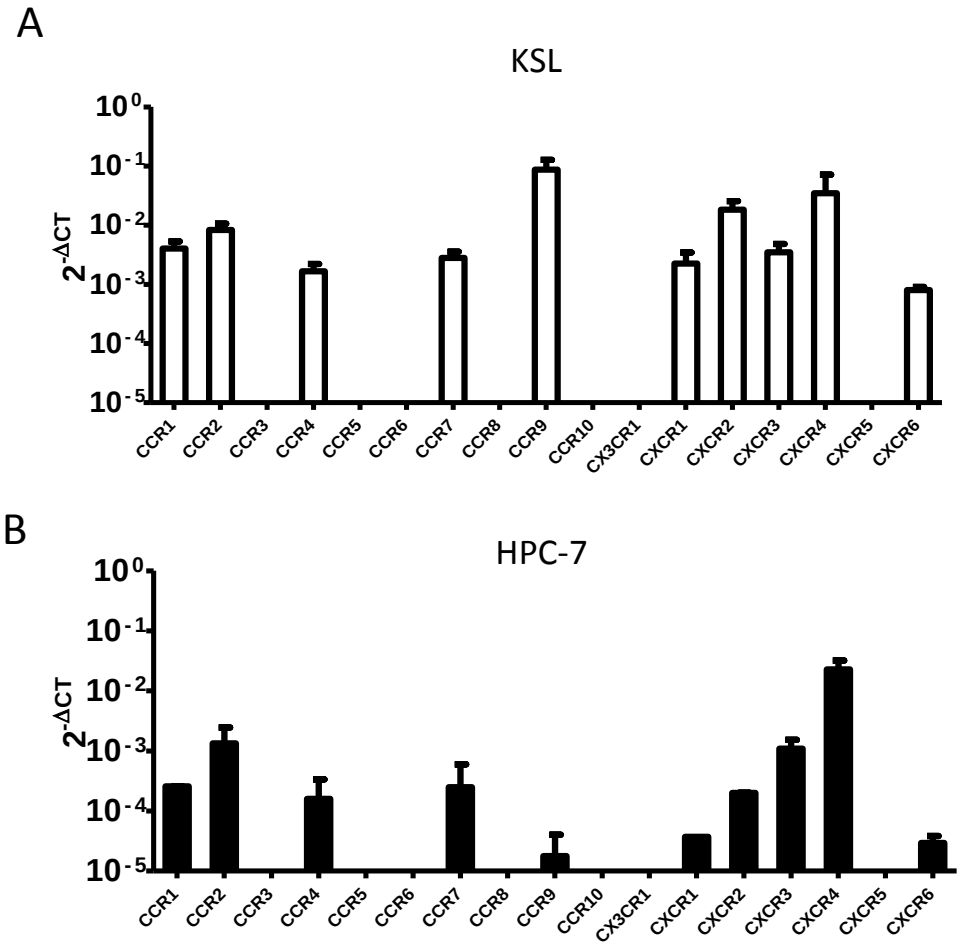


Figure 5.6
Chemokine Receptor Expression on primary KSL cells and HPC-7 cells

Figure 5.7

Cell Surface Expression of Chemoknie Receptors on KSL cells

(A)Cell surface chemokine receptor expression on freshly isolated KSL cells was determined by sequential gating of bone marrow cells to identify KSL cells and analysis of isotype (grey) and receptor (red line) expression of chemokine receptors. Plots demonstrate CXCR4 expression and are representative of multiple experiments. (B) Cell surface expression of chemokine receptors expressed as a percentage of total KSL cells staining positive for each receptor. Bars represent mean+/- SEM expression of 3 separate experiments.

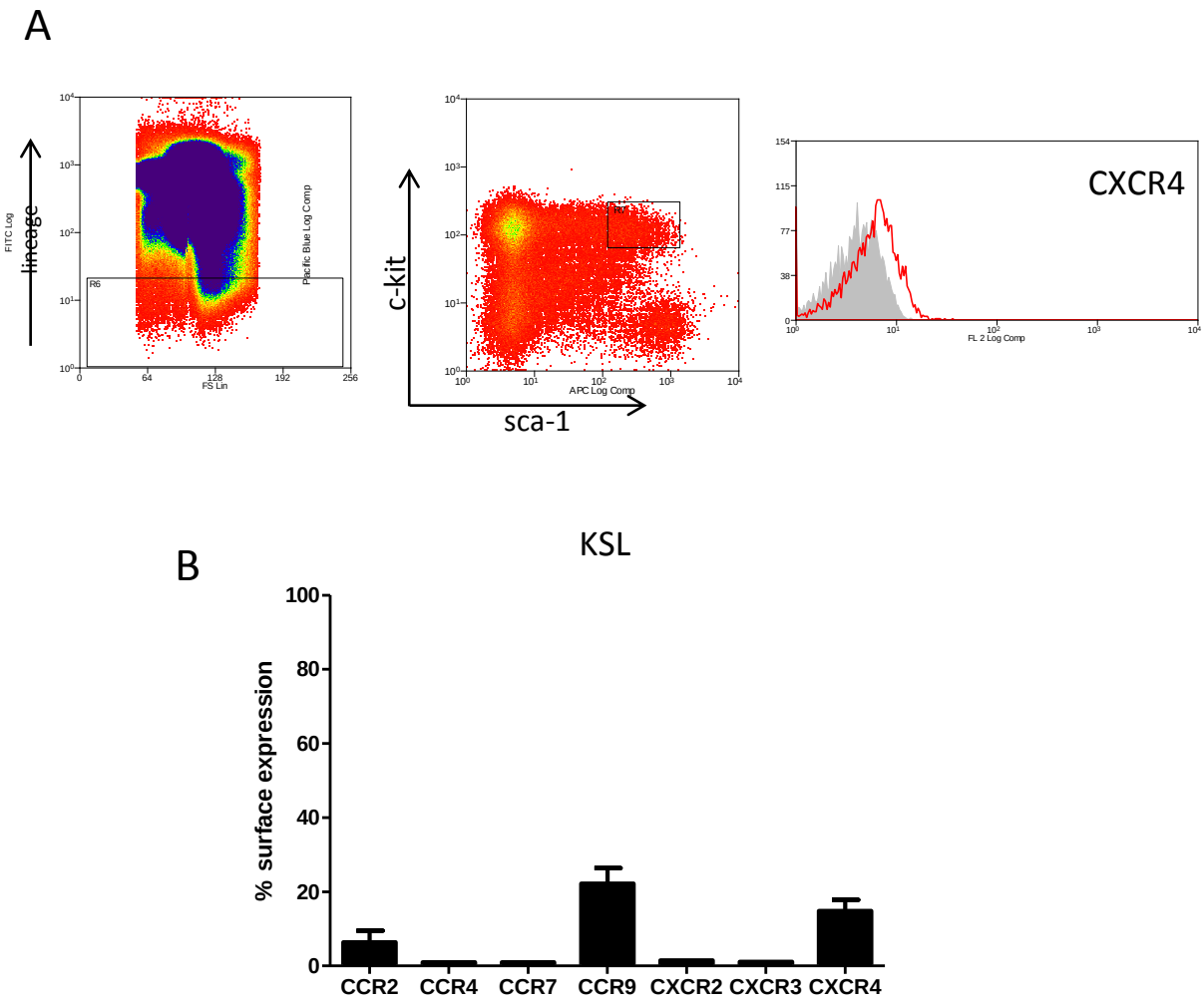


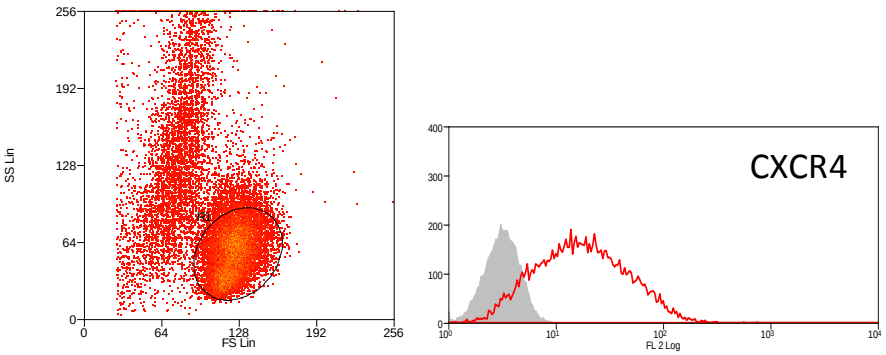
Figure 5.7
Cell Surface Expression of Chemokine Receptors on KSL cells

Figure 5.8

Cell Surface Expression of Chemoknie Receptors on HPC-7 cells

(A)Cell surface chemokine receptor expression on cultured HPC-7 cells was determined by gating on cell population to exclude debris and dead cells and analysis of isotype (grey) and receptor (red line) expression of chemokine receptors. Plots demonstrate CXCR4 expression and are representative of multiple experiments. (B) Cell surface expression of chemokine receptors expressed as a percentage of total KSL cells staining positive for each receptor. Bars represent mean+/- SEM expression of 3 separate experiments.

A



HPC-7

B

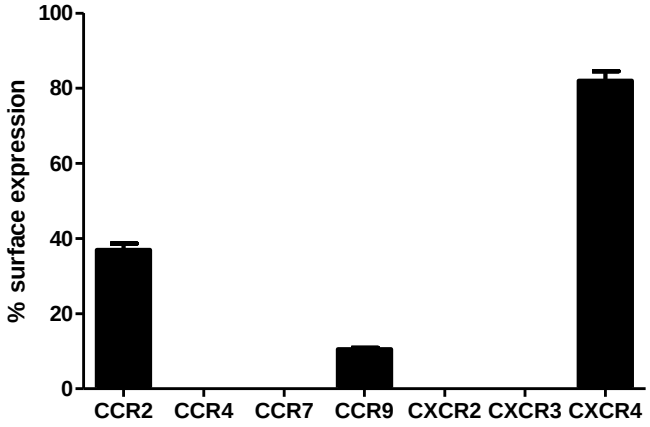


Figure 5.8
Cell Surface Expression of Chemokine Receptors on HPC-7 cells

ubiquitously expressed (81.97+/-4.39 % expression) and significant levels of CCR2 were also noted (36.96+/-7.93 % expression), CCR9 in contrast to KSL cells was present at much lower levels (10.49+/-0.66 % expression) on HPC7 cells (Fig 5.8B).

S1P and FTY720 have previously been shown to influence cellular migration through modulation of chemokine receptor signalling (Ryser *et al.* 2008),I studied the effect of S1P exposure on chemokine receptor expression. Freshly isolated KSL and cultured HPC7 cells were incubated with S1P and subsequent chemokine receptor expression compared with cells incubated in control media was determined. In KSL cells there was a two-fold increase in CXCR4 surface expression following incubation, however this was observed both in cells treated with S1P and in control treated cells (Baseline 14.84+/-2.96, Control media 28.35+/-3.7, S1P media 28.92+/-2.21 % expression)(Fig 5.9A). No other changes in chemokine receptor expression were observed in either KSL or HPC7 cells following exposure to S1P(Fig 5.9A,B).

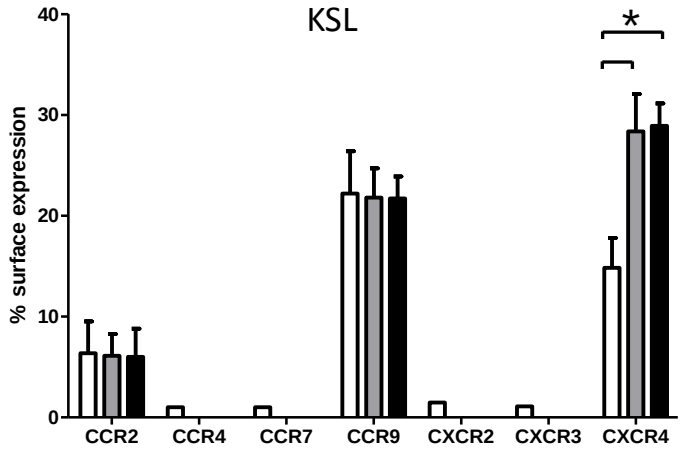
Figure 5.9

Alterations in Chemokine Receptor Expression on KSL and HPC-7 cells

after incubation with S1P

(A) Bone marrow cells were incubated in serum free media containing either 1μM S1P or dilutant control for 4 hours at 37^{o}C 5%CO_2. Cell surface chemokine receptor expression on incubated KSL cells was determined by sequential gating of bone marrow cells to identify KSL cells and analysis of chemokine receptor expression as previously shown. Chemokine receptor expression of KSL cells incubated in control media (grey) or 1μM S1P (black), compared with freshly isolated cells (white). Bars represent mean+/- SEM of 3 separate experiments, * $p<0.05$ (one way anova with bonferroni multiple comparison test). (B) HPC-7 cells were incubated in serum free media containing either 1μM S1P or dilutant control for 4 hours at 37^{o}C 5%CO_2. Cell surface chemokine receptor expression on incubated HPC-7 cells was determined by gating on cell population to exclude debris and dead cells and analysis of chemokine receptor expression as previously shown. Chemokine receptor expression of cells incubated in control media (white) and 1μM S1P (black). Bars represent mean+/- SEM of 3 separate experiments.

A



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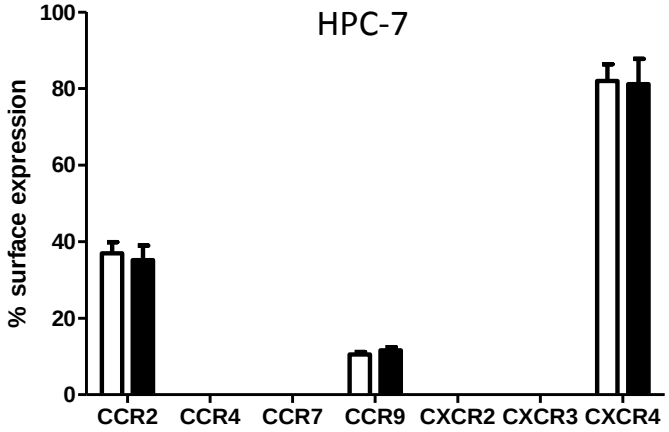


Figure 5.9
Alterations in Chemokine Receptor Expression on KSL and HPC-7 cells after incubation with S1P

5.6 FTY720 administration increases the number of HSC found in the liver during chronic liver injury

A significant increase in the number of bone marrow derived HSC in the liver during chronic liver injury was previously shown. There is published data from Massberg and colleagues demonstrating that FTY720 administration in vivo results in HSC accumulation in peripheral tissues. These findings lead me to investigate the effect of FTY720 on HSC accumulation within the liver during liver injury (Fig 5.10A).

FTY720 was administered to mice with chronic liver injury for 7 daysa this resulted in a significantly higher number of c-kit+ sca1+ lineage- (KSL) cells within the liver of mice receiving FTY720 compared with control animals (FTY720 1697+/-247 KSL cells per liver vs Control 982+/-110 KSL cells per liver, $p<0.05$)(Fig 5.10B). The number of HSC present in the peripheral blood was lower in mice receiving FTY720, although this was not statistically significant (FTY720 0.36+/-0.1 KSL cells per µl vs Control 0.43+/-0.14 KSL cells per µl, p=ns).(Fig 5.10C)

Figure 5.10

Accumulation of KSL cells within the liver following administration of FTY720 in vivo

(A)Chronic liver injury was induced in age and sex matched C57/BL6 mice by twice weekly intraperitoneal administration of carbon tetrachloride (1mg/kg) in mineral oil for 8 weeks, mice were then randomly allocated to receive either FTY720 (1mg/kg) or dilutant control by daily intraperitoneal injection for 7 days. HSC were defined as c-kit+ sca1+ lineage negative (KSL) and were identified in liver and peripheral blood by flow cytometry as previously described. Absolute cell numbers were quantified using counting beads and samples were run in duplicate. (B,C) Significantly higher numbers of KSL cells were detected in the livers of mice treated with FTY720 than controls, whilst in peripheral blood the numbers were unchanged. Data from individual mice is displayed (circles : control, squares : carbon tetrachloride treated) and means indicated by horizontal line. * $p<0.05$ (unpaired student's t-test)

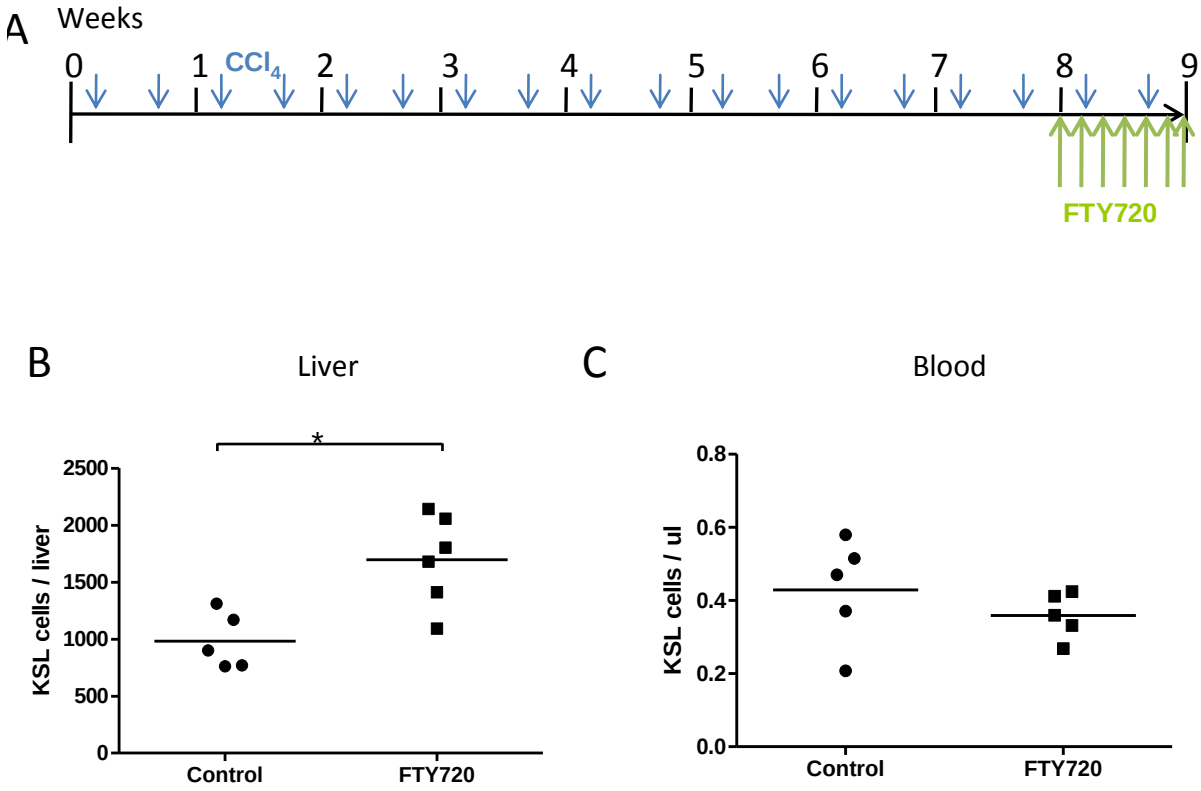


Figure 5.10
Accumulation of KSL cells within the liver following administration of FTY720 in vivo

5.7 FTY720 does not affect recruitment of HSC to the liver

Intra vital microscopy was used to determine the effect of FTY720 on HSC recruitment to the injured liver, W146 was also used to confirm the role of S1P1 in HSC recruitment. HPC7 cells were used given the difficulty obtaining sufficient numbers of freshly isolated KSL cells to perform the experiments. Cells were incubated with control media, FTY720 or W146 prior to injection and injected into the carotid artery as previously described.

