THE ROLE OF CLEVER-1 IN LYMPHOCYTE RECRUITMENT TO THE HUMAN LIVER

by

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Abstract

Lymphocyte infiltration of the liver drives the progression of all inflammatory liver diseases. Lymphocyte homing to the liver occurs within the hepatic sinusoids. The hepatic sinusoidal endothelial cells (HSEC) that line these channels have a unique phenotype and lack conventional adhesion molecules, making these cells a potential organ specific target for therapy. I carried out a detailed analysis of the expression of CLEVER-1 in the human liver and show that it is expressed on sinusoidal endothelium *in situ* and *in vitro* and at other sites of lymphocyte recruitment in the inflamed liver including neovessels, portal associated lymphoid tissue and vessels supplying hepatocellular carcinomas. The expression of CLEVER-1 on HSEC *in vitro* was upregulated by hepatocyte growth factor. Flow based adhesion assays with HSEC demonstrated that CLEVER-1 mediated lymphocyte transmigration and that this activity was subset specific with preferential activity for B cells and T regulatory (Treg) cells. Using confocal imaging I was able to show that CLEVER-1 mediates Treg migration via the transcellular route through HSEC. This is the first report of transcellular migration in the liver.

This study identifies CLEVER-1 as a novel lymphocyte recruitment signal to the liver and a potential target for therapy in chronic liver diseases.

This thesis is dedicated to my family, especially my wife, Malu and son, Zubin

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Abbreviations

AGEs Advanced Glycation End products

AIH Autoimmune Hepatitis

ALD Alcoholic Liver Disease

AP-2 Adaptor Protein 2

APC Allophycocyanin

APES Aminopropyltriethoxysilane

ATPase Adenosine Triphosphatase

BSA Bovine Serum Albumin

CDNA complementary DNA

CHO cells Chinese Hamster Overy cells

CLEVER-1 Common Vascular Ednothelial and Lymphatic Endothelial Receptor-1

CMFDA 5-chloromethylfluorescein diacetate

COLLA-R Collagen alpha chain Receptor

CRD Carbohydrate Recognition Domain

DAPI 4',6-diamidino-2-phenylindole

DC-SIGN Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-Integrin

DNA Deoxyribose Nucleic Acid

DNAse deoxyribonuclease

dNTP Deoxyribonucleotide triphosphate

EDTA ethylenediaminetetraacetic acid

ELISA Enzyme-Linked Immunosorbent Assay

ESAM Endothelial cell Selective Adhesion Molecule

FACS Fluorescence Activated Cell Sorting

FAM 6-carboxyfluorescein

FCS Fetal Calf Serum

FcγR Fc gamma Receptor

FEEL-1 Fasciculin, Epidermal Growth Factor-like, Laminin-type-Epidermal Growth Factor-like and Link domain containing Scavenger receptor

FITC Fluorescein Isothiocyanate

FOXP3 Forkhead box P3

GAGs Glycosoaminoglycans

GAPDH Glyceraldehyde 3-Phosphate Dehydrogenase

GGA Golgi localized, Gamma ear-containing Adenosine 5'-diphosphate-ribosylation factor-binding adaptor

GPCRs- G-Protein Coupled Receptors

GTP Guanosine Triphosphatase

HA Hyaluronan

HCC Hepatocellular carcinoma

HCV Hepatitis B

HCV Hepatitis C Virus

HDMEC Human Dermal Microvascular Endothelial Cell

HEVs High Endothelial Venules

HGF Hepatocyte Growth Factor

HLMVEC Human Lung Microvascular Endothelial Cell

HLyEC Human Lymphatic Endothelial Cell

HSEC Human Sinusoidal Endothelial Cell

HUVEC Human Umbilical Vein Endothelial Cell

ICAM-1 Intracellular Cell Adheision Molecule

IFNγ Interferon gamma

IL Interleukin

IMC Isotype Matched Control

JAM Junctional Adhesion Molecule

LAMP-1 Lysosomal-Associated Membrane Protein 1

LDL Low Density Lipoprotein

LLR Lectin Like Receptor

LOX-1 Lectin-likoe oxidised low-density lipoprotein receptor-1

LPS Lipopolysaccharide

LSECtin Liver and Lymph node Sinusoidal Endothelial cell C-type Lectin

L-SIGN Liver-Specific ICAM3-Grabbing Nonintegrin

LYVE-1 Lymphatic Vessel Endothelial Hyaluronan Receptor-1

mABs Monoclonal Antibodies

MAdCAM-1 Mucosal and Vascular Addressin Cell Adhesion Molecule-1

MHC Major Histocompatibility Complex

MR Mannose Receptor

mRNA Messenger RNA

NAFLD Non-Alcoholic Fatty Liver Disease

NASH Non-Alcoholic Steatohepatitis

NK cell Natural Killer Cell

NKT cell Natural Killer T cell

OPD o-Phenylenediamine dihydrochloride

PALT Portal tract Associated Lymphoid Tissue

PBC Primary Biliary Cirrhosis

PBS Phosphate Buffered Saline

PE Phycoerythrin

PECAM-1 Platelet/Endothelial Cell Adhesion Molecule-1

PE-Cy5 Phycoerythrin- CyChrome 5

PLC Phospholipase C

PS Phosphotidylserine

PSC Primary Sclerosing Cholangitis

PVR Poliovirus Receptor

qPCR quantitative PCR

RHO Ras Homologue

RNA Ribonucleic acid

RORγ Retinoid Related Orphan Receptor gamma

RTC Rat Tail Collagen

RT-PCR Reverse Transcription- Polymerisation Chain Reaction

SDS-PAGE Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SEM Standard Error of Mean

STAT Signal Tranducer and Activator of Transcription

T -bet T box expressed in T cells

T regs T regulatory cells

TAE Tris Acetate EDTA

TBS Tris Buffered Saline

TGFβ Transforming Growth Factor beta

TGN Trans Golgi Network

TLR Toll Like Receptor

TNFα Tumour Necrosis Factor alpha

v/v volume (solute) per volume (solvent)

VAP-1 Vascular Adhesion Protein-1

VCAM-1 Vascular Adhesion Molecule-1

VEGF Vascular Epidermal Growth Factor

w/v weight (solute) per volume (solvent)

CHAPTER 1

INTRODUCTION

1.1 Background

This thesis concerns the molecular mechanisms of lymphocyte recruitment to the human liver with emphasis on the role of the scavenger molecule CLEVER-1 and its potential as a therapeutic target. I begin with emphasising the need for new medical therapies for liver disease. This is followed by a description of the liver microvasculature and the changes that occur during liver injury that result in the establishment of a chronic hepatitis, the common pathway that links injury to scarring. Hepatitis is characterised by lymphocyte recruitment into the liver from blood, liver injury and if unresolved eventually collagen deposition (fibrosis) and culminating in nodule formation (cirrhosis). I then review our current understanding of lymphocyte recruitment to the liver and conclude with a review of the scavenger molecule CLEVER-1 and its specific role in lymphocyte recruitment.

1.2 The burden of liver disease

Liver disease is a huge and increasing global problem reflected in the United Kingdom where rates of chronic liver disease have been increasing, especially since the 1990s, to levels that are now amongst the highest in Europe (Leon and McCambridge, 2006). Unfortunately, despite this global burden, therapies for chronic liver disease remain limited and the main treatment strategies presently involve either removing the underlying trigger for the liver disease (Henderson and Iredale, 2007) or liver transplantation for end stage disease. Liver transplantation can be highly effective but there are several drawbacks. The major issue is the limited donor organ pool which

means a significant proportion of patients do not survive until an organ becomes available (El-Serag, 2001; Ubel and Caplan, 1998). Liver transplantation itself is a major operation and therefore co-existing medical and psychiatric issues can prevent patients from being considered for this procedure. Liver transplantation also carries with it the requirement for lifelong immunosuppression and problems consequent upon that as well as the problems of graft rejection and disease recurrence which can take an accelerated form in certain situations (Prieto et al., 1999). The current situation therefore requires further understanding of the mechanisms that underlie the progression of chronic liver disease so as to identify potential targets for treatment.

Chronic liver disease can develop in response to several different injurious factors that include chronic alcohol abuse, chronic infection with hepatitis B and C viruses, non alcoholic fatty liver disease and immune-mediated liver conditions (eg primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC)). Despite this variety of initiating injuries the progression of nearly all chronic liver diseases follows a common pathway that leads to fibrosis and scarring, termed cirrhosis (Iredale, 2007). Cirrhosis leads to further complications of liver failure, including portal hypertension and the development of hepatocellular carcinoma (Gines et al., 2004).

1.3 Liver function and architecture

The liver is the second largest organ of the body where it plays a critical metabolic and synthetic role. Its anatomical location allows it to directly

receive nutrients and compounds from the intestinal lumen. This close relationship to the intestinal tract allows the liver to process and redistribute fatty acids and glucose as well as modify and detoxify a range of compounds prior to release into the circulation. The liver can either store or release nutrients in response to hormonal regulation from endocrine organs, as well as neuronal stimuli. Glucose is an essential nutrient for the brain, erythrocytes and muscle and the supply of glucose is maintained by the ability of the liver to store glycogen and synthesise glucose (Pilkis and Granner, 1992). The liver also plays a role in fatty acid metabolism by regulating synthesis and transport to other organs in association with lipoproteins (Dixon and Ginsberg, 1992).

The liver is essential for the synthesis of a wide range of proteins. These include the acute-phase reactants which are present in acute and chronic inflammation and are thought to play a role in host defence, as well as blood coagulation factors (factor II, VII, IX and X). Another protein synthesised by the liver is albumin, which is the body's main serum binding protein as well as providing serum oncotic pressure.

Biotransformation of endogenous and exogenous compounds also occurs in the liver and is controlled by numerous transporter proteins which allow movement of these compounds into and out of the liver (Muller and Jansen, 1997). The excretion of these compounds can occur directly into the bile produced by the liver and thus to the intestine or into the blood for elimination by the kidney and lungs. The efficient manner by which this diverse range of

functions is performed is partly explained by the liver's vasculature and microarchitecture.

1.3.1 The hepatic vasculature

The liver has a unique dual blood supply which contributes to the liver's metabolic functions and impacts on hepatic immune regulation. Arterial blood from the hepatic artery comprises only 25% of the total blood supply, the rest coming from the portal vein which drains the gut and splanchnic organs. The hepatic artery and the portal vein both drain into the hepatic sinusoids and blood then flows from the portal area into the hepatic venules at the centre of the hepatic lobule (Figure 1-1). These venules connect to form the hepatic veins that drain into the inferior vena cava. The endothelial cells that line the different vessels comprising the hepatic vasculature are not uniform (Aird, 2007b). The portal venules and hepatic arterioles are lined by endothelium which is similar to vascular endothelium but there is still heterogeneity within these vessels. The portal venules are lined by spindle shaped endothelium which is non-fenestrated but covered by short microvilli. At the transition from the portal venule to the hepatic sinusoid, the endothelium is smooth and large and contains many actin fibres. This allows the endothelium to act as part of a sphincter which controls blood flow into the sinusoids. The hepatic sinusoids form a unique capillary bed lined by discontinuous endothelium with fenestrae; open pores 100-200nm in diameter which make up 6-8% of the endothelial surface, and which lack a classical basement membrane (Braet and Wisse, 2002). This unique

morphology has probably evolved in part as a consequence of the low flow sinusoidal environment, estimated to be 400-450mm/s compared with 500-1000mm/s in other capillary beds. This allows the sinusoidal endothelium to function as a sieve and the fenestrae to act as dynamic filters, for fluids, solutes and particles (Braet & Wisse, 2002).

The normal liver microarchitecture can be divided into subunits which are termed lobules. Each lobule consists of a terminal portal vein branch draining into terminal hepatic veins at the centre of the lobule with the gap between these vessels bridged by capillary-like sinusoidal channels. The lobule is divided into zones, in part relating to the oxygen supply (Figure 1-1 and Figure 1-2). Zone 1 comprises the portal tract area consisting of the terminal portal vein, terminal branches of the hepatic artery and bile duct and is thus supplied with highly oxygenated blood. Zone 3 is located around the terminal hepatic veins where oxygenation is poor and zone 2 represents the area between zone 1 and 3 (Saxena et al., 1999).

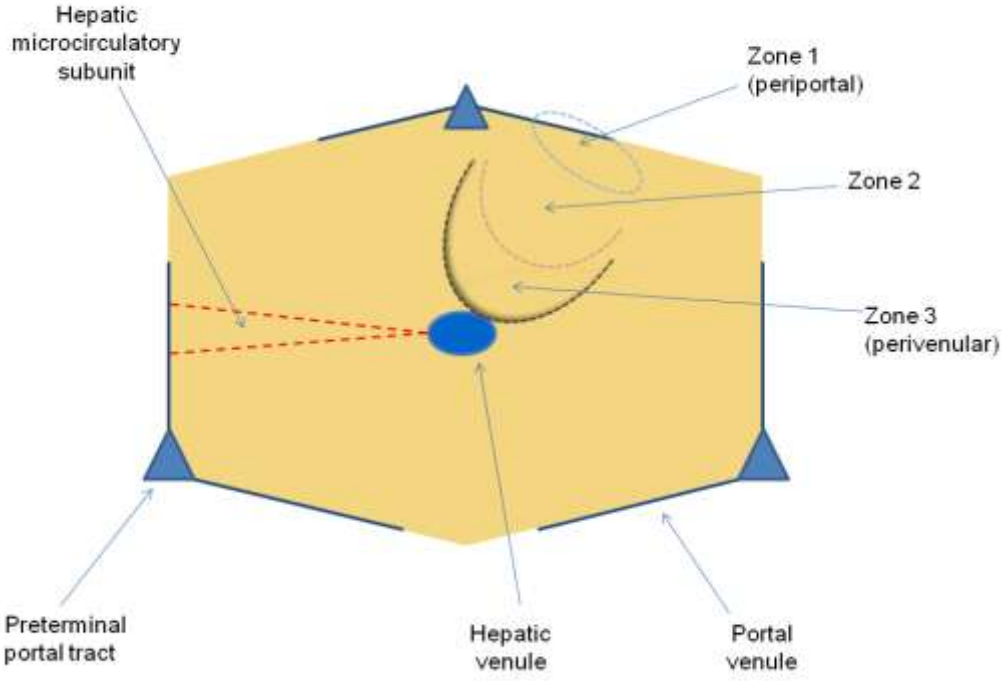

Figure 1-1 **Structure of the hepatic lobule**

Schematic representation of the hepatic lobule with zones 1 to 3 and the microcirculatory subunit outlined. The margins of each zone represent planes of equal blood pressure. The tissue in zone 1 receives blood that is higher in oxygen concentration than the tissue in zone 3.

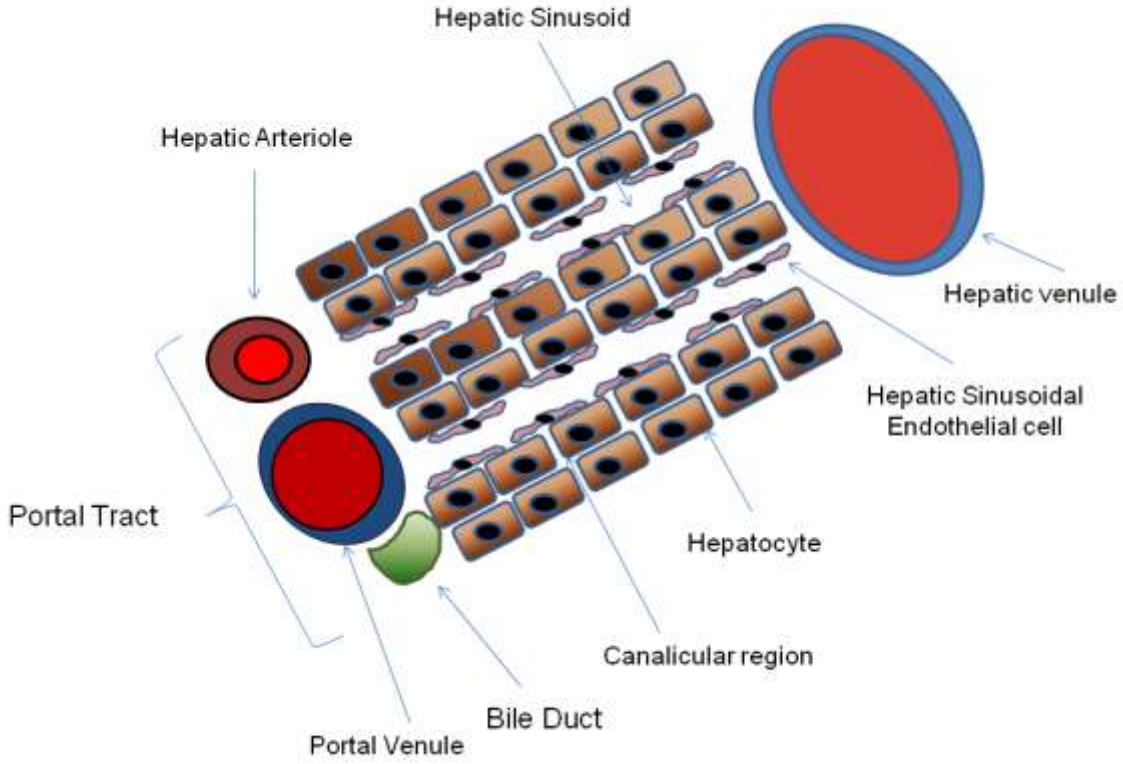

Figure 1-2 **The hepatic microcirculatory subunit**

Diagram of the hepatic microcirculatory subunit which is the smallest functional subunit. Blood from the portal venule drains into the proximal sinusoids (zone 1). Some arterioles drain directly into the proximal sinusoids. Local control of the microcirculation depends on arteriole sphincters and the state of contraction of the endothelial cells and stellate cells.

1.3.2 Cells of the Liver

The main epithelial cells of the liver are the hepatocytes which are polarized and arranged in plates that are one cell in diameter surrounded by blood filled sinusoids. The polarized nature of these cells is demonstrated by a canalicular region which consists of the lateral wall and numerous microvilli on the sinusoidal (basolateral) surface. The canalicular region of each hepatocyte is bound to the adjacent hepatocyte surface by tight junctions thereby forming bile canaliculi which drain into bile ducts. The sinusoidal surface is covered by the specialised hepatic endothelial bed which encloses the extracellular compartment called the Space of Disse. Hepatocyte microvilli allow the interchange of nutrients between the blood within the sinusoids and the hepatocytes. Liver resident macrophages, the Kupffer cells, are localised within the sinusoids where they have an important phagocytic role. The other major non-parenchymal cell is the stellate cell, also known as fat storage cells or Ito cells which are identified by their high Vitamin A and lipid content; they are the major site for Vitamin A storage. They have an important role in regulating sinusoidal blood flow and play a central role in fibrogenesis.

The healthy liver also contains a significant number of resident lymphocytes, estimated at 10^{10} (Racanelli and Rehermann, 2006). The lymphocyte population is a mixture of conventional lymphocytes (B cells, CD4 and CD8 cells) and unconventional cells (Natural Killer (NK) and Natural Killer T (NKT) cells). These cells are discussed in more detail in the next section.

1.4 Lymphoid cells

Lymphocytes are a vital arm of the human immune system and constitute 20-40% of the body's white blood cells. They are produced in the bone marrow by the process of haematopoeisis and then circulate into the blood, lymphatics and lymphoid tissue. They can be broadly divided into three groups, T cells, B cells and natural killer cells with each population having distinct functions and characterised by cell membrane components. T and B cells are the central cells of the adaptive immune system.

1.4.1 T cells

T cells arise in the bone marrow but mature in the thymus. They comprise of two well defined subpopulations defined by the nature of their TCR into alpha beta or gamma delta T cells. The majority of T cells in the circulation are alpha beta T cells and they can be divided into T helper cells and T cytotoxic cells. T helper cells are characterised by the presence of CD4 membrane glycoproteins and T cytotoxic cells are characterised by CD8 membrane glycoproteins. A CD4 or CD8 T cell which has not interacted with an antigen is unprimed and termed a naive T cell. If these naive T cells come across antigen they then differentiate into effector or memory cells, CD4 naive T cells differentiate into T helper cells and CD8 naive T cells into T cytotoxic cells. T helper cells function predominantly by secreting cytokines that modulate the local microenvironment to support specific types of immune responses. Cytokines are small proteins which are signalling molecules that

determine the immune response to antigens. The release of cytokines by T helper cells influences the function of other immune cells.

1.4.2 T helper subsets

We now know that CD4 cells can differentiate into a variety of T helper subsets. The type of T helper subset that develops will depend on signals received during antigen presentation including the nature of the cytokines present in the microenvironment, the strength of interaction of the antigen and T cell antigen receptor and the nature of the antigen presenting cell. (Boyton and Altmann, 2002). The first T helper subsets to be described were the Th1 and Th2 subsets. Th1 subsets are important for the control of cellular immunity against intracellular pathogens. They are characterized by expression of the transcription factor Tbet which promotes their production of interferon-γ. The cytokine trigger for Th1 differentiation is IL-12 produced by innate immune cells and interferon-γ production by NK cells and other T cells. Within the cell the differentiation requires activation of signal transducer and activator of transcription 4 (STAT4) and the transcription factor T-bet. The Th2 subgroup has a key role in humoral immunity and the defense against extracellular pathogens by producing IL-4, IL-5 and IL-13. The differentiation into this group is driven by IL-4 and STAT6 with the transcription factor GATA-3 (Zhou et al., 2009). More recently, a new group of effector cells have been identified termed Th17. These are an IL-17A, IL-17F and IL-22 producing subgroup which requires the transcription factor retinoid-related orphan receptor (ROR)γt being induced by TGFβ, along with

IL-6, IL-21 and IL-23 which activate STAT3 (Chen et al., 2007). The Th17 subgroup plays important roles in host defense against bacteria and fungi (Korn et al., 2007). Follicular T helper cells are a subset of T helper cells that have been shown to regulate the maturation of B cell responses and are dependent on the cytokine IL-21 (Breitfeld et al., 2000; Vogelzang et al., 2008). Further subsets including Th9 and Th22 cells have been proposed but whether these are truly distinct subsets remains to be determined.

1.4.3 Regulatory T cells

T helper cells are critical in mediating effector responses in antimicrobial immunity but the realisation that immune responses must also be controlled to prevent autoimmunity led to the search for cells capable of switching off or with suppressing immune responses. Sakaguchi and colleagues demonstrated that if T cells were depleted of the CD25+ population and transferred into animals they caused multiorgan autoimmunity (Sakaguchi et al., 1995). This CD25+ population was further identified as a CD4 subset and they were regulatory cells (T regs). They are vital in maintaining homeostasis of T effector functions and preventing pathological consequences (Shevach, 2009). This subset requires the transcription factor FOXP3 with induction by TGFβ, IL-2 and Retinoic acid (Josefowicz and Rudensky, 2009). The regulatory T cell subset can be further subdivided into two groups. The first group are natural occurring T regulatory cells that develop within the thymus during the fetal and neonatal stages of T cell maturation (Sakaguchi et al., 2001). This population is long lived, specific for

self antigens and characterised by expression of high levels of CD25, cytotoxic T-lymphocyte antigen 4 (CTLA-4) and glucocorticoid-induced TNF receptor family-related protein (GITR)(Bluestone and Abbas, 2003; Salomon et al., 2000; Shimizu et al., 2002). The second group are adaptive regulatory T cells which are generated from mature T cells by antigenic stimulation in certain conditions (Kingsley et al., 2002; Nakamura et al., 2001). In the *ex vivo* setting this has been demonstrated by culturing mature CD4 T cells in the presence of IL-10 (Barrat et al., 2002). The mechanism by which natural T regulatory cells suppress inflammation appears to be by direct contact with effector T cells or antigen presenting cells (Shevach, 2002). Whereas adaptive T regulatory cells appear to suppress inflammation by secreting cytokines such as IL-10 and TGF-β (Kingsley et al., 2002; Nakamura et al., 2001)

It is quite clear that T regulatory cells have suppressor function but there is debate whether these cells are a distinct subset of T helper cells or have plasticity to develop into other subsets and become effector cells. Recent *in vivo* studies have been able to track Foxp3 positive populations in physiological and inflammatory conditions (Rubtsov et al., 2010)**.** These experiments demonstrated that T regulatory cells were a stable population of CD4 cells in both physiological and inflammatory conditions.

The subsets of CD4 Tcells are summarised in Figure 1-3.

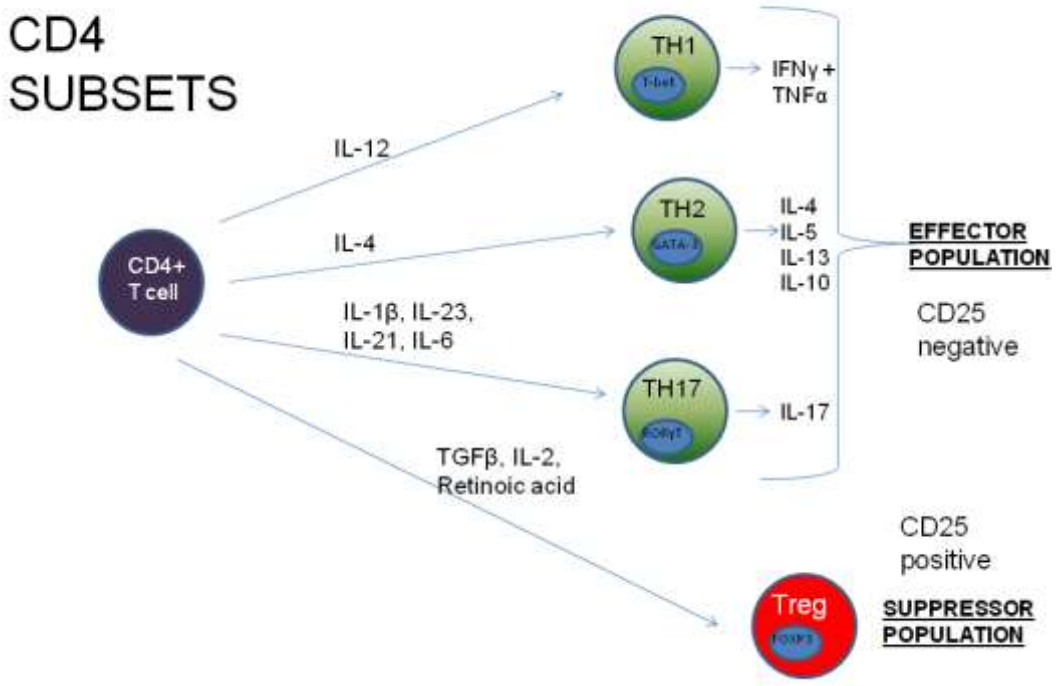

Figure 1-3 **CD4 T helper subsets**

A summary of the T helper subgroups and an outline of the cytokines which lead to each subset generation as well as the cytokines produces by each subset.

1.4.4 Cytotoxic T lymphocytes

Recognition of antigen-MHC complexes by a naive CD8 leads to a differentiation into an effector or memory cytotoxic T lymphocyte (CTL). CTLs recognise endogenous antigens that are processed and presented on MHC class I molecules. They monitor cells and bind to and then kill any that display foreign antigen in the context of the correct MHC class I complex. This allows them to recognise and mount an immune response against intracellular pathogens and tumour cells (de Visser et al., 2006; Wong and Pamer, 2003). Effector CTLs express cytokines such as tumour necrosis factor α (TNFα) and interferon γ as well as granzyme and perforin molecules which are cytolytic when released into apposing target cells. (Obar and Lefrancois, 2010).

1.4.5 B lymphocytes

B cells arise from the bone marrow and this is also the site of their maturation. They can be distinguished from other lymphocytes by the fact that they express membrane bound immunoglobulin (antibody) molecules which act as receptors for antigen. When a naive B cell encounters antigen that is recognised by its antibody it undergoes proliferation and differentiates into a memory cell and under the correct conditions long-lived plasma cells. Memory B cells are longer lived than naive B cells but express the same antibody as their parent B cell. Plasma cells are end-stage B cells and they have the ability to produce antibody which is secreted. Antibodies play important roles in complement activation, mediating opsonisation and

mucosal defences. It is important to mention that effective antibody production by B cells is dependent on interactions with T helper cells. B and T cells are present in peripheral lymphoid organs. When these populations are activated by an antigen, they migrate towards each other within the peripheral lymphoid organ. The B and T cell population interact and this leads to B cell proliferation and differentiation into antibody secreting cells. Subsequently the B cells migrate back into the follicles of the lymphoid tissue and proliferate rapidly to form germinal centres. The B cells within the germinal centre can perform isotype switching, leading to the production of different classes of antibody. B cells also undergo affinity maturation, where the antibodies generated have an increased capacity to bind antigens and therefore more efficiently bind to and neutralize foreign pathogens.

