# THE EFFECTS OF MESENCHYMAL STEM CELLS ON A MODEL OF AUTOIMMUNE LIVER DISEASE


**By**

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# ABSTRACT

End stage liver disease is the only one of the top five causes of death in the United Kingdom still increasing in prevalence year on year. Whilst disease specific treatments have shown promise in delaying progression to end stage liver disease, the only curative treatment once end stage liver disease has developed is liver transplantation. There are many causes of end stage liver disease but autoimmune disease remains a prevalent but poorly understood with limited treatment options to prevent progression to cirrhosis. Mesenchymal stem cells (MSC) are a novel therapeutic strategy which is gaining an evidence base in many conditions involving the immune system. This study describes a technique for the prospective isolation of MSC using PDGFRα and Sca-1 (PαS MSC) and following culture expansion these cells are administered in a pilot *in vitro* study using MSC in the OVA-Bil model of autoimmune liver disease, with the hypothesis that MSC will reduce liver inflammation in the OVA-Bil mouse model.

Male 8-12 week old OVA-Bil mice were injected IP with $1x10^7$ OT1 cells and $4x10^6$ OT2 cells. Control mice (n=3) were given phosphate buffered saline (PBS) whilst treated mice (n=3) were infused with $5x10^5$ PαS MSC via the tail vein on days 3 and 6. At day 10 mice were and serum alanine transaminase (ALT) and alkaline phosphatase (ALP) analysed. Liver tissue was fixed and paraffin sections were stained for Haematoxylin and Eosin (H&E).

Whilst no significant difference was shown in serum markers of liver injury between control and MSC treated groups, there was a trend towards a significant reduction in the treated mice in both ALT and ALP. Qualitative analysis of H&E sections demonstrated no difference in

lymphocytic infiltration in the portal regions of the liver tissue between the control and MSC treated mice.

This work has demonstrated the feasibility of an *in vivo* animal model of autoimmune hepatitis and will guide further study into efficacy and mechanism of action of PαS MSC in models of autoimmune liver disease.

# DEDICATION

This thesis is dedicated to my wife Molly, and daughter Florence, for their unwavering support in my academic pursuits, and to my parents, for supporting me through my studies and career.

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# LIST OF ABBREVIATIONS

(In alphabetical order)

| | |
|---|---|
| α-MEM | Alpha Modified Minimum Essential Medium (Eagle) |
| aCGH | Array Comparative Genomic Hybridization |
| AIH | Autoimmune Hepatitis |
| ALP | Alkaline Phosphatase |
| ALT | Alanine Transaminase |
| ANA | Anti-Nuclear Antibody |
| Anti-LC 1 | Anti-Liver Cytosol Type 1 |
| Anti-LKM 1 | Anti-Liver Kidney Microsomal Type 1 |
| Anti-SMA | Anti-Smooth Muscle Antibody |
| ASBT | Apical Sodium dependent Bile acid Transporters |
| AST | Aspartate Transaminase |
| CFU-F | Colony forming unit fibroblast |
| DBD | Donation after Brainstem Death |
| DCD | Donation after Cardiac Death |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DNA | Deoxyribonucleic Acid |
| dNTP | Deoxyribonucleotide Triphosphate |
| EDTA | Ethylenediaminetetraacetic acid |
| ESL | End stage Liver Disease |
| FBS/FCS | Foetal Bovine/Calf Serum |
| GFP | Green Fluorescent Protein |
| HBSS | Hank's Balanced Salt Solution |
| HGF | Hepatocyte growth factor |

| | |
|---|---|
| HLA | Human leukocyte antigen |
| HO1 | Haem Oxygenase 1 |
| IAIHG | International Autoimmune Hepatitis Group |
| IDO | Indoleamine 2,3-dioxygenase |
| IFNγ | Interferon gamma |
| IGF | Insulin like growth factor |
| IL | Interleukin |
| IP | Intra-peritoneal |
| IV | Intravenous |
| MHC | Major histocompatibility complex |
| MSC | Mesenchymal Stem/Stromal Cell |
| NASH | Non alcoholic steato hepatitis |
| PBC | Primary Biliary Cirrhosis |
| PC | Personal Computer |
| PCR | Polymerase Chain Reaction |
| PDC | Pyruvate dehydrogenase complex |
| PGE2 | Prostaglandin E2 |
| $PGE_2$ | Prostaglandin $E_2$ |
| PSC | Primary Sclerosing Cholangitis |
| PSG | Penicillin-Streptomycin-Glutamine |
| RNA | Ribonucleic Acid |
| SC | Subcutaneous |
| TBE | Tris/Borate/EDTA |
| $T_m$ | Melting Point |
| TNFα | Tumour necrosis factor alpha |
| Tris | 2-Amino-2-(hydroxymethyl)-1,3-propanediol |

| | |
|---|---|
| UC | Ulcerative Colitis |
| UDCA | Ursodeoxycholic acid |
| UDCA | Ursodeoxycholic acid |
| UK | United Kingdom |
| USA | United States of America |
| UV | Ultra Violet |

# 1. INTRODUCTION

## 1.1. End Stage Liver Disease

End stage Liver disease is currently responsible for 2% of deaths in the United Kingdom and is the only important cause of death still continuing to increase, with a rise in incidence of 20% in 2012[1]. The only proven curative treatment for end stage liver disease is liver transplantation, but with increasing donor waiting lists and the increasing demand due to rising prevalence of liver disease other therapeutic strategies are in dire need. In the UK in 2012-2013 there were 817 (53% of the total waiting list) patients receiving a liver transplant, 497 (32%) patients still waiting and 84 (5%) died whilst waiting for a liver transplant. The median number of days from listing to transplantation was 147 over the period 2008-2011[2]. The UK Chief Medical Officer recently highlighted end stage liver disease as a preventable cause of death which is continuing to increase in prevalence[3], with a clear need for new treatment options due to the limited number of organs available for transplantation.

There are a number of causes of liver cirrhosis and end-stage liver disease (Table 1). Whilst alcohol related liver disease and chronic hepatitis C infection are among the most common autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC) are important causes of liver disease[4].

| Causes of Liver Cirrhosis | |
|---|---|
| Alcohol related liver disease | Primary Biliary Cirrhosis |
| Viral Hepatitis B | Wilson's Disease |
| Viral Hepatitis C | 1-antitrypsin deficiency |
| Fatty Liver/NASH | Budd-Chiari Syndrome |
| Haemochromatosis | Cystic Fibrosis |
| Primary Sclerosing Cholangitis | Autoimmune Hepatitis |

**Table 1 A list of the most common causes of Liver Cirrhosis**

Liver disease is highly prevalent in young adults, and a higher proportion of younger people are affected by liver disease than the other major causes of death, leading to a large number of life years lost due to the terminal nature of many of the causes. Clearly novel therapeutic strategies are in great demand to reduce the global impact of this group of diseases.

## 1.2. Autoimmune Mediated Liver Disease

The concept of the immune system attacking the body is well established. Waldenström first described a series of young women who suffered from a form of fluctuating hepatitis over 50 years ago[5]. This was followed by an important early text that was published by Macaky and Burnet describing the process of immune tolerance and the consequences of a failure of the tolerance process[6]. This early but comprehensive description of autoimmune disease discussed approximately 30 known diseases including "Active chronic lupoid hepatitis", now known to be autoimmune hepatitis. In the 1980s AIH was recognised as a distinct entity named "Chronic active autoimmune hepatitis"[7], the term AIH being coined in the 1990s after international meetings in Brighton, UK and Los Angeles, USA[8].

Whilst the immune system can attack the liver in a number of ways leading to a variety of conditions such as Primary Biliary Cirrhosis (PBC) and Primary Sclerosing Cholangitis (PSC), the distinction between these conditions is not as well defined as once thought and there is almost certainly an overlapping spectrum of autoimmune diseases of the liver[9,10]. Attempts have been made to standardise the definition of these overlapping pathologies by the International Autoimmune Hepatitis Group (AIHG)[11].

There are phenotypic differences between the main causes of autoimmune liver disease (Table 2), but these clinical markers are not absolute and may exist to a greater or lesser degree further blurring the line between one distinct condition and the others.

| | AIH | PBC | PSC |
|---|---|---|---|
| **Age** | All age groups | >45 years | Usually >40 |
| **Gender** | Female:Male Type 1 4:1, Type 2 9:1 | Female:Male 9:1 | Female:Male 3:7 |
| **ANA** | 1:40 titre in 70-80% | Disease specific ANA in 30-50% | Non-specific ANA in 70-80% |
| **Anti-LKM 1** | 3-4% (categorises as type 2 AIH) | Not present | Note present |
| **pANCA** | 90% | Not present | 26-94% |
| **Interface hepatitis** | Characteristic | Occasionally present | Occasionally present |
| **Response to immunosuppression** | Yes | No | No |

**Table 2 A summary of the phenotypic markers of autoimmune liver disease**

The mechanisms by which liver injury is perpetrated in the 3 main autoimmune liver diseases (AIH, PBC and PSC) are not fully understood but appear to have similar aetiologies. Liver damage due to antibody mediated immune activation[12,13] and cellular immune activation[14,15] have both been implicated. Genetic predisposition coupled with environmental factors have also been shown to be contributory in all 3 conditions[16,17,18,19] leading to a persistent loss of immune tolerance and sustained ongoing liver injury by the immune system. The pattern of liver injury in AIH is mainly involving the portal and peri-portal areas, whereas in contrast to this, the damage seen in PBC and PSC is mainly found in the biliary epithelial cells. Patients with PBC and PSC are susceptible to hepatocyte injury and hepatic inflammation, but this is likely secondary to the biliary damage sustained, although the mechanisms underlying this have not been fully elucidated[20].

## 1.3. Autoimmune Hepatitis

### 1.3.1. Background

Autoimmune hepatitis (AIH) is one of the three main types of autoimmune disease which is specific to the liver and forms part of the overlap spectrum with PBC and PSC. AIH is characterised by a progressive inflammatory condition within the liver and on histological examination interface hepatitis is usually found[21]. There is no clear genetic or environmental trigger solely responsible for the development of AIH but there are a number of genes that have been implicated with the strongest associations being in genes encoding the Human Leukocyte Antigen (HLA)[22]. There are two distinct types of AIH, type 1, which is associated with HLA-DR3 and HLA-DR4[23], and type 2, which is associated with HLA-DR3 and HLA-DR7[24]. There is an increased incidence in females compared with males with a female:male ratio of 4:1 in type 1 AIH and 9:1 in type 2 AIH. A key feature of AIH is that it responds

favourably to immunosuppression with prednisolone and azathioprine compared with PBC and PSC which generally do not[25]. Whilst treatment is often effective overall, 20% of patients do not respond to conventional therapy[26].