In mice receiving control media cells, the number of adherent cells was highest in the first minute after injection with a gradual increase in the number of cells recruited over the subsequent 60 minutes (Fig 5.11A). Recruitment of cells treated with either FTY720 or W146 followed a similar pattern. Although there was some variation in individual experiments, the average number of adherent cells per field of view was not significantly different at any timepoint over the 60 minutes between control cells and cells treated with FTY720 or W146 (Adherent cells at 60 minutes: Control 10.3+/-0.66, FTY720 9.66+/-2.4, W146 9.0+/-1.15, p=ns)(Fig 5.11B).

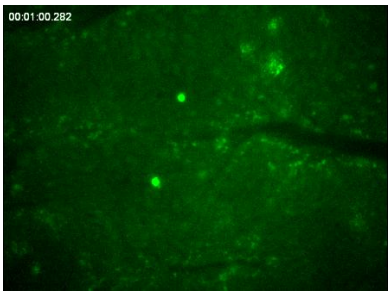
Tissue distribution of the infused cells at 60 minutes following injection was determined by immunofluorescent microscopic analysis of serial sections of central and peripheral regions of the liver. There were no differences observed in the numbers of fluorescently labelled cells per field of view between control cells and FTY720 or W146 treated cells (Fig 5.12A,B).

Figure 5.11

FTY720 and W146 do not affect recruitment of HPC-7 cells to injured liver

Chronic liver injury was induced in age and sex matched C57/BL6 mice by twice weekly intraperitoneal administration of carbon tetrachloride (1mg/kg) in mineral oil for 8 weeks. Under anaesthesia, individual mice underwent cannulation of the carotid artery followed by laparotomy and mobilisation of the liver. Mice were placed onto the stage of an inverted fluorescent microscope whereby a representative area of the liver could be monitored by intra vital microscopy. $1x10^6$ cultured HPC-7 cells were labelled with CFSE and treated for one hour with either FTY720-P (1µM), W146 (1µM) or control media and then washed and resuspended in PBS for injection. Cells were injected slowly into the carotid artery and a one minute recording of fluorescent cell recruitment to the imaged liver was recorded immediately and at 5 minute intervals for 60 minutes. (A) A representative image of intra vital microscopy of the liver demonstrating adherent fluorescent cells. (B) Mice were randomly allocated to receive cells treated with FTY720-P (blue line), W146 (green line) or control media (red line) and the number of adherent cells in the selected field of view at each time point counted. n=3 each group, mean+/-SD number of cells per field of view shown, p=ns (two way anova).

A



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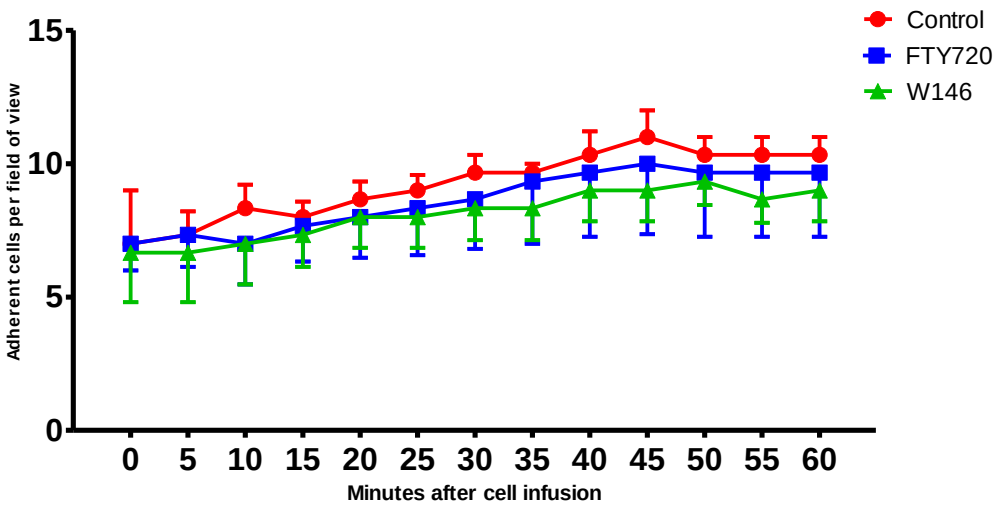

Figure 5.11
FTY720 and W146 do not affect recruitment of HPC-7 cells to injured liver

Figure 5.12

Distribution of cells treated with FTY720 and W146 following injection

After completion of intra vital microscopy experiments mice were sacrificed, individual organs removed and snap frozen in liquid nitrogen. Sections were cut from both the central and peripheral regions of the liver using a cryotome and directly analysed by fluorescent microscopy (FITC filter). The number of individual fluorescent cells were manually counted in 6 random, non-overlapping fields of view (x20 magnification) per section of (A) central liver tissue and (B) peripheral liver tissue. No difference in numbers of injected cells within the liver was seen between cells treated with FTY720-P, W146 or control media. n=3 each group, mean+/-SD number of fluorescent cells per field of view shown, p=ns (one way anova with bonferroni multiple comparison test)

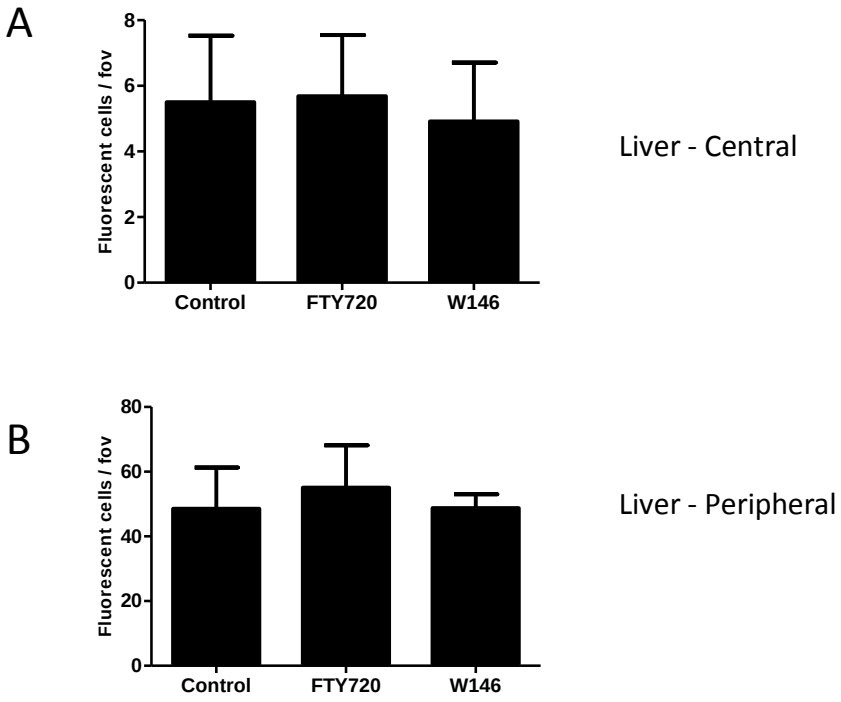

Figure 5.12
Distribution of cells treated with FTY720 and W146 following injection

5.8 Conclusions

S1P has been previously shown by the work of Massberg and colleagues to be a regulator of HSC trafficking(Massberg *et al.* 2007), and having shown the beneficial effects of HSC in liver injury and alterations in S1P levels during liver injury, I studied the effects of S1P receptor modulation on HSC trafficking during liver injury. In line with previous studies, I confirmed the expression of S1P receptors and demonstrated migration in vitro to S1P. FTY720 is an S1P receptor agonist at all subtypes except S1P2, resulting in receptor internalisation and degradation without G protein signalling rendering cells unresponsive to S1P(Oo *et al.* 2007). Loss of cell surface S1P1 following exposure to both S1P and FTY720 has also been shown in other cell types(Maeda *et al.* 2007). As expected FTY720 treatment of HSC resulted in loss of surface expression of S1P1 and impaired migration to S1P. Treatment with the competitive antagonist at the S1P1 receptor, W146, did not alter S1P1 expression but also resulted in a loss of S1P induced migration. These findings suggest that, in line with previous work, migration of HSC to S1P is mediated by the S1P1 receptor.

HSC are a rare population of cell within the bone marrow, limiting the repeated study of large numbers of cells in assays such as intravital microscopy. The HPC-7 cell line has previously been used in studies of HSC trafficking and recruitment. Similarities in the findings of initial studies of S1P receptor expression and migration confirmed that HPC-7 cells represent a suitable model for studying the effect of S1P receptor modulation on HSC trafficking.

Given that a role has been has shown for chemokine receptors in the trafficking of HSC to injured organs I investigated in more detail the expression profile of chemokine receptors on KSL cells. I confirmed previous observations regarding the expression of CXCR4 (Sasaki *et al.* 2009) and demonstrated increased cell surface expression on KSL cells following a period of culture(Sasaki *et al.* 2009). This increased expression was not altered by incubation with S1P and is likely to relate to the effect of culture. Exposure to constitutively high levels of SDF-1 within the bone marrow environment acts a retention signal for HSC within the bone marrow through CXCR4. In contrast, HPC-7 cells cultured in an environment free of SDF-1 exhibit almost universal CXCR4 expression, which is further increased by the presence of Stem Cell Factor in the culture media which has been shown to upregulate CXCR4 expression.

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progresses(Si *et al.* 2010) and the role of CCR2 in monocyte/macrophage migration is well established(Li *et al.* 2008). Expression of CCR9 on KSL cells was unexpected as previous reports have suggested that this is not expressed by HSC (Schwarz *et al.* 2007) but is expressed on less primitive haematopoietic progenitors and is required for thymic migration of lymphoid progenitor cells (Zlotoff *et al.* 2010). In my studies I have used KSL to define my cell population which includes both haematopoietic stem cells and multipotent progenitor cells(Adolfsson *et al.* 2001), and CCR9 expression has previously been demonstrated upon MPPs(Schwarz *et al.* 2007) and in particular lymphoid primed MPPs (Zlotoff *et al.* 2010) destined for the thymus.

Other than the increase in CXCR4 expression on KSL cells following culture there were no alterations in chemokine receptor expression after culture with S1P. Crosstalk between S1P receptors and chemokine receptors has previously been described and enhanced CXCR4 signalling has also been shown to occur without alteration in CXCR4 surface expression (Ryser *et al.* 2008), so my findings do not discount the possibility of enhanced signalling of other chemokine receptors following exposure to S1P. A major drawback of these findings are the absence of functional studies of chemokine receptors, namely migration assays to individual receptor ligands. These would be crucial to further understanding links between S1P receptor signalling and chemokine receptor signalling.

The findings of the chemokine receptor expression studies also further validate the use of the HPC-7 cell line in functional studies. Analysis of chemokine receptor expression revealed a similar repertoire of receptor expression albeit with some relative differences related to the more heterogeneous nature of the KSL cell population. Differences in cell surface

receptor expression were seen but as previously described are likely to be due to the effects of cell culture.

FTY720 has been shown to impair HSC migration in vitro and in vivo administration results in an increase in HSC within extramedullary tissues, having confirmed the effect of FTY720 on KSL cells in vitro I investigated the effect of FTY20 in vivo during liver injury. Having previously shown a significant increase in bone marrow derived KSL cells within the liver during injury, treatment of seven days with FTY720 further increased the number of KSL cells within the liver. Treatment with FTY720 during liver injury did not significantly alter the numbers of KSL cells in the peripheral blood and this is in contrast to studies administering FTY720 to normal mice where a reduction in circulating HSC has been described. In one study a profound reduction in peripheral blood HSC was seen(Massberg *et al.* 2007), although in a study by Juarez et al a significant decrease was only observed after increasing the sample sizes of the groups analysed (Juarez *et al.* 2012).

The proposed mechanism for the action of FTY720 on HSC in vivo is reduced egress into draining lymphatics rather than an effect on recruitment of HSC. Studies of HSC recruitment using intravital microscopy failed to show any effect of either FTY720, or the competitive S1P1 receptor antagonist W146, on recruitment to the injured liver. Based upon these findings I propose that the increase number of KSL cells within the liver following administration of FTY720 is as a result of accumulation due to reduced egress rather than an effect of FTY720 on cell recruitment.

6 MODULATION AND OPTIMISATION OF HSC TRAFFICKING WITH FTY720

6.1 Introduction

Although clinical studies of bone marrow derived stem cells for liver disease are at an advanced stage in testing of their safety and efficacy(Houlihan *et al.* 2008), a particular area of uncertainty remains whether the number of cells administered and indeed the number of cells reaching the injured liver dictates their therapeutic effect. Stem cells are rare populations of cells and strategies to improve trafficking to the liver may be of therapeutic benefit. The molecular mechanisms mediating the recruitment and retention of HSC to the liver are areas of particular interest however the tissue distribution of infused cells over time is not well described.

Injected bone marrow cells have been detected within the liver, predominantly localised to peiportal regions (Sakaida *et al.* 2004; Li *et al.* 2013). Sakaida and colleagues showed that injected GFP+ bone marrow cells were present in the periportal regions of the liver 7 days after infusion (Sakaida *et al.* 2004). Significantly higher numbers of injected cells were found in the livers of animals with either acute or chronic liver injury, compared with normal mice (Cho *et al.* 2011; Li *et al.* 2013). Li and colleagues have recently shown that following systemic injection of GFP+ HSC, the cell signal first appears in the lungs and then rapidly decreases by which time signal is apparent in the liver (predominantly) and spleen. Signal within the liver then continued to accumulate over the following days (Li *et al.* 2013). A similar effect was seen with the injection of fluorescently labelled cells into mice with acute liver injury, cell signal accumulated within the liver over 24 hours and then dropped over the

next 48 hours at which point the signal declined dramatically. Lower levels of signal were found in the spleen and kidney(Ezzat *et al.* 2012). Mesenchymal stem cells injected into mice with acute ischaemia reperfusion liver injury were detected only in the first 24 hours in the lungs, whilst the number of cells in the liver increased over 24 hours and then began to decline(Kanazawa *et al.* 2011).

Recent developments in optical imaging technologies have enabled real time in vivo imaging of mice treated with labelled cells. In vivo fluorescence imaging to track tissue distribution of injected cells can be performed by using fluorescent cell dyes to directly label cells (Kalchenko *et al.* 2006; Ezzat *et al.* 2012) or by using donor cells from mice expressing fluorescent proteins. Near infra red fluorescence is particularly attractive as at longer wavelengths there is increased contrast between the fluorescent dye and background tissue fluorescence which is predominantly due to water and haemoglobin and lower abruption of near infra red light results in greater tissue penetration and improved fluorescent signal resolution(Kalchenko *et al.* 2006; Zhang *et al.* 2012). Ushiki et al recently described the tissue distribution of NIR fluorescently labelled HSC immediately after injection in bone marrow transplantation experiments. They found that this technique was sensitive and accurate for the detection of very small numbers of labelled cells across a wide variety of organs(Ushiki *et al.* 2010). Both they and other authors found that ex vivo optical imaging of individual organs improved sensitivity and enabled greater discrimination of tissue distribution of injected cells(Ushiki *et al.* 2010; Ezzat *et al.* 2012; Li *et al.* 2013).

I have previously shown that repeated injections of HSC significantly improve liver injury and fibrosis and subsequently that administration of FTY720 in mice with liver injury increases

the accumulation of HSC within the liver. In this final section of work I will examine in more detail the tissue distribution of HSC following systemic injection over time and determine whether administration of FTY720 alters these dynamics. Ultimately I will determine whether the beneficial effect of repeated HSC injection can be enhanced by administration of FTY720 during liver injury.