1.4.6 Natural Killer cells and Natural Killer T cells

Natural Killer cells (NK cells) are large, granular lymphocytes. They are part of the innate immune system and do not have the T cell receptor or antibodies on their membrane. They have cytotoxic activity against tumour cells and virally infected cells. This is carried out by using NK cell receptors which have the ability to detect target cells by either the reduction in MHC class I expression or an altered range of surface antigens which can be characteristic of tumour cells or virally infected cells. Natural Killer T cells (NKT cells) are a subtype of lymphocytes which share characteristics of NK cells and T cells. NKT cells have T cell receptors but these receptors

recognise CD1 which is a MHC-like receptor. They also express several surface markers seen on NK cells and like NK cells they can kill target cells.

I have described the lymphoid cells of the liver, there is also a myeloid population of cells within the liver comprising of resident tissue macrophages.

There is gathering evidence that the response to antigens within the liver is skewed towards tolerance, in other words there is a lack of an immune response. The following section describes this in more detail and what happens if this tolerance is broken.

1.5 The liver as a lymphoid organ

The local population of lymphocytes in the liver are profoundly influenced by the hepatic microenvironment. Due to its vascular supply the liver is continually exposed to products of digestion as well as microbial products that originate from bacteria in the intestines. The hepatic immune system must strike a fine balance between tolerance of harmless antigens from the gastrointestinal system and the ability to rapidly respond to invading foreign pathogens.

The initial response to antigens is dictated by the innate immune system which is comprised of pattern recognition receptors such as Toll-like receptors (TLRs), cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptors and RNA helicases, including retinoic acid inducible protein-I (RIG-I) (Thompson and Locarnini, 2007).

The continual exposure of certain antigens to pattern recognition receptors seems to lead to a state of active tolerance. One of the best examples of this can be seen with lipopolysaccharide endotoxin (LPS). LPS is a component of gram-negative bacterial cell walls and it is found at relatively high levels in portal blood, up to 1ng/ml (Lumsden et al., 1988) but in the circulation the levels are almost undetectable. The removal of LPS from the circulation has been attributed to the cells of hepatic sinusoid which express the LPS receptor (Catala et al., 1999) but unlike other innate cells of the body the detection of LPS does not lead to a strong activating signal. This lack of activation by bacterial components is thought to be due to the continual exposure of these antigens to the liver. Pattern recognition receptors within the liver bind to these antigens which leads to intracellular signalling but the outcome is the release of tolerogenic cytokines such as interleukin 10 (IL-10) rather than pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFα) or interleukin-6 (IL-6) (Crispe, 2009). In contrast, the presence of viral components which are not constitutively exposed to the liver leads to an alternative pathway which leads to the release of cytokines with strong anti-viral properties such as Interferon α, and Inteferon β.

In addition to pattern recognition receptors there are also innate lymphocyte populations of NK and NKT cells which are both found at higher frequencies compared to other tissues (Norris et al., 1998; Tu et al., 2007). NK cells contribute to immunity against pathogens and increase in numbers during infection of the liver (McIntyre and Welsh, 1986). As seen with NK cells in other tissues they are activated by cytokine stimulation and triggered by

stronger signalling through activating receptors compared to inhibitory receptors, which leads to cytotoxicity and cytokine release (Lanier, 2008). In addition NK cells have the ability to promote T cell recruitment to the liver. Models of viral liver infection in mice demonstrate accumulation of NK cells in response to the chemoattractant cytokine CCL3 (Salazar-Mather et al., 1998). The NK cells synthesise interferon γ which promotes the secretion of CXCL9, which is responsible for T cell accumulation (Itoh et al., 2001). Through this cascade NK cells recruit T cells to the liver and promotes T cell immunity rather than tolerance.

NKT cells usually make up a small percentage of T cell populations in the body (<5%) but are enriched in the liver and have recently been shown to patrol the hepatic sinusoidal channels (Geissmann et al., 2005). Like NK cells, there is evidence that NKT cells promote hepatic immunity. This has been shown in the context of infections such as malaria and hepatitis B and tumour immunity (Gonzalez-Aseguinolaza et al., 2000; Kakimi et al., 2000; Toura et al., 1999). As described with NK cells above, NKT cells can also drive a cytokine/chemokine cascade to promote T cell homing to the liver. *In vivo* murine models have shown that activation of NKT cells leads to interferon γ production which leads to the production of the chemokine CXCL10. The induction of this chemokine by NKT cells was shown to promote the recruitment of a subset of T cells, specifically regulatory T cells (Santodomingo-Garzon et al., 2008).

The liver also has a large intravascular macrophage population, the Kupffer cells and contains liver dendritic cells. Kupffer cells express MHC class I and II molecules and co-stimulatory molecules making them potential antigen presenting cells. They have been shown to have tolerogenic properties by secreting IL-10 in response to LPS (Knolle et al., 1995). In contrast, they can also demonstrate anti-viral properties by presenting antigen in the context of hepatitis C infection (Burgio et al., 1998). The other myeloid group of cells are dendritic cells which have the most powerful antigen presentation properties (Nussenzweig and Steinman, 1980), both subgroups of plasmacytoid DCs (pDCs) and myeloids DCs (mDCs) are present within the liver. pDCs are known to produce high levels of IFN-α (Crispe, 2009), whereas the liver mDCs have been shown to produce IL-10 with an increased production of this cytokine in patients with chronic viral infections such as hepatitis C (Averill et al., 2007).

The sinusoidal endothelial cells of the liver express several surface markers which are associated with antigen presentation (MHC II, CD40, CD86). They can take up and process antigens and cross-present peptides on MHC class I to CD8 T cells. However, the results of both CD4 and CD8 T cell activation by LSEC tends to be tolerance rather than effector cell expansion allowing these cells to maintain homeostasis in the face of a large quantity of dietary antigens in the portal blood (Knolle et al., 1998). Antigen presentation has also been attributed to stellate cells and this is supported by their expression of MHC class I and II (Friedman, 2008). *Ex vivo* experiments suggested that stellate cells have the potential to activate T cells but most of the data

suggest they are poor T cell activators that tend to induce tolerance or regulatory T cell expansion rather than effector responses (Winau et al., 2007; Yu et al., 2004).

1.5.1 The liver's role in priming T cells and induction of tolerance.

The microanatomy of the liver and the range of antigen presenting populations make the liver an ideal site for lymphocyte activation. The priming and activation of lymphocytes are properties seen in lymphoid tissue and there is evidence that the liver shares these properties. Priming of lymphocytes requires direct contact with APCs and lymphocytes circulating through hepatic sinusoids can make direct contact with underlying hepatocytes, stellate cells as well as with HSEC and Kupffer cells. This has been confirmed with electron microscopy (Warren et al., 2006). Local activation and priming has been confirmed in the liver in murine models of chronic viral infections and liver transplants where bone-marrow APCs have been depleted (Ando et al., 1994; Klein and Crispe, 2006).

Despite the overlapping ability of the liver and lymphoid organs to activate and prime lymphocytes, the initial site of lymphocyte activation leads to opposing outcomes. The liver apears to induce tolerance, this is partly explained by the fact that antigen presentation in the liver is associated with the production of tolerogenic cytokines such as IL-10 as outlined above. In addition the liver also appears to have specific effects on the behaviour of CD8 and CD4 lymphocytes. This was clearly shown with CD8 lymphocytes where initial antigen presentation within lymph nodes in transgenic mice led

to a significant hepatitis, whereas initial antigen presentation in the liver led to defective CD8 cytotoxic activity and no hepatocellular damage (Bowen et al., 2004). It has been shown that CD8 lymphocytes activated in the liver appear to undergo apoptosis preventing an immune response (Bertolino et al., 1998; Bertolino et al., 1999). CD4 lymphocytes activated in the liver appear to undergo differentiation into a regulatory phenotype. The sinusoidal endothelial cell appears to play a central role in this process. CD8 T cells that are primed by sinusoidal endothelial cells do not differentiate into cytotoxic effector cells and CD4 T cells primed by these cells appear to differentiate into an anti-inflammatory/ regulatory phenotype where they secrete IL-4 and IL-10 (Knolle et al., 1999; Limmer et al., 2000).

The local environment of the liver induces tolerance but this also impacts on systemic tolerance. This was highlighted during the early experiments in organ transplantation where the transplantation of a liver with a kidney significantly improved the survival of the renal allograft (Calne et al., 1969). Tolerance of liver allografts has been suggested to be due to resident APCs of the transplanted liver inducing deletion of host T cells that have entered the graft. This may also be the explanation for oral tolerance, where antigen administered to the stomach induces systemic tolerance. When the venous drainage from the gut to the liver is diverted, oral tolerance is lost (Yang et al., 1994). The mechanism by which liver APCs could induce systemic tolerance is unclear but it is suggested that it could be due to peripheral deletion of CD8 cells or the induction of antigen specific Tregs (Crispe, 2009). Support for the deletion of CD8 cells in tolerance induction comes

from an influenza model of infection, where the influenza pathogen is limited to the respiratory tract but CD8 cells specific for influenza are found in the liver, associated with Kupffer cells (John and Crispe, 2004). Support for T regulatory cells contributing to systemic tolerance comes from mouse liver transplantation, where T reg numbers increased significantly after grafting. Depletion of these cells with anti-CD25 antibodies led to rejection of the graft. (Li et al., 2006)

It is quite clear that the liver is in a resting state of active tolerance, the mechanisms that switch the local microenviroment to promote an effector immune response are unclear. Type I inteferons (α/β) are likely to be involved, they are induced in hepatic viral infections and they are implicated in the recruitment of T cells during these infections (Liu et al., 2000; Salazar-Mather et al., 1998). It has also been hypothesised that DCs and sinusoidal endothelial cells must switch from their induction of tolerance within the liver to priming of T cells to deliver effector functions (Crispe, 2003).

1.6 Liver injury

In viral infections with Hepatitis A or E infected hepatocytes are killed by innate immune cells and antigen-specific CD8 T cells resulting in liver injury, inflammation followed by resolution, regeneration and repair with the restitution of normal hepatic microarchitecture. Chronic liver injury on the other hand takes a different pathway, here repeated insults lead to continuing liver injury, chronic inflammation that persists and an aberrant resolution phase characterised by chronic inflammation and scarring with collagen

deposition and fibrosis rather than regeneration. If this process progresses it leads to permanent tissue loss which is termed parenchymal extinction and the liver scar forms to heal the wound (Wanless et al., 2000). This is seen as fibrous septa which are flattened sheets of connective tissue containing remnants of damaged tissue, elements of healing and neovascularization. As the disease progresses these fibrous septa become more numerous and become confluent dividing the liver tissue into nodules that characterise established' cirrhosis (Wanless et al., 2000).

The development of cirrhosis leads to complications of portal hypertension due to distorted architecture, liver failure due to hepatocyte loss and carcinogenesis driven by the inflammatory and profibrotic environment. Thus understanding how chronic liver injury leads to cirrhosis may identify important therapeutic targets.

At the cellular level the response to injury by the liver leads the hepatic stellate cells to undergo a process of 'activation' and transdifferentiation which transforms them from quiescent cells to fibrogenic myofibroblasts (Friedman, 2000). This occurs under the influence of cytokines and growth factors secreted by other cells within the hepatic sinusoids including Kupffer cells, hepatocytes and sinusoidal endothelial cells. Hepatocytes and Kupffer cells are also a source of oxygen free radicals that have a paracrine effect on stellate cells (Maher, 1999) and endothelial cells activate stellate cells by depositing a splice variant of fibronectin and transforming the latent form of the cytokine transforming growth factor β1 (TGFβ1) into the fibrogenic form

(Friedman, 1999; Jarnagin et al., 1994). TGFβ1 is the main stimulant to matrix deposition by stellate cells (Friedman, 1999) and although it is secreted from several cells autocrine secretion is the main stimulus for stellate cell activation (Gressner, 1995). On activation stellate cells undergo proliferation and secrete matrix components and matrix modelling enzymes that result in ECM remodelling with replacement of the normal subendothelial ECM by fibril-forming collagen (Benyon et al., 1999; Theret et al., 1999). This is associated with profound morphological changes within the hepatic sinusoid. Hepatocytes lose their microvilli, sinusoidal endothelial lose their fenestrae which are characteristic of their normal phenotype and Kupffer cells become activated (Friedman, 2000). These morphological changes and deposition of scar within the sinusoids are responsible for the liver failure and portal hypertension seen in cirrhosis.

1.6.1 The importance of inflammation in the pathogenesis of cirrhosis

The inflammatory response is a vital part of the process by which the immune system deals with foreign organisms but if it persists and fails to resolve it can lead to continuing tissue injury and scarring and in the context of chronic liver disease this means progressive liver damage and cirrhosis (Iredale, 2007). There is evidence that the persistent inflammatory reaction that develops in response to recurrent liver injury and tissue death plays a central role in the development of fibrosis and progression to cirrhosis. During the early stages of fibrosis *in vivo* it can be seen that sites of inflammation are closely linked to deposition of fibrous material (Iredale, 2007) in both human

studies and animal models (Brunt, 2004; Constandinou et al., 2005; Sorensen et al., 1984; Tsukamoto et al., 1990). Thus histological evidence of necroinflammatory activity in liver biopsies correlates strongly with the subsequent development of fibrosis and cirrhosis in several liver diseases including alcoholic liver disease, viral and autoimmune hepatitis. The inflammatory process is characterised by activation of resident immune cells of the liver but also the recruitment of a lymphocyte-predominant infiltrate that constitutes chronic hepatitis (Lalor and Adams, 1999). This drives the deposition of collagen and the development of fibrosis leading to cirrhosis (Figure 1-4). The infiltrating lymphocytes are made up of various subsets which were discussed in section 1.4 and the following section summarises the evidence for their roles in liver disease.

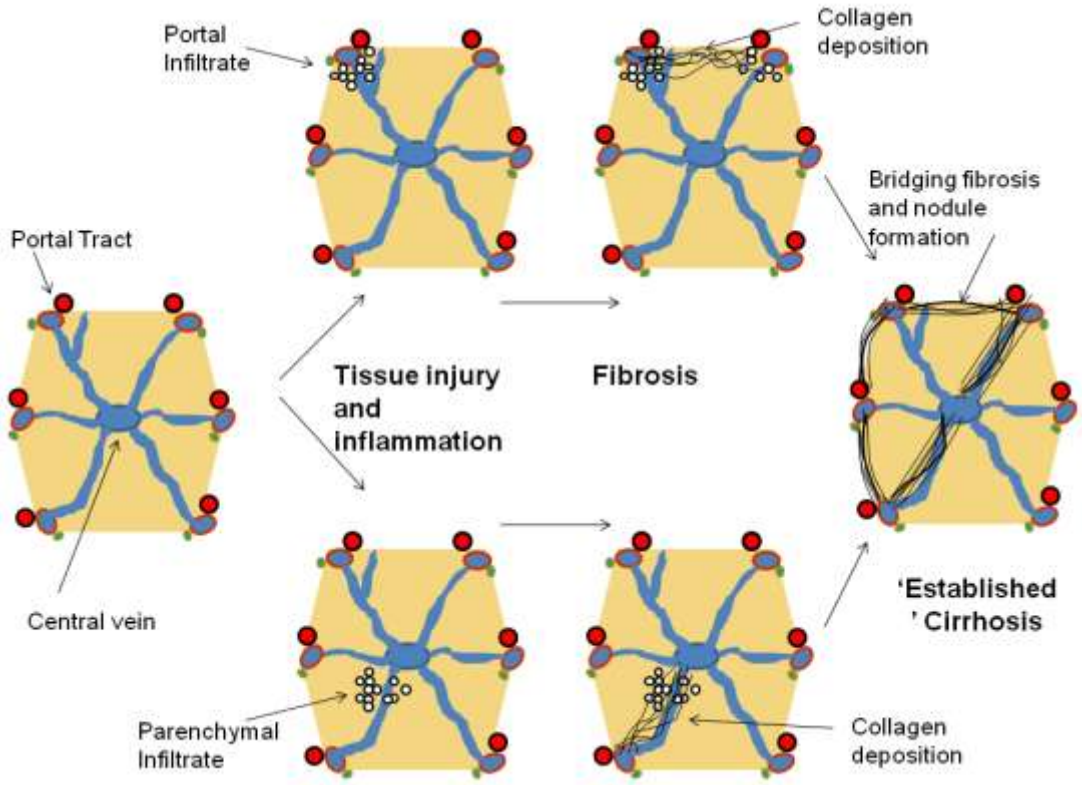

Figure 1-4 **The progression from liver injury to cirrhosis**

Inflammatory infiltration of immune cells in liver disease can occur around the portal tract or within the parenchyma. Irrespective of the pattern of infiltration the progression of disease follows a common pathway of collagen deposition/fibrosis leading to bridging fibrosis and nodule formation (cirrhosis).

1.7 Evidence for the role of lymphoid cells in liver disease

1.7.1 T cells

T cell infiltration into the liver has been shown to drive hepatocellular injury in various animal models of liver injury and is a characteristic feature of most progressive liver diseases including autoimmune hepatitis, viral infection, alcoholic liver disease and allograft rejection (Bogdanos et al., 2000; Chedid et al., 1993; Heneghan and Mcfarlane, 2002; Kita et al., 2001; Rehermann and Chisari; Rosen, 2008). A CD4 predominate T cell infiltrate is seen in autoimmune liver disease (Lohr et al., 1996) and in chronic viral hepatitis secondary to either hepatitis B or C, both CD4 and CD8 T cells have been implicated in liver injury (Chang et al.; Chisari, 1997; Napoli et al., 1996; Rosen et al., 2002). Experimental models of T cell mediated liver injury in mice by concavalin-A have shown that this process is influenced by STAT1 and STAT-3, where IFNγ-activation of STAT1 has detrimental effects by activating CD4 T cells while IL-6 activation of STAT3 has a protective role by suppression of IFNγ signalling (Hong et al., 2002). Concavalin A models have also demonstrated that IL-4 and TNFα play important roles in T cell mediated injury (Gantner et al., 1995; Jaruga et al., 2003). However, how well such animals reflect the situation in human disease is unclear where chronic iterative injury and persistent inflammation acts over months and years to drive fibrogensis and cirrhosis. There is also gathering evidence that the different functional T cell subsets may have important and distinct

roles in liver disease. The Th17 subpopulation of CD4 T cells has been noted to be increased in chronic inflammatory liver diseases (Lan et al., 2009; Lemmers et al., 2009), and both CD4 and CD8(Tc17) Il-17 secreting cells have been detected in chronic viral hepatitis (Billerbeck et al., 2010) and chronic inflammatory liver disease. These studies reported the presence in the liver of IL-22 secreting Th17 and Tc17 cells but the role of IL-22 is unclear because it can promote epithelial repair and regeneration through its effects on hepatocyte proliferation (Zenewicz et al., 2007).

1.7.2 T regulatory cells

Lymphocytes infiltrating liver tissue also contain populations of anti-inflammatory or suppressor cells including regulatory T cells (Oo et al., 2010). There is evidence that this population can influence the inflammatory microenvironment and thereby impact on disease progression. In viral hepatitis increased numbers of T regulatory cells within the liver may prevent an effective immune response allowing for viral persistence (Lan et al., 2006; Rushbrook et al., 2007). In chronic hepatitis C it has been shown that T regs specific for the Hepatitis C virus (HCV) can directly suppress the actions of HCV specific CD8+ cells (Ebinuma et al., 2008). T regulatory cells may also influence anti-tumour immunity. The proportion of Tregs infiltrating primary liver tumours, hepatocellular carcinomas, has been found to be increased and the numbers of tumour-infiltrating Tregs correlates with disease progression and survival (Fu et al., 2007).

Whilst increased T reg infiltration of the liver may promote viral infection and tumour development, decreased numbers or defective function may have other consequences. Autoimmune diseases develop because of a breakdown in immune tolerance to self-antigens. Tregs play an important role in preventing this autoreactivity. In autoimmune hepatitis it has been shown that circulating T regs are reduced in numbers and show functional impairment (Longhi et al., 2004; Longhi et al., 2005). Decreased frequency of T regulatory cells has also been reported in other autoimmune liver diseases such as primary biliary cirrhosis (Lan et al., 2006). It is possible that Tregs are defective in the face of severe inflammation or that they are overwhelmed by the effector response. However, work from our own group shows that the proportion of Tregs within the hepatic infiltrate is greatest in patients with severe inflammation. Indeed the highest levels were seen in seronegative fulminant hepatitis (Oo et al., 2010) . This suggests that recruitment of Tregs is maintained and either that they are defective or more likely that they are suppressed by the inflammatory microenvironment or more simply that they are swamped by the vigour of the effector response. These findings suggest that the balance between effector/suppressor subsets can have important implications for disease development and progression.

1.7.3 B cells

Whilst T cell populations have been shown to contribute to several inflammatory liver diseases, there is also gathering interest in B cells. The

majority of B cells are found within the spleen and secondary lymphoid tissue where interactions occur with T cells (Karrer et al., 1997). They also produce antibody and differentiate into memory B cells and plasma cells that migrate to the gut and bone marrow and secrete high affinity antibodies. B cell populations can also be found in peripheral tissues as part of the chronic inflammatory infiltrate although at much lower frequencies than T cells. In some forms of established chronic inflammation neolymphogenesis occurs leading to the development of tertiary lymphoid structures (Qin et al., 1998; Schroder et al., 1996). These tertiary lymphoid structures have been identified in inflammatory liver diseases (Grant et al., 2002; Murakami et al., 1999) and contain B cell rich areas arranged into lose aggregates that resemble the B cell follicles seen in secondary lymph nodes. Whether such structures are sites of local antibody production is less clear**.**

B cells have also been directly implicated in the progression of liver injury in murine models of liver diseases including autoimmune cholangitis and primary biliary cirrhosis. Experimental data has shown an important role for these cells in murine models of primary biliary cirrhosis (Moritoki et al., 2009a; Moritoki et al., 2009b). In a model of autoimmune cholangitis and colitis the depletion of B cells with anti CD20 antibodies led to an amelioration of liver disease but a worsening of gut inflammation. More recent studies in a murine model of primary biliary cirrhosis reported that B cell depletion led to a worsening of disease (Dhirapong et al., 2011). B cells have also been directly implicated in the development of liver fibrosis (Novobrantseva et al., 2005). These murine experiments demonstrated that

B cells isolated from the bone marrow homed to the liver and contributed to the progression of fibrosis in an antibody-independent manner. B cells are able to produce large amounts of cytokines and it may be that they promote a profibrogenic environment by cytokine secretion. In the context of hepatitis C infection, B cells have been shown to internalise a strain of the HCV virus and efficiently transmit it to hepatoma cells (Stamataki et al., 2008) suggesting that B cells could transmit HCV infection on homing to the liver. These diverse results suggest that the role of B cells in liver disease is complex but emphasise how the underlying mechanisms are poorly understood.

1.7.4 NK and NKT cells

NK cells are present at higher frequencies in the liver compared to other tissues (Norris et al., 1998; Tu et al., 2007). The human liver is also enriched for NKT cells, the numbers of these cells is highly variable ranging from 5% to 25% of the total lymphocyte population within the liver (Doherty and O'Farrelly, 2000). Both these cell types have been implicated in the pathogenesis of several chronic liver diseases including chronic viral hepatitis, alcoholic liver disease and non-alcoholic fatty liver disease (NAFLD). Both NK and NKT cells have the ability to kill virally infected cells and studies have shown that depleted numbers of these cells are associated with progression of chronic hepatitis C infection (Golden-Mason et al., 2007; Yamagiwa et al., 2008). NK cells are particularly important in mediating hepatocyte killing in hepatitis B infection (Dunn et al., 2007). However, the

role played by these cells is more complex and both cell types can have protective regulatory functions under certain circumstances. Studies in mice demonstrate that NKT cells are protective in some models of NAFLD and in con A hepatitis where they promote the recruitment of regulatory T cells (Li et al., 2005; Santodomingo-Garzon et al., 2008). The complexity is well-demonstrated by the situation in alcoholic liver disease where reduced numbers of NKT cells appear to be protective whereas activation of these cells exacerbates alcoholic liver injury (Minagawa et al., 2004). In primary biliary cirrhosis both cell types have been implicated in driving the liver injury associated with this condition (Chuang et al., 2006; Chuang et al., 2007).

All the lymphocyte subsets discussed above need to be recruited from the peripheral circulation to their site of action. The recruitment of these lymphocyte subsets into tissue is complex but understanding the molecular mechanisms that mediate this cell subtype specific process within the liver could identify both organ-specific and subset specific targets which are anti-inflammatory to halt progression to fibrosis and cirrhosis. The following section reviews the current understanding of lymphocyte recruitment, with emphasis on the liver.

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1.8 Lymphocyte trafficking

The immune system is able to respond to foreign antigen in a rapid and specific way. A major part of this process involves the trafficking of

lymphocytes from blood into various tissues and then into lymph and back to blood. The continual recirculation of lymphocytes involves migration into tissues where antigen is found since they are only activated by direct contact with antigens (von Andrian and Mackay, 2000). T cells that have not encountered antigen are termed naive T cells and they are programmed to migrate into secondary lymphoid organs (eg peripheral lymph nodes, Peyers patches, tonsils and spleen) where they encounter dendritic cells which present antigen that they have taken up in the periphery (Banchereau and Steinman, 1998). If the T cell recognises the antigen presented by the dendritic cell it becomes either an effector capable of migrating to the site of inflammation or it is programmed to migrate into B cell rich follicles to provide help to B cells (Morita et al., 2011). Once the antigen is cleared most of these activated cells die but subsets of primed, memory cells continue to patrol the peripheral tissue or remain in lymphoid tissue (Sallusto et al., 1999). These memory cells are then able to respond rapidly to repeated challenges by the same antigen thereby providing the basis of immunological memory.

To enter tissues from blood lymphocytes must first bind to and then cross the endothelial cells lining the vessel wall (Springer, 1995). Lymphocyte recirculation is not a random process, the endothelium plays an important role in controlling the access of lymphocyte subsets to particular tissues and this has an important effect on the local immune responses (Butcher and Picker, 1996; Kunkel and Butcher, 2002). The process is controlled by adhesion molecules on the surfaces of lymphocytes and endothelial cells and

the binding of chemoattractant cytokines, chemokines, to chemokine receptors on the surface of lymphocytes. The process involves a sequence of combinatorial interactions termed the 'adhesion cascade'.

1.9 The adhesion cascade

The first interactions between flowing lymphocytes and endothelium under conditions of blood flow are tethering interactions which lead to rolling on the vessel wall followed by arrest, intravascular crawling and transendothelial migration into tissue. In the initial step the lymphocytes are captured by selectins (Tedder et al., 1995) or members of the immunoglobulin superfamily (Alon et al., 1995; Berlin et al., 1995; Lalor et al., 1997) This capture serves to slow the cell down allowing it to roll on the vessel wall and interact via G-protein coupled receptors (GPCRs) on the lymphocyte surface with chemokines sequestered in endothelial glycocalyx (Adams and Lloyd, 1997; Campbell et al., 1996). Activation of these receptors leads to an intracellular signalling cascade which culminates in integrin receptors on the lymphocyte surface clustering at sites of contact and altering their confirmation to a 'high affinity' state allowing binding to the immunoglobulin family receptors on the endothelium which leads to arrest and firm adhesion to the vessel wall (Campbell et al., 1998; Tanaka et al., 1993). The activation of integrins involves an unfolding and extension of the integrin and an opening of the binding domain to promote interactions with counter-receptors (Shamri et al., 2005). The cell then undergoes intravascular crawling in which it migrates on the endothelial surface in search of sites where it can

undergo transendothelial migration into tissue. This last step is complex and poorly understood process and involves multiple adhesion molecules and chemokines(Imhof and urrand-Lions, 2004). Table 1-1 summarises the major adhesion molecules that play a role in the adhesion cascade and the individual stages in the ‘typical process’ are discussed below.