### 1.3.2. Autoimmune Hepatitis Pathophysiology

AIH can be sub-classified into two types based on the types of auto-antibodies present in the serum; type 1 is defined as positive for Anti-Nuclear Antibody (ANA) and/or Anti-Smooth Muscle Antibody Type 1 (Anti-SMA 1), and type 2, defined as positive for Anti-Liver Kidney Microsomal Antibody Type 1 (Anti-LKM 1) or Anti Liver Cytosol Antibody Type 1 (Anti-LC 1)[27].

Regulatory T-Cells almost certainly play a role in the pathophysiology of AIH and in particular the sub-group expressing the markers CD4, CD25 and FOXP3[28] ($CD4^+CD25^+FOXP3^+$), a type of regulatory T-Cell that has been shown to confer peripheral immune tolerance and have a role in the prevention of autoimmune disease[29]. These regulatory cells can suppress auto antibody production through the release of interleukin-10 (IL-10) and TGF-β. It has been shown that at the time of diagnosis both the number and the regulatory function of CD25+CD4+ cells is reduced, but increases following the induction of remission[30].

### 1.3.3. Therapeutic Strategies

It has long been recognised that suppression of the immune system leads to a more favourable outcome in patients with AIH[31]. Current guidelines split treatment of AIH into two distinct elements, induction of remission and maintenance of remission. Induction is achieved with

the use of high dose corticosteroids such as prednisolone in combination with the anti-proliferative immunosuppressant azathioprine, a drug which mainly targets B and T lymphocytes. Once remission is achieved maintenance schedules of either azathioprine alone or in combination with low dose prednisolone are used[32], although as with many diseases the aim is to minimise long term exposure to exogenous corticosteroids due to the significant side effect profile. Other treatment modalities are currently under investigation such as the inosine monophosphate dehydrogenase inhibitor mycophenolate mofetil[33], but whilst there are some modest benefits of other treatment strategies no one optimal medication regime has been found to be significantly better than the current treatment recommendations. There are limited numbers of patients with overlap syndromes therefore therapeutic strategies are difficult to research clinically but patients with features of AIH should be considered for immunosuppressive therapy[11]. Liver Transplantation is required in up to 20% of patients, either due to a hyper acute presentation not responding to steroids, or due to decompensated end stage liver disease[34].

## 1.4. Primary Biliary Cirrhosis

### 1.4.1. Background

Primary Biliary Cirrhosis (PBC) is one of the 3 main autoimmune diseases affecting the liver. The aetiology is unclear, however due to its association with other autoimmune disorders it is likely that it is also an autoimmune condition and hence why it is considered here. There are no clear genetic factors that have been discovered although up to 6% of patients with PBC have an effected family member[35].

Patients with PBC are often asymptomatic at diagnosis and the finding of a raised serum Alkaline Phosphatase (ALP), a hydrolase enzyme used as a marker for cholestasis, may be the only indicator[36].

### 1.4.2. Primary Biliary Cirrhosis Pathophysiology

PBC is characterised by destruction of bile ducts, and involves inflammation and disruption of the basement membrane of the bile duct cells[37]. The ducts become infiltrated with lymphocytes, macrophages and plasma cells which can lead to granuloma formation and cell loss occurs via necrosis rather than apoptosis[38]. Whilst the specific mechanisms responsible for damage to the liver have not been fully elucidated one of the most prevalent auto-antibodies that has been found is directed against the 2-oxo-acid dehydrogenase group of enzymes found in the mitochondria[39]. The main immune response effects the pyruvate dehydrogenase complex on the E2 binding site (PDC-E2). The continued activation of B-Cells reactive against PDC-E2 and their subsequent activation by T-Lymphocytes has been demonstrated using structurally similar compounds such as lipoic acid, and proposed as a mechanism of autoimmune injury in patients with PBC[40], however it has recently been demonstrated that patients with PBC possess $CD4^+$ T-Cells directed against PDC-E2 whereas healthy controls do not[41]. $CD8^+$ T-Cells reactive against PDC-E2 have also been shown to be found in the livers of patients with PBC[42]. It has also been shown that patients with PBC have functionally and phenotypically altered $CD8^+$ T regulatory cells[43]. Whilst many types of immune damage have been proposed it is still not clear the exact mechanism by which PBC develops or exerts its effects.

### 1.4.3. Therapeutic Strategies

Treatment of PBC is with ursodeoxycholic acid (UDCA), and in contrast to AIH there is no role for corticosteroids as PBC shows no response, although a small group of overlapping syndromes may see some benefit from their use. Survival is variable with treatment, as is the duration of time between diagnosis and development of liver failure. Approximately 25% of patients will develop liver failure within 10 years of diagnosis[44]. Liver transplantation is the only effective treatment for patients with late stage disease, but following liver transplant up to 30% of patients will have a recurrence of the disease[45], however this is usually at a histological level rather than being clinically significant.

## 1.5. Primary Sclerosing Cholangitis

Primary Sclerosing Cholangitis (PSC) is a chronic autoimmune disease predominantly affecting the liver. Characteristic histological changes include inflammation of the biliary tree effecting both the intra-hepatic and extra-hepatic regions. There is a male:female ratio of 2:1 in contrast to the other autoimmune disorders discussed which have a female preponderance[46]. There is also a strong link with Ulcerative Colitis (UC) with 70% of patients with PSC also suffering from UC[47]. Currently there is little in the way of treatment options for PSC other than liver transplantation with all the risks associated with it. Whilst there is considerable overlap and similarities between AIH, PBC and PSC the differences are sufficient that the animal models used in this study do not reflect the pathophysiology of PSC sufficiently well and so it will not be considered further.

## 1.6. The OVA-Bil model of Autoimmune Liver Disease

There are a wealth of animal models that represent different liver diseases. Models of PBC can be separated into spontaneous and induced. Spontaneous models would be impractical for this study due to the limited window but long expansion time required for PαS MSC. The OVA-Bil model was chosen due to familiarity, local availability of the model and following review of the literature it was decided that this model closely represented the disease processes being studied.

The Ova-Bil mouse model of autoimmune liver injury was developed by Buxbaum[48] in 2006 in order to allow researchers to explore the early immune responses following the recognition of antigen on biliary epithelium. Transgenic mice based on a background C57BL/6 were developed by injecting purified DNA constructs into oocytes. DNA constructs code for apical sodium dependant bile acid transporters with ovalbumin bound to their membranes. The ovalbumin is fused with a human transferrin receptor making the TFR-OVA complex specific for the biliary epithelium. Induction of liver injury is achieved following adoptive transfer of ovalbumin specific CD8+ (OT 1) and CD4+ (OT2) T-Cells. Transgenic mice are used to produce either OT1 or OT2 cells which are then extracted via splenectomy. Cells are injected into OVA-Bil mice via the intra-peritoneal route.

The extent of liver injury occurring in the OVA-Bil mice is dependent on the number of T-Cells transferred. Following adoptive transfer of $5x10^6$ OT1 cells and $2x10^6$ OT2 cells serum Alanine transaminase (ALT), an enzyme found in hepatocytes and used as a marker of hepatocyte injury, increases to 8 times the baseline level and peaks at day 10, but the greatest increase in ALT was following adoptive transfer of $1x10^7$ OT1 cells and $4x10^6$ OT2 cells (an

increase of 30 times the baseline). Serum ALP will double from baseline at the time of peak ALT rise. T-Cell mediated bile duct damage and hepatocellular inflammation and necrosis are seen.

The inflammatory response usually terminates after day 11 and ALT begins to decrease. The mechanisms behind this are complex and likely due to exhaustion of transplanted T-Cells. As OVA-Bil mice are tolerant to ovalbumin, adoptive transfer of ovalbumin specific T-Cells is required for induction of liver injury and hence this is an induced model rather than a spontaneous model.

## 1.7. Mesenchymal Stem Cells

There is a lot of controversy in the literature regarding Mesenchymal Stem Cells (MSC), what they really are, what their function is and how they can be used clinically. The term was first used in 1991 by Caplan[49], although earlier work by Friedenstein demonstrated a plastic adherent bone marrow cell termed the colony forming unit fibroblast (CFU-F)[50]. These non-haematopoietic stem cells are multipotent, and have been referred to as skeletal stem cells reflecting their differentiation potential[51], in other words they are capable of limited differentiation into particular cell lines rather than true pluripotency which is seen in embryonic stem cells. MSC have been shown to reside on the outer surface of bone marrow sinusoids and form part of the stromal compartment of the bone marrow[52], hence their alternative name, Mesenchymal Stromal Cells. Various markers have been suggested in order to define MSC, in humans they include; CD146, CD105, ALP, VCAM1 and STRO-1[53], and CD105, CD90 and VCAM1 in mice[54]. These markers demonstrate a heterogeneous population of cells and further studies have suggested the involvement of CD2, SSEA-1 and

SSEA-2 as markers of a hierarchy of MSC differentiation[55]. Until recently the isolation of MSC involved techniques utilising their plastic adherent properties. The development of an isolation technique for highly purified MSC using the markers PDGFR-α and Sca-1, so called PαS cells[80,81], has enabled the study of a subgroup of MSC that still retain all of the properties that make MSC an important therapeutic tool. MSC are capable of tri-lineage differentiation having the ability to form bone, cartilage and fat[56]. Whilst MSC have been thought of as stromal cells supporting the haematopoietic cells, their ability to self-renew has been recently shown[57]. The MSC transcriptome contains a combination of genes which characterise early but committed osteogenic cells such as the gene Runx2, as well as genes found in perivascular cells[58].

