INVESTIGATING THE IMMUNOMODULATORY ROLE OF MESENCHYMAL STROMAL CELLS IN PRIMARY SCLEROSING CHOLANGITIS

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Declaration

I, Ashnila Janmohamed confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Background and Aims: Liver-infiltrating T lymphocytes and high levels of tumour necrosis factor (TNFα) are implicated in the destruction of bile ducts in primary sclerosing cholangitis (PSC). Mesenchymal stromal cells (MSC) possess broad immunomodulatory properties, suggesting a potential therapeutic role in PSC. Herein, we sought to investigate the immunomodulatory role of umbilical cord derived MSC (UC-MSC) on T cells from patients with PSC by studying the effect of UC-MSC and UC-MSC derived conditioned media (UC-MSC-CM) on proliferation and activation of circulating and intrahepatic $CD4^+$ and $CD8^+$ T cells.

Method: Peripheral blood mononuclear cells (PBMC) were isolated from patients with PSC and hereditary haemochromatosis (controls). Intrahepatic $CD4^+$ and $CD8^+$ T cells were acquired by negative selection from explant livers of patients with PSC and primary biliary cholangitis (PBC). Cell trace violet labelled cells were co-cultured with UC-MSC at ratios; 1:1, 1:4, 1:16, 1:64 1:256 (UC-MSC: PBMC/T cells) and anti-CD3/anti-CD28 for either 4 (if intrahepatic T cells used) or 5 (if PBMC used) days. On the last day of culture, the PBMC/T cells were further stimulated with phorbol 12-myristate 13-acetate, ionomycin and brefeldin A for 4 hours. UC-MSC effect on $CD4^+$ and $CD8^+$ T cell proliferation/activation (TNFα, interferon (IFN)-γ, IL2 expression) was studied by flow cytometry. Experiments were repeated using UC-MSC-CM derived from both resting (untreated) and interferon (IFN) γ stimulated UC-MSC at concentrations ranging from 100%-50%.

Results: UC-MSC significantly suppressed proliferation of both circulating and intrahepatic PSC $CD4^+$and $CD8^+$T cells in a dose dependent manner. No significant differences in the UC-MSC inhibitory effect on T cell proliferation was seen between patients with PSC, PBC and hereditary haemochromatosis suggesting that the UC-MSC effect is not disease specific. UC-MSC significantly reduced both circulating and

intrahepatic PSC $CD4^+TNF\alpha^+$ and $CD4^+IFN\text{-}\gamma^+$expressing cells. In addition intrahepatic PSC $CD4^+IL2^+$ expressing cells were also reduced in the presence of UC-MSC. Conditioned media derived from untreated UC-MSC significantly reduced circulating PSC $CD4^+$ and $CD8^+$ T cell proliferation in a dose dependent manner confirming that cell-cell contact is not mandatory for UC-MSC to inhibit T-cell proliferation and that soluble factors are involved. UC-MSC-CM at 100% concentration reduced circulating PSC $CD4^+IFN\gamma^+$expressing cells however no effect on $CD4^+TNF\text{-}\alpha^+$, $CD4^+IL2^+$, CD8+TNF-α^+, $CD8^+IFN\gamma^+$, $CD8^+IL2^+$expressing cells was seen suggesting that the effect of UC-MSC-CM on T cell responses may not be as strong as when UC-MSC are in direct contact with T cells. No difference in the effect on T cell responses was seen between UC-MSC-CM derived from resting and IFNγ stimulated UC-MSC.

Conclusion: UC-MSC are able to suppress the proliferation and activation of PSC patient derived circulating and intrahepatic T cells. This effect of UC-MSC on T cells is not disease specific. UC-MSC can act directly and indirectly on circulating T cells. Our results support efforts to assess *in vivo* the immunomodulatory effects of UC-MSC in patients with PSC.

This thesis is dedicated to my loving family for their continued support and belief in me and for encouraging me to achieve the unthinkable

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Abbreviations

AE	Adverse event
AIH	Autoimmune hepatitis
AILD	Autoimmune liver disease
ALP	Alkaline Phosphatase
ALT	Alanine aminotransferase
APC	Antigen presenting cell
BEC	Biliary epithelial cells
BM-MSC	Bone marrow-derived mesenchymal stromal cells
CM	Conditioned media
CTV	Cell trace violet
DC	Dendritic cell
DDC	3,5-diethylcarbonyl-1, 4-dihydrocollidine
DLT	Dose limiting toxicity
DMC	Data monitoring committee
FBS	Fetal bovine serum
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FUT2	Fucosyltransferase 2
GGT	Gamma-Glutamyl transpeptidase
GVHD	Graft versus host disease
hAMSC	Human amnion-derived MSC
HFE	Hereditary haemochromatosis
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HLA-G5	Non-classic human leukocyte antigen class I molecule
IBD	Inflammatory bowel disease
ICAM-1	Intracellular adhesion molecule-1
IDO	Indoleamine 2,3-dioxygenase
IFNγ	Interferon γ
IgG	Immunoglobulin G
IL	Interleukin
iNOS	Inducible nitric oxide synthase
INR	International normalized ratio
ISCT	International society for cellular therapy
LBT	Liver blood test
LFT	Liver function test
LIMC	Liver-infiltrating mononuclear cell
LPS	Lipopolysaccharide
MAdCAM-1	Mucosal vascular addressin cell adhesion 1
MELD	Model for end-stage liver disease
MHRA	Medicines and Healthcare Products Regulatory Agency
MSC	Mesenchymal stromal cells
NHSBT	NHS Blood and Transplant
NK	Natural Killer
PAMPS	Pathogen associated molecular patterns
PBC	Primary biliary cholangitis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PGE2	Prostaglandin E2

PMA	Phorbol myristate acetate
PRR	Pathogen recognition receptors
P/S	Penicillin and streptomycin
PSC	Primary sclerosing cholangitis
RCT	Randomised controlled trial
SAE	Serious adverse event
SD	Study day
TCR	T cell receptor
TGFβ	Transforming growth factor β
Th	T helper
TLR	Toll like receptor
TNFα	Tumor necrosis factor α
Tregs	T regulatory cells
UC	Ulcerative colitis
UC-MSC	Umbilical cord derived mesenchymal stromal cells
UC-MSC-CM	Umbilical cord derived mesenchymal stromal cells conditioned media
UDCA	Ursodeoxycholic acid
ULN	Upper limit of normal
VAP-1	Vascular adhesion protein 1
VCAM	Vascular adhesion molecule
1-MT	1-methyltryptophan

CHAPTER 1.

INTRODUCTION

1.1 Primary sclerosing cholangitis (PSC)

Primary sclerosing cholangitis (PSC) is a cholestatic disease characterised by chronic bile duct destruction and progression to end-stage liver disease (1). Although PSC affects both sexes and all age groups, the majority of patients are men (65.5%) and young with mean age of diagnosis being 37 years *versus* 40 years in women (2). Clinically, PSC is characterised by the presence of intra- and extra-hepatic bile duct strictures, which can progress with time (Figure 1.1). Patients can be asymptomatic but many go on to develop recurrent episodes of cholangitis, biliary cirrhosis and eventually end-stage liver disease requiring liver transplantation.

PSC is strongly associated with inflammatory bowel disease (IBD) with some suggesting, common mechanistic pathways are involved in causing injury to both the liver and intestine. The majority of patients (~70%) with PSC will develop concomitant IBD at some point during their lifetime and 2.5-7.5% of patients with IBD may go on to develop PSC (2). The clinical phenotype of IBD in PSC is unique characterised by rectal sparing, back wash ileitis, pan colitis or predominately right-sided colitis (3). Although the liver disease and intestinal inflammation in PSC run an independent course, both PSC and IBD can also influence each other, recurrent PSC is more likely to occur in patients with either intact colon or ileo pouch-anal anastomosis (4). Also, patients with concomitant PSC and IBD are at an increased risk of developing colorectal cancer.

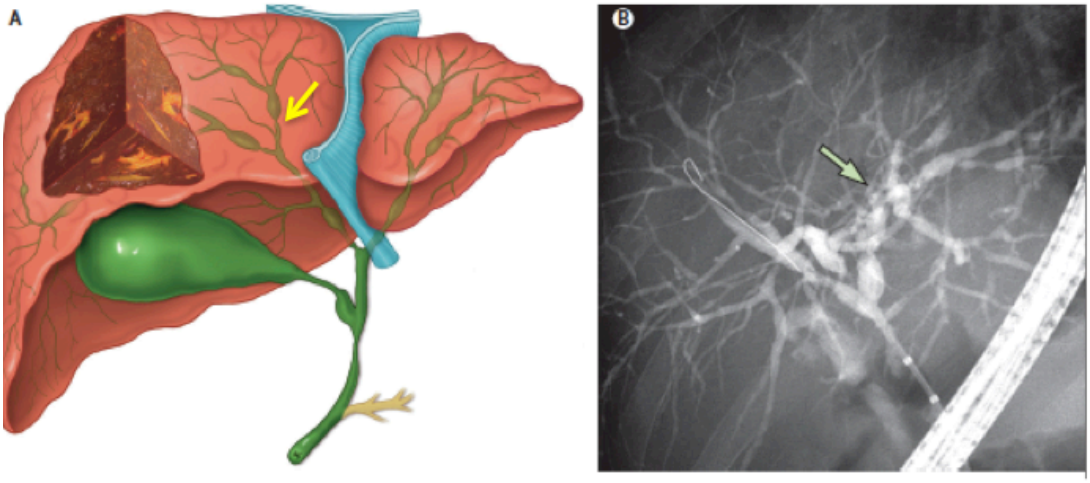

Figure 1-1: Clinical features of primary sclerosing cholangitis (PSC).

A: Presence of several strictures associated with dilatation of intra- and extra-hepatic bile ducts (yellow arrow) is a typical radiological finding in PSC. Strictures lead to cholestasis, patchy affection of peribiliary fibrosis and eventually biliary cirrhosis. **B:** Endoscopic retrograde cholangio-pancreatography image demonstrating typical features of PSC, there is presence of a dominant stricture associated with dilatation (green arrow). Figure from Hirschfield *et al* (1).

1.1.1 Burden of PSC

- The mean incidence and prevalence of PSC is reported to be up to 1.58 and 31.7 (in Finland) per 100,000 persons respectively (5-13). The highest incidence of PSC is reported in Northern Europe (Finland, 1.58 and Norway, 1.3 per-100,000 population, respectively) and North America (Minnesota 1.47) and lowest incidence is observed across the Mediterranean Basin (Italy, 0.1) (14). Reports suggest an increase in disease incidence and prevalence across North America and Northern Europe over time(14). In the United Kingdom (UK), the prevalence of PSC has reportedly increased over time from 3.23 per 100,000 patients in 1998 to 7.40 per 100,000 patients in 2014 (11). Despite being rare, PSC is disproportionately associated with complications and

mortality. There are currently no effective medical treatments available in PSC and liver transplantation remains the only curative option. More than 40% of patients with PSC require liver transplantation with the median transplant free survival ranging from 9.7 (United states) to 20.6 years (Netherland) (14). PSC accounts for 10-15% of the transplant activity in Europe and has become one of the leading indications for liver transplantation within the spectrum of cholestatic liver disease. Patients with PSC are reported to have excellent outcomes post-transplant with the European Liver Transplant Registry reporting 1-, 3- and 5-year survival rates of 86%, 80% and 77%, respectively, in patients transplanted between 1988 and 2005 (5). However, PSC can recur post-transplant leading to graft loss and subsequently re-transplantation. Variable rates of PSC recurrence are reported in the literature ranging from 10-60% of patients (15-20).

The incidence of PSC-related deaths and liver transplantation is reportedly increased in patients given a diagnosis of PSC younger than 40 years of age when the analysis is restricted to IBD patient populations specifically (14). In addition to risk of development of colorectal cancer in those with PSC and IBD, patients with PSC are at an increased risk of developing hepatobiliary malignancy, the vast majority being cholangiocarcinoma. Up to 50% of PSC related cholangiocarcinomas are diagnosed in the first year of presentation with PSC (21).

As with other liver diseases, patients with PSC-related cirrhosis can develop complications of cirrhosis and portal hypertension. A recent Mayo study by Bakhshi *et al* reported development of hepatic decompensation in 11 out of their 56 patients within 9.88 (interquartile range (IQR) 0.68–15.83) years of PSC diagnosis (22). Majority of patients in the study developed ascites (n=7), a further two patients developed variceal hemorrhage and hepatic encephalopathy. The respective 5-, 10- and 20-year

cumulative incidences of hepatic decompensation were estimated at 9.67% (IQR 4.06–21.30%), 12.90% (IQR 5.74–26.47%) and 31.58% (IQR 16.18–52.46%) respectively (22). PSC is reported to have significant impact on a patient's quality of life than healthy controls (23). Fatigue, anxiety and depression are frequently reported by patients in part due to unpredictability of disease course and lack of treatment.

Currently the demand for organs outweighs the supply and with rising liver disease, this demand will continue to increase. There is therefore an acute unmet need to develop an effective therapy in PSC that can halt the disease process and avoid development of clinical end points in PSC.

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1.1.2 Pathogenesis

Biliary epithelial cells (BEC) or cholangiocytes line the lumen of intra- and extra-hepatic bile ducts and are the primary sites of injury in PSC. The pathogenesis of PSC remains incompletely understood. It is thought that multiple genetic and environmental factors interact to cause relentless inflammation, cholestasis and fibrosis. Over time, there is progressive and chronic injury to small, medium and large bile ducts. Chronic bile duct injury eventually results in the development of concentric fibrosis around the bile ducts also defined as the 'onion-skin scar', this is pathognomonic in PSC (1).

Some of the proposed mechanisms implicated in the disease pathogenesis of PSC include genetic factors leading to immune dysregulation, homing of gut-derived T-cells to the liver, activation of the innate and adaptive immune system, microbial dysbiosis and disturbance in bile homeostasis. In early disease, only the portal tracts are involved with presence of a mixed inflammatory cell infiltrate consisting of T-cells,

plasma cells and neutrophils predominately around the bile ducts (1). Presence of inflammation, cholestasis and infection results in activation of cholangiocytes, which promote increased expression of adhesion molecules, pro-inflammatory/fibrogenic cytokines and chemokines further contributing to inflammatory as well as fibrotic responses (Figure 1.2) (24-26). There is cross- talk between activated cholangiocytes and immune cells such as T cells facilitating their recruitment to the portal areas (27). Persistent portal inflammation, bile duct destruction and periportal fibrosis eventually lead to bile duct loss, disorganised cholangiocyte proliferation and cirrhosis (1). Histologically, multiple different stages of disease processes typically occur side by side in the affected liver (28).

In this thesis, I will focus on whether umbilical cord derived mesenchymal stromal cells (UC-MSC) can modulate the activity of immune cells in particular T lymphocytes from patients with PSC thus understanding potential therapeutic approaches. With this in mind, the role of the immune system in the disease pathogenesis of PSC will be discussed in detail.

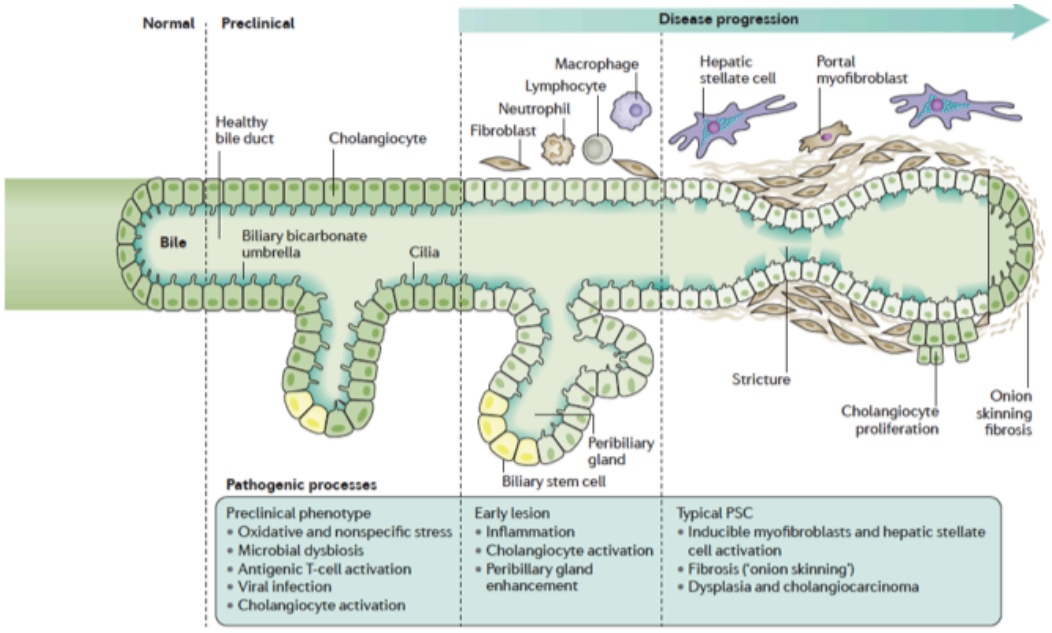

Figure 1-2: The principle aspects of bile duct lesions in primary sclerosing cholangitis (PSC).

PSC is a slow and progressive disease. The hallmark of PSC is an 'onion skin' lesion which reflects presence of concentric fibrosis around bile ducts. Histologically multiple different stages of biliary affections typically occur side by side in the diseased liver. Figure from Jiang *et al* (28).

1.1.3 Genetics and PSC

The factors responsible for initiating the process described in Figure 1.2 remain obscure. Siblings of patients with PSC are at increased risk of developing PSC (11-fold) indicating that genetic factors are possibly involved in the aetiology of PSC (29). Several susceptibility genes in PSC have been identified thus far through genome wide association studies however they contribute to a small fraction of disease susceptibility (30).

The strongest genetic associations in PSC lie within the human leucocyte antigen (HLA) region on chromosome 6 that encodes all major histocompatibility complex molecules. This region plays an important role in immune function. At least 31 non-HLA PSC risk loci have been identified (28). The overall genetic architecture in PSC is demonstrated to be similar to other autoimmune diseases such as type 1 diabetes mellitus, rheumatoid arthritis and coeliac disease more so than it is to ulcerative colitis (UC) and Crohn's disease implying that PSC is associated with other autoimmune diseases and not just IBD (28). Most of the non-HLA risk loci identified in PSC are associated with other immune mediated or autoimmune conditions. Only half of the PSC genes show an association with IBD and less than 10% of the IBD genes with PSC (28). The extent of comorbidity between PSC and IBD is not fully explained by shared genetic risk, other factors are thus likely to play a role (31). PSC is genetically more correlated to UC than Crohn's disease (31).

1.1.3.1 HLA type genes and PSC

The strong HLA association in PSC implies that the adaptive immune system is involved, with HLA class I and/or class II molecules most likely responsible. HLA class I which are expressed on all cells and HLA class II, expressed on antigen presenting cells (APCs), present endogenous and exogenous antigens to $CD8^+$ and $CD4^+$ T cells

respectively via a T-cell receptor (TCR). The antigen in PSC remains unknown. Early studies from Norway and the UK assessing the HLA association with PSC identified *HLA-B8* (*HLA-B*0801*) in the class I region and *HLA-DR3* (*DRB1*0301*) haplotypes in class II as important susceptibility markers (32, 33). The highest relative risk to developing PSC is conferred by the *DRB1*03, DQA1*0501* and *DQB1*02* homozygous genotype (16% of PSC patients versus 1% of controls; relative risk=16 $p<0.00001$ (34, 35).

1.1.3.2 Non-HLA type genes and PSC

The non-HLA findings in PSC indicate that the pathogenesis of PSC could be related to bile acid toxicity, immunological mechanisms and mechanisms associated with concomitant IBD.

Both the innate and adaptive immune systems are proposed to be involved in the disease pathogenesis of PSC. Abundant T-cells are found in close proximity to the bile ducts in PSC livers and are the predominant cell type in the portal inflammatory infiltrate in liver biopsies of PSC patients (36, 37).

PSC risk genes such as *PRDX5*, *TGR5*, *PSMG1*, *NFKB1* and *REL* may play a role in the innate immune responses through involvement in the lipopolysaccharide (LPS) induced toll like receptor 4 (TLR4)- NFκB signalling pathway (28). The LPS-TLR4-NFκB axis was demonstrated to result in biliary inflammation in the $CFTR^{-/-}$ mouse model (38). Exposure of cholangiocytes to gut-derived bacterial components such as LPS is proposed to cause disruption of tight junctions via TLR4 dependent mechanisms exposing cholangiocytes to a variety of toxic substances, such as bile acids, eventually causing injury and inflammation (39). In animal models, this mechanism is proposed to result in similar biliary injury seen in PSC (40).

Many T cell susceptibility genes have been identified such as *IL2/IL21*, *IL2RA*, *HDAC7*, *SIK2*, *PTPN2*, *SH2B3*, *CTLA4/CD28*, *IL2/IL21*, MMEL1/TNFRSF14, *CCL20*, *CD226*, *FOXP1*, *CCDC88B* and *PRKD2* which are crucial for the development and effector function of other T cell subsets and may play a role in adaptive immune responses in PSC (30). The practical utility of the identified genetic associations to biological pathways involved in disease pathogenesis of PSC remains speculative. For example an association with interleukin (IL)-2 signalling implies that T regulatory cells (Tregs) are involved in disease pathogenesis of PSC. The interleukin 2 receptor subunit alpha (*IL2RA*) forms one part of the IL2 high-affinity receptor and plays a key role in development and maintenance of Tregs. In addition it is involved in the adaptive effector response. In PSC, a reduction in the frequency of peripheral blood Tregs and intrahepatic $Foxp3^+$ T cells with impaired suppressive capacity has been reported and this correlated with homozygosity for the risk IL2RA allele (37, 41). This is presumed to contribute to loss of self-tolerance and perpetuation of chronic inflammatory damage. *CD28* is a co-stimulatory molecule required for T cell activation, survival and proliferation. The combination of TCR and CD28 increases IL2 production in activated T cells especially in $CD4^+$ T cells. Liver infiltrating T cells in PSC were found to lack CD28 and produce high levels of pro-inflammatory cytokines (37).

1.1.4 Environmental factors and PSC

The role of environmental factors in pathogenesis of PSC remains elusive. Smoking and coffee were found to be protective in PSC (42-44) although in the case of smoking, this may only be the case in patients with concomitant IBD (45). A positive association between recurrent urinary tract infections and PSC is reported (45). Interestingly, a higher frequency of urinary tract infections is also seen in patients with another

cholestatic liver disease namely, primary biliary cholangitis (PBC). In PSC, hormonal factors may play a role in influencing disease, as female PSC patients were less likely to receive hormone replacement therapy than controls (45). PSC is more common in men potentially implying increased oestrogen may explain the gender differences seen in PSC patients. Oestrogen is reported to possess anti-inflammatory properties by down-regulating the expression of adhesion and chemokine molecules (46). A study found that dietary habits and methods of food preparation differed in patients with PSC compared to controls suggesting alterations in intestinal microbiome may have a role to play in pathogenesis of PSC (45).

1.1.5 Innate immunity

The strong association between PSC and IBD makes a plausible suggestion that PSC shares similar pathogenic mechanisms with IBD. The latter is thought to result from an abnormal innate response to antigens of the intestinal flora with subsequent activation of the adaptive immune system. Similarly, it is proposed that development of PSC may be initiated by exogenous pathogens such as bacteria or pathogen-associated molecular patterns (PAMPs) (39). Figure 1.3 summarizes the proposed mechanism of tissue injury in PSC. In the presence of intestinal inflammation and increased gut permeability, microbes or metabolites of enteric microbiota from the gut enter the hepatic sinusoids via the entero-hepatic circulation and have the potential of activating the immune system resulting in inflammation (39). Sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells, hepatocytes, dendritic cells and monocytes recognize gut-derived microbial products via pattern recognition receptors (PRR) initiating a pro-inflammatory response (39). Similarly, PAMPs such as LPS, peptidoglycans and methylated bacterial dinucleotide motifs activate macrophages, dendritic cells and natural killer (NK) cells through PRR including TLRs and CD14 resulting in

inflammatory cytokine production (tumor necrosis factor (TNF) α and IL1β) and subsequently promoting recruitment and activation of lymphocytes (47).

Cholangiocytes express multiple TLRs (48). Under normal circumstances, gut commensals and pathogens are confined to the gut by mesenteric lymph nodes (49) moreover there is tight control of PRR on BECs preventing constant activation of the innate system from repeated exposure to intestinal endotoxins in bile. In PSC however there is hyper-responsiveness of the innate immune system and loss of immune tolerance to repeated endotoxin exposure (50). In PSC liver, cholangiocytes have increased TLR expression and there is activation of the MyD88/IRAK signaling complex; upon pathogen recognition pro-inflammatory mediators such as TNFα, INFγ, IL6 and IL8 are produced which promote further recruitment and activation of immune cells including T cells, macrophages, neutrophils, NK cells (50). The presence of abundant levels of TNFα and INFγ is proposed to lead to endotoxin hyper-responsiveness whilst absence of these inflammatory mediators results in cholangiocytes in PSC reverting to a phenotype capable of initiating endotoxin tolerance (39, 50).

The number of macrophages and monocyte-derived macrophages, which are peri-biliary and possess a pro-inflammatory phenotype are increased in livers of patients with PSC and murine models of sclerosing cholangitis (51-54). Depletion of monocytes improved disease severity in murine models of sclerosing cholangitis suggesting a possible role in the pathogenesis of PSC (52). Monocytes contribute to pathogen surveillance, antigen presentation, induce effector T cell function, produce pro-inflammatory cytokines and stimulation of epithelial cells (51). In a recent study by Kunzmann *et al* monocytes from patients with PSC were shown to produce high levels of Th17 inducing cytokines, IL1β and IL6 upon microbial stimulation with *Candida*

albicans compared to healthy controls (54). Microbe-activated monocytes induced the secretion of Th17 and monocyte recruiting chemokines CCL20 and CCL2 in human primary cholangiocytes in addition to pro-inflammatory cytokines IL6 and IL23. Both Th17 cells and $CD14^+$ monocytes/macrophages were found close to bile ducts within portal tracts of PSC patients. RNAscope® staining of liver tissue from PSC patients revealed mRNA expression of IL1β in both $CD14^+$ monocytes/macrophages within inflamed portal tracts and cholangiocytes of patients with PSC. The authors of this study propose that activated monocytes via positive feedback loop could be inducing the differentiation of Th17 cells, thereby amplifying their own recruitment and Th17 cells in to the periductular area of PSC liver (54).

1.1.6 Adaptive immunity

In PSC, a mixed inflammatory cell infiltrate comprising of lymphocytes, plasma cells, neutrophils, NK cells, Kupffer cells and perisinusoidal macrophages is present (39). T lymphocytes comprise of 80% of peri-portal mononuclear infiltration (55, 56). They are shown to produce high levels of TNFα in PSC livers classifying PSC as a predominantly T-helper 1 (Th1)- mediated disease (56). Liaskou *et al* demonstrated in their study that in PSC livers, $CD4^+$ T cells were localized mainly to the portal tracts and $CD8^+$ T cells in the portal areas and liver parenchyma (37). Both $CD4^+$ and $CD8^+$ T cells were located in close proximity to bile ducts (37). PSC lymphocyte mononuclear cells (LIMCs) were found to release pro-inflammatory cytokines specifically TNFα and IFNγ 24 hours post-culture without stimulation indicating that PSC patients have increased levels of TNFα and IFNγ (37). The same study found that continuous exposure to TNFα resulted in loss of expression of CD28 on PSC $CD4^+$ and $CD8^+$T-cells (37). $CD4^+CD28^-$ T-cells are chronically active pro-inflammatory Th1 cells implicated in the pathogenesis of inflammatory diseases (57-59). A significantly greater

proportion of $CD4^{+}CD28^{-}$ T cells releasing large amount of TNFα and IFNγ were found in PSC livers compared with PBC and non-alcoholic steatohepatitis livers and normal liver (37). This is proposed to result in an autocrine-like induction of further $CD28^{-}$ T cells and Th1 mediated immune injury to the bile ducts, where $CD4^{+}CD28^{-}$ T cells accumulate (37, 60). An elevated number of γδ T cells are also found in peripheral blood and portal areas of patients with PSC with these cells expressing CD45RO and IL2, suggesting an activated memory phenotype (61) and a role in immune-mediated liver injury. Using single cell RNA sequencing methods, Poch *et al* recently showed that, there is a greater proportion of tissue-resident naïve-like $CD4^{+}$ T cells in livers of patients with PSC (62). These tissue resident naïve-like CD4+ T cells were further demonstrated to have the propensity to acquire a Th17 polarisation state (62).

The role of Th17 cells and gut dysbiosis in PSC has been highlighted in a few studies (63, 64). Activated Th17 cells release IL17, IL21 and TNFα, which promote inflammation via recruitment of other immune cells and also contribute to epithelial repair by secreting IL22 (39). Patients with PSC were found to have the highest number of IL17 producing circulating $CD4^{+}$ T cells compared to other inflammatory liver diseases including PBC, healthy controls and IBD only patients indicating increased Th17 differentiation in vivo (54). Th17 cells play an important role in maintaining mucosal barriers and providing protection against pathogens at mucosal surfaces. Influx of gut microbiota upon breakdown of intestinal epithelial barrier for example in the presence of colonic inflammation in IBD can induce a Th17 immune response. PSC patients with or without IBD have impaired gut microbiota evidenced by reduced microbial diversity and altered composition (65) compared to those with UC alone (66, 67). Bile of patients with PSC is frequently colonized with bacteria. Ductal bile fluid of patients with PSC was found to have reduced biodiversity, altered microbiome and increased presence of Enterococcus faecalis (68). Katt *et al* showed an increased

production of IL17 by peripheral blood mononuclear cells (PBMC) from patients with PSC after pathogen stimulation and localisation of IL17 expressing lymphocytes around PSC bile ducts (63). An elegant study by Nakamoto *et al* (64) further confirmed that PSC patients had gut dysbiosis with identification of increased prevalence of *Klebsiella pneumonia*, *Proteus mirabilis* and *Enterococcus gallinarum* in faecal samples of PSC patients. The authors further studied the role of gut dysbiosis and pathogenesis of PSC by transplanting faecal samples derived from patients with PSC/UC (PSC/UC mice), UC alone (UC mice) or healthy controls (HC mice) in germ free mice. PSC/UC mice exhibited potent Th17 priming in the liver, increased IL17 expression both in the liver and colon compared to HC mice who had induction of Th17 priming in the colon only. PSC/UC mice exposed to 3,5-diethylcarbonyl-1, 4-dihydrocollidine (DDC), a toxic agent that generates biliary injury mimicking PSC were more susceptible to hepato-biliary injury as evidenced by increased level of cholestasis and peri-ductal fibrosis. Inoculation of the 3-mix-bacteria (*Klebsiella pneumonia*, *Proteus mirabilis* and *Enterococcus gallinarum)* in germ free mice resulted in the translocation of bacteria from the intestinal mucosa to mesenteric lymph nodes and enhanced Th17 induction as compared to when the bacteria was used individually or in combination. Use of a selective inhibitor of Th17 differentiation in the study, improved hepato-biliary injury and fibrosis of DDC-fed 3 mix bacteria germ free mice suggesting a role of Th17 activation in pathogenesis of biliary disease. Interestingly PSC derived-*Klebsiella pneumonia* was shown to disrupt the epithelial barrier by inducing pore formation to initiate bacterial translocation and hepatic inflammatory responses providing some mechanistic insight in to bacterial translocation and liver inflammation (64).

Impaired immune regulation is implicated in the pathogenesis of PSC. As highlighted earlier, circulating Tregs and intrahepatic $Foxp^{3+}$ cells are reduced in number and

function in patients with PSC (41). Tregs and Th17 cells share the same CD4 progenitor which in the presence of transforming growth factor β (TGFβ) only, differentiates in to Tregs and in the presence of both TGFβ and IL6 in to Th17 cells (39). Tregs can thus switch to Th1/Th17 lineage in an inflammatory environment (69). The hepatic microenvironment of immune mediated diseases including PSC is rich is pro-inflammatory cytokines such as IL6, IL8, IFNγ and IL1β and deficient in IL2 (70). IL2 is important for the survival of Tregs. Chen *et al* showed that liver infiltrating Tregs reside close to bile ducts and were susceptible to CD95-FASL medicated apoptosis either through contact with BEC or BEC supernatant (70). Schwinge *et al* highlighted the role of IL12 signalling and Tregs in the pathogenesis of PSC (71). IL12 has previously been reported to decrease Foxp3 expression (72), which is essential for the suppressive capacity of Tregs (73). $Mdr2^{-/-}$ (a mouse model of sclerosing cholangitis) and DDC-fed mice treated with IL2/IL2Ab complex but not saline increased the proportion of Tregs in the liver which were localized to inflamed portal tracts compared to spleen however this did not decrease the portal inflammation or fibrosis. Interestingly the same treatment in DSS-colitis, a control model improved the disease course highlighting that there may be organ specific differences in the function of Tregs. Enriched hepatic but not splenic Tregs in wild type mice following treatment with IL2/IL2Ab complex had reduced suppressive capacity and Foxp3 expression with increased IL12rb2 chain expression seen in hepatic but not splenic Tregs. Schwinge *et al* (71) further showed that liver derived Tregs from IL12 receptor beta 2 deficient mice after in vivo enrichment had increased suppressive capacity compared to wild mice after IL2-complex treatment and in vitro treatment of lymphocytes with IL12 resulted in phospho-Stat4 activation and reduced Foxp3 expression in Tregs. Interestingly increased expression of IL12 within the livers but not spleens of wild type and $Mdr2^{-/-}$ was seen following IL2/IL2Ab treatment highlighting that IL12 signalling may contribute to dysfunction of Tregs in murine models of sclerosing cholangitis (71).

Homing of gut-lymphocytes is another proposed mechanism in the pathogenesis of PSC. It is suggested that memory T lymphocytes activated in the inflamed gut and recruited to the liver via interaction with ectopically expressed adhesion molecules and chemokines contribute to portal inflammation in PSC (39). In PSC, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), an endothelium adhesion molecule normally restricted to the gut is found on the portal epithelium whilst CCL25, a chemokine is found on sinusoidal endothelium (74, 75). Dual-color immunofluorescence staining demonstrated adhesion of α4β7 lymphocytes (gut-derived lymphocytes) to MAdCAM-1-positive vessels and adhesion assays confirmed functionality of the interaction in inflamed livers from patients with PSC and IBD (76). Approximately 20% of liver infiltrating lymphocytes from PSC livers as compared to <2% in normal and PBC livers express CCR9, a receptor for CCL25 (75). $CCR9^+$ expression was strongly associated with co-expression of the gut integrin α4β7 (75). Most liver infiltrating $CCR9^+$ lymphocytes are $CD45RA^-CCR7^-CD11a^{hi}$ and secrete IFNγ in keeping with long-lived memory phenotype (75) which upon reactivation result in a T cell rich portal infiltrate within the liver, and thereafter an inflammatory response. The origin of $\alpha4\beta7^+CCR9^+$ lymphocytes being gut is supported by findings that showed hepatic dendritic and stellate cells failed to imprint these homing markers on $CD8^+$ T cells (77). PSC patients also have altered expression of CCL28, CXCL12 and CXCL16 (39). Both CCL28 and CXCL12 are capable of triggering α4β7-mediated adhesion of human lymphocytes to MAdCAM-1 in vitro (39, 78, 79). Once $\alpha4\beta7^+CCR9^+$ lymphocytes are in the liver, they may use other chemokines such as CXCL12 and CXCL16 to localize to biliary epithelium where they can destroy bile ducts (39). Some of the recruited $\alpha4\beta7^+$ T-cells may undergo local differentiation to express αEβ7 integrin providing another pathway to bind to biliary epithelium (76). Further evidence for the enterohepatic circulation of immune cells comes also from our TCR studies where we saw T cells with the same TCR overlapping between gut and liver tissue samples (80).

A combination of hepatic inflammation and activation of vascular adhesion protein-1 (VAP-1) by nutrients or bacteria in portal blood results in aberrant hepatic expression of MAdCAM-1 and CCL25 and subsequently recruitment of mucosal T cells to the liver (1, 81). VAP-1 is expressed on sinusoidal endothelium and is both an adhesion molecule as well as an ectoenzyme with potent amine oxidase activity (81). Hepatic VAP-1 enzyme activity is significantly increased in PSC and at similar levels to UC as compared with PBC, autoimmune hepatitis (AIH) and non-diseased livers (82). In vitro flow assay studies showed that activation of VAP-1 by cystaemine, a VAP-1 substrate that can be released by inflamed colonic epithelium and gut bacteria, in the presence of TNFα led to increased expression of MAdCAM-1 on the hepatic endothelium and adhesion of mucosal $\alpha4\beta7^+$ T cells (82).

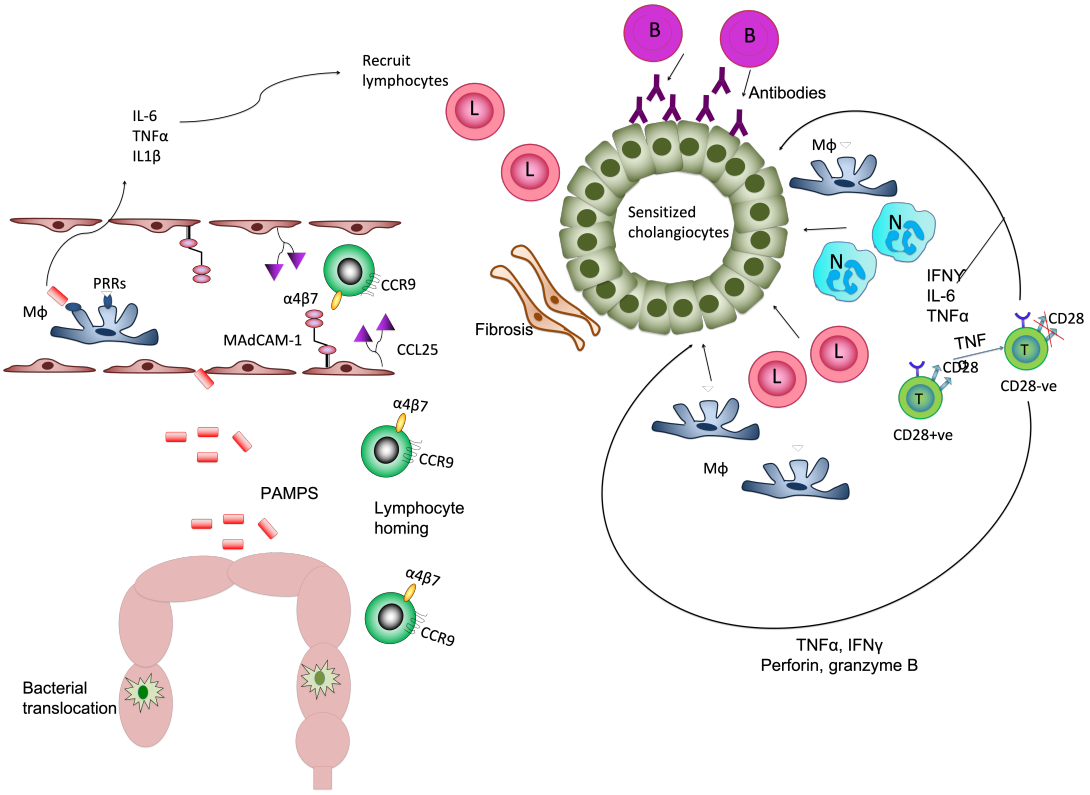

Figure 1-3: Proposed mechanism of tissue injury in primary sclerosing cholangitis (PSC)

a) Potential gut derived pathogens including microbes or metabolites of enteric microbiota enter the hepatic sinusoids via the entero-hepatic circulation. Sinusoidal endothelial cells Kupffer cells ,hepatocytes, hepatic stellate cells, dendritic cells and monocytes through pattern recognition receptors (PRRs) are able to recognize and respond to pattern-associated molecular patterns (PAMPs). Upon pathogen recognition, monocytes produce TNFα, IL6 and IL1β

b) Pro-inflammatory cytokines including TNFα, IL6 and IL1β promote the recruitment of T cells which contribute to further inflammation

c) Hepatic inflammation leads to up-regulation of MADCAM1 and CCL25 on endothelial cells thereby promoting the recruitment of gut derived α4β7+ effector T cells to the liver. Once $\alpha4\beta7^{+}$ lymphocytes are in the liver, they may use other chemokines such as CXCL12 and CXCL16 to localize to biliary epithelium where they can destroy bile ducts

d) High amount of TNFα in the microenvironment of the liver can result in loss of CD28 from T cells. CD28-T cells are pro-inflammatory cells that can release TNFα and IFNγ, perforin and granzyme leading to activation and death of biliary epithelial cells

e) Autoantibodies against biliary epithelial cells can activate the immune system by up-regulating toll like receptors, increasing expression of IL-6 and adhesion molecules such as CD44 that could thereby promote lymphocyte proliferation, Ig production and cell adhesion

Abbreviations: B- B-cells; Mo- Monocytes; N- Neutrophils; PAMPS- pattern-associated molecular patterns; PRR-pattern recognition receptor

1.2 Mesenchymal stromal cells (MSC)

1.2.1 Definition, biology and origins

Friedenstein *et al* was the first to describe isolation of mesenchymal stromal cells (MSC) (83), a heterogeneous population of plastic adherent fibroblast –like cells that have the ability to self-renew, proliferate and differentiate into tissues of mesodermal origin including osteocytes, adipocytes and chondrocytes.

Originally, MSC were identified and isolated from bone marrow however due to their low frequency (0.01 to 0.001% of total bone marrow cells) (84) and invasive process of acquisition of MSC from bone marrow, alternative sources of MSC were sought. Over the years, MSC have been successfully isolated from many human tissues including adipose tissue (85), synovial membrane (86), umbilical cord blood (87) and tissue (88), amniotic fluid (89) and placenta (90). Umbilical cord tissue is an attractive alternative source of MSC owing to high availability, non-invasive procedure and no ethical restrictions (no harm to mother or baby) of MSC acquisition as well as the higher expansion capacity compared to other tissue sources (91, 92). Umbilical cord derived MSC (UC-MSC) are more primitive and can be acquired from several components of the umbilical cord including arteries, veins, perivascular tissue and Wharton's jelly.

MSC possess both immunoregulatory and regenerative properties. It is their immunoregulatory property which has been exploited and extensively studied. MSC have been shown to act on a range of immune cells to exert an anti-inflammatory effect including T cells (reduce T cell responses including proliferation and pro-inflammatory cytokine production, induce Treg production), B cells (reduce B cell production and the ability of B cells to produce immunoglobulins) and monocytes (increase number of regulatory macrophage population (M2 macrophages) in the circulation and increase

IL10 (anti-inflammatory cytokine) expression. It is well reported that MSC from different sources are morphologically and immuno-phenotypically similar. There are however small differences reported in the level of tri-lineage differentiation (93) and functional capabilities (e.g. immunomodulation) potentially as a result of microenvironmental niche, local function (stromal support of haematopoiesis in the bone marrow and immune homeostasis by the adipose tissue), ontogenetic stage (birth associated in the case of umbilical cord tissue versus adult), isolation and culture procedure (94).

The heterogeneity of MSC and different experimental protocols makes it difficult to draw firm conclusions regarding differences between MSC derived from alternative sources and immunomodulation. In addition, most researchers use a single MSC source in experiments to enable consistency of data, comparative studies are therefore infrequent. The majority of studies have demonstrated no significant differences in the immunomodulatory potential of MSC from alternative sources although frequently stronger immunosuppressive capabilities than bone marrow-derived MSC (BM-MSC) have been reported (94). Li *et al* demonstrated that MSC from Wharton's Jelly was strongest at inhibiting T cell proliferation compared with adipose, bone marrow, and placenta derived MSC (95). Similarly Barcia *et al* showed UC-MSC have significantly greater potential to induce Tregs than BM-MSC (96). Najar *et al* in a comparative study demonstrated that both Wharton Jelly and adipose tissue derived MSC exerted greater immunosuppressive effect on T cell responses including prevention of lymphocyte activation and suppression of T cell proliferation compared to BM-MSC (97). Despite the limited comparative studies, MSC have great potential as therapy for immune-mediated diseases. Future work is however needed in understanding the immunomodulatory mechanisms and impact of heterogeneity of MSC from different sources. This will help in directing choice between different sources in the clinical setting and subsequently improve efficacy of MSC-based therapy.

1.2.2 Phenotypic characterization of MSC

Historically, lack of uniform criteria to define human MSC impacted on efforts to compare results derived from experimental and clinical studies prompting the International Society for Cellular Therapy (ISCT) to formulate a minimal criteria for defining human MSC (98). Dominici *et al* summarized three criteria to identify MSC including: 1) MSC should be plastic adherent in standard culture conditions, 2) MSC should exhibit positive surface expression for CD73, CD90, CD105 by flow cytometry and lack expression of CD45, CD34, CD14, CD11b, CD79α, CD19, HLA class II and 3) MSC must be able to differentiate in to adipogenic, osteogenic and chondrogenic lineages (98). It is widely reported that UC-MSC possess all three of the identification criteria set out by the ISCT (99). Figure 1.4 summarises the criteria to identify UC-MSC.

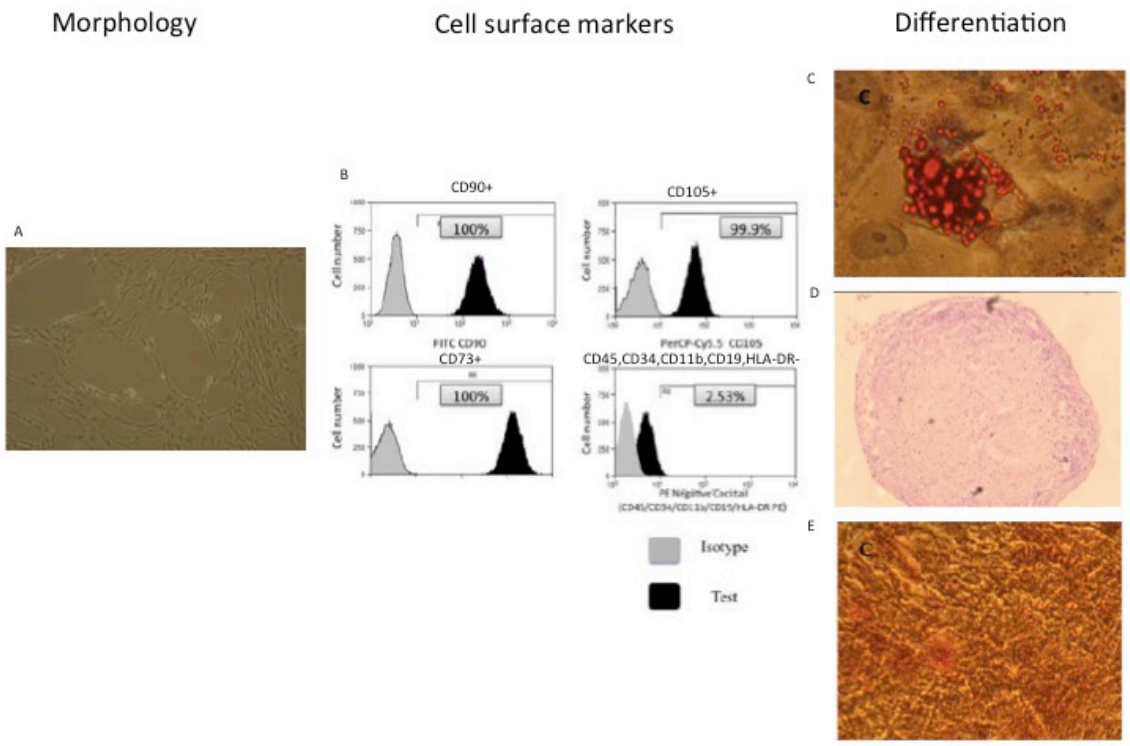

Figure 1-4: Phenotypic and functional characterization of umbilical cord derived mesenchymal stromal cells (UC-MSC). Data acquired with permission from Dr Mohamed Alfaifi.