Molecule	Expression	Role in Adhesion Cascade
L selectin	Leucocytes	Rolling
P selectin	Endothelial cells and Platelets	Rolling
E selectin	Endothelial cells	Rolling
Selectin Ligands		
Sialyl Lewis^x	Various cells regulated by fucosyl transferase VII	Ligand for all selectins which is presented on the following molecules :
PSGL-1	Leucocytes	Main ligand for P-selectin but binds all selectins
PNAd	High endothelial venules	Ligand for L-selectin
CLA	Skin homing leucocytes	Ligand for E-selectin
ß$_2$ Integrins		
α_L ß$_2$	Leucocytes	Firm adhesion and transmigration
α_M ß$_2$	Myeloid cells	
α$_4$ Integrins		
α_4 ß$_1$	Leucocytes except neutrophils	Rolling and Firm Adhesion
α_4 ß$_7$	Gut homing leucocytes	Rolling and Firm Adhesion
Immunoglobulin Superfamily		
ICAM-1	Increased expression on endothelial cells with cytokines	Ligand for ß$_2$ Integrins: firm adhesion and transmigration
ICAM-2	Constitutive on endothelium	Ligand for β_2 integrins: firm adhesion and transmigration
VCAM-1	Induced expression on endothelial cells with cytokines	Ligand for α_4 Integrins: rolling and firm adhesion
MAdCAM-1	High endothelial venules on gut associated lymphoid tissue	Ligand for L-selectin and α_4 ß$_{7:}$ rolling and firm adhesion
PECAM-1	Endothelial cells and leucocytes	Transmigration
JAM A,B,C	Endothelial cells	Transmigration

Table 1-1 **Summary of the molecular determinants of the adhesion cascade**

PSGL-1, P-selectin glycoprotein ligand 1; PNAd, Peripheral-node addressin; CLA, Cutaneous lymphocyte antigen; ICAM-1, Intercellular adhesion molecule 1; ICAM-2, Intercellular adhesion molecule 2; VCAM-1, Vascular cell adhesion molecule 1; MAdCAM-1, Mucosal vascular addressin cell adhesion molecule 1; PECAM-1, Platelet-endothelial cell adhesion molecule 1; JAM, Junctional adhesion molecule.

1.9.1 Rolling

Capture and rolling is classically mediated by molecules called selectins (L-selectin, P-selectin and E-selectin), which bind to glycosylated ligands ((McEver and Cummings, 1997). L-selectin is expressed on most lymphocytes, whereas E and P-selectin are predominantly expressed on endothelial cells. The selectin-ligand interaction involves a 'catch-bond' phenomenon in which each bond is strengthened with increasing shear stress (Marshall et al., 2003). In addition a transport phenomenon between selectin ligands and selectins allows the rolling motion of the cell to generate new bonds before old bonds are broken (Yago et al., 2007). There are situations where integrins can mediate rolling particularly α4 integrins and their ligands vascular cell adhesion molecule- 1 (VCAM-1) and mucosal vascular addressin cell adhesion molecule-1(MAdCAM-1) (Berlin et al., 1995; Lalor et al., 1997; Sigal et al., 2000)

1.9.2 Activation and Arrest

Following the rolling step, lymphocytes undergo activation by chemokines or other chemoattractants. This leads to integrins binding to immunoglobulin superfamily members such as intercellular adhesion molecule-1 (ICAM-1) or VCAM-1 on endothelial cells leading to arrest and firm adhesion (Campbell et al., 1996; Campbell et al., 1998). During inflammation, endothelial cells are activated by cytokines to express adhesion molecules and synthesise chemokines that are presented on their luminal surface via binding to

glycosoaminoglycans (GAGs) in the endothelial glycocalyx (Johnson et al., 2005). The binding of chemokines to GPCRs leads to a conformational change in the integrins from a bent low affinity state to an extended conformation which opens up the ligand binding pocket (Arnaout et al., 2005). This process is not completely understood but there appear to be three stages involving phospholipase C (PLC), activation of small GTPases and finally transitional integrin conformational changes through association with actin-binding proteins such as Talin-1(Ley et al., 2007).

1.9.3 Transmigration

Once leucocytes undergo firm adhesion they can migrate through the endothelial layer. The first step is crawling on the vessel wall which is integrin and ICAM-1 dependent (Phillipson et al., 2006; Schenkel et al., 2004). The initiation of transmigration may involve several mechanisms including chemoattractants acting under conditions of shear stress and interactions between integrins and endothelial cell ligands which stimulate endothelial cells to promote migration. The interaction between leucocytes and endothelial cells has been shown to involve the formation of 'docking sites' or' transmigratory cups' which are rich in ICAM-1 and VCAM-1(Barreiro et al., 2002; Carman and Springer, 2004a). Transmigration then occurs through the endothelial layer and this has been to shown to occur via a paracellular route or a transcellular route. In the paracellular route, ligation of adhesion molecules leads to weakening of intra-endothelial contacts. This has been shown for ICAM-1 ligation which leads to the release of intracellular

calcium and activation of p38 mitogen activated protein kinase and Ras homologue (RHO) GTPase which results in opening up of the endothelial junctions (Huang et al., 1993; Millan and Ridley, 2005). In addition endothelial junctional molecules which bind leucocytes such as platelet/endothelial cell adhesion molecule 1 (PECAM-1) and junctional adhesion molecule A (JAM A) are mobilized to junctions and thereby create a haptotactic gradient which guides leucocytes to the junctional zone (Muller, 2003a). The molecules directly implicated in migration include immunoglobulin superfamily members- PECAM-1, ICAM-1, ICAM-2, JAM A,B and C and endothelial cell-selective adhesion molecule (ESAM), as well as the non-immunoglobulin molecule CD99 (Ley et al., 2007). Other molecules implicated include poliovirus receptor (CD155) and several ectoenzymes, vascular adhesion protein-1 (VAP-1), CD157 and leucocyte specific protein 1(LSP 1) (Liu et al., 2005; Reymond et al., 2004; Salmi and Jalkanen, 2005). Transmigration involves several molecular interactions occurring in sequence as demonstrated by the fact that blockade of CD99 and PECAM-1 exerts an additive effect on leucocyte transmigration (Lou et al., 2007; Schenkel et al., 2002).

An alternative path for transmigration is the transcellular route. This route of leucocyte migration was described in several early *in vivo* studies (Azzali et al., 1990; Chamberlain and Lichtman, 1978; Feng et al., 1998; Lossinsky et al., 1989) but the inability to reproduce these findings *in vitro* led to controversy regarding the importance of transcellular migration (Luscinskas et al., 2002; Muller, 2003b). More recently with improved imaging techniques

the transcellular route has been confirmed in both *in vivo* and *in vitro* settings (Carman and Springer, 2004b; Cinamon et al., 2004; Engelhardt and Wolburg, 2004; Millan et al., 2006). The mechanism involves translocation of apical ICAM-1 to caveolae and F-actin rich regions and the transport of caveolin-1 to the basal membrane resulting in a channel through which the leucocyte can migrate (Cinamon and Alon, 2004; Millan et al., 2006). The proportion of paracellular versus transcellular migration appears to differ between endothelium in different tissues. Studies with human umbilical vein endothelial cells (HUVEC) have shown that transcellular migration occurs in only a small proportion of leucocytes (5-10%) (Carman & Springer, 2004b; Cinamon et al., 2004). A much larger proportion of leucocytes undergo transcellular migration across microvascular endothelium such as human dermal (HDMEC) and human lung (HLMVEC) microvascular endothelial cells and human lymphatic endothelial cells (HLyECs) (30-40%)(Carman et al., 2007; Millan et al., 2006). *In vivo* studies have demonstrated that 100% of lymphocytes that cross the blood-brain barrier underwent transcellular migration in a murine model of multiple sclerosis. Figure 1-5 summarises the two routes of leucocyte transmigration. To our knowledge the transcellular route of transmigration has never been reported in hepatic sinusoidal endothelium. The lack of tight junctions and a classical basement membrane in hepatic sinusoidal endothelium suggest that different mechanisms may apply in this bed.

The adhesion cascade that has been described above is the generally accepted paradigm for lymphocyte interactions with endothelium but it is clear that there are tissue-specific differences. Increasing evidence over the last 15 years has confirmed that specific molecular determinants regulate lymphocyte homing to specific tissues. This has been demonstrated by the molecular regulation of lymphocyte homing to the skin and the intestines which has been described in recent years (Butcher & Picker, 1996; Robert and Kupper, 1999). Tissue-specific signals may also play a role in lymphocyte trafficking to the liver but a true liver-specific adhesion receptor has yet to be found (Lalor et al., 2002b).

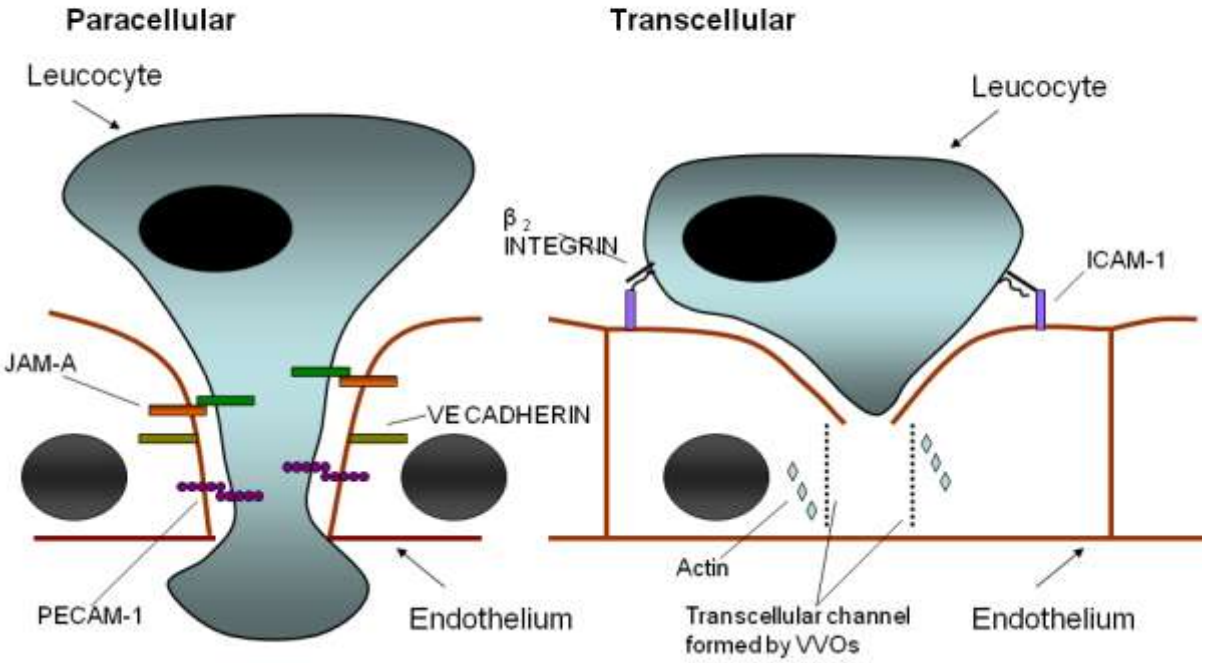


Figure 1-5 **Leucocyte transendothelial migration**

A schematic summary of the two possible routes that can be taken by transmigrating leucocytes. The paracellular route involves leucocytes binding to junctional molecules such as JAM-A and CD31 as well as the dissociation of tight junction molecules such as VE-Cadherin. Transcellular migration appears to be triggered by the binding of β2 integrins to ICAM-1 which leads to the formation of a transcellular channel comprising of VVOs and reorganisation of the actin cytoskeleton. JAM-A: Junctional adhesion molecule A, ICAM-1: Intracellular adhesion molecule- 1, VVO: Vesiculo-vacuolar organelle.

1.10 Lymphocyte recruitment to the liver

As mentioned in section 1.6.1, during the development of inflammatory liver diseases lymphocyte recruitment increases markedly and the intrahepatic localization of these infiltrating lymphocytes determines the nature and severity of disease. Parenchymal infiltrates lead to a lobular hepatitis as seen in viral infections, whereas portal infiltrates are seen in biliary diseases such as primary biliary cirrhosis and primary sclerosing cholangitis (Lalor et al., 2002b).

1.10.1 A unique environment for lymphocyte recruitment

Lymphocyte interactions with the hepatic microvasculature do not conform with the classical pathway of leucocyte adhesion described earlier (Lee and Kubes, 2008). Intravital experiments have shown that lymphocytes are capable of adhesion to different regions of the hepatic microvasculature but the majority adhere to the hepatic sinusoids (Wong et al., 1997). This is in contrast to most other organs where leucocyte extravasation takes place in the post-capillary venules. The hepatic sinusoids have several functions which set them apart from other vascular beds. They are the primary site for filtration in the liver and also display highly efficient scavenging function implicating the hepatic endothelium in the removal of soluble waste molecules and colloids from the circulation (Smedsrod, 2004). Thirdly they have an important role in antigen presentation being able to process and present antigens to CD4 and CD8 T cells and to tolerize immune responses to CD8 cells (Knolle and Limmer, 2001). In addition hepatic sinusoidal

endothelial cells (HSEC) display differences in adhesion molecule expression when compared to other endothelial cells. A fundamental example is the lack of expression of P-selectin under most circumstances *in vitro* or *in vivo* and a markedly reduced E-selectin expression (Adams et al., 1996). Furthermore there is no upregulation of these receptors on HSEC in diseases such as PBC (Steinhoff et al., 1993).

1.10.2 Classical endothelial adhesion molecules involved in lymphocyte recruitment to the liver.

Intravital experiments confirmed that lymphocyte recruitment within the hepatic sinusoids was selectin independent (Wong et al., 1997). In view of these findings, lymphocyte recruitment was initially thought to be due to 'physical trapping' but subsequent studies have demonstrated that lymphocytes do interact with sinusoidal endothelium via adhesion molecules. ICAM-1 is constitutively expressed on post-capillary venules and only minimally on true capillaries, in keeping with the fact that leucocyte recruitment occurs in the post-capillary venules (Iigo et al., 1997) but in the liver the density of ICAM-1 in the hepatic sinusoids is comparable to the central venules (Iigo et al., 1997). VCAM-1 is not expressed in normal liver tissue but it is markedly upregulated in inflammatory liver diseases (Volpes et al., 1992). Multiple strands of evidence implicate ICAM-1 and VCAM-1 in lymphocyte adhesion to the hepatic sinusoids. ICAM-1 deficient mice demonstrate reduced leucocyte adhesion to hepatic sinusoids (Wong et al., 1997) and VCAM-1 has been shown to mediate lymphocyte capture and

adhesion via α4 integrins. *In vitro* studies with human HSEC show reduced lymphocyte adhesion to hepatic sinusoids when VCAM-1 and ICAM-1 are blocked (Edwards et al., 2005; Lalor et al., 2002a).

1.10.3 Non-classical adhesion molecules involved in lymphocyte recruitment to the liver

ICAM-1 and VCAM-1 play a role in adhesion of leucocytes to endothelium, but the liver sinusoids also express another adhesion receptor known as vascular adhesion protein-1 (VAP-1) which appears to play a more specific role for lymphocyte recruitment to the liver. VAP-1 is a 170kDa homodimeric glycoprotein that has monoamine oxidase activity. It is expressed constitutively within hepatic sinusoids but is absent from other non-inflamed vessels in extralymphoid organs (Jalkanen and Salmi, 2001). *In vitro* VAP-1 mediates lymphocyte adhesion to human HSEC (Lalor et al., 2002a) and *in vivo* experiments on mice using intravital microscopy have suggested that VAP-1 has preferential activity for particular T cell subsets (Bonder et al., 2005). VAP-1 has also been implicated in lymphocyte recruitment to rodent liver transplants (Martelius et al., 2004). The precise role of VAP-1 is unclear. With regard to lymphocyte interaction with HSEC, VAP-1 mediates lymphocyte transmigration and intriguingly this transmigration can be inhibited with either blocking antibodies or enzyme inhibitors (Lalor et al., 2002a). How the enzyme activity of VAP-1 modulates recruitment is unclear. Provision of substrate to the amine oxidase of VAP-1 modulates the expression and function of ICAM-1 and VCAM-1 and MADCAM-1. Provision

of substrate to VAP-1 on monolayers of HSEC results in NFkB-dependent upregulation of ICAM-1 and VCAM-1 which results in increased lymphocyte adhesion from flow (Lalor et al., 2007). Some of these effects may be dependent on H_2O_2 generated by the catalytic activity of VAP-1 (Liaskou et al., 2011)

CD44 is another adhesion molecule which is widely expressed but appears to play a particular role in the liver. It has been shown to mediate sequestration of neutrophils in hepatic sinusoids during sepsis (McDonald et al., 2008) and is one of the receptors along with α4 integrins and VAP-1 which can mediate selectin-independent tethering in the liver sinusoids under flow. A recent study on neutrophil adhesion to hepatic sinusoids using intravital microscopy suggests that hyaluronan, a ligand of CD44, is increased on sinusoids in sepsis where it can support CD44-mediated adhesion. Moreover CD44 alone was sufficient and necessary for reversible neutrophil adhesion to hepatic sinusoids *in vivo* (McDonald et al., 2008).

MadCAM-1 is an adhesion molecule which is normally confined to mucosal endothelium in the bowel but can be induced on hepatic endothelium in some inflammatory situations (Grant et al., 2001). It plays a critical role in the compartmentalization of lymphocyte trafficking by recruiting gut specific mucosal lymphocytes. MadCAM-1 seems to be induced within the liver by as yet unknown factors, with the result that mucosal T cells are recruited to the liver where they drive immune-mediated liver disease (Adams and Eksteen, 2006).

1.10.4 The involvement of chemokines in liver disease

As described previously, chemokines play a critical role in several steps of the adhesion cascade involving triggering of integrin-mediated adhesion and migration responses (Rot and von Andrian, 2004). They also play a crucial role in the liver and chemokines secreted by liver cells contribute to the intrahepatic compartmentalization of lymphocyte recruitment. The CCR5 ligands CCL3-5 are strongly expressed on vascular endothelium within the portal tracts where they mediate lymphocyte recruitment in a range of inflammatory diseases including graft versus host diseases, immune mediated liver diseases and graft rejection (Goddard et al., 2001; Murai et al., 1999). Lymphocyte recruitment into the parenchyma via inflamed hepatic sinusoids is a feature of progressive liver injury in chronic viral infection and autoimmune disease and is associated with strong expression of the CXCR3 ligands CXCL9-11 on sinusoidal endothelium (Harvey et al., 2003; Shields et al., 1999). These chemokines are not only secreted by endothelium but by cholangiocytes, hepatocytes and stellate cells in the inflamed liver, but regardless of their source they can be presented on sinusoidal endothelium. For example the process of transcytosis (Middleton et al., 1997) allows chemokines secreted by hepatocytes or stellate cells to be transported to the luminal surface of the endothelial cell or chemokines secreted by cholangiocytes can be captured by retention in the proteoglycan-rich endothelial glycocalyx (Curbishley et al., 2005). *In vitro* experiments show that activation of CXCR3 on lymphocytes by its ligands promotes lymphocyte adhesion and transmigration across HSEC (Curbishley et al., 2005). *In vivo*

experiments suggest that CXCR3 and its ligands play a significant role in recruitment of virus specific CD8+ T cells to the murine liver (Hokeness et al., 2007).

Liver infiltrating lymphocytes also express the chemokine receptor CXCR6. This receptor is seen on CD4, CD8 and NKT cells (Boisvert et al., 2003). The CXCR6 ligand is a transmembrane chemokine, CXCL16 expressed by cholangiocytes in the inflamed liver and sinusoidal endothelium in the normal liver (Heydtmann et al., 2005). CXCR6 has been shown to mediate trafficking of NKT cells in murine sinusoids (Geissmann et al., 2005). The increased expression of CXCL16 on epithelial cells suggests that it may act to localize and retain effector lymphocytes at epithelial sites and biliary epithelium during viral hepatitis (Heydtmann et al., 2005). It has also been demonstrated that the chemokine CCL28 is present on inflamed endothelium, cholangicytes and hepatocytes. A high proportion of liver infiltrating T cells which expressed the receptor for CCL28 ,CCR10, were phenotypically and functionally T regulatory cells (Eksteen et al., 2006) which suggested that regulatory T cells were retained at bile ducts by the secretion of CCL28.

Aberrant expression of the gut chemokine CCL25 in the liver can promote the recruitment of mucosal T cells to the liver resulting in inflammatory disease in primary sclerosing cholangitis (PSC) (Eksteen et al., 2004). It promotes the homing of lymphocytes bearing the receptor CCR9 and suggests that

aberrant homing of mucosal T cells to the liver underlies the pathogenesis of PSC ((Adams & Eksteen, 2006).

1.10.5 Transendothelial migration across the hepatic sinusoids

The process of transmigration of lymphocytes across HSEC has several features which differ from that observed in other vascular beds. The tight junctions of vascular endothelium express PECAM/CD31 which has been shown to mediate leucocyte transmigration (Ley et al., 2007). Liver sinusoidal cells lack tight junctions, express lower levels of CD31 than vascular endothelium and blocking CD31 has no effect on neutrophil extravasation in the murine liver (Chosay et al., 1998). Additional experiments have demonstrated that junctional adhesion molecule-A (JAM-A) mediates neutrophil but not T cell extravasation into the liver (Khandoga et al., 2005) all of which suggests that novel mechanisms may apply. The precise molecular basis of lymphocyte transmigration across sinusoidal endothelium is not yet known. ICAM-1 and LFA-1 and VAP-1 have all been implicated in transmigration of lymphocytes and monocytes by our group using flow based adhesion assays and human HSEC (Aspinall et al., 2009) (Lalor et al., 2002a). VAP-1 is constitutively expressed and is not regulated by the same proinflammatory signals as ICAM-1 and VCAM-1. It is not yet clear how VAP-1 mediates adhesion and transmigration and nature of its ligand is not known. Interestingly, the enzymatic activity of VAP-1 can mediate NFk-B dependent activation of human HSEC leading to expression of adhesion molecules and secretion of chemokines (Lalor et al., 2007). The

fact that some of the VAP-1 substrates such as methylamine are constituents of food, wine and cigarette smoke suggest intriguing links between the gut and liver inflammation(Liaskou et al., 2011). Figure 1-6 summarises the major molecular determinants of lymphocyte recruitment within the hepatic sinusoids.

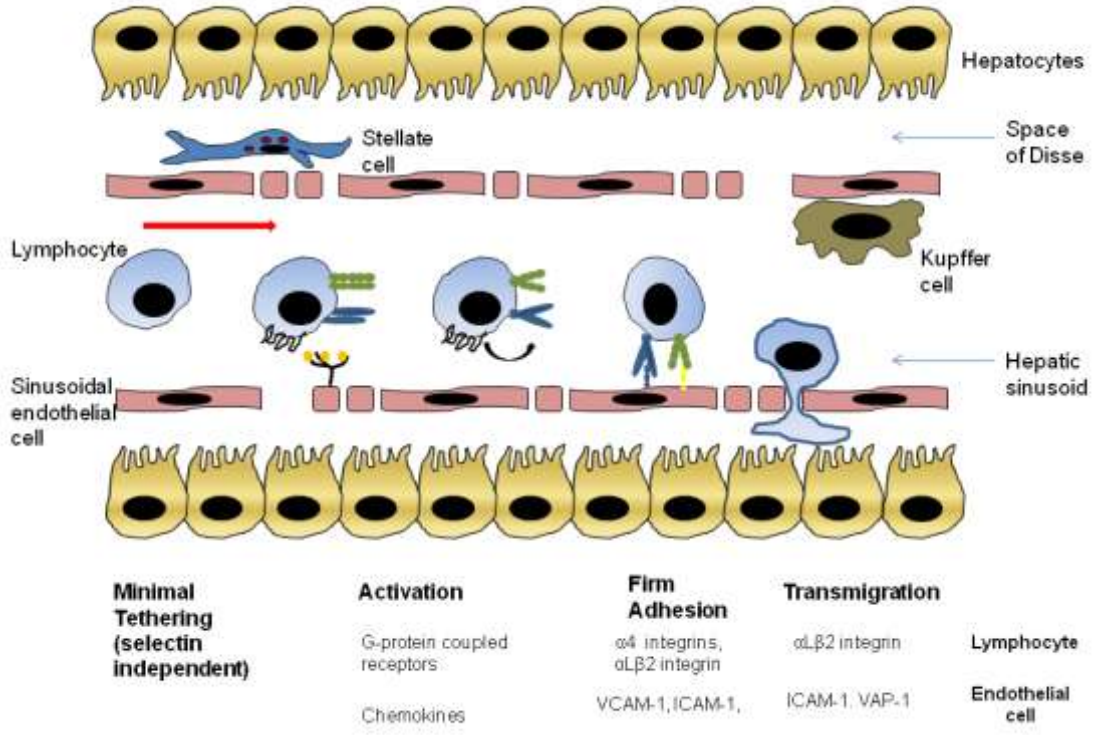


Figure 1-6 **Molecular determinants of lymphocyte recruitment within the hepatic sinusoids**

A schematic representation of lymphocyte recruitment via the hepatic sinusoids. Lymphocytes enter the hepatic sinusoids under conditions of flow. Following a brief tethering step they are exposed to chemokines on the endothelial surface. If they express the appropriate chemokine receptor they undergo activation which leads to firm adhesion and transmigration. The receptors involved each step are outlined on the bottom row.

1.10.6 The route taken by trafficking lymphocytes

Although, as discussed above, there has been a great deal of study into the molecular determinants of lymphocyte interactions with hepatic endothelium that promote homing to the liver, the precise anatomical route of lymphocytes trafficking through the liver is poorly understood. Recent *in vivo* studies have shed important light on this.

The trafficking of lymphocytes through the lymphatic system has been studied in detail, and in this situation naive lymphocytes continuously home to T cell areas via high endothelial vessels allowing them to interact with dendritic cells which have entered the lymph node via afferent lymphatic vessels. If lymphocytes are activated they are imprinted with receptors that allow them to migrate via blood to peripheral tissues whereas lymphocytes which do not recognise antigen leave the lymph node via the efferent vessels into the thoracic duct (von Andrian and Mempel, 2003). The liver has a large population of resident lymphocytes, which are continually patrolling from blood into the liver and back to hepatic lymph nodes via hepatic lymphatics (Xu et al., 2008). This is in keeping with the large antigen load that the liver receives from the gut and periphery. Recent studies in a rat model provide further insight into the route taken by lymphocytes within the liver (Xu et al., 2008). Using a model where the hepatic lymph nodes were either removed or ligated the authors showed that the transit time within the liver was approximately 3-4 hours, but lymphocytes appeared within the portal area within 30 minutes. Their studies suggest that most lymphocytes enter via the

sinusoids in the periportal regions with the subsequent route taken determined by the hepatic microenvironment. Lymphocytes may then exit the liver via lymphatic vessels located in the hepatic portal area to hepatic lymph nodes or be retained in the liver to carry out immune surveillance in the non-inflamed liver or as effector cells in the context of inflammatory disease.

The signals which mediate lymphocyte retention are unclear but there is evidence that other liver cell populations such as epithelial cells, stromal cells and kupffer cells play important roles. Kupffer cells, the resident liver macrophages, patrol the hepatic sinusoids ready to phagocytose foreign material. Interaction between Kupffer cells and other liver cell types profoundly effects their behaviour and secretion of cytokines (Alabraba et al., 2008). Stellate cells are fibroblasts situated in the Space of Disse and are central mediators of matrix formation during liver injury and fibrosis (Friedman, 2008). Our group has shown that stellate cells and activated liver myofibroblasts can support lymphocyte adhesion and promote migration suggesting to us that they may be critical regulators of post-endothelial migration and positioning of cells within the liver parenchyma (Holt et al., 2009). Hepatocytes have also been shown to directly influence the expression of adhesion molecules on sinusoidal endothelium in *in vitro* coculture models (Edwards et al., 2005). The levels of lymphocyte adhesion in these models were equivalent to those seen with stimulation by inflammatory cytokines. Once the lymphocytes have entered the liver, the mechanisms underlying their retention are still to be fully characterized. As

discussed above this may be as a result of interactions with epithelial cells or stromal cells but additional structures develop during some forms of chronic hepatic inflammation that may act as portals for ongoing recruitment and retention. Neovessels are formed in the portal tracts which are similar to HEVs seen in lymphoid tissue (Garcia-Monzon et al., 1995) and in both animals and humans they can become organized into 'portal tract associated lymphoid tissue' (PALT), (Grant et al., 2002; Yoneyama et al., 2001) tertiary lymphoid follicles within the portal tract which include discrete T and B cell areas and which contain dendritic cells and stromal cells. The development of this tissue provides an ideal environment for recruitment and retention of lymphocytes within the liver. This formation of tertiary lymphoid tissue appears to be partly explained by the presence of CCL19 and CCL21 which are expressed in lymphoid follicles in chronic inflammatory liver disease. In secondary lymphoid tissues CCL19 and CCL21 lead to the recruitment and organization of naïve T-cells and dendritic cells and they have been implicated in the formation of tertiary lymphoid tissues in chronic inflammation as part of the process of lymphoidneogenesis (Grant et al., 2002).