6.2 Near Infra Red fluorescent labelling of haematopoietic stem cells

Near infra red (NIR) fluorescent imaging has been used to study the trafficking of various cell types in vivo, low tissue background signal and greater tissue penetration makes it particularly suited to in vivo imaging. I used a commercially available cell dye (DiR, Invitrogen) and designed a series of initial experiments to optimise and validate my cell labelling strategy.

Cell labelling with increasing concentrations of DiR resulted in a concentration dependent increase in the fluorescent signal detected in vitro with IVIS imaging (Fig 6.1A,C), whilst median fluorescence intensity measured by flow cytometry did not greatly increase at concentrations above 5µM (Fig 6.1B). Toxicity of DiR labelling appeared to increase with concentrations of 10µM and above, but when labelled with 5µM there was no increase in the number of dead cells following labelling compared with unlabelled cells (Unlabelled 1.46+/-0.22, 5µM 1.32+/-0.16, 10µM 2.39+/-0.46, 20µM 3.7+/-0.47 % dead cells)(Fig 6.1D). Cells labelled with 5µM DiR maintained their proliferative ability with rates cell growth comparable with unlabelled cells (range 95.45-100% compared with unlabelled cells)(Fig 6.1E).

Accurate tracking of cells relies on the ability to determine cell number based upon fluorescence intensity and I was able to demonstrate a strong linear relationship (r^2=0.989) between total numbers of labelled cells and fluorescent signal when imaged in vitro by the IVIS system (Fig 6.2A,B).

Figure 6.1

Optimisation of DiR labelling of murine HSC

$1x10^6$ cultured HPC-7 cells were incubated with varying concentrations of DiR fluorescent dye for 10 minutes at room temperature, washed and resuspended in media. (A) Fluorescence of cells at different DiR concentrations was measured using the IVIS spectrum. (B) Representative IVIS image (fluorescence overlaid on photograph) of cells labelled with varying concentrations of DiR. (C) Median fluorescence intensity (MFI) of cells labelled with varying concentrations of DIR measured by flow cytometry. (D) Viability of labelled cells at different concentrations of DiR measured by viability dye exclusion determined by flow cytometry. n=3, Mean+/-SD % dead cells in each sample. (E)Proliferation of cells labelled with 5µM DiR, determined by comparison with proliferation of unlabelled cells at different timepoints. n=3, mean +/- SD % proliferation compared with unlabelled cells at each timepoint.

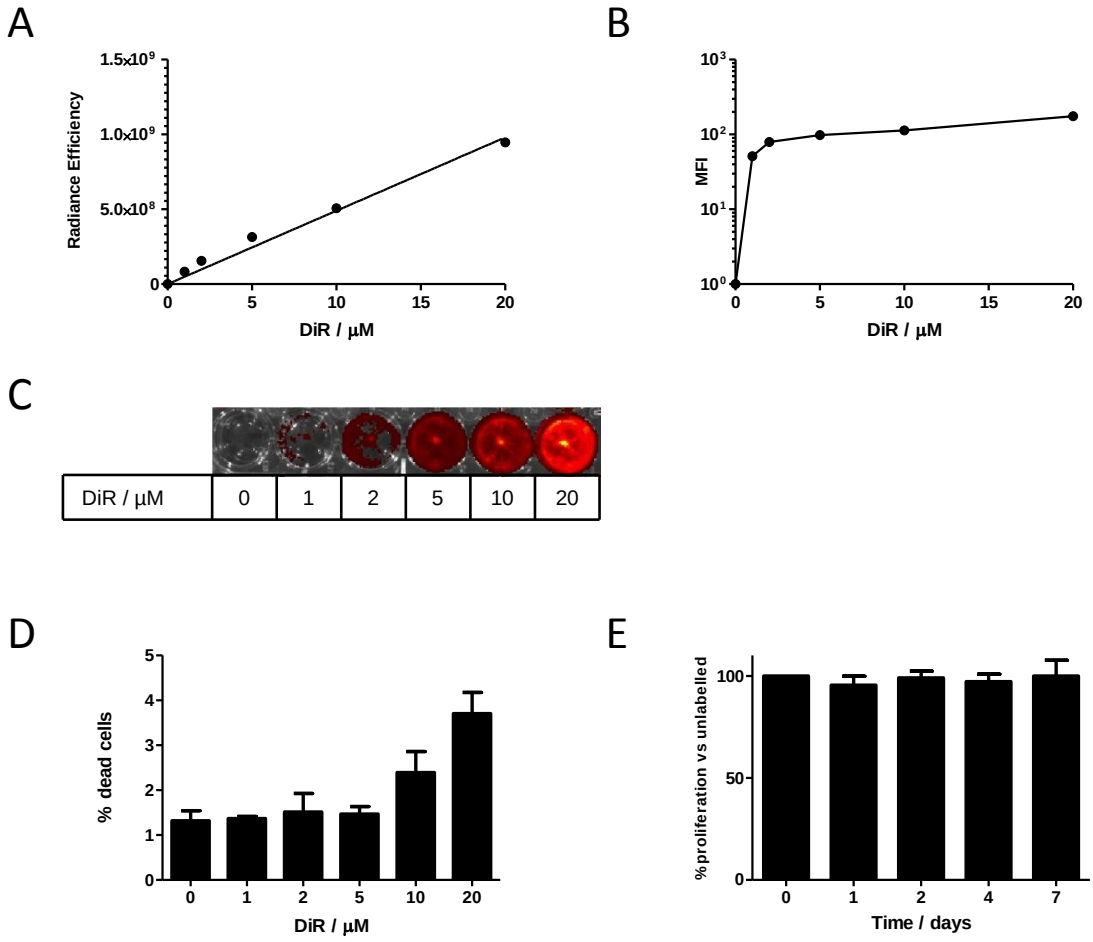


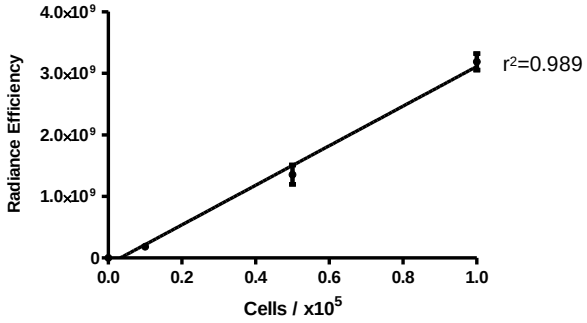
Figure 6.1
Optimisation of DiR labelling of HSC

Figure 6.2

Correlation between cell number and fluorescence intensity

HPC-7 cells were labelled with 5µM DiR for 10 minutes at room temperature, washed and resuspended in complete media. Cells were counted manually with a haemocytometer using trypan blue exclusion and varying numbers of cells added to individual wells of a 96 well plate as indicated. (A) Fluorescence intensity of varying numbers of cells labelled with 5µM DiR was measured using the IVIS imaging system. n=3, mean+/-SD fluorescence shown. (B) Representative IVIS image (fluorescence intensity overlaid on photograph) of indicated numbers of cells labelled with 5µM DiR

A



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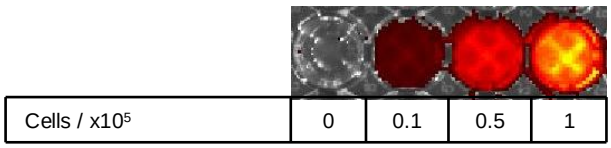


Figure 6.2
Correlation between cell number and fluorescence intensity

Having used HPC-7 cells to optimise and refine the strategy for cell labelling, I confirmed these findings using primary KSL cells. After labelling bone marrow preparations with 5µM DiR there was no appreciable cell toxicity as determined by the number of dead labelled KSL cells compared with unlabelled KSL cells (DiR labelled 0.81+/-0.09 vs Unlabelled 0.79+/-0.14)(Fig 6.3A). Proliferation also remained unaffected, with DiR labelled KSL cells retaining their colony forming ability (Fig 6.3B). Finally the median fluorescence intensity of labelled KSL cells was equivalent to the median fluorescence intensity of labelled HPC-7 cells (Fig 6.3C).

Over a period of seven days there was a 59% loss of fluorescent signal in vitro from labelled HPC-7 cells, however the fluorescent signal at day 7 remained 48-fold higher than unlabelled cells (Fig 6.4A).

It has been reported that NIR fluorescent dye transfer can occur from labelled cells and contaminate unlabelled cells(Lassailly *et al.* 2010). To investigate for this possibility HPC-7 cells were labelled with 5µM DiR and incubated mixed with unlabelled HPC-7 cells in a 1:1 ratio, at various timepoints cell samples were analysed by flow cytometry for the proportion of DiR positive cells. The proportion of DiR positive labelled cells remained constant at around 50% across all timepoints demonstrating minimal transfer of fluorescent dye between cells (Fig 6.4B).

Figure 6.3

Comparison of KSL and HPC-7 DiR staining

Freshly isolated KSL cells were incubated with 5µM DiR for 10 minutes at room temperature, washed and resuspended in media. (A) Cell viability of labelled cells (black bars) was measured by viability dye exclusion determined by flow cytometry and compared with unlabelled cells (white bars). n=3, mean+/-SD % dead cells shown. (B) Colony forming ability in labelled (black bars) and unlabelled (white bars) cells was measured by in vitro methylcellulose assay. n=3, mean +/- number of colonies formed shown. (C) Median fluorescent intensity of HPC-7 cells and KSL cells labelled with 5µM DiR was determined by flow cytometry. n=3, mean+/-SD MFI shown.

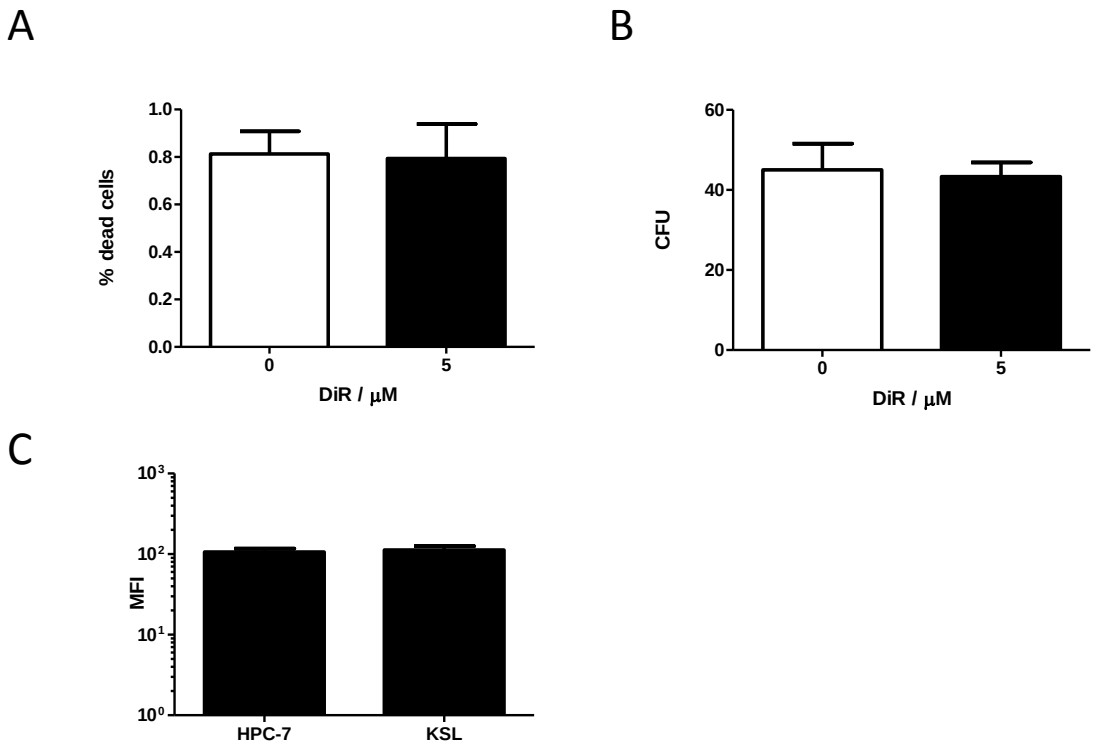


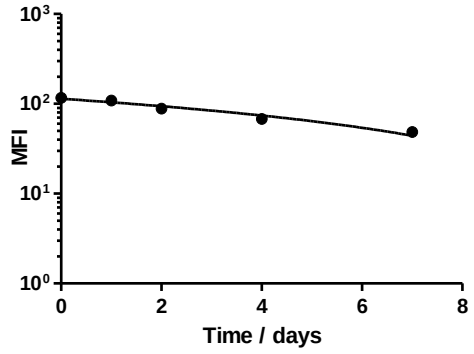
Figure 6.3
Comparison of KSL and HPC-7 DiR staining

Figure 6.4

Loss of fluorescent signal and fluorescent dye cell transfer

(A)$1x10^6$ cultured HPC-7 cells were labelled with 5µM DiR for 10 minutes at room temperature, washed and resuspended in complete media. A cell sample was taken at each timepoint and MFI determined by flow cytometry. (B) $1x10^6$ cultured HPC-7 cells were labelled with 5µM DiR for 10 minutes at room temperature, washed and resuspended in complete media together with $1x10^6$ unlabelled HPC-7 cells. A cell sample was taken at each timepoint and the percentage of fluorescent DiR labelled cells in the sample determined by flow cytometry. n=3, mean+/- SD % fluorescently labelled cells shown.

A



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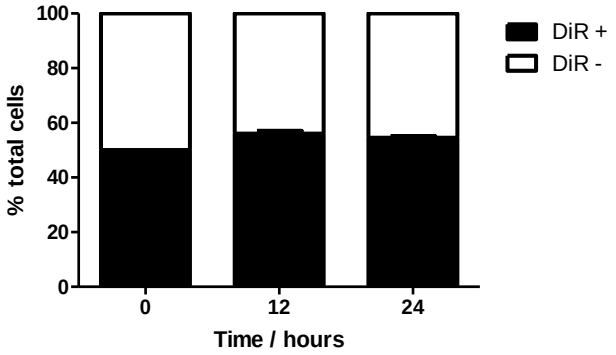


Figure 6.4
Loss of fluorescent signal and fluorescent dye cell transfer

6.3 Tracking of injected HSC by fluorescence imaging

My initial aim was to track the fate of injected HSC in real time through the use of the IVIS in vivo imaging system. Initial experiments involved whole animal in vivo fluorescent imaging following administration of $1x10^6$ DiR labelled HPC-7 via the tail vein. A fluorescent signal could be detected in the anatomical region of the liver, and a higher signal was detected in mice with liver injury compared with control mice in keeping with the hypothesis that liver injury results in increased recruitment of HSC to the liver. However only a weak fluorescent signal could be detected, possibly due to reduced tissue penetration and the presence of black fur despite extensive shaving, and it was difficult to accurately discriminate the organs from which the fluorescent signal was emitted (Fig 6.5A).

Ex vivo fluorescent imaging of the individual organs using the IVIS system provided much greater clarity on the fluorescent signal from each organ and enabled detection of fluorescent signal at lower intensities such as from the lungs and kidneys which had previously been undetectable with whole animal imaging (Fig 6.5B).

Ex vivo imaging of the liver demonstrated minimal fluorescence in mice which had received unlabelled cells, and again confirmed greater recruitment of labelled cells to injured liver compared with controls (Fig 6.5C).

Region of Interest (ROI) gating analysis with Living Image software permitted accurate quantification of fluorescence in individual organs and I performed further experiments to investigate levels of tissue autofluorescence and background fluorescence. In mice with liver injury tissue autofluorescence was significantly higher in the liver than in other organs , however this was negligible compared with tissue fluorescence following injection of DiR

labelled cells. Injection of unlabelled HPC-7 cells resulted in no appreciable change in fluorescence compared with untreated mice. There was a minor increase in fluorescence of the liver following administration of free DiR alone but levels remained lower than after injection of DiR labelled cells (Fig 6.6).

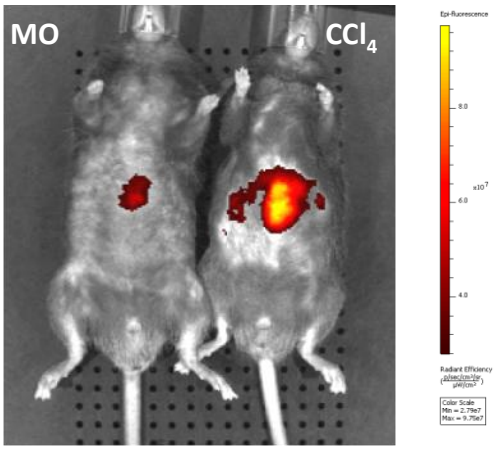
After injection of $1x10^6$ DiR labelled HPC-7 cells the fluorescence signal from the livers of mice with carbon tetrachloride induced liver injury was significantly higher than control mice, whilst the fluorescent signal from other organs remained unchanged (Fig 6.6). This confirms that recruitment of injected HSC to the liver is higher in liver injury than in normal liver.

Figure 6.5

Increased fluorescent signal and tissue discrimination with ex vivo imaging over in vivo imaging

(A)1x10^6 HPC-7 cells labelled with 5µM DiR were administered via tail vein injection to C57BL6 mice treated with twice weekly injections of either carbon tetrachloride(CCl_4, 1mg/kg) or mineral oil (MO) for eight weeks. Mice were euthansed after 1 hour and whole animal imaging was performed using the IVIS imaging system (B) Ex vivo imaging of individual organs. (C) Ex vivo imaging of livers alongside liver from an untreated age and sex matched C57BL6 mouse. All images are combined image of fluorescence intensity overlaid on photograph shown and are representative of multiple experiments

A



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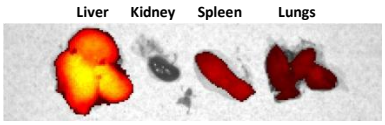

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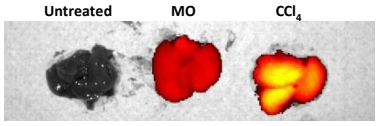

Figure 6.5
Increased fluorescent signal and tissue discrimination with ex vivo imging over in vivo imaging

Figure 6.6

Lack of significant background or tissue autofluorescence

C57BL6 mice were treated with either carbon tetrachloride (CCl4, 1mg/kg) or mineral oil (MO) for eight weeks and then received either $1x10^6$ unlabelled HPC-7 cells, 5µM free DiR or $1x10^6$ DiR labelled cells via tail vein injection. Mice were euthanased and ex vivo imaging performed using the IVIS imaging system. No significant tissue auto fluorescence or background fluorescence was seen compared with injection of DiR labelled cells. Tissue auto fluorescence was higher in the liver than other organs. All images are combined image of fluorescence intensity overlaid on photograph shown and are representative of multiple experiments.