Image demonstrating that UC-MSC fulfil all three criteria set out by the International society for cellular therapy including **A:** a) Plastic adherence and spindle shaped morphology. **B:** Flow cytometry plots demonstrating positive staining for typical MSC surface markers- CD105, CD90 and CD73. Differentiation in to C) Adipocytes; oil red staining of a lipid deposition cell D) Chondrocytes; cartilage matrix staining with alcian blue. E) Osteocytes; alizarin red staining of a calcium deposit

1.2.3 Functional characteristics of MSC

Physiologically, MSC play a role in maintenance of homeostasis. MSC provide structural support to most vertebrate organs through production and regulation of extracellular matrix via expression of matrix metalloproteinases. MSC support normal blood vessel structure and function with pericytes and perivascular smooth muscle cells, which regulate vasoconstriction and vascular permeability through paracrine or juxtracrine interactions with endothelial cells (100). MSC form perivascular niches/matrices where they regulate stem cell maintenance and differentiation (haematopoietic or epithelial) and are capable of self-renewal (101). Distinct MSC in different organs have organ specific roles for example lubrication of joints by synovial fibroblasts to enable movement (100).

MSC are known to play a role in wound healing when there is tissue damage. Tissue injury and damage results in activation of mesenchymal populations, release of pro-inflammatory mediators and initiation of an antifibrinolytic – coagulation cascade leading to further release of vasoactive factors, cytokines, chemokines, and recruitment of immune cells such as macrophages and leukocytes (100). There is interaction of fibroblasts, fibrocytes and resident cells including activated MSC inducing differentiation in to myofibroblasts, which synthesize extracellular matrix, produce angiogenic and growth factors including pro-fibrotic TGF β, α smooth muscle actin and vimentin to remodel the microenvironment and restore normal architecture (100). MSC revert to their original state or die through programmed cell death or immune mediated killing upon resolution of inflammation or tissue repair (102). Failure of tissue repair mechanisms or persistent inflammation/injury leads to further activation of MSC and subsequent increased production of extracellular matrix and fibrosis (100).

1.2.3.1 Immunomodulatory function of MSC

MSC are able to modulate immune responses either through direct cell-to-cell (MSC to immune cell) contact or indirectly through paracrine activity of secreted growth factors, cytokines, and hormones and release of extracellular vesicles that contain reparative peptides/proteins, mRNA, and microRNAs (103). MSC can influence the activity of both the innate and adaptive immune system and are deemed to be immunoprivileged due to low level of HLA class I expression, lack of expression of HLA class II and co-stimulatory molecules including CD40, CD80 and CD86 (104, 105).

1.2.3.1.1 MSC effect on the innate immune system

Macrophages differentiated from monocytes play an important role in the innate immune system. In addition to removing dying/dead cells and cellular debris monocytes/macrophages are also effector immune cells involved in cell-mediated immune responses. Depending on the microenvironment, macrophages are able to polarize into classically activated M1 macrophages, which possess strong antimicrobial activity or alternatively activated M2 (regulatory macrophage population) macrophages, which have anti-inflammatory properties. Polarization is driven by MSC ability to secrete soluble factors including IL10 and IL1Ra, which were shown to attenuate liver injury by promoting the number of M2 macrophages (106). Both *in vitro* and *in vivo* studies have demonstrated the capability of MSC to induce an immunoregulatory type of macrophages and improve survival of monocytes through upregulation of CCL18 (107). Co-culture of monocytes with human or mouse BM-MSC induced M2 macrophages associated with high IL10 and low levels of TNFα, IFNγ and IL12 production (108-110). Similarly infusion of both BM-MSC and umbilical cord perivascular cells in an experimental acute myocardial infarction mouse model led to

increased number of alternatively activated M2 macrophages in the circulation with decreased IL1β and IL6, and increased IL10 expression (111). Nemeth *et al* further demonstrated the role of MSC in reprogramming macrophages in a murine model of sepsis. The authors demonstrated that upon activation by LPS or TNFα, BM-MSC secreted prostaglandin E2 (PGE2) leading macrophages to release of anti-inflammatory IL10, which blocked further neutrophil infiltration into injured tissue preventing further damage and improving survival (112). Polarization to M2 macrophages by MSC has been shown to lead to generation of Tregs via production of CCL18 by MSC-induced M2 macrophages (107).

Dendritic cells (s) are important for antigen presentation to naïve T cells and subsequent initiation of a primary immune response as well as in tolerance. *In vitro* studies have shown that MSC can inhibit the maturation of monocytes, cord blood and $CD34^+$ haematopoietic progenitor cells in to DCs (101). Furthermore, MSC can modulate DC function by: a) impairing the ability of DCs to present antigen through decreased expression of co-stimulatory molecules CD80 and CD86 and presentation molecules HLA-DR (113) b) decreasing production of pro-inflammatory molecules including IL12, TNFα and IFNγ and upregulating production of anti-inflammatory IL10 by DCs (114, 115). Conditioned supernatants acquired from co-culture of murine MSC with splenocytes were found to have high levels of IL6 secreted by MSC (116). Bone marrow progenitors cultured in the presence of this conditioned supernatant were partly inhibited to differentiate into functional mature DCs with IL6 involved in the inhibitory action (116).

NK cells play an important role in the elimination of both tumour and virus-infected cells through their cytolytic activity and production of pro-inflammatory cytokines including TNFα and IFNγ. Their function is regulated by cell-surface receptors that transduce

either inhibitory or activating signal. Culturing in the presence of IL2 and IL15 enables proliferation of resting NK cells and enhances their cytolytic activity (101). Co-culture of MSC with pre-activated NK cells (NK cells cultured long term in IL2 for more than 7 days) and freshly isolated (resting/unactivated) NK cells in the presence of exogenous cytokines such as IL2 reduced the ability of NK cells to proliferate, exert cytotoxic activity and produce cytokines including IFNγ (101, 114, 117). MSC induced Inhibition of NK cell proliferation was found to be more profound with resting/unactivated NK cells as compared with pre-activated NK cells, which MSC had partial inhibitory effect (117). Both autologous and allogeneic MSC were killed by cytokine-activated NK cells however upon treatment with IFNγ, MSC were protected from NK- mediated lysis through upregulation of HLA class I molecules by MSC (117). This provides support to the notion that an inflammatory microenvironment rich with IFNγ may favour MSC induced inhibition of NK cell function whereas absence of IFNγ may promote elimination of MSC by activated NK cells (101). Soluble factors produced by MSC such as PGE2 and indoleamine 2,3-dioxygenase (IDO) were found to be key mediators in MSC induced inhibition of NK cell activity (118, 119). Both PGE2 and IDO are upregulated in the presence of IFNγ. The exact mechanism by which MHC class 1 expression on MSC protects against NK cell lysis is not fully understood. One explanation is expression of MHC class I on target cells prevents interaction with target antigen receptor on the NK cells (120). It is well described in the literature that low level HLA class 1 expression on MSC allows MSC to evade immune surveillance.

1.2.3.1.2 MSC effect on the adaptive immune system

A number of studies have demonstrated that MSC are able to inhibit T cell responses including T cell proliferation and effector cell function. MSC can inhibit T cell proliferation induced by mitogens (121-123), alloantigens (105, 121, 124) or specific

antigens (105). This inhibitory effect on T cell proliferation by MSC is not HLA restricted as both autologous and allogeneic MSC are effective (101). MSC are able to alter the outcome of an immune response for example through promotion of a Th1 to Th2 shift. Aggarwal *et al* have demonstrated using *in vitro* experiments that secretion of PGE2 by MSC induced DCs to upregulate IL10 production and reduce production of TNFα and IFNγ. This shifted the ratio of Th cells from a pro-inflammatory Th1 subtype to an anti-inflammatory Th2 subtype. The authors also demonstrated that presence of MSC reduced IFNγ and increased IL4 production by Th1 and Th2 effector cells respectively and that MSC promoted differentiation of naive T cells to an immunoregulatory (Treg) phenotype (114). The precise mechanism of immunomodulatory action of MSC on T cells is however not fully understood. Nicola *et al* showed that MSC inhibited the proliferation of T cells either by the secretion of soluble factors or by direct interaction of MSC with T lymphocytes (122). Direct interaction between MSC and T lymphocytes and subsequent suppression of T cell proliferation is proposed to require engagement with the inhibitory molecule programmed death 1 by its ligands (125). In addition to PGE2, other soluble molecules secreted by MSC implicated to play a role in the immunomodulatory effect of MSC on T-cell responses, include TGF β, hepatocyte growth factor (HGF) (122) and IDO (126). Notably, the production of these immunomodulatory molecules can vary according to the source of MSC; for example, Wharton jelly derived-MSC produce higher amounts of TGFβ than BM-MSC (126). Presence of an inflammatory environment in the host is important for activation of MSC and subsequent implementation of their immunomodulatory effect (127). Immunosuppressive effect of MSC including production of soluble factors can be induced and influenced by treatment with a combination of cytokines including IFNγ, TNFα and IL1β while IFNγ can independently induce IDO activity (128). Treatment of MSC with cytokines results in up-regulation of HLA class I, intracellular adhesion molecule 1 (ICAM1) and vascular adhesion molecule (VCAM) 1 on the surface of MSC

and enhances the expression of some chemokines by MSC (128). IDO, is an intracellular IFNγ dependent enzyme that induces tryptophan depletion from the local environment with tryptophan being an essential amino acid for lymphocyte proliferation. Some authors propose that the ability of MSC to suppress T cell proliferation is monocyte dependent; absence of monocytes in culture resulted in reduced inhibitory action of MSC on T cells (108, 129). Recently, Witte *et al* showed that phagocytosis of infused UC-MSC in lungs of mice induced an immunomodulatory phenotype in monocytes. The authors further proposed that circulating monocytes containing phagocytosed UC-MSC, mediated the immunomodulatory function of MSC in the rest of the organs (130).

Tregs play an important role in immune homeostasis. *In vitro* studies have demonstrated that MSC can induce the generation of $CD4^+CD25^+$ regulatory T cells from $CD4^+$ T cells through IL10 production (114, 131). Prevosto *et al* further demonstrated that the $CD4^+$ $CD25^+$ $Foxp3^+$ cells, generated from PBMC following co-culture with MSC, were functional and inhibited the proliferation of alloantigen activated T cells (132). Mechanisms involved in regulatory T cell induction are proposed to be both direct MSC-$CD4^+$T cell contact and soluble factors including PGE2 and TGFβ, which were shown to have a non-redundant role (133). Another molecule secreted by MSC implicated to contribute to MSC induced Treg generation is the non-classic human leukocyte antigen class I molecule (HLA-G5). Blocking experiments using neutralizing anti-HLA-G antibody demonstrated that HLA-G5, which is IL10 and cell contact dependent, contributed initially to the suppression of allogeneic T cell proliferation and then to the expansion of $CD4^+CD25^+FoxP3^+$ Tregs (134).

As highlighted in section 1.1.6, Th17 cells are proposed to be involved in the disease pathogenesis of PSC. MSC present at the early stages of naïve $CD4^+$ T cells

differentiating towards Th1 and/or Th17 cells suppress the proliferation and differentiation of Th1 and Th17 cells as well as induce generation of $CD4^+CD25^+Foxp3^+$ regulatory T cells (135). This mechanism is associated with an increase in IL10 production (135). Ghannam *et al* showed *in vitro* treatment with IFNγ and TNFα enhanced the expression of CD54 (ICAM1) by MSC thereby promoting the adherence of Th17 cells to BM-MSC in a CCR6-CCL20-dependent manner and enhancing their contact time. BM- MSC prevented both the differentiation of naïve $CD4^+$ T cells to Th17 cells and production of pro-inflammatory cytokines by Th17 cells including IL17 and IL22 (136). *In vivo* studies in animal models of autoimmune disease specifically multiple sclerosis and rheumatoid arthritis, have demonstrated that infused human or mouse BM-MSC led to improvement in disease activity and reduction in Th17 cell activity (137).

MSC can inhibit the proliferation of B cells and their ability to produce immunoglobulins. Murine studies have demonstrated that MSC can inhibit the proliferation of B cells stimulated with anti-CD40 and IL4 (138). Similar to murine studies, human BM-MSC co-cultured with purified B cells at a ratio of 1:1 (MSC: B cell) were found to inhibit proliferation of B cells and reduce both immunoglobulin production as well as expression of chemokine receptors such as CXCR4, CXCR5, and CCR7 without induction of apoptosis (139). CXCR4 was significantly reduced in the presence of BM-MSC even when B cells were co-cultured with BM-MSC at the lower ratio of 1:10 (MSC: B cell), suggesting that BM-MSC may preferentially target CXCR4, which has a role in the homing and fate of MSC (139). Transwell experiments separating MSC and B cells but allowing for exchange of secreted factors demonstrated that the effect of MSC on B cells is partly from the paracrine activity of soluble factors secreted by MSC (139). The importance of the cell- cell contact between MSC and T cells in MSC induced B cell inhibition has been highlighted in a study by Rosado *et al* who showed

that in the absence of $CD3^+$ T cells in culture, MSC were unable to inhibit B cell proliferation (140).

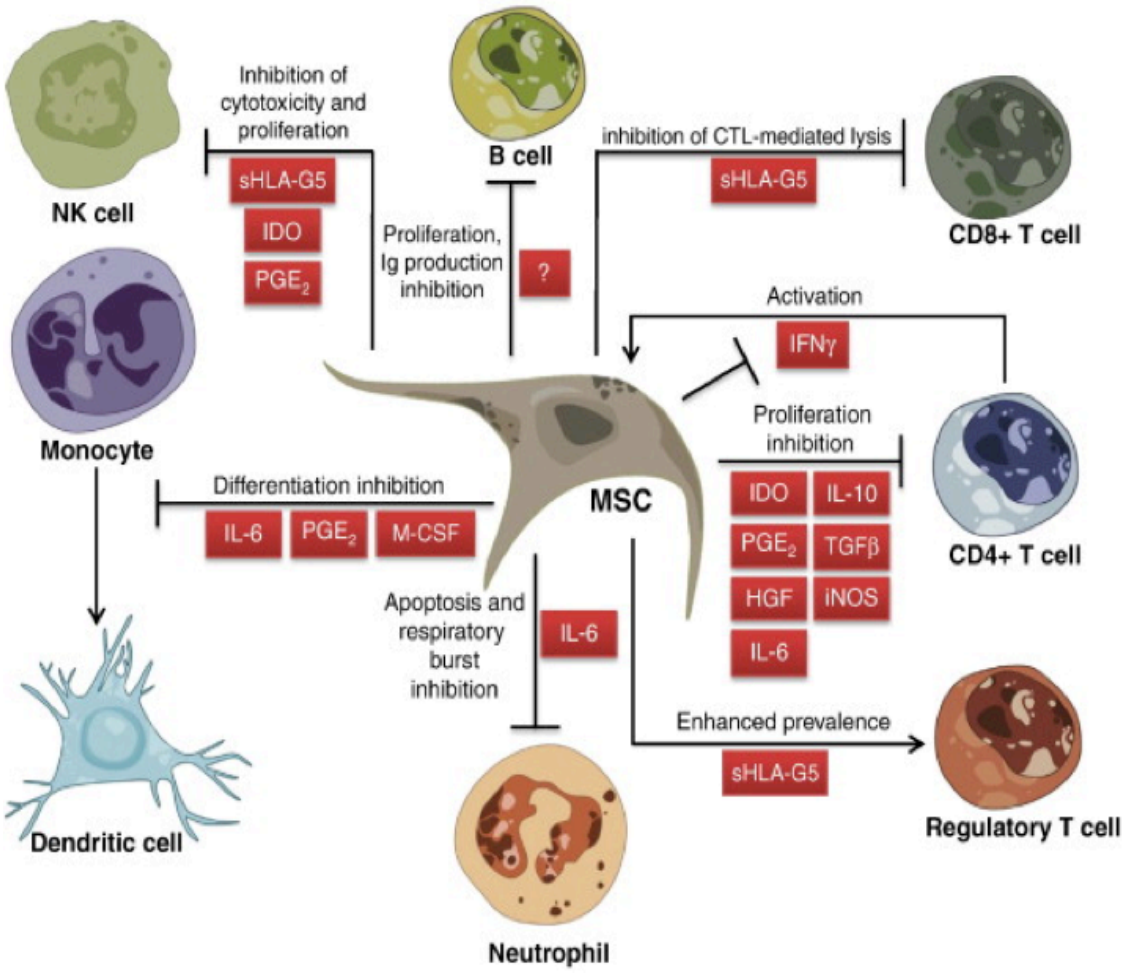

Figure 1-5: Immunosuppressive effect of mesenchymal stromal cells (MSC) on the immune system.

MSC can regulate the innate and adaptive immune system through interaction with various immune cells including T lymphocytes, B lymphocytes, NK cells, monocytes and dendritic cells. The exact mechanism of the immunomodulatory action by MSC is not fully understood however both direct contact of MSC with immune cells and paracrine activity are involved. Presence of an inflammatory microenvironment such as high levels of IFNγ is important for activation of MSC. MSC can inhibit T cell responses via both direct contact involving the engagement with the inhibitory molecule programmed death 1 by its ligands and release of soluble factors such as IDO, PGE2, HGF, IL6 and TGFβ. MSC can induce the generation and development of Tregs from $CD4^+$ T cells through production of IL10. Mechanisms involving generation of Tregs

likely involve direct MSC-$CD4^+$ T cell interaction or release of TGFβ, PGE2 and HLA-G5 by MSC. MSC are able to inhibit B cell proliferation, differentiation and function including expression of chemokine receptors such as CXCR4, CXCR5, and CCR7. MSC can inhibit the function of resting NK cells by reducing IL15 secretion either via cell-to-cell interaction or release of soluble factors, such as PGE2, IDO and HLA-G5. MSC have the capability of polarizing pro-inflammatory M1 macrophages to alternative M2 macrophages driven by the ability of MSC to secrete soluble factors including IL10. M2 macrophages further produce IL10, an anti-inflammatory cytokine which can induce the generation of Tregs. MSC can regulate dendritic cell function by blocking differentiation of monocytes to dendritic cells and impairing the ability of dendritic cells to present antigen. Secretion of IL6 by MSC inhibits respiratory burst and apoptosis of neutrophils, by activating STAT-3 transcription factor. **Abbreviations:** MSC- mesenchymal stromal cells; HGF- Hepatocyte growth factor; HLA-G5- non-classic human leukocyte antigen class I molecule; IDO- indoleamine 2,3-dioxygenase; IL- Interleukin; PGE2- Prostaglandin E2; TGFβ- Transforming growth factor β. Figure from Ben-Ami *et al* (141)

1.2.4 Enrichment of MSC

MSC are a heterogenous population of cells consisting of various subsets, each with a different surface marker and distinct therapeutic function including immunomodulatory function (142). Selection/sorting based on specific surface markers expressed on MSC allows for a homogenous and defined MSC subset with potentially enhanced efficacy including immunomodulation. It also reduces experimental variation and is an important quality control step when a vast number of MSC are being generated for experimental or pharmaceutical use. In addition to the surface markers identified by the ISCT, a number of molecules have been identified as MSC markers including:

1) CD271, also known as nerve growth factor receptor, is a marker for enrichment of BM-MSC possessing enhanced multi-potential differentiation capacity particularly for bone or cartilage repair (143). $CD271^+$ MSC have been shown to have greater immunosuppressive effect even at low concentrations compared to non-selected MSC (144). The majority of $CD271^+$ MSC were found to not co-express CD73 and CD90; percentage of CD73 and CD90 positive cells were found to be low in $CD271^+$ MSC from the BM (<10%) and adipose tissue (10-20%) (145).

2) Stro-1 antigen is present on fibroblast colony-forming unit cells in BM and potentially defines a MSC precursor subpopulation (146). Stro-1+ expanded MSC were demonstrated to have better migratory capacity to various organs/tissues than $Stro\text{-}1^-$ expanded MSC (146). Another study demonstrated that enrichment of $Stro\text{-}1^+$ MSC was associated with enhanced expression of cardiovascular-relevant cytokines (147).

3) CD146 is expressed on a number of MSC sources and is a key cell adhesion protein in vascular endothelial cell activity (145). $CD146^+$ MSC have been shown to have higher multipotency and proliferation potential, as well as supporting capacity for haematopoiesis (145).

4) $CD362^+$ (Syndecan-2) is a novel marker (patent number WO 2013117761 A1) representing a more homogeneous population of MSC with improved clonogenicity and enhanced immunomodulatory properties (148). $CD362^+$ MSC were shown to reduce liver inflammation specifically there was marked reduction in the expression level of inflammatory genes such as TNFα in animal models of liver injury (148). Syndecan-2, a heparin sulphate proteoglycan is expressed on haematopoietic cells and T cells (149). It is up-regulated during T cell activation and has been shown to reduce the surface expression and subsequent responsiveness of the TCR/CD3 complex (150). Enrichment of Syndecan-2 expression in MSC could thus play an important role in immunomodulation. All experiments involving UC-MSC undertaken during this period of research and presented in this thesis were performed using selected $CD362^+$ UC-MSC.

5) Other identified molecules in the literature include CD200, CD73 and aldehyde dehydrogenase (151).

1.2.5 Clinical application of MSC

MSC have been tested in humans since 1995. The first MSC phase I clinical study used autologous, culture-expanded MSC in patients with haematological malignancies in complete remission and demonstrated safety of MSC therapy with no reports of adverse events (152) . Since then over 5000 patients enrolled in more than 200 clinical trials involving MSC therapy have met safety end points. Clinical trials have assessed the safety and efficacy of MSC in the treatment of a range of diseases including graft versus host disease (153), diabetes (154, 155), inflammatory bowel disease (156), liver (151), kidney (157), lungs (158), cardiovascular (155), bone and cartilage (159, 160), neurological (161) and autoimmune diseases (154) (157) (161, 162). In 2012, the *SafeCell* systematic review and meta-analysis of clinical trials to examine the safety of

therapeutic intravenous MSC was published (163). The authors included trials from 1980 to 2011, which numbered eight randomised controlled trials (RCTs), 10 non-RCTs and 18 uncontrolled trials (36 in total). Study durations ranged from 0.5 to 60 months. Overall, *SafeCell* was unable to detect associations between MSC treatment and the development of acute infusion toxicity, organ system complications, infection, malignancy and death. Concerns about immunogenicity of allogeneic cell infusions were addressed and deemed to be unfounded.

In May 2009, the first large industry-sponsored phase III clinical trial involving the use of allogeneic, BM-MSC (Prochymal™) for treatment of steroid-refractory Graft vs Host Disease (GvHD) was completed by Osiris Therapeutic (USA). 244 steroid resistant GvHD (skin involvement n=144, gastrointestinal involvement n=179, liver involvement n=61) patients were enrolled and treated in addition to standard of care corticosteroid therapy: Prochymal™ (n=163), placebo (n=81) (Martin *et al*, 2010). The primary endpoint of the trial was complete remission of GvHD at ≥ 28 days. Patients in the MSC therapy group received 8 infusions of $2x10^6$ MSC/kg, over 4 weeks and further 4 infusions administered weekly after day 28 in those with a partial response. The study found no significant difference in the overall response rate between Prochymal™ and placebo (82% versus 73% respectively) (p=0.12) however patients treated with Prochymal were found to have less progression of liver GVHD at weeks 2 and 4 respectively (32% vs 59%, p=0.05; and 37% vs. 65%, p=0.05). Significant improvement in the response rate with Prochymal™ was observed in patients with liver (day 100 response rate: 76% versus 47%; odd ratio of 3.6) and gut (day 100 response rate: 82% versus 68%; odds ratio of 2.2) GvHD compared with placebo (Martin *et al*, 2010). Further analysis of the study suggested that children with GvHD responded better to MSC (164). On this basis, in May 2012, Health Canada issued marketing approval for Prochymal™ to treat children with acute GvHD (165). Prochymal™ has

since been acquired by Mesoblast (Australia) and licensed to JCR Pharmaceuticals in Japan. In November 2015, the Japanese Government approved TEMCELL (Prochymal™) for the treatment of acute GVHD after an allogeneic bone marrow transplant.

In 2016, Panés *et al* published the results of a phase 3 randomised double- blind multicentre trial involving the use of expanded allogeneic adipose derived stem cells (Cx601) in patients with Crohn's disease who had treatment refractory complex perianal fistulas (156). 212 patients were enrolled in to the study to receive either a single intra-lesional injection of 120 million Cx601 cells (n=107) or 24 mL saline solution (placebo) (n=105). The primary endpoint was combined remission at week 24 (clinical assessment of closure of all treated external openings that were draining at baseline, and absence of collections >2 cm of the treated perianal fistulas confirmed by imaging). Cx601 were found to be safe and efficacious (50% of patients who received Cx601 vs 24% of patients who received placebo; p=0.24 achieved remission 24 weeks after treatment) in treating complex perianal fistulas (156). These results were sustained for at least 1 year after treatment (166). The trial results resulted in the European Commission approving the first MSC pharmaceutical agent (Alofisel) to treat Crohn's related enterocutaneous fistula disease in March 2018.

1.2.5.1 MSC in animal models of PSC

To date there are only two pre-clinical studies in the literature that have explored the possibility of MSC therapy in animal models of sclerosing cholangitis. In a study by Suguira et al, sclerosing cholangitis in rats was induced by intragastric administration of alpha-naphthylisothiocynate, a toxin that targets intrahepatic bile ducts. Human

amnion-derived MSC (hAMSC) and hAMSC derived conditioned media injected intravenously via the penile vein of rats resulted in significant improvement in biliary hyperplasia with downregulated CK19 expression and fewer necrotic lesions caused by alpha-naphthlisothiocynate. Administration of hAMSC and hAMSC derived condition media also resulted in reduction in peri-biliary fibrosis and inflammatory reaction in the peri-biliary region as evidenced by reduced number of CD68-positive Kupffer cells as well as TNFα expression (167).

Recently, Angioni et al, demonstrated that administration of extracellular vesicles derived from human MSC reduced serum levels of alkaline phosphatase, bile acid, and alanine aminotransferase as well as decreased liver fibrosis in a mouse model of PSC. Extracellular vesicles are multi-signal messengers that have a key role in intercellular signalling by carrying cargo, such as mRNA, miRNA, and proteins. The authors further demonstrated that there was a reduction in accumulation of liver infiltrating $CD8^+$ T cells, granulocyte infiltration and VCAM 1 expression in mice that received extracellular vesicles derived from MSC (168). VCAM 1 expression by cholangiocytes is increased in PSC and has been shown contribute to persistent inflammation by conferring a survival signal to α4β1 expressing pro-inflammatory T lymphocytes (169).

1.2.5.2 MSC in humans

There have been a number of published phase I and II clinical trials that have used MSC to treat patients with varied aetiologies of liver disease including acute on chronic liver failure, autoimmune hepatitis and cirrhosis secondary to alcohol related liver disease, hepatitis B and C and PBC (151). Table 1 highlights pertinent clinical trials (n=15) involving MSC based therapy in liver disease from 2007 to July 2017 (151).

These trials have focused on safety, trial design, MSC sources, administration route, patient groups, and efficacy of MSC therapy. The studies in table 1 demonstrate heterogeneity in the dose (single versus multiple), source and route of administration of MSC. The majority of studies have used BM-MSC (n=12) with only 3 studies used UC-MSC. The first and most commonly used human MSC in studies are bone marrow followed by adipose tissue. These sources have therefore formed the foundation for most of the research in the MSC field. BM-MSC and adipose tissue derived MSC are harvestable human tissues that are thought to be renewable (bone marrow) or unwanted (adipose). Birth-associated tissue such as umbilical cord tissue and placenta, are excellent sources of human MSC, a significant advantage being that unlike BM-MSC, MSC from birth-associated tissue can be acquired non-invasively and is ethically non-problematic. Furthermore a number of studies including studies performed by our collaborators, Orbsen Therapeutics Limited have shown thaat MSC from birth-associated tissue in particular UC-MSC have high proliferative capacity compared to BM-MSC, life span and differentiation potential(170). Proliferation capacity of cells is important with regard to their application in cell therapy.

There is variation in the dose and frequency of MSC administration as well as efficacy outcomes which include assessment of improvement in the model for end-stage liver disease (MELD) score, liver blood tests, survival rates, histological fibrosis and reduction in ascites. Majority of the studies demonstrated that MSC therapy was safe and well tolerated in patients with liver disease. In a controlled trial involving 43 patients (24 patients were treated with multiple doses of UC-MSC infusion and 19 saline (control)) with acute on chronic liver failure secondary to hepatitis B virus infection, Shi *et al* showed that there was significant reduction in the MELD scores ($p=0.040$, 12 weeks) and improved survival rates at 72 weeks ($p=0.015$) and improvement in liver blood tests including albumin, bilirubin, serum alanine

aminotransferase in patients who received an infusion of UC-MSC compared to control (171).

Administration of UC-MSC was also found to be safe. Zhang *et al* further investigated the safety and efficacy of UC-MSC in patients with decompensated liver cirrhosis secondary to chronic hepatitis B in a randomised controlled trial (n=45). During a 1-year follow-up period there were no significant side effects or complications following UC-MSC infusion, significant reduction of ascites volume and improvement in liver blood tests were also noted in those that received UC-MSC compared to the control (saline) group ($p<0.05$) (172). In a pilot study (n=7), Wang *et al* investigated the safety and efficacy of UC-MSC in patients with PBC who were partially responsive to ursodeoxycholic acid treatment over a 48 week period (173). At the end of study follow up period, serum levels of liver enzymes had significantly reduced including alkaline phosphatase (474.29 ± 223.26 *vs* 369.86 ± 168.35 IU/L, $P = 0.044$) in patients who had received UC-MSC (173). In addition, UC-MSC infusion was well tolerated and found to be safe (173). Jang *et al* investigated the efficacy of repeated dosage of BM-MSC injected in to the hepatic artery at 4 and 8 weeks in a phase II clinical trial (n=11) involving patients with biopsy proven alcohol related cirrhosis and found improvement in the Child-Pugh score in 10 of the 11 patients (90.9% of patients from 7.1 ± 0.9 (pre-therapy) to 5.4 ± 0.7 (post-therapy); $P < 0.001$) (174). In addition the authors found histological improvement in liver fibrosis following BM-MSC infusion in six of the 11 patients (percentage collagen proportionate area decreased from 22.6 ± 8.4% to 17.7 ± 6.9% ($P < 0.001$) (174). In contrast, a comparison study (n=72) performed by Suk *et al* found that injecting two doses of BM-MSC in the hepatic artery of patients with biopsy proven alcohol related cirrhosis was not associated with an improvement in histological fibrosis in comparison with single BM-MSC dose transplantation (175). The route of administration of MSC therapy in the clinical trials highlighted in table 1 also differs with

infusion via the peripheral vein being the most common followed by injection in to the hepatic artery, one study injected MSC in the portal vein whilst another intra hepatic/intra-splenic. MSC were demonstrated to be efficacious irrespective of the route of administration (151).

Main improvement	MELD in 50% of patients and QoL	Decrease in MELD from 17.9±5.6 to 10.7 ±6.39 (p<0.05) and INR from 1.9±0.4 to 1.4±0.5 (p<0.05) at 6 months compared with baseline	Albumin, MELD and Child-Pugh score	Reduction in MELD sustained up to 36 weeks from 30±3.99 (baseline) to 16 ±1.73, bilirubin and PT sustained up to 12 weeks from 201±75.45 and 26.25±5.34 to 27.08±6.39 and 14.82±2.53 respectively	Reduction in MELD (p=0.040, 12 weeks), Improved survival rates at 72 weeks (p=0.015)	Improvement in MELD score (21 Vs 17; p=0.01), albumin (2.2 vs 2.6g/dl; p=0.013) and bilirubin (5.5 Vs 3.1mg/dl; p=0008) at 6 months	Reduction in MELD-Na, bilirubin and ascites and increase in albumin concentration at 48 weeks in the UC-MSC group (p<0.05)	No difference between groups	Improvement in albumin ($P = 0.000$), bilirubin ($P = 0.002$), INR ($P = 0.017$) at 6 months in the MSC treatment group compared to control
Primary outcome	Safety and feasibility	Feasibility, safety, and efficacy (LFT and MELD score)	Safety and short-term efficacy (LFT, MELD)	Improvement of MELD and LFT (short term) or development of HCC and mortality (long term)	Improvement in MELD score and LFT, adverse events and survival rates	Improvement in MELD and LFT	Safety and efficacy (LFT, MELD-Na	Safety and efficacy	Safety and efficacy
Disease aetiology (No of patients)	Cryptogenic; AIH (n=4)	HBV; HCV; ARLD; Cryptogenic (n=8)	HCV (n=10 intrahepatic; n=10 splenic and n=20 controls)	HBV (n=53 treatment; n=105 controls)	ACLF on a background of HBV (n=24 treatment; n=19 controls)	HCV (n=15 treatment; n=10 controls)	Decompensated cirrhosis secondary to HBV (n=30 treatment; n=15 controls)	Decompensated cirrhosis (n=14 treatment; n=11 controls)	HCV (n=20 treatment; n=20 controls)
Dose Route	Single Dose Peripheral Vein	Single Dose Portal Vein	Single Dose Intrahepatic or intra-splenic	Single Dose Hepatic artery	Multiple doses Peripheral Vein	Single Dose Peripheral Vein	Multiple doses Peripheral Vein	Single Dose Peripheral Vein	Single Peripheral vein
Cell Therapy	Autologous BM-MSC	Autologous BM-MSC	Autologous BM-MSC	Autologous BM-MSC	Allogeneic UC-MSC	Autologous BM-MSC	Allogeneic UC-MSC	Autologous BM-MSC	Autologous BM-MSC
Study F/U (Months)	Case series 12	Cohort 6	Case control 6	Case control 48	Case control 18	Case control 6	RCT 12	RCT 12	RCT 6
Reference	Mohamadnejad *et al* (176) 2007	Kharaziha *et al* (177) 2009	Amer *et al* (178) 2011	Peng *et al* (179) 2011	Shi *et al* (171) 2012	El-Ansary *et al* (180) 2012	Zhang *et al* (172) 2012	Mohamadnejad *et al* (181) 2013	Salama *et al* (182) 2014

Main improvement	Decrease in ALP (474.29 ± 223.26 *vs* 369.86 ± 168.35 IU /L, *P* = 0.044) and subjective improvement QoLat 48 weeks compared to baseline	MELD score at weeks 4,8,12 and 24 weeks in the MSC group c (p<0.05)	% collagen proportionate area decreased from 22.6 ± 8.4% to 17.7 ± 6.9% (P < 0.001)	histological fibrosis; reduction of 25% (19.5 ± 9.5% vs 14.5 ± 7.1%) in single dose group and 37% (21.1 ± 8.9% vs 13.2 ± 6.7%) in two doses group (p<0.001)..	None	Improvement in QOL
Primary outcome	Safety and efficacy	Efficacy (LFT, MELD)	Safety and efficacy	Safety and efficacy	Safety and Efficacy	Safety and Efficacy
Disease aetiology (No of patients)	UDCA-refractory PBC (n=7)	Cirrhosis secondary to HBV (n=20 treatment; n=19 control)	Alcohol-related liver cirrhosis (n=11)	Alcohol-related liver cirrhosis (n=18 single MSC dose; n=19 two MSC doses; n=18 control)	Alcoholic hepatitis (n=28 treatment; n=30 control)	ACLF secondary to HBV (n=56 treatment; n=52 control)
Dose Route	Multiple doses Peripheral vein	Single infusion Hepatic artery	Multiple doses Hepatic artery	Single or two doses Hepatic artery	Single dose Hepatic artery	Multiple doses Peripheral vein
Cell Therapy	Allogeneic UC-MSC	Autologous BM-MSC	Autologous BM-MSC	Autologous BM-MSC	Autologous BM-MSC	Allogeneic BM-MSC
Study F/U (Months)	Cohort 12	RCT 6	Cohort 6	RCT 12	RCT 1	RCT 6
Reference	Wang *et al* (173) 2014	Xu *et al* (183) 2014	Jang *et al* (174) 2014	Suk *et al* (175) 2016	Lanthier *et al* (184) 2017	Lin *et al* (185) 2017

Table 1: Clinical trials of mesenchymal stromal cell therapy in liver disease.

Abbreviations: ACLF- Acute on Chronic liver failure; AIH- Autoimmune hepatitis; ALP- Alkaline phosphatase; ARLD- Alcohol related liver disease; BM-MSC- Bone marrow derived mesenchymal stromal cells; CPS- Child-Pugh score; F/U- Follow-up; HBV- Hepatitis B virus; HCV- Hepatitis C virus; HCC- Hepatocellular carcinoma; LBT- Liver blood test; MELD- Model for End-Stage Liver Disease; MELD-Na- Addition of sodium to the MELD score; No- number; PBC- Primary biliary cholangitis; PT- Prothrombin time; QOL- Quality of life; RCT- Randomised controlled trial; UDCA- Ursodeoxycholic acid. Table adapted from Alfaifi *et al* (151).

1.3 Hypothesis

UC-MSC can suppress the activation and proliferation of peripheral blood and intrahepatic $CD4^+$ and $CD8^+$ T cells from patients with PSC.

1.4 Aims

- To investigate the direct effect of UC-MSC on PSC circulating and intrahepatic $CD4^+$ and $CD8^+$ T cell proliferation and cytokine production.
- To investigate if the effect of UC-MSC on $CD4^+$ and $CD8^+$ T cell proliferation and cytokine production is disease specific. This will be studied by comparing the effect of UC-MSC on T cell responses from peripheral blood of patients with hereditary haemochromatosis (HFE) *versus* PSC and explant livers of patients with PBC *versus* PSC.
- To investigate the indirect effect of UC-MSC on PSC circulating and intrahepatic T cell proliferation and cytokine production. This will be studied by using condition media derived from both resting and IFNγ stimulated UC-MSC. IFNγ stimulated UC-MSC conditioned media will be used to ascertain if the immunomodulatory action of UC-MSC on T cell responses is enhanced following IFNγ stimulation.
- To investigate the mechanism of action of UC-MSC on $CD4^+$ and $CD8^+$ T cell proliferation and cytokine production specifically the role of indoleamine 2.3-dioxygenase (IDO).

CHAPTER 2.

MATERIALS & METHODS

2.1 Isolation of selected CD362$^+$ umbilical cord derived mesenchymal stromal cells (UC-MSC)

2.1.1 Selected CD362$^+$ UC-MSC

For all the experiments involving MSC, selected CD362$^+$ UC-MSC sourced from Orbsen Therapeutics Limited (Ltd) (Galway, Ireland) were used. As previously highlighted the rationale for using a defined/selected population of MSC that is CD362$^+$ MSC was to reduce experimental variation. In addition data from our pre-clinical study was used to support the clinical trial investigating the safety and efficacy of a single infusion of selected CD362$^+$ UC-MSC in patients with PSC. MSC used in our experiments were sourced from umbilical cord tissue (Figure 2.1). All human umbilical cords used by Orbsen Therapeutics Ltd were acquired from caesarean section deliveries by tissue solutions Ltd (Glasgow, Ireland). Umbilical cords were obtained according to legal and ethical requirements of the UK with approval of the relevant ethical committee and with anonymous consent from the donor. In the results section CD362$^+$UC-MSC will be referred to as UC-MSC only.

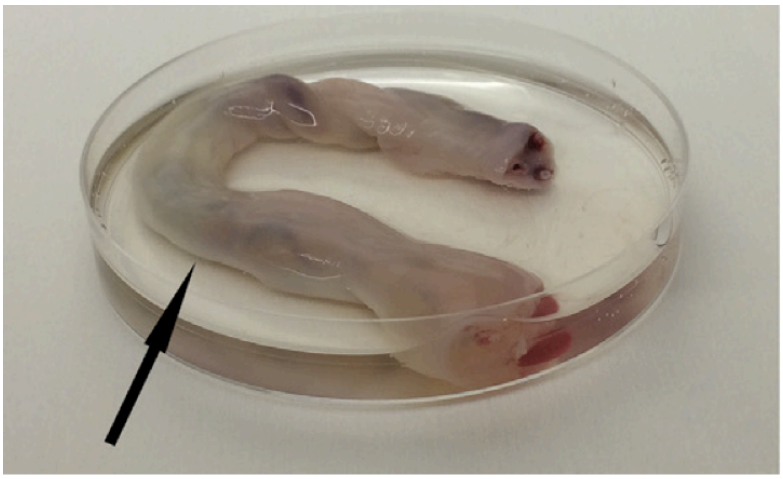

Figure 2-1: Human umbilical cord

Representative image demonstrating human umbilical cord (black arrow). Figure from Sadlick *et al (186).*

2.1.2 Isolation of selected CD362^{+} UC-MSC

Isolation and expansion of selected CD362^{+} UC-MSC was undertaken at Orbsen Therapeutics Ltd (Galway, Ireland). Briefly, umbilical cords were harvested within 48 hours of birth. The average length of cords used was 8.6 cm. The cords were initially disinfected with sterile 70/30 denatured alcohol then manually dissected in to 1cm^3 pieces and washed free of contaminating blood with PBS throughout the process. The dissected tissue was then transferred to 50 ml falcon tubes for enzymatic digestion using an enzymatic cocktail consisting of MEM alpha (Gibco, Thermo Fisher Scientific, Paisley, UK), 2 mg/ml collagenase 1, 1mg/ml hyaluronidase 1 and 0.1 mg/ml DNase (Sigma-Aldrich, Arklow, Ireland) and incubated for 2 hours at 37°C. After a series of washes to remove any undigested tissue, a cell suspension was obtained through filtration (100 µm) and centrifugation at 400 RCF for 5 minutes. The cells were then initially labelled with anti-CD362 antibody (Syndecan-2/ORB, APC, clone 305515, dilution 1:50, R&D Systems, Minneapolis, MN, USA) for 30 minutes at 4°C followed by subsequent incubation with anti-APC magnetic beads at a concentration of 20 µl/10^{7}cells (Miltenyi Biotec, GmbH, Bergish Gladbach, Germany) for 15 minutes at 4°C. The labelled cell fraction was then separated by MACS® column technology (Miltenyi Biotec) using magnetic separation columns and a MiniMACS™ separator. The resulting positive fraction and unsorted fraction were then analysed using flow cytometry (Figure 2.2). CD362^{+} enriched positive fraction was counted, seeded for expansion (discussed in section 2.2.3) and cryopreserved at passage 2 for shipment to the University of Birmingham.

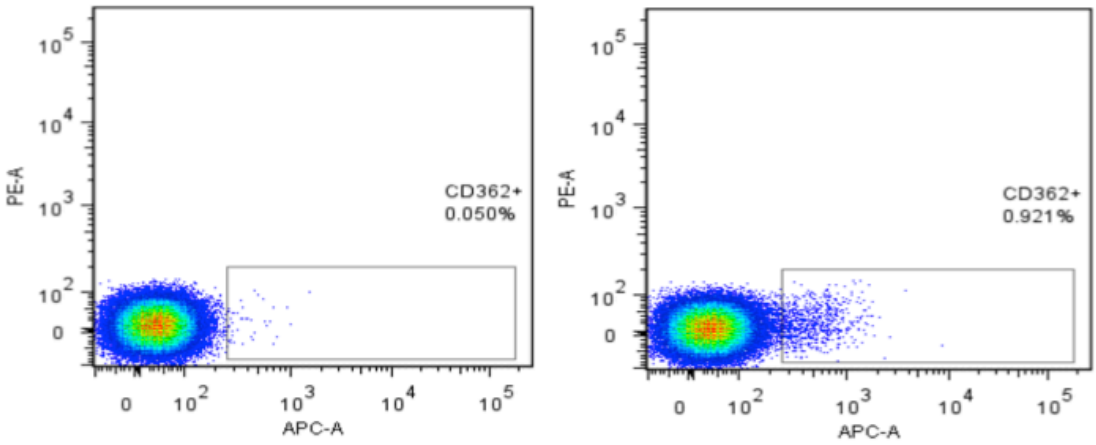

Figure 2-2: Flow cytometry analysis of umbilical cord derived mesenchymal stromal cells expressing CD362-APC in unsorted faction (left) and positive fraction (right).

Figure from MERLIN trial investigational medicinal prodossier.

2.2 Cell culture

2.2.1 Cell lines and human primary cells

The cells used in this study include $CD362^+$ umbilical cord derived mesenchymal stromal cells (UC-MSC) described in section 2.1, peripheral blood mononuclear cells (PBMC), liver infiltrating mononuclear cells (LIMCs), intrahepatic $CD4^+$ and $CD8^+$ T cells described in sections 2.3.1, 2.3.2 and 2.3.2.1 respectively.

2.2.2 Tissue culture plastic

Sterile 5, 10 and 20 ml pipettes, 75- and 175 cm^2 culture flasks, sterile 6-, 24- and 96-well plates, sterile 15 ml and 50 ml tubes and sterile cryovials were all purchased from Corning Costar Incorporated, Bucks, UK. 20ml plastic tubes and 5ml bijous were acquired from Ramboldi. Sterile plastic gloves used daily were from Premier Nitric. All tissue culture was carried out in a class II microflow safety cabinet. At all times aseptic

technique was followed by swabbing the cabinets with industrial methylated spirit (IMS; Adams Healthcare, Leeds, UK) before and after use.