A key problem with the current literature is that there is no clear definition that adequately describes MSC which can be used clinically due to the non-specific surface markers used in their isolation. A widely accepted definition proposed in a position statement by the International Society for Cellular Therapy[59], has been argued to be deeply flawed, and the loose set of properties described such as the ability to accumulate hydrophobic, mineralised and polyanionic material (such as fat, bone and cartilage) are properties that could be ascribed to a wide range of connective tissue cells[60]. This lack of clarity and specificity regarding the definition and subsequent isolation of MSC has clear implications for clinical trials and the reproducibility and predictability of results obtained. There is a clear need for a more specific definition of MSC and more selective isolation techniques. The advantages of a pure, well defined cell population likely includes increased potency and karyotypic stability, as key concerns levied at the more heterogeneous MSC populations is that of a lack of reproducibility in clinical trials, and of malignant transformation[61].

Tissue regeneration and reprogramming have been the mainstay of research areas for the use of MSC. It has been shown extensively that bone marrow derived MSC, administered into damaged tissue, can lead to the generation of new bone and associated tissues by enhancing the regenerative properties of cells local to the defect[62,63]. These MSC have a potential role in reducing local inflammatory cytokines by inhibiting the immune response[64]. Whilst the theory that MSC may exhibit pluripotency now seems to have little credence, there still remains a number of research groups looking into the effect of MSC in other non-skeletal tissues and their ability to regenerate these tissues, hypothesising that there are other mechanism at play not related to pluripotency[65]. There are a number of non-progenitor properties of MSC that have recently come to light and are undergoing study. Anti-inflammatory and immunoregulatory properties are important effects of MSC with great clinical potential, however these effects are not stem cell effects, moreover these properties of MSC are much more in keeping with those of fibroblasts to which they have been compared[66].

The ability MSC to suppress the immune system has gained popularity as a potential therapeutic intervention[67]. There is still work to be done to ascertain all of the mechanisms by which MSC can exert their immunosuppressive effects, but current mechanisms that have shown promise are those requiring close cell contact and honing to areas of active inflammation. Mediators responsible for MSC mediated immunosuppression include; TGF-β, IDO, PGE2, IGF, IFNγ, IL 10 and TNFα[68,69,70,71]. Whilst the bulk of the literature focuses on these direct mediators, requiring close cell contact, there has been a suggestion that MSC may be able to exert their effect remotely[72]. The whole picture with regards to MSC

immunosuppression has not been fully elucidated, it is likely that inhibition of T-Cell function is key[73], however there is growing evidence that MSC can also exert their effect via myeloid cell lines such as myeloid derived suppressor cells[74]. There are few clinical trials investigating the utility of MSC as immunosuppressant therapy, one notable area that has been studied is graft versus host disease, and whilst MSC have shown a lot of promise it is clear that a number of variables such as timing of infusions may determine whether MSC exert an immunosuppressive or pro-inflammatory effect[75,76,77].

Route of administration is also an area that requires study. The pharmacokinetics of MSC are not fully understood, but it has been clearly shown that when delivered via the intravenous route a large proportion of the cells adhere to the lungs which may cause endothelial cell damage[78]. However as positive effects at target sites have been shown *in vivo* using this route of administration without significant side effects, it seems that the clinical translation of intravenous administration is reasonable if carried out cautiously.

The various proposed effects of MSC, both in the field of regeneration and inflammation mean that these cells have a huge potential in many different areas of medicine and types of disease process[79]. Clearly MSC are a potential therapy for autoimmune disease, and this merits further study.

## 1.8. Aims and Hypothesis

Isolation of purified PαS MSC is key to enable the investigation of their mechanisms of action, as a highly purified cell line will remove the confounders that occur when using more heterogeneous cells types. The first aim of this study is to acquire the ability to consistently

perform the complex isolation of purified PαS MSC. The second aim is to test the ability of these PαS MSC to suppress autoimmune mediated liver injury in a mouse model of autoimmune liver disease.

# 2. METHODS

## 2.1. Literature Searching Techniques

The literature review for this thesis was carried out using systematic search methodologies. Multiple searches were performed for the different sections of this thesis over the period of the 7th of July 2014 to the 28th of July 2014. The following databases were used; Medline, EMBASE, Cochrane as well as searching relevant references from papers found and further searches using Google Scholar.

## 2.2. Cell Culture

Following cell sorting isolated purified PαS MSC were cultured in α-Modified Eagles Medium (α-MEM, Invitrogen). The medium was treated with 1% Penicillin-Streptomycin-Glutamine (PSG, Invitrogen) with the addition of 10% Foetal Calf Serum/Foetal Bovine Serum (FBS, Invitrogen). Media was changed every 3 days and cells were assessed under light microscopy daily. When cells reached 90% confluencey they were passaged and separated into larger containers. A minimum seeding density of $5x10^3$ cells per $cm^2$ was used.

## 2.3. Cell Passage

Cell passage was performed when signs of confluence were detected. Medium was removed and the cells were washed 3 times with Phosphate Buffered Saline (PBS) whilst still adherent to the container or plate they were currently housed in. A combination of trypsin and EDTA (TrypLE, Invitrogen) was added to the container and left covered in an incubator at 37°C for 3 minutes. Loss of adherence was confirmed by microscopy and then the trypsin mixture was neutralised by the addition of an equal volume of the treated α-MEM (with added PSG and

FBS). The cell suspension was transferred by pipette into either a 20ml or 50ml Falcon™ Conical Centrifuge tube depending on the volume of suspension being separated. Cells were spun by centrifuge at 2000rpm for 5 minutes. The supernatant was discarded and the cells were then resuspended by vigorous tapping of the conical tube until a homogenous cell suspension remained. An appropriate volume of medium was then added and the suspension agitated to achieve a homogenous mixture. The cells were then seeded onto larger plates to allow for continued growth.

## 2.4. Prospective Isolation Of Purified Murine PαS Mesenchymal Stem Cells

The following methodology was developed by Morikawa et al[80] and then expanded on by Houlihan et al[81]. The technique required a number of years of optimisation by my colleagues Diarmaid Houlihan and Shankar Suresh in order to be consistent and reproducible in the laboratory environment at the University of Birmingham. The technique allows for the isolation of highly purified (99% pure) PαS MSC which so far has not been achieved by any other technique. The learning of this isolation technique has formed a large part of the work I have undertaken during my MRes research period and its complexities have meant that other students have struggled to reproduce this technique. As such the methods used in order to isolate purified PαS MSC will be described in detail

### 2.4.1. Animal Husbandry

Wild type C57BL/6 mice were used from stock maintained at the University of Birmingham. All mice were housed in the [redacted] and cared for using the well established care protocols in that facility.

All scientific procedures performed on animals were carried out in accordance with the Animals (Scientific Procedures) Act 1986, UK, and all procedures underwent ethical review prior to being performed.

### 2.4.2. Removal and preparation of long bones

Wildtype mice (C57BL/6) were used aged between 8 and 12 weeks. Numbers of mice used varied depending on stock but usually between 10 and 15 were required in order to achieve a suitable yield of PαS MSC. Mice were [redacted]. Mice were immediately sprayed with a solution of 70% ethanol. A transverse incision in the lower abdomen/pelvic region was performed to allow access to the leg bones. Skin and muscle was retracted using forceps and care was taken not to contaminate the working area with hair and faeces. The skin was retracted over the ankle and an incision made through the ankle joint with sharp scissors removing the foot which was discarded. The tibia was exposed using a blunt dissection technique and a further incision through the knee joint performed in order to free the tibia. The bone was then cleaned using tissue paper to remove all loosely adherent tissues before placing the clean bone into a container of PBS stored on ice. The hip joint was fixed with forceps and further blunt dissection carried out in order to release the femur. A further incision was then made through the hip joint freeing the femur. This was also cleaned with tissue paper and stored in PBS as described above. This procedure was repeated for both legs on every mouse being used.

Following the surgical stage of the isolation all bones were washed three times by agitating in fresh PBS. The bones were then placed into a sterile mortar and a single break made with a pestle by gentle compression at the centre of each bone individually. Bones were then cut

using sharp, sterile scissors into small fragments for approximately 5 minutes until there was minimal resistance to cutting from the resulting paste. The bony paste was then washed in PBS and the supernatant discarded. The bone paste were then placed into a 50ml Falcon™ Conical Centrifuge tube containing 20ml of Dulbecco's Modified Eagles Medium (DMEM, Invitrogen) preheated to 37°C with 0.2% collagenase (Wako) and PSG. The tube was placed onto a shaker in an incubator at 37°C with an oscillatory setting of 125rpm for 1 hour.

### 2.4.3. Preparation of Cell Suspension From Bone Paste

On removal from the incubator the tube was immediately placed on ice to stop the collagenase reaction. The bone fragments were filtered using a 70µm sterile filter (BD Falcon) into a new conical tube and kept on ice. The remaining bone fragments/bone paste are returned to the mortar. A solution of Hank's Balanced Salt Solution (HBSS) is prepared with the addition of 2% FBS, 1% PSG and 10mM of HEPES buffer (Sigma). 2.5ml of this $HBSS^{+}$ solution is added to the bone fragments. The bone fragments were then gently crushed using the pestle with repeated light tapping for 100 taps. This was followed by the addition of a further 2.5ml of the $HBSS^{+}$ solution was added using a pipette. The total of 5ml of solution was pipetted up and down 3 times to aid with cell release. Gentle swirling of the bone fragments and liquid was carried out using the pestle for 30 seconds. Further mixing with the pipette was carried out with repeat up and down transfer. The solution was then carefully drawn up into the pipette being sure to leave the remaining bone fragments and the solution filtered through a 70 µm filter into the conical tube containing the previous filtrate, remaining on ice at all times. This process was repeated a further 5 times until a total of 50ml of solution had been collected in the conical tube.

The solution was subsequently spun in a pre-chilled centrifuge at 4°C for 7 minutes at 280g (1350rpm). Following centrifugation the supernatant was discarded and the cell pellet resuspended as previously described but no medium added.

### 2.4.4. Red Blood Cell Lysis

Red blood cell lysis was required in order to remove all of the red blood cells, which are not being collected, and therefore improve the efficiency of the later sorting step. Whilst the technique described is a non-standard technique it has been found through meticulous optimisation to give the greatest yield of PαS MSC compared with other methods of red cell lysis.