A

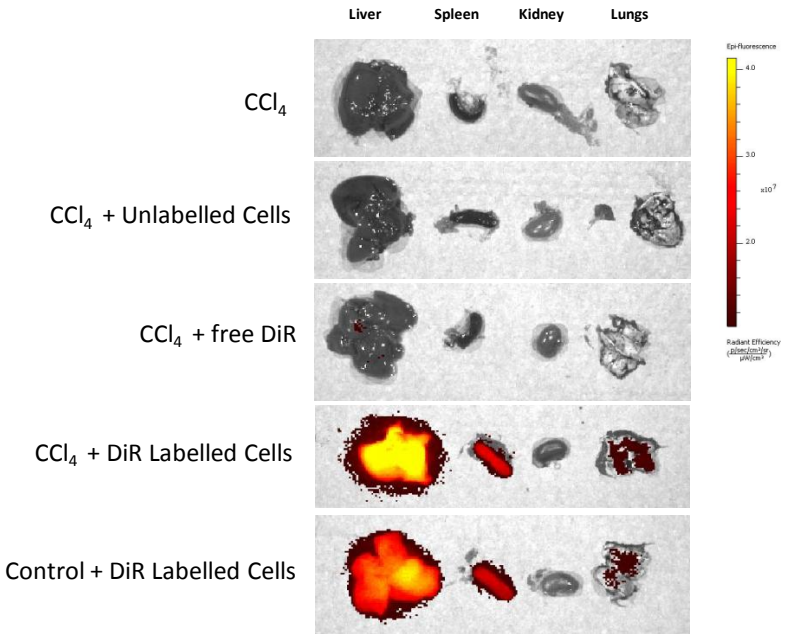

Figure 6.6
Lack of significant background or tissue autofluorescence

6.4 Increased hepatic accumulation of HSC with FTY720 administration

I had previously demonstrated that administration of FTY720 to mice with liver injury resulted in increased accumulation of bone marrow derived HSC within the liver, this lead me to hypothesize that the administration of FTY720 would increase the accumulation of injected HSC within the injured liver. Given the number of cells required for such experiments and the relative scarcity of primary KSL cells I elected to use HPC-7 cells for initial experiments to determine the tissue distribution of injected cells at various timepoints and study the effect of FTY720 administration.

High levels of fluorescent signal were detected in the liver and lungs shortly after cell injection, and whilst the signal in the lungs rapidly declined over the following 48 hours, the signal in the liver accumulated over the first 48 hours and then gradually declined (Fig 6.7A,B). Fluorescent signal was detected in the spleen after 24 hours and remained constant but fluorescent signal was virtually undetectable in the kidney (Fig 6.7B).

A similar pattern was observed in mice treated with FTY720, significantly however the fluorescent signal within the liver of FTY720 treated mice was 27% higher than untreated mice after 48 hours (p=ns) and the difference increased to 50% and 86% higher than control at day 4 and day 7 respectively ($p<0.01$ at both timepoints)(Fig 6.7A,B).

In order to reinforce the data on tissue distribution of injected cells I also quantified the numbers of fluorescent DiR labelled cells within selected organs by preparing single cell suspensions for analysis and quantification by flow cytometry (Fig 6.8A). Given the scarcity of injected cells in the populations analysed, accuracy of analysis was improved by co-staining for CD45 to exclude non-haematopoietic cells with subsequent gating of the

resultant cell population according to the expected size and granularity of HPC-7 cells (Fig 6.8A).

Figure 6.7

Administration of FTY720 increased the number of injected HSC within the liver – Fluorescence Intensity

C57BL6 mice received twice weekly administration of carbon tetrachloride via intraperitoneal injection for 8 weeks. $5x10^5$ DiR labelled HPC-7 cells were then injected via the tail vein followed by daily administration of either FTY720 (1mg/kg) or vehicle control by intraperitoenal injection for 7 days. Mice were then euthanased either 1 hour following injection (time 0) or 1,2,4,7 days after injection and ex vivo imaging of organs performed using the IVIS imaging system . (A) Representative combined photographic and fluorescence intensity images of livers from mice treated with either FTY720 (FTY720) or vehicle (Control) at timepoints following cell injection. (B) Fluorescence was measured by ROI analysis in organs from mice treated with either FTY720 (blue line) or vehicle (red line) and quantified as radiance efficiency following background subtraction. Graphs represent fluorescence in individual organs at timepoints studied. Points represent mean +/-SD, n=3 per group at each timepoint, * $p<0.05$ ** $p<0.01$ (student's unaired t-test at each timepoint).

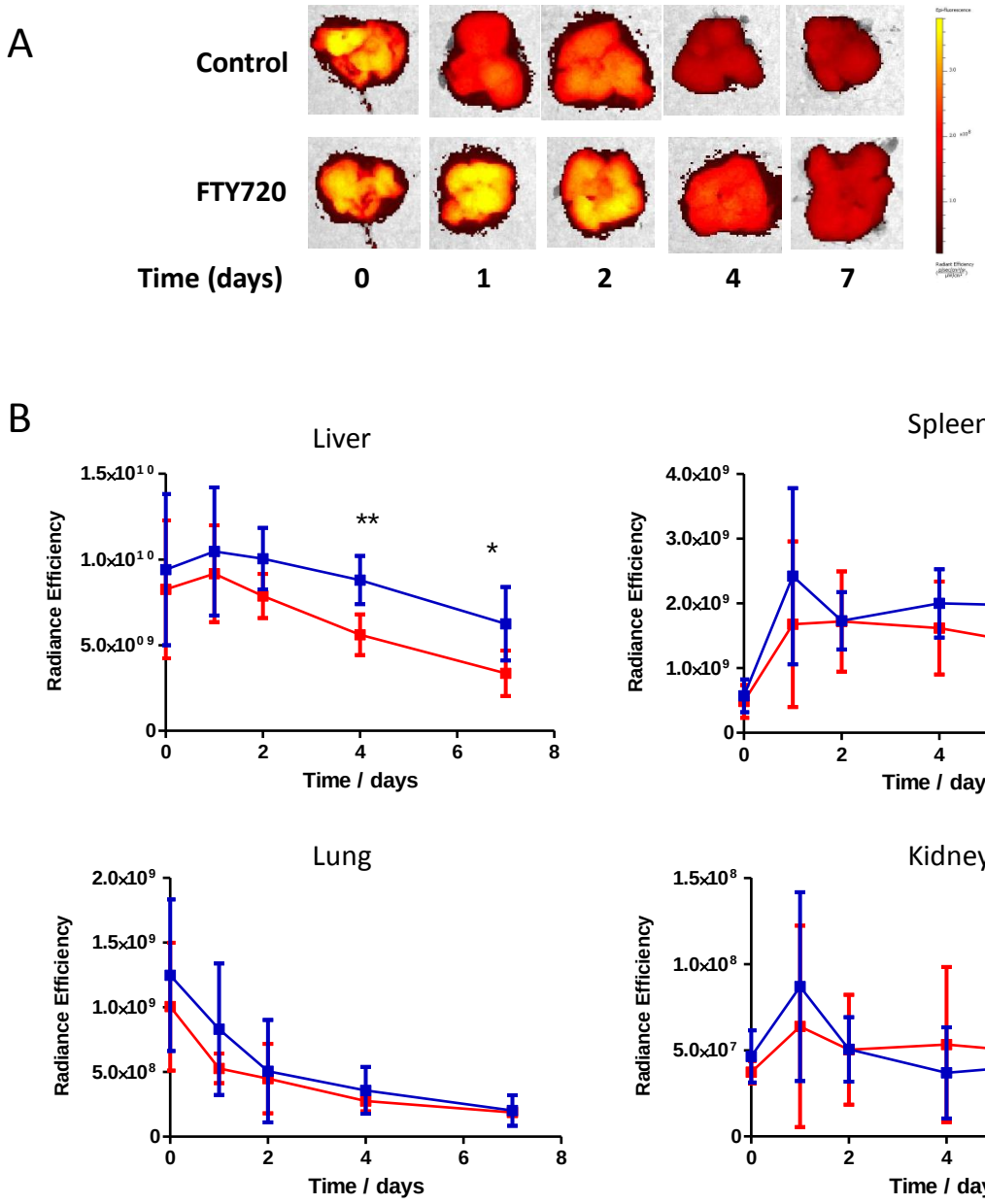

Figure 6.7
Administration of FTY720 increased the number of injected HSC within the liver – Fluorescence Intensity

Figure 6.8

Administration of FTY720 increased the number of injected HSC within the liver – Cell quantification

C57BL6 mice received twice weekly administration of carbon tetrachloride via intraperitoneal injection for 8 weeks. $5x10^5$ DiR labelled HPC-7 cells were then injected via the tail vein followed by daily administration of either FTY720 (1mg/kg) or vehicle control by intraperitoenal injection for 7 days. Mice were then euthanased either 1 hour following injection (time 0) or 1,2,4,7 days after injection and DiR labelled cells within individual organs quantified by flow cytometry as previously described. (A) Sequential gating strategy used to identify injected DiR labelled cells. (Initial forward scatter – side scatter gate to eliminate cell debris and dead cells followed by identification of CD45 positive DiR positive cells to eliminate cellular contamination of non haematopoietic cells and back gating of this population on forward scatter – side scatter to confirm expected size and granularity) (B) Absolute numbers of injected cells within individual organs analysis from mice treated with either FTY720 (blue line) or vehicle (red line) was quantified by flow cytometry at each timepoint. Points represent mean +/-SD number of cells within each organ at timepoint studied, n=3 per group at each timepoint. *$p<0.05$ **$p<0.01$ (students unpaired t-test at each timepoint).

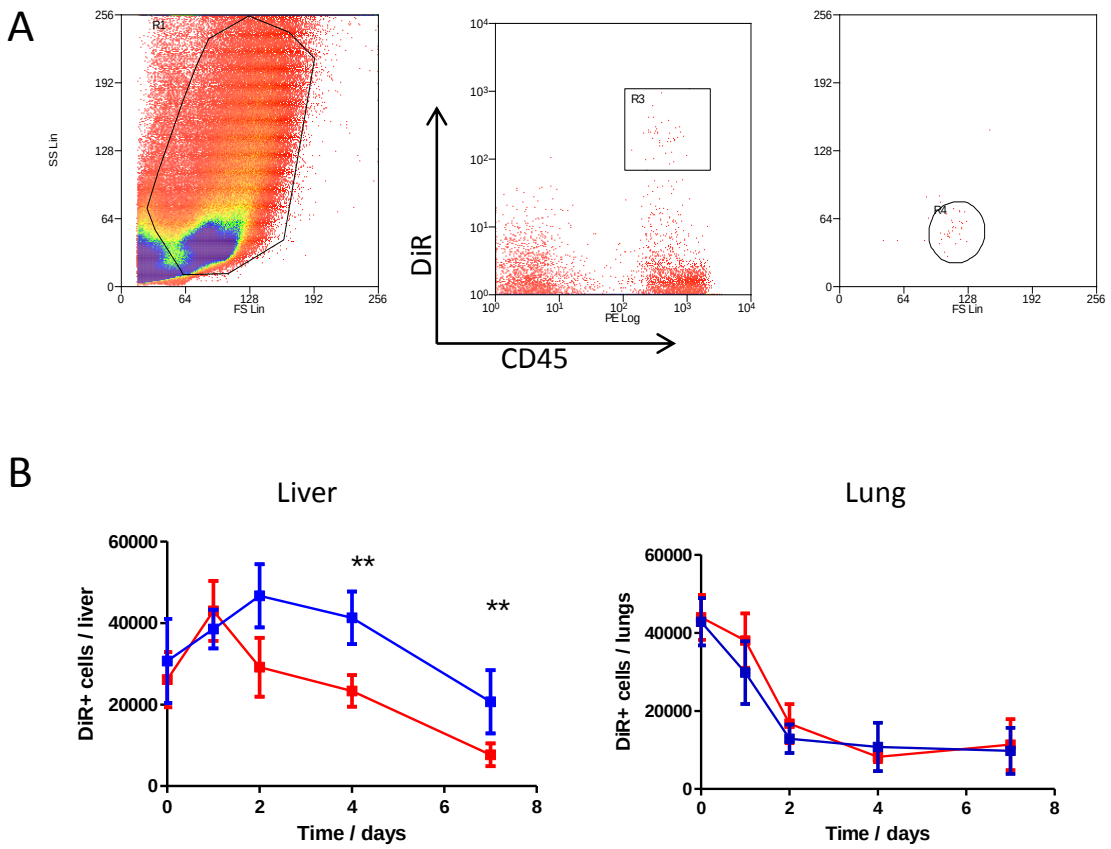

Figure 6.8
Administration of FTY720 increased the number of injected HSC within the liver – Cell quantification

This analysis confirmed the distribution of injected cells over time within the liver and lungs. In mice treated with FTY720 there significantly higher numbers of injected cells within the liver at day 2 (FTY720 46671 cells per liver vs Control 29155 cells per liver, $p<0.05$) and day 4 (FTY720 41294 cells per liver vs Control 23349, $p<0.05$), with a trend to higher numbers of cells at day 7 (Fig 6.8B).

To confirm that the findings observed following injection of HPC-7 cells were also true of primary KSL cells I undertook a repeat experiment but given the scarcity of primary KSL cells chose to examine only one timepoint. I chose day 4 following injection as the timepoint to test given the marked difference between FTY720 treated and untreated mice with HPC-7 cells.

Mice treated with FTY720 had 1.4-fold increase in fluorescent signal in the liver at day 4 following cell injection (FTY720 $2.057x10^9$ vs Control $1.45x10^9$, $p<0.05$)(Fig 6.9A,B). This was confirmed with quantification of DiR labelled cells by flow cytometry demonstrating a 75% increase in DiR positive cells in the livers of FTY720 treated mice(FTY720 13514+/-1506 vs Control 7687+/-1556, $p<0.05$)(Fig 6.9B).

Figure 6.9

Administration of FTY720 increased the number of injected KSL cells within the liver

C57BL6 mice received twice weekly administration of carbon tetrachloride via intraperitoneal injection for 8 weeks. $1x10^5$ DiR labelled KSL cells were then injected via the tail vein followed by daily administration of either FTY720 (1mg/kg) or vehicle control by intraperitoenal injection for 7 days. Mice were euthanased on day 4 after cell injection and ex vivo imaging of organs performed using the IVIS imaging system. (A) Representative combined photographic and fluorescence intensity images of organs from mice treated with either FTY720 (FTY720) or vehicle (Control) at timepoints following cell injection. (B) Fluorescence was measured by ROI analysis and quantified as radiance efficiency following background subtraction. Graphs represent fluorescence intensity of the liver. Data from individual mice is displayed (circles : control, squares : FTY720 treated) and means indicated by horizontal line. * $p<0.05$ (unpaired student's t-test). (C) Absolute numbers of injected cells, identified as CD45 positive DiR positive were quantified as previously described (Fig 6.8) ,within the liver were quantified by flow cytometry. Data from individual mice is displayed (circles : control, squares : FTY720 treated) and means indicated by horizontal line. * $p<0.05$ (unpaired student's t-test).

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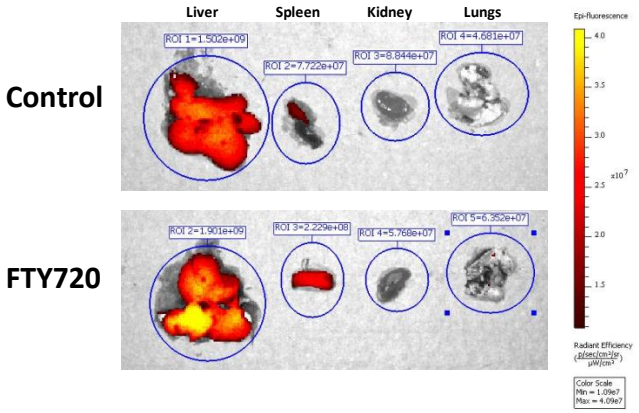

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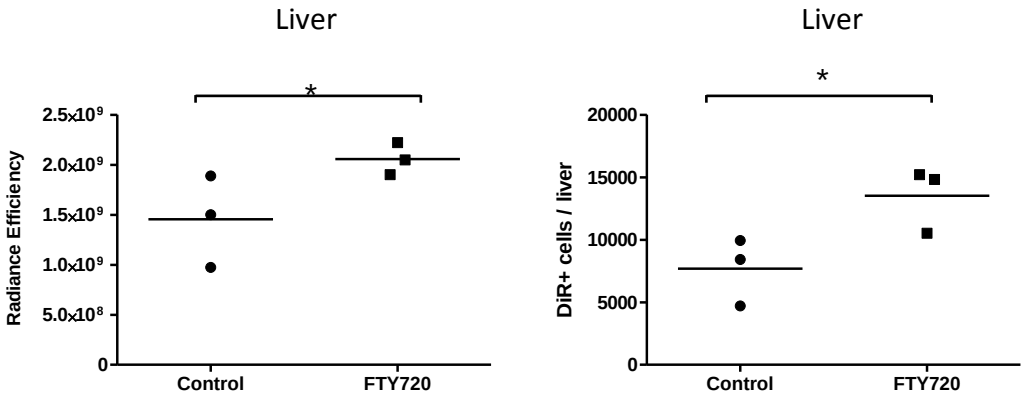

Figure 6.9
Administration of FTY720 increased the number of injected KSL cells within the liver

6.5 Enhanced anti fibrotic effect of HSC with FY720 administration

Having previously demonstrated the anti fibrotic effect of repeated injection of purified HSC and subsequently an increase in the number of injected cells within the liver with administration of FTY720, the ultimate aim of this work was to determine whether administration of FTY720 enhanced or improved the anti fibrotic effect of HSC in liver injury.