1.11 Novel aspects of hepatic endothelial function which may provide additional mechanisms for leucocyte recruitment

Despite this body of work there is still a requirement for further studies to elucidate the molecular determinants of lymphocyte recruitment to the hepatic microvasculature. In previous sections I have discussed the

importance of lymphocyte interactions within the unique sinusoidal vascular bed and the receptors which have been shown to be important. However, the unusual nature of the hepatic sinusoidal endothelium provides alternatives which may have functional significance. The sinusoidal endothelial layer is discontinuous, lacks a basement membrane and the cells themselves have fenestrae (Braet & Wisse, 2002). Within these cells there are numerous endocytic vesicles and lysosome-like vacuoles in comparison to other endothelial cells (Aird, 2007b). These morphological properties are associated with the unique functional properties of HSEC. The fenestrae allow HSEC to acts as a communication route between the sinusoidal lumen and the Space of Disse. They are organised in groups of structures known as 'sieve plates' and the filtration they permit is dynamic allowing passage of fluids, solutes and particles in a bidirectional manner (Braet & Wisse, 2002). Along with this filtration capacity, HSEC also have the ability to take up waste molecules from blood via endocytic vesicles. Many cells have the ability to perform endocytosis but HSEC are unique in that they can endocytose a large number of different ligands in a highly efficient manner (Smedsrod, 2004). It is important to make a distinction between the uptake of particles by HSEC and Kupffer cells, especially since they are in such close proximity. Kupffer cells perform phagocytosis which involves the uptake of particles from the blood above a certain size (approximately 1 μm or greater), whereas HSEC perform receptor mediated endocytosis of colloids and soluble macromolecules (Smedsrod, 2004). These physiological functions of HSEC are essential for the maintenance of homeostasis in terms of degradation of

macromolecules and as well as allowing hepatocytes to interact with the peripheral circulation. The ability to perform such efficient endocytosis and scavenge a diverse array of ligands is partly explained by the C-type lectins that are expressed on these cells.

1.11.1 C-Type Lectins

The C-type lectin-like receptors (LLRs) comprises a diverse family of proteins. They are related by a structural motif which was originally identified in the carbohydrate–recognition domain of the mannose-binding lectin. Whilst many proteins in this family bind specific sugar structures that mediate biological events such as cell-cell adhesion and innate immune responses in a calcium dependent manner, the motif in question has subsequently been identified in proteins which do not demonstrate calcium binding or carbohydrate specificity (Robinson et al., 2006). The groups within this family are listed in Table 1-2.

Table 1-2 **C Type Lectin Groups**

Group I	Proteoglycans
Group II	Type 2 receptors-eg Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) and Liver/Lymph node-specific intercellular adhesion molecule 3- grabbing non-integrin (L-SIGN)
Group III	Collectins eg Mannose binding Proteins
Group IV	Selectins
Group V	NK- cell receptors group- NK cell receptors and dectin-1, C-type lectin-like receptors (CLEC)-1 and -2).
Group VI	Mannose receptor family-eg DEC205 and mannose receptor
Group VII	Free CTLD
Group VIII	Simple type 1 receptors eg Layilin
Group IX	Tetranectin family
Group X	Polycystin
Group XI	Attractin family
Group XII	CTLD with acidic neck/ Eosinophil major basic protein
Group XIII	Integral membrane protein
Group XIV	Endosialin family

They are expressed on a variety of cell-types but predominantly on myeloid cells (dendritic cells and macrophages), the receptors in group V are specifically found on NK cells. Consistent with this expression profile, they play important roles within the immune-system, especially with regard to inflammation and immunity towards tumour and virally-infected cells. They have the ability to bind exogenous ligands and like toll-like receptors (TLRs) can recognise pathogen-associated molecular patterns. Their expression also extends to specialised vascular beds including lymphatic endothelium and the hepatic sinusoidal endothelium, where they have been associated with a homeostatic role. Importantly, unlike TLRs, these receptors do not discriminate between self and non-self and readily bind endogenous ligands (Cambi and Figdor, 2003).

Receptor mediated endocytosis by HSEC utilises several members of the LLR family. These receptors include the collagen-alpha chain receptor (COLLA-R) which binds free alpha chains and therefore allows HSEC to take up collagen waste molecules (Ostgaard et al., 1995) as well as the Fc-gamma receptors (FcgRs) which bind the Fc domain of Immunoglobulin G (Johansson et al., 1996; Skogh et al., 1985). One of the most extensively studied C-type lectins on HSEC is the mannose receptor (MR). It has been shown to bind several ligands, many of which are endogenous waste molecules, including lysosomal hydrolases, denatured collagen and c-terminal procollagen peptides. (Malovic et al., 2007; Martinez-Pomares et al., 2001; Smedsrod et al., 1990).

Two more recently discovered LLRs are stabilin-1 and stabilin 2 which are both expressed on sinusoidal endothelium (Politz et al., 2002). These receptors are homologues consisting of multi domain proteins and a C-type lectin-like hyaluronan-binding link domain. The presence of this link domain suggests that these receptors should bind hyaluronan (HA), this is indeed true of Stabilin 2 which has been shown to be the major HA binding receptor of HSEC (McCourt et al., 1999). Stabilin-1, on the other hand, does not appear to bind HA but has also been separately identified as FEEL-1 (fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1) and CLEVER-1 (common lymphatic endothelium and vascular endothelium receptor-1) and unlike stabilin-2 it is expressed on macrophages (Martens et al., 2006). As well as binding HA, stabilin-2 is also the receptor for several other blood borne scavenger ligands (McCourt et al., 2004). These include amino-terminal propeptides of types I and III collagen (McCourt et al., 1999; Melkko et al., 1994) and advanced glycation end products (AGEs) (Hansen et al., 2002) all of which are endocytosed by stabilin-2 in studies of rat liver sinusoidal cells.

Other LLRs which are expressed on HSEC come from the type-2 receptor subclass and are called dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) and liver/lymph node-specific intercellular adhesion molecule 3- grabbing non-integrin (L-SIGN) (Bashirova et al., 2001; Lai et al., 2006). LLRs in the type 2 group have a single C-terminal C-type lectin domain which is separated from the transmembrane domain by a neck region. The members of this group are involved in varied functions from cell-

cell interaction to uptake of serum glycoproteins. DC-SIGN was originally identified on dendritic cells and has been shown to internalize antigen and promote antigen presentation as well as cell-cell interaction via ICAM-3 (Engering et al.). There is now increasing evidence that DC-SIGN and L-SIGN may also play a role in the uptake of blood-borne viruses into the liver. Both these receptors have been shown to capture the human immunodeficiency virus (HIV) by binding to envelope glycoprotein gp120 (Bashirova et al., 2001). They have been implicated in the capture of Hepatitis C via interaction with the hepatitis C virus glycoprotein E2 (Lai et al., 2006). A third lectin with a similar structure to DC-SIGN and L-SIGN has been identified and designated the liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin) (Liu et al., 2004). This lectin has been shown to be co-expressed with L-SIGN and is encoded in the same cluster of lectin encoding genes as DC-SIGN and L-SIGN (Liu et al., 2004). Whereas DC-SIGN and L-SIGN have an almost identical ligand binding specificity, LSECtin exhibits important differences despite expression and structural similarities. LSECtin has been shown to interact with proteins from the Ebola virus and the SARS coronavirus but, unlike DC-SIGN and L-SIGN, does not interact with HIV or Hepatitis C glyocproteins (Gramberg et al., 2005).

1.12 A role for C-type lectins in lymphocyte adhesion and recruitment.

More recently, there has been interest in the ability of scavenger receptors to act as cell adhesion molecules. The selectins which are classed as group IV LLRs are well known for their role in mediating lymphocyte adhesion and homing to lymph nodes (Ley et al., 2007), but other LLRs have also been shown to have leucocyte binding properties. An example of this is the oxidised low-density lipoprotein receptor-1 (LOX-1) which is a type II membrane glycoprotein with a C-type lectin like structure in the extracellular domain. It is an endocytosis receptor for atherogenic oxidized low-density lipoprotein (LDL) (Sawamura et al., 1997). LOX-1 has been shown to support rolling and adhesion of mononuclear leucocytes in static and flow conditions and to promote leucocyte recruitment in an *in vivo* model of endotoxin induced inflammation (Honjo et al., 2003). In this model blockade of LOX-1 led to an amelioration of endotoxin induced uveitis.

Whilst LOX-1 is a scavenger receptor expressed on vascular endothelium, scavenger receptors expressed preferentially on lymphatic endothelium have also been implicated in lymphocyte recruitment. The mannose receptor has been shown to play an important role in lymphocyte binding to lymphatic endothelium and its ligand in this process has been identified as L-selectin (Irjala et al., 2001). Lymphatic endothelium expresses several LLRs that are found on HSEC including the mannose receptor, CLEVER-1 and stabilin-2 (Martens et al., 2006). Stabilin-2 has been reported to mediate the firm

adhesion of lymphocytes to HSEC via interaction with the integrin αMβ2 (Jung et al., 2007). Aside from binding lymphocytes stabilins have also been implicated in other cell adhesion processes. Stabilin-2 is present in the sinusoidal endothelial cells of the bone marrow where it plays a scavenging role but also mediates adhesion of stem and progenitor cells by a hyaluronan dependent mechanism (Qian et al., 2009).

As discussed earlier, stabilin-2 has a clear scavenging function in endothelial cells but the role of its close homologue stabilin-1 (also known as CLEVER-1 or FEEL-1) in endothelial cells is less well understood. The following section summarises the current understanding of this receptor's function and regulation.

1.13 Common Lymphatic Endothelial and Vascular Endothelial Receptor-1 (CLEVER-1)

CLEVER-1 was originally identified as the MS-1 antigen which was shown to be a marker of sinusoidal endothelium of the spleen, lymph node, liver and adrenal cortex (Goerdt et al., 1991). Ultrastructural analysis of splenic sinusoidal endothelial cells by electron microscopy demonstrated the antigen in these cells as small clusters of cytoplasmic reactivity deposited at zones of contact between sinusoidal endothelial cells (Goerdt et al., 1991). Further studies of skin lesions demonstrated that MS-1 antigen can be identified on endothelial cells of continuous origin in certain pathological conditions such as psoriasis, T cell lymphomas and melanoma metastasis (Goerdt et al., 1993). These studies also identified the presence of MS-1 antigen on

dendritic cells and macrophages *in vivo* and *in vitro*. Interleukin 4 and dexamethasone were shown to strongly induce the expression of MS-1 on cultured monocytes (Goerdt et al., 1993).

1.13.1 Structure and function of CLEVER-1

The antigen recognised by MS-1 antibody was subsequently identified as a 280kDa type 1 transmembrane protein with multiple epidermal growth factor-like repeats, seven fasciculin domains and a single Link domain and a short cytoplasmic tail (Politz et al., 2002) (Figure 1-7). The protein was named stabilin-1 and a homologous protein was also cloned in parallel named stabilin-2 which, as mentioned before, is the hyaluronan receptor on hepatic sinusoidal endothelium (McCourt et al., 1999; Politz et al., 2002). Further analysis of stabilin-1 localised it to chromosome 3 and scanning for sorting signals identified a a tyrosine-based motif and a dileucine motif involved in protein kinase C/ kinase CK2-dependent sorting into the endosomal compartment (Politz et al., 2002). The authors suggested that these three sorting signals indicated that stabilin-1 shuttled between the plasma membrane and endosomal compartments. Further studies of alternatively activated macrophages have demonstrated that stabilin-1 is involved in two intracellular trafficking pathways and in early endosomes and the trans Golgi network (Kzhyshkowska et al., 2004).

The upregulation of stabilin-1 in alternatively activated macrophages has led several groups to try and understand its role in this subset of macrophages. Classical activation of macrophages is mainly induced by LPS or TH1-related

cytokines whereas alternative activation is mediated by glucocorticoids or TH2-related cytokines such as IL-4, IL-10, IL-13. Macrophages are generally known to orchestrate immune responses to pathogens but they also play an important role in tissue homeostasis and wound healing (Hume, 2006). The homestatic functions of macrophages are performed by the alternatively activated subset and involve clearance of exogenous components in the extracellular environment. These macrophages are characterised by the expression of molecules that lead to tolerance and the resolution of inflammation and have a high phagocytic capacity (Gratchev et al., 2005). It has been suggested that stabillin-1 contributes to this process by mediating the uptake of certain breakdown products as well as separate intracellular sorting of other molecules. Subsequent functional studies have shown that stabilin-1 performs endocytosis of substances such as acetylated LDL and secreted protein acidic and rich in cysteine (SPARC) as well as the intracellular sorting of a novel chitinase-like protein (Kzhyshkowska et al., 2005; Kzhyshkowska et al., 2006b; Kzhyshkowska et al., 2006c). It has also been shown implicated in endocytosis of placental lactogen by alternatively activated macrophages (Kzhyshkowska et al., 2008). These homeostatic functions may be coupled with the ability of stabilin-1 to prevent excess tissue damage during inflammation or autoimmune responses. This is highlighted by a recent study which demonstrated that stabilin-1 functions as a receptor on macrophages for the removal of cell corpses (Park et al., 2009). Here Stabilin-1 was shown to bind to phosophatidlyserine (PS) presented on the surface of these cell corpses.

The sequences corresponding to stabilin-1 were cloned separately by another group who, in view of its structure named it FEEL-1 (fasciculin, EGF-like, laminin-type EGF-like link domain- containing scavenger receptor-1) (Adachi and Tsujimoto, 2002). FEEL-1 was shown to bind acetylated LDL and AGEs as well as gram negative and gram positive bacteria (Adachi & Tsujimoto, 2002; Tamura et al., 2003). These binding studies were performed in CHO cells transfected with FEEL-1. A monoclonal antibody to FEEL-1 also influenced the vascular remodelling of human umbilical vein endothelial cells suggesting that FEEL-1 played a role in angiogenesis (Adachi & Tsujimoto, 2002). Subsequent studies broadened the repertoire of FEEL-1 by demonstrating that it also binds heat shock protein 70 (HSP70) (Theriault et al., 2006). HSP70 is an intracellular chaperone that can be released from tumour cells and therefore may have a role in anti-tumour immunity (Calderwood et al., 2005).

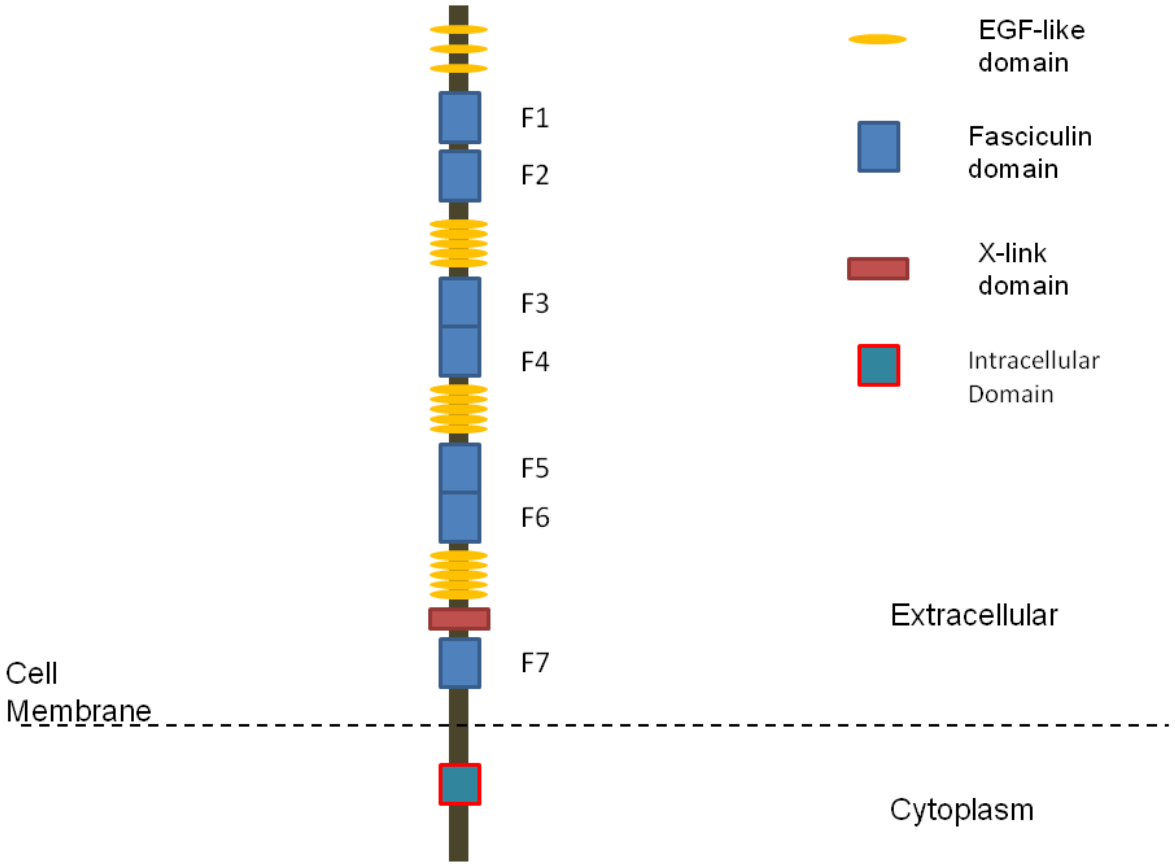


Figure 1-7 **The structure of CLEVER-1**

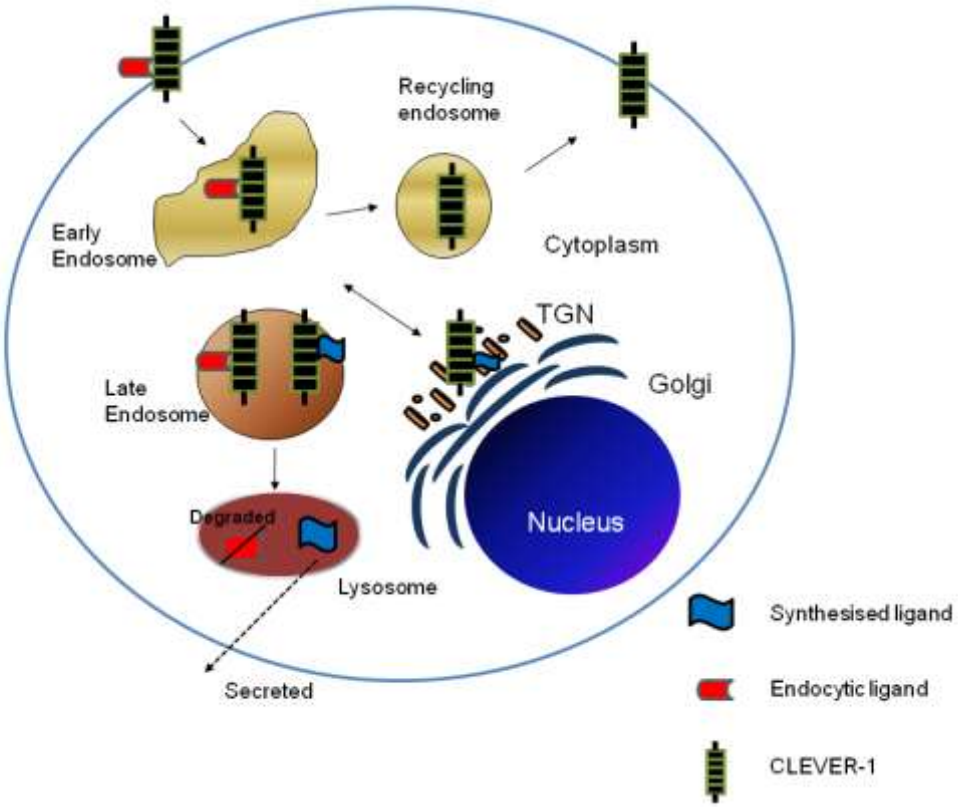
Schematic outline of the structure of the Common Lymphatic and Vascular Endothelial Receptor-1 (CLEVER-1) also known as Stabilin-1 and FEEL-1.

1.13.2 The intracellular trafficking of CLEVER-1

Functional studies of CLEVER-1/stabilin-1 identified its involvement in two intracellular pathways 1) as a classical endocytic receptor undergoing endocytosis and recycling and 2) as an intracellular sorting receptor shuttling between the endosomal compartment and the trans-Golgi network (**Error! eference source not found.**). The role of CLEVER-1/Stabilin-1 in these two pathways is regulated by different factors.

A model utilising transfected CHO cells demonstrated that CLEVER-1/stabilin-1 internalised extracellular ligands and then translocated them from early endosomes to late endosomes. Treatment with wortmannin led to a block of receptor trafficking to the late endosomal compartment (Kzhyshkowska et al., 2005). Wortmannin is a specific inhibitor of phosphatidylinositide-3 (PI3K) and therefore the trafficking of CLEVER-1/stabilin-1 from early to late endosomal compartment is PI3K dependent. More recently, sorting nexins (SNXs) have been implicated in regulating the intracellular trafficking of CLEVER-1/stabilin-1. SNXs are a family of proteins which are involved in intracellular membrane trafficking (Carlton et al., 2005). Specifically, SNX17 directly interacts with the cytoplasmic domain of CLEVER-1/stabilin-1 and is an important regulator of CLEVER-1/stabilin-1 shuttling between the plasma membrane and early endosomes. Knockdown of SNX17 led to decreased surface expression of CLEVER-1/stabilin-1 and increased degradation (Adachi and Tsujimoto, 2010)**.**

In contrast, the intracellular sorting pathway of CLEVER-1/stabilin-1 appears to be mediated by Golgi-localized, γ-ear-containing Arf (ADP-ribosylation factor)-binding proteins (GGAs). GGAs are monomeric clathrin receptors that mediate the intracellular sorting of several receptors (Ghosh et al., 2003; He et al., 2005) The CLEVER-1/stabilin-1 cytoplasmic tail contains distinct binding sites for GGAs and this interaction appears to be specific for intracellular sorting. Deletion of these binding sites on CLEVER-1/Stabilin-1 led to the abrogation of intracellular sorting by this receptor but had no effect on receptor mediated endocytosis (Zhang et al., 2009).



1-8 CLEVER-1 Trafficking Pathways

Schematic representation of the current understanding of CLEVER-1 trafficking. CLEVER-1 can bind endocytic ligand and as shown transports this to early and late endosomes before dissociation which leads to ligand degradation. Ligand free receptor can recycle back to the plasma membrane. CLEVER-1 can also shuttle between the transgolgi network (TGN) and late endosomes.It has been suggested that the ligands bound in this pathway are destined for secretion via the lysosomal pathway.

These CLEVER-1/Stabilin-1 functional and regulation studies have concentrated on alternatively activated macrophages or transfected cell lines, parallel studies in endothelial cells have given less conclusive results. Studies in pig and rat endothelial cells showed that CLEVER-1/stabilin-1 was mainly found within the early endosomes but was also on the cell surface (Hansen et al., 2005a). CLEVER-1/Stabilin-1 did not recognise classical scavenger ligands such as advanced glycation end products (AGEs) and collagen N-terminal propeptides, whilst its homologue, stabilin-2, had a clear scavenger profile, (Hansen et al., 2005a). Studies in human lymphatic endothelium demonstrated that stabilin-1 underwent rapid cycling between the plasma membrane and early endosomes and confirmed that the stabilin-1 Link-domain did not bind hyaluronan (Prevo et al., 2004).

1.13.3 The role of CLEVER-1 in lymphocyte recruitment

An antibody against CLEVER-1, 3-372, was initially shown to clearly stain lymphatic endothelium both in afferent and efferent lymphatics (Irjala et al., 2003b). The antibody was developed from a programme that raised monoclonal antibodies against isolated efferent lymphatic vessels of human lymph nodes. The molecule identified by 3-372 was also present on vascular endothelium in high endothelial venules (HEVs), hence it was named the common lymphatic endothelial and vascular endothelial receptor (CLEVER-1). Static adhesion assays on lymph node sections demonstrated that 3-372 blocked lymphocyte adhesion to HEVs and lymphatic endothelium. 3-372 also cross reacted with rabbit CLEVER-1 allowing i*n vivo* studies to be

carried out. In these studies rabbits were challenged with keyhole limpet hemocyanin injected into the footpad which led to an immune response and enlargement of draining lymph nodes. The animals given anti-CLEVER-1 antibody had significantly smaller draining lymph nodes and lymphatic sections showed significantly fewer lymphocytes in comparison to control animals (Irjala et al., 2003b).

Further analysis by immunoblotting demonstrated that CLEVER-1 was a molecule of approximately 270-300 kDa with several isoforms. Affinity chromatography allowed purification of the protein and after cleavage with trypsin and mass spectrometry the sequences identified were identical to stabilin-1 (Irjala et al., 2003b). Tissue lysate analysis for CLEVER-1 protein showed that there were three different isoforms seen in lymph node, tonsil and synovium. These isoforms varied in intensity suggesting the existence of splice variants or different glycoforms (Irjala et al., 2003b).

Expression of CLEVER-1 *in vivo* was studied in normal and diseased states by analysing normal skin samples and inflamed samples from psoriatic lesions. This demonstrated that CLEVER-1 was present on lymphatic vessels in the normal state but in the context of inflammation CLEVER-1 was also seen on chronically inflamed HEV- like neo blood vessels (Salmi et al., 2004). It was clear from these findings that CLEVER-1 was regulated differently on lymphatic and vascular endothelium.

Although CLEVER-1 supported lymphocyte adhesion in static assays and *in vivo* blockade of CLEVER-1 resulted in a reduced inflammatory response in

lymph nodes (Irjala et al., 2003b), the exact role of CLEVER-1 was not known. This led to studies of lymphocyte interactions with human lymphatic endothelial monolayers under the conditions of physiological flow which revealed that CLEVER-1 contributes to the rolling and transmigration steps of lymphocyte recruitment to lymphatic endothelium (Salmi et al., 2004). Expression studies on human tissue demonstrated that CLEVER-1 was expressed on lymphatic endothelium constitutively and only on vascular endothelium when it was inflamed. Thus it was proposed that CLEVER-1 may play a dual role: 1) in the diapedesis of lymphocytes into non-lymphoid tissue during inflammation and 2) the egress of lymphocytes from secondary lymphoid organs via efferent lymphatic endothelium (Irjala et al., 2003b; Salmi et al., 2004).

Recent studies have provided more *in vivo* evidence for the role of CLEVER-1 in lymphocyte homing. Blocking antibodies to CLEVER-1 efficiently prevented migration of lymphocytes into draining lymph nodes in both murine and rabbit models. The role of CLEVER-1 on inflamed vascular endothelium was also investigated using a mouse model of peritonitis. In these experiments, treatment with a blocking antibody to murine CLEVER-1 led to significant reduction of lymphocyte trafficking at the site of inflammation (Karikoski et al., 2009a).

In conclusion, CLEVER-1 is a lectin-like receptor which has been identified in alternatively activated macrophages and the specialised vascular beds of lymph nodes and the liver. In macrophages there is evidence that CLEVER-

1 contributes to tissue homeostasis and scavenging. In lymphatic endothelium there is *in vitro* and *in vivo* evidence that CLEVER-1 plays a role in lymphocyte recruitment, specifically contributing to transmigration.

This thesis tested the hypothesis that CLEVER-1 is involved in the recruitment of lymphocytes via human hepatic sinusoidal endothelium.

Specific Aims

1. Document the expression of CLEVER-1 in normal and diseased human liver tissue.
2. Determine the factors responsible for the expression at these sites.
3. Determine the contribution of CLEVER-1 to lymphocyte adhesion to HSEC and assess if CLEVER-1 had preferentially activity for particular lymphocyte subsets.

CHAPTER 2

MATERIAL AND METHODS

2.1 Human liver tissue

We obtained normal liver tissue from margins of hepatic resections carried out for hepatocellular cancer and donor tissue that was not used or excess to requirement for transplantation. Diseased liver tissue from explanted livers was obtained from transplantation explants as part of the Birmingham liver transplant programme. All tissue was obtained with prior consent and ethically approved.