2.2.3 Cell culture and passage

All cells were cultured on tissue culture plastic in appropriate media (as indicated in the respective sections) and maintained at 37^{o}C in a humidified 5% CO_2 incubator. Cell viability and growth were monitored throughout using an inverted phase contrast microscope (Olympus IX50) where live cells appeared phase bright. Adherent CD362^{+} UC-MSC formed projections and flattened upon attachment to the growth surface whilst PBMC, LIMC and intrahepatic CD4^{+} and CD8^{+} T cells typically remained round and symmetrical. All cultures were examined daily and an assessment of cell morphology, colour of culture medium and cell density was undertaken. Adherent CD362^{+} UC-MSC were passaged using an appropriate volume of the proteolytic enzyme TrypLE^{TM} (i.e. 2.5 ml in a 75cm^2 and 5 ml in a 175 cm^2 flask, enough to cover the cell monolayer). TrypLE$^{™}$ Express (1x) stable Trypsin-like enzyme with Phenol Red purchased from Thermo Fisher Scientific was used. Initially, used media was discarded, and cells were washed with Phosphate buffered saline (PBS) which was prepared from tablets (Oxoid, UK) according to the manufacturers' instructions, pre-warmed TrypLE™ express was then added to the cells which were incubated at 37^{o} C for 5 minutes to enable loosening of the cells. Subsequently, the flask was agitated gently to help cell detachment. Once detachment was complete, culture medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermofisher Scientific) was added to the cell suspension and the cells were collected by centrifugation at 400 g for 5 minutes. The pellet was re–suspended in appropriate media, cells were then counted using a haematocytometer (CAMLAB, Cambridge, UK) and re–seeded in new flasks for further

culture. 1 x10^6 UC-MSC were cultured per 175 cm^2 flask. Where applicable, cell viability was assessed using trypan-blue.

2.2.4 Maintenance of cells by freezing and storing

For long–term storage, $CD362^+$ UC-MSC were cryopreserved in liquid nitrogen. After detachment and pelleting, cells were re–suspended in chilled CryoStor cell cryopreservation media containing 10% dimethyl sulfoxide (Sigma-Aldrich). 1 x 10^6 UC-MSC were re-suspended in 1 ml of CryoStor and subsequently aliquoted in to one cryovial. The cryovials were then placed into a Mr FrostyTM freezing container (Wessington Cryogenics, Tyne & Wear, UK), which was transferred to –80^{o}C to allow gradual cooling at a rate of 1^{o}C per minute, thus preventing ice crystal formation in the cell cytoplasm. Once the container had reached –80^{o}C (after 4 hours or more conveniently overnight), the vials of cells were immediately placed into liquid nitrogen storage. When required, cells were taken out of liquid nitrogen on ice, thawed as quickly as possible and transferred to a 15 ml tube containing media. Cells were then centrifuged in order to remove any debris. Following centrifugation, cells were counted and viability was assessed with trypan–blue exclusion before re–suspension and culture in the appropriate media.

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2.2.5 Culture of CD362^{+} UC-MSC

All *in vitro* experiments were repeated with multiple CD362^{+} UC-MSC donors. UC-MSC from passage 2-4 were seeded either in 75cm^2 or 175cm^2 flasks containing 10 mls and 30 mls of culture medium respectively and kept at 37^{o}C in a humidified 5% CO2 incubator. The culture medium consisted of minimum essential medium with glutaMAX^{TM} and phenol red (MEM-α) (Gibco, Thermofisher Scientific) supplemented with 10% heat inactivated FBS, 1% penicillin/streptomycin (P/S) solution (10,000 units/ml penicillin, 10,000 µg/ml streptomycin (Gibco, Thermofisher Scientific) and 25µg/ml recombinant human basic fibroblast growth factor (FGF) (Peprotech, UK). The culture medium was changed every 2-3 days until the cells reached the desired confluency (70-80%). Cells were detached after incubation at 37^{o} C for 5 minutes with appropriate volume of TrypLE express (i.e. 2.5 ml in a 75cm^2 and 5 ml in a 175 cm^2 flask, enough to cover the cell monolayer) (Gibco, Thermofisher Scientific). Following dilution with the culture medium containing 10% FBS, the cells were centrifuged at 400g for 5 minutes, counted and either re-seeded or re-suspended at the desired concentration depending on the experiment.

2.2.6 Characterization of CD362^{+} UC-MSC

Characterisation of MSC was performed using criteria set out by the ISCT by Orbsen Therapeutics Ltd and validated at University of Birmingham by laboratory members of our study group including Mohammed Alfaifi and Dr Debashis Haldar (PhD students at University of Birmingham). The criteria that define MSC are:

Plastic adherence: MSC were confirmed to be highly plastic adherent when cultured and demonstrated to have a fibroblast like appearance (Figure 2.3).

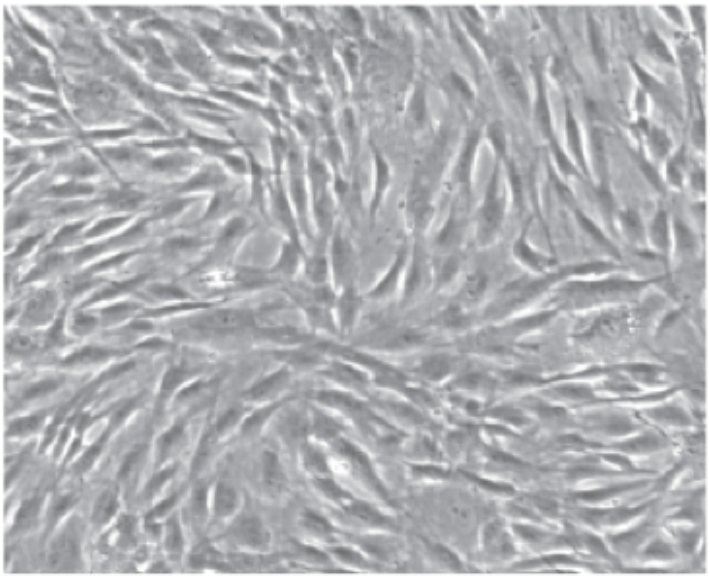


Figure 2-3: Fibroblast appearance of CD362^{+} umbilical cord derived mesenchymal stromal cells (UC-MSC)

Representative image of cultured MSC isolated from umbilical cord. 1x10^{6} UC-MSC from passage 2 were cultured in 175cm^{2} flasks containing 30 mls of culture media (AMEM+10%FBS+1% P/S+ 25 μ g/ml FGF) for 5 days. The culture medium was changed every 2-3 days until cells reached the desired confluency (70-80%). Image was acquired from a light microscope (Olympus IX50 inverted microscope) using 10x magnification. **Abbreviations:** FBS- Fetal bovine serum; FGF- Fibroblast growth factor; P/S- Penicillin and streptomycin; UC-MSC- Umbilical cord derived mesenchymal stromal cells.

Phenotypic characterization of CD362^{+} UC-MSC: Immunophenotyping of CD362^{+} UC-MSC was performed by labelling with standard MSC markers according to the ISCT criteria and analysed by flow cytometry (Cyan, flow cytometer analyser). A human MSC analysis kit (BD bioscience) was used and details of antibodies in the kit are found in Table 2. Labelling of MSC was undertaken according to the manufacturers' instructions.

A

Product	**Contents**
Positive marker cocktail	CD105 PerCP-Cy5.5, CD73 APC and CD90 FITC
Additional positive drop-in marker	CD44 PE
Negative marker cocktail	CD45, CD34, CD11b, CD19 and HLA-DR PE
Isotype controls	1) mIgG1 PerCP-CY5.5, mIgG1 APC and mIgG1 FITC for positive control. 2) IgG1 and IgG2b PE for negative cocktail. 3) IgG2b PE for CD44 drop in.
Compensation controls	1) CD105 PerCP-Cy5.5 2) CD73 APC 3) CD90 FITC 3) CD44 PE

B

Tube number	**Add**	**Concentration**
1	FITC Mouse Anti-Human CD90	5µl
2	PE Mouse Anti-Human CD44	5µl
3	PerCP-Cy™5.5 mouse anti-human CD105	5µl
4	APC mouse anti-human CD73	5µl
5	Nothing	
6	hMSC positive isotype control cocktail + PE hMSC negative isotype control cocktail	20µl 20µl
7	hMSC positive control + PE hMSC negative cocktail	20+20µl
	And/or	
8	hMSC positive isotype control cocktail + Drop in isotype control (PE mouse IgG2b)	20µl 5µl
9	hMSC positive cocktail + PE drop in (PE mouse anti-human CD44)	20µl 5µl

Table 2: A) Human analysis kit content B) Concentration of antibodies used.

Table courtesy of Dr Mohamed Alfaifi. Abbreviation: hMSC- Human mesenchymal stromal cells

MSC tri-lineage differentiation: The tri-lineage differentiation potential of $CD362^+$ UC-MSC was confirmed by culturing under standard adipogenic, chondrogenic and osteogenic differentiation conditions. Briefly, for both adipogenic and osteogenic differentiation, cells from passage 3 were cultured in a 12-well plate at a seeding density of 1 $x10^4$ cells/cm^2 for 14 and 21 days respectively. The cells were initially cultured in standard MSC culture medium consisting of MEM-α (Gibco, Thermofisher Scientific) supplemented with 10% heat inactivated FBS (Gibco, Thermofisher Scientific), 1% P/S solution (Gibco, Thermofisher Scientific) and 25µg/ml recombinant FGF (Peprotech, UK) until they reached 80% confluence following which, the media was replaced by either adipogenic or osteogenic differentiation media (Gibco, Thermofisher scientific), according to the manufacturer's instructions. Normal complete media was used as negative control.

Adipogenic differentiation was visualized by Oil red O staining (Sigma-Aldrich), which stains lipids in red colour. Osteogenic differentiation was assessed using 1% alizarin red staining (Sigma-Aldrich), the 1% alizarin red is an organic dye that stains calcium deposits. For chrondrogenic differentiation, 2.5 x 10^5 UC-MSC were cultured as a pellet and placed in chondrogenic differentiation media. After 21 days, pellets were snap frozen and the presence of proteoglycans and glycosaminoglycan examined by toluidine blue staining. Proteoglycan deposits stained purple in colour.

From here on and in the results section $CD362^+$UC-MSC will be referred to as UC-MSC only.

2.2.7 Generation of conditioned media derived from UC-MSC (UC-MSC-CM)

Condition media (CM) was generated from both resting and interferon gamma (IFNγ) (R&D systems UK) stimulated UC-MSC. 50,000 UC-MSC were plated in flat Corning® Co-star® 6-well tissue culture treated plates (Sigma-Aldrich) in 2 mls of culture media consisting of MEM-α (Gibco, Thermofisher Scientific) supplemented with 10% heat inactivated FBS (Gibco, Thermofisher Scientific) and 1% P&S solution (Gibco, Thermofisher Scientific) in the presence or absence of 50 ng/ml IFNγ. Culture media alone in the absence of UC-MSC was used as control. When the cells reached 70-80% confluence (approximately at day 4), the culture media from all the wells was removed, cells were washed twice with PBS and the culture media was refreshed without the presence of IFNγ in all the wells at a volume of 1ml. After 24 hours, all the CM was collected, centrifuged at 4000 rpm for 10 minutes, then filtered using a 0.22μm filters (Sysmex Partec) and stored at -80°C in 1.5 ml eppendorf tubes for future experiments.

2.3 Human tissue and blood

Human liver tissue was obtained from explant diseased livers, or from normal tissue that was either surplus to transplantation requirements or from excised tumor margin. Human liver tissue was collected at the Queen Elizabeth Hospital, Birmingham, UK. Explant diseased livers of patients with either PSC or PBC were used in experiments. Peripheral blood was obtained from patients with PSC and HFE. All human tissue and blood samples were collected with local research ethics committee approval and patient consent (Local Research and Ethics Committee Birmingham references: 2003/242, renewed 2012; 06/Q2702/61 and Dyfed Powys Research Ethics Committee, 13/WA/0392; and NRES Research Ethics Committee North West Preston, 14/NW/1146).

2.3.1 Peripheral blood mononuclear cell isolation (PBMC)

Peripheral blood was collected into Ethylenediaminetetraacetic acid tubes. PBMC were isolated by density gradient centrifugation over lympholyte (Cedarlane Laboratories) according to manufacturer's instructions. Briefly, blood was layered on top of lympholyte at 1:1.5 ratio (lympholyte: blood) and centrifuged for 20 minutes at 800 x g with no brake. The cell layer was collected from the density gradient interface and washed with PBS twice. The pellet containing PBMC were re-suspended in 1 ml of PBS and further studied for their phenotype and function.

2.3.2 Liver infiltrating mononuclear cell (LIMC) isolation

LIMCs were isolated from fresh human explant liver tissue. Liver tissue was washed in ice-cold PBS to remove residuals of blood, then cut into small pieces and enzymatically digested using 10mg/ml of collagenase type II (Life technology, UK) in the presence of RPMI1640 and heat inactivated 10% fetal calf serum (FCS) in an incubator at 37°C for 1 hour. Thereafter the tissue was mechanically digested in RPMI1640 using a Stomacher400 Circulator (Seward Stomacher400 Circulator [Cole-Parmer Instrument Co Ltd, London, UK]) for 330 seconds at 260 speed, filtered and the supernatant distributed in 50 ml falcon tubes. After several washes with cold PBS and centrifugation at 2000rpm for 5 minutes, LIMCs were isolated by density gradient centrifugation using lympholyte (Cedarlane Laboratories) for 20 minutes at 800 × g with no brake. The cell layer was collected from the density gradient interface and washed with PBS twice. The pellet containing LIMCs was re-suspended in 1 ml of PBS and further studied for their phenotype and function. For some experiments, $CD4^+$ and $CD8^+$ T cell enrichment was performed from the LIMCs, for this the pellet containing LIMCs was re-suspended at a concentration of 5 x 10^7 cells/ml in MACS buffer instead of PBS. The method used

is described in the following section 2.3.2.1. MACS buffer was made using heat inactivated 2% FCS, 2 mM Ethylenediaminetetraacetic acid (Sigma Aldrich) and 976 ml PBS. Thereafter the prepared MACS buffer was filtered, stored at 4℃ until use and kept sterile throughout.

2.3.2.1 $CD4^+$ and $CD8^+$ intrahepatic T-cell enrichment

$CD4^+$ and $CD8^+$ intrahepatic T-cell enrichment was performed using EasySep™ negative selection kits (Stemcell Technologies) according to the manufacturers instructions. In brief, LIMCs at a concentration of 5 x 10^7 cells/ml in MACS buffer were placed in two 5ml Falcon™ polystyrene round-bottom tube (BD Biosciences) each tube containing 5 x 10^7 cells/ml in the presence of 50 µL/mL EasySep™ human $CD4^+$ and 50 µL/mL $CD8^+$ T cell enrichment cocktail (Stemcell Technologies) in the respective 5ml Falcon™ polystyrene round-bottom tube. The LIMCs were mixed well and incubated at room temperature for 10 minutes. Following this and after vortexing the EasySep™ D magnetic particles well, the magnetic particles were added to the LIMCs at a concentration of 100 µL/mL for $CD4^+$ T cell enrichment and 150 µL/mL for $CD8^+$ T cell enrichment. The cells were further incubated at room temperature for 5 minutes. Each cell suspension was then brought up to a total volume of 2.5 mls using MACS buffer, mixed well by gently pipetting 2-3 times and each tube containing LIMCs was placed in an EasySep™ magnet (Stemcell Technologies) for 5 minutes. $CD4^+$ and $CD8^+$ T cells were acquired by inverting the magnet in one continuous motion in to new 5 ml Falcon™ tubes. Magnetically labelled unwanted cells remained bound in the original tube. Acquired $CD4^+$ and $CD8^+$ T cells were resuspended in 500 µl PBS after centrifugation and further studied as detailed in sections 2.3.3.

2.3.3 Circulating and intrahepatic CD4$^+$ and CD8$^+$ T-cell proliferation and activation assays; direct and indirect culture with UC-MSC and UC-MSC derived CM respectively

2.3.3.1 PBMC/LIMCs/intrahepatic CD4$^+$ and CD8$^+$ T-cells and UC-MSC co-culture assays

UC-MSC acquired using the method described in section 2.2.3 were re-suspended at a concentration of 1 x10^6 cells/ml for use in the co-culture experiments. The UC-MSC were plated 24 hours prior to addition of PBMC/T-cells to enable adherence in 96-well Corning® Co-star® round bottom tissue culture treated wells (Sigma-Aldrich) at PBMC: MSC ratios of 1:1, 1:4, 1:16, 1:64 and 1:256. Total volume in each well was kept at 200µl and culture media used consisted of MEM-α, 10% heat inactivated FBS and 1% P/S. The PBMC/T-cell number was always kept constant in experiments whilst the MSC number was varied. For example, for the well containing PBMC: MSC ratio of 1:4, 100,000 PBMC and 25,000 UC-MSC were co-cultured.

Independent experiments were carried out using PBMC, LIMCs or intrahepatic CD4$^+$ and CD8$^+$ T cells. Cells acquired using the methods described in section 2.3 were re-suspended at a concentration of 7 x 10^6 cells/ml in PBS after two washes with PBS. The cells were then labelled with cell trace violet (CTV) (Thermofisher, Life Technologies) at a concentration of 1ul/1ml cells and incubated at 37°C for 20 minutes. Thereafter, media containing FCS was added to the top of the tube to quench any unbound CTV and the cells further incubated for 5 minutes at 37°C. After 2 washes, the cells were then re-suspended in culture media comprising MEM-α, 10% heat inactivated FBS and 1% P&S at a concentration of 2 million cells/ml ready for co-

culture with the plated UC-MSC for a total of 5 days if PBMC were used and 4 days if LIMCs and intrahepatic $CD4^+$ and $CD8^+$ T cells were used. Old media in the wells containing adherent MSC was removed and replaced with media containing PBMC/LIMC/intrahepatic $CD4^+$ and $CD8^+$ T-cells. Each well contained 100,000 PBMCs/LIMCs/$CD4^+$/$CD8^+$ T-cells in the presence or absence of 0.5µg/ml anti-CD3 (OKT3; e-bioscience) and 1µg/ml anti-CD28 (L293; BD bioscience). Total volume in each well was kept at 100µl. CTV labeled cells only (without MSC) in presence or absence of anti-CD3 (OKT3; e-bioscience) and anti-CD28 (L293; BD bioscience) were used as controls. At day 5, if PBMC and day 4, if LIMCs and intrahepatic T cells, the cells were further stimulated for 4-hours with phorbol 12-myristate 13-acetate (PMA; 50ng/ml, Sigma), ionomycin (1 µM, Sigma) and Brefeldin A (3 µg/ml, Sigma) to allow for intracellular cytokine accumulation; UC-MSC effect on T cell proliferation and cytokine expression of cells was assessed by flow cytometry as described in section 2.4.

2.3.3.2 PBMC/LIMCs/intrahepatic $CD4^+$ and $CD8^+$ T-cells and UC-MSC derived condition media (UC-MSC-CM)

Independent experiments were performed using resting and IFNγ UC-MSC derived CM (UC-MSC-CM). These experiments were performed in the same manner as described in section 2.3.3.1 with the exception that the PBMC/intrahepatic $CD4^+$ and $CD8^+$ T-cells were used at a concentration of 3.4 $x10^6$ cells/ml instead of 2 $x10^6$ cells/ml which was the concentration used in the co-culture experiments. Frozen CM was thawed on the day of the experiment and used at the following concentrations: 100%, 90%, 80%, 70%, 60% and 50% in the presence of PBMC/T-cells. To make concentrations 50%-90%, the CM was diluted using fresh culture media consisting of MEM-α, 10% heat inactivated FBS and 1% P/S solution. Similar to the co-culture experiments, the final

volume in each well was always kept at 100 µl. The effect of UC-MSC-CM on T cell proliferation and cytokine expression was studied by flow cytometry as described in section 2.4.

2.3.3.3 Assessing viability of cultured UC-MSC

In some experiments, the viability of cultured UC-MSC was determined. In the co-culture experiments, once the PBMC/LIMCs/T-cells were removed the 96 culture plate wells containing adherent UC-MSC were washed initially with 200 µl of PBS followed by 200 µl MACS buffer. Thereafter 50 µl of pre-warmed TrypLE™ express was added to the UC-MSC which were incubated for 5 minutes at 37°C. After the incubation, 150 µl of culture media containing 10% FBS was added and gentle pipetting was performed to dislodge the adherent cells. The dislodged UC-MSC were then transferred to a FACS tube containing 1ml of culture media comprising 10%FBS and centrifuged at 2000rpmi for 5 minutes. The pellet of UC-MSC was stained for viability using a viability kit (zombie NIR™ dye APC-CY7; Biolegend) as per manufacturers instructions. Following incubation with the viability dye, the cells were washed once with MACS buffer, re-suspended in 500 µl MACS buffer and analysed by flow cytometry.

2.4 Flow cytometry

Intracellular cytokine staining was performed on CTV labelled PBMC/LIMCs/intrahepatic T cells on day 5 (if PBMC) and day 4 (LIMCs and intrahepatic T cells) respectively of culture to determine the effect on UC-MSC on $CD4^+$ and $CD8^+$ T cell on T cell activation via TNFα, IL-2 and IFNγ assessment. Following a 4-hour stimulation with PMA, ionomycin and Brefeldin A PBMC/LIMCs/intrahepatic T cells were collected in FACS tubes, washed with PBS and stained first for viability

using a viability dye (zombie NIR™ dye APC-CY7; Biolegend) as per manufacturers instructions. Briefly the zombie NIR™ dye was diluted at 1: 1000 in PBS. From this solution, 100 µl was added per FACS tube to stain 1-10 x 10^6 cells. The cells were then incubated for 20 minutes at room temperature protected from light. After washing x1 with FACS buffer (7.5 mls of heat inactivated 10% FBS in 492.5 mls PBS), the cells were fixed using 100µl of intracellular fixation buffer (fixation/permeabilization solution kit; BD biosciences) and incubated in the dark for 20 minutes at 4°C. The cells were then washed twice with 1ml of 1x permeabilisation buffer, then stained in the recommended amount of mouse anti-human fluorochrome labelled antibody for detection of the following antigens; CD3, CD4, CD8, TNFα, IL-2 and IFNγ (Table 3) and incubated for 30 minutes at 4°C. Control samples were labelled with isotype–matched control IgG1 Ab conjugated to PerCP, peCF594, peCY7, FITC, PE and APC. Other control samples included CTV labelled cells only (no presence of anti-CD3 and CD28) and cells stimulated with Brefeldin A only. After the incubation, the cells were washed once with 1 ml of 1x permeabilisation buffer then re-suspended in 500 µl FACS buffer and acquired on the flow cytometer (Cyan, flow cytometer analyser).

ANTIBODY	CLONE	VOLUME	SOURCE
CD3 PerCP	SK7	7µL in a total volume of 100 µL	BD bioscience
CD4 peCF594	RPA-T4	5µL in a total volume of 100 µL	BD bioscience
CD8 pecy 7	RPA-T8	3µL in a total volume of 100 µL	BD bioscience
TNF FITC	MAb11	3µL in a total volume of 100 µL	BD bioscience
IFN γ APC	B27	0.3µL in a total volume of 100 µL	BD bioscience
IL2 PE	5344.111	3µL in a total volume of 100 µL	BD bioscience
FITC Mouse IgG1, K	MOPC-21	2µL in a total volume of 100 µL	BD bioscience
APC Mouse IgG1, K	MOPC-21	2µL in a total volume of 100 µL	BD bioscience
PE Mouse BALB/c, IgG1, K	MOPC-21	2µL in a total volume of 100 µL	BD bioscience
PE-CF394 Mouse IgG1 K	X40	2µL in a total volume of 100 µL	BD bioscience
Pecy7 Mouse IgG1, K	MOPC-21	2µL in a total volume of 100 µL	BD bioscience
Mouse IgG1 PerCP	X40	2µL in a total volume of 100 µL	BD bioscience

Table 3: Antibodies used for phenotypic surface marker and intracellular cytokine expression.

2.5 Inhibition of indoleamine 2,3-dioxygenase

The role of indoleamine 2,3-dioxygenase (IDO) as one of the factors implicated as a mechanism in which UC- MSC inhibit T-cell proliferation and activation was investigated. For these experiments, CM from resting and IFNγ stimulated UC-MSC was acquired as described in section 2.2.5. In some wells containing resting and IFNγ stimulated UC-MSC, an IDO inhibitor namely 1-methyltryptophan (1-MT) (Sigma) was added from day 0 at a concentration of 1mM and CM collected and stored in the same manner described in section 2.2.5. CM acquired from the following conditions: **a)** untreated (resting) UC-MSC **b)** IFNγ treated UC-MSC **c)** resting + 1-MT treated UC-MSC (control) **d)** IFNγ + 1-MT treated UC-MSC was subsequently used in experiments as previously described in section 2.3.3.1 and 2.3.3.2 to determine the effect on $CD4^+$ and $CD8^+$ T cell proliferation/activation in peripheral blood of patients with PSC.

2.5.1 Measuring Kynurenine metabolite levels

Kynurenine and tryptophan levels as markers of IDO activity were measured using liquid chromatography-mass spectrometry. These experiments were undertaken by the Phenome centre, University of Birmingham. Briefly the experiments were undertaken as follows:

Sample preparation: After thawing the frozen CM samples, 50 µl of each sample was aliquoted into a new microfuge tube. A pooled quality control sample was created by mixing a further 50 µl of each sample together in a new microfuge tube, after vortexing 50 µl of the sample was re-distributed in to 8 new microfuge tubes. To each 50 µl CM an aliquot 150 µl methanol (containing internal standard – D8-phenylalanine at 10 µM) was added and vortexed. The samples were then centrifuged at 13,000g 4°C for 10 minutes. Supernatants acquired were loaded in to a high-performance liquid

chromatography vial and kept at 4°C during analysis. An extract blank was prepared as above but using 50 µl water instead of 50 µl cell media.

Standard curve preparation: Standard samples were made at various concentrations through serial dilutions of tryptophan and kynurenine dissolved in water. Thereafter, 150 µl methanol (containing internal standard – D8-phenylalanine at 10 µM) was added to 50 µl of each standard concentration solution. Standard sample preparation then continued as described earlier. See Figure 2.4 for the standard curves used in Kynurenine and Tryptophan assays.

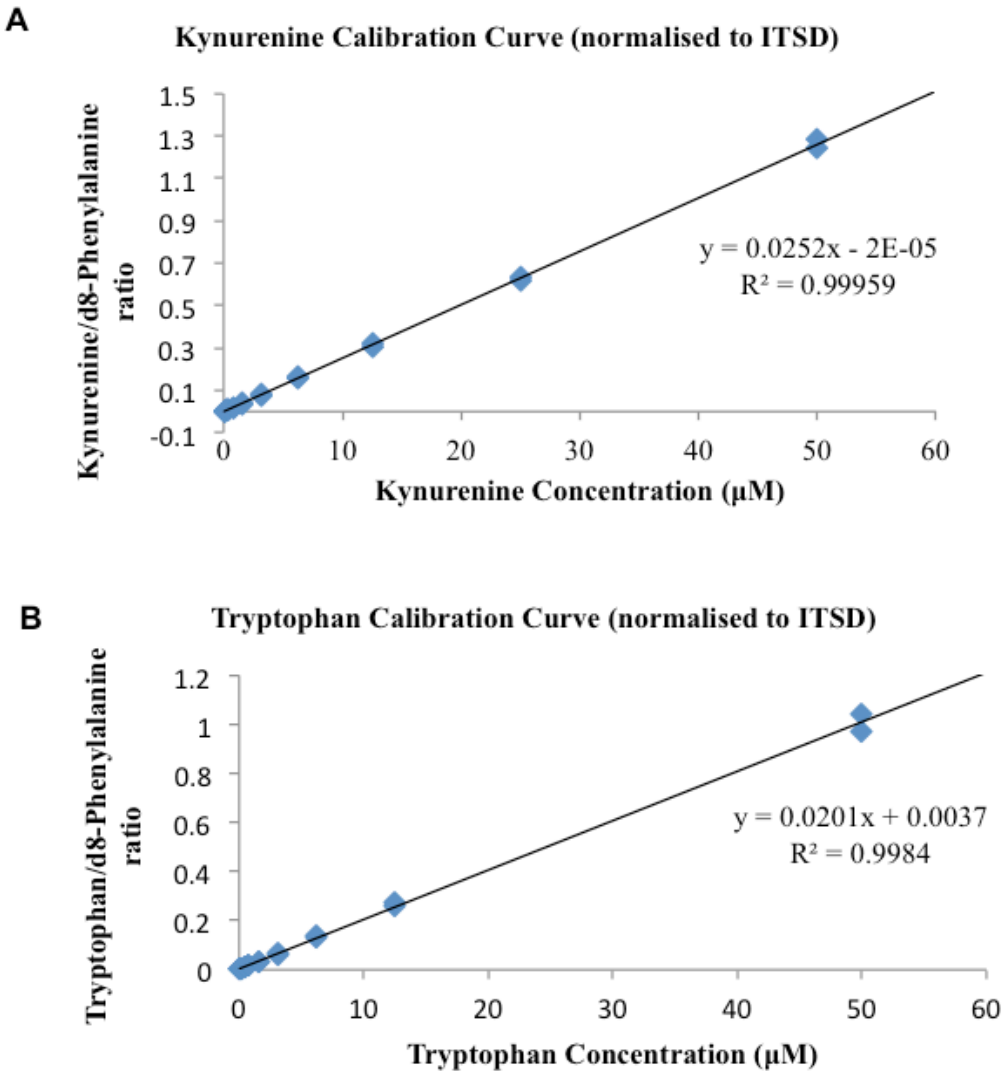


Figure 2-4: Standard curve used in Kynurenine (A) and Tryptophan (B) assays

Standard samples were made at various concentrations through serial dilutions of tryptophan and kynurenine dissolved in water.150 µl methanol (containing internal standard – D8-phenylalanine at 10 µM) was added to 50 µl of each standard concentration solution. After centrifugation at 13,000g 4°C for 10 minutes the acquired supernatants were analysed using liquid chromatography-mass spectrometry. Each data point was the mean of duplicate measurements.

Liquid chromatography-mass spectrometry analysis: Samples were analysed using the standard polychlorinated biphenyls C18-reversed phase assay. Mobile phase A = water 0.1% formic acid; mobile phase B = methanol 0.1% formic acid in positive ion mode only. Mass spectrometry fragmentation events were triggered on tryptophan, kynurenine and D8-phenylalanine to confirm identity. Standards were acquired in duplicate, biological samples in singlet.

2.6 Assessing effect of UC-MSC derived condition media (UC-MSC-CM) on $CD28^-$ T cell frequency

As highlighted in chapter 1, CD28-T cells are immune-pathogenic T cells found localised around bile ducts and associated with pro-inflammatory cytokine production. We undertook preliminary experiments to investigate the effect of UC-MSC-CM on CD28- T cell frequency. $CD4^+CD25^-$ T cells were separated from PBMC using a negative selection antibody cocktail specifically EasySep® $CD4^+CD127^{lo}CD25^+$isolation kit (StemCell Technologies, Manchester, United Kingdom). The isolated $CD4^+CD25^-$T cells were re-suspended at $2x10^6$ cells/ml and cultured in 96 round bottom wells at a concentration of 50 ul/ well (100,000cells) in RPMI (10% FBS, 2mM L-glutamine and 1% penicillin and streptomycin) for a total of 21 days as previously described (37). At the start of the culture period, all the cells were stimulated with anti-CD3/anti-CD28 dynabeads (1 ul/well), (Life Technologies, Paisley, United Kingdom), IL2 (50 U/mL; Peprotech, London, United Kingdom), in the presence/absence of TNFα (10 ng/mL;Peprotech), with or without 90% UC-MSC-CM (acquired as described in section 2.2.5) and 1,25(OH)2D3 (10 nmol/L; ENZO Life Sciences, Exeter, United Kingdom); UC-MSC-CM and 1,25(OH)2D3 were added in at days 7 and 14 of culture. At day 4, the dynabeads were removed using a magnet and

cells split to 0.5 x 10^6 cells/mL. Cultures were assessed every 3–4 days and maintained at 0.5–1x 10^6 cells/ml. Cytokines, UC-MSC-CM and 1,25(OH)2D3 were also re-supplemented at these times, the concentration of IL2 was increased to 100 U/mL after 2 weeks. The final volume in each well was always maintained at 200 µl. At days 14 and days 21 the cells were collected and the CD28- frequency assessed by flow cytometry using methodology described in 2.4. The antibodies used for these experiments are as shown in Table 4.

ANTIBODY	CLONE	FINAL CONCENTRATION	SOURCE
CD 3 FITC	UCHT1	2 µL/106 cells	BD bioscience
CD4 APC	RPA-T4	2 µL/106 cells	BD bioscience
CD28 PE	CD28.2	2 µL/106 cells	BD bioscience
Mouse IgG1-FITC	MOPC-21	3 µL/106 cells	BD bioscience
Mouse IgG2b, k-PE	27-35	5 µL/106 cells	BD bioscience
Mouse IgG2a-APC	X39	4 µL/106 cells	BD bioscience

Table 4: Antibodies used to assess CD28-T cell frequency.

2.7 Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (GraphPad, US). Normality tests were applied by using D'Agostino-Pearson omnibus or Shapiro-Wilk normality tests. Data was expressed as mean (+/- standard deviation) when normally distributed and as median (+/- interquartile range) when not normally distributed. The significance of differences between means was tested using Kruskal-Wallis test for non-parametric data and one-way ANOVA for parametric data. Two-way ANOVA and Mann-Whitney test was used to assess the differences between means when comparing data between two independent groups. A value of $P \leq 0.05$ was considered statistically significant. Generally statistical data are expressed as follows: *≤0.05, **≤0.01, ***≤0.001, **** p≤0.0001. Flow cytometry data analysis was performed using the FlowJo 8.7 software.

CHAPTER 3.

DIRECT EFFECT OF UMBILICAL CORD-DERIVED MESENCHYMAL STROMAL CELLS ON CIRCULATING AND INTRAHEPATIC T CELLS FROM PATIENTS WITH PSC

3.1 Introduction

PSC is an immune-mediated biliary disease lacking effective therapy. Evidence suggests that T lymphocytes play a significant role in the immune-pathogenesis of PSC. There is high T- cell infiltration comprising of both $CD4^+$ and $CD8^+$ T cells in livers of PSC patients (37) with both $CD4^+$ and $CD8^+$ T cells demonstrated to be located in close proximity to bile ducts, the site of injury in PSC (37). Studies have further showed that expression of pro-inflammatory cytokines in particular TNFα and IFNγ is significantly higher in PSC livers than normal liver and there is a high presence of pro-inflammatory $CD4^+$ and $CD8^+$ T cells that have lost expression of CD28 (37). $CD4^+CD28^-$ T-cells are proposed to be important drivers of persistent chronic inflammation given their reduced susceptibility to regulatory mechanisms.

As previously highlighted in the section 1, there is extensive evidence that MSC possess broad immunomodulatory function through either direct cell-cell interaction or via release of paracrine factors. Many studies have focused on the effect of MSC on T-cell responses. *In vitro* studies have demonstrated that proliferation and activation of $CD4^+$ and $CD8^+$ T-cells stimulated with polyclonal mitogens, allogeneic cells or specific antigen is inhibited by MSC (105, 114, 122, 124, 126, 187). MSC are shown to suppress pro-inflammatory cytokine secretion such as IFN ϒ and TNFα production, $CD8^+$ cytotoxic T-lymphocyte-mediated cytotoxicity and regulate the balance between T-helper-1/T-helper-2 (114, 188). MSCs also promote the generation and development of T- regulatory cells, which can positively influence the balance of immune related damage during tissue injury (132). Several reports on culture systems have highlighted that cell-cell contact is a pertinent for the immunomodulatory action of MSC (189, 190). Direct contact between MSCs and T-cells is needed for T-regulatory cell induction (133). Cell-cell contact activates MSC to secrete cell adhesion molecules such as

programmed death ligand 1, proposed to be one of the mechanisms involved in inhibiting T-cell proliferation (125). Similarly, secretion of prostaglandin E2 by MSC, a molecule involved in immunomodulation is enhanced upon co-culture with PBMC (105).

Majority of the studies assessing MSC effect on T- cell responses in humans have been performed using peripheral blood. In PSC, the site of injury is the biliary system within the liver. T-cell phenotype differs in peripheral blood and livers of PSC patients in that intrahepatic $CD4^+$ and $CD8^+$ T-cells are predominately activated/memory T cells and may also possess an exhausted phenotype whereas circulating $CD4^+$ and $CD8^+$ T cells are naïve. It is thus of importance to know whether MSC are equally effective at suppressing both circulating and intrahepatic T-cell effector function from PSC patients. Our study thus differs from others in that we assessed the effect of MSC on $CD4^+$ and $CD8^+$ T cells isolated from both diseased explant liver tissue and peripheral blood of patients with PSC. The results of these *in vitro* studies will hopefully lend support to our clinical trial aimed at assessing the safety and efficacy of MSC in patients with PSC or autoimmune hepatitis.

3.1.1 Chapter Aims

- To determine the optimal conditions for UC-MSC and T-cell co-culture experiments
- To assess the direct effect of UC-MSC on circulating and intrahepatic $CD4^+$ and $CD8^+$ T cell proliferation and activation (TNFα, IFNγ and IL-2 assessment) from patients with PSC
- To determine whether the effect of UC-MSC is specific to PSC by using peripheral blood from patients with HFE and explant livers from patients with PBC

3.2 Results

3.2.1 Optimisation of co-culture conditions

3.2.1.1 Culture media

PBMCs/T cells and UC-MSC are grown in different culture medias (RPMI 1640 and AMEM respectively) .We initially studied, which culture media best supported the survival and growth of UC-MSC co-cultured with T cells. The growth and morphology of cultured UC-MSC was assessed visually. Our methodology is described in section 2.2.3, in brief, UC-MSC were seeded in 24-well flat bottom plates at various densities (1000, 10,000, 20,000, 40,000, 80,000) and in 3 different types of media: 1) AMEM, 10% FCS and 1% P/S 2) RPMI 1640, 10% FCS and 1% P/S 3) 50% AMEM + 50% RPMI 1640, 10% FCS and 1% P/S for 1,3 and 5 days. We found that UC-MSC were able to adopt their normal phenotype (spindle, fibroblastoid) in all three culture medias. However, UC-MSC cultured in AMEM reached target confluency (70-80%) faster compared to when cultured in the other two medias (Figure 3.1 A-C). Stimulated PBMCs cultured in AMEM were able to achieve a mean proliferation of 89% (n=6 PSC blood experiments, Figure 3.4B) indicating that the use of AMEM culture media did not affect the ability of stimulated PBMCs to proliferate. Based on this, all future co-culture experiments were performed using AMEM, 10% FCS and 1% P/S as the culture media.

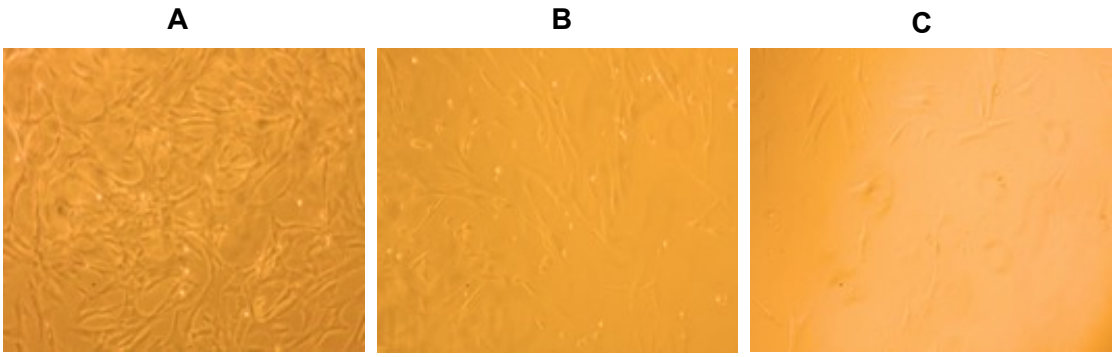


Figure 3-1: Umbilical cord derived mesenchymal stromal cells (UC-MSC) cultured in AMEM media grew faster compared to when cultured in RPMI and RPMI+AMEM.

Representative images of UC-MSC cultured for 5 days at a similar seed density (20 x10^4) in (**A)** AMEM+10%FCS+1% P/S, **(B)** RPMI + 10% FCS + 1% P/S and **(C)** 50% AMEM + 50% RPMI + 10% FCS + 1% P/S The effect on UC-MSC growth and morphology at day 1,3 and 5 was assessed visually. Images were acquired from a light microscope (Olympus IX50 inverted microscope) using 10x magnification

3.2.1.2 Culture plates

Given that UC-MSC are adherent cells, they are usually cultured on flat surfaces so that they can adopt their fibroblastoid appearance whereas PBMCs/T-cells are usually cultured in round bottom well plates that favour cellular contact. To determine which type of culture plates retains UC-MSC immunomodulatory function, the effect of UC-MSC on T cell proliferation was studied by co-culturing UC-MSC in a) flat and b) round bottom well plates. CTV labelled PBMC isolated from blood of patients with HFE (n=2 donors) were co-cultured UC-MSC (n= 2 UC-MSC donors) at the following UC-MSC: PBMC ratios- 1:1, 1:2, 1:4, 1:8, 1:16 in the presence of concomitant anti-CD3 (0.5µg/ml) and anti-CD28 (1µg/ml) in round and flat bottom 96 well plates. The effect of UC-MSC on $CD4^+$ and $CD8^+$ T cell proliferation was assessed using flow cytometry at day 5. I found that the UC-MSC were able to exert a suppressive effect on $CD4^+$ and $CD8^+$ T cell proliferation irrespective of the shape of the wells (Figure 3.2 A and B). All subsequent experiments were performed in round bottom well plates due to the need of close cell-cell contact.

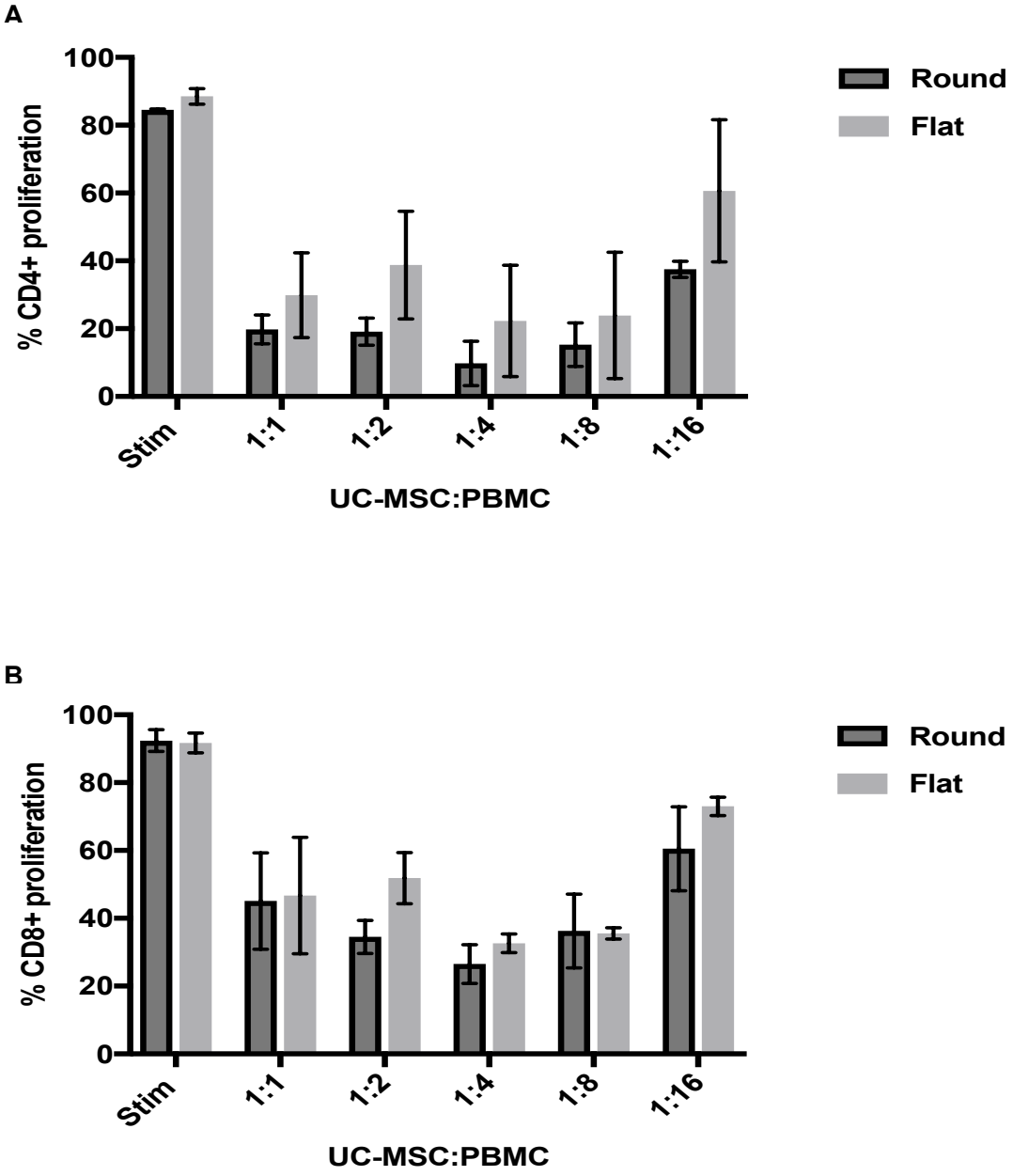


Figure 3-2: Umbilical cord derived mesenchymal stromal cells (UC-MSC) are able to exert a suppressive effect on $CD4^+$ and $CD8^+$ T cell proliferation irrespective of the shape of the wells in tissue culture plates.

UC-MSC were co-cultured with CTV labelled PBMC in round and flat bottom well plates at various ratios and in the presence of 0.5 μg/ml anti-CD3/1 μg/ml anti-CD28; the PBMC number

was kept constant at 100,000 cells/well whilst the UC-MSC number varied. At day 5, effect on $CD4^+$ and $CD8^+$ T cell proliferation was assessed with flow cytometry. Bars show the percentage of **(A)** $CD4^+$ and **(B)** $CD8^+$ T cells that have proliferated in the absence of UC-MSC (Stim, stimulated PBMC only) and in the presence of UC-MSC at different ratios in round and flat bottom well plates. Data shown as median and interquartile range in n=2 peripheral bloods from patients with hereditary heamochromatosis. **Abbreviations:** Stim- stimulated PBMC only; PBMC- peripheral blood mononuclear cells, UC-MSC- umbilical cord derived mesenchymal stromal cells

3.2.1.3 Assessing UC-MSC viability in co-culture experiments

To ensure that the viability of UC-MSC is unaffected when in co-culture with T- cells and subsequently that the effect seen on T cell proliferation is not a consequence of UC-MSC death, the viability of UC-MSC at day 5 of co-culture period was assessed. UC-MSC were collected from the round bottom well plates, after T cell removal, stained with a viability dye and their viability assessed by flow cytometry (method description in section 2.4). UC-MSC cultured in the absence of T cells were used as control. UC-MSC co-cultured with T cells (across all ratios) and UC-MSC cultured alone were found to be similarly viable as indicated by the percentage viability (1:1 (100000 UCMSC: 100000 PBMC) median % viability of 88.3% and UC-MSC alone median % viability of 92.8%) (Figure 3.3). This data confirms that the viability of UC-MSC remains high and not affected by T cell co-culture.