I utilised a similar experimental design to my previous experiments examining the anti fibrotic effect of HSC (Fig 6.10) . Four experimental groups were included: i) control mice with liver injury alone, ii) mice with liver injury receiving FTY720 alone to control for the effect of FTY720 on liver fibrosis and function, iii) mice with liver injury receiving repeated injections of purified KSL cells and iv) mice with liver injury receiving repeated injections of KSL cells together with FTY720 administration for duration of cell injections.

Figure 6.10

Investigation of the effects of FY720 in conjunction with repeated infusions of purified HSC in a murine model of chronic liver injury - Experimental timeline

Chronic liver injury was induced in age and sex matched C57/BL6 mice by twice weekly intraperitoneal administration of carbon tetrachloride (1mg/kg) in mineral oil for 6 weeks, mice were then randomly allocated to a treatment group. (A) Mice in the control group continued to receive twice weekly injections of intraperitoneal carbon tetrachloride until week 10 and were sacrificed 72 hours following the final injection of carbon tetrachloride. (B) Mice in the FTY720 treatment group received twice weekly carbon tetrachloride in the same way as control mice but in addition received thrice weekly FTY720 (1mg/kg) via intraperitoneal injection from the start of week 7, mice were sacrificed at the start of week 10, 72 hours after the final injection of carbon tetrachloride. (C) Mice in the cell treatment group received twice weekly carbon tetrachloride in the same way as control mice but in addition received a tail vein injection of $5x10^4$ purified KSL cells isolated from C57/BL6 donor mice on the first day of weeks 7,8 and 9, mice were sacrificed at the start of week 10, 72 hours after the final injection of carbon tetrachloride, 7 days following the final cell injection. (D) Mice in the cell treatment plus FTY720 group received twice weekly carbon tetrachloride in the same way as control mice but in addition received a tail vein injection of $5x10^4$ purified KSL cells isolated from C57/BL6 donor mice on the first day of weeks 7,8 and 9 and thrice weekly FTY720 (1mg/kg) via intraperitoneal injection from the start of week 7, mice were sacrificed at the start of week 10, 72 hours after the final injection of carbon tetrachloride, 7 days following the final cell injection.

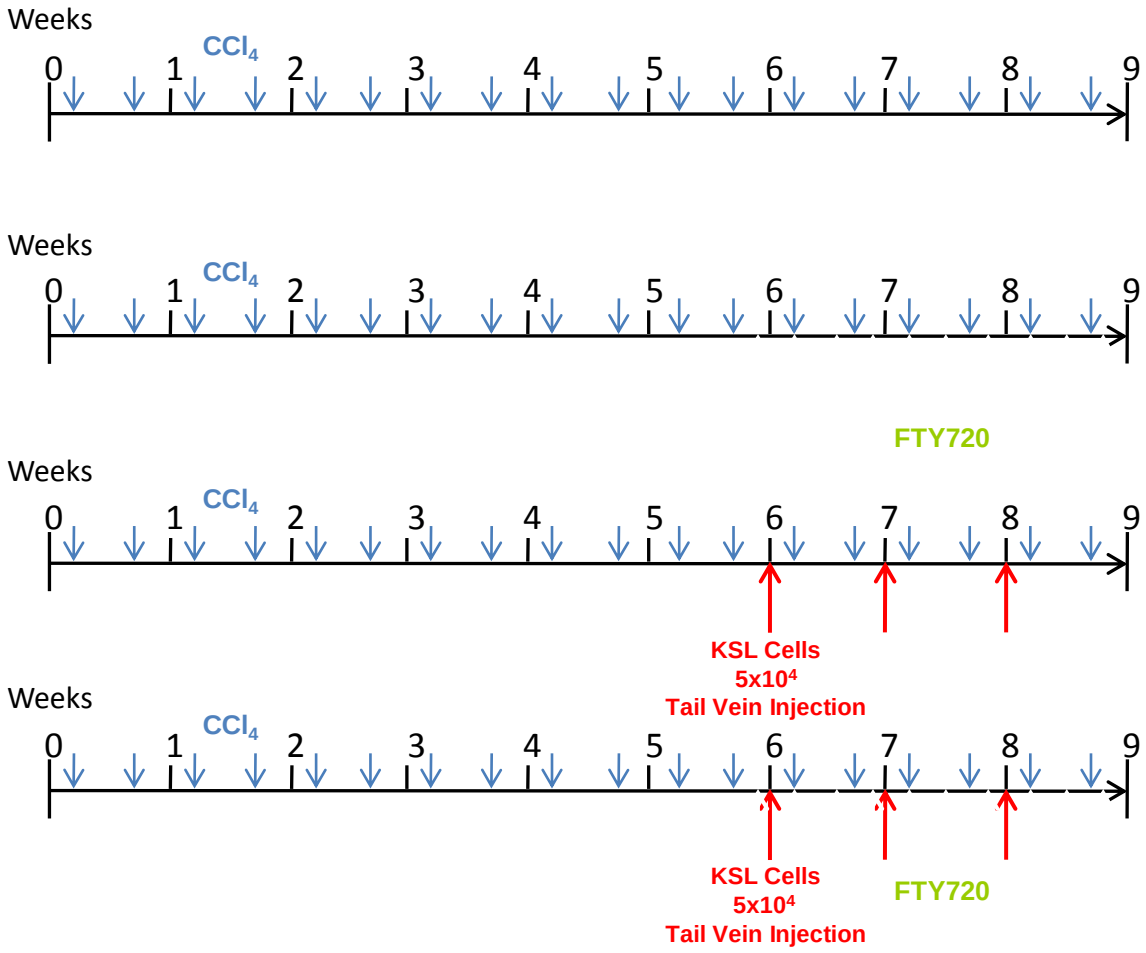

Figure 6.10

Investigation of the effects of FY720 in conjunrtion with repeated infusions of purified HSC in a murine model of chronic liver injury - Experimental timeline

There was no effect on hepatic fibrosis with administration of FTY720 alone. No differences in picrosirius red staining (Control 4.19+/-0.63 % staining vs FTY720 4.38+/-0.74 % staining, p=ns) or hepatic hydroxyproline content (Control 436.5+/-26.6 mg per g protein vs FTY720 462.0+/-35.5 mg per g protein, p=ns) were seen. αSMA staining and gene expression of myofibroblast activation was similar in both groups. Serum albumin levels showed no significant change.

Fgure 6.11

Further reduction in hepatic fibrosis in mice treated with FTY720 in conjunction with repeated KSL cell injections

(A) Formalin fixed paraffin embedded liver tissue sections from mice treated with KSL cell infusions (white bars) and with KSL cells plus FTY720 (grey bars) were stained for picrosirius red. 6 random, non-overlapping images were obtained from each section and staining was quantified as a percentage of the image positive for picorsirius red using ImageJ software. Lower levels of picrosirius red staining was detected in mice treated with FTY720 and KSL cels compared with KSL cell infusions alone. n=12-13 each group, mean +/-SD % area stained shown, *p<0.05 (two tailed, unpaired students t-test). (B) Representative photomicrograph images (x20 magnification) of picrosirius red staining (red, no counterstain) from control mice and mice treated with FTY720 alone, KSL cells alone and KSL cells plus FTY720. (C) Hydroxyproline content in samples of liver tissue from KSL cell treated mice (white bars) and from KSL cells plus FTY720 treated mice(grey bars) was determined and expressed as mg of Hydroxyproline per gram of liver tissue. Lower amounts of hydroxyproline were found in livers from mice receiving KSL cell injections plus FTY720 compared with livers from mice receiving KSL cells alone. n=12-13 each group, mean+/-SD hydroxyproline content shown. (D) Serum samples were obtained by centrifugation of whole blood from mice treated with KSL cell injections (white bars) and from mice treated with KSL cells plus FTY720 (grey bars) and serum albumin concentration was measured by bromocresol green colorimetric assay. n=12-13 each group, mean+/-SD serum albumin concentration shown.

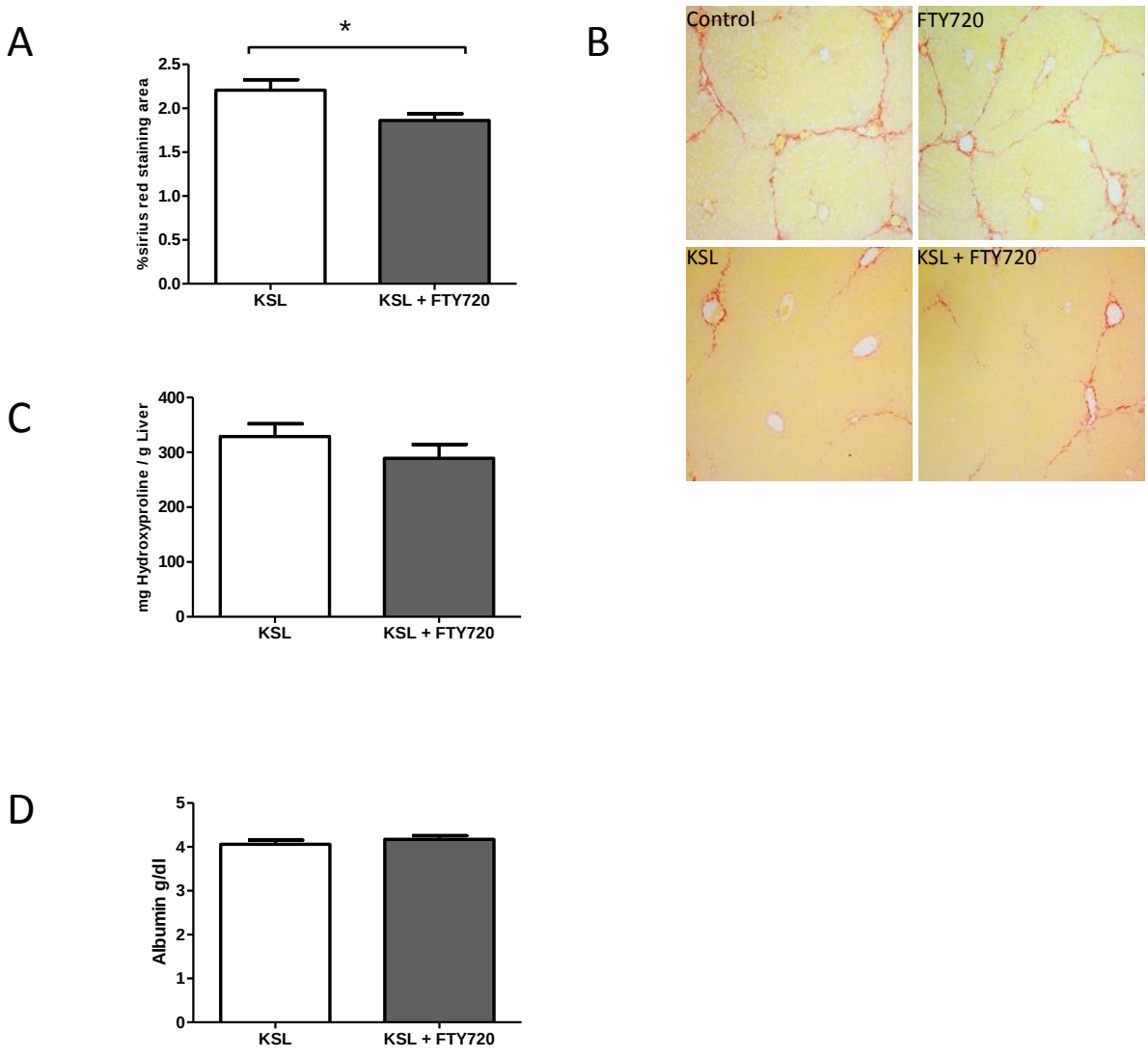

Figure 6.11
Further reduction in hepatic fibrosis in mice treated with FTY720 in conjunction with repeated KSL cell injections

Figure 6.12

Further reduction in activated hepatic myofibroblasts in mice treated with FTY720 in conjunction with repeated KSL cell injections

(A) Formalin fixed paraffin embedded liver tissue sections from mice treated with KSL cell infusions (white bars) and with KSL cells plus FTY720 (grey bars) were stained for aSMA. 6 random, non-overlapping images were obtained from each section and staining was quantified as a percentage of the image positive for aSMA using ImageJ software. Significantly lower levels of aSMA staining was detected in mice treated with FTY720 and KSL cell infusions than those treated with KSL cells alone. n=12-13 each group, mean +/-SD % area stained shown, *p<0.05 (two tailed, unpaired students t-test). (B) Representative photomicrograph images (x20 magnification) of aSMA staining (brown, DAB substrate, haematoxylin counterstain) from control mice and mice treated with FTY720 alone, KSL cells alone and KSL cells plus FTY720. (C,D) RNA was extracted from liver tissue from mice treated with KSL cell infusions (white bars), KSL cells plus FTY720 (grey bars) and control mice (black bars) and using quantitative RT-PCR gene expression of (C)col1a1 and (D) aSMA were determined, normalised to GAPDH expression and relative to control mice using the 2^{-ddCT} method. Hepatic gene expression of both col1a1 and aSMA was significantly lower in both KSL cell treated and KSL cells plus FTY720 mice than control mice, but no difference was seen between the two treatments was seen. n=12-13each group, mean +/-SD fold change shown, *p<0.05 (wilcoxon signed rank test).

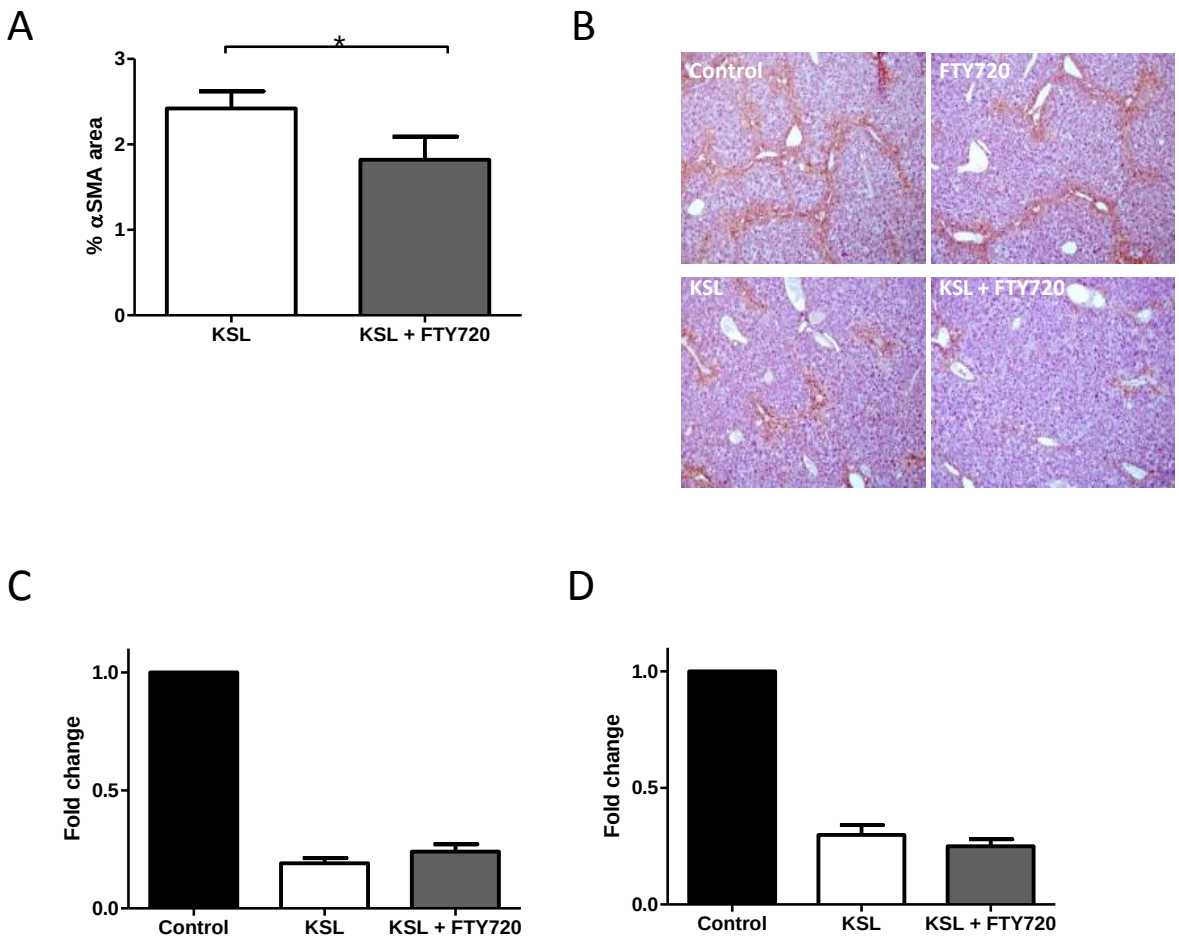

Figure 6.12
Further reduction in activated hepatic myofibroblasts in mice treated with FTY720 in conjunction with repeated KSL cell injections

The administration of FTY720 in conjunction with KSL cell injections did result in further improvements in some of the hepatic fibrosis markers, above the improvement seen with KSL cell injection alone. There was a further reduction in picosirus red staining (KSL 2.21+/-0.11 %staining vs KSL+FTY720 1.86+/-0.10 %staining, $p<0.05$)(Fig 6.11A,B) and although a 12% reduction in hepatic hydroxyproline content (KSL 328.9+/-83.63 vs KSL+FTY720 289.2+/-90.94, p=ns)(Fig 6.11C) was observed this did not reach significance. αSMA staining for activated myofibroblasts was reduced in FTY720 treated mice (KSL 2.31+/-0.22 %staining vs KSL+FTY720 1.82+/-0.19 %staining, $p<0.05$)(Fig 6.12 A,B) but as gene expression of αSMA and col1α1 had been profoundly suppressed in mice treated with KSL cell injection, no further improvements were observed with the addition of FTY720 (Fig 6.12C,D). Serum albumin levels were not significantly higher in mice treated with FTY720 compared with those treated with KSL cell injections alone (Fig 6.11D).