2.2 Cell culture and isolation of Human Sinusoidal Endothelial Cells (HSEC)

2.2.1 Plastics used for cell culture

Sterile pipettes, 25 cm^2 and 75cm^2 flasks, sterile 15ml and 50 ml tubes and sterile cryovials were all purchased from Corning CoStar Incorporated, Bucks. Sterile 96 well plates were purchased from Becton Dickinson Labware. 20ml plastic tubes and 5ml bijous were purchased from Ramboldi .

2.2.2 Cell culture work

All tissue culture was performed in class II microflow safety cabinet. At all times aseptic technique was followed and cabinets were sprayed with industrial methylated spirit (IMS;Adams Healthcare, Leeds UK) before and after use.

2.2.3 HSEC isolation

I used a protocol established in our laboratory to isolate HSEC (Lalor et al., 2002a). Approximately 50g of liver tissue was taken from diseased explanted livers or normal resections, mechanically chopped using sterile scalpels, enzymatically digested with 0.2% collagenase Type 1A (Sigma, UK) filtered and further purified by density gradient centrifugation over Percoll^{TM} (Amersham Biosciences, Bucks UK). HSEC were isolated using negative magnetic selection with HEA-125 antibody (50µg/ml, 1:10 Progen Biotec, GMBH, Germany) to deplete biliary epithelial cells, followed by positive selection with CD31 antibody (10µg/ml, DAKO, UK).and dynalbeads conjugated with goat anti-mouse mAbs (Dynal,UK.) The cells were maintained in human endothelial basal medium (Invitrogen, Paisley, UK) supplemented with 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin (Sigma, UK) 10% Human serum, 10ng/ml of vascular epidermal growth factor (VEGF Peprotech UK) and 10ng/ml of hepatocyte growth factor (HGF Peprotech UK). The cells were transferred to a rat tail collagen (Sigma, UK) 25cm^2 flask and cultured at 37^{0}C. Once the cells were confluent they were passaged to a 75cm^2 flask.

2.2.4 Cell culture and passage

Cells were cultured in standard tissue culture plastic flasks in complete media (as detailed above) and maintained at 37^{0}C in a humidified 5% CO_2 incubator. Cell growth was assessed using an inverted phase contrast microscope (Olympus IX50). Adherent cells formed projections when

attached to the plastic surface and cells in suspension remained rounded, phase-bright and symmetrical. The cultures were monitored daily for normal cell morphology, media colour and cell density. Where appropriate, cells were passaged using proteolytic enzymes (TrypLE™ Express (x1) stable trypsin like enzyme with Phenol red :Invitrogen, UK). Initially, the media in the flasks was discarded and cells were washed in PBS. An appropriate volume of TrypLE was added and the cells were agitated gently until they detached. PBS was then added to the cells and the suspension was centrifuged at 550g for 5 minutes. The pelletted cells were then resuspended in media and transferred to new culture flasks.

2.2.5 Freezing and storing Cells

For long term storage cells were preserved in liquid nitrogen. After detachment and pelleting the cells were resuspended in freezing media consisting of 95% FCS+5% DMSO (Sigma, UK). Cells were transferred to cryovials and placed in a MrFrosty™ freezing container (Wessington Cryogenics, UK) which was transferred to -80^0C to permit gradual cooling to prevent crystal formation within the cell cytoplasm. Once cells had been stored at -80^0C overnight they were transferred to liquid nitrogen for long-term storage. When required cells were taken out of liquid nitrogen, thawed and washed in PBS and centrifuged to remove cellular debris. Cells were counted and viability was assessed using trypan blue followed by resuspension and culture in appropriate media.

2.3 Isolation of peripheral blood lymphocytes and lymphocyte subsets.

2.3.1 Isolation of peripheral blood lymphocytes

Venous blood was collected from healthy volunteers or patients undergoing venesection for haemochromatosis, at the Queen Elizabeth Hospital Birmingham, Liver Outpatients Clinic. The mononuclear fraction was purified by density gradient centrifugation over lympholyte (Lympholyte-H, VH Bio, UK) at 550g for 25 minutes. Cells were then incubated on plastic for 1 hour to allow adherence of monocytes before aspiration of the lymphocyte-enriched supernatant. Lymphocytes were then resuspended to a known cell count in media appropriate for the desired application.

2.3.2 Isolation of CD4 and CD8 lymphocyte subsets

Peripheral blood mononuclear cells were purified as described above in section 2.3.1. The cell fraction was washed, pelleted and viability was confirmed following which 5x 10^7 cells were suspended in 500µl of isolation buffer (PBS containing 0.1% (v/v) BSA and 2 mM EDTA) with 100µl of heat inactivated FCS and 100 µl of Antibody mix from either the human CD8 T cell isolation kit (Dynabeads® Untouched™ Human CD8 Tcells, Invitrogen, UK) or CD4 T cell isolation kit (Dynabeads® Untouched™ Human CD4 Tcells, Invitrogen, UK) depending on which population was being isolated. This was followed by an incubation step for 20 minutes at 4^0C on a mixer which provided tilting and rotation. The cells were then washed and centrifuged at

350g for 8 minutes. The cells were resuspended in 500µl of isolation buffer followed by addition of 500µl of magnetic beads supplied with the isolation kits (pre-washed as per manufacturer's instructions). The cells were incubated with the beads for 15 minutes at room temperature with gentle tilting and rotation. Following this the cells were vigorously pipetted followed by addition of 5mls of isolation buffer and placed in a magnet for 2 minutes. The supernatant containing the untouched T cell population was transferred to another tube. The negatively isolated cells were washed at 350g for 8 minutes and then resuspended in RPMI with 10% (v/v) FCS.

2.3.3 Isolation of CD4 effector and CD4 regulatory T cell subsets

Peripheral blood mononuclear cells were freshly prepared as described above and viability was confirmed following which $2x10^8$ mononuclear cells were resuspended in 1 ml of cold isolation buffer (PBS containing 0.1% (v/v) BSA and 2 mM EDTA) with 400 µl FCS and 400µl of antibody mix from the $CD4^+CD25^+$ T cell isolation kit (Dynabeads® Regulatory $CD4^+CD25^+$ T cell Kit, Invitrogen, UK). The cells were incubated for 20 minutes at 4^0C on a mixer with tilting and rolling followed by a wash and centrifugation for 8 minutes at 350g. The cells were resuspended in 4 ml of cold isolation buffer with the addition of 2 ml of depletion magnetic Dynabeads® from the isolation kit and incubated for 15 minutes at room temperature on a mixer with rolling and tilting. The bead bound cells were vigorously pipetted, added to 6 mls of isolation buffer and placed in a magnet for 3 minutes. The supernatant, containing the bead-free CD4 T cell fraction, was removed,

washed and centrifuged at 350g for 8 minutes. The cell pellet was resuspended at a concentration of 1.5 $x10^7$ cells ml^{-1}. 400µl of Dynabeads® CD25, supplied with the isolation kit, were added to 2 ml of CD4 cell suspension and incubated for 25 minutes at 4^0C on a mixer which provided tilting and rotation. The cell suspension was then placed in a magnet for 1 minute and the supernatant was removed. This supernatant contained the $CD4^+CD25^-$ T cell fraction which was washed pelletted and resuspended in RPMI 10% v/v FCS. The bead bound cells were resuspended in 1 ml of RPMI 1% v/v FCS and incubated with 160 µl of DETACHaBEAD®, supplied with isolation kit, for 45 minutes at room temperature with gentle tilting and rotation. The cell suspension was placed in a magnet for 1 minute and the supernatant containing the $CD4^+CD25^+$ population was removed, washed and centrifuged at 350g for 8 minutes followed by resuspension in RPMI 10% v/v FCS. The CD25- fraction generated was used as the effector population and the CD25+ fraction was used as the regulatory population.

2.3.4 Isolation of B cells

Mononuclear cells were freshly prepared from venous blood as above and were resuspended to 2.5 $x10^7$cells ml^{-1} in isolation buffer (PBS containing 0.1% v/v BSA 2 mM EDTA). 100µl of pre-washed Dynabeads (as per manufacturer's instructions) from the B cell isolation kit (Dynabeads® CD19 pan B, Invitrogen, UK) were added to 4 mls of cell suspension. Cells were incubated for 20 minutes at 4^0C with gentle tilting and rolling. The cell suspension was then placed in a magnet for two minutes and the

supernatant discarded. The bead bound cells were washed four times, on each occasion 4 ml of buffer was added to the cells and the solution was placed in a magnet for 1 minute following which the supernatant was removed. The cells were then resuspended in 1ml of RPMI 1%v/v FCS and incubated with 40µl of bead detachment solution (DETACHaBEAD® CD19, Invitrogen, UK) for 45 minutes with gentle tilting and rotation at room temperature. Following this the cell solution was pipetted vigorously and placed in a magnet for 1 minute. The supernatant containing the cells was removed, washed in RPMI 1%v/v FCS, centrifuged for 6 minutes at 400g and resuspended in RPMI 10% v/v FCS.

2.3.5 Assessment of cell purity using flow cytometry

The purity of HSEC isolates and lymphocyte subsets isolations was confirmed by flow cytometry. HSEC were grown to confluence in a T25 flask and then trypsinised followed by washing in ice cold PBS and centrifugation. The cell pellet was resuspended in ice cold wash buffer (PBS containing 10% (v/v) FCS) at $1x10^6$ cells/ml. 100µl of cell suspension was incubated with mouse anti-human CD31 (R and D systems) at 5µg/ml for 30 minutes at 4^0C in the dark. Control samples were labelled with an isotype matched control IgG1 antibody (DAKO, UK) at 5µg/ml. The cell suspensions were washed and incubated with a goat anti mouse FITC conjugated secondary antibody (Cambridge Biosciences,UK) 1 in 50 dilution of stock for 30 minutes at 4^0C in the dark followed by a further wash and resuspension in wash buffer prior to

flow cytometry. Samples were run on a Cyan flow cytometer and results were analysed using a Summit Software (Dako Cytomation, UK).

Purity of CD4, CD8 and B cell isolations was assessed by resuspending lymphocyte samples in ice-cold wash buffer at $1x10^6$ cells ml^{-1}. 100µl of cell suspension was incubated with appropriate fluorochrome-labeled antibody for 30 minutes at 4^oC. Control samples were labeled with a fluorochrome-labeled isotype matched controls. The cell suspension was washed and resuspended in wash buffer prior to flow cytometry.

Purity of T regulatory cell isolations was assessed by surface and intracellular staining. Lymphocytes were resuspended in ice-cold wash buffer at $1x10^6$ cells ml^{-1}. 100 µl of cell suspension was incubated with conjugated antibodies against CD4, CD25 and CD127 for 30 minutes at 4^0 C. Intracellular staining was performed by incubating lymphocytes in 100µl of FoxP3 fixation solution (eBioscience, UK) for 20 minutes at room temperature in the dark. The cells were then washed twice in permeabilisation buffer (eBioscience, UK) followed by incubation in 100µl of permeabilisation buffer and appropriate amount of fluorochrome labeled antibody against FoxP3 or isotype matched control for 20 minutes in the dark at room temperature. Samples were then washed in permeabilisation buffer. Finally, the cell suspension was washed and resuspended in wash buffer prior to flow cytometry.

Antibodies and the concentrations they were used at for flow cytometry are summarized in Table 2-1.

Table 2-1 **Antibodies used for Flow cytometry**

Conjugated Monoclonal Antibodies	**Clone**	**Source**	**Final Concentration**
Pacific Blue Mouse anti Human CD4	RPA-T4	BD Bioscience	1 in 20 dilution of stock
Pacific Blue Mouse IgG1 control	MOPC-21	BD Bioscience	1 in 20 dilution of stock
FITC Mouse anti Human CD8	RPA-T8	BD Bioscience	1 in 10 dilution of stock
FITC Mouse anti Human IgG1 control	MOPC-21	BD Bioscience	1 in 10 dilution of stock
APC Mouse anti Human CD19	HIB 19	BD Bioscience	1 in 10 dilution of stock
APC Mouse anti Human IgG1	MOPC-21	BD Bioscience	1 in 10 dilution of stock
PECy5 Mouse anti Human CD25	IM2646	Beckman Coulter	1 in 10 dilution of stock
PECy5 Mouse IgG2a control	A09148	Beckman Coulter	1 in 10 dilution
Alexa 488 Mouse anti Human CD127	HCD127	Biolegend	1 in 20 dilution
Alexa 488 Mouse IgG1 control	MOPC 21	Biolegend	1 in 20 dilution
PE Rat anti Human FoxP3	PCH101	eBioscience	1 in 5 dilution
PE Rat IgG2a control	Ebr2A	eBioscience	1 in 5 dilution

2.4 Immunohistochemistry and Immunofluorescence

CLEVER-1 expression in human liver tissue was visualized by immunohistochemistry and immunofluorescence (Crosby et al., 2009). Fresh liver was cut into 1cm^2 cubes, snap frozen in liquid nitrogen and stored at -70^{0}C. Livers were embedded in OCT TissueTek mount and sections 5 microns thick were cut using a cryostat (Bright OTF) and mounted on glass microscope slides (BDH UK) coated in 0.01% Poly-L-Lysine (stock 0.1% w/v from Sigma UK). Once mounted, sections were fixed in acetone (NHS supplies), wrapped in foil and stored at -20^{0}C before use.

2.4.1 Immunohistochemical staining.

Liver sections were obtained from four representative samples of normal liver, or tissue from patients with alcoholic liver disease, primary biliary cirrhosis, primary sclerosing cholangitis and autoimmune hepatitis. All monoclonal antibodies used for immunohistochemical staining are summarized in Table 2-2. Tissue sections were initially re-fixed in acetone for 5 minutes, and endogenous peroxide activity was then quenched by placing sections in 0.3% H_2O_2 in methanol for 30 minutes followed by washing in water. This was followed by a blocking step with 2% casein for 20 minutes. Tissue sections were incubated with 100µl of mouse anti human primary mAbs (monoclonal antibodies) directed against the molecule of interest for 1 hour. Mouse IgG antibody was used as a negative control antibody at equivalent concentrations. Samples were incubated at room temperature in a humidified chamber and placed on a rocker to ensure complete coverage

of samples in the antibody solution. The sections were then washed in tris buffered saline/0.1% Tween (TBS/Tween) for 5 minutes, followed by incubation with ImmPRESS^{TM} Universal anti-mouse/rabbit IgG reagent (Vector Labs, Burlingame CA) at room temperature for 30 minutes. This was followed by a further washing step in TBS/Tween for 5 minutes. The sections were then incubated with a Vector® VIP substrate kit for 5 minutes. Sections were finally rinsed in tap water and counterstained with haematoxylin. Images were analysed on a Zeiss Axioscope microscope in conjunction with Axiovision software (Carl Zeiss, UK).

Table 2-2 **Primary and secondary monoclonal antibodies used for immunohistochemical staining**

Monoclonal Antibodies	Clone	Source	Final Concentration
Mouse IgG1 control	DAK G01	DAKO	10µg/ml
Mouse anti Human CLEVER-1	3-372	Gift from S Jalkanen Turku Finland	10µg/ml
Anti mouse/rabbit secondary Immpress Universal kit	-	Vector Labs	Use stock solution

2.4.2 Immunofluorescence

Prior to staining, all samples were removed from -20^{0}C to equilibriate to room temperature before being re-fixed in acetone for 5 minutes. After air drying, samples were labeled, a wax pen was used to draw a well around the sections to retain antibody solutions, and each section was pre-wetted with staining buffer (PBS/10%FCS/0.1% sodium azide). All monoclonal antibodies used for immunofluorescent staining are summarized in Table 2-3. If the antibodies used were biotinylated then the sections were preincubated using an avidin/biotin blocking kit (Vector Laboratories, UK) as per manufacturer's instructions. The tissue sections were then incubated with 100µl of mouse anti-human primary mAbs for 1 hour in a dark humidified box

for 1 hour. This was followed by a washing step with PBS for 30 minutes. The sections were then stained with the appropriate fluorescent secondary antibodies for 30 minutes with a final washing step in PBS for 1 hour. The sections were then mounted with Fluorescent Immunomount (DAKO,UK). Throughout these steps the sections were protected from light.

Table 2-3 **Primary and secondary monoclonal antibodies used in immunofluorescent studies in liver tissue**

Monoclonal Antibodies	**Clone**	**Source**	**Final Concentration**
Mouse IgG1 negative control	DAK GO1	DAKO	10µg/ml
Mouse IgG2b negative control	73009	R and D Systems	5µg/ml
Mouse IgG1 biotin negative control	-	AbD Serotec	2.5µg/ml
Mouse anti Human CLEVER-1	3-372	Gift from S Jalkanen, Turku, Finland	10µg/ml
Mouse anti Human L-SIGN	120604	R and D Systems	5µg/ml
Mouse anti Human CD68	Y1/82A	BD Biosciences	5µg/ml
Mouse anti Human CD34 Biotinylated	QBEND/10	AbD Serotec	2.5µg/ml
Streptavidin-Alexafluor 488	-	Invitrogen	25µg/ml
Goat anti Mouse IgG1 Texas Red	-	Cambridge Biosciences	1/50 Manufacturers stock
Goat anti Mouse IgG2b FITC	-	Cambridge Biosciences	1/50 Manufacturers stock

For fluorescent staining of HSEC, cells were grown on RTC coated coverslips (Biosciences, University of Birmingham, UK) and when confluent they were fixed in 4% paraformaldehyde for 15 minutes. The cells were then permeabilised in 0.3% Triton for 15 minutes and then blocked with 10% goat serum (Sigma Aldrich, UK) for 20 minutes. This was followed by incubation with mouse anti-human monoclonal antibodies for 60 minutes at room temperature. Summary of antibodies used in these studies are listed in Table 2-4. The cells were then washed in PBS and incubated with appropriate isotype and species specific secondary antibody for 30 minutes at room temperature protected from light. Following this the cells were washed with PBS and mounted with fluorescent mounting media (DAKO,UK).

In other experiments cells were incubated with anti CLEVER-1 antibody (3-372) or an isotype matched control for 15 minutes or 30 minutes prior to fixation. This was followed by fixation and permeabilisation as described above and stained with an appropriate isotype and species specific secondary antibody for 30 minutes at room temperature protected from light. Cells were then washed and mounted as described above.

All staining was assessed using Axiovision software as before (section2.4.1) or imaged by confocal microscopy on an LSM 510 microscope equipped with 63x1.32 objective. Images were acquired and analysed by LSM software. LSM software was also used to create profiles of fluorescent intensity of each molecule which enabled graphical demonstration of overlapping fluorescent signals to confirm colocalisation.

Table 2-4 **Monoclonal antibodies used for immunofluorescent studies in HSEC**

Monoclonal Antibodies	**Clone**	**Source**	**Final Concentration**
Mouse IgG1 negative control	DAK GO1	DAKO	10µg/ml
Mouse IgG2b negative control	73009	R and D	5µg/ml
Mouse anti Human CLEVER-1	3-372	Gift from Sirpa Jalkanen	10µg/ml
Mouse anti Human LYVE-1	264725	R and D	5µg/ml
Goat anti Mouse IgG2b fluorescein isothiocyanate	-	Cambridge Biosciences	1/50 dilution of stock
Goat anti Mouse IgG1 fluorescein isothiocyanate	-	Cambridge Biosciences	1/50 dilution of stock

2.5 Western Blotting of Liver Tissue

Whole liver tissue blocks were weighed and homogenized in CellLytic™ MT buffer (Sigma, UK) with protease inhibitor and DNase-I (5U(Sigma, UK) according to manufacturer's instructions. Mechanical homogenization was performed with a scalpel followed by a hand-held homogenisor. The samples were then placed on a Vibraplatform at 4^0C overnight, centrifuged at 13,000 RPM for ten minutes and the supernatant was transferred to a separate tube. The protein concentration of each sample was calculated using a Sigma® Total Protein Kit (Micro Lowry, Onishi & Barr modification) according to the manufacturer's instructions. Bovine Serum Albumin (Sigma, UK) was used as a protein concentration standard. Samples were stored at -20^0C prior to use.

The protein samples were separated by SDS polyacrylamide gel electrophoresis using standard protocols. Briefly, stacking and resolving gels were prepared as described in Table 2-7 and then poured between two glass plates and left to set. The protein samples (20 µg) were diluted in 5xSDS-PAGE loading buffer, with or without β-mercaptoethanol and boiled for ten minutes at 100^0C, depending on the antibody used. The gel apparatus was assembled and gel tank was set up by pouring in 1x running electrophoresis buffer to cover the electrodes. 25 µl of each sample was loaded onto the stacking gel along with 10µl of a molecular weight marker (Colorburst Marker; Sigma,UK). Electrophoresis was performed at 200V for 30 minutes ensuring that the dye front reached the bottom of the gel.

2.5.1 Western blot transfer

The proteins were then transferred from the gel to a nitrocellulose membrane (Amersham Biosciences,UK). The nitrocellulose membrane, sponges and filter paper were pre-wetted by immersion in transfer buffer. A 'sandwich' was then formed with the membrane and gel between the filter paper and sponges which were placed in a transfer block. The transfer blocks with an ice pack were then placed in an electrophoresis tank filled with transfer buffer. The blot was then run at 100V for 1 hour. The transfer of protein was checked by adding Ponceau Red (0.1% ponceau in 5% acetic acid; Sigma) to the membrane for 5 minutes before washing off with water.

2.5.2 Western blot development

The protein-coated membranes were blocked with 5% non-fat milk (Marvel) in PBS/0.02% Tween for 1 hour at room temperature with continuous agitation, and then probed with primary antibodies diluted in 5% non-fat milk overnight at 4^0C on a rocker. antibodies used for western Blotting are summarized in

Table 2-8. The membrane was washed three times with PBS-0.02% Tween for five minutes, followed by incubation with a peroxidase-conjugated secondary antibody in 5% non-fat milk for 1 hour at room temperature on a rocker. The membrane was then washed for thirty minutes with PBS-0.02%Tween. Chemiluminescence reagent (ECL plus: Amersham Pharmacia, Biotech, UK) was added to the membrane as per manufacturer's instructions, and the membrane was then exposed with enhanced

chemiluminescence detection film (Amersham,UK) and developed using a Kodak X–Omat 1000 processor (University of Birmingham). For reprobing,, the membrane was stripped by incubation with stripping buffer for 45 minutes at 50^0C followed by several washes with distilled water. The membrane was then blocked with 5% non-fat milk and re-probed with anti β-actin antibody as described earlier.

Table 2-5 **Source and Molecular weights of reagents used for Western Buffers**

Reagent	**Molecular Weight (FW)**	**Source**
Trisma Base	121.14	Sigma
Glycine	75.07	Sigma
Sodium dodecyl sulfate (SDS)	288.38	Sigma
Glycerol	92.09	Sigma
Methanol	32.04	Fisher Scientific
Tween 20	-	
β-mercaptoethanol	78.13	Sigma
Non-fat milk	-	Marvel

Table 2-6 Buffers used in SDS PAGE

Buffers	Constituents
10 x Electrophoresis Buffer	Per litre- 30.3g Trisma base, 144g glycine, 10g SDS
Transfer Buffer	Per 2 litres- 28.8g glycine, 6g Trisma base, 400ml methanol, 1g SDS
Resolving gel Buffer	1.5M Trisma Base pH8.8 (pH adjusted with HCl)
Stacking gel Buffer	0.5M Trisma Base pH6.8 (pH adjusted with HCl)
5XSDS-PAGE Sample buffer	200mM Trisma Base pH 6.8, 20%glycerol, 10% SDS 0.05% bromophenol blue, 10mM β-mercaptoethanol
Stripping Buffer	Per 100ml:20.0ml 10% SDS, 12.5ml 0.5M Trisma base pH6.8, 67.5 dH_20, 0.8ml β-mercaptoethanol
Blocking Buffer	PBS +0.02% Tween-20, 5% non-fat milk

Table 2-7 **Components of SDS PAGE gels**

	Stacking Gel (4%)	**Resolving Gel (6%)**
ddH_20	6.1ml	5.4ml
30% Degassed Acrylamide/Bis (BioRad)	1.3ml	2ml
Stacking Gel buffer	2.5ml	-
Resolving Gel buffer	-	2.5ml
10% w/v SDS (Sigma)	0.1ml	0.1ml
10% w/v ammonium persulfate (APS) Sigma	60µl	60µl
N,N,N^{I},N^{I}-tetramethylethylenediamine	60µl	60µl

Table 2-8 **Antibodies used for Western Blotting**

Antibody	**Clone**	**concentration**	**Source**
CLEVER-1	3-372	1µg/ml	Gift from S Jalkanen, Turku, Finland
β-actin	Clone AC-15	1µg/ml	Sigma
Goat anti Mouse HRP	-	1:2500	DAKO

2.6 PCR

2.6.1 Conventional PCR

CLEVER-1 and LYVE-1 gene expression by HSEC were analysed by RT-PCR. HSEC were grown to confluence before trypsinisation and pelletting. Total RNA was purified using the RNeasy kit (Qiagen, UK) as recommended by the manufacturer. The concentration of RNA was measured using a NanoDrop spectrometer (ThermoFisher scientific,UK). An OD 260/280 of greater than 2 was taken as acceptable purity. One microgram of total RNA reverse transcribed to generate cDNA with Supersript II (Invitrogen,UK) according to manufacturers instructions. All samples were treated with DNase I (Sigma, UK) prior to cDNA synthesis. The concentration of cDNA was measured using a NanoDrop spectrometer. An OD 260/280 of greater than 1.8 was taken as acceptable cDNA purity. This method of RNA purification and cDNA generation was also performed for cells in quantitative PCR studies (see section2.6.1) and were pre-treated as described in sections 2.7.3, 2.7.4, 2.7.5 prior to trypsination and pelleting.

Primer sequences for CLEVER-1 and LYVE-1 were designed based upon Genbank sequence information and GAPDH was used as an endogenous control.

Table 2-9 **CLEVER-1 Primers for Conventional PCR**

Forward Primer	5’-ACTCTGTCCTGGACAGCG-3’
Reverse Primer	5’-CAGCCGCTCATGGACACC-3’
Amplicon size	289 bp

Table 2-10 **LYVE-1 Primers for Conventional PCR**

Forward Primer	5’-GCCTGTAGGCTGCTGGGACTAAG-3’
Reverse Primer	5’-CCCAGCAGCTTCATTCTTGAATG-3’
Amplicon size	518 bp

Table 2-11 **GAPDH Primers for Conventional PCR**

Forward Primer	5’-AGAAGGCTGGGGCTCATTTG-3’
Reverse Primer	5’-AGGGGCCATCCACAGTCTTC-3’
Amplicon size	259 bp

For CLEVER-1 PCR, the initial PCR reaction was set at thirty five cycles under the following conditions 95˚C for 1min, 50˚C for 1min, 72˚C for 2min and a final extension at 72˚C for 5min. The PCR reaction was further optimized by developing a touchdown protocol which was run under the following conditions : thirty five cycles under the following conditions 95˚C for

1min, followed by an annealing step for 1 minute with a temperature range from 55˚C to 48˚C, 72˚C for 2min and a final extension at 72˚C for 5min.

For the LYVE-1 reaction, thirty five PCR cycles were run under the following conditions: 95˚C for 1 min, 61˚C for 1min, 72˚C for 2min and a final extension at 72˚C for 5min, and for GAPDH primers thirty five cycles were run under the following conditions: 94˚C for 30 seconds, 55˚C for 30 seconds, 72˚C for 45 seconds and a final extension at 72˚C for 10 minutes. All amplified products were analysed on 2% agarose gel containing ethidium bromide (Sigma) diluted in 1 x TAE. The PCR products were identified by their respective sizes. Further details of the components and concentrations of reagents used for PCR are described in Table 2-12 and Table 2-13.