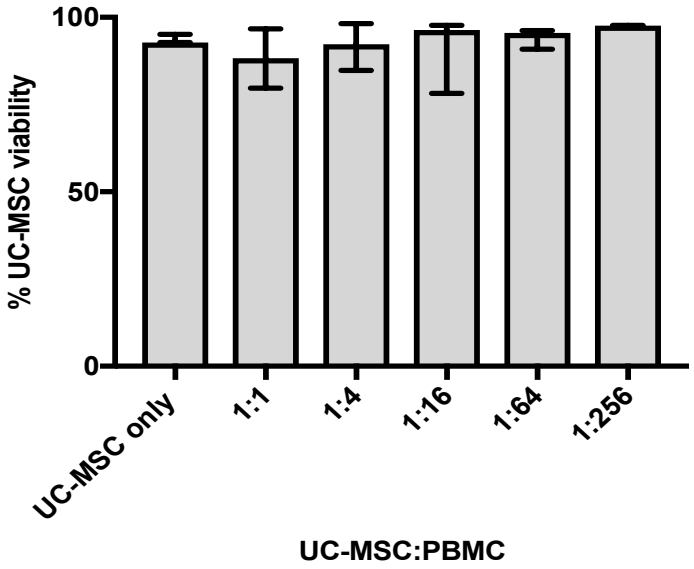


Figure 3-3: Umbilical cord derived mesenchymal stromal cells (UC-MSC) remain viable in co-culture with T cells at all ratios.

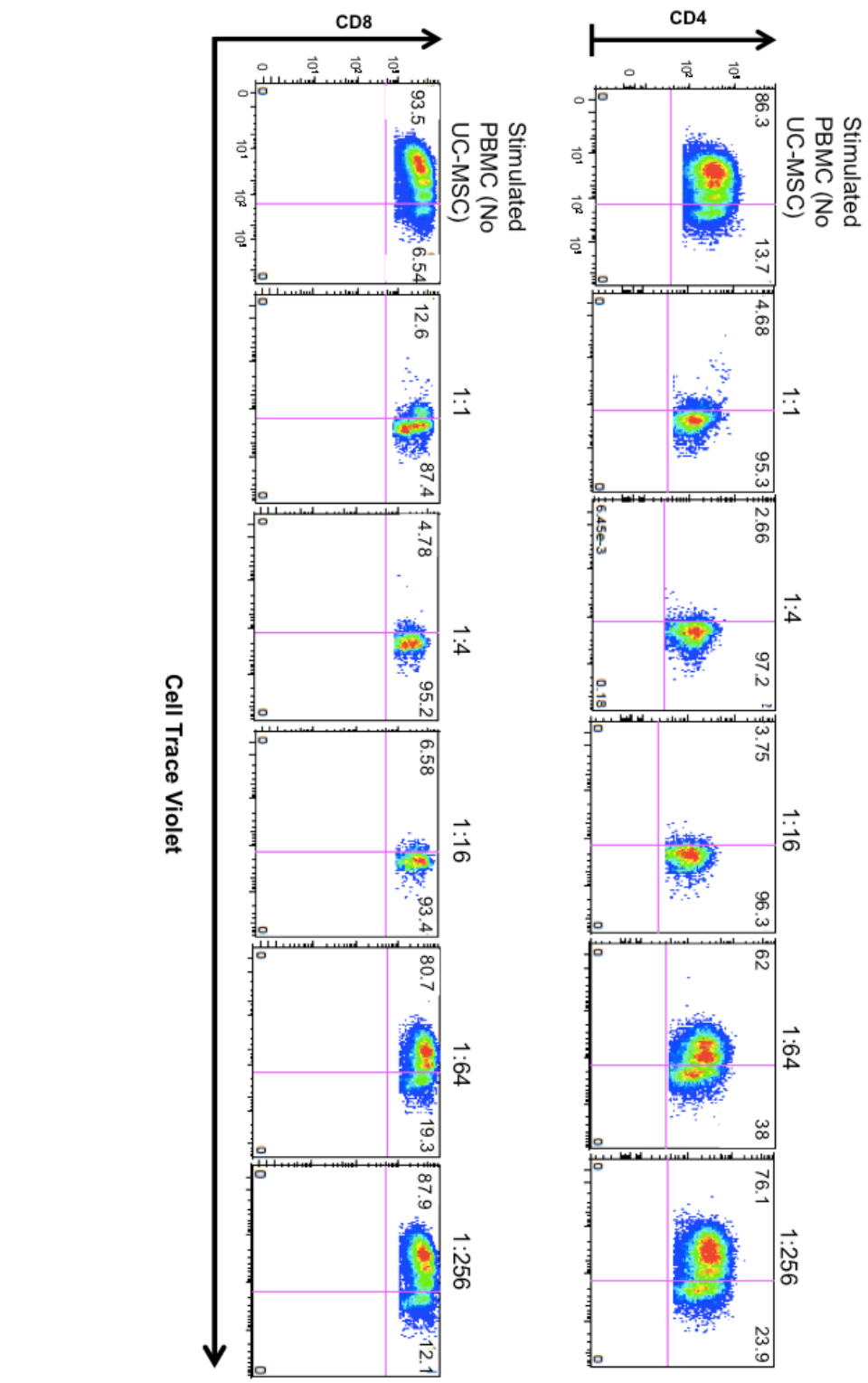
UC-MSC were co-cultured with PBMC at various ratios and in the presence of 0.5μg/ml anti-CD3/1 μg/ml anti-CD28; the PBMC number was kept constant at 100,000 cells/well whilst the UC-MSC number varied. At day 5, adherent UC-MSC were trypsinised and stained with a viability dye. Viability of UC-MSC was then assessed using flow cytometry. UC-MSC cultured only was used as control. Data shown as median with interquartile range and analysed by Kruskal-Wallis test n=3 independent experiments. **Abbreviations:** PBMC- peripheral blood mononuclear cells; UC-MSC- umbilical cord derived mesenchymal stromal cells

3.2.2 Effect of UC-MSC on circulating $CD4^+$ and $CD8^+$ T cell proliferation

3.2.2.1 Proliferation of T-cells from patients with PSC

To study the effect of UC-MSC on circulating $CD4^+$ and $CD8^+$ T cell proliferation, CTV labeled stimulated PBMC isolated from peripheral blood of PSC patients (n=6) were co-cultured with UC-MSC at various ratios and at day 5 effect on T cell proliferation assessed by flow cytometry (method description in sections 2.3.3.1 and 2.4). Co-culture of UC-MSC with stimulated PBMCs resulted in suppression of $CD4^+$ T cell proliferation of the latter from 90.25% when UC-MSC were absent to 27.8% at UC-MSC: PBMC ratio of 1:16 ($p \leq 0.01$). Similarly there was suppression of $CD8^+$ T cell proliferation from 93.5% when UC-MSC were absent to 51.5% at UC-MSC: PBMC ratio of 1:16 ($p \leq 0.05$) (Figures 3.4B and 3.4C). Moreover, the suppressive effect of UC-MSC was dose dependent, with the highest level of suppression of T cell proliferation seen when UC-MSC were cultured at 1:1 ratio with $CD4^+$ (% proliferated $CD4^+$ T cells alone median: 90.25% vs $CD4^+$ T cells + UC-MSC at 1:1: 27.2%, $p \leq 0.01$) and $CD8^+$ T cells (% proliferated $CD8^+$ T cells alone median: 93.5% vs $CD8^+$ T cells + UC-MSC at 1:1: 39.3%, $p \leq 0.05$) as compared to when UC-MSC were cultured with T cells at 1: 256 ratio. There was no significant effect in the percentage T cell proliferation when UC-MSC were cultured at both 1:64 and 1:256 ratio (lowest number of UC-MSC) with $CD4^+$ (% proliferated $CD4^+$ T cells alone: 90.25% vs $CD4^+$ T cells + UC-MSC at 1:64: 79.5% vs $CD4^+$ T cells + UC-MSC at 1:256: 90.5%) and $CD8^+$ (% proliferated $CD8^+$ T cells alone: 93.5% vs $CD8^+$ T cells + UC-MSC at 1:64: 81% vs $CD8^+$ T cells + UC-MSC at 1:256: 87.9%) T cells.

A


CD4
CD8
Cell Trace Violet
Stimulated PBMC (No UC-MSC)
Stimulated PBMC (No UC-MSC)
1:1
1:4
1:16
1:64
1:256
86.3
13.7
4.68
95.3
2.66
97.2
3.75
96.3
62
38
76.1
23.9
93.5
6.54
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95.2
6.58
93.4
80.7
19.3
87.9
12.1

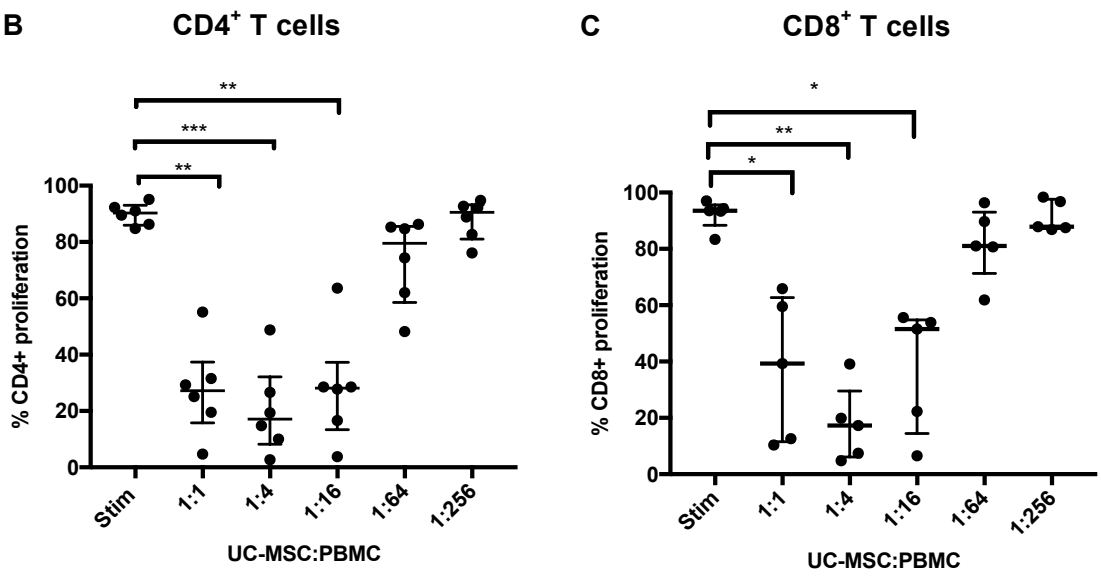


Figure 3-4: Umbilical cord derived mesenchymal stromal cells (UC-MSC) suppress circulating $CD4^+$ and $CD8^+$ T cell proliferation from patients with primary sclerosing cholangitis (PSC) in a dose dependent manner.

UC-MSC were co-cultured with CTV labelled PBMC at various ratios and in the presence of 0.5µg/ml anti-CD3 and 1 µg/ml anti-CD28; the PBMC number was kept constant at 100,000 cells/well whilst the UC-MSC number varied. At day 5, effect on $CD4^+$ and $CD8^+$ T cell proliferation was assessed with flow cytometry. **A:** Representative flow pseudocolour plots demonstrating the effect of UC-MSC on $CD4^+$ and $CD8^+$ T cell proliferation. CTV labelled $CD4^+$ and $CD8^+$ T cells were gated to generate the pseudocolor plots of $CD4^+$ and $CD8^+$ T cell counts (Y axis) versus CTV intensity (X axis) **B and C:** Data showing % of $CD4^+$ and % of $CD8^+$ T cell proliferation in n=6 and n=5 PSC blood samples respectively. Lines indicate median values and bars the interquartile range. Data is statistically significant using Kruskal-Wallis test *p≤0.05, **p≤0.01, ***p≤0.001 **Abbreviations:** PBMC- peripheral blood mononuclear cells; Stim- stimulated PBMC only (no UC-MSC); UC-MSC- umbilical cord derived mesenchymal stromal cells

3.2.2.2 Proliferation of T-cells from patients with hereditary haemochromatosis (controls)

To assess if the suppressive effect of UC-MSC on $CD4^+$ and $CD8^+$ T cell proliferation was specific to PSC, peripheral blood from patients with hereditary haemochromatosis (HFE) was used as controls and the same experiments described in section 3.2.2.1 was repeated. It is noteworthy that we did not know whether patients with HFE had underlying chronic liver disease. UC-MSC were also able to suppress $CD4^+$ and $CD8^+$ T cell proliferation in HFE controls, with no statistically significant differences in the effect between patients with PSC and HFE controls (Figure 3.5 A-B) confirming that the UC-MSC effect on T-cell proliferation is not disease-dependent/specific.

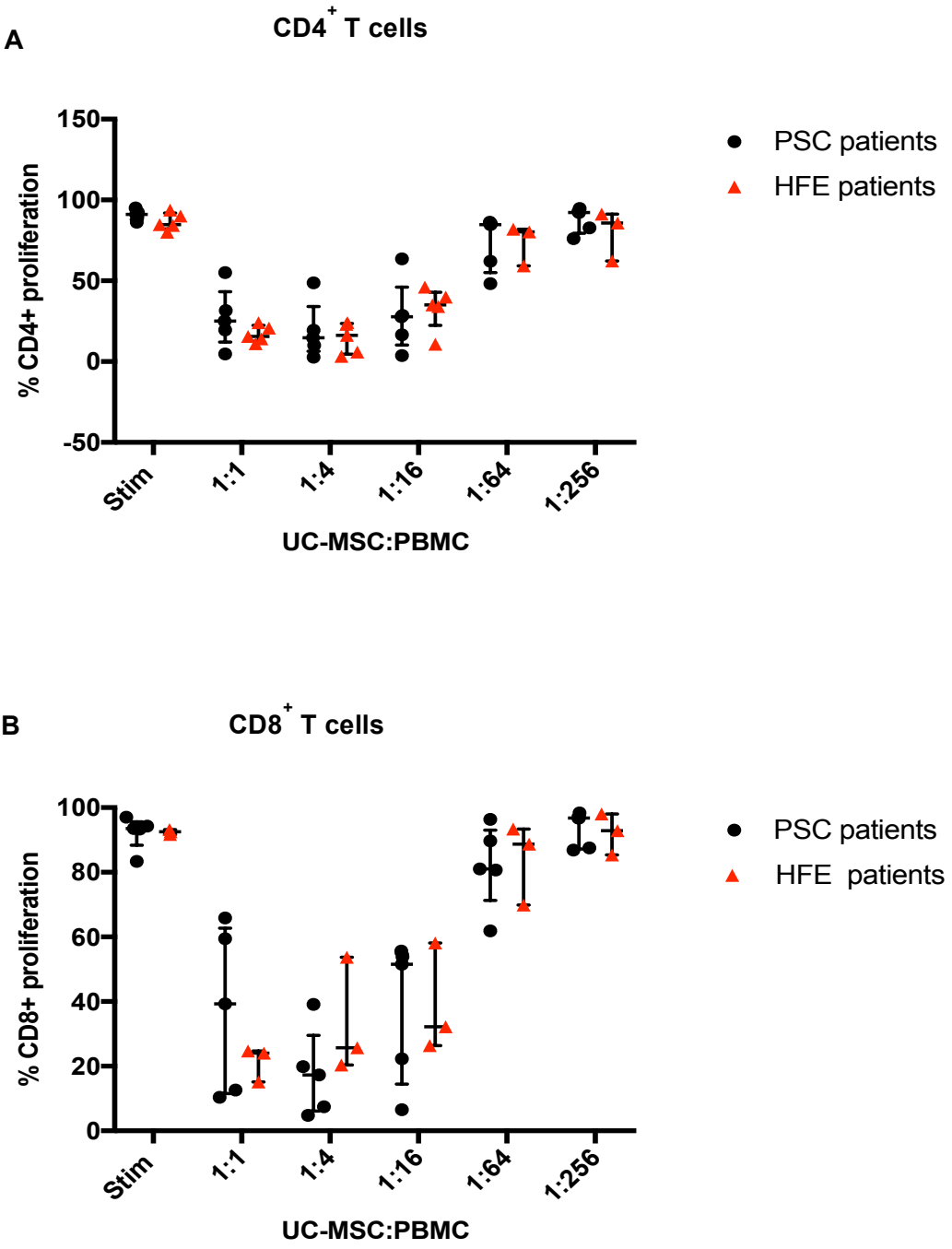


Figure 3-5: The suppressive effect of umbilical cord derived mesenchymal stromal cells (UC-MSC) on proliferation of circulating $CD4^+$ and $CD8^+$ T cell is not disease specific.

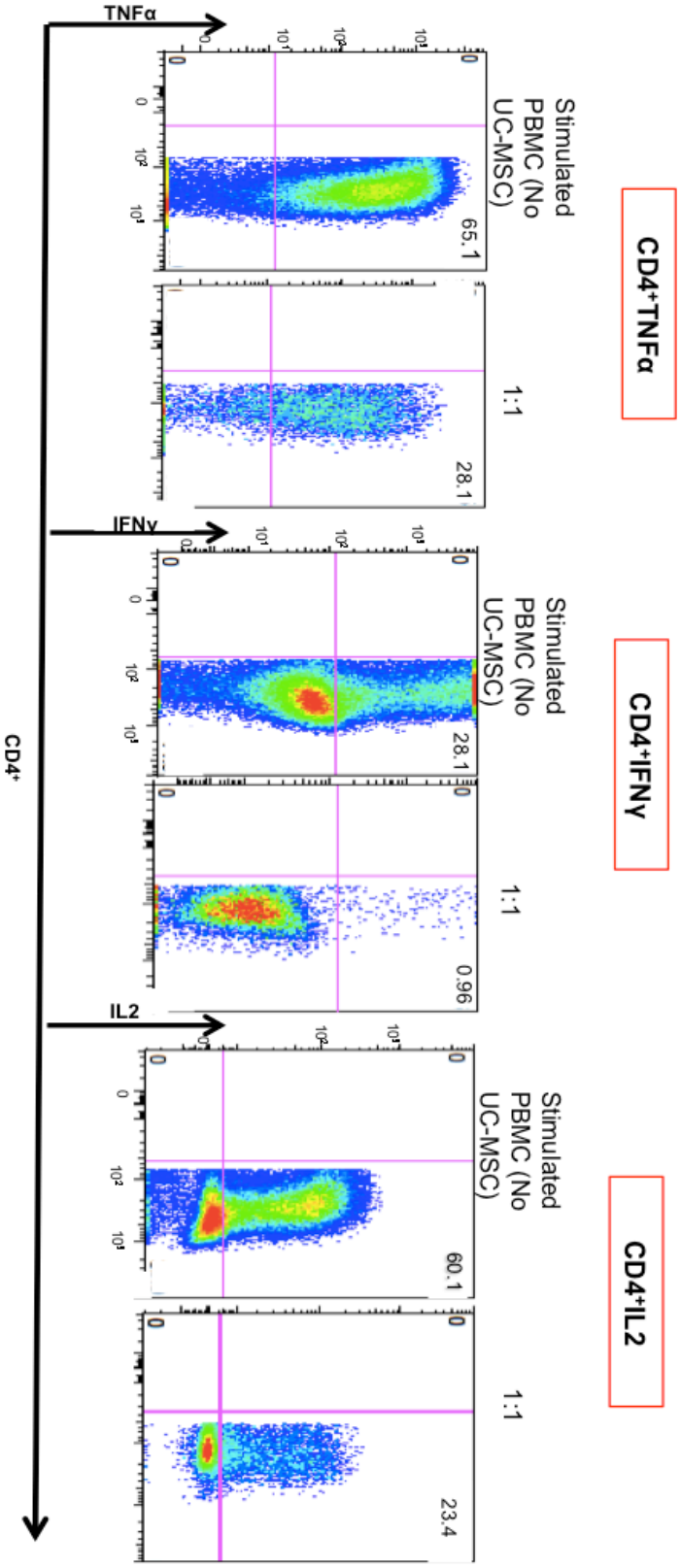
UC-MSC were co-cultured with CTV labelled PBMC at various ratios and in the presence of 0.5µg/ml anti-CD3 and 1 µg/ml anti-CD28; the PBMC number was kept constant at 100,000 cells/well whilst the UC-MSC number varied. At day 5, effect on T cell proliferation was

assessed with flow cytometry. Peripheral blood from patients with hereditary haemochromatosis (HFE) was used as controls. **A:** Data showing % of $CD4^+$ T cell proliferation in n=6 PSC and n=5 HFE controls except for the ratios 1:64 and 1:256 which includes data from n=4 HFE controls. **B:** Data showing % of $CD8^+$ T cell proliferation in n=5 PSC and n=3 HFE controls. Lines in graphs A and B indicate median values and bars the interquartile range . Data analysed using two-way ANOVA. **Abbreviations:** HFE- Hereditary Haemachromatosis PBMC- peripheral blood mononuclear cells; Stim- stimulated PBMC only (no UC-MSC); UC-MSC- umbilical cord derived mesenchymal stromal cells

3.2.3 Effect of UC-MSC on circulating $CD4^+$ and $CD8^+$ T cell activation from patients with PSC

As previously discussed in sections 1.1.6 and 3.1 significantly high TNFα and IFNϒ levels are present in PSC livers. To further investigate the immunomodulatory role of UC-MSC in PSC, their effect on circulating $CD4^+$ and $CD8^+$ T cell activation was studied. On day 5 of co-culture (UC-MSC and anti CD3/anti CD28 stimulated PBMC), PBMC were further stimulated with 50ng/ml PMA, 1 μM ionomycin and 3 μg/ml Brefeldin A for 4 hours and the intracellular expression of TNFα, IFNγ and IL 2 cytokines was assessed using flow cytometry. UC-MSC reduced the percentage of circulating $CD4^+$TNFα expressing cells (PBMC only median: 69.45% vs PBMC+ UC-MSC at 1:1; 41.6%, $p<0.05$) and percentage of $CD4^+$IFNγ expressing cells (PBMC only median; 23.8% vs PBMC+ UC-MSC at 1:1; 1.035% vs PBMC+ UC-MSC at 1:4; 2.35%, $p<0.05$) (n=4 PSC). There was a trend towards reduction in the percentage of circulating $CD4^+IL2^+$ expressing cells however this did not reach statistical significance (Figures 3.6 B-D). Similarly although there appears to be a trend towards reduction in the percentage of circulating $CD8^+$TNFα and $CD8^+$ IFNγ expressing cells when UC-MSC were present, this did not reach statistical significance (n=4 PSC) (Figures 3.6 E-F). It was not possible to analyse data showing effect of UC-MSC on the percentage of $CD8^+$IL2 expressing cells with confidence due to there being very few $CD8^+$IL2 expressing T cells in the absence of UC-MSC (control).

A.


CD4+TNFα
CD4+IFNγ
CD4+IL2
TNFα
IFNγ
IL2
CD4+
Stimulated PBMC (No UC-MSC)
1:1
65.1
28.1
28.1
0.96
60.1
23.4

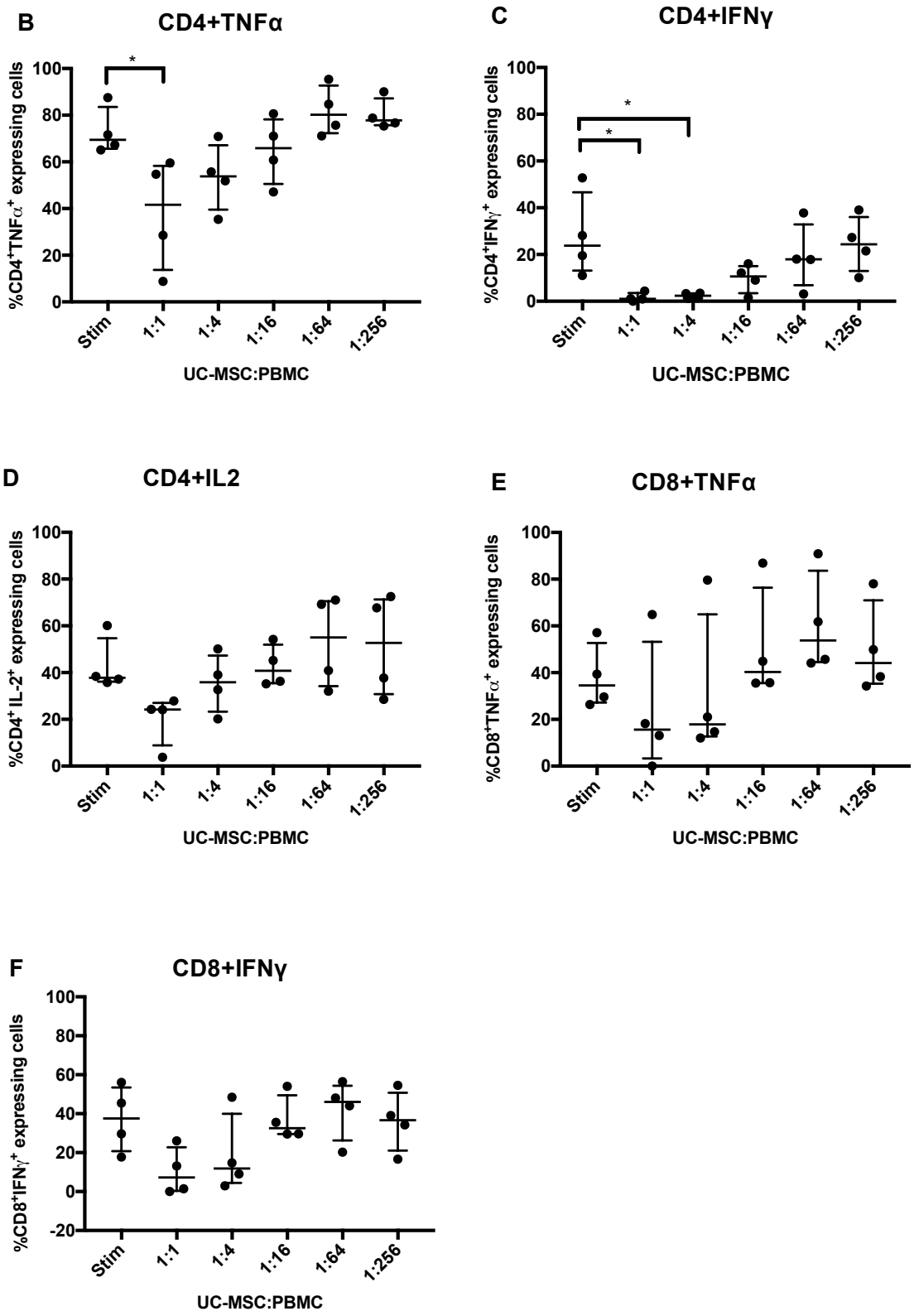


Figure 3-6: Umbilical cord derived mesenchymal stromal cells (UC-MSC) affect the ability of circulating $CD4^+$ and $CD8^+$ T cells from patients with primary sclerosing cholangitis (PSC) to produce inflammatory cytokines.

UC-MSC were co-cultured with PBMC at various ratios in the presence of 0.5μg/ml anti-CD3/1μg/ml anti-CD28. At day 5, PBMC were further stimulated with 50ng/ml PMA, 1 μM ionomycin and 3 μg/ml Brefeldin A for 4 hours and expression of TNFα, IFN γ and IL2 assessed by flow cytometry. **A:** Representative flow cytometry pseudocolour plots demonstrating the effect of UC-MSC on %$CD4^+$TNFα, IFN γ and IL2 expressing cells**. B:** Data presented as median with interquatile range and analysed by one-way ANOVA; n=4. **C:** Data presented as median with interquatile range and analysed by Kruskal-Wallis test; n=4. **D-F:** Data presented as median with interquartile range and analysed by Kruskal-Wallis test; n=4. Lines indicate median values and bars the interquartile range. *$p<0.05$. **Abbreviations:** PBMC- peripheral blood mononuclear cells; Stim- stimulated PBMC only (no UC-MSC); UC-MSC- umbilical cord derived mesenchymal stromal cells

3.2.4 Effect of UC-MSC on proliferation of intrahepatic $CD4^+$ and $CD8^+$ T cell cells

3.2.4.1 Optimisation of UC-MSC and intrahepatic T-cell co-culture experiments

The effect of UC-MSC on intrahepatic $CD4^+$ and $CD8^+$ T cell proliferation and activation was studied. Unexpectedly, at the end of the 5-day co-culture period, the total number of stimulated LIMCs retrieved was very low (14, 497 cells) in comparison to the number that was initially seeded in the wells (100,000 cells) and to stimulated PBMC retrieved at the end of 5 day culture period (mean number of flow cytometry events gated on lymphocytes: LIMC; 14,497 ± 6798 cells versus PBMC: 206,040 ± 33113 cells, $p \leq 0.05$) from patients with PSC (n=4) (Figure 3.7A and B). This prevented confident analysis of intrahepatic T cell proliferation and activation. Considering the fact that the LIMCs were T cell receptor stimulated thus should have divided, several concerns were raised: a) the culture period may be too long for the intrahepatic T cells and b) T cells may compete with other mononuclear cells for nutrients. To address these concerns $CD4^+$ and $CD8^+$ T cells were negatively selected and the culture time was reduced to 4 days.

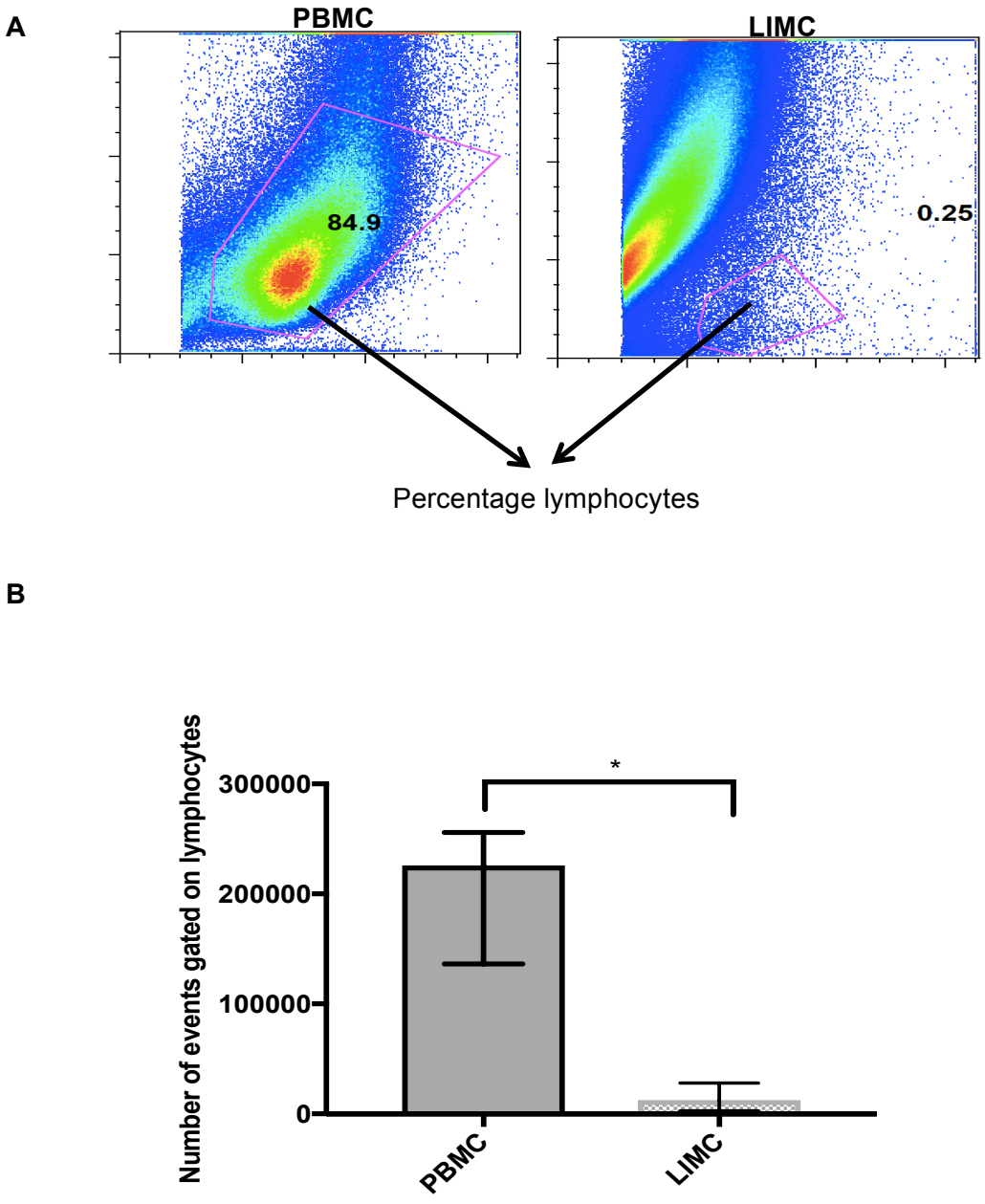


Figure 3-7: Liver infiltrating mononuclear cells do not survive during the 5-day culture period. There are differences between cultured peripheral blood mononuclear cells (PBMC) and liver infiltrating mononuclear cells (LIMCs).

LIMCs and PBMC/well were cultured in the presence of 0.5 µg/ml anti-CD3 and 1 µg/ml anti-CD28 for 5 days. **A:** Representative flow cytometry pseudocolour plot demonstrating PBMC and LIMCs with gating on lymphocytes. **B:** Data showing total number of flow cytometry events

gated on lymphocytes presented as median with interquatile range and analysed using Mann-Whitney test; LIMC data: n=4 explant livers including 2 PSC, 1 PBC, and 1 normal resection; PBMC data: n=4 PSC peripheral blood. * $p \leq 0.05$. **Abbreviations:** LIMC- liver infiltrating mononuclear cells, PBMC- peripheral blood mononuclear cells.

3.2.4.2 Proliferation of intrahepatic CD4+ and CD8+ T cells from patients with PSC

To determine the effect of UC-MSC on intrahepatic T cells, $CD4^+$ and $CD8^+$ T cells were isolated using negative bead selection from LIMCs of patients with PSC and co-cultured with UC-MSC at the same ratios used in the peripheral blood experiments i.e. UC-MSC: T-cell 1:1, 1:4, 1: 16, 1:64, 1:256 in the presence of anti-CD3 (0.5µg/ml) and anti-CD28 (1µg/ml) for 4 days. At day 4, flow cytometry was used to assess the effect of UC-MSC on intrahepatic T cell proliferation. Addition of UC-MSC suppressed $CD4^+$ (% proliferated $CD4^+$ only median; 86.9% vs $CD4^+$ + UC-MSC at 1:1; 19% vs $CD4^+$ + UC-MSC at 1:4; 17.45% vs $CD4^+$ + UC-MSC at 1:16; 24.8%, $p<0.05$) and $CD8^+$ (% proliferated $CD8^+$ only median; 85.3% vs $CD8^+$ + UC-MSC at 1:1; 17.2% vs $CD8^+$ + UC-MSC at 1:4: 7.93%; $p\leq 0.05$) T cell proliferation at ratios of up to 1:16 and 1:4 respectively compared to stimulated $CD4^+$ (n=6) and $CD8^+$ (n=5) T cells only (UC-MSC absent) (Figure 3.8 A and B). As with peripheral blood the suppressive effect of UC-MSC on intrahepatic T cells was dose dependent. In the $CD8^+$ T cell data set, although cells were isolated from 5 PSC livers, on some occasions it was not possible to culture $CD8^+$ T cells with UC-MSC at all the ratios due to low numbers of $CD8^+$ T cells initially acquired from LIMCs.

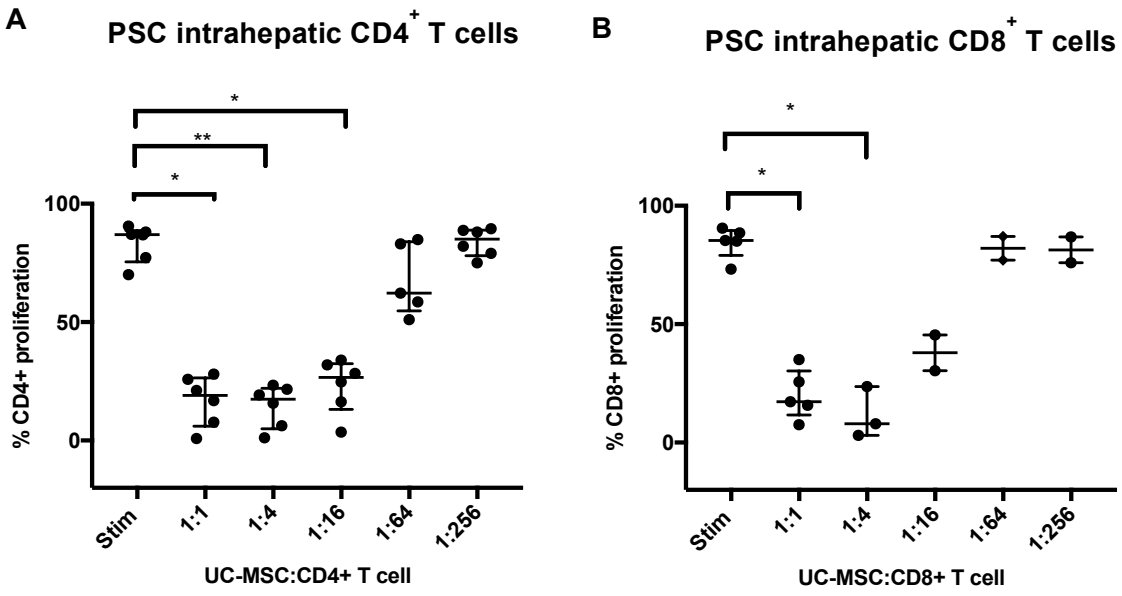


Figure 3-8: Umbilical cord derived mesenchymal stromal cells (UC-MSC) suppress intrahepatic CD4$^+$ and CD8$^+$ T cell proliferation from patients with primary sclerosing cholangitis (PSC).

Negatively selected intrahepatic CD4$^+$ and CD8$^+$ T cells were co-cultured with UC-MSC at 1:1,1:4,1:16, 1:64, 1:256 for 4 days in the presence of 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28. The T cell number was kept constant at 100, 000 cells/well whilst the UC-MSC number varied. At day 4, effect of UC-MSC on intrahepatic T cells was assessed using flow cytometry. **A:** Data showing % CD4$^+$ proliferation in n=6 PSC livers. Lines indicate median and bars the interquartile range. Data analysed by Kruskal-Wallis test. **B:** Data showing % CD8$^+$ proliferation in n=5 PSC livers for 1:1, n=3 PSC livers for 1:4 and n=2 PSC livers for 1:16, 1:64, 1:256. Lines indicate median and bars the interquartile range. Data analysed by Kruskal-Wallis test. * $p<0.05$ ** $p \leq 0.01$. **Abbreviations:** Stim- stimulated T cells only (no UC-MSC); UC-MSC- umbilical cord derived mesenchymal stromal cells.

3.2.4.3 Proliferation of intrahepatic CD4$^+$ and CD8$^+$ T cells from patients with PBC

To determine whether the observed effects of UC-MSC on intrahepatic T cell proliferation were specific to PSC, explanted liver tissue from patients with PBC were used as controls. The rationale for using PBC livers as controls is based upon the fact that both PSC and PBC are cholestatic liver diseases (affecting the biliary tree) and share similar disease characteristics. Addition of UC-MSC resulted in a trend towards reduction of the percentage of intrahepatic CD4$^+$ proliferating cells (% proliferated CD4+ alone median: 87.95% vs CD4$^+$ + UC-MSC at 1:1: 16.75%) from patients with PBC although these results did not reach statistical significance (Figure 3.9 A). There was a trend in reduction in the percentage of intrahepatic CD8$^+$ T cell proliferation on addition of UC-MSC at ratios of up to 1:4 compared to stimulated CD8$^+$ T cells only (UC-MSC absent) (% proliferated CD8$^+$ only median: 85% vs CD8$^+$ + UC-MSC at 1:1; 5.94% vs CD8$^+$ + UC-MSC at 1:4; 6.4%, $p \leq 0.01$) (Figure 3.9B). No statistically significant difference in the suppressive effect of UC-MSC on intrahepatic CD4$^+$ (Figure 3.9C) and CD8$^+$ (Figure 3.9D) T cell proliferation between livers of patients with PSC and PBC was observed confirming that the effect of UC-MSC is not disease specific.

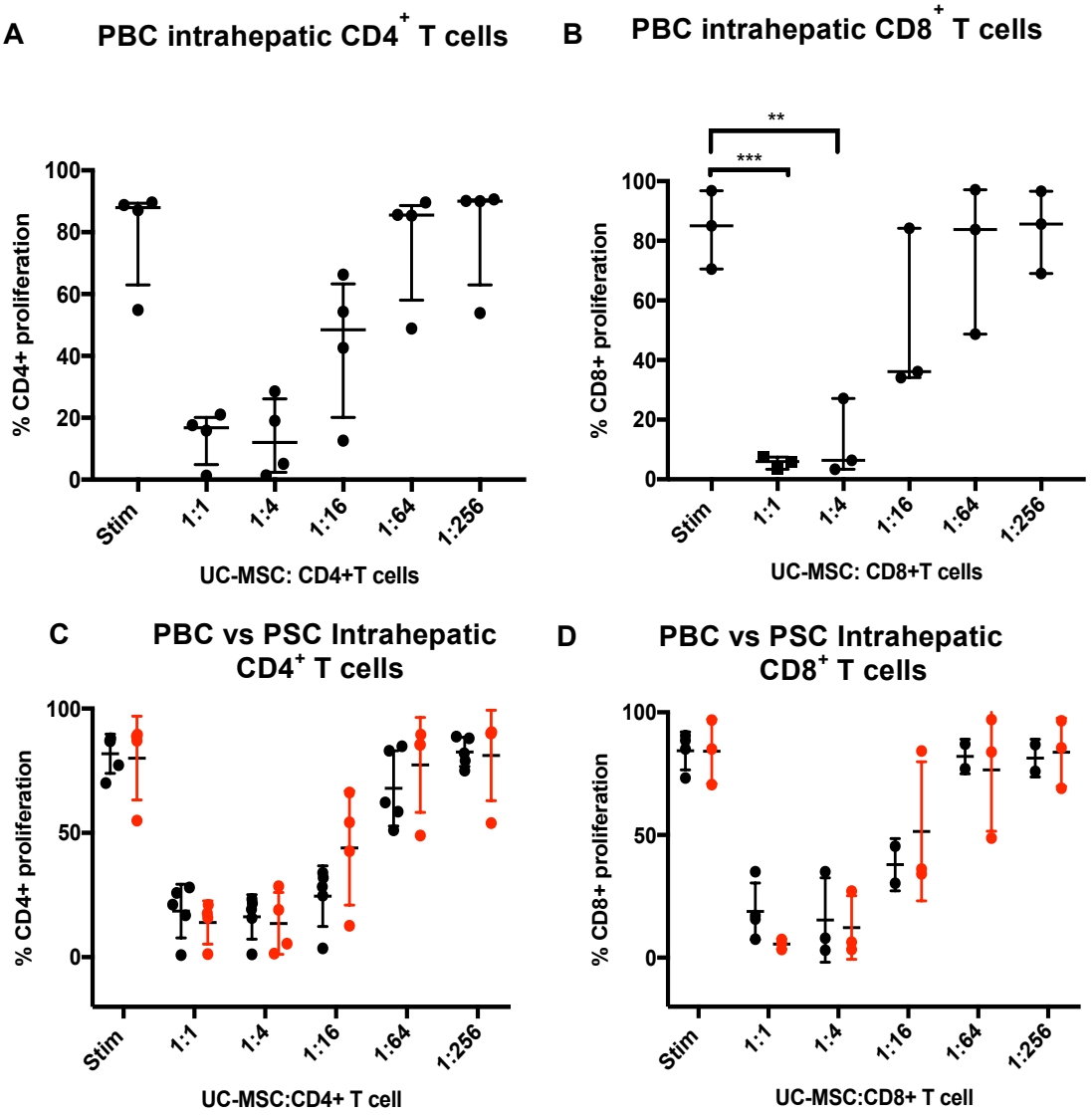


Figure 3-9: Umbilical cord derived mesenchymal stromal cells (UC-MSC) had a tendency to suppress intrahepatic CD8$^+$ T cell proliferation from patients with primary biliary cholangitis (PBC). The effect of UC-MSC on suppression of intrahepatic T cell proliferation is not disease specific.

Negatively selected intrahepatic CD4$^+$ and CD8$^+$ T cells from PBC livers were co-cultured with UC-MSC at 1:1,1:4,1:16, 1:64, 1:256 for 4 days in the presence of 0.5μg/ml anti-CD3 and 1μg/ml anti-CD28. The T cell number was kept constant at 100, 000 cells/well whilst the UC-

MSC number varied. At day 4, effect of UC-MSC on intrahepatic T cells was assessed using flow cytometry. **A:** Data showing % $CD4^+$ proliferation in n=4 PBC livers. Lines indicate median and bars the interquartile range. Analysis performed by Kruskal Wallis test **B:** Data showing % $CD8^+$ proliferation in n=3 PBC livers. Lines indicate median and bars the interquartile range. Analysis performed by one-way ANOVA. **C:** Comparison of PBC (n=4) and PSC (n=5) % $CD4^+$ proliferation using two-way ANOVA analysis. **D:** Comparison of PBC (n=3) and PSC (n=4 for 1:1, n=3 for 1:4, n=2 for 1:16, 1:64 and1:256 ratios) % CD8+ proliferation using T test ** $p \leq 0.01$ *** $p \leq 0.001$. **Abbreviations:** Stim- stimulated T cells only (no UC-MSC); UC-MSC- umbilical cord derived mesenchymal stromal cells.