6.6 Conclusions

In earlier sections of this work I demonstrated the anti fibrotic effect of repeated injection of haematopoietic stem and progenitor cells, these cells were injected into a peripheral vein rather than directly into the portal circulation. It has been shown previously that cells injected through a peripheral vein are recruited to the liver(Sakaida *et al.* 2004; Cho *et al.* 2011), which is confirmed by the therapeutic effect observed with peripheral vein injection. The dynamics of cell trafficking and tissue distribution after injection is not well understood.

In the initial studies of this section of work I optimised a method of labelling HSC with a near infra red fluorescence lipophilic membrane dye – DiR. A near infra red dye was used to reduce the effects of tissue autofluorescence and improve tissue penetration when tracking injected cells through tissues. At the concentration used, 5µM, the dye did not affect cell viability or impair proliferation in either primary KSL cells or cultured HPC-7 cells. The fluorescence intensity of DiR labelled cells showed a slight decline over time but maintained significantly higher signal than unlabelled cells and over tissue background fluorescence. When comparing the effect of different treatments on cell trafficking, the decline in fluorescence would be consistent between groups and allow relative comparisons to be made.

The fluorescent signal from DiR labelled cells was directly proportional to the concentration of labelled cells within samples in vitro. This was confirmed during the in vivo experiments where tissue fluorescent intensity following injection of DiR labelled cells was approximately proportional to the number of cells injected. This confirmed that use of tissue fluorescence intensity was an appropriate method of quantifying DiR labelled cells within different organs.

Concerns have previously been raised regarding the possibility of dye transfer between labelled and unlabelled cells(Lassailly *et al.* 2010), however I did not observe any significant transfer of dye between labelled and labelled HPC-7 cells in culture. When quantifying DiR labelled cells within tissues using flow cytometry I used additional antibody staining and sequential gating as a precautionary measure to attempt to eliminate any contamination effect.

Attempts at whole animal in vivo imaging were thwarted by low fluorescent signal, which may have been due to impaired tissue penetration and increased light absorbance in mice with black fur. The fluorescent signal observed could not be well discriminated and did not allow adequate assessment of cell trafficking to different organs, in particular signal from the lungs was not detected. Ex vivo fluorescent imaging of individual organs, as used in other studies of HSC trafficking (Ushiki *et al.* 2010; Li *et al.* 2013), allowed detection of much lower fluorescent intensities and clear discrimination of the signal from individual organs. The liver exhibited higher background tissue autofluorescence than other organs but this was insignificant compared with fluorescent signal after labelled cell injection.

Studies of the fate of HSC after injection have shown rapid clearance from the circulation with uptake in a variety of different organs and only minimal homing to the bone marrow initially. Use of the HPC-7 cell line enabled study of larger cells numbers and repeated experiments. My studies have shown that immediately after injection, uptake of labelled cells into the liver is greater in mice with liver injury compared with control mice, a finding consistent with established data showing increased recruitment of HSC to injured liver (Kavanagh *et al.* 2010; Cho *et al.* 2011; Li *et al.* 2013).

I studied the subsequent tissue distribution of injected cells over the course of seven days. There was an even distribution to HSC accumulation between the lungs and liver immediately after injection allaying concerns over the trapping of injected cells within the pulmonary vasculature. Over the subsequent 24-48 hours cells cleared from the lungs but continued to accumulate in the liver and subsequently the spleen. Maximal cell accumulation in the liver was at 48 hours with subsequent slow decline following this. I did not examine homing of injected cells to the bone marrow in these studies due to low levels of uptake. This findings indicate that administration of HSC by tail vein injection does result in recruitment of cells to the liver, but the significant numbers of cells in the lungs may mean greater cell recruitment to the liver would occur after portal vein injection although I did not study this. Work by Dr.Dean Kavanagh (PhD 2010) suggests that portal vein injection results in greater recruitment of cells to the liver compared with peripheral injection, however the difference in cell recruitment between normal and injured mice is lost with portal vein injection. The implications of this include the possibility of a maximal threshold of cell recruitment which cannot be exceeded, or a mechanical component to cell recruitment after portal vein injection rather than being adhesion molecule mediated.

The techniques used in these studies have shown to be more sensitive and consistent for tracking the trafficking of injected cells given that the whole organ is assessed rather than methods such as tissue section analysis where only a fraction of the total organ can be analysed**.** This explains why in previous studies KSL cells are difficult to identify in tissue sections at 7 days after injection, as approximately 20,000 or more sections could be generated per liver and from my whole organ studies only 10,000 cells were found in the liver at day 7.

I previously observed that administration of FTY720 affects HSC migration and results in an accumulation of bone marrow derived HSC within the injured liver. The studies in this section of work have shown that administration of FTY720 following cell injection also results in an increased accumulation of injected cells in the liver compared to untreated mice. The number of cells within the liver was no different over the first 24 hours but a difference became apparent at 48 hours which was maintained until day 7. There was increased numbers of cells within the spleen after 48 hours with the most pronounced difference seen at day7 but this did not reach significance. These findings are consistent with the observations from Massberg that tissue dwell time is approximately 36-48 hours and that FTY720 results in tissue accumulation due to impaired tissue egress(Massberg *et al.* 2007). These studies had been conducted using cultured HPC-7 cells given the scarcity of primary KSL cells, in order to confirm these findings with KSL cells I chose a single timepoint to assess accumulation of injected cells within the liver, this was day 4 when the difference between cells in the liver of FTY720 treated and untreated mice was most pronounced. Both fluorescent intensity of the liver and quantified cell numbers in the liver were greater in the FTY720 treated mice than the controls, confirming the observations made using the cell line.

From the published data on the effect of bone marrow derived stem cells on liver injury it is not clear whether the numbers of cells injected correlates with the beneficial effect seen. There have been no studies examining the effect of different 'doses' of cells and direct comparison of independent studies is complicated by the variety of experimental designs in terms of cell type, cell number, route of delivery and outcome measures(Houlihan *et al.* 2008). Two previous studies using KSL cells have used significantly higher numbers of

(cultured) cells than in my studies and shown a beneficial effect, and although I studied repeated injections, the effect was not proportional to the higher numbers of cels used(Cho *et al.* 2011; Li *et al.* 2013). In general human studies have attempted to standardise protocols by using fixed numbers of cell per kg body weight . I hypothesized that increasing the number of injected cells within the liver would enhance their therapeutic effect, this would be of particular benefit in optimising the effect of the scarce population of KSL cells and studied the effect of FTY720 administration combined with KSL cell injections on liver fibrosis.

FTY720 administration in conjunction with repeated KSL cell injections lead to a further reduction in hepatic fibrosis compared with KSL cell injections alone. Significant reductions in ECM collagen deposition measured by picosirius red staining and in numbers of αSMA+ myofibroblasts were noted, a reduction in hepatic hydroxyproline content did not achieve statistical significance which may have been related to variability in assessing the low levels of hydroxyproline in the assay.

Treatment with FTY720 alone without KSL cell injections did not significantly alter any marker of hepatic fibrosis. In some animal models of organ injury, for example renal ischemia reperfusion injury and experimental autoimmune encephalitis, FTY720 treatment has resulted in either reduced severity or improved recovery from injury. These have predominantly been immune mediated injury rather than chronic fibrosis models and it is possible that treatment with FTY720 may be beneficial in an immune mediated liver injury or even if given prior to the onset of fibrosis rather than to reduce fibrosis. It would appear that

treatment with FTY720 alone lacks the postulated regenerative stimulus provided by KSL cells and it is this effect that is required to initiate the resolution of fibrosis.

Taken in conjunction with the observations that FTY720 treatment results in an increase in the numbers of injected cells in the liver, these findings suggest that increasing the numbers of injected KSL cells in the liver results in an enhanced anti fibrotic effect. The observation that FTY720 treatment alone does not impact upon fibrosis implies that the increased numbers of KSL cells are the reason for improved fibrosis rather than increased numbers of KSL cells in the liver as result of improved fibrosis due to FTY720 treatment.

It remains unclear however whether there is indeed an optimum 'dose' for KSL cell injection and whether a plateau of cell recruitment to the liver is reached as the Kavanagh data (Kavanagh *et al.* 2010)suggests or whether a maximal stimulus to endogenous repair is reached which cannot be improved upon. However, autologous HSC are not a limitless resource partly due to impaired bone marrow function in cirrhosis (Lorenzini *et al.* 2008)and partly due to the risks of the side effects of mobilising agents such as GCSF, and therefore approaches to optimise their therapeutic effect are prudent.

Modulation of S1P signalling represents a potential mechanism for optimising the effect of HSC as it results in increased HS within the liver.

7 DISCUSSION

7.1 Background

There is a growing need for new therapies for chronic liver disease. The incidence of liver disease is rapidly rising, driven by increases in obesity and alcohol related liver disease (Leon *et al.* 2006). Current treatments for liver disease consist of treatment of the underlying disease where possible, for example anti viral therapy for Hepatitis C, and treatment of the complications of liver disease, such as diuretics for ascites and beta-blockers for varices. Liver transplantation is the only curative treatment for end stage liver disease, however this is limited by the availability of donor organs, post operative complications and recurrence of underlying liver disease. There are no alternative treatments for underlying hepatic fibrosis and cirrhosis that are common to all forms of chronic liver disease.

Since the first reports of the contribution of the bone marrow to hepatic regeneration by Petersen et al, there has been growing interest in the potential role of bone marrow derived cells as a treatment for liver disease (Petersen *et al.* 1999). There has been rapid progress to clinical studies of bone marrow cells in patients with liver disease despite a relative lack of convincing evidence for either the effect or mechanism of effect from pre-clinical models. Sakaida and colleagues described a reduction in hepatic fibrosis following infusion of bone marrow cells, however subsequent studies have reported mixed findings(Sakaida *et al.* 2004; Thomas *et al.* 2011) (Cho *et al.* 2011). A difficulty in interpreting these studies is the heterogeneous nature of bone marrow cells and the uncertainty over the cell population responsible for the anti fibrotic effect. Although a therapeutic effect has been demonstrated the mechanism of action remains to be determined. Initially bone marrow

cell transdifferentiation or cell fusion resulting in hepatocyte repopulation had been proposed but it is now generally accepted not to be a frequent occurrence and would not account for an improvement in liver function or a reduction in fibrosis.

Haematopoietic stem cells represent a rare population of cells within the bone marrow and engraftment of injured organs occurs at low frequency. The mechanisms by which HSC engraft the bone marrow have been well studied(Mazo *et al.* 2011), although understanding of the mechanisms of tissue homing is more limited and tissue specific mechanisms may be important. Attempts to optimise the therapeutic potential of HSC through modulation of surface adhesion molecules in murine models of colitis and kidney injury have been reported (Kavanagh *et al.* 2013; White *et al.* 2013). Sphingosine 1-phosphate has been shown to be an important regulator of HSC egress from tissues in draining lymphatics, and modulation of S1P receptor signalling by FTY720 resulted in reduced egress and an accumulation of HSC within peripheral tissues(Massberg *et al.* 2007).

7.2 Summary of Main Findings

The work presented in this thesis has generated new insights into the benefits of haematopoietic stem cell therapy for liver disease, the role of S1P in liver injury and in HSC trafficking, and the potential for enhancing the therapeutic effects of HSC through modulation of S1P signalling.

The main findings arising from this work include:

- The numbers of bone marrow derived haematopoietic stem cells found within the liver and peripheral blood are higher during liver injury
- Chronic liver injury increases the level of S1P within the liver and serum
- The rise in S1P within the liver and serum is accompanied by an increased hepatic expression of sphingosine kinase 1, predominantly in liver sinusoidal endothelial cells
- Increased sphingosine kinase 1 expression is also seen human liver disease
- Administration of FTY720 further increases the number of HSC within the liver during chronic liver injury which does not appear to be mediated by increased recruitment to the liver
- Repeated injection of purified haematopoietic stem and progenitor cells improves hepatic fibrosis
- Increased numbers of endogenous macrophages and neutrophils are found in the injected liver after HSC injection

- The anti fibrotic effect is not dependent upon myeloid differentiation of injected HSC
- Administration of FTY720 increases the accumulation of injected HSC within the liver, and augments the anti fibrotic effect of injected HSC

7.3 Role of S1P in HSC Mobilisation during Liver Injury

The novel use of a bone marrow chimera demonstrated increased mobilisation of HSC into the circulation and increased HSC within the liver during liver injury. These findings, together with evidence of the alterations that occur in S1P concentrations within the liver and blood during liver injury, support the emerging evidence that S1P is an important regulator of HSC mobilisation and trafficking (Fig 7.1).

It has been shown that administration of the sphingosine lyase inhibitor THI results in higher serum S1P concentration and increased mobilisation of HSC(Schwab *et al.* 2005; Golan *et al.* 2012), and that DOP, which inhibits sphingosine lyase within the bone marrow resulting in higher bone marrow S1P levels, reduces mobilisation (Juarez *et al.* 2012). It is not clear whether S1P levels in the blood correlate with the severity of liver injury or with the numbers of HSC mobilised into the circulation and this would be worthy of further study in both animal models and in patients. It is interesting that a solitary study of patients with HCV cirrhosis demonstrated reduced serum S1P concentrations(Ikeda *et al.* 2010) and more work in humans is required to understand better changes in S1P across the spectrum of fibrosis and cirrhosis given the consistent findings from this work and other studies in animal models.

My work confirms the observations from other studies of S1P induced HSC migration in vitro (Seitz *et al.* 2005), and Rataczajk and colleagues have shown that GCSF induced HSC mobilisation from the bone marrow is mediated by chemotaxis to S1P and is independent of SDF-1 levels (Rataczjak *et al*. 2010). They have shown that GCSF induced activation of the complement system results in the release of lytic enzymes by granulocytes which disrupt the

Figure 7.1 Proposed Effect of Liver Injury and FTY720 on HSC Trafficking

Under normal conditions there is continual low level mobilisation and circulation of HSC which is mediated in part by response to an S1P gradient between bone marrow and blood (Rataczjak et al 2010). During liver injury the gradient increases due to increased S1P levels in the blood which occurs in conjunction with increased hepatic SphK1 expression and increased hepatic S1P concentration. Increased HSC mobilisation from the bone marrow and recruitment into the liver occurs during liver injury. The mechanisms by which injected HSC are recruited to the liver are not fully understood but have been shown to include chemokine receptor CXCR4 and CCR2(Kollet el al 2003, Si et al 2010) and integrin α4β1 (Kavanagh et al 2010) signalling. Administration of FTY720 does not affect recruitment of HSC to the liver but results in an accumulation of bone marrow derived HSC within the injured liver. The effect of FTY720 on HSC is to reduce surface expression of S1P1 receptor making cells unresponsive to the S1P gradient and reducing egress of HSC from the liver.