Table 2-12 **10xTAE Electrophoresis Buffer**

Reagents	**Molecular Weight**	**Final Concentration**	**Source**
Trisma Base	121.14	40mM	Sigma
Acetic acid	60.05	20mM	Sigma
$Na_2EDTA_2H_20$	372.24	1mM	Sigma
ddH_20		Fill up to 1 litre	

Table 2-13 **PCR reaction components**

5x Green GoTaq®Flexi Buffer	1x	Promega, Southampton
$MgCl_2$ (25mM)	1.5mM	Promega, Southampton
Forward Primers	0.4uM	Altabiosciences
Reverse Primers	0.4uM	Altabiosciences
dNTPs	0.2mM (total)	Invitrogen
GoTaq®DNA polymerase	1.25u	Promega, Southampton
cDNA	10µg/ml	Primary human hepatic sinusoidal endpthelial cells
Nuclease free Water	Make up to 50µl	Sigma

2.6.2 Quantitative PCR

Quantitative analysis of relative mRNA expression of CLEVER-1 in HSEC was performed by Taqman® Gene expression assays with FAM labeled probes for CLEVER-1(alternate gene name stabilin-1: Hs01109068_m1,Applied Biosystems,UK). GAPDH was used as the housekeeping gene (FAM labeled probe :Hs99999915_m1, Applied Biosystems,UK). Assays were run in a MicroAmp® Optimal 96 well reaction plate-0.1ml (Applied Biosystems,UK) on an ABI prism 7900 sequence detector (University of Birmingham). The cycle threshold value (Ct) for CLEVER-1 was normalized against the housekeeping gene. The relative

expression was calculated by the $2^{-\Delta\Delta Ct}$ method. Components of the PCR reaction are described in.Table 2-14.

Table 2-14 **Components of quantitative PCR reaction**

Component	Final Concentration
Taqman 2x master mix (Applied Biosystems)	1x
20x Assay mix(Applied Biosystems)	0.5x
cDNA	100ug/ml
RNAse free water	Upto 10µl

2.7 Stimulation of primary HSEC cultures.

2.7.1 Cytokine Stimulation of Cell Monolayers

All stimulations were performed in appropriate complete culture media. Cells were cultured to confluence as described previously in RTC-coated 96-well plates (Becton Dickinson, Oxford, UK). For test samples all culture medium was removed and replaced with unstimulated media (control) or stimulation media either alone or concominant with interferon-gamma (IFN-γ) (Table 2-15). Cytokine concentrations used were previously determined optimal concentrations used by our group. All stimulations were for 24 hours at 37^0C in a humidified incubator with a 5% CO_2 atmosphere.

Table 2-15 **Agents used for stimulation of cultured monolayers**

Cell Stimulant	Final Concentration	Source
TNF-α	10ng/ml	Pepro Tech
IFN-γ	10ng/ml	Pepro Tech
TGF-β	10ng/ml	Pepro Tech
LPS	1µg/ml	Sigma Aldrich
IL-4	10ng/ml	Pepro Tech
IL-10	10ng ml^{-1}	Pepro tech

2.7.2 Measurement of adhesion molecule expression by primary cell cultures

Expression of adhesion molecules on primary cell cultures was investigated by ELISA (Enzyme-linked Immunosorbent assay). Cells were cultured and stimulated as described before being fixed in 100% methanol (BDH, US) for 5min and washed in buffer (PBS/0.1% gelofusin). Cells were incubated with normal goat serum (Sigma, UK) for 60 min at 37^{o}C to reduce non-specific secondary antibody binding before being washed twice with PBS prior to addition of primary antibodies in triplicate wells (60 min incubation at 37^{o}C). Following two further washes, cells were incubated with a relevant secondary antibody conjugated to horseradish peroxidase for 45min at 37^{o}C. Cells were then washed a further three times before development of the

peroxidase enzyme label using an OPD-based substrate (1,2-phenylenediamine dihydrochloride in tablet form; DAKO, UK) in accordance with manufacturer's instructions. Colour development was allowed to continue for 3-5 min before being stopped by the addition of 0.5M sulphuric acid (H_2SO_4; Fisher Scientific). Absorbance at 490nm was measured on an automated plate reader (Dynax laboratories, MRX). Background absorbance was corrected by subtracting absorbance values of an isotype-matched control antibody (DAKO,UK) and mean absorbance values were calculated from triplicate tests. All antibodies used are detailed in Table 2-16 .

Table 2-16 **Antibodies used for ELISA studies**

Monoclonal Antibodies	**Source**	**Final Concentrations**
Mouse anti Human CLEVER-1 (3-372)	Gift from Sirpa Jalkanen	10 µg/ml
Mouse IgG1 control	R and D	10 µg/ml
Goat anti Mouse-HRP	DAKO	1:5000

2.7.3 Cytokine and growth factor stimulation of cultured HSEC.

All stimulations were performed in appropriate complete culture media. Cells were grown to confluence in 75cm^2 flasks as described previously in section 2.2.1. For test samples all culture medium was removed and replaced with unstimulated media (control) or stimulation media containing cytokine of

choice (Table 2-17). Stimulations were performed for either 4 or 24 hours at 37^0C in a humidified incubator with a 5% CO2 atmosphere. To study the effect of vascular epidermal growth factor (VEGF) and hepatocyte growth factor (HGF) all culture medium was removed from and replaced with media without growth factor supplementation (control) or media with either VEGF or HGF supplementation or a combination of both growth factors summarized in Table 2-17. Studies were performed for 24 hours at 37^0C in a humidified incubator with a 5% CO2 atmosphere.

Table 2-17 **Cytokines and growth factors used to stimulate cultured HSEC for qPCR**

Cell Stimulant	**Final Concentration**	**Source**
TNF-α	10ng/ml	Pepro Tech
IFN-γ	10ng/ml	Pepro Tech
LPS	1µg/ml	Sigma Aldrich
IL-4	10ng/ml	Pepro Tech
IL-10	10ng/ml	Pepro Tech
Vascular Epidermal Growth Factor	10ng/ml	Pepro Tech
Hepatocyte Growth factor	10ng/ml	Pepro Tech

2.7.4 Stimulation of HSEC with Bile.

Bile was collected from resected gall bladders at the time of liver transplantation. HSEC was cultured to confluence in 75cm2 culture flasks. For test samples all culture medium was removed and replaced with unstimulated media (control) or media containing bile at a dilution of 1 in 100. Studies were performed for 24 hours at 37^0C in a humidified incubator with a 5% CO2 atmosphere.

2.7.5 Exposing HSEC to hypoxic conditions

HSEC were grown to confluence in $75cm^2$ and test samples were maintained in either normoxia or incubated in hypoxic conditions for 24 hours. Hypoxia was achieved by placing cells in an airtight incubator (RS Mini Galaxy A incubator, Wolf Laboratories, UK) which was flushed with 5% CO2 and 95% Nitrogen until oxygen content within the chamber reached 0.1%. The level of oxygenation was checked by a dissolved oxygen monitor (DOH-247-KIT, Omega Engineering, UK). To ensure culture media was appropriately oxygenated it was pre-incubated in a sterile container, which allowed gas exchange for 8 hours prior to study. Oxygenation of the media was checked by the dissolved oxygen monitor.

2.8 Bafilomycin treatment of primary HSEC

Trafficking of CLEVER-1 was assessed by inhibiting the vacuolar type H+-ATPase with Bafilomycin A1 (Sigma UK) (Yoshimori et al., 1991). HSEC grown on RTC-coated coverslips were treated with Bafilomycin A1 at a concentration of 10nM for 24 hours. The cells were fixed and fluorescently stained as described in section 2.4.2. Details of the antibodies used are in

Table 2-18.

Table 2-18 **Primary and secondary antibodies used for intracellular trafficking studies**

Monoclonal Abs	**Clone**	**Source**	**Final Concentration**
Mouse IgG1 control	DAK GO1	DAKO	10 µg/ml

Mouse IG2b control	73009	Rand D Systems	5 µg/ml
Rabbit IgG control	-	DAKO	5 µg/ml
Mouse anti Human CLEVER-1	3-372	Gift from S Jalkanen, Turku,Finland	10 µg/ml
Mouse anti Human Lamp-1	25	BD Biosciences	5 µg/ml
Mouse anti Human Rab5	1/Rab5	BD Biosciences	5 µg/ml
Rabbit anti Human Trans-Golgi network protein	-	Sigma	5 µg/ml
Goat anti Mouse IgG1 Alexa fluor 546	-	Invitrogen	10 µg/ml
Goat anti Mouse IgG2b FITC	-	Cambridge Biosciences	1/50 dilution of stock
Goat anti Rabbit IgG Alexa fluor 488	-	Invitrogen	1/500 dilution of stock
Lectin GS-II from Griffonia simplicifolia, Alexa fluor 488	-	Invitrogen	10 µg/ml

2.9 Static adhesion assays

Static adhesion assays were performed on frozen liver sections with peripheral blood lymphocytes in order to screen for effects of function-blocking antibodies on adhesion to intact tissue.

2.9.1 Static adhesion assay

I used an established method (McNab et al., 1996) of adhesion assay. Frozen sections from different diseased liver specimens were obtained as previously mentioned, although sections were cut at 10 micron thickness. Where appropriate, tissue sections were incubated with 100µl of optimally diluted mouse anti human function-blocking antibody directed against the molecule of interest for 1 hour on a rocker in a humidified chamber. Isotype-matched mouse immunoglobulins were used as controls. The slides were then washed with PBS for 5 minutes, prior to addition of 100µl of peripheral blood lymphocytes resuspended in RPMI-1640/10%(v/v) FCS (Invitrogen,UK) at $1x10^6$/ml for 30 minutes and allowed to bind in static conditions at room temperature. The slides were then gently washed with PBS and then fixed in acetone for 5 minutes. This was followed by two 5 minute washes in PBS. The slides were then stained with haematoxylin and mounted with glass coverslips and immunomount solution (Thermoscientific UK). Slides were analysed by manual counting of adherent lymphocytes to sinusoids in 10 representative high power fields. Assays were counted blind.

Table 2-19 **Blocking antibodies used in static adhesion assays**

Monoclonal Abs	**Clone**	**Source**	**Final Concentration**

Mouse IgG1 control	DAK GO1	DAKO	20 µg/ml
Mouse anti Human CLEVER-1	3-372	Gift from S. Jalkananen, Turku, Finland	20 µg/ml

2.10 Flow based adhesion assay

In order to determine the physiological significance of CLEVER-1 expression on HSEC under physiological levels of flow I carried out flow-based adhesion using a method established in our laboratory (Lalor et al., 2002a). Assays used freshly isolated lymphocyte subsets and measured interactions with HSEC grown in glass capillary tubes (microslides) or IBIDI chambers as discussed below.

2.10.1 Preparation of microslides

Microslides are flattened glass capillary tubes, 5cm in length with a rectangular cross-section of 0.3 x 0.03 cm (Figure 2.2B). The microslides were initially acid-washed overnight (70% v/v nitric acid;Sigma,UK) before

being rinsed in excess of water with a final wash in distilled water and dehydration in anhydrous acetone. Microslides were then coated for 10 mins at room temperature with a 4% (v/v) solution of 3-aminopropyltriethoxysilane (APES; Sigma UK) prepared in anhydrous acetone. After a final wash in anhydrous acetone, microslides were rinsed in distilled water to remove any residual acetone before being blotted and dried in a glass oven overnight at 60^0C. To allow connection of microslides to a custom designed multi-port culture dish (Glassblower, School of Chemistry, University of Birmingham, UK) 1cm long sections of silicone tubing (3mm bore and 1mm wall thickness; Portex Ltd., Kent, UK) were attached at one end and the slides were autoclaved at 121^0C prior to use.

2.10.2 Culture of endothelial cells in microslides

Sterile microslides were coated with RTC for 2 hours at 37^0C and washed thoroughly with PBS. HSEC were cultured (as described in 2.3) in a T75 flask until confluent before being dissociated in trypsin-EDTA, washed in PBS and resuspended at 0.5 $x10^6$/ 100µl in complete medium. Microslides were then attached to a 1ml pipette and 50µl of the cell suspension was aspirated into each slide before being incubated at 37^0C for 1 hour to allow the cells to settle and adhere to the internal lower surface. Individual microslides were then attached to each of the six ports within the culture dish via the previously attached silicone tubing and covered in approximately 40ml of complete medium. The culture dish was placed in a humidified incubator at 37^0C with a 5% CO_2 atmosphere and attached via silicone tubing to an

external pump, timed to replace spent media in the microslides at 2 hourly intervals. The endothelial monolayers in microslides were treated with cytokines (TNFα at 10ng/ml and IFN γ at 10ng/ml) for 24 hours prior to adhesion assay.

2.10.3 Culture of endothelial cells in ibidi slides

Whilst the microslide system is effective for use in flow assays, it is technically challenging to perform. Thus during the course of my studies we also used an alternative, commercially available multi channel culture system in the form of IBIDI chambers. Here, endothelial cells were grown in Ibidi ® µ-Slide VI 0.4 channel slides (Thistle Scientific, UK). These slides were precoated with RTC by injecting 30 µl of liquid into each channel and incubating for 2 hours at 37^0C and followed by washing thoroughly with PBS. HSEC were cultured (as described in section 2.2.4) in a T75 flask until confluent before being dissociated in trypsin-EDTA, washed in PBS and resuspended at 3 $x10^6$ ml^{-1} in complete medium. 30 µl of cell suspension was injected directly into each channel. The cells were left to adhere to the slides for one hour at 37^0C following which complete medium was added to fill the ports on either side of the channel. The cells were grown to confluence in a humidified incubator at 37^0C with a 5% CO_2 atmosphere and by placing the slides on a Ibidi ® µ-Slide rack (Thistle Scientific,UK) to allow gas exchange between the medium within the slide and the incubator atmosphere. Once cells were confluent they could be stimulated with

cytokines as described in 2.10.2 by directly injecting cytokine solution into the slides.

2.10.4 Flow based adhesion assays

All manipulations of microslides and IBIDI chambers were at room temperature immediately prior to assay. For microslides containing a confluent HSEC monolayer, the slides were fixed to a glass microscope slide using cyanoacrylate glue and the two ends of the microslide were coated in double-sided adhesive tape (Scotch ™ pressure sensitive tape; 3M, Bracknell, Berks, UK) to allow an airtight seal. One end of the microslide was then attached via a silicone tubing to Harvard syringe pump (Harvard Apparatus, South Natic, MA, USA) and the other to an electronic solenoid valve (Lee Products Ltd., Gerrards Cross, Bucks, UK) which permitted the alternation between a cell free wash buffer (Endothelial medium/0.1% (v/v) BSA) and a lymphocyte suspension (typically 1 x 10^6/ml in endothelial media/0.1% (v/v) BSA). The withdrawal rate of the syringe pump was altered such that the flow rate within the microslide maintained a shear stress of 0.5 dyne/cm^2 (0.05Pa). In the case of Ibidi® microslides the attachment to the silicone tubing was made using adaptors from the Ibidi ® µ-Slide VI 0.4 flow kit (Thistle Scientific). Here the withdrawal rate of the syringe pump was altered according to the Ibidi ® instructions to maintain a shear stress of 0.5 dyne/cm^2 (0.05Pa).

Once attached to the flow system, chambers were enclosed in a thermostatically controlled transparent chamber maintained at 37^0C. Cultured endothelial cells were perfused with a 5 min bolus of lymphocytes at a constant wall shear stress of 0.05Pa, which approximates to the shear stress observed in the post-capillary venules *in vivo*. Some experiments involved HSEC monolayers or lymphocytes being pre-treated with function blocking antibodies or an isotype matched control. Here antibodies were prepared in endothelial media/0.1% (v/v) BSA and 50 µl was aspirated into the relevant microslide or lymphocyte suspensions were resuspended in an appropriate dilution of antibody for 30mins prior to assay (Table 2-20). All incubations were at 37^0C, and lymphocytes were resuspended in endothelial medium/0.1% (v/v) BSA to a final concentration of 1 $x10^6 ml^{-1}$ prior to flow adhesion assay. To investigate the effect of blocking chemokine activity via G-protein coupled receptors, lymphocytes were incubated with 200ng ml^{-1} of Pertussis Toxin (a Giα protein inhibitor, PTX; Sigma UK) for 30 minutes at 37^0C. Lymphocytes captured from flow were visualized by phase-contrast microscopy using an Olympus IX50 inverted microscope (Olympus (UK)Ltd., Southall Middlesex,UK) and video microscope recordings were made along the length of the microslide. Offline analysis of videos allowed the pattern of adhesion to be assessed. Lymphocytes adherent to the upper surface of the HSEC monolayer appeared phase-bright whilst those that had migrated through the monolayer appeared phase dark. Adherent cells were classified as exhibiting ‘static adhesion’ (non-migrated), ‘shape changed’/activated morphology or as ‘migrated’ and individual categories were expressed as

percentage of total adhesion. Total adhesion was normalized for adhesive surface area and number of lymphocytes perfused i.e. adherent cells/mm^2/10^6 perfused.

Table 2-20 **Blocking antibodies used in flow adhesion assays**

Monoclonal Antibodies	CLONE	Source	Final Concentration
Mouse anti Human CLEVER-1	3-372	Gift from S. Jalkanen, Turku, Finland	20µg/ml
Mouse anti Human ICAM-1	BBIG-I1	R and D	10µg/ml
Mouse anti Human VCAM-1	BBIG-V1	R and D	10µg/ml
Mouse anti Human VAP-1	TK-814	Biotie, Finland	10µg/ml
Mouse anti Human IgG1	11711	R and D	20 µg/ml
Mouse anti Human IgG2a	20102	R and D	10µg/ml

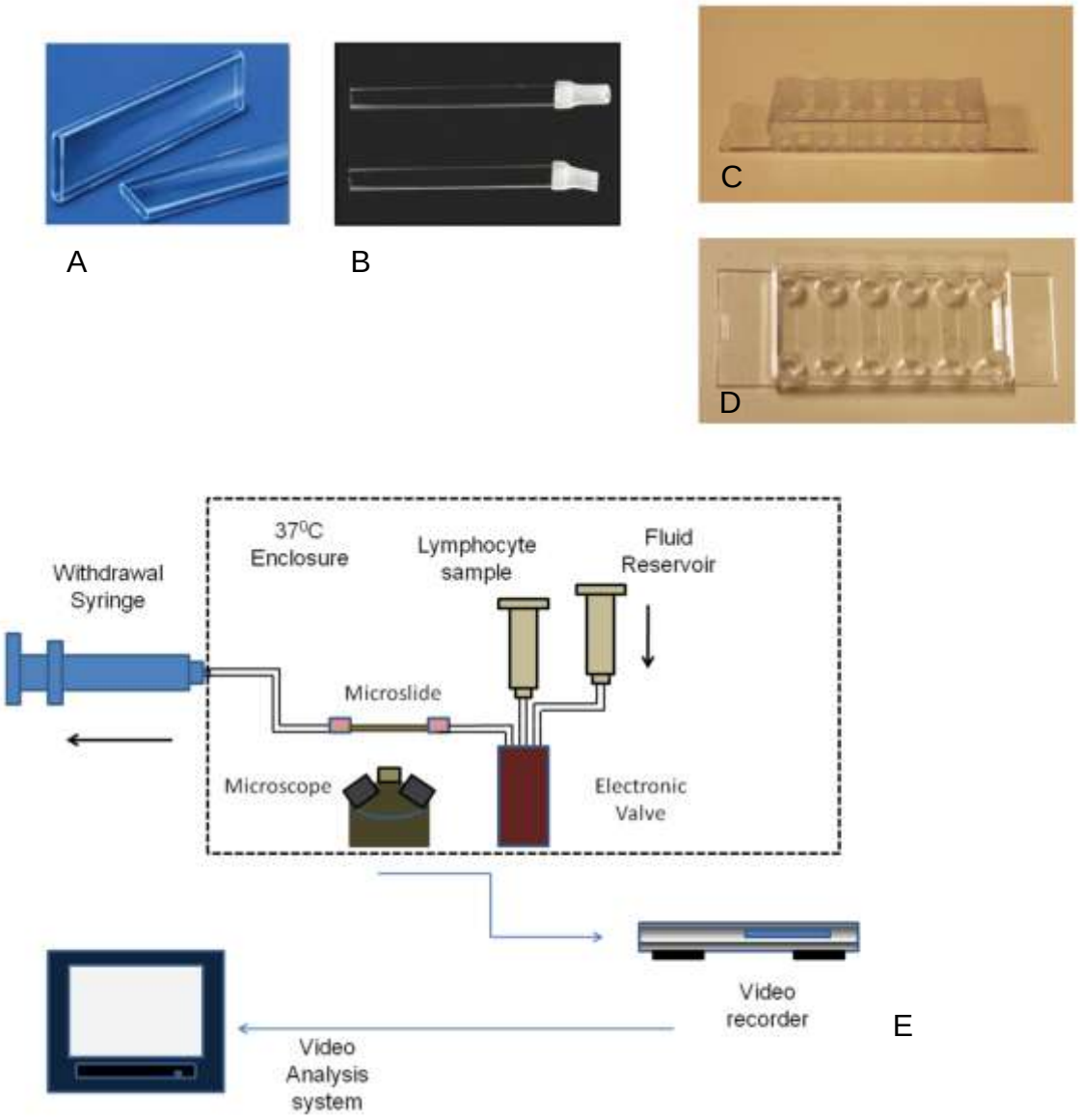


Figure 2-1 **Microslides and flow assay set-up**

HSEC were cultured in microslides (panel A) with silicone tubing attachments (Panel B) in microdishes before being used in flow assays. Alternatively HSEC were cultured in Ibidi chamberslides (lateral view in panel C and view from above panel D). These microslides were then attached to flow assay tubing and lymphocytes perfused over HSEC. Interactions were recorded using a phase contrast microscope and analysed offline (Panel E).

2.11 Manual Tracking of Lymphocytes under conditions of flow

To investigate the motility of lymphocytes on endothelial monolayers, flow adhesion assays were carried as described in section 2.10.4. The assay involved timelapse video recordings of a randomly selected single field of view (10x objective) for a period of five minutes under conditions of flow. Offline analysis of the video recordings was carried out using Image J software and a manual tracking plug-in (version 1.42q, NIH, Bethesda, USA), allowing the position of each lymphocyte to be tracked throughout the period of recording. The data was then analysed using the Ibidi Chemotaxis plug-in for Image J (http://www.ibidi.de/applications/ap_chemo.html) which facilitated the measurement of lymphocyte migration over the course of each recording and represented the data graphically for ease of interpretation. These analyses were performed with B cell populations which were then compared to a mixed T cell population, comprising both CD4- and CD8-positive T cells.

2.12 Confocal imaging of lymphocytes undergoing transendothelial migration

Flow based adhesion assays were performed followed by immunofluorescent staining and confocal microscopy to capture lymphocytes transmigrating across HSEC.

Initially, HSEC were seeded and cultured in ibidi ® µ-Slide VI 0.4 channel slides (Thistle Scientific, UK) as described in 2.10.3. Cells were then stimulated for 24 hours with TNFα and IFNγ (both 10ng/ml) for 24 hours. Prior to flow assay HSEC were incubated with cell tracker green (Invitrogen,UK) diluted in serum free endothelial basal media as per manufacturer's instructions for 30 minutes at 37^0C followed by a washing step with endothelium basal medium containing 10% human serum. Depending on which subset was being studied, CD4 lymphocyte purification or T regulatory cell purification was performed from whole blood as described in sections 2.3.2 and 2.3.3. The lymphocyte subsets were diluted to $1x10^6$ cells/ml and prepared as described in 2.10.4. If live cell imaging was being performed lymphocytes were stained with cell tracker violet for 30 minutes followed by a washing step and then resuspended in endothelial medium/0.1% (v/v) BSA prior to flow assay.

Flow adhesion assays were then performed as described in section 2.10.4. Initially cultured endothelial cells were perfused for 5 min with a bolus of CD4 lymphocyte fractions at a constant wall shear stress of 0.05Pa. To perform live cell imaging, ibidi slides were removed from flow assay chamber and

gently flushed with PBS followed by staining with cell mask orange (Invitrogen) for 15 minutes at 37^0C.

In other experiments, CD4 or T regulatory cell fractions were perfused for 10 minutes over HSEC and then fixed in 4% paraformaldehyde for 15 minutes followed by permeabilisation with 0.1% Triton for ten minutes. FoxP3 fixation/permeabilisation kit (eBioscience,UK) was used in experiments with T regulatory cells. A blocking step was performed with 10% goat serum (Sigma,UK) for twenty minutes. Cells within the ibidi slides were then incubated with mouse anti-human or rat anti-human monoclonal antibodies for 60 minutes at room temperature. The cells were then washed in PBS and incubated with appropriate isotype and species specific secondary antibody for 30 minutes at room temperature protected from light. A further washing step was performed before staining with DAPI (Invitrogen,UK), washing with PBS and mounting with fluorescent mounting media (DAKO,UK).

In all experiments lymphocyte/ endothelial interactions were imaged by confocal microscopy on an LSM 510 microscope equipped with 63x1.32 objective. Images were acquired and analysed by LSM software. LSM software was also used to create Z-stack profiles of images.

Table 2-21 **Antibodies and cellular stains for cells in Ibidi microslides**

Monoclonal Antibodies	CLONE	Source	Final Concentration
Mouse anti Human CLEVER-1	3-372	Gift from S. Jalkanen, Turku Finland	10µg/ml
Mouse IgG1 control	DAK G01	DAKO	10µg/ml
Mouse anti Human ICAM-1	BBIG-I1	R and D	10µg/ml
Rat anti Human FoxP3	PCH101	eBioscience	5µg/ml
Rat IgG2a control	-	eBioscience	5µg/ml
Goat α Mouse IgG1 Alexa fluor 546	-	Invitrogen	10 µg/ml
Goat α Rat IgG Alexa fluor 633	-	Invitrogen	5 µg/ml
DAPI nucleic acid stain	-	Invitrogen	1 in 2000 of stock solution
Cell Tracker™Green CMFDA	-	Invitrogen	1 in 1000 of stock solution
CellMask™ Orange plasma membrane stain	-	Invitrogen	5µg/ml
Cell Tracker™ violet BMQC dye	-	Invitrogen	1 in 1000 of stock dilution

2.13 Statistical Analysis

Calculated means of data are given together with standard error of mean SEM. Data means were compared using paired sample T-tests or the independent sample T-tests as appropriate. A value of $p \leq 0.05$ (two tailed) was considered statistically significant. Statistics were performed using the software package GraphPad Prism 5. Where possible, statistics used the raw data rather than the calculated data. For example, actual numbers of cells per high power field were used in adhesion assays to calculate statistical significance rather than percentage of maximal adhesion.

CHAPTER 3

THE EXPRESSION OF CLEVER-1 IN HUMAN HEPATIC TISSUE

3.1 Introduction: The expression of CLEVER-1 in normal and diseased human liver tissue by immunohistochemistry

CLEVER-1 is known to be expressed by tissue macrophages (Kzhyshkowska et al., 2006a) and on the sinusoidal endothelium (non-continuous endothelium) of spleen, lymph node and adrenal cortex (Goerdt et al., 1991). The presence of CLEVER-1 is thus restricted to sinusoidal endothelium in health but in certain disease states it is upregulated on continuous endothelium. This has been shown in malignant conditions such as T-cell lymphomas, melanoma metastasis and also in psoriasis, a chronic inflammatory condition of the skin, and on a background of wound healing (Goerdt et al., 1993; Salmi et al., 2004). The presence of CLEVER-1 in macrophages was initially demonstrated within placental tissue and subsequently found in tissue macrophages of the colon, stomach, skin and lymph node ((Goerdt et al., 1991; Martens et al., 2006; Prevo et al., 2004).

These studies used various antibodies which include MS-1 (a mouse mAB against human spleen) (Goerdt et al., 1991), F4 (a rabbit polyclonal Ab against the recombinant cytoplasmic tails of human CLEVER-1) (Politz et al., 2002), and 1.26 (mouse Mab against N-terminal fragment of CLEVER-1) (Schledzewski et al., 2006). Previous studies in the normal human liver using the MS-1 antibody confirmed that CLEVER-1 was restricted to the sinusoidal regions in liver sections. The staining did not outline the entire channel but appeared to stain the perinuclear region (Goerdt et al., 1991). Double immunohistochemical staining with CD11b and CD45 was used to

demonstrate that Kupffer cells were negative for CLEVER-1 but a sinusoidal specific marker was not used to confirm presence on sinusoidal endothelium. A further study using the MS-1 antibody and the F4 antibody demonstrated that, whilst CLEVER-1 was present on both the lymph node endothelial cells and a lymph node macrophage population, it was restricted to the sinusoidal endothelium in the human liver (Martens et al., 2006). There have been no reported studies of the expression of CLEVER-1 within diseased human liver tissue or in the context of malignant liver disease such as hepatocellular carcinoma.

Therefore the aims of our tissue expression studies were to

1. Identify the cell-specific expression of CLEVER-1 within the normal human liver *in vivo*.
2. Identify differences in CLEVER-1 expression between normal and diseased liver and any differences between specific aetiologies ie parenchymal diseases vs biliary diseases.
3. Study the expression of CLEVER-1 in hepatocellular carcinomas (HCC).