3.2.5 Effect of UC-MSC on activation of intrahepatic $CD4^+$ and $CD8^+$ T cell cells

3.2.5.1 Activation of PSC T cells

The effect of UC-MSC on ability of intrahepatic T cells from patients with PSC to produce inflammatory cytokines was assessed next. On day 4, the co-cultured intrahepatic T cells were further stimulated with 50ng/ml PMA, 1 μM ionomycin and 3 μg/ml Brefeldin A for 4 hours and the expression of TNFα, IFN γ and IL 2 assessed using flow cytometry. The presence of UC-MSC reduced the percentage of $CD4^+$TNFα ($CD4^+$ alone median: 77.9% vs $CD4^+$ + UC-MSC at 1:1: 21.2% vs $CD4^+$ +UC-MSC at 1:4: 19.1% vs $CD4^+$ + UC-MSC at 1:16: 45%, $p<0.05$) (Figure 3.10 A), $CD4^+$IFNϒ ($CD4^+$ only median: 23.1% vs $CD4^+$ + UC-MSC at 1:1: 7.91% vs $CD4^+$ + UC-MSC at 1:4: 7.62%, $p\leq0.01$) (Figure 3.10 B) and $CD4^+$IL2 ($CD4^+$ only median: 57.5% vs $CD4^+$ + UC-MSC at 1:1: 16.8% vs $CD4^+$ + UC-MSC at 1:4: 15.9%, $p\leq0.01$) (Figure 3.10 C) expressing cells compared to stimulated $CD4^+$ T cells only (UC-MSC absent) at ratios of up to 1:16, 1:4, 1:4 respectively (n=5). No trend in reduction in the percentage of $CD8^+$TNFα (Figure 3.10 D), $CD8^+$ IFNϒ (Figure 3.10 E) and $CD8^+$ IL2 (Figure 3.10 F) expressing cells was seen on addition of UC-MSC.

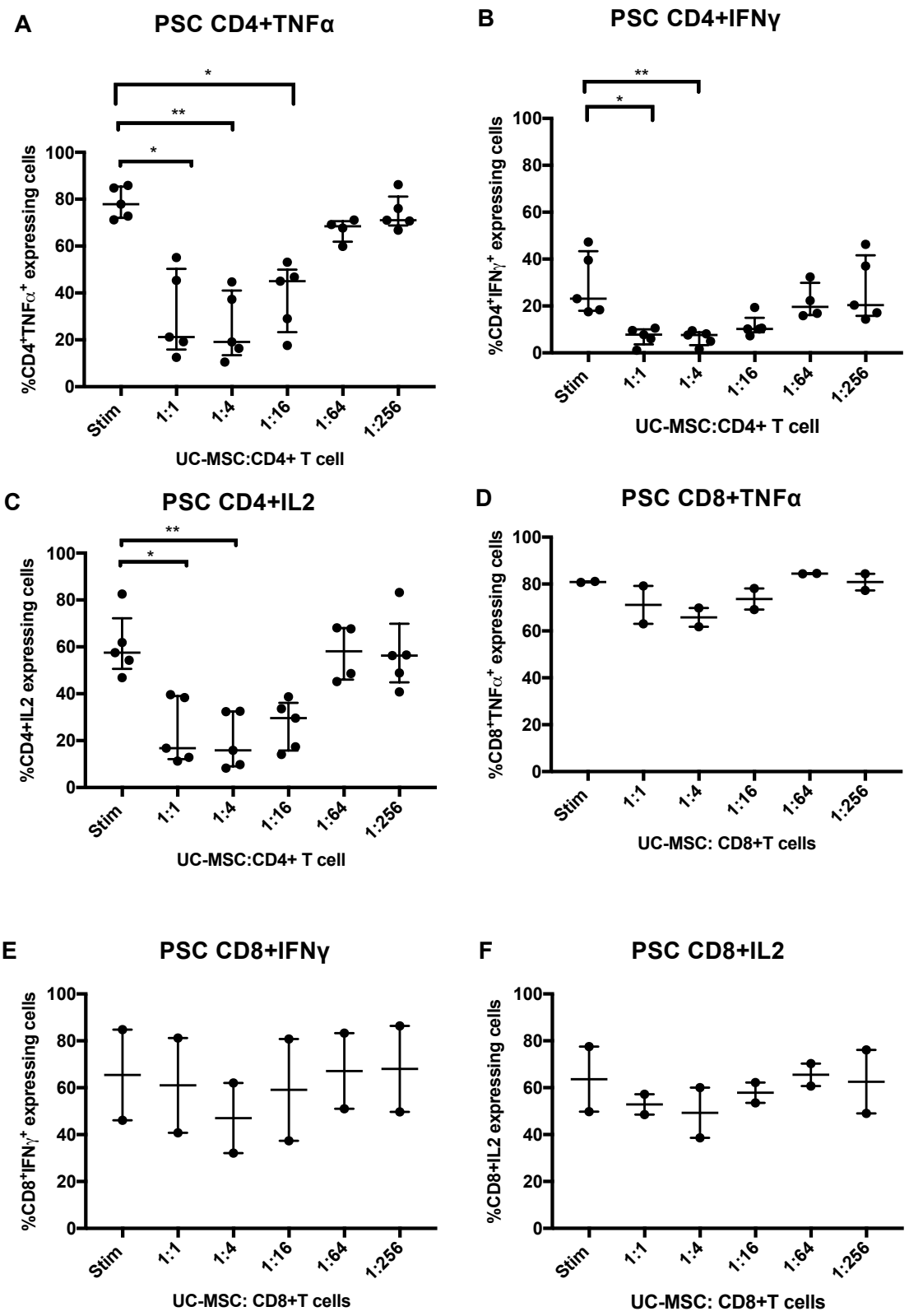

A
PSC CD4+TNFα
%CD4+TNFα+ expressing cells
UC-MSC:CD4+ T cell
B
PSC CD4+IFNγ
%CD4+IFNγ+ expressing cells
UC-MSC:CD4+ T cell
C
PSC CD4+IL2
%CD4+IL2 expressing cells
UC-MSC:CD4+ T cell
D
PSC CD8+TNFα
%CD8+TNFα+ expressing cells
UC-MSC: CD8+T cells
E
PSC CD8+IFNγ
%CD8+IFNγ+ expressing cells
UC-MSC: CD8+T cells
F
PSC CD8+IL2
%CD8+IL2 expressing cells
UC-MSC: CD8+T cells
Stim
1:1
1:4
1:16
1:64
1:256

Figure 3-10: UC-MSC reduce the ability of intrahepatic $CD4^+$ T cells, from patients with PSC, to produce inflammatory cytokines.

UC-MSC were co-cultured with $CD4^+$ and $CD8^+$ T cells at various ratios in the presence of 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28. The T cell number was kept constant at 100, 000 cells/well whilst the UC-MSC number varied. At day 4, T cells were further stimulated with 50ng/ml PMA, 1 µM ionomycin and 3 µg/ml Brefeldin A for 4 hours and expression of TNFα, IFN γ and IL2 assessed by flow cytometry. **A-C:** Data showing $\%CD4^+$TNFα, IFNγ, IL-2 expressing cells in n=5 PSC livers except for the ratio 1:64 which represents data of n=4. Lines indicate median and bars the interquartile range. Analysis performed using Kruskal-Wallis test. **D-F:** Data showing $\%CD8^+$ TNFα, IFNγ, IL-2 expressing cells in n=2 PSC livers. Lines represent median and bars the interquartile range. *$p<0.05$ **$p\leq0.01$. **Abbreviations:** Stim- stimulated T cells only (no UC-MSC); MSC- umbilical cord derived mesenchymal stromal cells.

3.2.5.2 Activation of PBC T cells

$CD4^+$ and $CD8^+$ T cells isolated from the explanted livers of patients with PBC were used as controls to assess the effect of UC-MSC on activation of intrahepatic T cells. Addition of UC-MSC resulted in a trend in reduction of the percentage of $CD4^+$IL2 ($CD4^+$ only median: 43.8% vs $CD4^+$ + UC-MSC at 1:1: 12.5% vs $CD4^+$ + UC-MSC at 1:4: 23.5%,) expressing cells compared to stimulated $CD4^+$ T cells only at ratios of up to 1:4 (Figure 3.11C). There was also a trend towards reduction of percentage $CD4^+$TNFα (Figure 3.11A) and IFNγ (Figure 3.11B) expressing cells. No trend in reduction in the percentage of $CD8^+$TNFα, IFNγ and IL-2 expressing cells from patients with PBC was observed on addition of UC-MSC (Figure 3.11D-F).

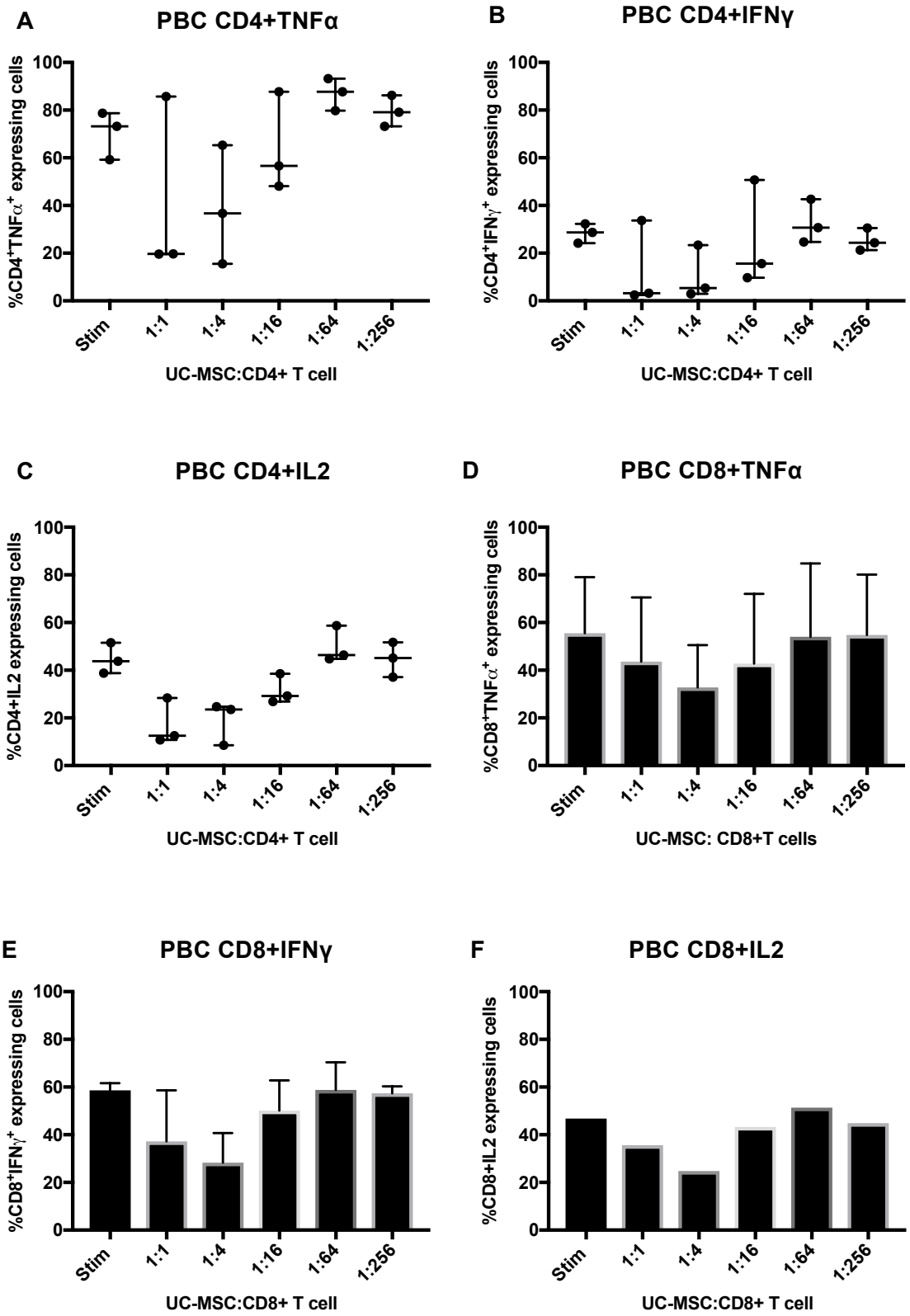

A
PBC CD4+TNFα
%CD4+TNFα+ expressing cells
100
80
60
40
20
0
Stim
1:1
1:4
1:16
1:64
1:256
UC-MSC:CD4+ T cell
B
PBC CD4+IFNγ
%CD4+IFNγ+ expressing cells
100
80
60
40
20
0
Stim
1:1
1:4
1:16
1:64
1:256
UC-MSC:CD4+ T cell
C
PBC CD4+IL2
%CD4+IL2 expressing cells
100
80
60
40
20
0
Stim
1:1
1:4
1:16
1:64
1:256
UC-MSC:CD4+ T cell
D
PBC CD8+TNFα
%CD8+TNFα+ expressing cells
100
80
60
40
20
0
Stim
1:1
1:4
1:16
1:64
1:256
UC-MSC: CD8+T cells
E
PBC CD8+IFNγ
%CD8+IFNγ+ expressing cells
100
80
60
40
20
0
Stim
1:1
1:4
1:16
1:64
1:256
UC-MSC:CD8+ T cell
F
PBC CD8+IL2
%CD8+IL2 expressing cells
100
80
60
40
20
0
Stim
1:1
1:4
1:16
1:64
1:256
UC-MSC:CD8+ T cell

Figure 3-11: UC-MSC also has an effect on the ability of intrahepatic CD4+ T cells from patients with PBC to produce pro-inflammatory cytokines.

UC-MSC were co-cultured with $CD4^+$ and $CD8^+$ T cells at various ratios in the presence of 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28. The T cell number was kept constant at 100, 000 cells/well whilst the UC-MSC number varied. At day 4, T cells were further stimulated with 50ng/ml PMA, 1 µM ionomycin and 3 µg/ml Brefeldin A for 4 hours and expression of TNFα, IFN γ and IL2 assessed by flow cytometry. **A-C:** Data showing $\%CD4^+$TNFα, IFNγ and IL2 expressing cells in n=3 PBC livers. For graphs **A-C,** lines indicate median and bars the interquartile range. Analysis performed using Kruskal-Wallis test. **D-E:** Data showing %CD8+ TNFα and IFNγ expressing cells in n=2 PBC livers. Lines indicate median and bars the Interquartile range. **F:** Data showing %CD8+IL2 expressing cells in n=1 PBC liver. **p≤0.01. **Abbreviations:** Stim- stimulated T cells only (no UC-MSC); UC-MSC- umbilical cord derived mesenchymal stromal cells.

3.3 Conclusion

The immunomodulatory role of MSC in humans has been extensively studied, however, only few studies have used diseased tissue to study the effect of MSC on T cell responses. Majority of the studies investigating the effect of MSC on T cell responses have used T cells derived from peripheral blood. In PSC, it is reported that peripheral blood comprises predominately naïve T cells whilst intrahepatic T cells are of activated/memory phenotype (37). With this in mind, it is important to study the effect of MSC on both circulating and intrahepatic T cells to assess if there are differences in the effect of UC-MSC. In this chapter, our *in vitro* experiments have demonstrated that the addition of UC-MSC suppresses circulating and intrahepatic $CD4^+$ and $CD8^+$ T cell proliferation from patients with PSC. In keeping with reported literature, this effect is dose dependent with the highest number of UC-MSC at the ratio of 1:1 exerting maximum suppressive effect and lowest number of UC-MSC at the ratio of 1:256 exerting no suppressive effect. We found no significant differences in the suppressive effect of UC-MSC on circulating $CD4^+$ and $CD8^+$ T cells between patients with PSC and those with hereditary haemochromatosis (HFE controls) confirming that the effect of UC-MSC on $CD4^+$ and $CD8^+$ T cell proliferation is not disease dependent. Explanted liver tissue from patients with PBC was used as control as PBC and PSC belong to the same family of autoimmune liver diseases and share overlapping clinical features. Similar to intrahepatic T cells from patients with PSC, addition of UC-MSC suppressed intrahepatic $CD8^+$ T cell proliferation from patients with PBC. This confirmed that the effect of UC-MSC on T cell responses is not disease specific. In PBC livers, there was a trend in reduction of intrahepatic $CD4^+$ proliferation however our results did not reach statistical significance (n=4 PBC livers). An explanation of this could be the small sample numbers and sample variation. On assessing effect of UC-MSC on T cell activation, we found that UC-MSC reduced both circulating and intrahepatic $CD4^+$ T

cell activation from patients with PSC evidenced by reduction in the percentage of $CD4^+$TNFα, $CD4^+$IFNγ and $CD4^+$IL2 expressing cells in the presence of UC-MSC. In patients with PSC, we found no statistically significant reduction in circulating and intrahepatic $CD8^+$ T cell activation on addition of UC-MSC, which is unexpected given the suppressive effect of UC-MSC on $CD8^+$ T cell proliferation. One reason for this could be as previously highlighted due to small sample numbers (n=2 PSC liver) as well sample variation. Of the 8 PSC livers that we isolated $CD8^+$ T cells from, we were only able to utilise data from 2 PSC livers. Experiments involving $CD8^+$ T cells were limited partly due to acquiring low number of $CD8^+$ T cells from liver tissue at the start of the experiment which meant that all the UC-MSC: $CD8^+$ ratios could not be studied. .This is contrary to findings of our group who have previously demonstrated there is reversal of CD4:CD8 ratio (1:1.4) in PSC livers as compared to peripheral blood (37). It is thus likely that operator technique of isolating intrahepatic $CD8^+$ T cells may have affected cell numbers. In addition, there were some co-culture experiments involving UC-MSC and intrahepatic $CD8^+$ T cells whereby analysis with confidence was not possible due to low $CD8^+$ T cell numbers in the control at the end of culture period. An explanation for this could be the lack of addition of growth factors such as IL-2 and IL-15, which are deemed to be important for the generation and maintenance of memory $CD8^+$ T cells (191). As highlighted previously $CD8^+$ T cells in PSC livers are mostly effector memory T cells. PSC livers have been shown to lack IL-2 (37). Generation and homeostasis of memory $CD8^+$ T cells is also reportedly heavily dependent on presence of macrophages and dendritic cells expressing IL-15 RA (192). For our liver experiments, we had to use purified $CD4^+$ and $CD8^+$ T cells as the use of LIMCs impacted significantly on cell survival and therefore analysis. Our justification of not adding growth factors was based upon the need to standardise experiments so as to allow comparison of the UC-MSC effect on T cell responses derived from both blood and liver. Lastly, when assessing effect of UC-MSC on T cell activation in PBC livers,

we reported similar to PSC livers, addition of UC-MSC resulted in a significant reduction in the percentage of CD4+IL2 expressing cells again suggesting again that the effect of UC-MSC on T cells is not disease specific.

CHAPTER 4.

INDIRECT EFFECT OF UMBILICAL CORD-DERIVED MESENCHYMAL STROMAL CELLS ON CIRCULATING AND INTRAHEPATIC T CELLS FROM PATIENTS WITH PSC

4.1 Introduction

MSCs have been widely reported to mediate their immunosuppressive effect indirectly via release of soluble factors. A number of studies have demonstrated that separation of MSC and PBMC by a semi permeable membrane (for example a transwell) or using MSC- derived condition media does not prevent inhibition of proliferation of T cells by MSC (105, 122, 193). This is of relevance as animal studies have demonstrated, a large proportion of intravenous MSC are trapped in the lung in the first passage (194, 195). After 24 hours, most of the MSC are reported to be dead and there is rapid clearance of viable MSC with translocation to other organs such as the liver and spleen (130). At 72 hours, no living or dead MSC are found to be detectable (130). The short survival of MSC however does not impact their effectiveness as beneficial effects of MSC have been reported in a number of clinical trials long after the MSCs have been cleared (156, 196-199) implying that soluble factors may be involved in the MSC mechanism of action. Zanotti *et al* demonstrated that encapsulated MSC in mice were able to reduce the percentage of $CD4^+$ T proliferation concluding that MSC do not require homing to specific organs or cell–cell contact to control inflammation and that their immunosuppressive action is based on the release of soluble factors that may act systemically (200). Several different molecules secreted by MSCs are reported in the literature to have an immunomodulatory effect on T cell responses including TGF-β1, HGF (122), PGE2 (114) and IDO (126). Pro-inflammatory cytokines such as IFNγ alone or in combination with TNFα, IL1α or IL1β stimulate MSC to enhance secretion of various enzymes and soluble factors such as nitric oxide, cyclooxygenase 2, PGE2 (involved in inhibition of T- cell proliferation) and IDO (inhibits growth and function of immune cells) (118, 128, 148, 201, 202). The efficacy of soluble factors in experiments remains debatable with some studies demonstrating that the inhibitory effect of MSC on T cell responses in vitro is more enhanced when there is cell-cell contact as

compared to when MSCs are separated by a transwell (122) whilst others have shown that inhibition of T cell proliferation by murine MSC requires cell-cell contact (125, 189).

4.1.1 Chapter aims

- To determine the optimal timing for generation of condition media from UC-MSC
- To assess the indirect effect of UC-MSC on circulating and intrahepatic $CD4^+$ and $CD8^+$ T cell proliferation and activation (TNFα, IFNγ and IL-2 assessment) from patients with PSC using UC-MSC derived condition media
- To compare the effect of conditioned media derived from untreated UC-MSC versus IFNγ stimulated UC-MSC on circulating and intrahepatic $CD4^+$ and $CD8^+$ T cell proliferation and activation (TNFα, IFNγ and IL-2 assessment)

4.2 Results

4.2.1 Optimum timing for generation of UC-MSC- conditioned media (UC-MSC-CM)

Published studies have reported acquisition of conditioned media (CM) from cultured MSC at various time points including at day 3 (203), 4 (105) and 5 (204). For these experiments, 50,000 untreated UC-MSC/well were plated in flat 6- well plates in culture media and the efficacy of the CM collected at days 3, 4, and 5 was compared using T cell proliferation assays. CTV labelled PBMC isolated from patients with PSC (n=3) were cultured in 100% CM collected at days 3,4, 5 from UC-MSC and in the presence of anti-CD3 (0.5µg/ml) and CD28 (1µg/ml). At day 5, the effect of 100% CM on $CD4^+$ and $CD8^+$ T cell proliferation was assessed by flow cytometry. Stimulated PBMC only in culture media were used as controls. CM collected at day 5 had a more pronounced effect at suppressing both $CD4^+$ (*$p<0.05$) and $CD8^+$ (**$p\leq0.01$; *$p<0.05$) T cell proliferation as compared with CM collected at day 3 and day 4 (Figure 4.1 A and B). Therefore for all future experiments, CM collected from cultured UC-MSC at day 5 was used. It is noteworthy that at day 5, the cultured UC-MSC were 70-80% confluent.

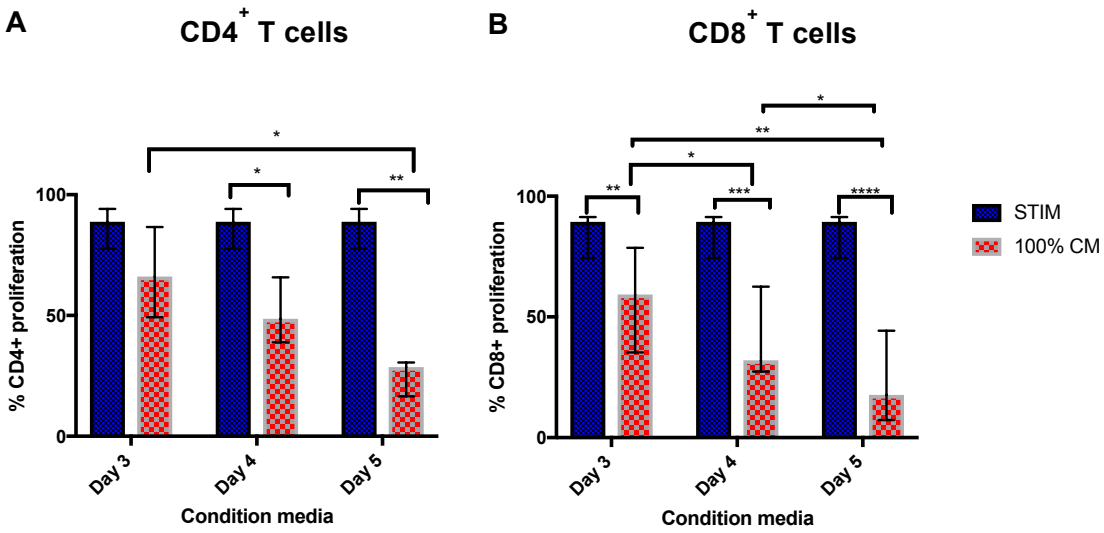


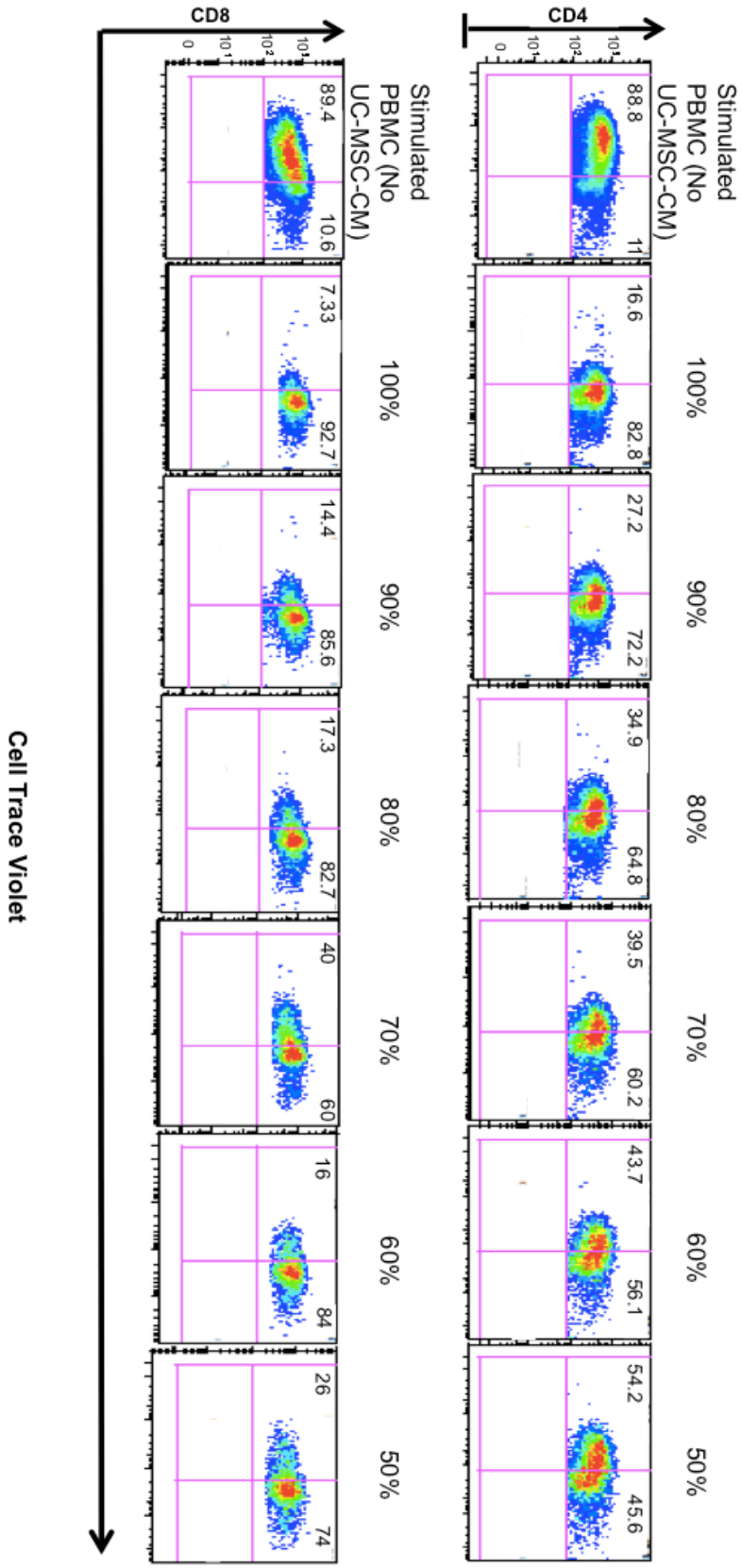
Figure 4-1: Conditioned media (CM) collected from cultured untreated Umbilical cord derived mesenchymal stromal cells (UC-MSC) at day 5 had a more pronounced effect on suppressing T cell proliferation compared with CM collected at days 3 and 4.

CTV labelled PBMC isolated from patients with PSC were cultured in 100% CM collected from UC-MSC at days 3,4, 5 in the presence of 0.5µg/ml anti-CD3/1µg/ml anti-CD28. At day 5, the effect of CM on $CD4^+$ and $CD8^+$ T cell proliferation was assessed by flow cytometry. **A:** Data showing % $CD4^+$ proliferation in n=3 PSC peripheral blood presented as median and interquartile range. Analysis performed using two-way ANOVA. **B:** Data showing % $CD8^+$ proliferation in n=3 PSC peripheral blood presented as median and interquartile range. Analysis performed using two-way ANOVA. * $p<0.05$ ** $p\leq0.01$ *** $p\leq0.001$**** ≤0.0001. **Abbreviations:** CM- conditioned media derived from UC-MSC; Stim- stimulated PBMC only in control media (no UC-MSC-CM)

4.2.2 Effect of conditioned media derived from untreated UC-MSC (UC-MSC-CM) on proliferation of circulating $CD4^+$ and $CD8^+$ T cells from patients with PSC

To assess whether the effect of UC-MSC on inhibition of T cell proliferation is contact dependent, we used CM generated from untreated UC-MSC to culture peripheral blood T cells from patients with PSC. CTV labelled PBMC isolated from patients with PSC were cultured at various concentrations of CM ranging from 100% to 50% in the presence of anti-CD3 (0.5μg/ml) and anti-CD28 (1μg/ml). At day 5, the effect of CM on $CD4^+$ and $CD8^+$ T cell proliferation was assessed by flow cytometry. Presence of UC-MSC-CM was associated with dose response inhibition of T cell proliferation (Figure 4.2 A-C). $CD4^+$ (% proliferated $CD4^+$ only median: 85.8% vs $CD4^+$ + UC-MSC-CM at 100%: 5.68% vs $CD4^+$ + UC-MSC-CM at 90%: 27.2% vs $CD4^+$ + UC-MSC-CM at 80%: 44.3%; $p \leq 0.01$) (Figure 4.2 B) and $CD8^+$ (% proliferated $CD8^+$ only median: 88.65% vs $CD8^+$ + UC-MSC-CM at 100%: 5.3% vs $CD8^+$ + UC-MSC-CM at 90%: 32.45% vs $CD8^+$ + UC-MSC-CM at 80%: 59.5%; $p \leq 0.01$) (Figure 4.2 C) T cell proliferation was inhibited by the presence of CM at concentrations of up to 80% compared with stimulated $CD4^+$ and $CD8^+$ T cells only confirming that cell-cell contact is not required to achieve inhibition of T cell proliferation.

A


CD4
CD8
Stimulated PBMC (No UC-MSC-CM)
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90%
80%
70%
60%
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88.8
11
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27.2
72.2
34.9
64.8
39.5
60.2
43.7
56.1
54.2
45.6
89.4
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Cell Trace Violet

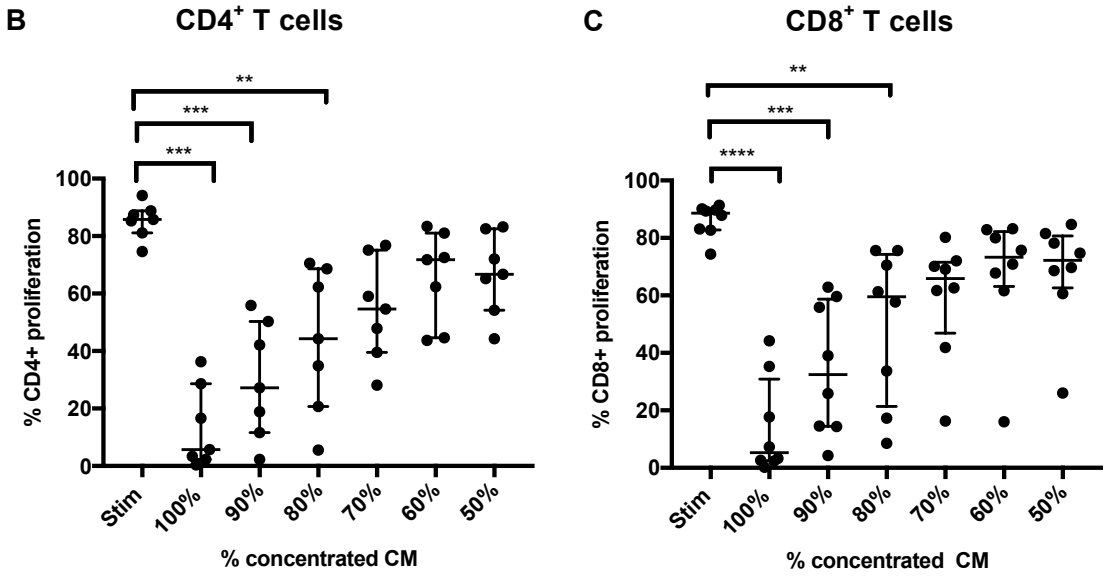

Figure 4-2: Cell-cell contact is not required to achieve inhibition of $CD4^+$ and $CD8^+$ T cell proliferation by umbilical cord derived mesenchymal stromal cells (UC-MSC).

CTV labelled PBMC isolated from patients with PSC were cultured in various concentrations of CM in the presence of 0.5μg/ml anti-CD3/1 μg/ml anti-CD28. At day 5, effect of CM on $CD4^+$ and $CD8^+$ T cell proliferation was assessed by flow cytometry. **A:** Representative flow pseudocolour plots demonstrating the dose response effect of CM on $CD4^+$ and $CD8^+$ T cell proliferation. **B:** Data showing % $CD4^+$ proliferation in n=7 PSC peripheral bloods. Lines indicate median and bars the interquartile range. Data analysis performed using Kruskal-Wallis test. **C:** Data showing % $CD8^+$ proliferation in n=8 PSC peripheral bloods. Lines indicate median and bars the interquartile range. Data analysis performed using Kruskal-Wallis test. **p ≤ 0.01 ***p≤0.001****≤0.0001. **Abbreviations:** CM- conditioned media derived from UC-MSC; Stim-stimulated PBMC only in control media (no UC-MSC-CM)

4.2.3 Effect of conditioned media derived from untreated UC-MSC (UC-MSC-CM) on activation of circulating $CD4^+$ and $CD8^+$ T cells from patients with PSC

Next, the effect of UC-MSC-CM on T cell activation from patients with PSC was studied. At day 5 stimulated PBMC cultured in 90% and 100% UC-MSC-CM were further stimulated with 50ng/ml PMA, 1 μM ionomycin and 3 μg/ml Brefeldin A for 4 hours following which expression of TNFα, IFN γ and IL 2 was assessed using flow cytometry. The two highest concentrations of CM were selected to assess the effect on T cell activation on the basis that, if there was any effect, it would be most likely observed with the higher concentrations of CM given earlier findings in section 4.2.2. The presence UC-MSC-CM reduced the percentage of $CD4^+$IFNγ expressing cells (% $CD4^+$ IFNγ expressing cells only median: 28.3% vs $CD4^+$ + UC-MSC-CM at 100%: 12.25%; $p \leq 0.01$) at concentrations of up to 100% compared to stimulated PBMC only (Figure 4.3 B). UC-MSC-CM had no effect on the percentage of $CD4^+$TNFα (Figure 4.3A), $CD4^+$IL2 (Figure 4.3C), $CD8^+$TNFα, $CD8^+$IFNγ and $CD8^+$IL2 expressing cells (Figures 4.3 D-F).

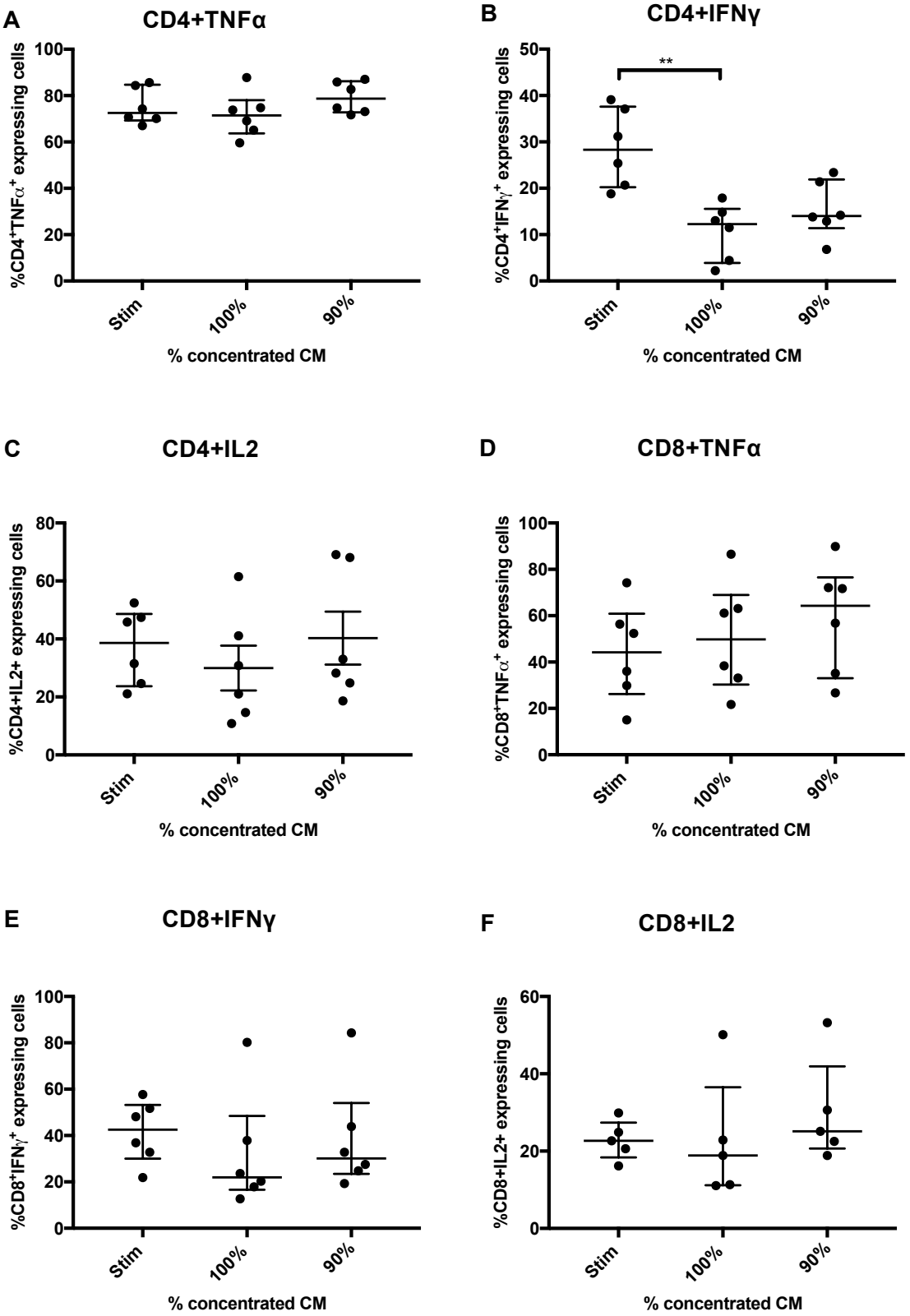
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Figure 4-3: Presence of umbilical cord derived untreated mesenchymal stromal cells conditioned media (UC-MSC-CM) reduces the percentage of circulating $CD4^{+}IFN\gamma$ expressing cells.

PBMC were cultured with 90% and 100% concentrated UC-MSC-CM in the presence of 0.5µg/ml anti-CD3/ 1 µg/ml anti-CD28. At day 5, the PBMC were further stimulated with 50ng/ml PMA, 1 µM ionomycin and 3 µg/ml Brefeldin A for 4 hours and expression of TNFα, IFN γ and IL2 assessed by flow cytometry. **A-C:** Data showing %CD4+ TNFα, IFN γ and IL2 expressing cells in n=6 PSC peripheral bloods. Lines indicate median and bars the interquartile range. Data analysis performed using Kruskal-Wallis test. **D-F:** Data showing % CD8+ TNFα, IFN γ and IL2 expressing cells in n=6 PSC peripheral bloods. Lines indicate median and bars the interquartile range. Analysis performed using Kruskal-Wallis test. **p ≤ 0.01 **Abbreviations:** CM-conditioned media derived from UC-MSC; Stim: stimulated PBMC only in control media (no UC-MSC-CM)

4.2.4 Effect of conditioned media derived from untreated UC-MSC (UC-MSC-CM) on intrahepatic $CD4^+$ and $CD8^+$ T cell proliferation from patients with PSC

Having demonstrated that UC-MSC-CM is able to inhibit circulating $CD4^+$ and $CD8^+$ T cell proliferation, the next step was to determine whether UC-MSC-CM would have a similar effect on intrahepatic $CD4^+$ and $CD8^+$ T cells from patients with PSC. $CD4^+$ and $CD8^+$ T cells were isolated using negative bead selection from LIMCs of patients with PSC and cultured with various concentrations of UC-MSC-CM ranging from 100% to 50% and in the presence of anti-CD3 (0.5µg/ml) and anti-CD28 (1µg/ml) for 4 days. At day 4, the effect of UC-MSC-CM on intrahepatic $CD4^+$ and $CD8^+$ T cell proliferation was assessed using flow cytometry. No inhibition of intrahepatic $CD4^+$ T cell proliferation in the presence of UC-MSC-CM (n=4 PSC livers; Figure 4.4 A) was found at all concentrations. Similar to peripheral blood, a trend towards dose related inhibition of $CD8^+$ T cell proliferation in the presence of UC-MSC-CM was observed (n=2 PSC livers; Figure 4.4 B).

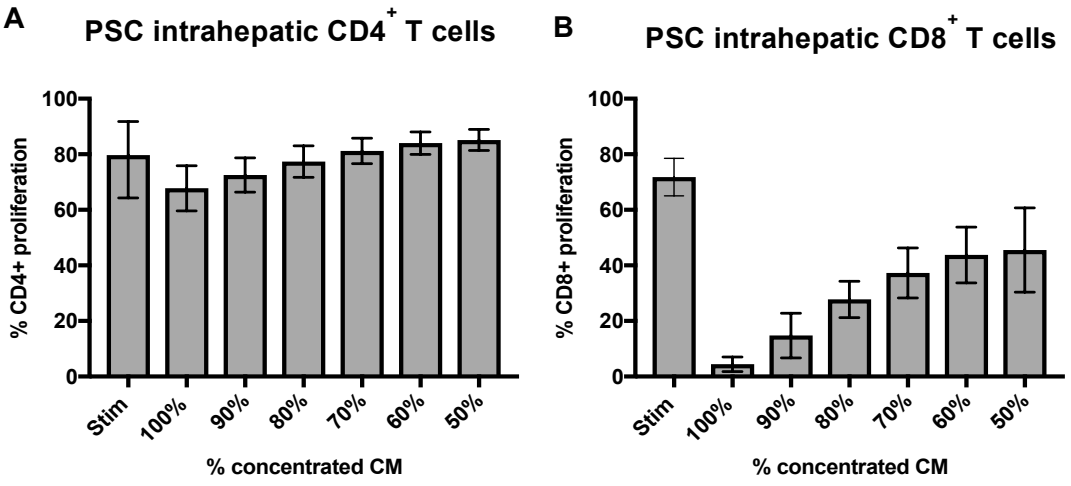
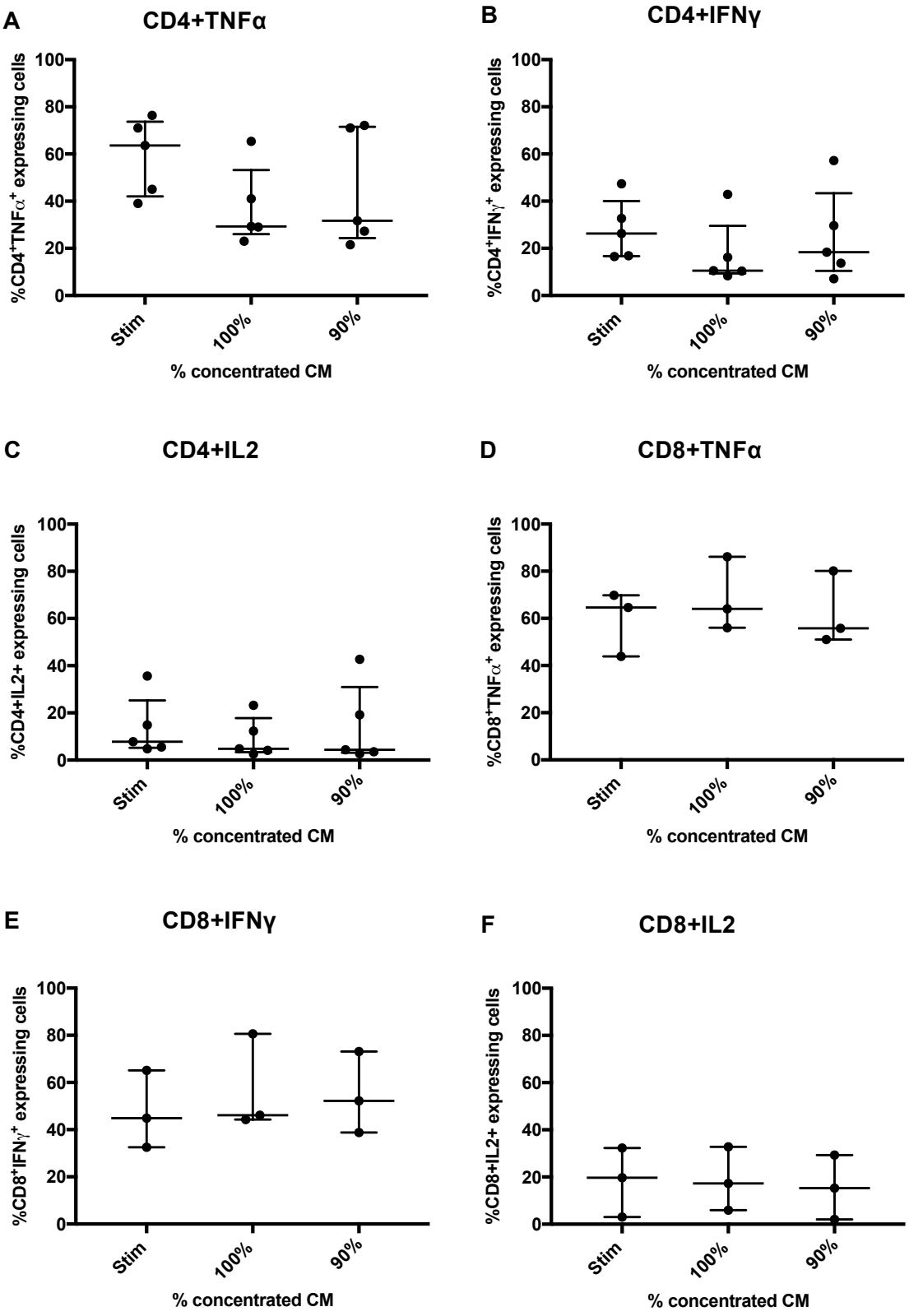


Figure 4-4: Presence of umbilical cord derived mesenchymal stromal cells conditioned media (UC-MSC-CM) results in a trend towards dose-related inhibition of intrahepatic $CD8^+$ T cell proliferation from patients with primary sclerosing cholangitis (PSC).