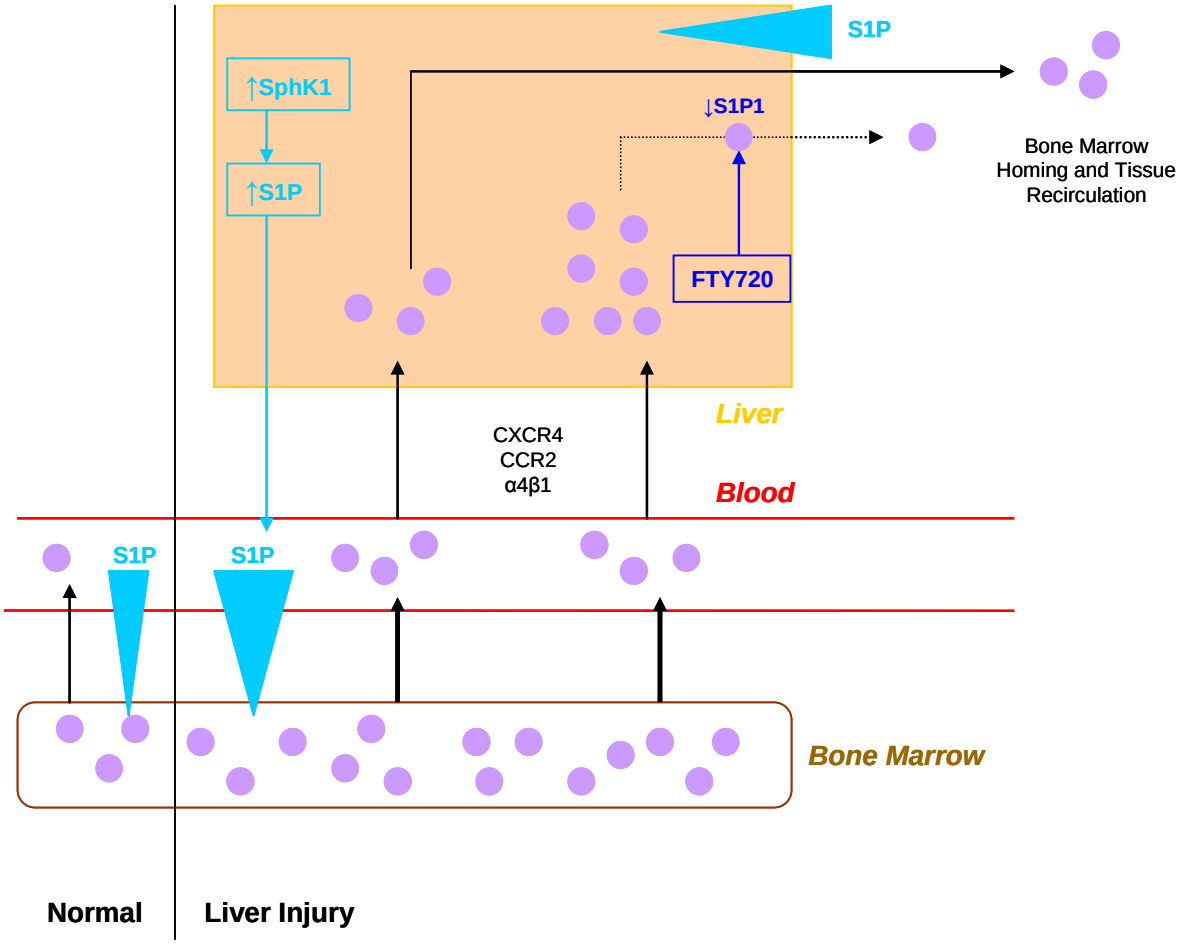

Figure 7.1

Proposed Effect of Liver Injury and FTY720 on HSC Trafficking

SDF-1 – CXCR4 interaction on HSC within the bone marrow, and that levels of S1P are increased within the blood through complement mediated lysis of erythrocytes containing large stores of S1P and subsequently HSC migration occurs across the exaggerated S1P gradient between the bone marrow and circulation(Ratajczak *et al.* 2010). My work has shown an increase in the S1P gradient between bone marrow and blood during liver injury which I propose as a potential mechanism for the increased HSC mobilisation that occurs during liver injury.

Drug induced mobilisation of HSC from the bone marrow in patients with cirrhosis is not as effective as in healthy controls and may occur for a variety of reasons including impaired bone marrow function and splenic sequestration of cells, but this data raises the possibility that impaired responsiveness to the S1P gradient, or reduced serum S1P levels in patients with cirrhosis are potential mechanisms.

In contrast to the role of S1P in bone marrow mobilisation, this data does not show a role for S1P receptor signalling in the recruitment of HSC to the injured liver. Neither FTY720 or the competitive antagonist to S1P1 W146 affected recruitment of HSC (albeit a cell line) in an in vivo assay over 60 minutes. Furthermore, an increase in S1P in both the liver and serum was observed during liver injury which provides no evidence to suggest that there is a reversal of the expected 'high to low' S1P gradient from blood to liver. The mechanisms responsible for HSC recruitment to the injured liver are not completely understood and include SDF-1:CXCR4 and VLA4:α4β1 interactions and chemokine receptors CXCR4 and CCR2 (Kollet *et al.* 2003; Si *et al.* 2010; Mazo *et al.* 2011). My studies do not provide any evidence that S1P receptor

signalling is involved either directly or indirectly, through modulation of chemokine receptor signalling, in the recruitment of HSC to the injured liver.

7.4 Proposed Mechanisms Mediating the Anti Fibrotic Effect of HSC

A significant improvement in hepatic fibrosis following repeated injection of a purified population of KSL cells (including both haematopoietic stem and progenitor cells) is a novel finding and represents the first time this has been tested in a robust and clinically relevant model.

Previous studies of KSL cells have used either cultured cells with no clear phenotype or less robust models of liver fibrosis(Cho *et al.* 2011; Li *et al.* 2013). This work has used a smaller number of cells than previous studies but taken the approach of repeated injection rather than a one off treatment. The numbers of cells used in these studies are likely to be more physiologically relevant, particularly given the data on impaired stem cell mobilisation in patients with cirrhosis, and are higher than the number of KSL cells found in an injection of 10^8 bone marrow cells.

Although a significant reduction in fibrosis was observed, this must be interpreted with the observation that the murine carbon tetrachloride model of liver injury is probably more analogous to an advanced fibrotic stage of liver disease seen in humans rather than overt cirrhosis with marked architectural disruption and regenerative nodule formation.

A further concern with the extrapolation of these results to humans relates to the source of the injected cells, in these studies transgenic donor mice were used to isolate the cells whereas autologous cells would be most appropriate in human studies. A report from Mannheimer et al found that injections of bone marrow cells isolated from rats with liver injury did not exert the same anti fibrotic effect as those from healthy donors(Mannheimer *et al.* 2011).

This work was designed to address the question of therapeutic efficacy rather than to specifically to examine the mechanisms responsible for the anti fibrotic effect. Several findings have, however, provided some insight into the mechanisms involved – the low number of injected cells present within the liver at seven days after injection, the increase in endogenous cell populations and the consistent effect of both myeloid and lymphoid progenitors are suggestive of a regenerative paracrine effect mediated by stem and progenitor cells rather than an effect mediated directly by differentiation of injected cells. The paracrine effect has previously been defined as '*a form of communication between two different cells, where one cell releases chemical mediators to its immediate environment, which results in a change in the behaviour of a cell in its adjacent environment*' (Anthony *et al.* 2013).

The resolution of fibrosis involves a reduction in ECM production, increased MMP activity and a reduction in activated myofibroblasts, through senescence, apoptosis or reversion to inactive stellate cells (Iredale *et al.* 1998; Bataller *et al.* 2005; Kisseleva *et al.* 2012). These processes are interlinked as collagen 1 within ECM promotes myofibroblast survival and increases matrix secretion and TIMP production(Ramachandran *et al.* 2012). A reduction in scar tissue is required to reduce myofibroblast activation as mice with non degradable collagen 1 fail to resolve fibrosis (Issa *et al.* 2003). As expected with resolution of fibrosis these studies demonstrate a marked reduction in both activated myofibroblasts and hepatic collagen deposition.

The vital role of macrophages in both the progression and resolution of hepatic fibrosis is increasingly recognised(Fallowfield *et al.* 2007; Ramachandran *et al.* 2012). This was demonstrated in work from Duffield and colleagues showing that depletion of macrophages during the resolution of fibrosis resulted in impaired breakdown of scar tissue but also that depletion during the development of fibrosis reduced the formation of scar tissue (Duffield *et al.* 2005). Potential mechanisms involved include myofibroblast apoptois induced by macrophage secretion of TWEAK and MMP(Bird *et al.* 2013) , phagocytosis of pro inflammatory signalling molecules from damaged hepatocytes, increased MMP 9 and 13 production (Fallowfield *et al.* 2007) and the recruitment of additional anti fibrotic cells such as neutrophils expressing MMP9 and MMP8 (Mantovani *et al.* 2004; Harty *et al.* 2008).

The mechanisms by which HSC influence the resolution of fibrosis remain unclear but based upon these observations and the currently limited evidence I would propose that further investigation of the effect of cytokines and growth factors secreted by HSC within the environment of the fibrotic liver warrant further investigation. HSC are capable of secreting cytokines and chemokines at concentrations far in excess of other cells of the immune system(Granick *et al.* 2012) and display effector properties within immune regulation through cytokine stimulation of other cells – a further example of a paracrine effect.

The response of HSC to pro inflammatory and pro fibrotic stimuli within the injured liver have not been studied and there are particular molecules that merit further consideration. This work has demonstrated the recruitment of endogenous cells expressing macrophage and neutrophil markers and a study of bone marrow derived macrophages demonstrated

increased hepatic expression of both macrophage (MCP-1, MIP1α) (Fallowfield *et al.* 2007) and neutrophil (MIP2, KC) chemokines after cell injection (Thomas *et al.* 2011).

TWEAK, secreted by macrophages (Bird *et al.* 2013) induces myofibroblast apoptosis and stimulates hepatic progenitor cell activation(Kallis *et al.* 2011). This occurs following bone marrow cell injection even in normal liver and represents a potential mechanism for effect either directly through HSC secretion of TWEAK or indirectly through the recruitment of increased numbers of macrophages.

IL-10, an anti inflammatory cytokine which also promotes ECM breakdown (Cheong *et al.* 2006), was elevated in patients with liver disease who had received bone marrow cell infusion and was associated with a reduction in TGFβ and IL-6(Suh *et al.* 2012). Subsequently the same study demonstrated that bone marrow cells from IL-10 knockout mice had no effect on hepatic fibrosis, despite upregulation of MMP9 and MMP 13, whereas wild type bone marrow improved fibrosis (Suh *et al.* 2012). Treatment with bone marrow derived stem cells with adenoviral induced expression of IL-10 in rats with liver injury suppressed inflammation, reduced myofibroblast activation and resulted in an improvement in liver function(Lan *et al.* 2008).

The mechanisms by which HSC induce resolution of fibrosis, are therefore, likely to be multifactorial and involve complex interactions between signalling molecules rather than an individual signal. Future studies should examine the repertoire of signalling molecules, such as chemokines and cytokines, secreted by HSC in response to liver injury and studies using cells from appropriate knock out mice may help clarify the role of individual molecules.

7.5 Role of S1P in Liver Injury

The increased S1P concentration and upregulation of hepatic sphingosine kinase observed in this work has previously been described in various models of murine liver injury. I confirmed that similar changes in enzyme expression can be seen in human liver injury, however further evaluation in serum S1P levels should be considered as it has been reported that serum S1P levels in patients with hepatitis C cirrhosis were lower than healthy controls (Ikeda *et al.* 2010).

The significance of the alterations in S1P related to cellular trafficking and stem cell mobilisation have been discussed, although the significance of S1P in the development of fibrosis requires further study.

A possible role for S1P in the development of fibrosis has been suggested by Li and colleagues following observations that the S1P3 receptor mediates both myofibroblast and mesenchymal stem cell migration and treatment with a selective S1P3 antagonist reduced myofibroblast activation and hepatic fibrosis(Li *et al.* 2009; Li *et al.* 2009). A direct effect of S1P in stimulating hepatic stellate cell motility and proliferation has been shown and myofibroblast activation was reduced and hepatocyte proliferation increased in S1P2 receptor knock out rats with liver injury, whilst no S1P induced proliferation was seen in S1P2 knockout myofibroblasts in vitro.(Ikeda *et al.* 2000; Ikeda *et al.* 2009). The importance of S1P2 receptor signalling in the development of hepatic fibrosis was also proposed by studies showing reduced αSMA gene expression together with a reduction in TIMP-1 and TGFβ in S1P2 knock out mice with liver injury. (Serriere-Lanneau *et al.* 2007). In a rat study S1P2 was shown to mediate an increase in portal pressure through contractility of

myofibroblasts(Ikeda *et al.* 2004). Furthermore the existence of a feedback loop whereby S1P2 signalling by exogenous S1P upregulates sphingosine kinase 1 expression and results in increased exogenous S1P, was proposed by the observation that sphingosine kinase 1 was downregulated in S1P2 knockout mice (Serriere-Lanneau *et al.* 2007).

The observations from this work show that FTY720 treatment alone did not affect hepatic fibrosis. However, FTY720 has been shown to be beneficial in injury to other organs, and may be explained by factors such as those relating to choice of dose and timing of administration in relation to onset of injury. However, the previously described importance of S1P2 and S1P3 signalling in the development of hepatic fibrosis may also explain these findings. FTY720 is an S1P receptor functional antagonist with no activity on the S1P2 receptor and with markedly less activity on the S1P3 receptor than the S1P1 receptor (Brinkmann *et al.* 2002), and it is possible that an increasing the dosage of FTY720 would activate S1P2 and S1P3 as well as S1P1.

Endothelial cells are a significant source of extracellular S1P, particularly within the vascular and lymphatic circulation (Venkataraman *et al.* 2008). I have demonstrated for the first time a significant expression of sphingosine kinase 1 in liver sinusoidal endothelial cells(LSEC) and in particular the preferential upregulation of sphingosine kinase 1 in LSEC during liver injury compared with hepatocytes and mononuclear cells. Sinusoidal endothelial cells are activated by cytokines produced during liver injury such as TNFα, inducing changes in adhesion molecule expression and modulating cell recruitment (Lalor *et al.* 2002). Increased S1P production through stimulation of sphingosine kinase activity is also mediated through TNFa(Xia *et al.* 1998) and other stimulants of sphingosine kinase include PDGF, NGF, EGF,

FGF, VEGF, IL1b and LPS – all of which may be upregulated within the liver during liver injury(Takabe *et al.* 2008). Further examination of the specific role of these cytokines in the upregulation of Sphingosine kinase in sinusoidal endothelium may help better understand the role of S1P in the pathogenesis of hepatic fibrosis.

Cells other than those of the liver that may contribute to the increase in S1P seen in both serum and liver during liver injury include platelets and peripheral blood mononuclear cells(Iannacone *et al.* 2005; Oo *et al.* 2010). Platelets in particular possess large intracellular stores of S1P which can be released upon activation(Yatomi *et al.* 1997; Yang *et al.* 1999), as has been demonstrated in models of cardiac injury. Platelets are recruited to the liver, particularly during the development of immune mediated liver damage(Iannacone *et al.* 2005), and this may represent a source of increased S1P during liver injury. Peripheral blood mononuclear cells express high levels of sphingosine kinase, although this data suggests a lack of upregulation during liver injury. This is supported the observation that peripheral blood mononuclear cells do not secrete S1P in response to stimulation in vitro (Yatomi *et al.* 1997), however increased numbers of mononuclear cells are found within the liver during injury and may contribute to increased S1P. Further investigation could involve generation of bone marrow chimerism using bone marrow from sphingosine kinase knock out mice to study the contribution of sphingosine kinase and S1P derived from haematopoietic cells to liver and serum S1P during liver injury. Ultimately this may also help determine whether S1P derived from extra hepatic cells, particularly in the case of platelets, contributes to the progression of fibrosis.

7.6 The Effect of FTY720 on HSC Trafficking and Enhancing the Anti Fibrotic Effect

Observations from these studies suggested a role for S1P in the mobilisation of HSC from the bone marrow during liver injury, and whilst administration of FTY720 resulted in accumulation of HSC within the injured liver this was not mediated by altered HSC recruitment. These findings, together with those of Massberg, lead me to study the effect of FTY720 on the fate of injected HSC.

The findings from the therapeutic studies of repeated HSC injection demonstrating an anti fibrotic effect suggested that injection of cells into a peripheral vein did not prevent efficacy. Previous studies had suggested that injected cells may become trapped in the pulmonary vascular bed, thus hindering their homing to injured organs (Kavanagh *et al.* 2010). In this comprehensive study I have shown that accumulation of injected cells does occur initially in the lungs, cells quickly dissipated and ongoing recruitment was seen in the liver and spleen at subsequent timepoints. This pattern of tissue distribution is similar to that seen with injections of other cell populations, such as dendritic cells (personal communication, Dr Stuart Curbishley).

Increased accumulation of endogenous HSC during liver injury following FTY720 treatment within the liver was demonstrated in this study and confirmed the findings in normal mice(Massberg *et al.* 2007). For the first time I show that a similar phenomenon is observed with injected HSC, the findings at multiple timepoints using the HPC-7 cell line were confirmed with injection of KSL cells at a single timepoint. This provides encouraging evidence for the concept of optimising the beneficial effects of this rare population of cells (Fig 7.2).

Figure 7.2 Proposed Mechanisms Mediating and Augmenting the Anti Fibrotic Effect of HSC

(A) The observed effects of injected HSC include a reduction in hepatic collagen, reduced myofibroblast activation and recruitment of F4/80+ and Ly6G+ cells. Egress of HSC from the liver is mediated by migration across an S1P gradient between liver and draining lymphatics, followed by bone marrow homing and tissue recirculation (Massberg et al 2007).

(B) FTY720 administration does not alter recruitment of injected HSC to the liver, however increased numbers of injected HSC accumulate within the liver as a result of reduced surface expression of S1P1 receptor rendering HSC less responsive to the S1P gradient meditating egress from the liver. The increased numbers of injected HSC within the liver are associated with further reductions in hepatic collagen and myofibroblast activation.