The resuspended pellet present in a 50ml conical tube had 1ml of cold (approximately 4°C) sterile water ($H_2O$) added whilst continuous rotating of the tube was carried out by hand. After 5 seconds of contact with the water 1ml of double strength PBS solution (with 4% FBS added) was added followed by 13ml of $HBSS^+$ solution to make a total volume of 15ml in order to quench the reaction. The cell suspension was then filtered through a sterile 70 µm filter before further spinning in a pre-chilled centrifuge at 4°C for 5 minutes at 280g (1350rpm). Following this the supernatant was again discarded and the cell pellet resuspended as described previously in 1ml of the $HBSS^+$ solution.

### 2.4.5. Fluorescent Staining of PαS Mesenchymal Stem Cells

The staining colours were determined after careful analysis of the requirements of the MoFlo™ Cell sorter at the University of Birmingham along with the availability of stains for the relevant markers. The antibody panel used was as described in the literature[80,81].

The antibodies used were as follows; CD45-PE (eBioscience), Ter-119-PE (eBioscience), Sca-1-FITC (eBioscience) and PDGFRα-APC (eBioscience). Propidium Iodide (PI, Sigma) solution was also used as described later.

Sterile FACS tubes were used for all samples and controls. Staining of the main sample was carried out in the conical tube and transferred to a cuvette as described later. All samples were kept on ice during the staining process. Staining was carried out in a standard hood with the light switched off and all antibodies were protected from light exposure.

Control tubes were as follows; negative control (cells only), positive control for PE (cells and PE antibody), positive control for FITC (cells and FITC antibody), and positive control for APC (cells and APC antibody). Each control tube (4 in total) had 100µl of $HBSS^{+}$ solution, 4µl of the cell suspension and 1µl of the relevant antibody.

The main sample tube staining was achieved with the addition of 1µl per mouse of each of the 4 antibodies (CD45-PE, Ter119-PE, Scar-1-FITC and PDGFRα-APC). All samples were then incubated on ice in the dark for 30 minutes.

Following incubation the control samples were washed by the addition of a further 1ml of $HBSS^{+}$ solution. The control samples were then spun at 2000rpm for 5 minutes. The supernatant was discarded and the resulting cell pellets resuspended as described earlier. The main sample conical tube was spun in a pre-chilled centrifuge at 4°C for 5 minutes at 280g

(1350 rpm). Following this the supernatant was discarded and the resulting cell pellet resuspended.

A solution of PI stain and HBSS was made with a concentration of 2µl of PI per ml of HBSS. The control tubes each had 500µl of PI/HBSS solution added. The main sample had 1ml PI/HBSS solution per mouse added (10-15ml). Each control tube was filtered into a new FACS cuvette through a sterile 35µm filter. The main sample was filtered into multiple FACS cuvette tubes through a sterile 35µm filter aiming for 3-4ml in each cuvette. All samples were then stored on ice in the dark ready for cell sorting.

### 2.4.6. Cell Sorting of PαS Mesenchymal Stem Cells

A MoFlo™ XDP (Beckman Coulter) Cell Sorter (Figure 1) was used for cell sorting. The

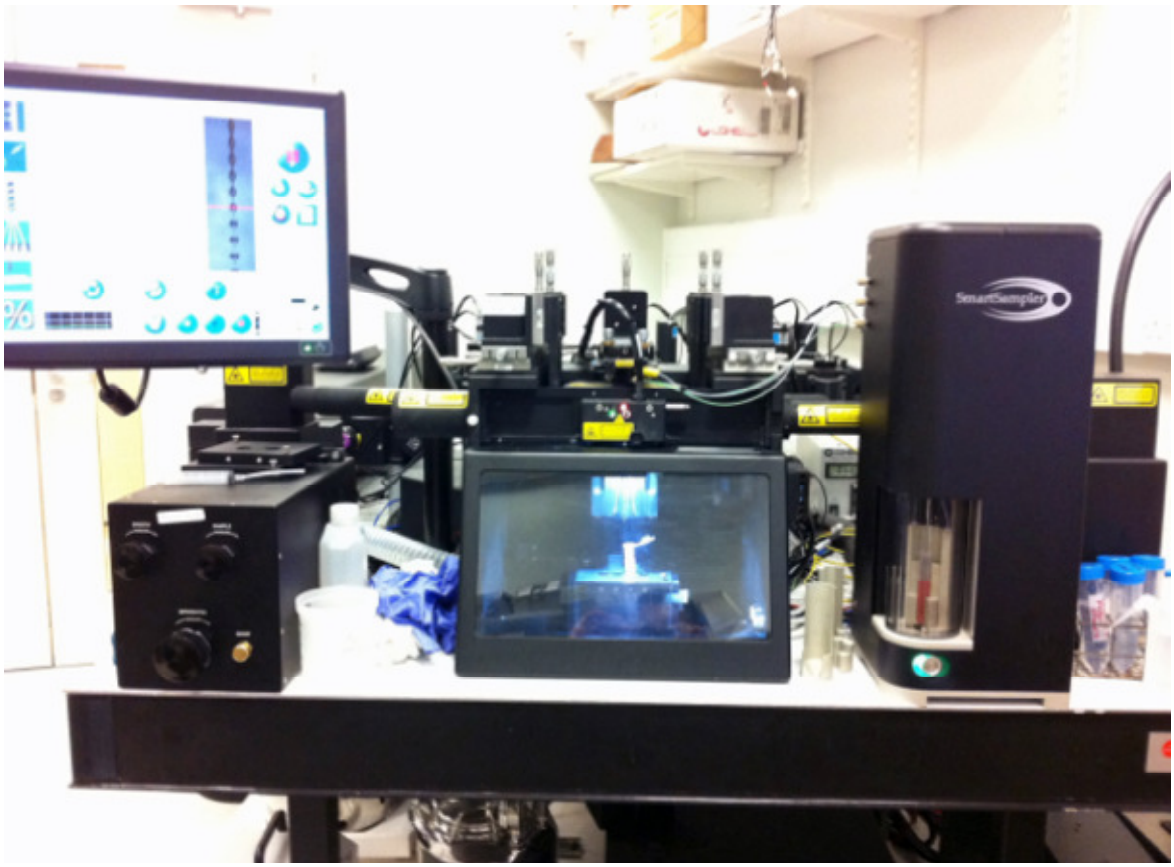

**Figure 1 A photograph depicting the MoFlo™ XDP Cell Sorter setup at the University of Birmingham**

cell sorter was configured to the manufacturer's exact specifications and calibrated extensively prior to use and the machine allowed to reach a stable working temperature. Whilst many lasers are available on this particular machine only the 488nm and 647nm lasers are used in this protocol. Voltage, fanning, laser alignment and drop delay were all calibrated as per manufacturer specifications. The negative control was run through to ensure detection of events without any significant detection by the lasers. Following this each single antibody control/compensation tube was run starting with PE, then FITC then APC. Each of these channels was compensated against the others in order to prevent significant colour bleed between the fluorescent antibodies. Once compensation was complete gating was carried out.

Gating on PI allowed for the live cells to be selected. Negative gates for PE and Terr119 were created and positive gates for PDGFRα and Sca-1 in order to select the PαS cells. Finally a gate was drawn in order to select the central population of PαS cells based on previous optimisations and experience from the literature[80,81]. The positively selected cells (PαS) were collected in a small collecting tube containing αMEM media with added PSG and 10% FCS. The mixture was then transferred into a FACS cuvette following completion of the sort and spun in a centrifuge at 2000rpm for 5 minutes. The resulting pellet was resuspended and the appropriate amount of culture medium added for seeding onto 6 well plates.

## 2.5. Growth Factor Priming of PαS Mesenchymal Stem Cells

Growth factor priming of MSC has effects on differentiation and function. In order to prepare PαS MSC primed with different growth factors freshly isolated PαS MSC were cultured either in standard medium (α-MEM + PSG + FCS), or standard medium with added growth factors. A concentration of 10ng/ml of Transforming Growth Factor β (TGF-β) was added to the standard medium and cells cultured as described earlier (2.1).

## 2.6. OVA-Bil Mouse Model

The OVA-Bil mouse model is a model of autoimmune liver disease where OVA Specific T-Cells are directed at membrane bound ovalbumin expressed on apical sodium dependent bile acid transporters (ASBT) located on hepatocytes in a transgenic mouse. This model was used to assess *in vivo* efficacy of the purified PαS MSC as described below.

### 2.6.1. Animal Husbandry

The OVA-Bil mouse model, a model of Primary Biliary Cirrhosis, had been previously used by Dr Bertus Eksteen in our facility and frozen embryos were available, therefore the colony was re-derived from these. The background mice used were C57BL/6 mice and heterozygotes for OVA-Bil were used and maintained. Transgenic mice with the OT1 and OT2 ova-specific T-Cell phenotype were purchased from Jackson Laboratories and maintained at [redacted].

All mice were housed in the [redacted] and cared for using the well established care protocols in that facility.

All scientific procedures performed on animals were carried out in accordance with the Animals (Scientific Procedures) Act 1986, UK, and all procedures underwent ethical review prior to being performed.

### 2.6.2. Induction of Liver Injury

Male transgenic mice of both the OT1 (OVA-specific $CD8^+$ T-Cells) and OT2 (OVA-specific $CD4^+$ T-Cells) T-Cell receptor type were sacrificed using schedule 1 methods (cervical dislocation) aged between 8 and 12 weeks. Splenectomy was performed immediately following confirmation of death. Suspensions of splenocytes were created by passing the explanted spleens through a 70µm mesh. Red cell lysis was performed using sterile water and the resulting suspension was washed and resuspended in PBS. Cell counts were performed using a haemocytometer and suspensions were made with a cell count of $1x10^7$ OT1 splenocytes consisting of OVA-specific $CD8^+$ T-Cells and $4x10^6$ OT2 splenocytes consisting

of OVA-specific $CD4^+$ T-Cells. These were combined into single suspensions in a volume of 200µl of PBS. The splenocyte suspension was administered to 10 C57BL/6 OVA-Bil mice by intraperitoneal injection using a 25 gauge needle, each mouse receiving $1x10^7$ OT1 cells and $4x10^6$ OT2 cells in 200µl of PBS. Blood samples were taken by cardiac puncture at days 6-11 under terminal anaesthesia with isoflurane 2% and oxygen as the carrier gas, with [redacted]. Analysis of serum ALT and ALP was carried by the clinical biochemistry department at the Birmingham Women's Hospital. Hepatectomy was performed immediately following confirmation of death and the liver samples were stored in liquid nitrogen. This experiment was repeated 3 times to ensure consistency of results.