Negatively selected $CD4^+$ and $CD8^+$ T cells isolated from PSC livers were cultured in various concentrations of UC-CM-MSC in the presence of 0.5µg/ml anti-CD3/1µg/ml anti-CD28. At day 4, the effect of UC-MSC-CM on T cell proliferation was assessed using flow cytometry. **A:** Data showing $\%CD4^+$ proliferation in n=4 PSC livers presented as median with interquartile range. Data analysis performed using Kruskal-Wallis test. **B:** Data showing $\%CD8^+$ proliferation in n=2 PSC livers presented as median with interquartile range. **Abbreviations:** CM- conditioned media derived from UC-MSC; Stim: stimulated T cells only in control media (no UC-MSC-CM).

4.2.5 Effect of conditioned media derived from untreated UC-MSC (UC-MSC-CM) on intrahepatic $CD4^+$ and $CD8^+$ T cell activation from patients with PSC

We next investigated whether UC-MSC-CM was effective in reducing intrahepatic $CD4^+$ and $CD8^+$ T cell activation in PSC livers. At day 4, stimulated intrahepatic T cells cultured in 90% and 100% UC-MSC-CM were further stimulated with 50ng/ml PMA, 1 μM ionomycin and 3 μg/ml Brefeldin A for 4 hours following which expression of TNFα, IFN γ and IL 2 was assessed using flow cytometry. Presence of untreated UC-MSC-CM at 100% concentration resulted in a trend towards reduction in the percentage of $CD4^+$TNFα and $CD4^+$ IFNγ expressing cells however these results did not reach significance (n=5 PSC livers) (Figure 4.5 A). There was also no observed reduction in $CD4^+$ IL 2 (figure 4.5 A), $CD8^+$ TNFα, $CD8^+$ IFN γ and $CD8^+$ IL 2 expressing cells (n=3 PSC livers; Figure 4.5 B)) in the presence of UC-MSC-CM.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%CD4+IFNγ+ expressing cells
100
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Figure 4-5: Umbilical cord derived mesenchymal stromal cells conditioned media (UC-MSC-CM) does not affect the ability of intrahepatic $CD4^+$ and $CD8^+$ T cells from patients with primary sclerosing cholangitis (PSC), to produce inflammatory cytokines.

Negatively selected $CD4^+$ and $CD8^+$ T cells were cultured with 90% and 100% concentrated UC-MSC-CM in the presence of 0.5µg/ml anti-CD3/1µg/ml anti-CD28. At day 4, the T cells were further stimulated with 50ng/ml PMA, 1 µM ionomycin and 3 µg/ml Brefeldin A for 4 hours and expression of TNFα, IFN γ and IL2 assessed by flow cytometry. **A-C:** Data showing $\%CD4^+$ TNFα, IFN γ and IL2 expressing cells in n=5 PSC livers. **A-C:** Lines indicate median and bars the interquartile range. Data analysis performed using Kruskal-Wallis test. **D-F:** Data showing $\%CD8^+$ TNFα, IFN γ and IL2 expressing cells in n=3 PSC livers. Lines indicate median and bars the interquartile range. Data analysis performed using Kruskal-Wallis test. **Abbreviations:** CM: conditioned media derived from UC-MSC; Stim: stimulated $CD4^+$/$CD8^+$ T cells only in control media (no UC-MSC-CM).

4.2.6 Comparing the effect of conditioned media derived from untreated (UC-MSC-CM) versus IFNγ-stimulated UC-MSC (IFNγ UC-MSC-CM) on proliferation and activation of circulating $CD4^+$ and $CD8^+$ T cells from patients with PSC

4.2.6.1 T cell proliferation

Pro-inflammatory cytokines such as IFNγ have been shown to enhance the immunosuppressive potential of MSC. To investigate this, the effect of untreated UC-MSC-CM versus IFNγ stimulated UC-MSC-CM on circulating $CD4^+$ and $CD8^+$ T cell proliferation from patients with PSC was compared. Acquisition of conditioned media derived from IFNγ stimulated UC-MSC (IFNγ UC-MSC-CM) is discussed in the materials and methods section 2.2.5. Results from 4 independent experiments demonstrated similar findings to untreated UC-CM-MSC in that there is a dose response effect in reduction of $CD4^+$ and $CD8^+$ T cell proliferation in the presence of IFNγ stimulated UC-MSC-CM (Figures 4.6A and B). IFNγ stimulated UC-MSC-CM inhibited $CD4^+$ (% proliferated $CD4^+$ only median: 85.5% vs $CD4^+$+ IFNγ UC-MSC-CM at 100%: 11.98% vs $CD4^+$ + IFNγ UC-MSC-CM at 90%: 40.5% vs $CD4^+$+ IFNγ UC-MSC-CM at 80%: 51% vs $CD4^+$ + IFNγ UC-MSC-CM at 70%: 63.8%; $p \leq 0.01$) and $CD8^+$ T cell (% proliferated $CD8^+$ only median: 88.8% vs $CD8^+$ + IFNγ UC-MSC-CM at 100%: 13.48% vs $CD8^+$ + IFNγ UC-MSC-CM at 90%: 51.6% vs $CD8^+$ + IFNγ UC-MSC-CM at 80%: 61.9% vs $CD8^+$ + IFNγ UC-MSC-CM at 70%: 70.7%; $p \leq 0.01$) proliferation at concentrations of up to 70% compared to stimulated PBMC only. Importantly, there was no statistical difference in the effect on circulating $CD4^+$ (Figure 4.6 A) and $CD8^+$ (Figure 4.6 B) T cell proliferation between untreated UC-MSC-CM and IFNγ stimulated UC-MSC-CM at all concentrations.

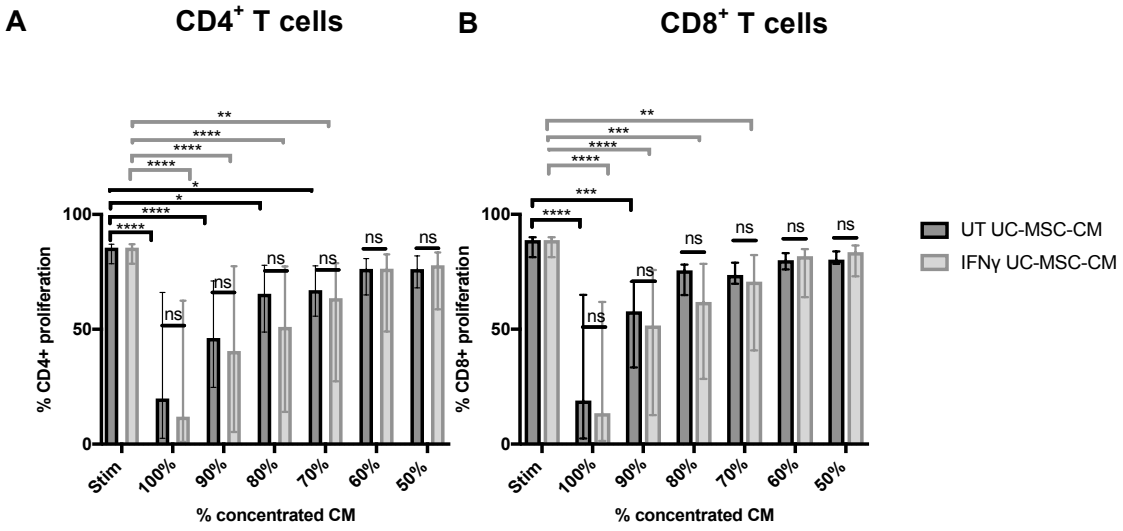
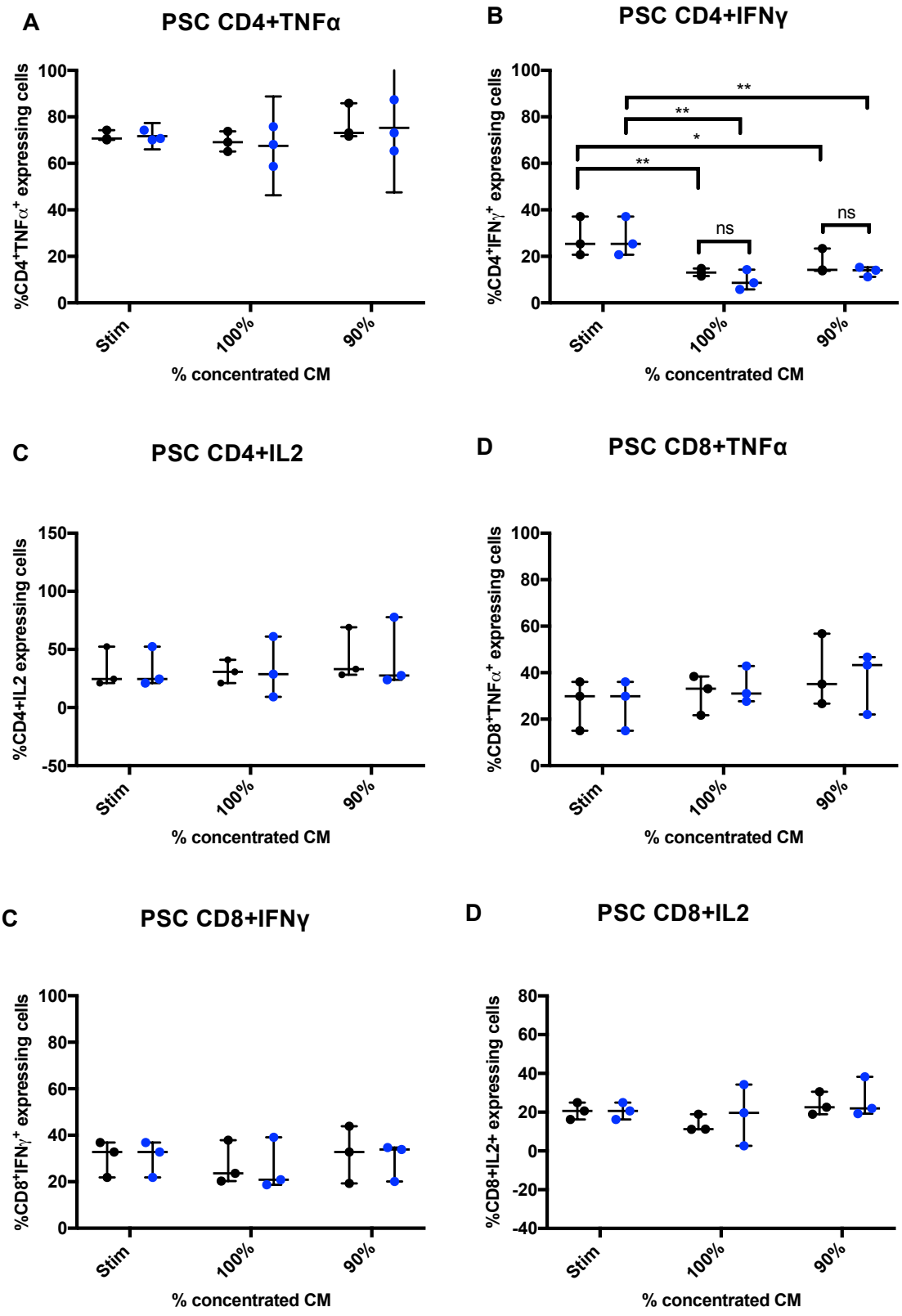


Figure 4-6: There are no differences in the inhibitory effect of untreated and IFNγ stimulated umbilical cord derived mesenchymal stromal cells conditioned media (UC-MSC-CM) on circulating T cell proliferation from patients with primary sclerosing cholangitis (PSC).

CTV labelled PBMC isolated from patients with PSC were cultured in various concentrations of untreated and IFNγ stimulated UC-MSC-CM in the presence of 0.5µg/ml anti-CD3/1µg/ml anti-CD28. At day 5, the effect of CM on $CD4^+$ and $CD8^+$ T cell proliferation was assessed by flow cytometry. IFN γ UC-MSC-CM was obtained by stimulating UC-MSC with IFN γ for 72 hours thereafter washing and culturing in fresh medium for 24 hours. **A:** Data showing $\%CD4^+$ proliferation in n=4 PSC peripheral bloods presented as median with interquartile range. Data analysis performed by two-way ANOVA. **B:** Data showing % $CD8^+$ proliferation in n=4 PSC peripheral bloods presented as median with interquartile range. Data analysis performed by two-way ANOVA. * $p<0.05$ **$p\leq0.01$ ***$p\leq0.001$****≤0.0001. **Abbreviations:** CM- conditioned media derived from UC-MSC; IFNγ UC-MSC-CM- conditioned media derived from IFNγ stimulated UC-MSC; NS- not significant; Stim- stimulated PBMCs only in control media (no UC-MSC-CM); UC-MSC- umbilical cord derived mesenchymal stromal cells; UT- un-treated

4.2.6.2 T cell activation

Next the effect of untreated UC-MSC-CM versus IFNγ stimulated UC-MSC-CM on circulating $CD4^+$ and $CD8^+$ T cell activation from patients with PSC was studied. IFNγ stimulated UC-MSC-CM significantly reduced the percentage of $CD4^+$ IFNγ expressing cells at up to 90% concentration (% $CD4^+$ IFNγ expressing cells only median: 25.4% vs $CD4^+$ + IFNγ UC-MSC-CM at 100%: 8.6% vs $CD4^+$ + IFNγ UC-MSC-CM at 90%: 11.2%; $p \leq 0.01$) compared to stimulated PBMC only (Figure 4.7B). There was no statistical difference in the effect of reducing the percentage of $CD4^+$ IFNγ expressing cells between untreated UC-MSC-CM and IFNγ stimulated UC-MSC-CM (Figure 4.7B). Similar to untreated UC-MSC-CM, presence of IFNγ stimulated UC-MSC-CM did not reduce the percentage of $CD4^+$TNFα (Figure 4.7A), $CD4^+$IL2 (Figure 4.7C), $CD8^+$TNFα, $CD8^+$IFNγ, $CD8^+$IL2 expressing cells (Figures 4.7 D-F).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%CD4+IFNγ+ expressing cells
**
**
*
**
ns
ns
C
PSC CD4+IL2
%CD4+IL2 expressing cells
150
100
50
0
-50
D
PSC CD8+TNFα
%CD8+TNFα+ expressing cells
C
PSC CD8+IFNγ
%CD8+IFNγ+ expressing cells
D
PSC CD8+IL2
%CD8+IL2+ expressing cells
80
60
40
20
0
-20
-40

Figure 4-7: IFNγ stimulated umbilical cord derived mesenchymal stromal cells conditioned media (IFNγ UC-MSC-CM) results in reduction of percentage circulating $CD4^{+}$IFNγ expressing cells from patients with primary sclerosing cholangitis (PSC) however no differences in the effect on circulating T cell activation is seen between untreated and IFNγ UC-MSC-CM.

PBMC were cultured in 90% and 100% concentrated untreated and IFNγ stimulated UC-MSC-CM in the presence of 0.5μg/ml anti-CD3/1 μg/ml anti-CD28. At day 5, the PBMCs were further stimulated with 50ng/ml PMA, 1 μM ionomycin and 3 μg/ml Brefeldin A for 4 hours and expression of TNFα, IFN γ and IL2 assessed by flow cytometry. IFN γ UC-MSC-CM was obtained by stimulating UC-MSC with IFN γ for 72 hours thereafter washing and culturing in fresh medium for 24 hours. **A-C:** Data showing %$CD4^{+}$TNFα, IFNγ and IL2 expressing cells in n=3 PSC peripheral bloods. **D-F:** Data showing %$CD8^{+}$TNFα, IFNγ and IL2 expressing cells in n=3 PSC peripheral bloods. Lines indicate median and bars the interquartile range. Data analysis performed by two way ANOVA.* $p<0.05$ **$p\leq0.01$. **Abbreviations:** CM- conditioned media derived from UC-MSC; IFNγ UC-MSC-CM- conditioned media derived from IFNγ stimulated UC-MSC; NS- not significant; Stim- stimulated PBMCs only in control media (no UC-MSC-CM); UC-MSC- umbilical cord derived mesenchymal stromal cells; UT-un-treated

4.2.7 Comparing the effect of conditioned media derived from untreated (UC-MSC-CM) versus IFNγ-stimulated UC-MSC (IFNγ UC-MSC-CM) on proliferation and activation of intrahepatic $CD4^+$ T cells from patients with PSC

4.2.7.1 T cell proliferation

Having demonstrated that untreated UC-MSC-CM did not inhibit intrahepatic $CD4^+$ T cell proliferation in PSC livers, we next sought to determine whether IFNγ stimulated UC-MSC-CM would be effective in inhibiting intrahepatic $CD4^+$ T cell proliferation in PSC livers. Results from 3 independent experiments demonstrated that similar to untreated UC-MSC-CM, IFNγ stimulated UC-MSC-CM had no statistically significant effect in inhibiting $CD4^+$ T cell proliferation in PSC livers (Figure 4.8 A).

4.2.7.2 T cell activation

The effect of untreated UC-MSC-CM versus IFNγ stimulated UC-MSC-CM on intrahepatic $CD4^+$ T cell activation from patients with PSC was studied. Similar to the effect seen when using untreated UC-MSC-CM, there is a dose response trend towards reduction in percentage of $CD4^+$TNFα (Figure 4.9 A) and $CD4^+$IFNγ (Figure 4.9 B) expressing cells in the presence of IFNγ stimulated UC-MSC-CM however this has not reached statistical significance. There were no statistical differences between untreated UC-MSC-CM and IFNγ stimulated UC-MSC-CM in the effect on PSC intrahepatic T cell activation.

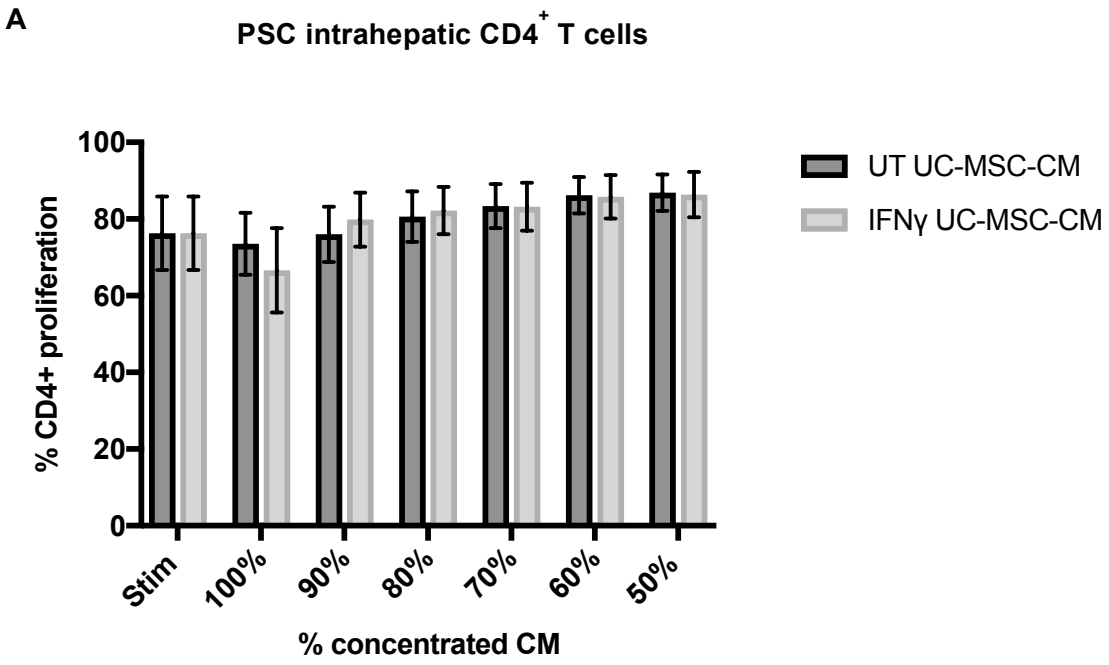


Figure 4-8: Similar to untreated umbilical cord derived mesenchymal stromal cells conditioned media (UC-MSC-CM), IFNγ stimulated UC-MSC-CM has no effect in inhibiting intrahepatic $CD4^+$ T cell proliferation from patients with primary sclerosing cholangitis (PSC).

Negatively selected $CD4^+$ T cells from PSC livers were cultured in various concentrations of UC-CM-MSC and IFNγ stimulated UC-MSC-CM in the presence of 0.5µg/ml anti-CD3/1µg/ml anti-CD28. At day 4, the effect of CM on T cell proliferation was assessed using flow cytometry. IFN γ UC-MSC-CM was obtained by stimulating UC-MSC with IFNγ for 72 hours thereafter washing and culturing in fresh medium for 24 hours. **A:** Data showing $\%CD4^+$ proliferation in n=3 PSC livers presented as median with interquartile range. Data analysis performed using two-way ANOVA. **Abbreviations:** CM- conditioned media derived from UC-MSC; IFNγ UC-MSC-CM- conditioned media derived from IFNγ stimulated UC-MSC; Stim- stimulated PBMC only in control media (no UC-MSC-CM); UC-MSC- umbilical cord derived mesenchymal stromal cells; UT-un-treated

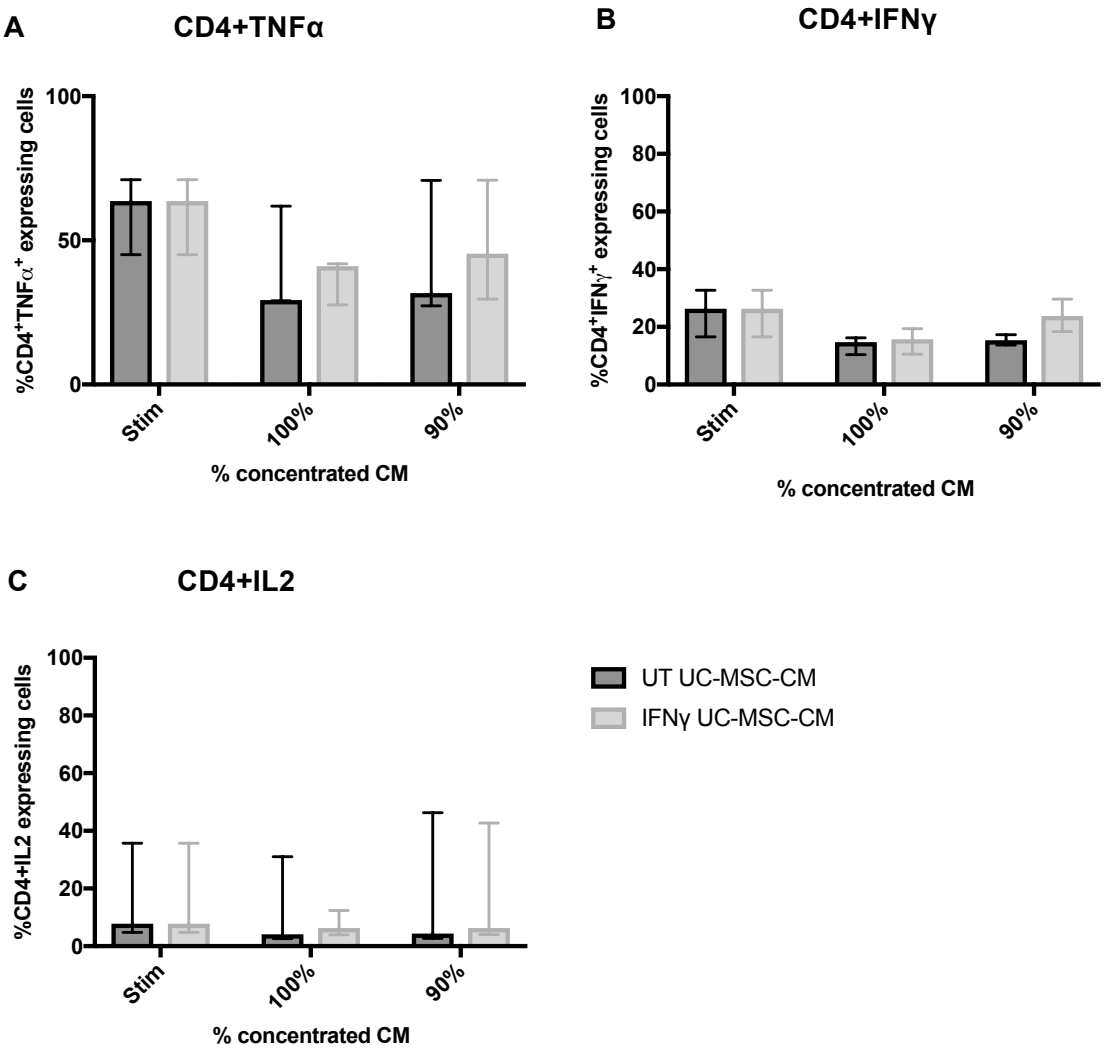


Figure 4-9: There are no differences in effect on CD4$^+$ intrahepatic T cell activation from patient with primary sclerosing cholangitis (PSC) between untreated umbilical cord derived mesenchymal stromal cell conditioned media (UC-MSC-CM) and IFNγ stimulated UC-MSC-CM.

Negatively selected CD4$^+$ T cells from PSC livers were cultured in 90% and 100% concentrated UC-MSC-CM and IFNγ UC-MSC-CM in the presence of 0.5μg/ml anti-CD3/1μg/ml anti-CD28. At day 4, the T cells were further stimulated with 50ng/ml PMA, 1 μM ionomycin and 3 μg/ml Brefeldin A for 4 hours and expression of TNFα, IFN γ and IL2 assessed by flow cytometry. IFN γ UC-MSC-CM was obtained by stimulating UC-MSC with IFN γ for 72 hours thereafter washing and culturing in fresh medium for 24 hours. **A-C:** Data showing % CD4$^+$TNFα, IFNγ and IL2

expressing cells in n=3 PSC livers presented as median with interquartile range. Data analysis performed using by two-way ANOVA. **Abbreviations:** CM- conditioned media derived from UC-MSC; IFNγ UC-MSC-CM- conditioned media derived from IFNγ stimulated UC-MSC; Stim-stimulated PBMC only in control media (no UC-MSC-CM); UC-MSC- umbilical cord derived mesenchymal stromal cells; UT-un-treated

4.3 Conclusion

In this chapter, we have shown that conditioned media derived from untreated UC-MSC significantly reduces circulating $CD4^+$ and $CD8^+$ T cell proliferation from patients with PSC in a dose dependent manner. These results confirm that cell-cell contact is not mandatory for UC-MSC to inhibit circulating T-cell proliferation and that soluble factors are involved. Untreated UC-MSC-CM significantly reduces the percentage of circulating $CD4^+$IFNγ expressing cells from patients with PSC however unlike when UC-MSC were in direct contact with PBMC, untreated UC-MSC-CM did not reduce the percentage of circulating $CD4^+$TNFα and $CD4^+$IL2 expressing cells. No reduction of circulating $CD8^+$ T cell activation was seen in the presence of untreated UC-MSC-CM. In PSC liver, untreated UC-MSC-CM did not inhibit $CD4^+$T cell proliferation. There was a dose response reduction of intrahepatic $CD8^+$T cell proliferation in the presence of untreated UC-MSC-CM. With respect to effect on intrahepatic T cell activation, although a trend in reduction of percentage intrahepatic $CD4^+$TNFα and $CD4^+$IFNγ expressing cells was observed in the presence of untreated UC-MSC-CM, these results did not reach statistical significance. One explanation could be low sample numbers (n=5) and sample variation however the results from the same number of samples shown in section 3.3.5.1 demonstrated that direct co-culture of UC-MSC with PSC intrahepatic $CD4^+$ T cells significantly reduced the percentage of $CD4^+$TNFα, $CD4^+$IFNγ and $CD4^+$IL2 expressing cells. Our experiments have further shown that the presence IFNγ stimulated UC-MSC-CM did not result in enhanced inhibition of circulating and intrahepatic T cell proliferation and activation given that there were no statistically significant difference in the effect on circulating and intrahepatic T cell proliferation and activation between untreated UC-MSC-CM and IFNγ stimulated UC-MSC-CM.

Data involving intrahepatic $CD8^+$ T cells is from a small number of samples. Of the 5 PSC liver used in the condition media experiments, we could only utilise data involving $CD8^+$ T cells from 3 livers. This is because as discussed previously I acquired a low number of $CD8^+$ T cells from PSC livers at the start of experiments limiting the number of experiments that could be performed. Similarly when comparing untreated UC-MSC-CM and IFNγ stimulated UC-MSC-CM, experiments using intrahepatic $CD8^+$ T cells could not be perfromed due to the number of intrahepatic T cells required to allow comparison. Experiments using T cells isolated from PBC livers (n=2) had been performed however it was not possible to analyse the results with confidence therefore data has not been shown. Due to the rarity of PBC and that patients with PBC are infrequently transplanted given availability of effective therapy, I was not able to perform more experiments within a certain time period to allow confident analysis.

Our results using UC-MSC-CM are variable and suggest that the effect of UC-MSC-CM may be less profound than direct co-culture of UC-MSC with PBMC/T cells at reducing T cell proliferation and activation. Tse *et al* concluded similar findings (105). One explanation of this could be related to the methodology. We used conditioned media derived from UC-MSC to investigate the indirect effect of UC-MSC on T cell responses instead of a transwell system where although MSC are not in direct contact with PBMC/T cells, MSC are still present in the system thus there is 'cross talk' between PBMC and MSC which in turn is thought to enhance the inhibitory factor of MSC. Tse et al found that MSC cultured in the lower chamber of a transwell system were able to exert a greater suppressive effect on T cell proliferation compared to MSC- conditioned media (105). In both cases, the authors demonstrated increased suppression on addition of third party PBMC to the MSC cultures (105). It is also likely that increased concentrations of cell supernatants (conditioned media) are required compared to MSC numbers used in co-culture so as to exert an inhibitory effect on T cell responses. In

the transwell system MSC continue to proliferate therefore constantly produce high concentrations of soluble factors as compared to when cell supernatant is used alone. MSC: T cell ratios have been shown to be critically important for the immunomodulatory functions of MSC. Najar *et al* found that MSC cultured alone at low concentrations or the corresponding CM improved lymphocyte proliferation rather than induce T-cell inhibition (205).

IFNγ has been reported to be paramount in immunosuppression by human MSC (118, 206). Contrary to some (207), we were unable to demonstrate that IFNγ stimulated UC-MSC-CM enhanced inhibition of T cell proliferation and activation. An explanation for this could be found in a study by Ren *et al* who demonstrated that the addition of recombinant IFNγ alone to mixed co-cultures of MSC and T cell blasts did not induce immunosuppression. However the concomitant addition of either one of TNFα, IL1α or IL1β along with IFNγ resulted in immunosuppression by MSC. The authors concluded that although IFNγ was required, it was insufficient alone in inducing MSC to become immunosuppressive and that it synergized with TNFα, IL1α or IL1β whilst TNFα, IL1α or IL1β acted interchangeably (128).

CHAPTER 5.

ROLE OF INDOLEAMINE 2,3- DIOXYGENASE (IDO) AS POTENTIAL MECHANISM OF MESENCHYMAL STROMAL CELL ACTION

5.1 Introduction

One factor that has been shown to play a role in MSC ability to exert an immunomodulatory effect is Indoleamine 2,3-dioxygenase (IDO). IDO is an IFNγ inducible intracellular enzyme that has a primary role in immunoregulation (108, 208). In human cells, IDO, which is produced by a number of antigen presenting cells including macrophages and dendritic cells, degrades tryptophan in to kynurenine and other metabolites. IDO is involved in tolerance induction via depletion of tryptophan thereby sensitizing activated T cells to apoptosis. Tryptophan catabolites can act potently and directly on T cells to control their proliferation and/or survival in vivo (208). Uptake of tryptophan is important for cell proliferation, depletion results in termination of proliferation whilst kynurenine, a ligand for the aryl hydrocarbon receptor promotes differentiation of $Foxp3^+$ T-regulatory cells (209).

IDO is proposed to be one of the factors involved in MSC-mediated T cell suppression (Figure 5.1) (108, 126). Expression of IDO at gene level is absent in naïve/un-stimulated MSC however exposure to IFNγ induces MSC to express IDO. Some authors have demonstrated that blocking IDO in MSC-PBMC/T-cells co-culture abrogated the immunosuppressive effects of MSC on T cells thereby concluding that IDO is the main mechanism of T cell inhibition by activated MSC (114, 124, 131). François *et al* showed in their paper that the level of IDO expression correlated with the suppressive potential of a given MSC donor and that IDO activity was involved in the differentiation of monocytes to IL10 secreting M2 immunosuppressive macrophages ($CD14^+/CD206^+$) which themselves are indirectly involved in suppressing T cell proliferation (108). Conversely, Tse et al could not find an effect of IDO in their experimental system (105).

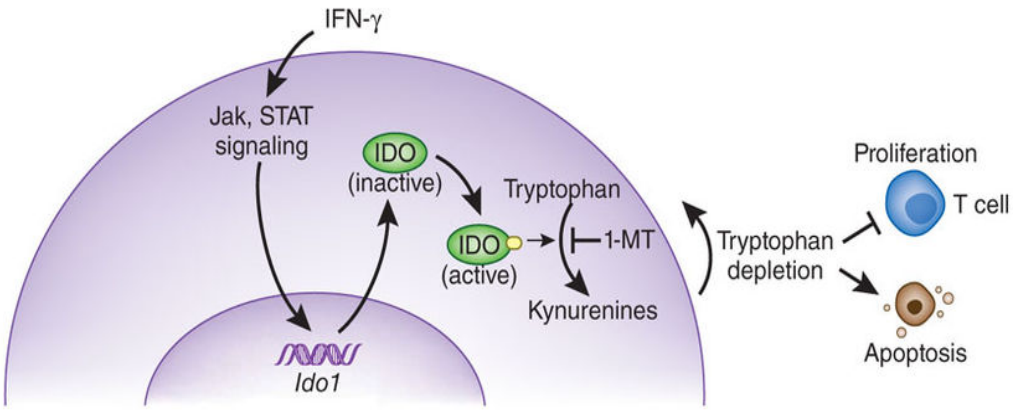


Figure 5-1: Schematic diagram representing the proposed role of Indoleamine 2,3-dioxygenase (IDO) in mesenchymal stromal cells mediated T cell suppression.

IFNγ activates STAT1-mediated signalling cascade that causes de-novo mRNA transcription and protein expression of IDO1, which activates the IDO enzyme leading to degradation of tryptophan and production of kynurenine and its metabolites. Tryptophan depletion impairs antigen dependent T cell activation, suppresses T cell function and increases generation of T regulatory cells. Typtophan depletion and several kynurenine metabolites also activate the stress response kinase GGN2 leading to T cell arrest and apoptosis (108). **Abbreviations:** IDO- Indoleamine 2,3-dioxygenase; 1-MT- 1-Methyl-D-Tryptophan. Diagram from Chen W, 2011 (210).

5.1.1 Chapter aims

The main aim of this chapter is to confirm whether the effect of UC-MSC on T cell proliferation and activation from patients with PSC is IDO driven. This was investigated using an IDO inhibitor- 1-Methyl-D-Tryptophan (1-MT). I aimed to:

- Determine the concentration of 1-Methyl-D-Tryptophan (1-MT), an IDO inhibitor, for use in conditioned media experiments
- Assess the effect of 1-MT on circulating T- cell proliferation and activation from patients with PSC using UC-MSC conditioned media
- Measure kynurenine and Tryptophan concentrations as a marker of IDO activity in UC-MSC conditioned media

5.2 Results

5.2.1 Optimisation of concentration of 1-Methyl-D-Tryptophan (1-MT), a specific IDO inhibitor

Variable concentrations of 1-MT have been used in the literature ranging from 0.5mM-1.5mM. We initially started by using 1.5mM 1-MT in experiments based on our research group's experience. A detailed explanation of the methodology is provided in the materials and methods section 2.5. In brief, 50,000 UC-MSC/well were plated in 6 well plates. On day 1, culture media in all the wells was replaced and some wells were treated with either 50 ng/ml of IFNγ + 1.5mM 1-MT or 1.5mM 1-MT only for 72 hours. At day 4, after washing the UC-MSC, fresh media was added for 24 hour. After 24 hours, the following four CM groups were acquired: a) untreated (UT) UC-MSC-CM only b) UT + 1.5mM 1-MT UC-MSC-CM c) IFNγ UC-MSC-CM only and d) IFNγ + 1.5mM 1-MT UC-MSC-CM. In the first two experiments, we found through visual observation that there was marked reduction in adherent UC-MSC in all the wells treated with IFNγ + 1.5mM 1-MT compared with untreated +1.5mM 1-MT (Figure 5.2B).

Given the reduction in adherent UC-MSC seen in the IFNγ + 1.5mM 1-MT treated wells, there was concern that 1-MT at a concentration of 1.5mM may be either killing or preventing proliferation of UC-MSC. To investigate this, two different donors of UC-MSC were first labelled with CTV for assessment of proliferation and thereafter cultured following treatment with various doses of 1-MT+/-IFNγ as appropriate in a continuous live cell imaging and analysis system that is Cell-IQ for 4 days. At the end of the culture period (day 5), all UC-MSC in the wells were stained with a viability dye. The percentage proliferation and viability of UC-MSC was assessed using flow cytometry. The same methodology as for generation of UC-MSC-CM was used in terms of culture media changes. Treatment groups included: a) UT UC-MSC only b)

UT+0.5mM 1-MT c) UT+1mM 1-MT d) UT+1.5mM 1-MT e) IFNγ UC-MSC only, f) IFNγ+0.5mM 1-MT g) IFNγ+1mM 1-MT and h) IFNγ+1.5mM 1-MT. For this set of experiments, 12,500 UC-MSC were cultured in duplicates per treatments in 24 well plates. We found addition of 1-MT at all the tested concentrations did not affect proliferation or viability of UC-MSC (Figure 5.3 A-C). For all future experiments involving 1-MT, a concentration of 1mM was used as many authors in published literature have used this concentration to assess the role of IDO in MSC induced T-cell suppression.

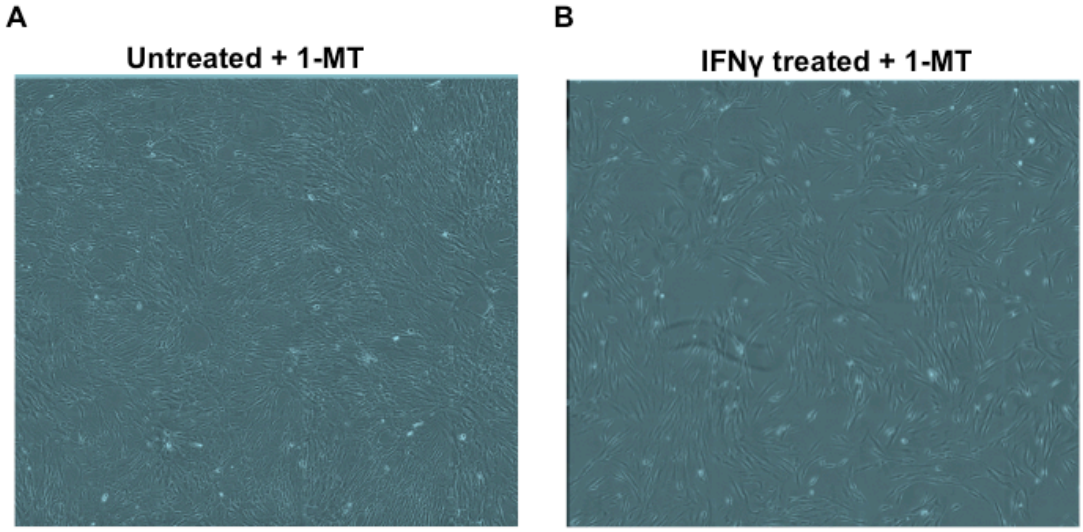
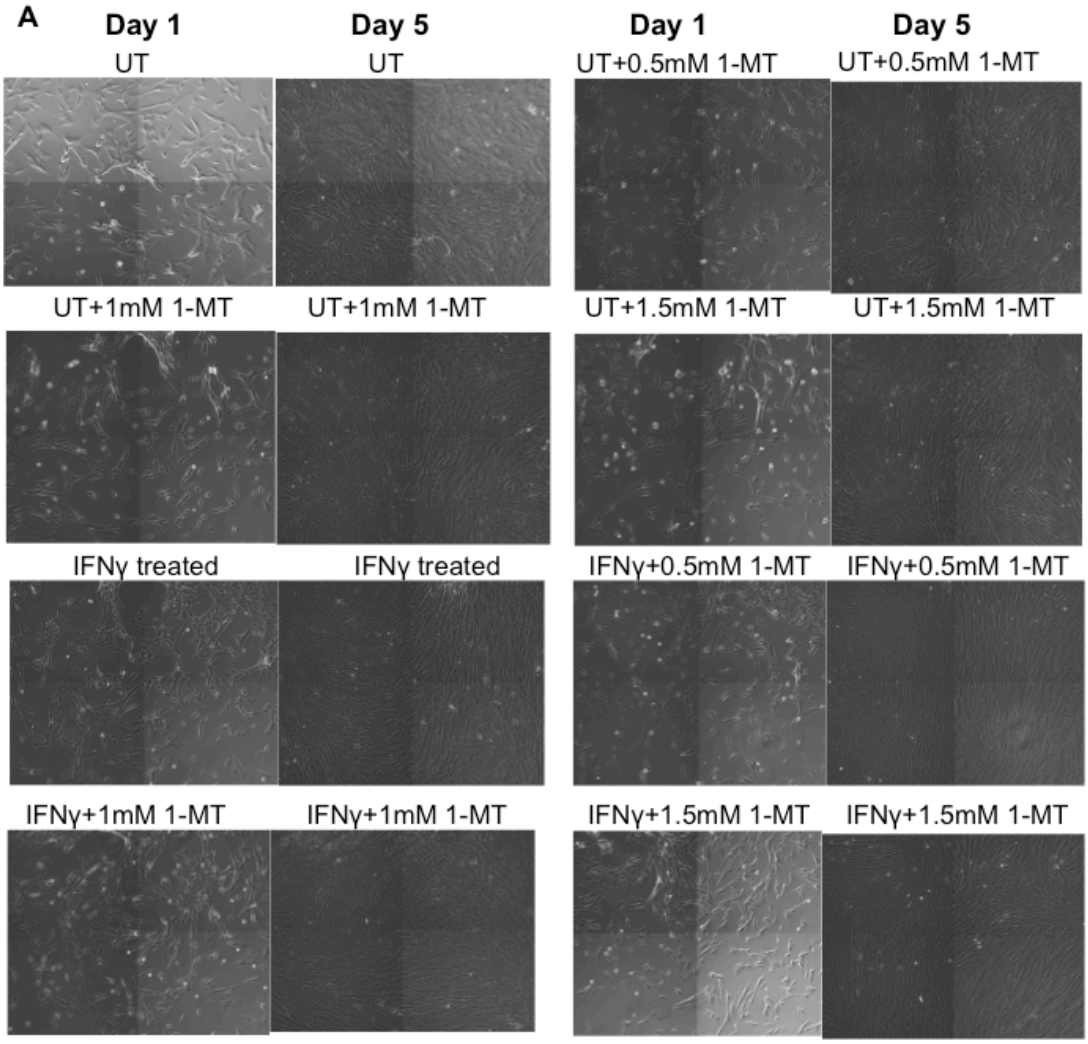


Figure 5-2: Marked reduction in adherent umbilical cord derived mesenchymal stromal cells (UC-MSC) seen in wells treated with IFNγ + 1.5mM 1-Methyl-D-Tryptophan (1-MT) compared with untreated + 1.5mM 1-MT.

Untreated + 1-MT and IFN γ treated + 1-MT UC-MSC-CM was obtained by stimulating UC-MSC with IFN γ and/or adding 1-MT for 72 hours thereafter washing and culturing in fresh medium for 24 hours. **A and B:** Representative images demonstrating adherent UC-MSC in wells treated with 1-MT. Images acquired from a light microscope (Olympus IX50 inverted microscope) using 10x magnification. **Abbreviations;** 1-MT- 1-Methyl-D-Tryptophan; UC-MSC-CM- Conditioned media derived from UC-MSC; UC-MSC- Umbilical derived mesenchymal stromal cells


A
Day 1
Day 5
Day 1
Day 5
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UT
UT+0.5mM 1-MT
UT+0.5mM 1-MT
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UT+1.5mM 1-MT
UT+1.5mM 1-MT
IFNγ treated
IFNγ treated
IFNγ+0.5mM 1-MT
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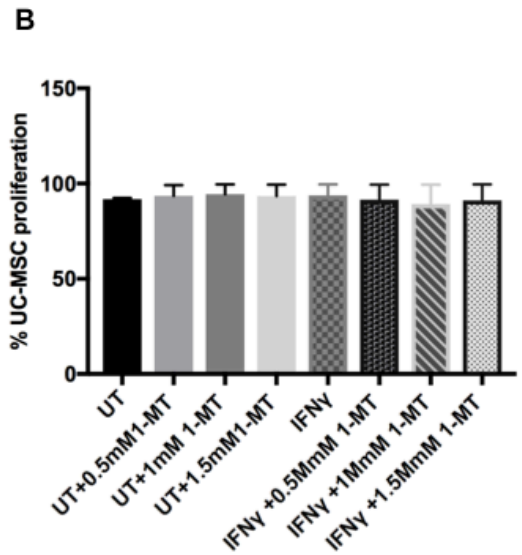
IFNγ+1.5mM 1-MT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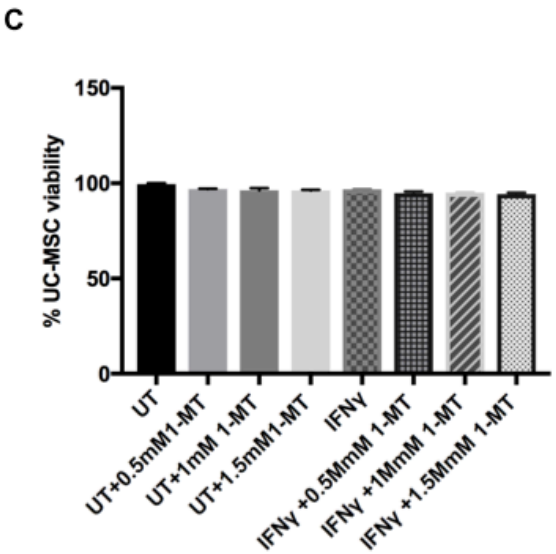
IFNγ +1.5MmM 1-MT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IFNγ +1.5MmM 1-MT

Figure 5-3: Addition of 1-Methyl-D-Tryptophan (1-MT) has no effect on the proliferation and viability of umbilical cord derived mesenchymal stromal cells (UC-MSC).