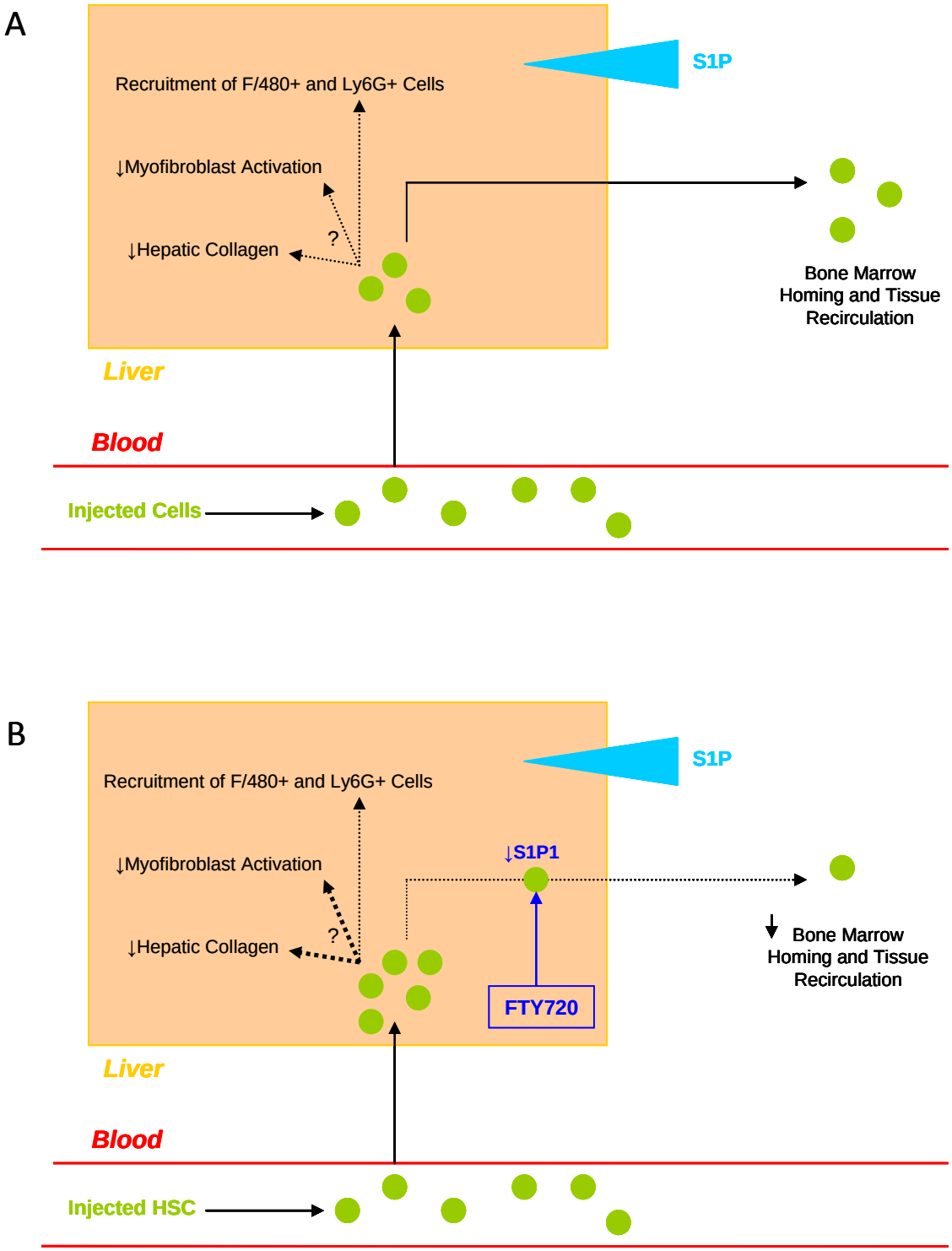

Figure 7.2

Proposed Mechanisms Mediating and Augmenting the Anti Fibrotic Effect of HSC

When designing these studies, the dose of FTY720 administered was the same as that used in previous studies which involved normal mice rather than those with liver injury and the frequency of administration was chosen predominantly for practical purposes to allow consistency throughout treatments. Given that this approach to FTY720 dosing resulted in no change in numbers of HSC within the peripheral blood during liver injury rather than the marked reduction seen in normal mice (Massberg *et al.* 2007; Juarez *et al.* 2012) it is possible that this did not represent an optimum strategy. Further investigation of the effects of alternative FTY720 dosing strategies will determine whether this effect could be further enhanced.

The ultimate aim of increasing the numbers of injected KSL cells within the liver was to improve upon the therapeutic effect. In general these studies provide 'proof of concept' for this approach. Although not all markers of fibrosis significantly improved, this may be in part due to insufficient sample sizes to allow confirmation of this effect. As previously discussed the dosing regime for FTY720 could be examined in more detail. I propose that the observations from these studies demonstrate that administration of FTY720 results in increased hepatic accumulation of injected KSL cells resulting in an enhanced anti fibrotic effect, particularly as administration of FTY720 alone had no effect on hepatic fibrosis. These findings suggest that there may be a relationship between the number of cells injected (and thus the number of cell engrafting the liver) and the therapeutic effect observed. It remains unclear whether there is an optimum 'dose' of cells, above which no further effect is seen. Two studies using $1x10^6$ KSL cells demonstrated a reduction in picosirius red staining (Cho *et al.* 2011, Li *et al*. 2013)whilst I demonstrated a reduction in this study using $5x10^4$ KSL cells on 3 occasions. Although the experimental design of all three studies varied and it is not

clear whether there is a continual relationship between number of cells injected and anti fibrotic effect observed, or whether a 'plateau effect' exists whereby any further increases in cell numbers does not increase the anti fibrotic effect. Further studies utilising different experimental protocols are required to determine the optimum strategy for cell administration and the role of FTY720 in maximising this effect.

7.7 Proposed Future Work

The work presented in this study has demonstrated the anti fibrotic effect of repeated injections of purified HSC, together with the observations that modulation of S1P receptor signalling with FTY720 increases the number of injected cells within the injured liver and augments the anti fibrotic effect. These studies were designed to help inform and optimise the use of HSC as a therapy for liver disease, however I would propose that there are three key areas worthy of further study to understand better the therapeutic role of HSC in liver repair: i)an improved understanding of the molecular and cellular mechanisms responsible for the observed anti fibrotic effect, ii) determination of the optimum regime for cell therapy (numbers of cells to infuse and frequency of administration) and iii) further validation of the role of S1P receptor signalling in HSC trafficking during liver injury.

This work was designed to demonstrate the anti fibrotic effect of HSC, and although not specifically designed to determine the mechanisms responsible has suggested that paracrine mechanisms are involved. A greater understanding of the mechanism of action involved in liver repair after HSC injection will help with developing their use as a clinical therapy. I would propose further in vitro experiments to investigate the profile of cytokines, chemokines and growth factors synthesised and secreted by HSC (for example, IL-10 has been described as a potential anti fibrotic cytokine (Huang *et al.* 2006)).

I have performed some initial experiments using qPCR to examine the gene expression profile of a panel of cytokines, chemokines and growth factors expressed by HSC, however this preliminary data requires replication and further validation. This approach could be followed by the quantitative analysis of such molecules secreted by HSC into conditioned

media, using a multiplex array approach. Subsequent experiments would examine alterations in the expression profile following stimulation of HSC by cytokines, such as TGFβ, present during liver injury, with the aim of identifying potential targets for further study. Although the mechanisms underlying the anti fibrotic effect are likely to be multifactorial the role of individual molecules could be studied by repeating this experimental protocol using either HSC isolated from knockout mice deficient in the molecule of interest or by in vitro genetic manipulation of wild type HSC (eg viral transduction of shRNA coding for the gene of interest).

Further in vitro investigation of the effects of HSC on myofibroblast activation using co-culture models would be of value. In particular, determining whether the effect of HSC on myofibroblasts alters following stimulation of HSC. The effect of HSC conditioned media alone, rather than direct cell contact, induces similar effects on myofibroblast activation could also be studied.

This study demonstrated the beneficial anti fibrotic effect of three injections of 50,000 HSC, this experimental approach was selected for reasons of practicality and clinical applicability. There does remain uncertainty as to the optimum approach for cell administration, particularly the optimum number of cells to inject and the frequency and timing of injections to maximise the therapeutic effect. I would propose similar experimental protocols as used in this work to examine the effect of varying concentrations of cells in injections on hepatic fibrosis and of alternative timings and frequency of cell delivery. As I have previously described HSC are a rare population of cells, so significant benefits may be gained from seeking to maximise their therapeutic effect and avoiding superfluous use of cells.

It is also worth noting that in this study the injected HSC were harvested from mice without liver injury, however clinical applications of stem cell therapy rely on use of autologous cells harvested from patients with liver disease. A recent animal study has suggested that injecting bone marrow cells harvested from animals with liver injury does not improve hepatic fibrosis(Mannheimer *et al.* 2011). Future studies could address this in more detail by isolating HSC from mice with chronic carbon tetrachloride liver injury and determining whether the anti fibrotic effect is reduced or lost altogether.

The findings from this work suggest that administration of FTY720 results in increased accumulation of injected cells within the injured liver through modulation of S1P signalling at the S1P1 receptor. I would propose that further study of this is required to validate this proposed mechanism.

Systemic injection of FTY720 may induce confounding effects, such as alterations in lymphocyte migration, that modulate the trafficking of HSC through alternative mechanisms. As the effect of FTY720 on HSC is to render the cells unresponsive to the effects of S1P, I questioned whether a similar effect would be observed in HSC that did not express S1P1 receptors. The S1P1 knockout mouse is embryonically lethal at day 14.5, and therefore I attempted to use a lentivirally delivered shRNA vector to knockdown S1P1 on HSC. Unfortunately I was unable to succeed with this approach although further optimisation may be possible. An alternative approach would be the use of HSC isolated from fetal liver of S1P1 knockout mice to study this in greater detail and clarify the role of S1P1 in the trafficking of injected HSC.

7.8 Implications for Clinical Studies

There have been many reports of clinical studies describing the use of bone marrow stem cells and haematopoietic stem cells in patients with chronic liver disease (Fig 1.5). These have predominantly involved small numbers of patients and made no comparisons with untreated controls. The general message from these studies is that stem therapy appears to be a safe and feasible therapeutic option, however efficacy has yet to be proven. There is a clear need for larger, randomised, controlled studies of stem cell therapy for liver disease.

The work performed in this study was designed to have some relevance to clinical applications, demonstrating the anti fibrotic effect of small numbers of purified HSC injected into a peripheral vein. This approach is clinically relevant and is currently being studied in a large clinical trial which I helped design, set up and manage.

The REALSTIC (Repeated Autologous Infusions of Stem cells In Cirrhosis) trial is the first phase II, randomised, controlled trial of autologous CD133 stem cells in patients with liver disease. In this trial CD133+ haematopoietic stem cells are isolated from the peripheral blood of patients with cirrhosis by leukopheresis following administration of the mobilising cytokine Granulocyte Colony Stimulating Factor (G-CSF). Using Good Manufacturing Practice (MHRA, UK) compliant immunomagnetic separation techniques, a population of CD133+ bone marrow cells which are highly enriched for haematopoietic stem cells is isolated. These cells are then injected back to the patient through a peripheral vein on 3 occasions at monthly intervals. Patients enrolled in the trial are randomised to one of three treatment groups – a control group receiving standard care, a group receiving G-CSF alone and a group

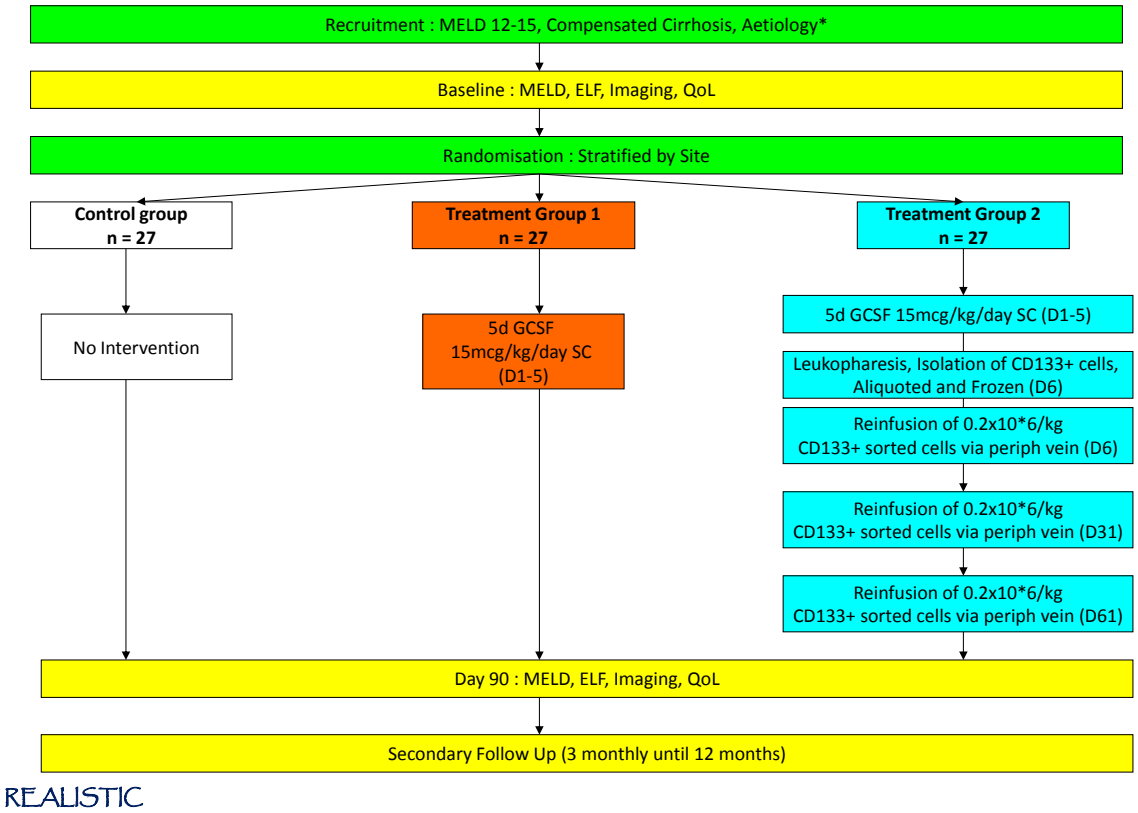

Figure 7.3 Overview of the REALISTIC Trial

Schematic representation of the REALISTIC trial design. Inclusion criteria for the trial include MELD score 12-15 and the presence of compensated cirrhosis, certain liver diseases such as autoimmune hepatitis and primary sclerosing cholangitis are excluded. Recruited patients are randomised into one of three trial groups – a control group receiveing standard care alone, treatment group 1 receiving G-CSF treatment alone and treatment group 2 receving G-CSF followed by leukopheresis, CD133 cell isolation and repeated cell reinfusion. The primary outcome measure of change in MELD score is determined at day 90, secondary outcome measures include non invasive markers of liver fibrosis (ELF, FIbroscan) and quality of life scores (CLDQ).(MELD Model for End stage Liver Disease, ELF Enhanced Liver Fibrosis test, QoL Quality of Life, GCSF Granulocyte Colony Stimulating Factor, SC Subcutaneous).

receiving G-CSF and cell injections(Fig 7.1). The inclusion of a group receiving G-CSF alone is to control for the effects of stem cell mobilisation alone being the beneficial mechanism.

The aim of this trial is to demonstrate the beneficial effects of CD133 stem cell therapy in liver disease compared with standard treatment which will be determined by the primary outcome measure at day 90 after enrolment. The choice of primary outcome measure is vital as it is this which will determine the relative success or failure of a study. Many of the small studies to date have reported biochemical data, such as improvements in serum albumin levels, and whilst these are encouraging give little indication of the effect of therapy on prognosis or survival. Histological markers again provide useful quantitative data when analysing efficacy but are not validated as markers of liver related complications or survival. Transplant free survival is clearly the most definitive outcome measure, but patients with compensated cirrhosis have 1 and 2 year survival rates of 95 and 90%(D'Amico *et al.* 2006) and may require studies with large numbers of participants over long time periods to detect significant benefit. Prognostic scoring systems, such as the Model for End stage Liver Disease (MELD) score, have been long established in hepatology for predicting survival(Malinchoc *et al.* 2000; Ripoll *et al.* 2007). The MELD score is calculated from laboratory measurements of serum bilirubin, serum creatinine and international normalised ratio (INR) (http://optn.transplant.hrsa.gov/resources/MeldPeldCalculator). The MELD score represents a standardised, easily obtainable outcome measure that has been validated to predict survival. Of particular relevance to clinical trials is emerging evidence that the change in MELD score (deltaMELD) between timepoints is of greater prognostic significance than the absolute MELD score(Bambha *et al.* 2004; Huo *et al.* 2005). The primary outcome measure used in the REALISTIC trial is the change in MELD score between day 0 and day 90,

the trial is powered to detect a difference in change in MELD score between groups of 1.0 thus requiring sample sizes of 27 participants per treatment group. Secondary outcome measures used include non invasive markers of hepatic fibrosis (transient elastography (Fibroscan) and serum markers (ELF test)), a further prognostic scoring system for liver disease (United Kingdom model for End stage Liver Disease, UKELD) and quality of life assessment (CLDQ questionnaire).

The work in this thesis has demonstrated the dynamics of tissue distribution of injected cells following peripheral vein injection. My work suggests that peripheral vein injection is a valid approach to cell delivery and that the need to inject cells via the hepatic artery or portal vein, which is technically difficult and carries significant risk, is not required. There have been no studies in patients examining in detail the fate of injected cells and further clinical studies addressing this issue are required. Techniques that could be utilised include radionucleotide labelling of cells with whole body nuclear imaging, or newer techniques such as magnetic resonance imaging (MRI), in conjunction with super paramagnetic iron oxide (SPIO) labelling of cells, which may provide the accuracy and definition to trace relatively small numbers of in injected cells.

7.9 Final Conclusions

This thesis contains the novel findings that repeated injections of purified c-kit+ sca1+ lineage- haematopoietic stem and progenitor cells, delivered via a peripheral vein exert a significant anti fibrotic effect in a murine model of liver injury. This provides encouraging evidence for their use in human studies. Although not designed to investigate the mechanisms mediating this effect, these data would suggest that a paracrine mechanism stimulating endogenous repair is involved and propose further study in this area.

Furthermore I also propose a mechanism by which the therapeutic efficacy of these cells can be enhanced. Sphingosine 1-phosphate regulates both the egress of HSC from tissues into the draining lymphatics and also stimulates mobilisation of HSC from the bone marrow. As demonstrated here, alterations seen in liver injury may be involved in liver injury induced HSC mobilisation. FTY720 resulted in an increase in the number of injected HSC within the liver and improved the therapeutic effect. I propose that this mechanism may warrant further investigation as a means of optimising cell therapy.

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