### 2.6.3. Genotyping of OVA-Bil Mice

OVA-Bil mouse genotyping was carried out in order to confirm carriage of the OVA transgene and therefore susceptibility to the OT1 and OT2 lymphocytes. In order to control expression levels of ovalbumin within the liver it was decided that heterozygote male mice would be used. OT1 and OT2 mice were also genotyped in order to confirm expression of the ova-specific T-Cells. A polymerase chain reaction (PCR) technique was used as described below.

#### 2.6.3.1. Sample Preparation

Ear clippings were taken from mice at weaning by staff in the animal house using local protocols and were provided in labelled sample tubes which were immediately frozen for storage and subsequent analysis.

### 2.6.3.2. Polymerase Chain Reaction

A hot start PCR technique was used which avoids the non-specific amplification of DNA at low temperatures due to the inactive nature of the Taq DNA polymerase enzyme and increases the yield of DNA.

Tissue samples were mixed with 100µl of a 50 millimolar solution of Sodium Hydroxide and placed in a heating block at 95°C for 15 minutes. Following heating samples were removed from the heating block an 10µl of a 1 Molar solution of 2-Amino-2-(hydroxymethyl)-1,3-propanediol (TRIZMA$^{TM}$) base (Sigma-Aldrich) and mixed thoroughly.

A master mix was created with a total volume of 18µl per mouse analysed. The mixture contained 10µl of Qiagen Fast Cycling PCR master mix (Qiagen) which contains HotStarTaq polymerase, PCR buffer, Magnesium ions and deoxyribonucletide triphosphates (dNTP), 4µl of RNAase free water (Qiagen), 2µl of CoralLoad Dye (Qiagen), 1µl of Forward Primer (Qiagen) and 1µl of Reverse Primer (Qiagen). New 200µl PCR eppendorfs were used and individually labelled. 18µl of the master mix described above was added followed by 2µl of digested mouse ears (processed DNA) was added. Negative controls were used where the processed DNA was replaced with 2µl of water. All tubes were briefly spun to ensure samples sat at the bottom of the tube.

A G-storm GS1 PCR thermocycler (G-storm, UK) was used to process the samples. An optimised protocol for hot start fast cycling was used based on a fragment length of 994 base pairs (Table 3).

| Step | Duration | Temperature | Notes |
|---|---|---|---|
| **Initial activation step** | 5 minute | 95°C | HotStarTaq and DNA Polymerase activated |
| **Denaturation** | 5 seconds | 96°C | |
| **Annealing** | 5 seconds | 55°C | 5°C below $T_m$ of the primers |
| **Extension** | 30 seconds | 68°C | 3 seconds per base pair |
| **Repeat** | 35 cycles | | |
| **Final Extension** | 1 minute | 72°C | |
| **Holding** | As required | 8°C | Can be stored longer at -20°C if required |

**Table 3 A Thermocycler protocol for HotStart PCR based on a base pair length of 994. This protocol was carried out on a G-Storm GS1 PCR thermocycler running for 35 cycles and 45 minutes. This was used in the HotStart PCR technique for the preparation of OVA-Bil DNA of gel electrophoresis.**

The protocol was run for 35 cycles taking approximately 45 minutes to complete. Following completion the thermocycler stored the samples at 8°C as per protocol until required.

### 2.6.3.3. PCR Gel Preparation

A solution of 1.1% Agarose was made by dissolving 1.5g of agarose (Sigma-Aldrich) into 75ml of Tris/Boric Acid/EDTA buffer (TBE, Sigma-Aldrich). This was thoroughly mixed until all of the agarose had dissolved and then heated at high power using an 800w microwave for 80 seconds. 7.5µl of SYBR safe (Life technologies) was added to the agarose solution and mixed. The mixture was then poured into a PCR setting block and a comb added to provide 18 channels. The solution was then left to cool form a gel (gelling point 36°C). Once set the comb was removed and 750ml of TBE buffer poured carefully on top to ensure no bubbles were created as a running buffer.

### 2.6.3.4. PCR Gel Electrophoresis

Following gel preparation the samples were added as follows. The first well was designated as the ladder and 5µl of HyperLadder™ 100BP (Formerly HyperLadder™ IV, Bioline) and subsequent wells for the DNA samples, 10µl of which was added to each well. The electrodes on the PCR block were attached to a regulated DC bench power supply with the anode connected to the end nearest to the sample wells and the cathode connected to the end furthest away. The voltage is set to 100mV to start the sample separation. The samples were run for 90 minutes in order to allow for adequate separation. On completion the gel was viewed on an Ultra Violet transilluminator connected to a Personal Computer.

## 2.6.4. Use of PαS Mesenchymal Stem Cells in OVA-Bil Model

Purified PαS MSC were isolated as described previously. Cells were cultured in α-MEM to passage 4-6. Cytokine stimulation was carried out as described earlier. Cells were trypsinised and then washed and resuspended in PBS. Cell counting was performed and $5x10^5$ cells were suspended in a total volume of 200µl of PBS. The cell suspension was infused into the tail vein of the OVA-Bil Mice on days 3 and 6 following induction of liver injury with OT1/OT2 OVA Specific T-Cells. Control mice were infused with 200µl of PBS alone. All mice were [redacted] on day 10 to enable analysis of serum ALT and ALP. Blood samples were obtained by cardiac puncture following general anaesthesia with isoflurane. [redacted]. Hepatectomy was performed immediately following confirmation of death and the liver was placed in PBS ready for either fixing or further study.

### 2.6.5. Immunohistochemistry and Staining of Murine Solid Organs

Solid organs were removed immediately following confirmation of death and placed in formalin for fixation. Following fixation the organs were embedded in paraffin wax. Paraffin blocks were sectioned when required into 4µm sections and secured to microscope slides ready for staining. Haematoxylin and Eosin staining was carried out using local protocols by [redacted].

# 3. RESULTS

## 3.1. Cell sorting of PαS Mesenchymal Stem Cells

Extensive optimisation work had already been carried out by colleagues using this isolation technique as previously discussed. Gating as described in the methods section yielded between 10,000 and 12,000 cells per mouse, equating to a yield of 100,000 to 120,000 cells for a 10 mouse isolation run. The time taken to achieve this would be in the region of 10-12 hours. Figure 2 shows an ideal PαS sort on the Cell Sorter with a flow cytometric profile looking at Sca-1 and PDGFR positive cells with the ideal gating location marked. A yield of approximately 0.05% of the total number of events detected by the sorter would be expected.

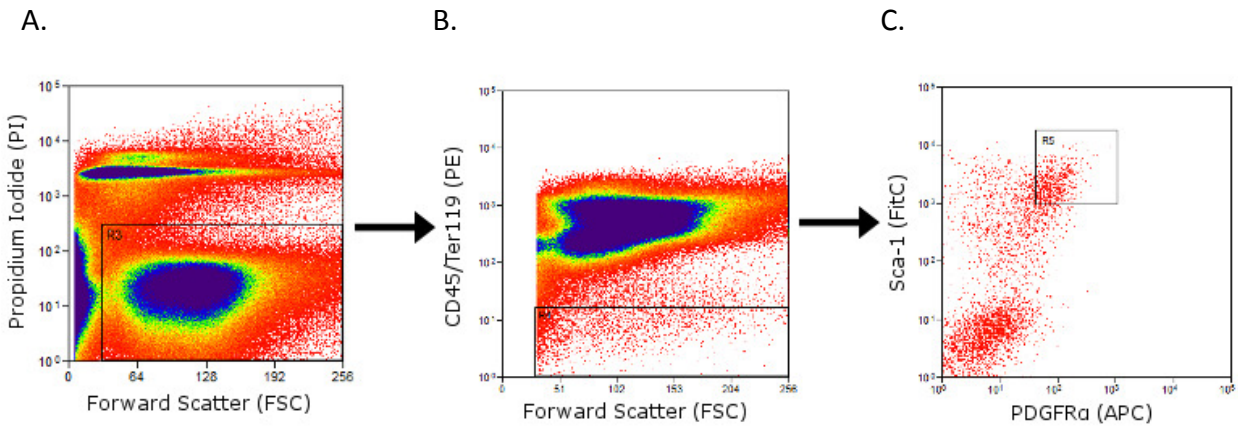


**Figure 2 Representative scatter plots demonstrating the gating strategy used during cell sorting of long bone derived cells suspension. 10 male 10 week old C57BL/6 mice were used in this experiment and long bones extracted, finely chopped into a paste and digested with collagenase. Following red cell lysis antibody labelling was carried out for CD45, Ter-119, Sca-1, PDGFRα and a live dead marker. Live cells were gated A) and then the non-haematopoietic cells were selected B). The final gate was set to isolate PDGFRα and Sca-1 positive cells (PαS) C). Roughly 100,000 PαS cells were isolated, approximately 0.05% of the total cell suspension.**

This gating strategy also excluded haematopoietic cells marked with Ter-119 and CD45 as well as dead cells which were stained with PI.

## 3.2. Gel Electrophoresis of PCR of OVA-Bil DNA

In order to confirm the genotype of the OVA-Bil mice, PCR and gel electrophoresis were carried out on samples from all offspring taken immediately following completion of weaning. In order to facilitate best use of the mouse colony and control ovalbumin expression it was decided that heterozygote males would be used in the OVA-Bil experiments. A Hot Start PCR technique was used in order to avoid low temperature non-specific amplification and aiming to increase the DNA yield. Minimal optimisation was required as the technique was familiar and the protocol already optimised by the manufacturer. Example PCR gels following 90 minutes of electrophoresis at 100mV and transillumination on a UV camera are shown below (Figure 3).