CTV labelled UC-MSC were cultured following various treatments for 5 days in a continuous live cell imaging and analysis system. Where applicable, 50ng/ml IFN γ and/or various concentrations of 1-MT was added at day 1 for 72 hours following which the UC-MSC were washed and cultured in fresh medium for a further 24 hours. At day 5, following trypsinisation, UC-MSC in all the wells were stained with a viability dye, flow cytometry was used to assess UC-MSC proliferation and viability. **A:** Representative images of UC-MSC cultured following various treatments at day 1 and day 5. **B:** Data showing % proliferation of UC-MSC in n=2 UC-MSC donors presented as mean ± SEM. **C:** Data showing % viability of UC-MSC in n=2 UC-MSC donors presented mean ± SEM. **Abbreviations;** 1-MT- 1-Methyl-D-Tryptophan; CTV- cell trace violet; UC-MSC- umbilical cord derived mesenchymal stromal cells, UT- untreated.

5.2.2 Effect of IDO inhibition on UC-MSC-conditioned media (UC-MSC-CM) suppression of circulating $CD4^+$ and $CD8^+$ T cell proliferation from patients with PSC

To determine whether IDO is the main mechanism involved in UC-MSC induced inhibition of T cell proliferation in peripheral blood of PSC patients, we used conditioned media derived from IFNγ (50ng/ml) stimulated UC-MSC containing 1mM l-MT in suppression assays. As previously mentioned 1-MT is an IDO inhibitor. Conditioned media derived from untreated UC-MSC containing 1mM 1-MT was used as control. We observed that there was no difference in the inhibitory effect of 1-MT on suppression of circulating $CD4^+$ T cell proliferation by conditioned media.

5.2.3 Effect of IDO inhibition on UC-MSC-conditioned media (UC-MSC-CM) reduction of circulating $CD4^+$ and $CD8^+$ T cell activation from patients with PSC

The role of IDO as a mechanism involved in UC-MSC-CM reducing circulating T cell activation from patients with PSC was studied. Presence 1-MT in UC-MSC-CM did not have an effect on $CD4^+$ T cell activation (Figure 5.5 A-C).

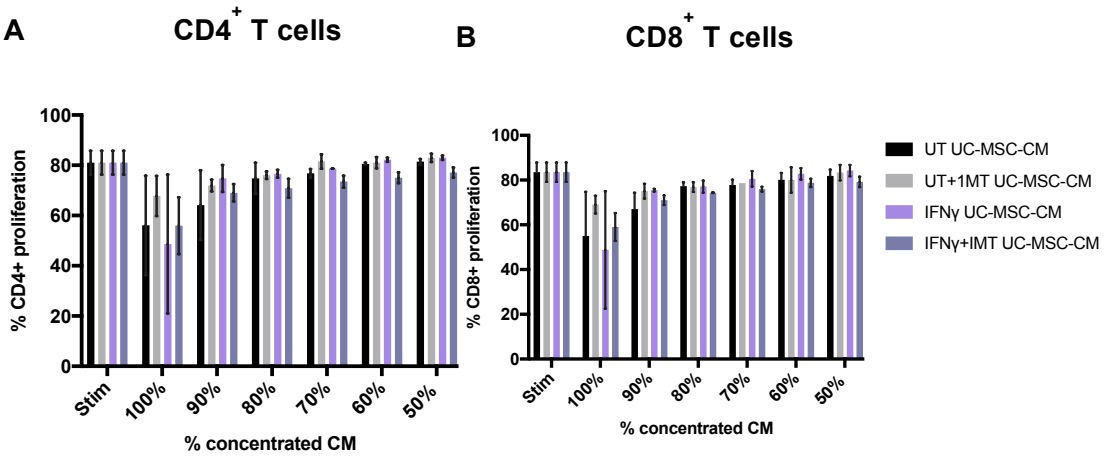
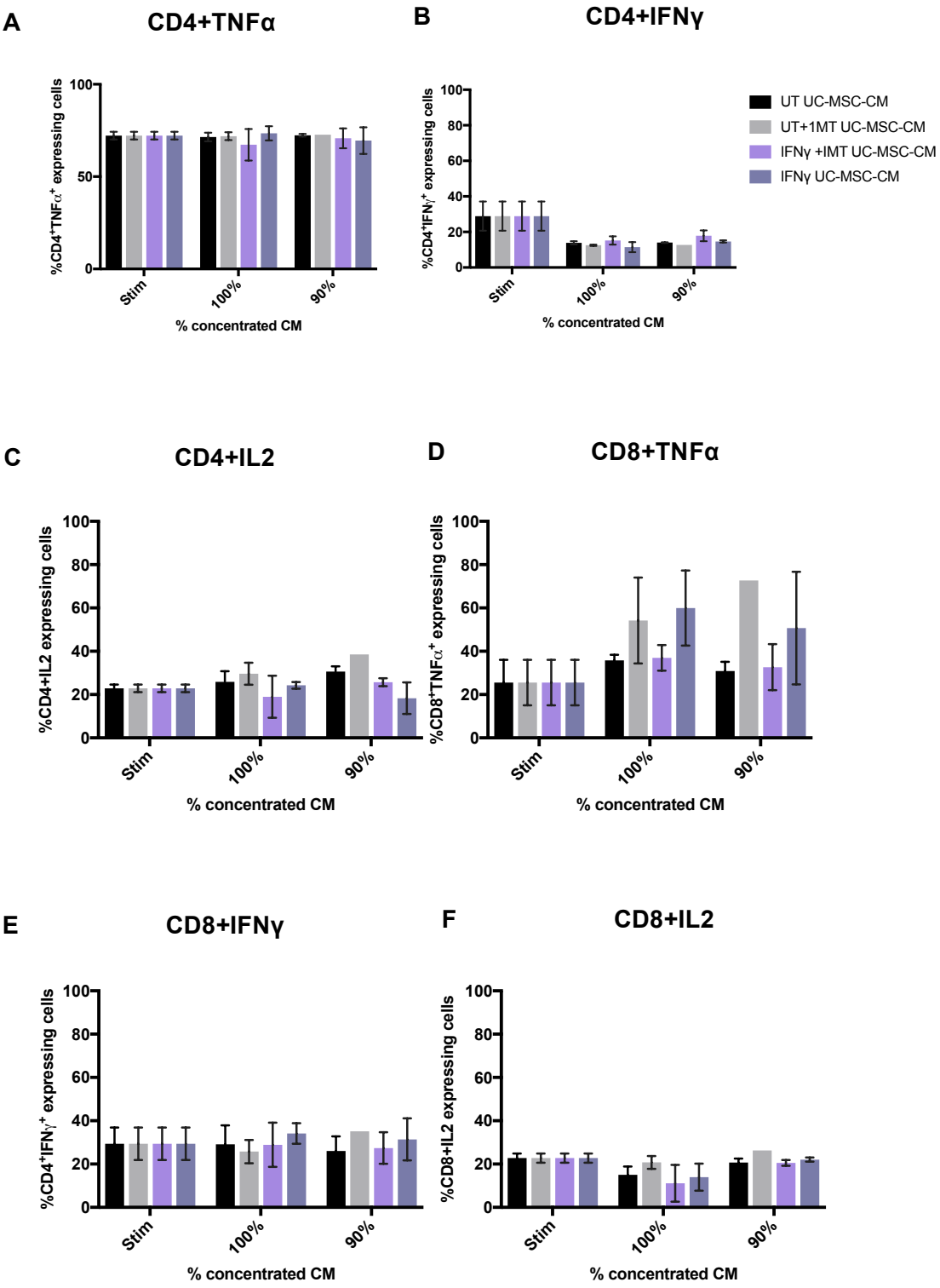


Figure 5-4: Presence of 1-Methyl-D-Tryptophan (1-MT) in untreated and IFNγ stimulated umbilical cord derived mesenchymal stromal cells conditioned media (IFNγ UC-MSC-CM) had no effect on inhibiting suppression of circulating T cell proliferation from patients with primary sclerosing cholangitis (PSC).

CTV labelled PBMC were stimulated with 0.5µg/ml anti-CD3/ 1µg/ml CD28 and cultured in various concentrations of UC-MSC-CM for 5 days. At day 5, effect on T cell proliferation was assessed using flow cytometry. CM was obtained by culturing the UC-MSC for 5 days. At day 1 of culture where appropriate the UC-MSC were stimulated with 50ng IFNγ and/or 1mM 1-MT was added for 72 hours. At day 4, after washing, culturing in fresh media for 24 hours, CM was acquired and frozen until use. **A:** Data showing %$CD4^+$ proliferation in n=2 PSC peripheral bloods presented as median with interquartile range. **B:** Data showing % $CD8^+$ proliferation in n=2 PSC peripheral bloods presented as median with interquartile range. **Abbreviations:** CM-conditioned media derived from UC-MSC, CTV- cell trace violet; IFNγ UC-MSC-CM-conditioned media derived from IFNγ stimulated UC-MSC, Stim- stimulated PBMC only in control media (no UC-MSC-CM), UC-MSC- umbilical cord derived from mesenchymal stromal cells, UT-un-treated, 1-MT- 1-Methyl-D-Tryptophan.


A
CD4+TNFα
%CD4+TNFα+ expressing cells
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100
80
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20
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Stim
100%
90%
% concentrated CM

Figure 5-5: Presence of 1-Methyl-D-Tryptophan (1-MT) in umbilical cord derived mesenchymal stromal cells conditioned media (UC-MSC-CM) has no effect on the ability of circulating $CD4^+$ T cells from patients with primary sclerosing cholangitis (PSC), to produce pro-inflammatory cytokines.

PBMC were stimulated with 0.5µg/ml anti-CD3/ 1µg/ml CD28 and cultured in various concentrations of UC-MSC-CM for 5 days. At day 5, the PBMC were further stimulated with 50ng/ml PMA, 1 µM ionomycin and 3 µg/ml Brefeldin A for 4 hours and expression of TNFα, IFN γ and IL2 assessed by flow cytometry. CM was obtained by culturing the UC-MSC for 5 days. At day 1 of culture where appropriate the UC-MSC were stimulated with 50ng IFNγ and/or 1mM 1-MT was added for 72 hours. At day 4, after washing, culturing in fresh media for 24 hours, CM was acquired and frozen until use**. A-C:** Data showing % $CD4^+$ TNFα, IFNγ and IL2 expressing cells in n=2 PSC peripheral bloods presented as median with interquartile range. **D-F:** Data showing % $CD8^+$ TNFα, IFNγ and IL2 expressing cells in n=2 PSC peripheral bloods presented as median with interquartile range. **Abbreviations:** CM- conditioned media derived from UC-MSC, IFNγ UC-MSC-CM- conditioned media derived from IFNγ stimulated UC-MSC, Stim-stimulated PBMC only in control media (no UC-MSC-CM), UC-MSC- umbilical cord derived from mesenchymal stromal cells, UT-un-treated, 1-MT- 1-Methyl-D-Tryptophan.

5.2.4 Kynurenine and tryptophan concentrations as a marker of IDO activity in UC-MSC-conditioned media (UC-MSC-CM)

Given that our experiments involving 1-MT in UC-MSC-CM did not have the expected effect on both circulating $CD4^+$ and $CD8^+$ T cell proliferation and activation, the efficacy of 1-MT was investigated by measuring kynurenine and tryptophan concentrations as markers of IDO activity. As mentioned in section 5.1, presence of IFNγ induces MSC to express IDO, which exerts its immunosuppressive action by catalysing tryptophan in to kynurenine and other metabolites. An IDO inhibitor blocks the breakdown of tryptophan thereby preventing the immunosuppressive action of IDO. To determine kynurenine and tryptophan concentrations, frozen UC-MSC-CM from the following conditions: a) untreated b) IFNγ stimulated c) untreated + 1mM 1-MT (control) d) IFNγ stimulated + 1-MT was analysed at the Phenome centre, Birmingham (University of Birmingham) using mass spectrometry. The analysed conditioned media was the same media used in suppressions assays described in section 5.2.2. The results demonstrated, that IFNγ UC-MSC-CM was associated with increased IDO activity as evidenced by a significant 10-fold increase in kynurenine (kynurenine concentration in UT UC-MSC-CM mean: 2.6μM vs IFNγ UC-MSC-CM: 26.5μM; $p<0.05$) (Figure 5.6A) and reduction of tryptophan (tryptophan concentration in UT UC-MSC-CM mean: 36.6μM vs IFNγ UC-MSC-CM: 5.9μM; $p\leq 0.01$) (Figure 5.6B) concentrations. However, unexpectedly, there were no significant differences in the kynurenine (Figure 5.6 A) and tryptophan (Figure 5.6 B) concentrations between IFNγ UC-MSC-CM and IFNγ +1-MT UC-MSC-CM confirming that the 1-MT used in experiments was not effective at inhibiting IDO activity. Conditioned media from un-treated UC-MSC in the presence or absence of 1-MT was used as controls and as expected were associated with similarly high tryptophan and low kynurenine concentrations (Figures 5.6 A and B).

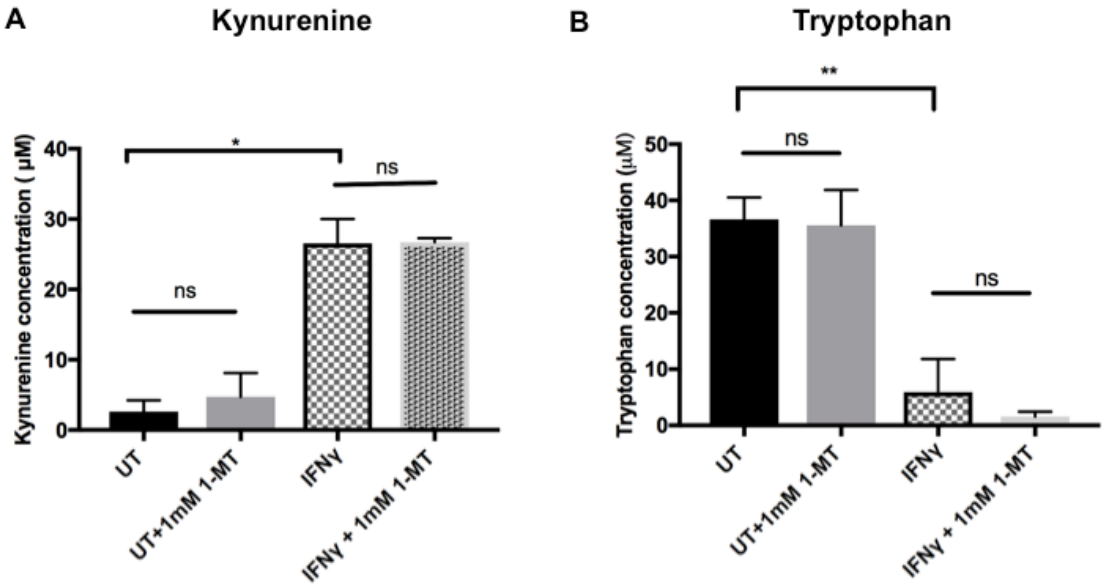

Figure 5-6: The 1-Methyl-D-Tryptophan (1-MT) used in experiments was ineffective at inhibiting Indoleamine 2,3-dioxygenase (IDO) activity.

Mass spectrometry was used to measure tryptophan and kynurenine concentrations in UC-MSC-CM treated in various conditions. UC-MSC-CM was obtained by culturing the UC-MSC for 5 days. At day 1 of culture where appropriate the UC-MSC were stimulated with 50ng IFNγ and/or 1mM 1-MT was added for 72 hours. At day 4, all the UC-MSC were washed and cultured in fresh media for 24 hours. UC-MSC-CM acquired was frozen until use in experiments. **A:** Data showing kynurenine concentration in n=3 UC-MSC-CM samples presented as mean ± SEM. Analysis performed using Friedman test. **B:** Data showing tryptophan concentration in n=3 UC-MSC-CM samples presented as mean ± SEM. Analysis performed using one-way ANOVA. * $p<0.05$ ** $p\leq0.01$. **Abbreviations:** NS- not significant; UC-MSC-CM- conditioned media from umbilical cord derived mesencyhmal stromal cells; UT- untreated, 1-MT- 1-Methyl-D-Tryptophan.

5.3 Conclusion

IDO plays a pertinent role in MSC induced T cell suppression. As previously highlighted a number of studies have shown that IDO is one of the main mechanisms of T cell inhibition by activated MSC. In keeping with studies, a specific IDO inhibitor, 1-Methyl-D-Tryptophan (1-MT) was used to assess whether IDO is the main mechanism involved in suppressing circulating T cell proliferation and activation by UC-MSC-CM. The results are mixed in that presence of 1-MT had no effect on inhibiting circulating $CD4^+$ T cell proliferation by conditioned media. Given these mixed results, the efficacy of 1-MT was determined by measuring kynurenine and tryptophan concentrations as markers of IDO activity. This confirmed that the 1-MT used in our experiments was ineffective at inhibiting IDO activity as demonstrated by the markedly increased kynurenine and reduced tryptophan concentrations similar to when 1-MT was not present (IFNγ UC-MSC-CM only).

1-MT is a synthetic tryptophan analogue that acts as a competitive inhibitor of IDO. It exists in the enantiomeric forms L- methyl-tryptophan (L-1-MT) and D-methyl-tryptophan (D-1-MT). In order to function as a potent inhibitor, it is essential to reach similar or higher levels than tryptophan in the target tissue or to have a higher affinity to IDO than the natural substrate, tryptophan (211). Based on my results, the tryptophan levels in the controls, which included untreated UC-MSC-CM only and untreated UC-MSC-CM +1-MT were similar (Figure 5.6B). 1-MT has been researched extensively in tumour immunology. It is described as being a less potent inhibitor of IDO enzyme activity both in vivo and in vitro (211, 212). The L-isomer is reported to be a weak substrate for IDO1 and the D-isomer neither binds nor inhibits the purified IDO enzyme however interestingly greater potency of D-1MT in reducing IDO mediated suppression of T cell proliferation is reported (212).

In our experiments, 1mM of D-1MT was used it may be that both a higher concentration of 1-MT and 1-Methyl-DL-Tryptophan (both isomers) should have been used in order to generate a higher tryptophan concentration in the conditioned media to enable inhibition of IDO activity. We used 1-MT as the IDO inhibitor at a concentration of 1mM given it has been used extensively to demonstrate the role of IDO in MSC experiments. Interestingly many have used 1-MT in the context of co-culture experiments (108, 128) whereas we used conditioned media, which may provide another explanation for the lack of efficacy of 1-MT in our experiments given that we have previously demonstrated that conditioned media derived from UC-MSC has a less potent immunomodulatory effect than UC-MSC directly co-cultured with PBMC/T cells. Further work is thus needed to investigate the role of IDO as a mechanism in MSC action.

CHAPTER 6.

INVESTIGATING THE SAFETY AND ACTIVITY OF THE USE OF A SINGLE INFUSION OF SELECTED MESENCHYMAL STROMAL CELLS TO REDUCE LIVER INFLAMMATION IN THE TREATMENT OF PATIENTS WITH PSC AND AIH (MERLIN); A STUDY PROTOCOL FOR AN ADAPTIVE, SINGLE ARM, MULTI-CENTRE PHASE IIA MULTI-DISEASE CLINICAL TRIAL

6.1 MERLIN clinical trial overview

Our pre-clinical in-vitro studies have demonstrated that UC-MSC are able to suppress circulating and intrahepatic T cell proliferation and activation from patients with PSC thus providing evidence of their effectiveness in PSC. A clinical trial (MERLIN) was designed to infuse UC-MSC in patients with PSC or AIH and assess the safety and efficacy of UC-MSC in a clinical setting. MERLIN is a 24-month single arm multi-centre, multi-disease clinical trial of treatment with a single infusion of selected UC-MSC (ORBCEL-C^{TM}) in adult patients with either PSC or AIH. I contributed to the setting up of the clinical trial. My role included writing trial related documents required for medicines and healthcare products regulatory agency (MRHA) and ethical approval. This included the trial protocol (detailed in section 6.5.1), investigator's brochure, investigational medicinal product dossier, patient information sheet and advanced therapy medicinal product manual. I undertook a pilot study to understand the immune profile of patients with PSC, PBC and AIH in collaboration with Erasmus, University medical center, Rotterdam. For the pilot study, I identified appropriate patients, collected samples and isolated PBMCs as well as plasma, which were frozen until shipment to Erasmus where immune profiling was performed. My work helped us attain MHRA and ethical approval on the December 2018. Thus far 7 patients have been recruited to the trial and have received an infusion of ORBCEL-C^{TM}. Phase 1 of the trial, is expected to be completed by the end of 2021 and phase 2 will commence in 2022 and expected to be completed by 2024. Preliminary results are expected by 2022.

6.2 Trial protocol

6.2.1 Introduction

Primary sclerosing cholangitis (PSC) and autoimmune hepatitis (AIH) belong to a family of rare diseases that is autoimmune liver diseases (AILD). Despite being rare, these diseases contribute disproportionately to the mortality and morbidity of chronic liver diseases.

PSC is characterised by chronic inflammation and fibrosis leading to bile duct stricturing and progression to end-stage liver disease (1). The incidence and prevalence of PSC is on the rise (8, 13, 213). More than 60% of patients diagnosed with PSC are men and median age at diagnosis is 30-40 years (2). It is associated with inflammatory bowel disease (IBD) and is a risk factor for hepatobiliary and colorectal malignancy. More than 50% of patients require liver transplantation within 10-15 years of symptomatic presentation and in the United Kingdom PSC has become the leading indication of liver transplantation among patients with AILD (214), a reflection of the lack of effective therapies in PSC.

AIH is a heterogenous liver disease characterised by hepatitis of varying severity and risk of development of end-stage liver disease if not treated promptly and judiciously (215). The incidence of AIH is also rising; In England the incidence of AIH had doubled to 2.56 per 100 000 populations/year over an 18- year study period (Grøenbæk et al, EASL, 2019). Standard immunosuppressive treatment namely corticosteroids and azathioprine are effective in treating the majority of patients however treatment is limited by side effects particularly with long-term corticosteroid use. Approximately 10-15% of patients discontinue treatment due to intolerable side- effects (216). Corticosteroid use is reported to be associated with a significant decrease in health-

related quality of life (HR-QOL) of AIH patients (217). Despite guidance to taper corticosteroids at 12-18 months post introduction of corticosteroid sparing agents and remission, a UK study found more than 55% of patients continued taking long-term corticosteroids as part of their treatment regimen (218).

Both PSC and AIH represent chronic immune mediated liver injuries. The aetio-pathogenesis is not fully elucidated however it is proposed that environmental factors on a background of genetic defects in immune-regulation allows for persistent inflammation and breakdown of self-tolerance (39). There is strong evidence supporting the role of T cell mediated damage across both diseases with the innate and adaptive immune systems involved. Both diseases are strongly linked with IBD generating several pathogenic theories in which gut commensals, pathogens and intestinal antigens are implicated in causing liver injury. For example, presence of pro-inflammatory tumour necrosis factor α activates vascular adhesion protein 1 VAP-1 in the liver, inducing MADCAM-1 and promoting recruitment of α4β7+ mucosal effector cells to the liver (76, 81). An imbalance in immune-regulatory pathways is also present in both diseases; Tregs in the peripheral blood and livers of both PSC and AIH patients are impaired in frequency and function (37, 41, 219). Thus far, in PSC no treatment has been able to combat the inflammatory disease process and in AIH, current therapies do not specifically target the biological pathways involved representing large unmet needs in developing safe, effective therapy. This and common mechanistic themes of liver injury justifies our proposal to use allogeneic mesenchymal stromal cells (MSC) as an immunotherapy to treat both PSC and AIH.

MSC are fibroblast like cells that possess the ability to differentiate down mesendermal cell lineages (220) and can be isolated from a number of tissues.

Many clinical trials have utilised MSC to treat immune-mediated inflammatory diseases, some with success. A phase 3 randomised double- blind multicentre trial demonstrated expanded allogeneic adipose derived stem cells (Cx601) were safe and efficacious (50% of patients who received Cx601 vs 24% of patients who received placebo; p=0.24 achieved remission 24 weeks after treatment) in treating complex perianal fistulas in patients with Crohn's disease who were refractory to conventional/biologic treatment (156). Over 400 clinical trials have confirmed safety of allogeneic MSC therapy with no reported major adverse in various clinical settings including some involving patients with liver disease (151). The majority have recruited patients with advanced liver disease in whom MSC therapy was not only was found to be safe but some have reported clinical efficacy (171-173). More robust large-scale clinical trials are however needed to gain confidence regarding MSC efficacy in liver disease. In addition, long-term safety and efficacy data is lacking.

Given the successful use of MSC in IBD, scientific evidence regarding the safety profile of MSC and their ability to regulate the immune and inflammatory response we hypothesised that a single intravenous (IV) infusion of selected MSC would be safe and efficacious in reducing liver inflammation in patients with either PSC or AIH. To test this hypothesis, we designed an adaptive, single arm, multicentre phase IIa multi-disease clinical trial entitled 'selected Mesenchymal Stromal Cells to reduce liver inflammation in the treatment of patients with PSC or AIH (MERLIN).'

ORBCEL-C^{TM}

For our trial, we are using a selected and enriched preparation of MSC (CD362^{+}MSC or ORBCEL-C^{TM}) sourced from allogeneic umbilical cord tissue and expanded ex vivo by NHS Blood and Transplant (NHSBT) in conjunction with Orbsen therapeutics Limited. We have used selected MSC because a homogenous population of cells will

aid in maintaining consistency of treatment results. No safety concerns in the literature regarding administration of MSC at doses as high as 10 $x10^6$ cells/kg have been reported and efficacy is seldom seen below a dose of 2.0 x 10^6 cells/kg. In our trial, a target dose of 2.5 x 10^6 cells/kg was chosen on the grounds that this is the dose, from the literature, which is most likely to be effective and feasible from a manufacturing process.

6.2.2 Method

Study design overview

The sample size will be a maximum of 25 patients for both stages and each cohort (PSC and AIH). The screening period to assess for eligibility and collect baseline data is 28 days prior to patients receiving one infusion of ORBCEL-C™, which marks study day (SD) 0 of the trial. After patients have reached SD56 (primary safety/efficacy evaluation time point), all patients from both the cohorts will be followed long-term up to 22 months (D720). Visits after SD56 will be in accordance with standard clinical practice and align with routine clinic visits.

There are two main stages in the trial and the open-label trial design will apply to both disease cohorts. Recruitment in both cohorts will occur independently and simultaneously. Stage 1 (dose safety stage) will determine the highest safe dose in the trial over a 14-day period to take forward to Stage 2. Stage 2 (activity and safety stage) will determine safety and tolerability over a longer period of 56 days and efficacy of ORBCEL-C™. There are three ORBCEL-C™ doses that will potentially be evaluated in the trial 1) Dose level A: 1 $x10^6$ cells/kg 2) Dose level B: 2.5 $x10^6$ cells/kg 3) Dose level C: 0.5 $x10^6$ cells/kg (See Figure 6.1 for trial design). As this is a clinical trial aimed at safety, we will recruit patients initially to the lower dose of 1 $x10^6$ cells/kg, providing this

dose is deemed safe, further patients will be recruited to the target dose of 2.5 $x10^6$ cells/kg. Detailed explanation is provided in Figure 6.2.

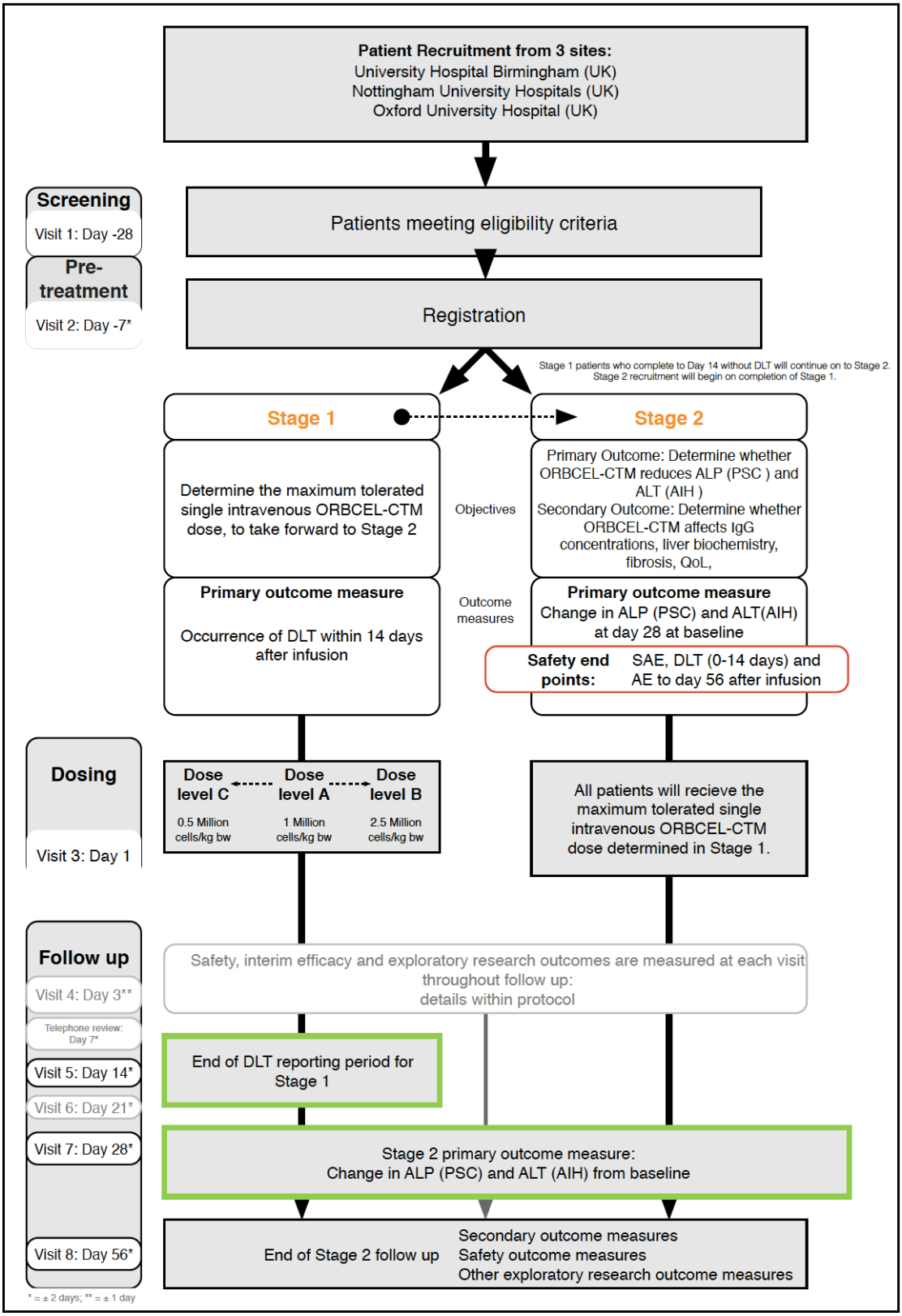
Patient Recruitment from 3 sites:
University Hospital Birmingham (UK)
Nottingham University Hospitals (UK)
Oxford University Hospital (UK)
Screening
Visit 1: Day -28
Patients meeting eligibility criteria
Pre-treatment
Visit 2: Day -7*
Registration
Stage 1 patients who complete to Day 14 without DLT will continue on to Stage 2.
Stage 2 recruitment will begin on completion of Stage 1.
Stage 1
Stage 2
Determine the maximum tolerated single intravenous ORBCEL-CTM dose, to take forward to Stage 2
Objectives
Primary Outcome: Determine whether ORBCEL-CTM reduces ALP (PSC) and ALT (AIH)
Secondary Outcome: Determine whether ORBCEL-CTM affects IgG concentrations, liver biochemistry, fibrosis, QoL,
Primary outcome measure
Occurrence of DLT within 14 days after infusion
Outcome measures
Primary outcome measure
Change in ALP (PSC) and ALT(AIH) at day 28 at baseline
Safety end points:
SAE, DLT (0-14 days) and AE to day 56 after infusion
Dosing
Visit 3: Day 1
Dose level C
0.5 Million cells/kg bw
Dose level A
1 Million cells/kg bw
Dose level B
2.5 Million cells/kg bw
All patients will recieve the maximum tolerated single intravenous ORBCEL-CTM dose determined in Stage 1.
Follow up
Visit 4: Day 3**
Telephone review: Day 7*
Visit 5: Day 14*
Visit 6: Day 21*
Visit 7: Day 28*
Visit 8: Day 56*
* = ± 2 days; ** = ± 1 day
Safety, interim efficacy and exploratory research outcomes are measured at each visit throughout follow up:
details within protocol
End of DLT reporting period for Stage 1
Stage 2 primary outcome measure:
Change in ALP (PSC) and ALT (AIH) from baseline
End of Stage 2 follow up
Secondary outcome measures
Safety outcome measures
Other exploratory research outcome measures

Figure 6-1: Merlin trial schema

Abbreviations: AE- adverse event; AIH- autoimmune hepatitis, ALT- alanine transaminase, ALP- alkaline phosphatase, DLT- dose limiting toxicity, IgG- immunoglobulin G, PSC- primary sclerosing cholangitis, SAE- serious adverse event

In Stage 1, initial safety will be evaluated over a dose limiting toxicity (DLT) (see later for definition) assessment period of 14 days from start of treatment, using cohorts of 3 patients based on a Bayesian safety-monitoring rule, similar to the conventional 3+3 cohort design. Initially the first patient recruited to a dose level in each cohort will be assessed for DLT for 14 days. Provided there are no DLTs for this patient, further patients to that dose level per cohort will be recruited as per description in Figure.6.2.

The highest safe dose determined in Stage 1 for a cohort will be selected for recruitment of subsequent patients in Stage 2 until a total of 19 evaluable patients (maximum 25 patients) for both stages combined have been recruited for each disease cohort.

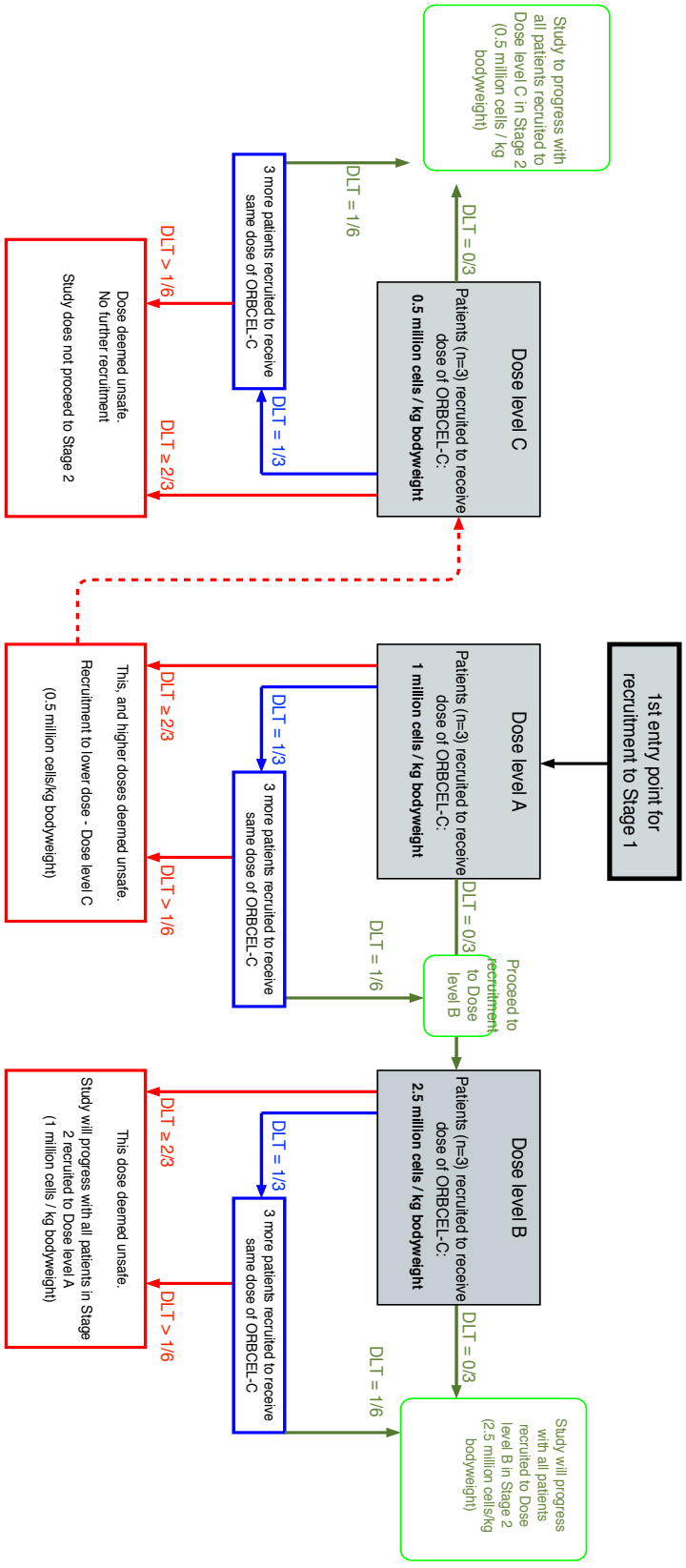

Figure 6-2: Flow diagram showing the recruitment pathway for the dose confirmatory stage (Stage 1)

Abbreviation: DLT- dose limiting toxicity

6.2.3 Treatment

A single arm rather than placebo-controlled design was chosen to allow for efficient enrolment of patients into the study as the strict inclusion and exclusion criteria would likely mean that a significant number of patients may not be eligible, a placebo group may therefore act as a substantial barrier for enrolment. Furthermore in AIH it would be unethical to have a placebo-controlled trial as previous controlled trials have showed high mortality rates in controls.

Our trial is based on a bucket trial design. Similar conceptual approaches to trials have been used in oncology with success where a single agent has been used to treat a number of cancer settings. Such bucket trial designs provide greater power to discern the effect of interventions on common mechanisms by virtue of combining patients groups, as well as providing additional data on safety. Moreover, such approaches confer efficiencies as regards to regulatory approvals and operational management of such clinical studies.

6.2.3.1 Patient selection

Currently recruitment has commenced at the Queen Elizabeth Hospital, Birmingham. It is anticipated that recruitment will soon begin at other UK trial centres including the John Radcliffe-Oxford University Hospital (Oxford) and Queens Medical Centre (Nottingham). Informed consent to participate in the trial will be obtained by appropriately trained members of the research team at each site.

PSC patients: The main inclusion criteria are patients aged ≥ 18, ≤70 years with a diagnosis of PSC as evidenced by chronic biochemical cholestasis (defined by an

elevated serum alkaline phosphatase (ALP) and/or gamma-glutamyl transpeptidase (GGT) above the upper limit of normal (ULN)) of more than 6 months duration and either radiological or histological evidence of PSC. Patients must have a serum ALP of at least 1.5 times the ULN both at Visit 1 (SD-28) and Visit 2 (SD-7). Patients on ursodeoxycholic acid (UDCA) will be asked to stop taking UDCA and a 8-week washout period prior to the first screening visit is required. Those with concomitant IBD must have stable disease evidenced by the absence of clinically significant flares requiring intensification of therapy from baseline maintenance or oral prednisolone greater than 10 mg or biologic therapy. Patients should also have endoscopic evidence of stable disease within the past 24 months prior to the planned ORBCEL-C^{TM} infusion and no evidence of high-grade dysplasia.

AIH patients: The main inclusion criteria are patients aged ≥ 18, ≤70 years with an established pre-existing biopsy proven diagnosis of AIH consistent with the simplified international AIH group criteria (http://www.mdcalc.com/simplified-scoring-autoimmune-hepatitis-aih/). Patients must have evidence of active AIH defined by a serum alanine aminotransferase (ALT) level of at least 1.5 times ULN at the time of both screening visits and have been on at least 24 weeks of standard-of- care AIH therapy (except biologics) at the time of screening. The doses of immunosuppressive therapy should be stable for a minimum period of 4 weeks at the time of screening with no planned changes during the trial period.

A detailed exclusion criteria is provided in Table 5.

Generic exclusion criteria that apply to both patients with PSC and AIH

1. Refusal or lacks capacity to give informed consent to participate in trial
2. Patient who is unable to participate in follow up assessment
3. Participation actively, or within 5 half-lives, of another interventional clinical trial
4. Known hypersensitivity to the investigational product or any of its formulation excipients
5. Evidence of active malignancy (within 3 years of visit 1 (screening)), other than non-melanomatous skin cancer and cervical dysplasia in situ
6. Major surgical procedure within 30 days at visit 1 (screening)
7. Prior organ transplantation
8. Active harmful alcohol consumption as evaluated and documented by the investigator
9. Creatinine >133 µmol/L or being treated with renal replacement therapy at the time of Visit 1 (screening)
10. AST or ALT > 10 x ULN
11. ALP > 10 x ULN
12. Platelets < 50 x 10^9/L
13. Total Bilirubin > 2 x ULN
14. INR > 1.3 (in the absence of concomitant use of Warfarin or equivalent anti-coagulant therapy)
15. Albumin < 35 g/litre
16. Haemoglobin < 10 g/dl
17. Past or present evidence of decompensated chronic liver disease:
    - **a.** Radiological or clinical evidence of ascites
    - **b.** Hepatic encephalopathy
    - **c.** Endoscopic evidence for portal hypertensive bleeding
18. Any active treatment with biologic therapy (monoclonal antibodies)
19. Clinically severe cardiovascular disease as evaluated by the Investigator
20. Pregnancy or breast-feeding
21. Women of child bearing potential who are unwilling to practice effective contraception (I.e. barrier, oral contraceptive pill, implanted contraception, or previous hysterectomy, bilateral oophorectomy) for the duration of the trial up to 90 days after the trial drug is administered. If using hormonal agents the same method must have been used for at least 1 month before study dosing and subjects must

use a barrier method during that time period

22. Non-vasectomised men, sexually active with women of child bearing potential, who are not willing to practice effective contraception (condom with spermicide) for the duration of the trial up to 90 days after the trial drug is administered

23. Patients with a history of hepatitis C (present or past infection), known positivity for antibody to HIV or any evidence of current or past hepatitis B infection

24. Presence of an acute/chronic infection or illness that, at the discretion of the Investigator, might compromise the patient's health and safety in the trial

25. Receipt of live vaccination within six weeks prior to visit 1 (screening)

Exclusion criteria specific to patients with PSC

1. Documented alternative aetiology for sclerosing cholangitis (i.e. secondary sclerosing cholangitis)

2. A dominant (as determined by Investigator) alternative chronic or active liver injury other than PSC at the time of visit 1 (screening); Patients with possible overlap syndrome with AIH are excluded from the PSC cohort if the Investigator considers AIH as the dominant liver injury

3. UDCA use within 8 weeks of the first screening visit (if a patient was taking UDCA a washout period of at least 8 weeks prior to the first screening is required)

4. ALP > 10 x ULN

5. Evidence of cholangitis within 90 days of visit 1 (screening)

    a. Documented evidence of cholangitis by physician

    b. Need for any antibiotics for presumed cholangitis

6. Any patient taking prophylactic antibiotics to combat recurrent cholangitis

7. Presence of percutaneous biliary drain, or internal biliary stent

8. Diagnosed hepatocellular carcinoma or cholangiocarcinoma or high clinical suspicion thereof

9. Dominant stricture clinically suspicious of cholangiocarcinoma (as determined by Investigator)

For those with IBD

1. Unstable disease as evidenced by:

    a. Documented clinically significant flare within 90 days of enrolment requiring any marked intensification of therapy from baseline maintenance (maintenance therapy = thiopurines, 5-aminosalicylates, or oral prednisolone <10mg/day; biologics therapy is an exclusion criteria

    b. Requirement for daily prednisolone >10mg

    c. Mayo Clinic Score ≥ 2 AND clinician assessment of active disease requiring

up-titration of treatment; last colonoscopy within last year used for endoscopic component. 2. Any colonoscopic evidence of clinically significant dysplasia at last colonoscopy 3. Patients who have not had their routine colonoscopy within 24 months prior to planned MSC infusion and are unable to have their screening colonoscopy examination as per standard care prior to study visit 3 (treatment)
Exclusion criteria specific to patients with AIH 1. A dominant (as determined by Investigator) alternative chronic or active liver injury other than AIH at the time of visit 1 (screening); Patients with possible overlap syndrome with PSC are excluded from the AIH cohort if the Investigator considers PSC as the dominant liver injury 2. AST or ALT > 10 x ULN 3. Patients on a prednisolone dose of > 20 mg at the time of screening 4. Treatment with biologic therapy within 24 weeks of the time of screening 5. Patients with a history of poor compliance with medication 6. Diagnosed hepatocellular carcinoma or cholangiocarcinoma or high clinical suspicion thereof

Table 5: Exclusion criteria

Abbreviations: AIH- autoimmune hepatitis, ALT- alanine transaminase, ALP- alkaline phosphatase, AST- aspartate transaminase, INR- international normalised ratio, PSC- primary sclerosing cholangitis, UDCA- ursodeoxycholic acid, ULN- upper limit of normal

6.2.3.2 Concomitant therapy

AIH patients will continue on their standard-of-care immunosuppressive therapy. This includes any AIH therapy with the exception of biologic therapy (an exclusion criteria). No changes in medication doses will be allowed during the trial unless clinically indicated. If the clinical disease warrants intervention, treatment will be as per standard-of-care and at the investigator's discretion. If during the trial period, a patient's prednisolone dose exceeds 20 mg, that patient will be excluded from further trial follow up. Relevant members of the trial team will be required to document any antibiotic use, over the counter preparations and herbal remedies throughout the trial period.

In PSC patients, initiation or clinically significant change of doses during the trial period of any therapy that can potentially change serum ALP e.g. prednisolone at a dose of greater than 10 mg, methotrexate, fenofibrate or bezafibrate, will not be permitted.

6.2.3.3 Preparation of ORBCEL-C™

Manufacturing, storage and distribution of ORBCEL-C™ will be undertaken by NHSBT, Birmingham, UK and licensed in accordance with the quality and safety regulations of the Human Tissue Authority and MHRA. Once manufactured, the ORBCEL-C™ cells will be cryopreserved, stored in CryoMACS bags (Miltenyi, Biotech) and in storage vessels at temperatures of less than -150°C in the vapour phase of liquid nitrogen. The process of cryopreservation and storage of ORBCEL-C™ cells has been validated by NHSBT to ensure the MSC phenotype and cell viability is preserved. A weight based dosing regime of ORBCELC™ has been employed in the trial; there will be 2 bag units of ORBCELC™ available for use – Bag A (80 $x10^6$ cells) and Bag B (40 $x10^6$ cells). When ORBCEL-C™ is required for administration, frozen bags of cells will be

transferred and delivered via dry shippers directly to the trial patient's bedside in the clinical area at the recruiting centre. The cells will be administered on the day of release and directly after thawing in a water bath. Whole bags will be used based on calculated doses for any given weight. Thirty minutes prior to the intravenous infusion of ORBCEL-C™ trial patients will receive intravenous chlorpheniramine 10 mg. Standard and research blood tests will be taken 1 hour pre-infusion, following this, each bag of ORBCEL-C™ will be given over 10-15 minutes. Safety investigations will be completed pre- and post infusion including vital sign monitoring and clinical assessment. Further standard and research bloods will be taken 4 hours post infusion.