Immunohistochemistry was performed with an ImmPRESS^{TM} Universal anti-mouse/rabbit IgG reagent (Vector Labs, Burlingame CA) and a Vector VIP substrate kit method to detect the localisation of CLEVER-1 in liver tissue. At least four cases of normal liver and tissue from chronically inflamed parenchymal diseases (alcoholic liver disease and autoimmune liver disease) and biliary diseases (primary sclerosing cholangitis and primary biliary

cirrhosis) were studied. CLEVER-1 expression in malignant liver disease was studied in sections from hepatocellular carcinomas.

3.1.1 Expression of CLEVER-1 in normal liver

In normal liver tissue CLEVER-1 expression was localised within the sinusoidal channels (Figure 3-1). The staining was uniform across the hepatic lobule but confined to the sinusoids, other hepatic structures including hepatocytes, bile ducts, portal vessels and central veins were negative. Examination of the normal tissue at increased magnification demonstrated that the staining did not outline the entire channel but was more prominent in the perinuclear region of positive staining cells (Figure 3-2). With this technique it was not possible to determine whether the positive staining was within Kupffer cells or HSEC. Staining with isotype matched control was negative in all tissues.

3.1.2 CLEVER-1 expression in diseased liver tissue.

Analysis of CLEVER-1 staining in tissue from patients with alcoholic liver disease or autoimmune hepatitis demonstrated CLEVER-1 staining within the hepatic sinusoids but the expression was patchy compared to that seen in normal sections. Sinusoids that were CLEVER-1 positive demonstrated increased intensity of staining in comparison to normal liver tissue (Figure 3-3). Similar findings were also seen in sections from biliary diseases (Figure 3-4). Higher magnification of the sections demonstrated that more of the outline of the sinusoidal channel was stained with CLEVER-1 suggesting that

there was a change in the localisation of CLEVER-1 expression to the membrane of the cells (Figure 3-5). CLEVER-1 expression was also present in other structures apart from the sinusoids in diseased tissue. Strong staining was seen within the fibrous septa and on the luminal aspect of neovessels within the septa (Figure 3-6A). CLEVER-1 was also detected on endothelium within portal associated lymphoid follicles**.** More specifically, positive CLEVER-1 staining was seen in the vessels supplying these follicles, which are characterised by their high endothelial venule-like structure (Figure 3-6C, E).

3.1.3 CLEVER-1 expression in malignant disease.

CLEVER-1 was detected within tumour sinusoids in tissue taken from HCC arising on the background of different types of chronic liver disease. The staining of CLEVER-1 in some tumours was similar in pattern to that found within normal liver sinusoids whereas in other tumours the staining was more continuous within the sinusoids (Figure 3-7 A, C). Interestingly, in contrast to normal livers, CLEVER-1 staining was detected in the walls of vessels within the tumour (Figure 3-7 E).

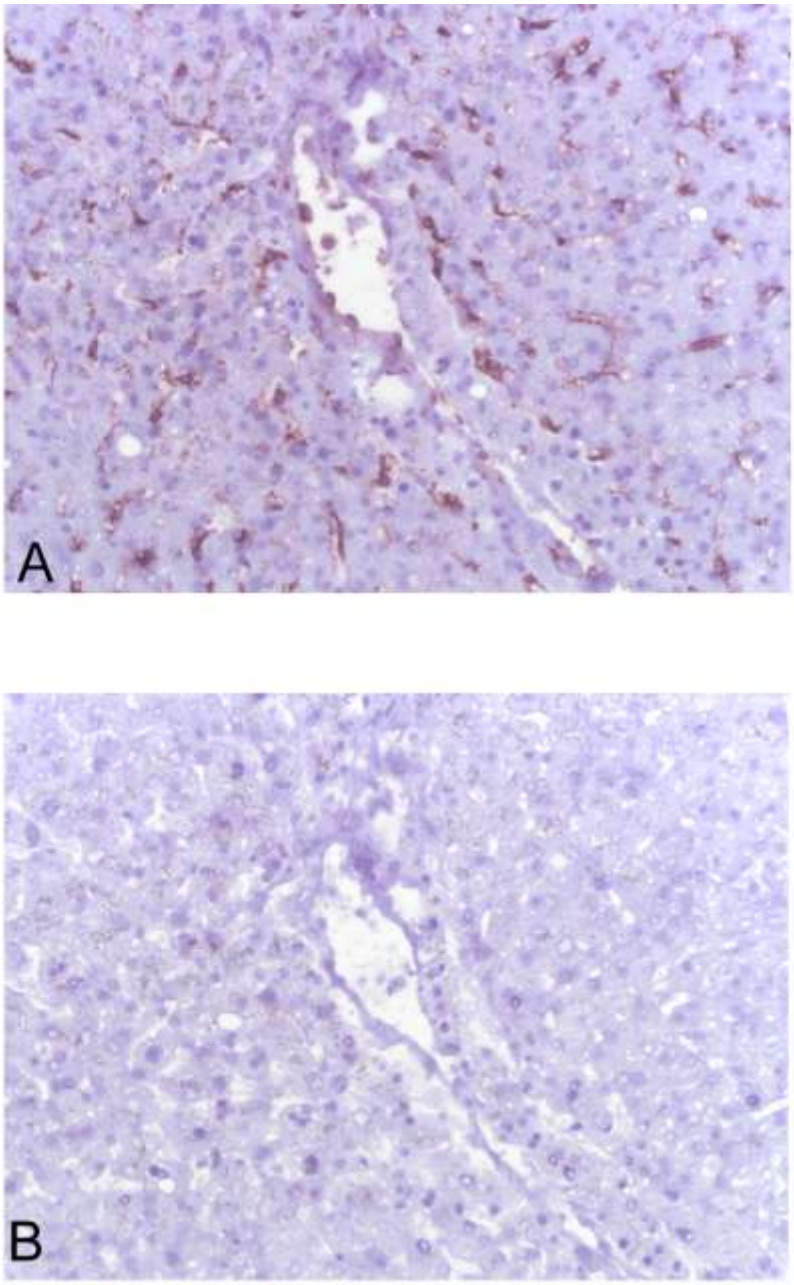


Figure 3-1 **Expression of CLEVER-1 in normal human liver**

Representative images from sinusoidal fields in normal liver samples which were stained with an antibody raised against CLEVER-1 (A) or isotype matched control antibody (B) Fields were captured by 20x objectives. CLEVER-1 staining (purple) can be seen within the sinusoids.

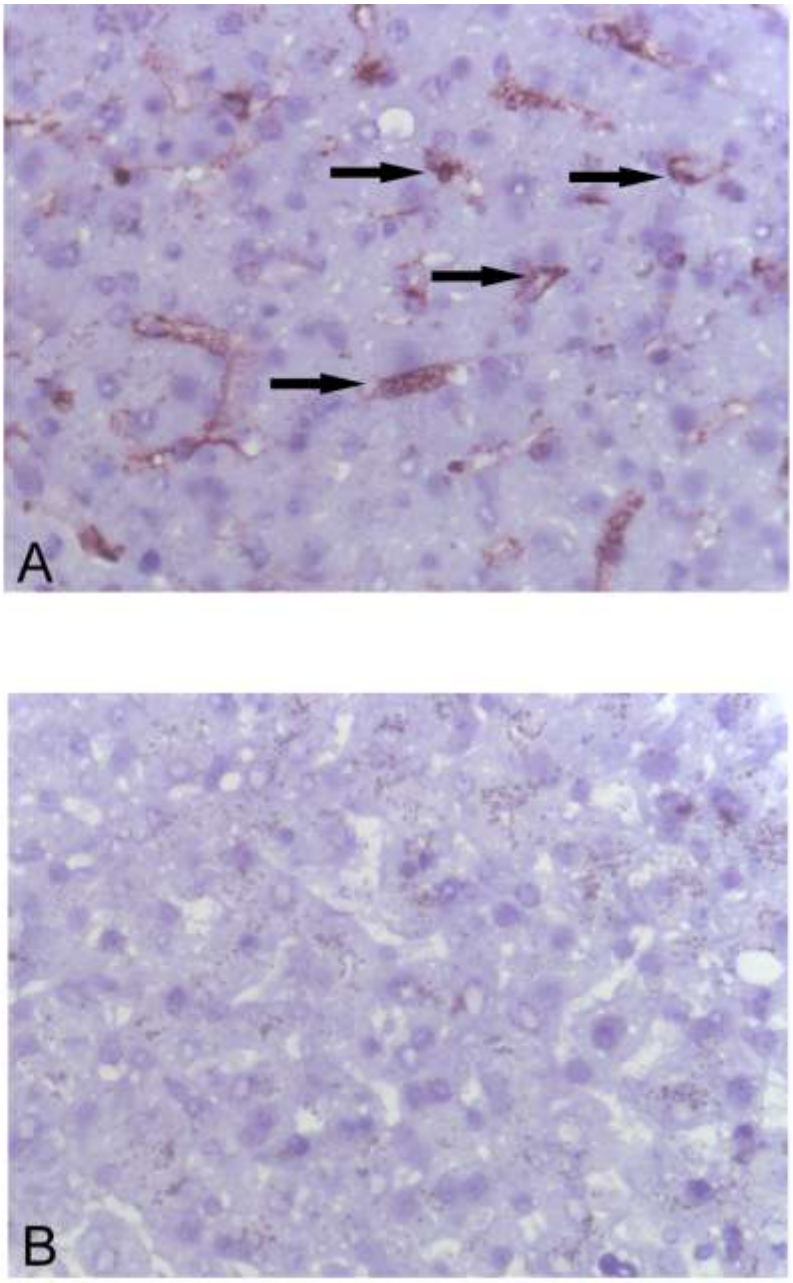


Figure 3-2 **Expression of CLEVER-1 in normal hepatic sinusoids**

Representative images from sinusoidal fields in normal liver samples which were stained with an antibody raised against CLEVER-1 (A) or isotype matched control (B Fields were captured by 40x objectives. Arrows denote areas of prominent perinuclear staining of CLEVER-1 positive cells within the sinusoids.

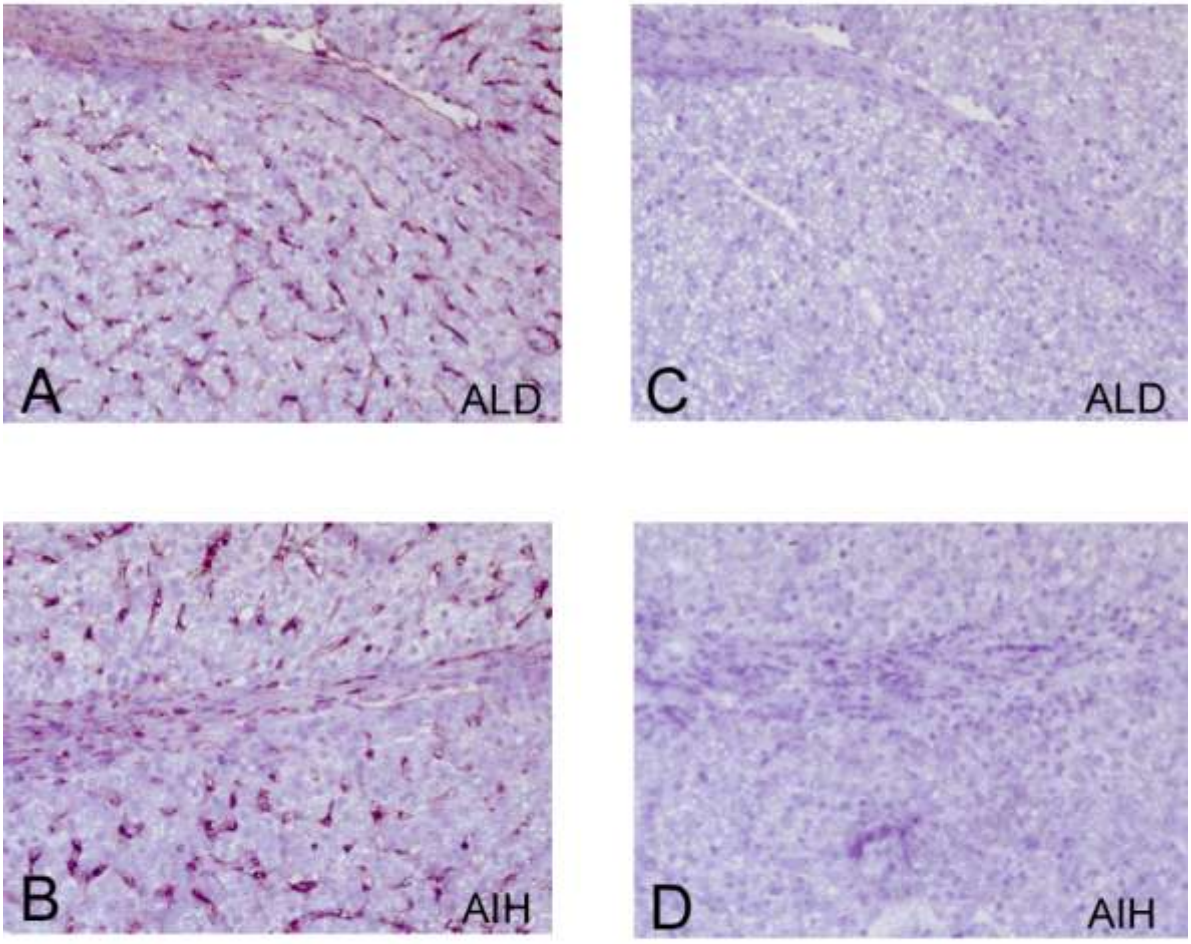


Figure 3-3 **Expression of CLEVER-1 in chronic parenchymal liver disease**

Representative images from samples of explanted livers from patients with chronic parenchymal liver disease which were stained with an antibody raised against CLEVER-1 (A and B) or isotype matched control (C and D). Fields were captured by 20x objectives. ALD (alcoholic liver disease), AIH (autoimmune hepatitis)

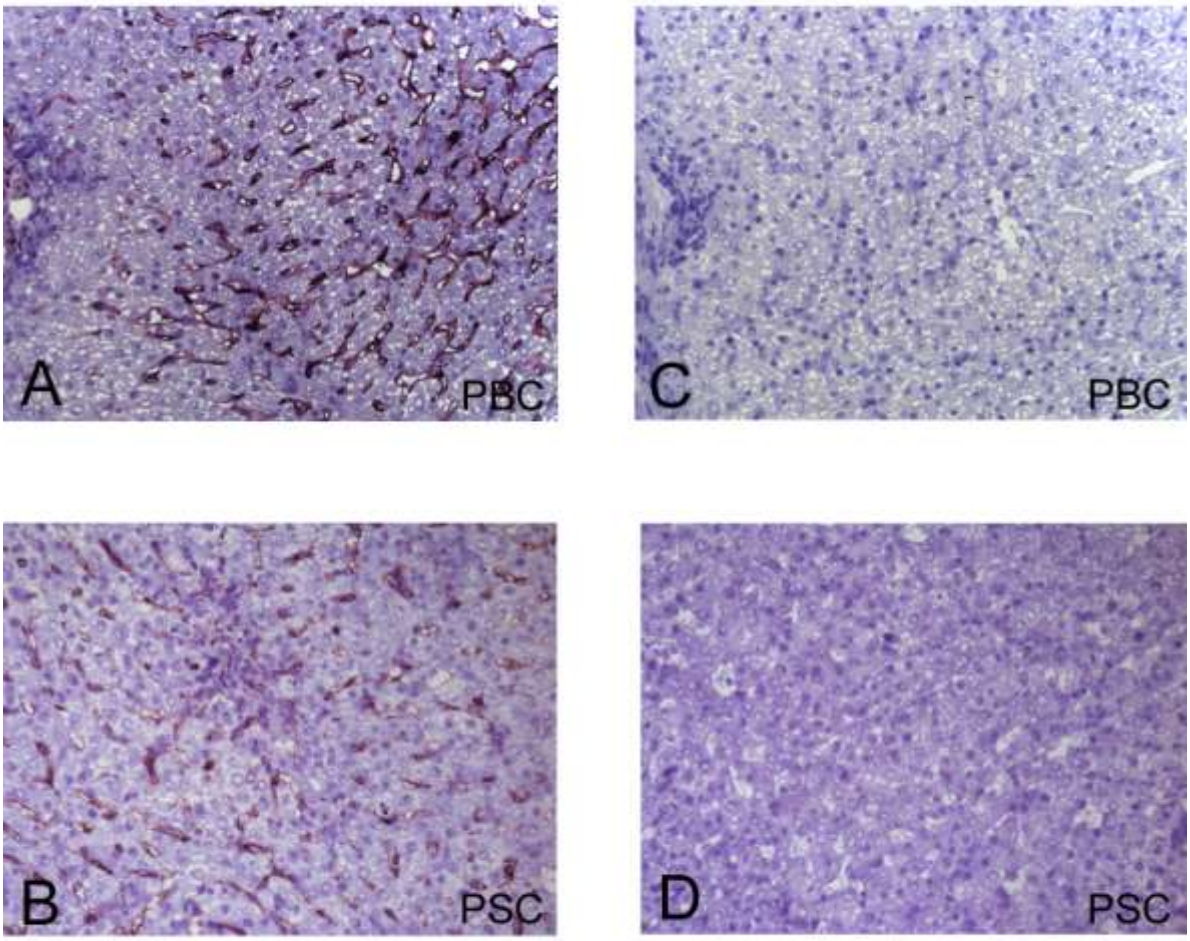


Figure 3-4 **Expression of CLEVER-1 in chronic biliary liver disease**

Representative images from samples of explanted livers from patients with chronic biliary liver disease which were stained with an antibody raised against CLEVER-1 (A and B) or isotype matched control (C and D). Fields were captured by 20x objectives. PBC (primary biliary cirrhosis), PSC (primary sclerosing cholangitis)

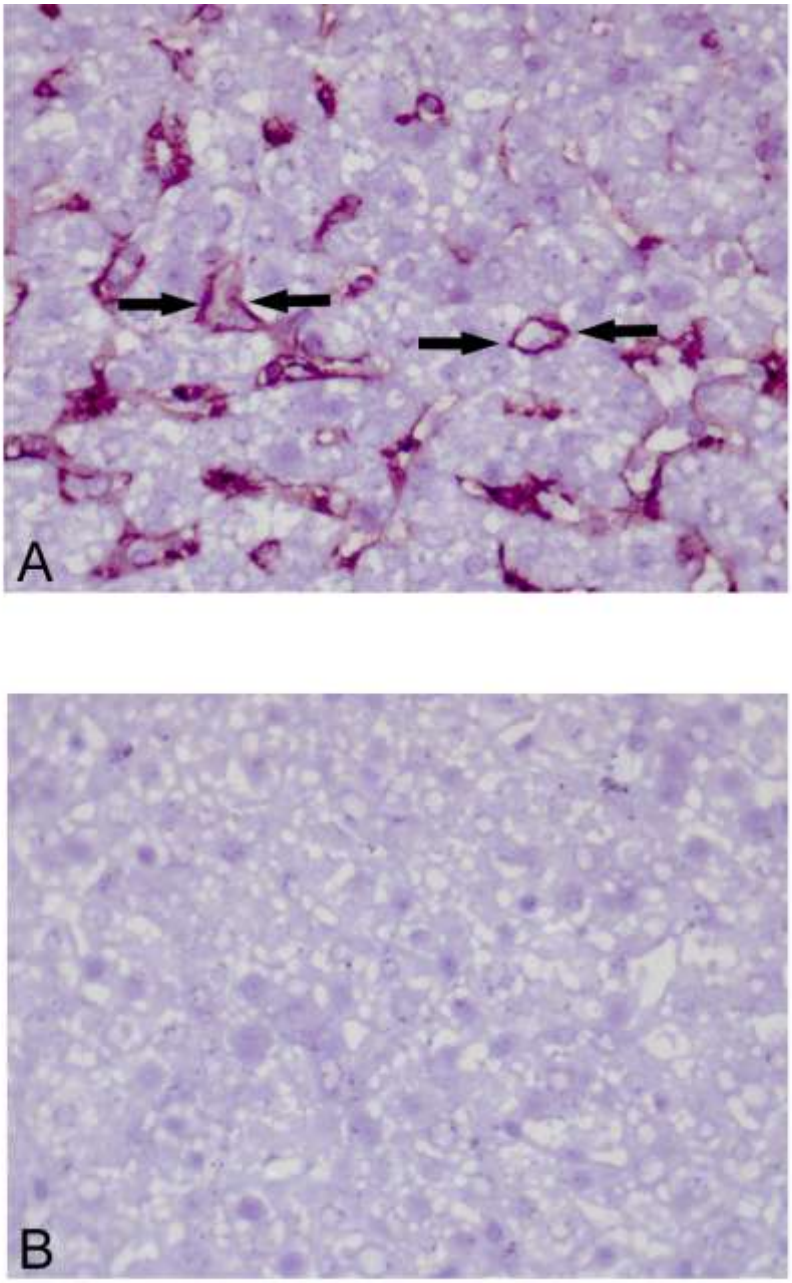


Figure 3-5 **Expression of CLEVER-1 within diseased hepatic sinusoids**

Representative images from sinusoidal fields in diseased liver samples which were stained with an antibody raised against CLEVER-1 (A) or isotype matched control (B). Arrows denote areas of CLEVER-1 staining where it outlines the sinusoidal channels. Fields were captured with a 40x objectives. The sample is from a patient with ALD.

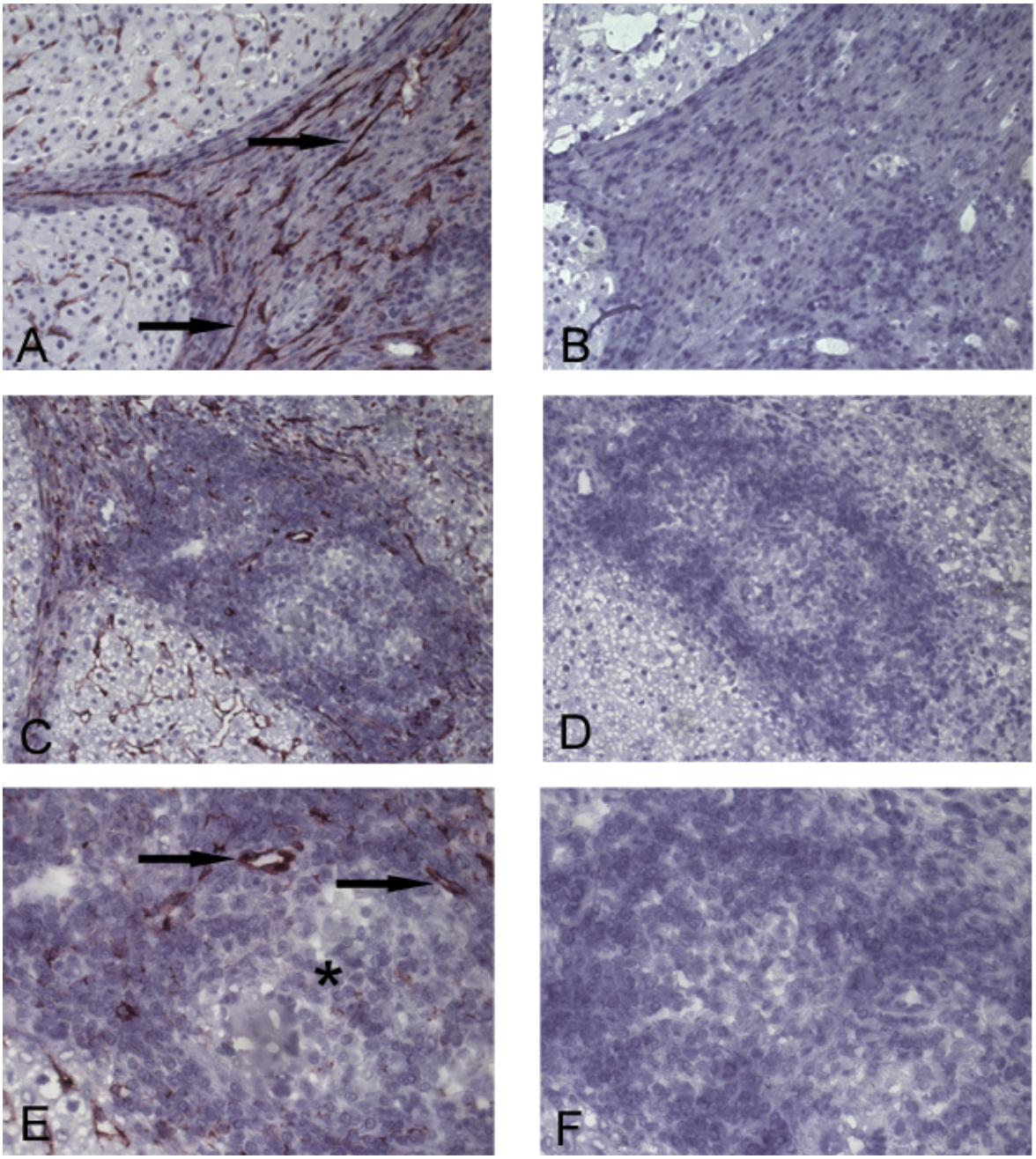


Figure 3-6 **Expression of CLEVER-1 within fibrous septum and portal associated lymphoid tissue**

Representative images of fibrous septa (A,B) and portal associated lymphoid tissue in chronically inflamed liver tissue (C-F) which were stained with an antibody raised against CLEVER-1 (A and C), or isotype matched control (B and D). E is inset of panel C and F is inset of panel D. Arrows highlight vessels staining positive for CLEVER-1 and asterisk denotes a germinal centre. Fields were captured with a 20x objective, apart from Panels E,F which were captured with 40x objective. Liver tissue from patients with ALD.

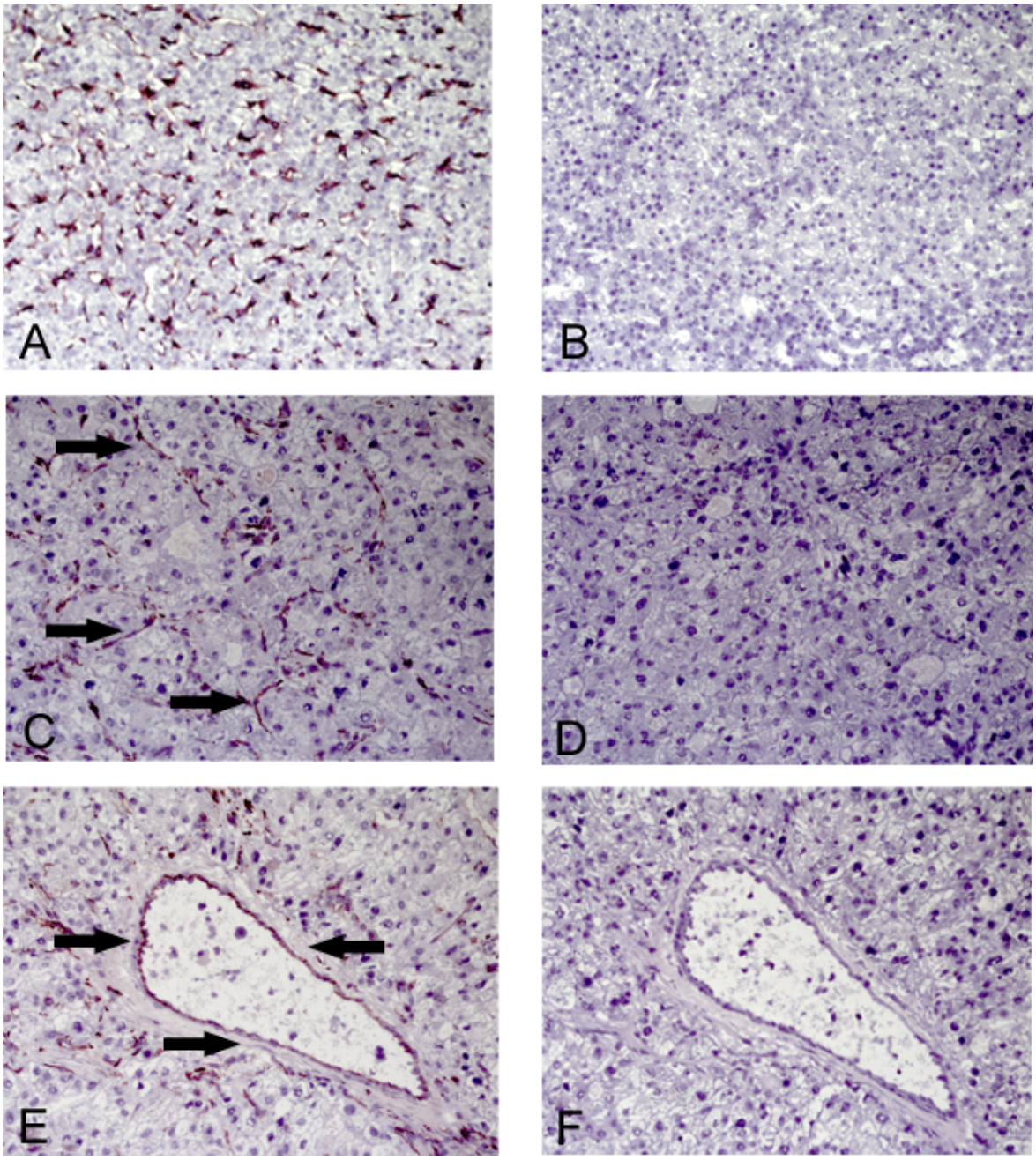


Figure 3-7 **Expression of CLEVER-1 within hepatocellular carcinoma**

Representative images of sinsusoidal fields (A –D) and tumour-associated vessel (E,F) in hepatocellular carcinoma samples which were stained with an antibody against CLEVER-1 (A,C,E), or isotype matched control (B,D,F). Fields captured with a 20x objective.