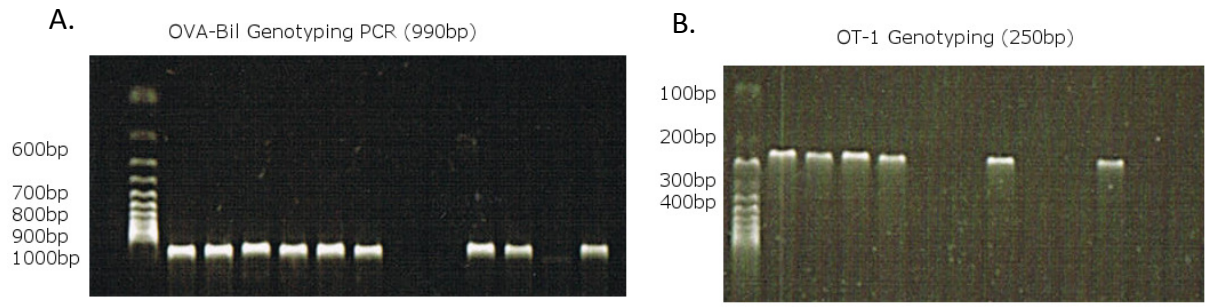


**Figure 3 PCR genotyping of OVA-Bil and OT 1 mice using a HotStart Taq polymerase and gel electrophoresis. OVA-Bil mice A) produce a band of 990bp size. Mice in lanes 8, 9 and 12 were all negative for the OVA gene whereas 1-7, 10-11 and 13 were all positive. OT-1 mice B) produce a band at 250bp and mice in lanes 5-6, 8-9 and 11-12 were all negative for the OT-1 gene whereas lanes 1-4, 7 and 10 were all positive.**

Mice with no OVA-Bil expression were [redacted]. Homozygotes were retained as breeding stock where needed [redacted].

## 3.3. Liver Damage using the OVA-Bil Mouse Model

The clinical importance of autoimmune hepatitis and PBC is described earlier. The OVA-Bil model originally described in 2006[48], is a model in which transgenic mice expressing ovalbumin on apical sodium channels in the hepatobiliary system undergo induced hepatobiliary injury following inoculation with ova specific T lymphocytes. This immune mediated biliary injury demonstrates features of the autoimmune condition PBC.

Induction of liver injury was achieved by the intra-peritoneal administration of $1x10^7$ OT1 splenocytes consisting of OVA-specific $CD8^+$ T-Cells and $4x10^6$ OT2 splenocytes consisting of OVA-specific $CD4^+$ T-Cells administered in a single suspension. These cells will cause specific hepatobiliary injury due to the selective expression of ovalbumin in the recipient transgenic mice. It was decided to use ALT and ALP as markers of liver damage as well as histological examination based on the literature and on the transferability to the clinical environment[48]. These baseline experiments were repeated multiple times in order to quantify the degree of liver injury at various time points. In keeping with the literature a consistent rise in both ALT and ALP from baseline levels was observed with a peak ALT at day 10 of 635 IU/L and a peak ALP of 500 IU/L at day 11. At day 11 the ALT levels began to fall in keeping with the literature. On day 10 following adoptive transfer of OT1 and OT2 cells OVA-Bil mice were [redacted] and all solid organs removed and fixed in formalin for sectioning and histological analysis. Following sectioning slides were prepared and stained with haematoxylin and eosin (H&E). There were no significant findings in the solid organs apart from the liver, which is to be expected as the ovalbumin expression is specific to the hepatobiliary system and parallel experiments carried out by other group members confirmed that RNA expression of ovalbumin was limited to liver tissue. Liver sections showed

lymphocytic infiltration throughout the biliary system with the greatest levels of infiltration effecting the biliary cells which encase the portal tracts (Figure 4).

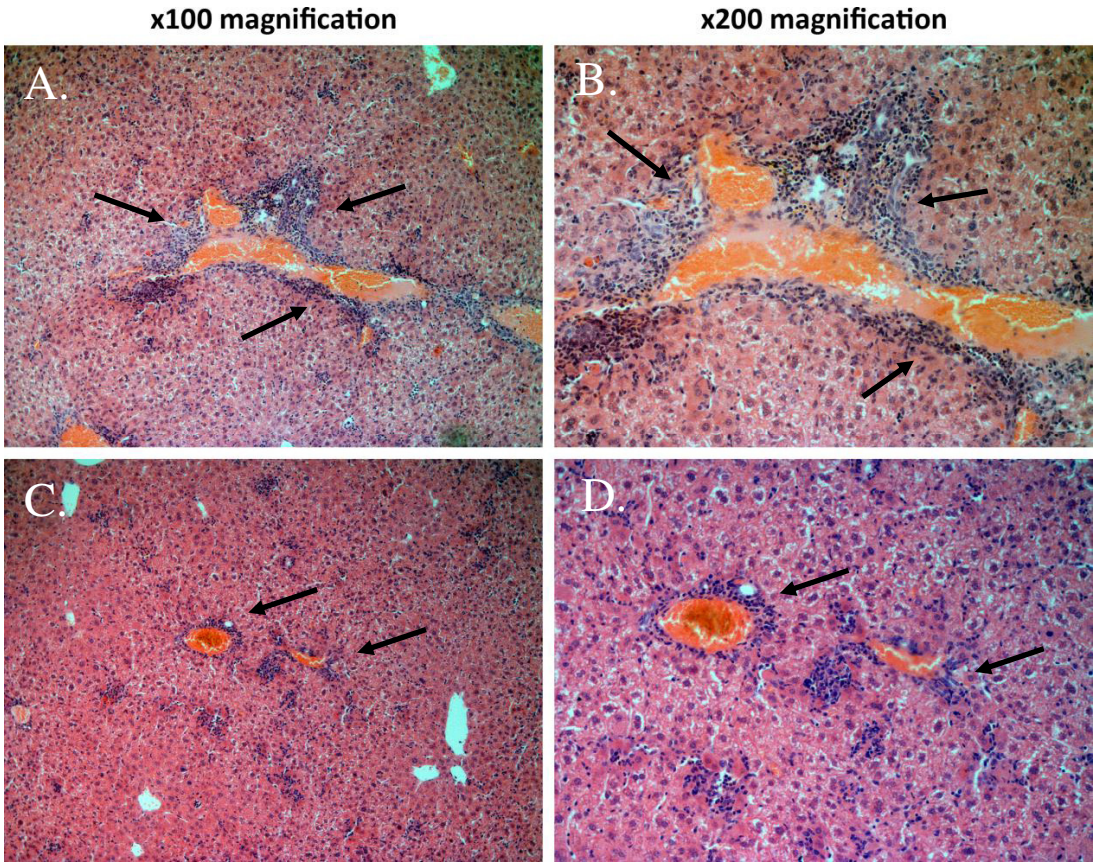


**Figure 4 H&E Stained sections of liver tissue from injured OVA-Bil Mice at day 10 post adoptive transfer of $1x10^7$ OT1 cells and $4x10^6$ OT2 cells demonstrating lymphocytic infiltration into the biliary cells encasing the portal tracts. Qualitative analysis was carried out on these sections. Male 8-12 week old OVA-Bil mice were injected IP with OT1 and OT2 cells and culled at day 10, Livers were removed, fixed in formalin and embedded in paraffin wax and 5µm sections were cut for staining. Sections from two different animals demonstrate 100x (A,C) and 200x (B,D) magnification.**

Interface hepatitis was also observed with clear spread of lymphocytes into the liver parenchyma. Hepatocyte necrosis and apoptosis were also observed. This pattern of injury clearly explains the concomitant ALT and ALP rises observed on serum testing.

## 3.4. Effect of PαS Cells on Injured and Uninjured Mice

Parallel unpublished *in vitro* work carried out in the liver laboratories by Diarmaid Houlihan has shown that PαS MSC are able to suppress T-Cell proliferation. Further unpublished parallel work has shown that cytokine priming increases the rate of growth of PαS MSC but without detrimental effect on their function. It has also been shown that senescence is reduced without significant karyotypic transformation and no malignant gain of function. Because of this ongoing work and the parallel *in vitro* work carried out by Shankar Suresh which showed that T-Cell proliferation was suppressed more with cells cultured in TGF-βs than those cultured in α-MEM it was decided that PαS MSC cultured in TGF-β would be the best choice of cells to show a dampening down of the immunologic response in the OVA-Bil model. It is clear from the literature and other work in our research group that the timing of the administration of MSC is crucial in order for them to have an immunosuppressive effect rather than the pro-inflammatory effect seen in some studies. It was decided that PαS MSC should be administered on days 3 and 6 based on a review of the current literature and previous *in vitro* work.

As this was a pilot investigation 3 control and 3 treatment mice were used. A total of $5x10^5$ PαS MSC were administered via the tail vein, on day 3 and again on day 6 following induction of liver injury in OVA-Bil mice, and the mice [redacted] at day 10 to enable examination of ALT, ALP and liver histology.

There was a fall in mean serum ALT and ALP levels following administration of PαS MSC. The decrease in ALT (Control ALT 1145 IU/L, PαS Treated ALT 523 IU/L) was more

marked than the decrease in ALP (Control ALP 203 IU/L, PαS Treated ALP 183 IU/L), however neither of these results reached statistical significance (Figure 5). It should be noted however that there was a wide variation in serum ALT and ALP in the control group which will have affected the results.

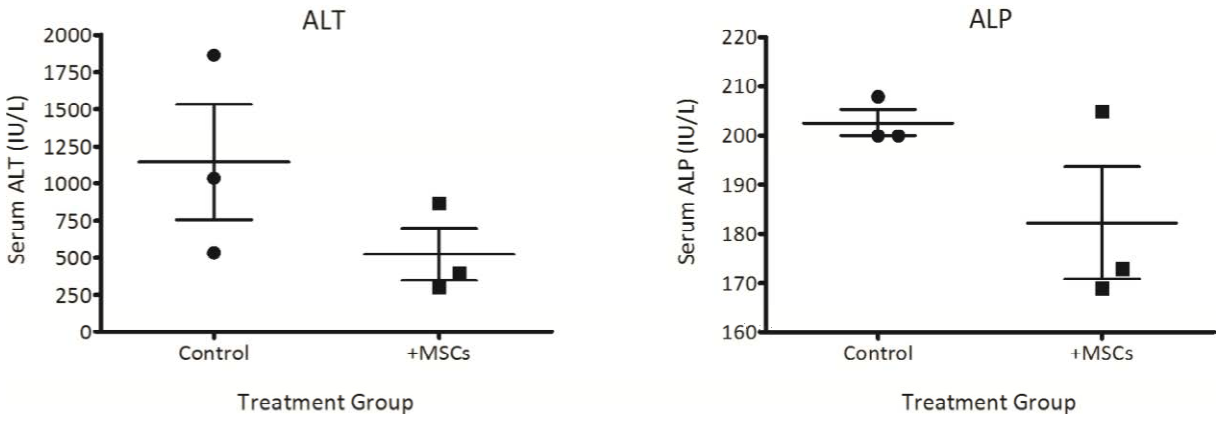


**Figure 5 Serum ALT and ALP Levels Day 10 following adoptive transfer of $1x10^7$ OT1 cells and $4x10^6$ OT2 cells in OVA-Bil mice treated with PαS MSC demonstrate non-significant decreases. 3 control and 3 intervention male 8-12 week old OVA-Bil mice were injected IP with $1x10^7$ OT1 cells and $4x10^6$ OT2 cells. Treated mice were injected via the tail vein with $5x10^5$ PαS MSC at day 3 and again at day 6 following adoptive transfer, control mice were given a PBS injection on the same days. Mice were culled at day 10 and serum samples sent for analysis. Student's t-test was performed and did not demonstrate significance.**

Day 10 livers were removed and fixed in paraffin for staining as described previously. Histological analysis provided by Professor Stephen Hubscher demonstrated no difference in the number of lymphocytes infiltrating the hepatobiliary system with similar numbers found in both liver parenchyma and in the portal architecture (Figure 6).