6.2.3.4 Study visit overview

There are eight patient-related visits at their nearest trial sites. The study is divided in to six stages 1) screening and enrolment (Visits 1 and 2, over a maximum period of 28 days) 2) ORBCEL-C™ treatment (Visit 3, SD0) 3) end of DLT reporting period for Stage 1 (Visit 5, 14 days post infusion) 4) primary end point assessment (Visit 7, 28 days post infusion) 5) secondary endpoint assessment (Visit 8, 56 days post infusion) 6) long- term follow-up ((D180-D720) 6 months post trial up to 22 months). In the event of an adverse event (AE), an unscheduled visit will be arranged.

The schedule for the study visits and data collection is summarized in tables 6 and 7. Given the fluctuating nature of both PSC and AIH as well as the natural variation of serum ALP levels, there will be two screening visits (21 days apart) to ensure eligibility of the patient. At the second screening visit (Visit 2, SD-7), in PSC patients we will confirm that the serum ALP has not changed by more than 25% from visit 1 and in AIH patients, that the serum ALT remains at least 1.5 times ULN.

PSC patients

			Informed consent	Eligibility assessment[1]	Clinical assessment[2]	Vital signs[3]	Electrocardiogram[4]	Registration
Screening	Visit 1	SD 28***	X					
	Visit 2	SD 7**		X	X			
Treatment	Visit 3	SD 0*			X	X		
Follow up	Visit 4	SD 3			X			
	Telephone	SD7*			X			
	Visit 5	SD 14**			X			
	Visit 6	SD 21**			X			
	Visit 7	SD 28**			X			
	Visit 8	SD 56**			X			
Long term safety follow up	Month 6	D180 ± 30 days			X			
	Month 9	D270 ± 30 days			X			
	Month 12	D360 ± 30 days			X			
	Month 18	D540 ± 30 days			X			
	Month 24	D720 ± 30 days			X			

[5] X

PSC Patients		Pregnancy test[6]	Review of concomitant medication[7]	Screening blood tests[8]	Standard blood tests[9]	Non-invasive fibrosis serum markers: ELF[11]	Fibroscan®	Exploratory research blood and serum sampling[12]
Screening	Visit 1	SD 28***	X	X	X	X	X	X
	Visit 2	SD 7**	X	X				X
Treatment	Visit 3	SD 0*	X	X		X[10]		X[13]
Follow up	Visit 4	SD 3		X		X		X
	Telephone	SD7*		X				
	Visit 5	SD 14**		X		X		X
	Visit 6	SD 21**	X	X		X		X
	Visit 7	SD 28**		X		X	X	X
	Visit 8	SD 56**		X		X		X
Long term safety follow up	Month 6	D180 ± 30 days		X		X		
	Month 9	D270 ± 30 days		X		X		
	Month 12	D360 ± 30 days		X		X		
	Month 18	D540 ± 30 days		X		X		
	Month 24	D720 ± 30 days		X		X		

PSC Patients	Exploratory research blood and serum sampling[14]	Serum total bile acid levels[16]	Anti- human leukocyte antigen (HLA) antibody testing[17]	Questionnaires[18]	IBD Assessment[19]	MSC infusion	Adverse / Clinical event monitoring[20]
							X
							X
							X
							X
	X						X
							X
	X						X
							X
	X	X	X	X	X		X
	X	X	X	X	X	X	X
	X[15]	X	X	X	X	X	X
	X						X
							X
	X						X
							X

Table 6: Study schedule for PSC patients

Key

SD: study day; D-day *: ± 1 day **: ± 2 days; *: ± 7 days**

1. Eligibility will be assessed against pre-determined inclusion and exclusion criteria
2. Clinical assessments**-** consists of a complete history and examination at screening (visit 1), 7, 8 and focussed history and examination performed during the other visits .The trial clinician will perform a standard physical assessment at Visit 1, 2, 7 and 8 to include examination of the main and specific systems: Respiratory, cardiovascular, skin, lymph nodes*,* abdomen. At other visits with the exception of visit 1, 2, 7, 8 and follow up at 6-24 months (see above), a focussed examination of specific systems prompted by clinical suspicion will be performed
3. Vital signs- Heart rate, blood pressure, body temperature, oxygen saturation (SpO2), Respiratory rate. On the day of infusion, intense monitoring of vital signs (as described above): 5 minutes pre-infusion, at cessation of infusion, then at 30, 60, 120 and 240 minutes post cessation; any additional monitoring or assessment is at the discretion of clinical staff
4. To be performed additionally beyond Visit 1 if deemed clinically relevant
5. Registration to be done within 7 days of visit 2
6. To be performed by urine human chorionic gonadotrophin assessment (urine dipstick test) on women with child bearing potential
7. All medications at the time or within 3 months (90 days) prior to screening (Visit 1) should be recorded. Any new medication or changes to current medication during the trial should also be recorded at each visit or telephone review
8. Hepatitis B serology (HBsAg), hepatitis C antibody (HCV Ab), HIV antibody (HIV Ab). If not previously performed then Ferritin, Transferrin saturation, anti-mitochondrial antibody (AMA), anti-nuclear antibody (ANA), anti-smooth muscle antibody (ASMA), caeruloplasmin, alpha 1 antitrypsin (α1AT)
9. Full Blood Count (FBC), Urea and Electrolytes (UE), Liver Function tests (LFT), INR, C-reactive protein (CRP), immunoglobulins (Igs). At sites other than the coordinating site,

serum ALP measurements will be processed locally and a further whole blood sample will be frozen and sent to the coordinating centre for analysis

10. Taken 1 hour before infusion, and 4 hours after infusion
11. Blood panel for Enhanced Liver Fibrosis (ELF)
12. Frozen serum and peripheral blood mononuclear cells (PBMCs) will be stored and batch analysed by flow cytometry, cytokine and chemokine analysis will be performed by cytokine array, microRNA and RNA analysis by qPCR or equivalent technology and endothelial cell activation markers measured using flow assays.
13. Taken 1 hour before infusion, and 4 hours after infusion
14. Additional research blood will be taken to allow isolation of PBMCs which in addition to plasma will be stored and batch analysed in Rotterdam (ERASMUS University) by cell flow cytometry, cytokine and chemokine analysis by cytokine array
15. Taken 1 hour before infusion, and 4 hours after infusion
16. Fasting bloods for serum total bile acid levels will be taken 1 hour before infusion visit 3 (SD 0) and visit 7 (SD28)
17. Sample taken at Visit 3 (pre-infusion) and Visit 7
18. SF36v2 , nine point fatigue severity scale and Pruritus Visual Analogue Scale
19. Mayo IBD Score (non-endoscopic aspects) will be used to assess the severity of IBD, in those who have co-existent IBD.
20. Adverse events / bloods and clinical events will be monitored continuously until completion of follow up

AIH patients

Long term safety follow up	Month 24	D720 ± 30 days					
	Month 18	D540 ± 30 days		X			
	Month 12	D360 ± 30 days		X			
	Month 9	D270 ± 30 days		X			
	Month 6	D180 ± 30 days		X			
Follow up	Visit 8	SD 56**		X			
	Visit 7	SD 28**		X			
	Visit 6	SD 21**		X			X
	Visit 5	SD 14**		X			
	Telephone	SD7*		X			
	Visit 4	SD 3		X			
Treatment	Visit 3	SD 0*		X	X		X
Screening	Visit 2	SD 7**	X	X	X	X[5]	X
	Visit 1	SD 28***	X	X	X	X	X
			Informed consent	Eligibility assessment[1]	Clinical assessment[2]	Vital signs[3]	Electrocardiogram[4]	Registration	Pregnancy test[6]

AIH patients			Review of concomitant medication[7]	Screening blood tests[8]	Standard blood tests[9]	Non-invasive fibrosis serum markers: ELF[12]	Fibroscan®	Exploratory research blood and serum sampling[13]
Screening	Visit 1	SD 28***	X	X	X	X	X	X
	Visit 2	SD 7**	X		X			X
Treatment	Visit 3	SD 0*	X		X[10,11]			X[14]
Follow up	Visit 4	SD 3	X		X			X
	Telephone	SD7*	X					
	Visit 5	SD 14**	X		X			X
	Visit 6	SD 21**	X		X			X
	Visit 7	SD 28**	X		X	X	X	X
	Visit 8	SD 56**	X		X			X
Long term safety follow up	Month 6	D180± 30 days	X		X			
	Month 9	D270± 30 days	X		X			
	Month 12	D360± 30 days	X		X			
	Month 18	D540± 30 days	X		X			
	Month 24	D720± 30 days	X		X			

AIH patients	Exploratory research blood and serum sampling[15]	Serum total bile acid levels[17]	Anti- human leukocyte antigen (HLA) antibody testing[18]	Questionnaires[19]	MSC infusion	Adverse / Clinical event monitoring[20]
						X
						X
						X
						X
						X
						X
						X
	X		X	X		X
	X	X	X	X	X	X
	X[16]	X	X	X	X	X
				X		X
						X

Table 7: Study schedule for AIH patients

Key

SD: study day; D:day *: ± 1 day **: ± 2 days; *: ± 7 days**

1. Eligibility will be assessed against pre-determined inclusion and exclusion criteria
2. Clinical assessments**-** consists of a complete history and examination at screening (visit 1), 7, 8 and focussed history and examination performed during the other visits .The trial clinician will perform a standard physical assessment at Visit 1, 2, 7 and 8 to include examination of the main and specific systems: Respiratory, cardiovascular, skin, lymph nodes*,* abdomen. At other visits with the exception of visit 1, 2, 7, 8 and follow up at 6-24 months (see above), a focussed examination of specific systems prompted by clinical suspicion will be performed
3. Vital signs- Heart rate, blood pressure, body temperature, oxygen saturation (SpO2), Respiratory rate. On the day of infusion, intense monitoring of vital signs (as described above): 5 minutes pre-infusion, at cessation of infusion, then at 30, 60, 120 and 240 minutes post cessation; any additional monitoring or assessment is at the discretion of clinical staff
4. To be performed additionally beyond Visit 1 if deemed clinically relevant
5. Registration to be done within 7 days of visit 2
6. To be performed by urine human chorionic gonadotrophin assessment (urine dipstick test) on women of child bearing potential
7. All medications at the time or within 3 months (90 days) prior to screening (Visit 1) should be recorded. Any new medication or changes to current medication during the trial should also be recorded at each visit or telephone review. Clinicians should particularly enquire and record the use of antibiotics, herbal remedies and over the counter preparations
8. Hepatitis B serology (HBsAg), hepatitis C antibody (HCV Ab), HIV antibody (HIV Ab). If not previously performed then Ferritin, Transferrin saturation, anti-mitochondrial antibody (AMA), anti-nuclear antibody (ANA), anti-smooth muscle antibody (ASMA), caeruloplasmin, alpha 1 antitrypsin (α1AT)

9. Full Blood Count (FBC), Urea and Electrolytes (UE), Liver Function tests (LFT), INR, C-reactive protein (CRP), immunoglobulins (Igs). At sites other than the coordinating site, serum ALT measurements will be processed locally and a further whole blood sample will be frozen and sent to the coordinating centre for analysis
10. Taken 1 hour before infusion, and 4 hours after infusion
11. Immunoglobulin G concentrations taken 1 hour before infusion, at visit 7 and 8
12. Blood panel for Enhanced Liver Fibrosis (ELF)
13. Frozen serum and peripheral blood mononuclear cells (PBMCs) will be stored and batch analysed by flow cytometry, cytokine and chemokine analysis will be performed by cytokine array, microRNA and RNA analysis by qPCR or equivalent technology and endothelial cell activation measured by Flow assays.
14. Taken 1 hour before infusion, and 4 hours after infusion
15. Additional research blood will be taken to allow isolation of PBMCs which in addition to plasma will be stored and batch analysed by our Rotterdam collaborators by cell flow cytometry, cytokine and chemokine analysis by cytokine array
16. Taken 1 hour before infusion, and 4 hours after infusion
17. Fasting bloods for serum total bile acid levels will be taken 1 hour before infusion visit 3 (SD 0) and visit 7 (SD28)
18. Sample taken at Visit 3 (pre-infusion) and Visit 7
19. SF36v2, pruritis visual analogue scale and nine point fatigue severity score
20. Adverse events / bloods and clinical events will be monitored continuously until completion of follow up

6.2.4 Outcome measure and analysis

6.2.4.1 Primary outcome measure

Stage 1

The primary outcome measure for Stage 1 is the occurrence of DLT over a 14-day period after ORBCEL-C™ infusion (SD0).

Stage 2

The primary outcome measure for Stage 2 includes 1) safety and tolerability via occurrence of DLT (SD0-14 only), serious adverse events (SAE) and AE throughout the trial period up to SD56 and 2a) **PSC patients**: a change in serum ALP at SD28 from baseline and over multiple time points before and after OBCEL-C™ infusion from SD-28 to SD56 2b) **AIH patients**: change in ALT trend over multiple time points before and after ORBCEL-C™ from SD-28 to SD56.

6.2.4.2 Secondary outcome measure

PSC patients

Secondary outcome measures include a change from baseline at 28 days, 56 days and throughout the trial period following ORBCEL™ infusion in; 1) individual markers of liver biochemistry and function including aspartate aminotransferase, GGT, bilirubin, albumin, international normalised ratio (INR) and composite risk scores including Mayo PSC risk score and Model for End Stage Liver Disease 2) non-invasive clinical markers of fibrosis including the enhanced liver fibrosis test and transient elastrography 3) HR-QOL measured by the pruritus visual analogue scale, fatigue severity scale and SF-36 V2.0) 4) IBD severity measured by the non-endoscopic aspects of the Mayo IBD score.

AIH patients

In the AIH cohort, similar secondary outcomes to PSC patients will be measured in addition we will determine a change from baseline at 28 days, 56 days and throughout the trial period following ORBCEL-C™ infusion in immunoglobin G (IgG) concentrations.

6.2.4.3 Exploratory research outcomes

Our exploratory research outcomes include a change throughout the trial period of the following measured parameters 1) markers of immune activation- immunoglobulin values and C-reactive protein concentration 2) markers of biliary injury- fasting total bile acid levels (measured at SD0 and SD28) 3) circulating inflammatory cells profiles in particular phenotypic expression of Tregs (our common primary mechanistic end point) 4) endothelial cell activation markers such as vascular adhesion protein-1 and intracellular adhesion molecule 1.

6.2.4.4 Statistical justification and outcome analysis

In the dose safety stage, we anticipate to recruit between 6-12 patients for each cohort. This sample size is based on a Bayesian safety-monitoring rule, similar to the conventional 3+3 cohort design. The underlying true toxicity rate has been assessed using Bayesian methodology with a beta-binominal conjugate.

The primary end-point for activity in the PSC cohort is based on the percentage reduction in serum ALP 28 days after an ORBCELC™ infusion. We will aim to recruit a sample size of 13 patients in the activity stage. This number is based on power calculations that demonstrate there will be at least 81% power to detect a mean percentage drop of at least 20% in serum ALP with 13 patients, using a one-sided

paired t-test with an alpha of 0.05. In the AIH cohort, the primary end-point of change in serum ALT trend will not be powered to investigate a specific change in serum ALT. A pragmatic size of 13 patients for the activity stage was chosen primarily due to feasibility of recruitment within the trial duration and as a means for assessing the trend in serum ALT trend over time.

For the entirety of the trial, we anticipate to recruit 19 (maximum 25) evaluable patients for both Stage 1 and Stage 2 combined, per cohort. Allowing for a dropout rate of approximately 10%, this results in an overall recruitment target of 38-44 patients (maximum 56 patients).

6.2.4.5 Analysis of outcome measures

All patients who receive an infusion of ORBCEL-C^{TM} at Visit 3 (SD0) will be included in the safety population. An evaluable patient is a patient who reaches at least day 14 for DLT assessment or a patient who experiences a DLT at any time post infusion but prior to day 14. Any patient that withdraws or dies due to consequence not related to treatment prior to day 14 will be replaced.

Analysis of outcome measures related to activity in both disease cohorts will be undertaken on modified intention-to-treat populations. In the PSC cohort, an evaluable patient is a patient that has both visit 3 and visit 7 serum ALP measurements. In the AIH cohort, analysis will be conducted on all patients who have primary outcome data at baseline and at least 1 clinical data set after the infusion.

In the PSC cohort, for the primary outcome measure of change in serum ALP, co-primary analyses will be performed on the defined evaluable population examining 1)

change in ALP at day 28 from baseline using a t-test or non-parametric test if appropriate and 2) changes over multiple time-points before and after the infusion using a repeated measures (longitudinal) analysis. In the AIH cohort, measurements of serum ALT will be taken at multiple time points from visit 1 to visit 8. Change in trend of serum ALT will be measured using repeated measures analysis.

The secondary outcomes in the PSC cohort will be analysed as per the primary outcome where appropriate, however these analyses will not be powered. In the AIH cohort, the secondary outcome of change in liver biochemistry and function, IgG concentration and enhanced liver fibrosis test results will be analysed using the t-test or non-parametric methodology where applicable.

We will evaluate delta changes in phenotypic expression of Tregs, which was chosen as the primary mechanistic end point. A historical dataset concerning the percentage of Tregs in PSC patients at a given time point was used to estimate the power for an analysis of percentage Tregs for both groups combined.

6.2.4.6 Adverse event (AE) reporting

Dose limiting toxicity (DLT)

DLT is defined as an AE (including abnormal biochemical tests) determined to be related to ORBCEL-C^{TM}. The DLT reporting period is from Visit 3 (SD0) to end of Visit 5 (SD14) for both Stages 1 and 2. All DLTS will be reported immediately upon the investigator/study staff becoming aware of the event. DLT rate in the trial is expected to be <15%. If there is a high chance (>80%) that the probability of a true DLT is ≥ 15% taking in to account the number of DLTs observed, it will be recommended that the trial is stopped early for toxicity in consultation with the Data Monitoring Committee (DMC).

In Stage 1, the DMC will review the available safety data separately for each of the patient cohort at each decision stage (after a cohort has been treated) and make dose recommendations for the next cohort of patients.

AE reporting and analysis

The AE reporting period is between visit 3 (SD0) and Visit 8 (SD56). SAE's will be reported throughout the trial period up to Visit 8 (SD56) and thereafter for 22 months during the long-term follow-up of trial patients. All AE's and SAE's will be evaluated by the investigators and recorded. Each AE will be graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events V4.0. The central trial office (Birmingham) will keep detailed records of all reported AE's and perform an evaluation with respect to seriousness, causality and expectedness. As with Stage 1, the DMC will review safety data in Stage 2 after 6 (if not already assessed) and 9 patients. The DMC will advice accordingly with regards to trial conduct and if extra data monitoring is required for the remainder of the trial. The DMC operates in accordance with a trial specific charter based on a template created by the Damocles Group.

6.2.5 Conclusion

PSC and AIH are progressive inflammatory liver diseases characterised by immune-mediated tissue injury leading to cholestasis in PSC and in both fibrosis eventually resulting in end-stage liver disease requiring liver transplantation. Defects in immune-regulatory pathways, contribute to the relentless chronic inflammation and liver injury. There are huge unmet needs with regards to therapy in both PSC and AIH. In PSC there is lack of treatment that can combat the inflammatory process involved and in AIH, current therapies are untargeted/blunt, plagued by side- effects and have remained unchanged for many decades. MSC are shown to be safe in a numerous

clinical trials including those involving patients with liver disease. They possess broad immunomodulatory properties and can potentially target many immune cells e.g. T cells, B-cells, monocytes, Tregs as well as pathways implicated in the disease pathogenesis of PSC and AIH.

There are some limitations in the clinical trial design. The unpredictability and fluctuating nature of PSC and AIH pose particular challenges in trial design. In PSC, there are no established surrogate endpoints for regulatory approval. Serum ALP can fluctuate during the natural course of the disease, which may limit its usefulness as a primary end point (Trivedi et al, EASL 2019). However several studies have shown serum ALP is a surrogate marker of transplant-free survival, it is therefore accepted as a surrogate endpoint by the international PSC study group. In AIH, placebo-controlled trials would be unethical due to the high efficacy of standard-of-care therapy in population terms making direct comparisons between novel agents and standard-of-care therapy difficult. Histological remission lags behind biochemical remission in AIH. Histological assessment is regarded as the direct way to gauge disease severity in many liver diseases however frequent liver biopsies are impractical in a trial setting.

Our unique trial design is aimed at assessing safety of ORBCEL-C^{TM} and early biochemical activity signals with a view of justifying larger scale trials. We are assessing a number of non-invasive surrogate measures of outcomes and have incorporated a long-term follow-up period (total 24 months) to better understand the effect of MSC therapy in PSC or AIH.

CHAPTER 7.

DISCUSSION

7.1 Overview

MSC possess broad immunomodulatory properties that could potentially make them therapeutic candidates to treat PSC. The safety of intravenous MSC infusion in autoimmune diseases has already been confirmed in several phase I/II clinical trials, with some promise of efficacy (153, 161, 221-223). A phase III trial demonstrated that Alfosil (expanded allogeneic adipose-derived mesenchymal stem cells) was safe and efficacious in treating complex peri-anal fistulas in patients with Crohn's disease one-year post treatment (166) leading to Alfosil becoming the first allogeneic stem cell therapy to receive central marketing authorization approval in Europe. As highlighted in chapter 1, IBD is strongly associated with PSC with some suggesting that both diseases may share common pathogenic mechanisms. With this in mind, we are currently running a clinical trial (MERLIN) at the University of Birmingham investigating the safety and efficacy of a single intravenous infusion of UC-MSC in patients with PSC and AIH. Many in vitro and in vivo studies have shown that MSC are able to inhibit T- cell responses however majority of the in vitro studies have used peripheral blood mainly from healthy subjects. In patients with PSC, it is well described that T cells differ between peripheral blood and liver, it is thus of importance to study tissue derived T cells when assessing the immune-modulatory function of MSC. In PSC, there are no ideal animal models that truly depict all the disease characteristics. The aim of this study was therefore to investigate *in vitro* whether UC-MSC can affect the proliferation and activation of T cells from patients with PSC. Our study is unique in that we investigated not only circulating T cells taken from patients at an "early" stage but most importantly we studied intrahepatic T cells that are present at the site of actual inflammation. Our findings from these pre-clinical studies would lend support to MERLIN clinical trial.

7.1.1 Direct effect of UC-MSC on circulating and intrahepatic T cells

In this study we investigated the direct effect of UC-MSC on proliferation and activation of circulating and intrahepatic T cells from patients with PSC. Our data showed findings similar to Di Nicola *et al* (122) in that UC-MSC were able to suppress the proliferation of circulating $CD4^+$ and $CD8^+$ T cells from patients with PSC in a dose dependent manner. UC-MSC equally inhibited the percentage proliferation of both $CD4^+$ and $CD8^+$ T cells (% proliferation of $CD4^+$: 28% vs $CD8^+$: 38% at UC-MSC: PBMC ratio of 1:1) (see chapter 3.3.2). We further demonstrated that UC-MSC reduced circulating $CD4^+$ T cell activation as evidenced by the diminished proportion of $CD4^+$ T cells expressing TNFα and IFNγ when UC-MSC were present. A trend towards reduction of $CD8^+$ TNFα and $CD8^+$ IFNγ expressing cells was also seen although this effect was not statistically significant (see chapter 3.3.3). Our results are in contrast to a recent in vitro study investigating the effect of bone marrow-derived MSC (BM-MSC) on peripheral blood T cells from patients with rheumatoid arthritis, an autoimmune disease. The authors demonstrated that BM-MSC equally inhibited production of IL-2, IFNγ and TNFα by $CD4^+$ and $CD8^+$ T cells and that this inhibitory effect was transversal for all T cell functional compartments among both rheumatoid arthritis patients and healthy controls (224). The same authors however in an earlier study using peripheral blood from healthy subjects showed that the inhibitory effect of BM-MSC on production of IL2, IFNγ and TNFα differentially affected $CD4^+$ and $CD8^+$ T cells as well as T cell functional compartments in particular the authors noted similar to our findings that the effect of MSC over the frequency of TNFα and IL2 cells was more pronounced in $CD4^+$ T cells compared to $CD8^+$ T cells and in naïve and central memory compartments for both $CD4^+$ and $CD8^+$ T cells (225).

UC-MSC were equally effective at suppressing intrahepatic PSC $CD4^+$ and $CD8^+$ T cell

proliferation in a dose dependent fashion. Similarly to peripheral blood, there was reduction in intrahepatic $CD4^+$ T cell activation in the presence of UC-MSC as shown by percentage reduction in the percentage of $CD4^+$ TNFα, $CD4^+$ IFNγ and $CD4^+$IL2 expressing cells. No reduction of PSC intrahepatic CD8+ T cell activation was observed on addition of UC-MSC. These findings are in concordance with other in vitro studies involving MSC and tissue from patients with immune mediated diseases such as Crohns disease (226), rheumatoid arthritis (224) and immune thrombocytopenia (227), which have also confirmed the effectiveness of MSC in inhibiting T cell proliferation and cytokine production.

Notably, we observed that the UC-MSC effect on T cell proliferation is not disease specific as there were no significant differences between peripheral blood and livers of patients with PSC versus patients with hereditary haemochromatosis and PBC respectively. Bartholomew *et al* showed that inhibition of T cell responses by MSC is not antigen-specific (124) and that MSCs target both primary and secondary T-cell responses (189) . This property of MSCs makes them an attractive therapy to treat a broad range of immune mediated diseases.

Although there is always concern that in vitro data will not translate to efficacy in human disease, our preliminary data provides optimism regarding a potential immunosuppressive role of MSC in PSC and justification for undertaking the MERLIN clinical trial.

7.1.2 Indirect effect of UC-MSC on circulating and intrahepatic T cells

The precise mechanism by which MSC regulate immune responses remains unclear however both cell-cell contact and soluble factors released by MSC are involved. We were further interested to study the indirect effect of UC-MSC on circulating and intrahepatic T cell proliferation and activation from patients with PSC; precisely to evaluate the effect of UC-MSC conditioned media (UC-MSC-CM) (secretome) at various concentrations (50-100%) on T cell function. Presence of UC-MSC-CM resulted in a dose response inhibition of circulating $CD4^+$ and $CD8^+$ T cell proliferation from patients with PSC (see chapter 4.3.2). These findings are concordant with published literature and confirm that MSC cell-to-cell contact is not essential to achieve inhibition of T cell proliferation and soluble factors are involved (122, 200). We found UC-MSC-CM reduced the proportion of circulating $CD4^+$ T cells that expressed IFNγ however no effect on percentage reduction of $CD4^+$TNFα, $CD4^+$IL2 expressing cells and $CD8^+$ T cell activation was seen. We detected a dose related inhibition of PSC intrahepatic $CD8^+$ T cell proliferation in the presence of UC-MSC-CM. No significant reduction of intrahepatic $CD4^+$ T cell proliferation and $CD4^+$/$CD8^+$ T cell activation was observed in the presence of UC-MSC-CM. This is surprising given the effect of CM on peripheral blood T cell proliferation and may imply that MSC effect on intrahepatic T cell responses may require cell-cell contact. Although comparative experiments between cell to cell contact and indirect effect of UC-MSC were not performed due to feasibility of acquiring the required sample numbers for all the conditions, our results suggest that the effect of UC-MSC-CM is variable and not as strong as when UC-MSC are in direct contact with immune cells. There are conflicting reports in the literature regarding the efficacy of soluble factors secreted by MSC. Tse *et al* have also found that the suppressive effect on T cells is variable and less profound when MSC-conditioned media is used than that observed in transwell experiments where the MSC

are separated from T cells by a chamber, but still in close proximity. In both cases suppression of the responder PBMC was enhanced when irradiated third-party PBMC were added to the MSC culture, indicating that physical interaction of PBMC and MSC increased production of the suppressive activity (105). We used conditioned media as opposed to the transwell system to study the indirect effect of MSC on T cells because we wanted to mimic physiology. It is described in animal studies that MSC do not remain viable for more than 72 hours upon administration (130) but yet remain efficacious in clinical studies indicating that soluble factors released by MSC are involved. Majority of published in vitro studies demonstrating successful suppression of T cell proliferation using soluble factors secreted by MSC have used either a transwell system (121, 122, 124) or a similar system to inhibit cell contact but maintaining MSC presence (200, 228) indicating that 'cross talk' between PBMC and MSC is relevant for MSC to exert inhibitory action. Alternatively, increased concentrations of conditioned media may be required compared to MSC numbers in co-culture to exert an inhibitory effect on T cell responses (228). MSC: T cell ratios are pertinent for the immunomodulatory functions of MSC as shown by Najar *et al* who found that MSC cultured alone at low concentrations or the corresponding CM improved lymphocyte proliferation rather than induced T-cell inhibition (205). Another possible explanation for the differences in UC-MSC-CM efficacy between peripheral blood and livers of patients with PSC could be the use of PBMC and enriched T cells respectively. Although the mechanism of immunosuppression by MSC remains unclear, MSC are known to influence other immune cells including $CD8^+$ T cells, monocytes, B cells, natural killer cells and dendritic cells, it is thus plausible that aspects of MSC mechanism of action may be mediated through interaction with $CD4^-$ T cells which in turn may act upon $CD4^+$ T cells (228).

In this chapter we also compared the effect of conditioned media derived from untreated UC-MSC versus IFNγ stimulated UC-MSC on circulating and intrahepatic T cell proliferation/activation. IFNγ enhances the immunosuppressive potential of MSC and induces changes in MSC immunophenotype. It is known to upregulate HLA-class 1, ICAM-1, VCAM 1 on the surface of MSC, induce IDO activity (128, 148) and secrete important immunomodulatory molecules, such as PGE2, HGF, TGF-β, and CCL2. Priming/pre-conditioning of MSC with IFNγ inhibits T cell effector function (229), suppresses T cell proliferation (108), increases suppression of NK cells and reduces NK mediated cytotoxicity (230). Our study demonstrated no significant differences in circulating and intrahepatic T cell proliferation and activation between the conditioned media derived from untreated UC-MSC and IFNγ stimulated UC-MSC (see sections 4.3.6 and 4.3.7). Ren *et al* also found that stimulation of MSC with IFNγ alone was insufficient to induce immunosuppression and only when TNFα, IL1α or IL1β was added along with IFNγ to the mixed co-cultures of MSC and T cells did immunosuppression by MSC occur (128). Similarly Liang *et al* showed that addition of exogenous IFNγ to MSC co-cultured with stimulated PBMC did not affect the immunosuppressive capacity of MSC (231). The authors further demonstrated that addition of neutralizing antibody against IFN-γ partially restrained the rate of T cell proliferation (231) again highlighting that although IFNγ is required for immunosuppressive effect of MSC, synergism with other inflammatory cytokines induces the ability of MSC to inhibit T cell proliferation.

As highlighted earlier, IFNγ induces IDO production. IDO is one of the mechanisms involved in MSC induced immunosuppression and its production is proposed to be variable depending on the MSC donor, it is thus possible that this was the case with our MSC donor. For this part of the experiments, we were only able to use one UC-MSC donor due to lack of availability of other UC-MSC donors from the manufacturing

company. It is plausible that our UC-MSC donor was unable to produce high concentrations of IDO upon IFNγ stimulation, to enable enhancement of MSC effect. It is also possible that IDO may not be the main mechanism involved in the inhibitory effect of UC-MSC on T cell responses observed in our experiments involving PSC tissue. Other factors released by MSC independent of IFNγ stimulation such as human leukocyte antigen G5, HGF, TGF-β and iNOS may have a bigger part to play in immunosuppression either individually or in combination. Lastly my methodology of using conditioned media as opposed to the transwell system may explain the lack of enhanced inhibition of circulating T cell proliferation/activation on addition of IFNγ stimulated UC-MSC-CM. MSC production of suppressive molecules may be dependent on the cross talk between MSC and activated T cells (123).

7.1.3 Role of IDO as a possible mechanism of MSC action

The exact mechanism of MSC action remains unknown. Some authors report that IDO is the main mechanism involved in the immunosuppressive effect of MSC whilst others contraindicate this. The potential role of IDO in the pathogenesis of PBC was highlighted in an early study by Oertelt-prigione *et al* who proposed impairment of IDO induction in circulating monocytes of patients with PBC and an atypical pattern of IDO expression in bile ducts of PBC patients with IDO expression localized at the luminal surface of cholangiocytes and within aggregates of damaged cholangiocytes in early stage PBC (232).

We investigated the role of IDO using the addition of an IDO inhibitor, 1-MT to UC-MSC-CM and assessing its effect on circulating T cell proliferation and activation from patients with PSC. A trend towards partial restoration of $CD4^+$ and $CD8^+$ T cell proliferation was observed at 100% concentration however no effect on $CD4^+$ T cell

activation was seen when IFNγ UC-MSC-CM in combination with 1-MT was used. Interestingly a trend towards increase in the percentage of $CD8^{+}TNF\alpha$ expressing cells was observed at 100% concentration in the presence of IFNγ UC-MSC-CM and 1-MT. Given the mixed results, functional IDO enzyme activity was studied in conditioned media containing 1-MT through measurement of kynurenine and tryptophan concentrations using mass spectrometry. We found that IFN γ stimulation was appropriately associated with enhanced IDO activity however the 1-MT used in our experiments was not efficacious at inhibiting IDO enzyme activity (tryptophan concentration remained low and kynurenine concentration high at similar levels to IFN γ UC-MSC-CM without 1-MT). Our finding of enhanced IDO activity in conditioned media derived from IFN γ stimulated UC-MSC further suggests that it is likely that IDO is not the main mechanism involved in the demonstrated immunosuppression effect on addition of UC-MSC because we saw no differences in effect between IFNγ UC-MSC-CM and untreated UC-MSC-CM. Other soluble factors such as those discussed earlier in section 7.1.2 may be involved.

Possible explanations for the lack of efficacy of 1-MT in our experiments could be the use of 1-MT as an IDO inhibitor. 1-MT has been extensively studied in tumour immunology and is reported to be a less potent inhibitor of IDO enzyme activity both in vivo and in vitro (211, 212). We used 1-MT in our experiments because it is widely used by researchers to investigate the role of IDO in MSC induced immunosuppression. It is a synthetic tryptophan analogue and acts as a competitive inhibitor of IDO existing in two enantiomeric forms; L- methyl-tryptophan (L-1-MT) and D-methyl-tryptophan (D-1-MT). In order to function as a potent inhibitor, 1-MT needs to reach similar or higher levels than tryptophan in the target tissue or to have a higher affinity to IDO than the natural substrate, tryptophan (211). Our results showed that the tryptophan concentration in untreated UC-MSC-CM plus 1-MT was similar to that of untreated UC-

MSC-CM containing no 1-MT (control) perhaps indicating that the 1-MT we used was not potent. In addition, we used the D-1-MT isomer in our experiments; there remains a debate as to which of the isomers are more effective at suppressing T cell proliferation. Qian *et al* used tissue from ovarian cancer patients and compared the efficacy of L-1-MT, D-1-MT and D/L 1-MT (233). The authors demonstrated that L-1-MT and D/L 1-MT but not D-1-MT restored $CD4^+$ and $CD8^+$ T cell proliferation suppressed by IDO in stimulated PBMC, tumour associated lymphocytes and tumour infiltrating lymphocytes (233). Furthermore, both L-1-MT and D/L 1-MT were more efficient in inhibiting kynurenine production and restoring tryptophan by IDO-expressing cells as compared to D-1-MT. In the study, D-1-MT achieved less inhibition of kynurenine production and did not restore tryptophan even at high concentrations (233). Future experiments should be performed using either D/L 1-MT or L-1-MT following comparison of efficacy.

7.2 Limitations and future work

Several of the limitations and potential future work have been highlighted in chapter 3-5 and sections 7.1.1-7.1.3. This section discusses the main limitations and future work.

7.2.1 Limitations

- Sample numbers: Both PSC and PBC are rare diseases and thus availability of explanted liver tissue was infrequent. This is particularly the case with PBC as there is effective treatment available. There were also logistical issues for the co-culture experiments involving liver tissue and UC-MSC in that it was necessary to have UC-MSC cultured all the time in the eventuality that an explant liver may be available. Variability of MSC growth rates meant that sometimes at the time of availability of an explanted liver, there may not have been enough UC-MSC to carry out experiments. Unfortunately, time and funding constraints limited further experiments. The number of intrahepatic T cells required for various conditions was another limitation particularly in the case of $CD8^+$ T cells. Reduced numbers of $CD8^+$ T cells were often acquired from explanted liver tissue pre culture due to potential operator technique and post culture possibly due to the absence of growth factors during the culture period. For our co-culture experiments, we acquired $CD8^+$ T cells from 8 PSC livers but were only able to obtain data for all the conditions from 2 livers. Our rationale for not adding growth factors was that we wanted to replicate our peripheral blood experiments.

- Peripheral blood and liver experiments: Direct comparison of experiments involving peripheral blood and explant tissue was not possible. This is due to

the variability in the timing of explant liver tissue, it was not possible to coordinate this with peripheral blood as peripheral blood was acquired from patients scheduled to attend the PSC clinic. It is noteworthy that tissue from patients at various spectrum of liver disease was acquired. For example explanted liver tissue was likely acquired from patients with end-stage liver disease whereas the peripheral blood samples were acquired mostly from patients who were clinically stable and not cirrhotic. For the peripheral blood experiments, HFE blood was used in some cases, we want to highlight that we did not know whether patients with HFE had underlying chronic liver disease. Healthy controls were not used in this study, as we did not have ethics to carry out experiments involving healthy individuals.

- Translation of in vitro experiments: All in vitro studies are subject to the legitimate criticism that they cannot be considered to truly reflect events in the human condition. Nevertheless, having used explanted liver tissue from patients with PSC patients, our study provides optimism that UC-MSC may be efficacious in inhibiting T cell responses in PSC. It further lends support to the MERLIN clinical trial, which will hopefully provide an insight in to safety and efficacy of UC-MSC in the clinical setting.

- A number of factors can influence the functionality of MSC including donors, culture conditions, cryopreservation and passage numbers. Variation in study methodology involving MSC makes it difficult to elucidate the precise mechanism involved in suppression of T cells. For example some use mixed lymphocyte culture to study effect of MSC on proliferation whilst others use PBMC or enriched T cells. Similarly when studying indirect effect of MSC some

have used conditioned media whilst others the transwell system.

7.2.2 Future work

In this study, we have demonstrated that UC-MSC are effective at suppressing circulating and intrahepatic T cell proliferation and activation from patients with PSC. However it should be noted that there are several outstanding questions that need to be addressed in particular understanding the mechanism of action of MSC in immunomodulation. Future work should thus be directed at:

1. **Investigating the role of UC-MSC in generation of T regulatory cells (Tregs)**

Tregs are regulators of immune tolerance. Defects in immunoregulatory pathways/mechanisms are implicated in disease pathogenesis of PSC. Circulating $CD4^{+}CD25^{high}FOXP3^{+}CD127^{low}$ lymphocytes are low in frequency and less able to suppress T cell proliferation in patients with PSC compared with controls (41). Reduced numbers of intrahepatic $CD3^{+}FOXP3^{+}$ lymphocytes are also detected in liver biopsies of patients with PSC (41). MSC can exert their immunosuppressive action via inhibition of pro-inflammatory T cells and induction of T cells with a regulatory phenotype. Further work to investigate the effect of UC-MSC on polarization of $CD4^{+}$ T cells towards Tregs and functionality of Tregs in PSC patients would contribute towards understanding mechanisms of MSC action and provide further optimism as to the role of MSC as potential therapy in PSC.

2. **Characterization of soluble factors released from MSC and investigation of the main soluble factors involved in the immunosuppressive action of UC-MSC**

Significant efforts are being made to understand mechanism of MSC action including pathways involved in cell-cell interaction and involvement of paracrine factors. Identification of key mechanisms and factors is important as manipulation could enhance MSC action. Future work using advanced techniques/technology is required to determine the exact nature of soluble factors released from UC-MSC followed by identification of the key soluble factors involved in MSC induced immunosuppression. Future experiments investigating the indirect effect of UC-MSC should compare the use of conditioned media versus the transwell system. Experiments comparing the direct effect of MSC vs conditioned media on circulating and intrahepatic T cell responses are also warranted.

3. Investigating the effect of UC-MSC on PSC CD28- T cells

There is a high frequency of CD28-T cells that release pro-inflammatory cytokines in livers of PSC patients (37). Loss of CD28 occurs following continuous antigenic stimulation and TNFα exposure. Vitamin D has been shown to markedly suppress the development of CD28-T cells in vitro even in the presence of TNFα (37). Given the proposed role of CD28-T cells in immune-pathogenesis of PSC, future experiments involving MSC in PSC should investigate the effect of MSC on CD28- T cells. Preliminary results from one experiment demonstrated a trend towards reduction in the percentage frequency of CD28- T cells when cells in the presence of TNFα were treated with 90% UC-MSC-CM for 7 days compared to T cells in the presence of TNFα only (Figure 7.1 A). However conversely and un-expectantly we observed a trend towards increase in the percentage frequency of CD28- T cells when cells in the presence of TNFα were treated with 90% UC-MSC-CM for 14 days compared to T cells in the presence of TNFα only (Figure 7.1 B). Further experiments following optimisation are thus required to make robust conclusions regarding the effect of MSC on CD28-T cells from patients with PSC.

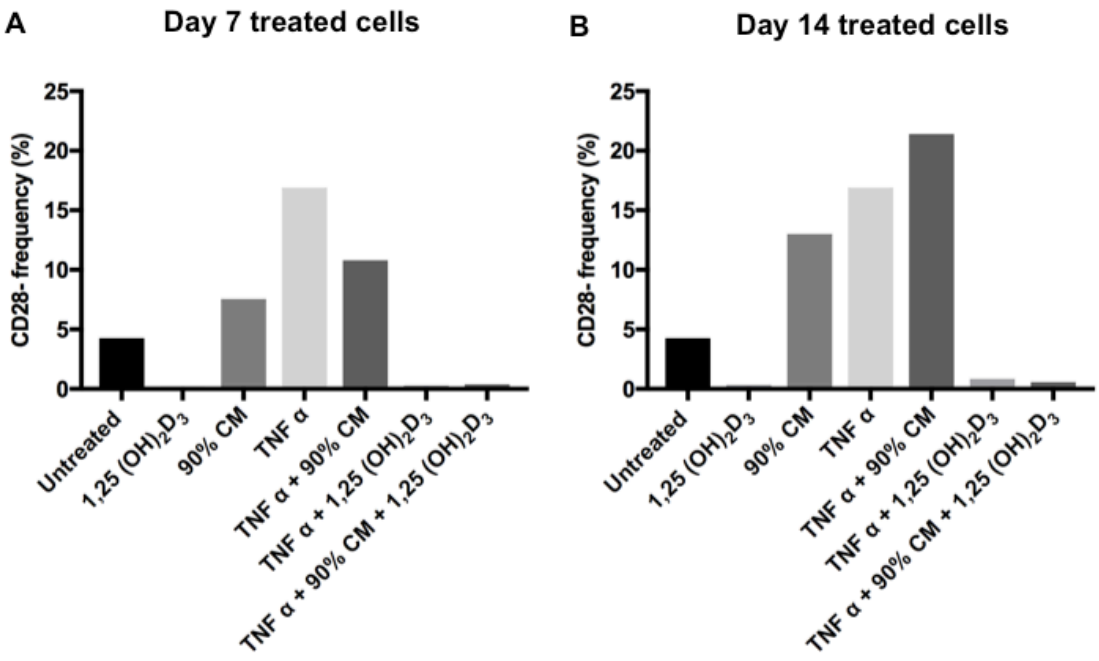

Figure 7-1: TNFα enhances emergence of CD28-T cells and treatment with 90% umbilical cord derived mesenchymal stromal cell conditioned media for 14 days results in a trend towards increase in percentage CD28- T cell frequency.

$CD4^+$ T cells from peripheral blood of patients with hereditary haemochromatosis were stimulated with anti-CD3/anti-CD28 (1µl/well) beads and cultured for 21 days in the presence or absence of TNFα (10ng/ml) with or without $1,25(OH)_2D_3$ (10nM) and with or without 90% UC-MSC conditioned media . $1,25(OH)_2D_3$ and 90% conditioned media were added at days 7 and 14 of the experiment. The frequency of CD28- cells was measured at 14 (day 7 treated cells), and 21 (day14 treated cells) days by flow cytometry. **A** and **B** shown as % frequency of CD28-T cells following 7 (A) and 14 (B) days of treatment in n=1 donor. **Abbreviations;** CM-conditioned media, D- day, UC-MSC- umbilical cord derived mesenchymal stromal cells, $1,25(OH)_2D_3$ – vitamin D.

4 MSC as a therapeutic tool in inflammatory disease

MSC have been used successfully in clinical trials to treat immune mediated/inflammatory diseases including graft versus host disease, multiple sclerosis, IBD, osteo- and rheumatoid arthritis. Many phase I/II clinical trials have demonstrated safety and tolerability of MSC as therapy. However data regarding efficacy remains ambiguous. There is an unmet need for larger phase II/III trials with long term follow up data to enable robust conclusions regarding efficacy of MSC. There are a number of explanations for the ambiguous results including lack of standardisation during the MSC extraction, expansion, culture conditions, administration procedures/routes and inter-individual MSC donor differences. To maximize clinical benefit, a number of issues need to be addressed before MSC can be widely accepted as cell therapy to treat patients. These include MSC source, number of cells, optimal passage time in culture before use, cell sub-population and standardization process of cell production. The success of clinical trials and the outlook of MSC therapy as an entirety will be defined from effective and informative clinical trial designs with adherence to hard efficacy end points. Key issues need to be addressed prior to the design of such trials including: adjunct immunosuppressive therapy (in the case of AIH), appropriate MSC dose, route (systemic versus localized) and frequency (single versus multiple doses) of administration, trafficking properties of infused MSC and a thorough immune monitoring plan in order to gain mechanistic insight on the function of MSC in patients. With MERLIN, we hope to contribute and add to existing data on MSC cellular therapy. Although our trial is primarily aimed at assessing safety of an infusion of MSC in PSC and AIH, we will also speculate on the relative therapeutic efficacy with aims of progressing to larger phase III studies in the near future.

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