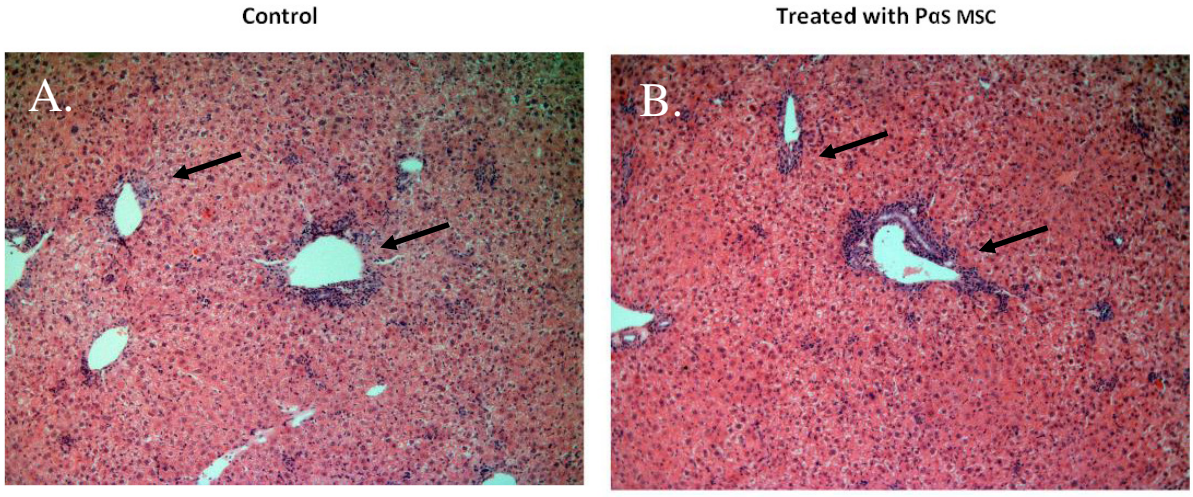


**Figure 6 Liver histology of treated and untreated OVA-Bil livers day 10 post adoptive transfer of $1x10^7$ OT1 cells and $4x10^6$ OT2 cells demonstrating no difference in lymphocytic infiltration in the portal regions. Qualitative analysis was carried out on these sections. Male 8-12 week old OVA-Bil mice were injected IP with $1x10^7$ OT1 cells and $4x10^6$ OT2 cells. A) Treated mice were injected via the tail vein with $5x10^5$ PαS MSC at day 3 and again at day 6 following adoptive transfer, B) control mice were given a PBS injection on the same days. Mice were culled at day 10, Livers were removed, fixed in formalin and embedded in paraffin wax and 5µm sections were cut for staining.**

Whilst this analysis was quantitative it did not take into account the relative size differences in mouse livers and further work would be needed in order to determine if there is a true difference.

# 4. DISCUSSION

The isolation of purified PαS MSC is a complex technique initially described by Morikawa[80], and further refined by Houlihan[81]. This technique has proven difficult to replicate meaning that the mouse MSC literature is still very much based on the use of plastic adherent MSC, a more heterogeneous population of cells, or the use of human MSC administered to mice. A large part of the focus of this research has been on the mastery of the technique of prospective isolation of highly purified PαS MSC using novel cell markers to identify and sort them. This has then created the environment to enable pilot *in vivo* work to show potential efficacy of these cells.

## 4.1. Limitations of the Experimental Techniques

Isolation of PαS MSC is a rewarding but timely process. Whilst the accuracy and reproducibility of this technique is good, the low cell yield requires that cells be culture expanded in order to produce enough cells exert an effect. Whilst it has not been fully elucidated what the correct dose or route of administration may be, it is clear that a much greater number is required than can be achieved at early passage with this technique. The benefit of using a purified population of cells which have no contaminating haematopoietic or fibroblastic cell types outweighs the difficulties encountered. Culture expansion could be potentially be improved by growth factor supplementation but this would require further study in order to demonstrate safety and efficacy.

This study was designed as a pilot study in order to demonstrate the feasibility of prospectively isolating PαS MSC and administering them in a mouse model of autoimmune liver disease. It is therefore not surprising that the results found are not significant as the

study was not powered to achieve significance, although encouraging that a non-significant reduction in markers of liver injury has been demonstrated. Further study is required with increased numbers of animals in order to ascertain if there is a true effect present.

The choice of ALT as a marker for liver injury is backed up by the literature and by the clinical correlation in humans. In retrospect the choice of ALP is perhaps less wise. Whilst changes in ALP were seen this is likely related to the different age of mice as in C57BL/6 mice there is limited expression in the liver and therefore serum levels are influenced much more by bone turnover which will increase serum ALP and is greater in younger mice.

The assessment of liver histological sections did not yield a significant difference, however this was a qualitative assessment only. Quantitative assessments may yield a different result. Whilst there may not be a visible overall difference in the infiltrative cells demonstrated specific staining for immune cells using CD45 or F4/80 should be carried out along with quantitative analysis in order to look at more specific markers of immune cell infiltration. Flow cytometric analysis of liver cell digest would also yield more specific information regarding the immune cell infiltration.

## 4.2. Purified PαS Mesenchymal Stem Cells

The MSC literature is littered with contrasting studies and a lack of clarity regarding the mechanisms of action of MSC, mainly because as yet this has not been fully elucidated. Concerns have been raised regarding the need to grow in culture what is a very rare population of cells for a prolonged period of time in order obtain the large numbers required for therapy, and the inherent risks of DNA copying abnormalities and tumour formation[82].

These concerns have also been raised when considering murine MSC[83]. The cell populations considered here are heterogeneous and it cannot be clear exactly what make up of cells there are as the current definition of an MSC and its accompanying cell surface markers are still not specific enough to yield a purified population. Prior to the development of a prospective isolation technique MSC were isolated based on their plastic adherence properties which led to a heterogeneous population of cells with contamination from haematopoietic cells and various fibroblastic cells making it very difficult to study mechanistic properties of MSC. A key advantage of the isolation technique described in this study is that it reliably produces a highly purified set of cells with a unique combination of surface markers, namely PDGFRα and Sca-1, so called PαS cells. The amount of clonal expansion required will also be significantly less with a purified cell population therefore reducing the risk of malignant transformation. Further work is required to confirm the karyotypic stability of PαS MSC. It will be important to use sensitive techniques for assessing for genetic stability as it has been shown that traditional techniques such as G-banding analysis of metaphase chromosomes is not adequate to pick up abnormalities in MSC and more advanced techniques such as array comparative genomic hybridization (aCGH) are required[84]. Assessment of tumour formation following multiple *in vivo* infusions would also be useful in assessing the relative safety of PαS MSC.

## 4.3. OVA-Bil Mouse Model of Autoimmune Liver Disease

The OVA-Bil mouse model used in the experiments in this study requires the adoptive transfer of $CD4^+$ and $CD8^+$ T-Cells specific for ovalbumin by modified OVA-Bil mice who express ovalbumin on the apical sodium channel of their biliary epithelium. This leads to a bile duct specific injury pattern and an associated injury to the surrounding hepatocytes[48].

The baseline liver injury seen in the mice injured in this study showed a 25-30 fold increase in serum ALT from the uninjured controls at day 10, but only a doubling in ALP levels following adoptive transfer of $1x10^7$ OT1 cells and $4x10^6$ OT2 cells. Whilst it is possible that the PBS carrier could have an effect on serum markers of liver injury, this is not a recognised phenomenon and the use of the same volume of PBS in the control and treated groups in this study will mitigate any effect that may have occurred due to carrier use. Lymphocytic infiltration particularly around the bile ducts was also seen on histology. This is entirely consistent with the results found in the original description of the OVA-Bil model by Buxbaum et al[48]. The histological findings of interface hepatitis and leakage of lymphocytes into the surrounding liver parenchyma were also consistent with the findings of Buxbaum. Due to the amount of necrosis seen in these areas it is likely that the release of cellular contents will further drive the immune system to damage hepatocytes and lead to hepatocyte apoptosis[85]. As in other models of liver disease such as the $MDR2^{-/-}$[86], leakage of bile acid and cholestasis will further contribute to hepatocyte necrosis and liver injury. In particular, the presence of CD8+ T-Cells within the hepatic parenchyma can lead to hepatic inflammation due to the release of TNF-α and IFNγ by the T-Cells[87]. Targeting T-Cells would therefore be a potential therapeutic strategy in these types of liver injury. The key advantages of the OVA-Bil model of liver injury used are its clear T-Cell mediated injury pattern which is not as well defined in other models and the ability to induce liver injury at a convenient time rather than using a spontaneous model.

## 4.4. Mesenchymal Stem Cells In Autoimmune Disease

Autoimmune disease is an important clinical entity as whilst it is not the most common of diseases encountered, a lot of autoimmune disease has limited treatment options. Treatments

that do exist often involve either long courses of corticosteroids with the accompanying side effects (Table 4), or immunosuppressant drugs with risk of infection and a dose and duration related risk of renal failure.

| Side Effects | |
|---|---|
| Weight Gain | Hypertension |
| Osteoporosis | Glaucoma |
| Cushing's Syndrome | Cataracts |
| Proximal Muscle Wasting | Delayed Wound Healing |
| Steroid Induced Diabetes | Susceptibility to Infection |
| Growth Retardation in Children | Depression |
| Steroid Psychosis | Insomnia |

**Table 4 A summary of the common side effects seen in patients taking regular corticosteroids**

Autoimmune liver diseases are a group of conditions that fit the above description with the caveat that liver transplant remains an option for late stage disease. Unfortunately the demand for donor organs is far outstripped by the number of patients on the transplant waiting list with just over half of patients who need a liver transplant actually receiving one2. MSC appear to be an attractive option to treat autoimmune liver disease due to their ability to modulate the immune system whilst preserving the response to infection[88], as well as avoiding the side effects mentioned above. The pilot *in vivo* work in this study showed no significant difference in OVA-Bil mice with induced liver injury following adoptive transfer of OT1 and OT2 splenocytes at day 10 post injury either with or without treatment with $5x10^6$ PαS MSC at day 3 and 6 when comparing ALT, ALP and liver histology. It is worth noting however,

that there was a definite trend showing a reduction in ALT in the mice treated with PαS MSC when compared with the mice that did not undergo treatment. This is encouraging and further study with larger numbers of mice is required in order to achieve a definite answer as a clear limitation of this series of experiment is the low numbers of mice used in order to generate the pilot data. Timing of infusions of MSC is also key and their immune regulating properties as has been shown in models of kidney transplant[89], where early administration led to an immunosuppressive actions, but later administration led to a pro-inflammatory effect and early graft dysfunction. Understanding this ability to have both pro and anti-inflammatory effects may yield a greater understanding of how MSC exert their effects and could lead to improving their therapeutic potential. Further work modifying the timings of MSC infusions may yield more significant results in the OVA-Bil model of autoimmune disease, as the current timings were decided upon based on a review of the literature, but as no studies using these highly purified cells have been carried out in autoimmune liver disease, there is room for refinement.

## 4.5. Mechanisms Of Mesenchymal Stem Cell Immunosuppression

A large number of possible mechanisms by which MSC interact with the immune system and exert their effect have been suggested, the main route by which MSC are thought to work is by cell to cell contact and the secretion of anti-inflammatory cytokines local to the area of inflammation (Table 5).

| Cytokine Levels Altered By Mesenchymal Stem Cells | |
|---|---|
| **HO1** | IDO |
| **TGF-β** | $PGE_2$ |
| **IL-10** | HLAG5 |
| **IL-6** | Reduce IL-12 |
| **IL-4** | Reduce TNF |
| **HGF** | Reduce IFNγ |

**Table 5 A summary of the inflammatory cytokines modulated by MSC**

A key mechanism in MSC induced immunosuppression is due to their effects on T Lymphocytes[90,91] and parallel work in the Newsome Laboratory in our group has confirmed this *in vitro*. Whilst the lymphocytic infiltration was analysed using histological sections in this study no account was made for the relative sizes of the livers and so a more quantitative measure may yield different results. Lymphocyte isolation from hepatic tissue and lymphocyte counting using flow cytometry with markers specific to CD4 and CD8 would give a more accurate indication of the relative amounts of lymphocytes when correct for size of organ and may show an improved effect in the MSC treated group.

The effect of MSC on infiltrating lymphocytes in the hepatic parenchyma is likely to be key and will require further study to ascertain the significance of this. The OVA-Bil model used in this experiment involves the adoptive transfer of exogenous lymphocytes targeted specifically at ovalbumin. In order to quantify the amount of liver injury cause by the OVA-Bil mouse's own lymphocytes following stimulation from hepatic necrosis the next step would

be to used tagged OT1 and OT2 cells with, for example Green Fluorescent Protein (GFP) in order to distinguish the relative contributions to the hepatitis seen.

It is still not clear whether MSC only exert a direct effect on immune cells such as T Lymphocytes or whether they have effects on other immune cells. MSC have been shown to exert an immunosuppressive effect on Dendritic Cells[92], B Cells[93] and natural killer cells[94]. MSC can inhibit the differentiation of dendritic cells, in particular the differentiation from monocytes to immature dendritic cells, a cytokine mediated process. This inhibition not only prevents the expression of dendritic cell surface markers but also leads to a functional inhibition of dendritic cell cytokine release, in particular the inhibition of IL-12 release, a potent stimulator of T-Cells[94]. This could represent a mechanism by which MSC exert their effect on T-Cell stimulation. Further study into the mechanisms behind this process and the relative contribution to MSC mediated immunosuppression are needed. One such experiment could be to knock out macrophages using clodronate, to compare the immunosuppressive effect in the absence of this pathway.

## 4.6. Localisation of Mesenchymal Stem Cells During Immunosuppression

The current accepted position in the literature is that MSC need to access an area of inflammation in order to exert their immunosuppressive effect[95], although increasingly there is evidence that a systemic effect may also play a role[96]. In this study systemically administered MSC have a local effect on liver inflammation, reducing the amount of hepatocyte damage as demonstrated by serum ALT levels, however it is not clear from these experiment whether the MSC were acting systemically, locally or a combination of the two. Further work needs to be carried out to show, not only where MSC migrate in models of

inflammation, but also more importantly where they are functionally immunosuppressive. The use of labelled MSC with GFP in models of inflammation would address the first part of this question. Novel imaging techniques such as CryoViz™ could be used to give a high resolution (single cell level) image of a whole animal at different time points following IV

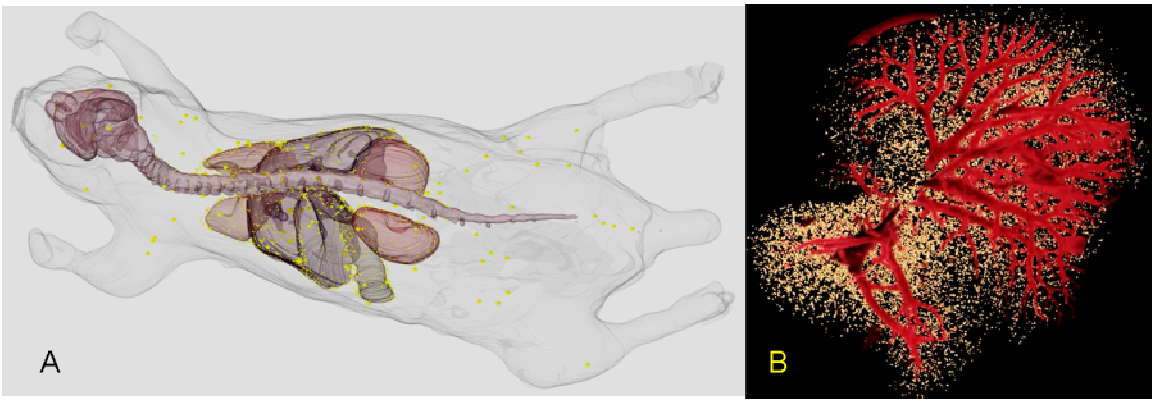


**Figure 7 Demonstration images using CryoViz™ tracking MSC with a fluorescent tag; a) whole mouse image, b) organ specific views (Provided by BioInVision)**

infusion of MSC (Figure 7)[97].

In order to address the question of where MSC exert their functional suppression it would be necessary to look at markers of MSC suppressive activity. One marker that has been found to be key in MSC induced immunosuppression is IDO and work in the Newsome laboratory so far unpublished has confirmed this. Using an RNA knockdown technique such as lentiviral transfection, markers such as IDO could be tagged with a fluorescent protein which would be expressed on translation of the RNA linked to IDO, therefore allowing tracking of functionally active MSC rather than all MSC. This could be carried out for other candidates for MSC related immunosuppression to give a rounded picture of what is expressed and where during models of inflammatory diseases.

It has recently been shown that MSC can exert a completely distant immunosuppressive effect without migrating to areas of active inflammation. This effect has been shown to be superior when encapsulated MSC are injected subcutaneously into mouse models of autoimmune liver disease and graft versus host disease[72], when compared with IV administration. So far this is the only study to suggest that MSC may have an immunosuppressive effect when encapsulated. The importance of further work looking at encapsulated MSC is twofold; from a mechanistic standpoint it enables further study into the remote effects of MSC by preventing the local effects due to the cells being trapped, secondly encapsulated cells survive longer than cells infused IV and may have a longer and more substantial effect[72].

## 4.7. Future Uses of Mesenchymal Stem Cells

This study has focussed on the use of MSC in models of autoimmune liver disease, the aim being to provide a novel treatment strategy that reduced the amount of immunosuppressant drugs required and their accompanying complications, and also to aim to reduce the number of patients going on to develop end stage liver disease and ultimately require a liver transplant. There is however another use that could be considered for MSC and that is to improve outcomes from liver transplant itself. Whilst a lot of liver diseases are treatable it is inevitable that some either do not respond to therapy, or there is no curative therapy other than liver transplantation for example PSC. Donor livers undergo an ischaemic insult prior to transplantation. This ischaemic injury is more profound in the Donation after Cardiac Death (DCD) when compared with Donation after Brainstem Death (DBD)[98], as there is usually a much longer warm ischaemia time due to the nature of the dying process. There is a considerable contribution to the resultant damage to the liver by the immune system which is functionally connected to the ischaemia reperfusion injury seen, each one mutually

reinforcing the other[99]. The immune mediated damage seen in explanted livers is a candidate for MSC therapy, but the efficacy and timing of this will require considerable further study. There may also be a role for MSC in acute and chronic rejection, another T-Cell mediated non-suppurative cholangitis[100]. Further work looking into efficacy of MSC in models of liver transplantation may yield interesting and useful opportunities for clinical translation.

# 5. CONCLUSIONS

The aims of this study were to acquire the ability to isolate purified MSC in a reproducible manner which was achieved, and to test the ability of MSC to suppress autoimmune mediated damage in a model of autoimmune hepatitis, which was partially achieved. This study was a pilot study and has demonstrated feasibility in carrying out the described techniques. The burden of end stage liver disease remains great and novel therapeutic strategies are required. Mesenchymal stem cells may have a role to play in the treatment of diseases due to autoimmunity and further study into both the efficacy and mechanism of action of these cells is required.

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