3.2 CLEVER-1 expression in the liver by immunofluorescence

3.2.1 Dual staining of CLEVER-1 with sinusoidal endothelial cell and kupffer cell markers.

Although our initial immunohistochemical investigations clearly showed expression of CLEVER-1 within normal and diseased liver sinusoids, we could not conclusively identify the specific cell which expressed CLEVER-1 using this technique (see section 3.1). The two major cell populations within the hepatic sinusoids are the Kupffer cells and sinusoidal endothelial cells. Dual-colour immunofluorescence was therefore used to identify which of these two cell-types within the hepatic sinusoids was CLEVER-1 positive. CLEVER-1 colocalised with L-SIGN (a sinusoidal endothelial cell specific marker) confirming that CLEVER-1 was expressed by HSEC (Figure 3-8 A-C). Colocalisation was confirmed with confocal microscopy (Figure 3-8 D and E). CLEVER-1 did not colocalise with CD68 (Kupffer cell marker) positive cells. The absence of CLEVER-1 from Kupffer cells was noted in normal and diseased livers (Figure 3-9).

3.2.2 Dual staining with vascular endothelial cell markers

Our immunohistochemical studies demonstrated CLEVER-1 expression within fibrous septa of chronically inflamed livers (Figure 3-6). The positive staining appeared to be present within the walls of inflammatory neovessels which are known to express the vascular endothelial marker CD34. Dual

colour immunofluorescence was used to demonstrate colocalisation of CLEVER-1 with CD34 within the fibrous septa confirming the expression of CLEVER-1 on neo-vessels (Figure 3-10).

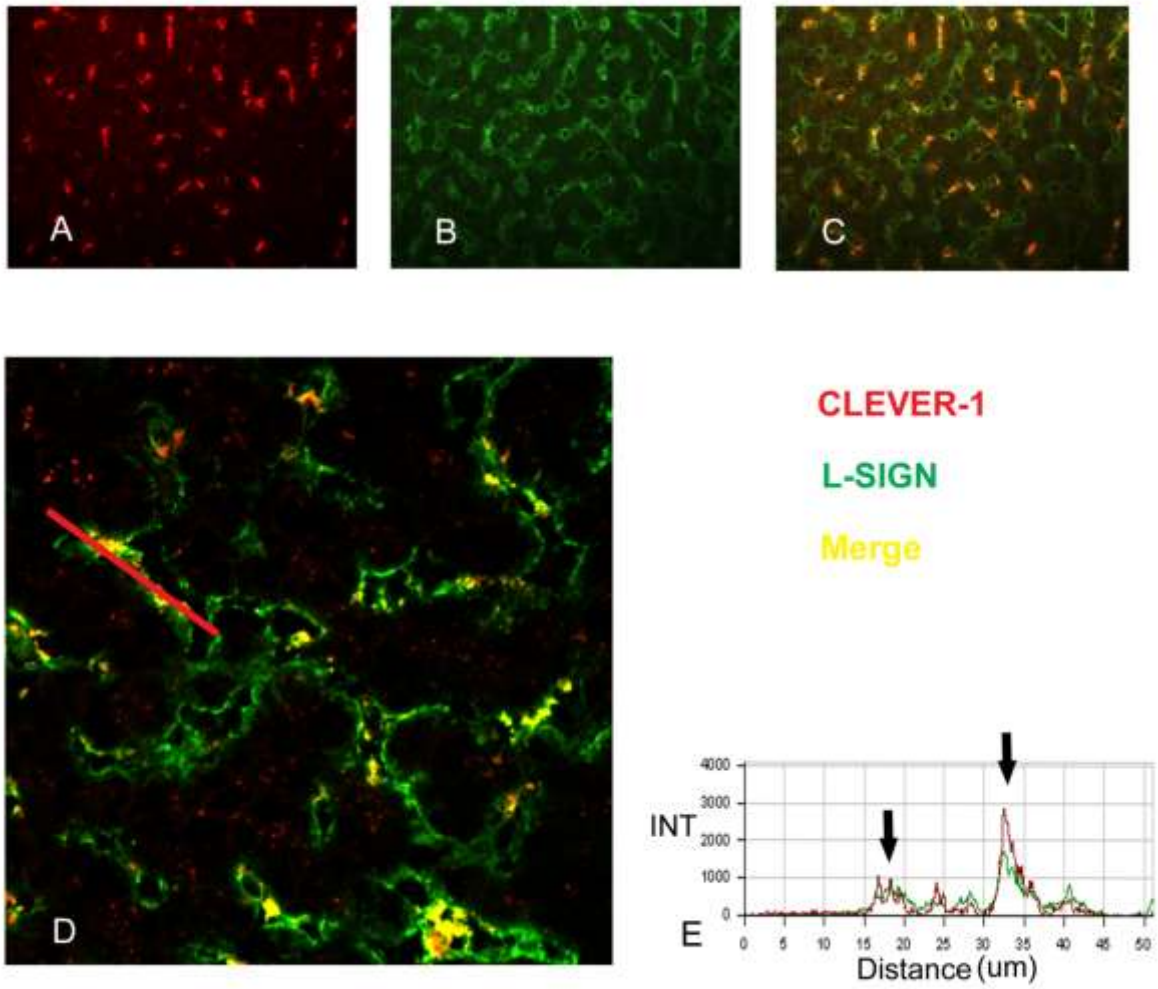


Figure 3-8 **CLEVER-1 is expressed by HSEC**

Normal liver samples were stained using dual-colour immunofluorescence. Representative images are shown of a normal liver stained with CLEVER-1 antibody and texas red secondary (A), and L-SIGN antibody with FITC secondary (B). Panel C demonstrates merged image of Panel A and B. Panel D demonstrates confocal imaging of a normal liver sample stained with CLEVER-1 antibody (Alexa Fluor 546 secondary) and L-SIGN antibody (FITC secondary. Colocalisation is seen as yellow. Panel E shows two colour intensity profile of line in Panel D (INT=Fluorescent intensity). Fields captured by 40x objective except Panel D which was captured by 63x objective.

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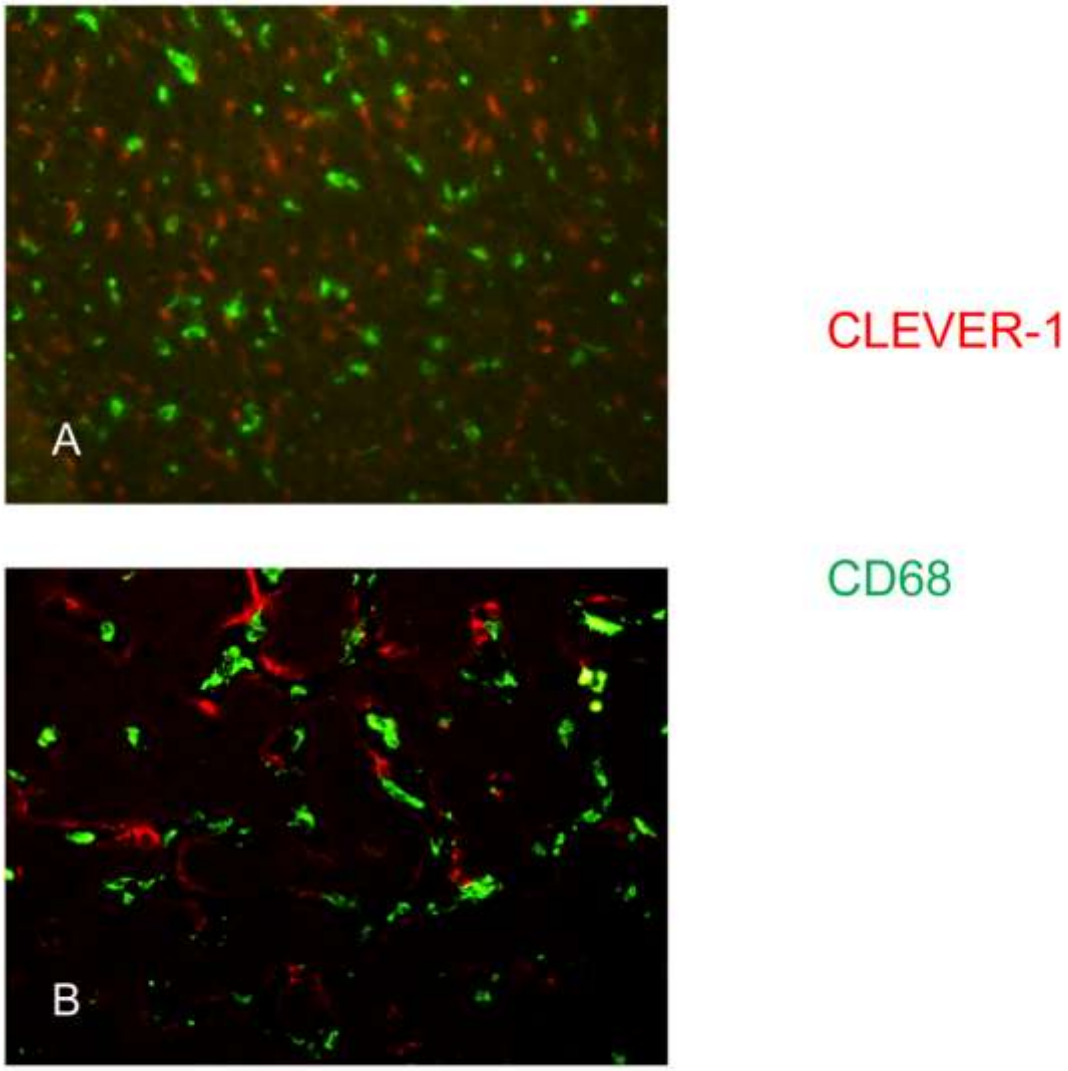

Figure 3-9 **CLEVER-1 is not expressed by Kupffer cells**

Liver samples were stained using dual-colour immunofluorescence. Representative images are shown from samples of normal liver (A) and from samples of explanted liver from a patient with primary biliary cirrhosis (B). Sections were stained with CLEVER-1 antibody (Texas red secondary) and CD68 antibody (FITC secondary). Panel A is a field captured by 20x objective. Panel B is a field captured by 40x objective.

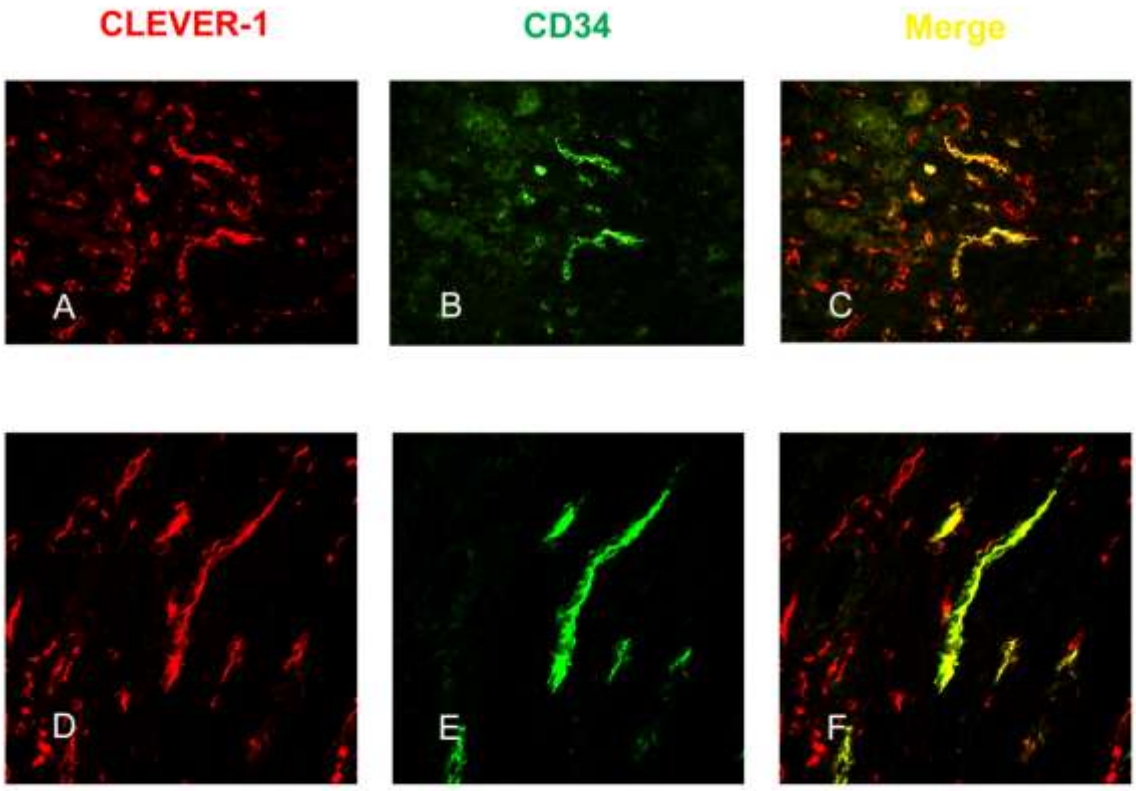

Figure 3-10 **CLEVER-1 is expressed by neovessels**

Liver samples were stained using dual colour immunofluorescence. Representative images of explant liver samples from a patient with alcoholic liver disease which were stained with CLEVER-1 antibody and Texas red secondary (A), CD34 antibody and FITC secondary (B). Images were then merged (C). Fields were captured by 40x objective. Representative confocal images were also taken of explanted liver sections which were stained with CLEVER-1 antibody and Alexafluor 546 secondary (D), CD34 antibody with FITC secondary (E). Images were then merged (F). Fields were captured by 63x objective.

3.2.3 The quantification of CLEVER-1 protein in liver tissue

Semi-quantititative assessment of CLEVER-1 protein in normal liver tissue compared to diseased liver tissue was performed by western blotting. Protein lysates were obtained from normal liver and explanted livers from patients with a range of chronic inflammatory liver diseases. Initial western blots using the 3-372 antibody to label CLEVER-1 were negative (not shown). The assay was repeated without reducing the protein lysates with beta-mercaptoethanol. Following this alteration to the assay, protein bands for CLEVER-1 were detected between molecular weight markers for 300kDa and 220kDa consistent with the known molecular weight of CLEVER-1 of 270kDa. The results demonstrated the presence of protein in, and in contrast with immunohistochemical data we noted no obvious difference in intensity between normal liver and diseased livers (Figure 3-11).

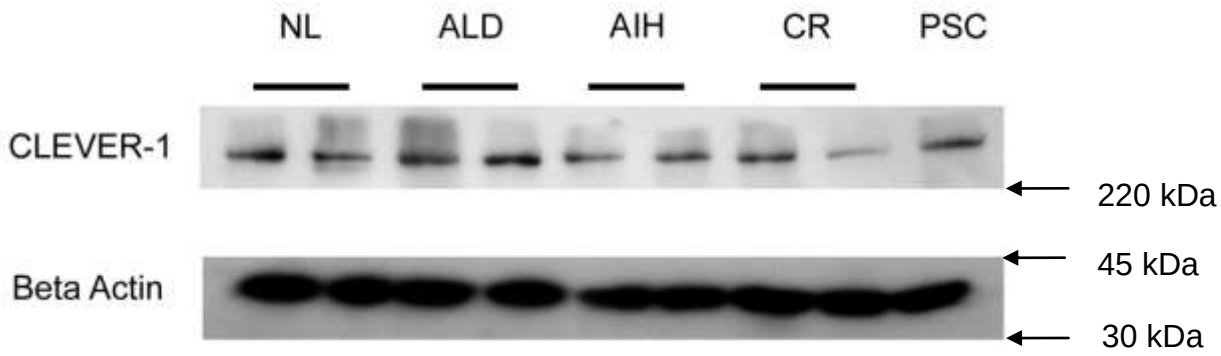

Figure 3-11 **CLEVER-1 Protein Expression in Normal and Diseased Liver Tissue**

The top row represents a western blot for CLEVER-1 of normal and diseased liver tissue. A blot for β Actin was performed for the same samples to ensure equal protein loading. NL- Normal liver, ALD-Alcoholic liver disease liver, AIH- Autoimmune hepatitis liver, CR- Chronic rejection, Primary Sclerosing Cholangitis. Data shows blots from two samples of each condition apart from PSC which is a single sample.

3.3 The expression of CLEVER-1 in primary human hepatic sinusoidal endothelial cells

Several groups have studied the expression of CLEVER-1 on isolated human cells *in vitro*. An early report studying human macrophages, confirmed basal expression of CLEVER-1 in these cells by immunoprecipitation and demonstrated a strong stimulatory effect of IL-4 and dexamethasone on protein expression (Goerdt et al., 1993). Further studies confirmed the expression of CLEVER-1 in monocyte derived macrophages from humans and bone marrow derived macrophages from mice (Kzhyshkowska et al., 2004; Politz et al., 2002; Schledzewski et al., 2006). Studies by FACS and immunofluorescence with cultured endothelial cells from lymph node and skin lymphatics confirmed that these cells also expressed CLEVER-1 *in vitro* (Prevo et al., 2004; Salmi et al., 2004). CLEVER-1 has also been shown to be expressed by primary human hepatic sinusoidal endothelial cells and pig and rabbit liver sinusoidal endothelial cells (Hansen et al., 2005a; Prevo et al., 2004). Thus we wished to confirm and extend the available data for expression in human primary hepatic endothelial cells in the current study. To this end we confirmed the purity and phenotype of our cell populations and then we investigated i)The expression of CLEVER-1 in the isolated HSEC from normal and diseased livers by RT-PCR from normal and diseased livers and ii) the expression and distribution of CLEVER-1 on HSEC by immunofluorescence.

3.3.1 Purity and sinusoidal phenotype of isolated hepatic endothelial cells.

HSEC were isolated from normal and diseased liver tissue as described in 2.2.3. Cultured HSEC displayed a characteristic morphology (Figure 3-12) and endothelial cell purity was assessed by CD31 expression using flow cytometry, shown to be 99%. (Figure 3-12). LYVE-1 is expressed on sinusoidal endothelial cells and this marker was used to confirm the phenotype of the endothelial cells from HSEC isolates (Mouta et al., 2001). LYVE-1 expression was demonstrated by immunofluorescence and the presence of LYVE-1 messenger RNA was confirmed by reverse transcriptase PCR in HSEC isolates from normal and diseased livers (Figure 3-13 C).

3.3.2 Expression of CLEVER-1 in isolated human hepatic sinusoidal endothelial cells

Once we had confirmed the phenotypic identity of our cell isolates we studied CLEVER-1 expression by semiquantative RT-PCR in HSEC isolates from normal liver specimens. Agarose gels demonstrated PCR products for CLEVER-1 at the expected size in both early and late passage samples (**Error! Reference source not found.**Panel A). However as is evident in his figure, several non-specific bands were also amplified during the PCR reaction. To increase the specificity of the PCR reaction a touchdown protocol was set up which commenced at a high annealing temperature and subsequently decreased the temperature with each cycle. The touchdown protocol resulted in a clear specific band (**Error! Reference source not**

ound. panel B). The touchdown method was therefore used in any further PCR reactions to detect CLEVER-1.

During chronic liver injury HSEC are known to undergo significant morphological and phenotypical changes (Couvelard et al., 1993; Mori et al., 1993). Our staining results suggested that HSEC from diseased livers maintained their CLEVER-1 expression *in vivo* (see section 3.1.2). To confirm these findings *in vitro* semiquantative RT-PCR for CLEVER-1 was performed in HSEC isolates from explanted livers from patients with alcoholic liver disease, primary biliary disease. Figure 3-15 shows CLEVER-1 mRNA was detected in all these samples confirming that HSEC from diseased liver maintained CLEVER-1 expression.

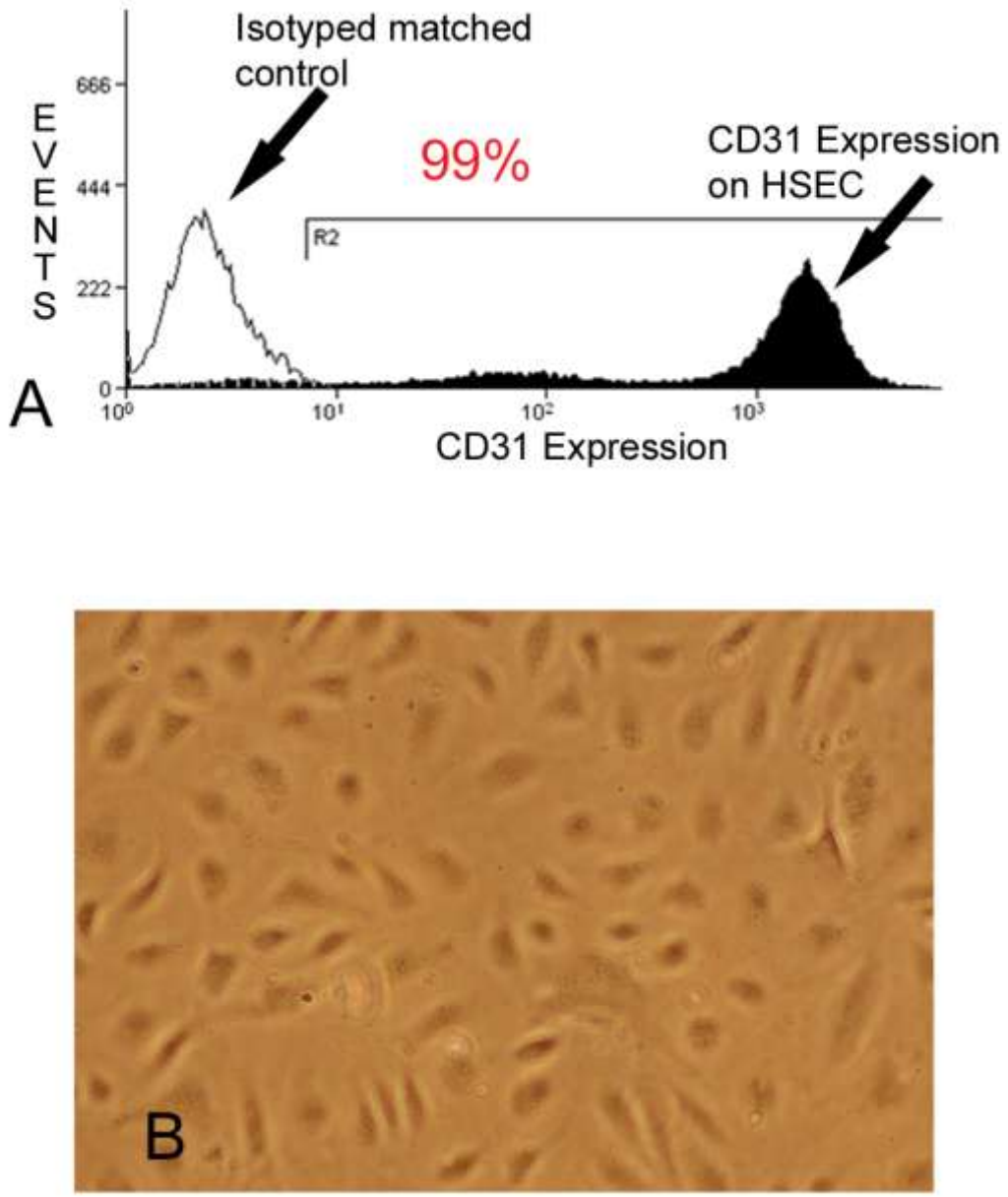

Figure 3-12 **Purity and morphology of isolated HSEC**

Panel A- Isolated human hepatic sinusoidal endothelial cells (HSEC) from liver samples were stained for CD31 and isotype matched control and analyzed by flow cytometry. Panel B – Photograph of isolated HSEC in culture.

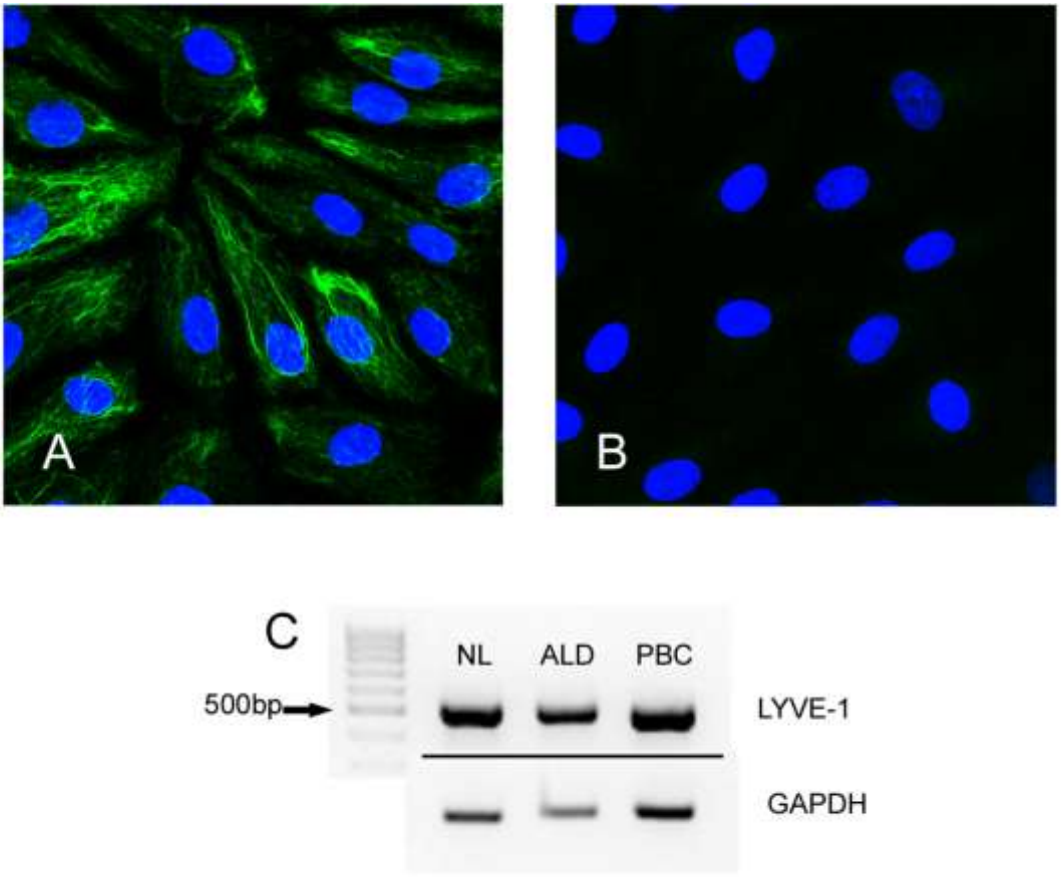

Figure 3-13 **Sinusoidal phenotype of isolated HSEC from normal and diseased livers**

Representative images of isolated human hepatic sinusoidal cells (HSEC) stained for LYVE-1 (FITC secondary) (Panel A) and isotype matched control (Panel B). Nucleii were stained with DAPI(Blue). Representative agarose gel of HSEC isolates from normal liver (NL), alcoholic liver disease (ALD) and primary biliary cirrhosis (PBC).

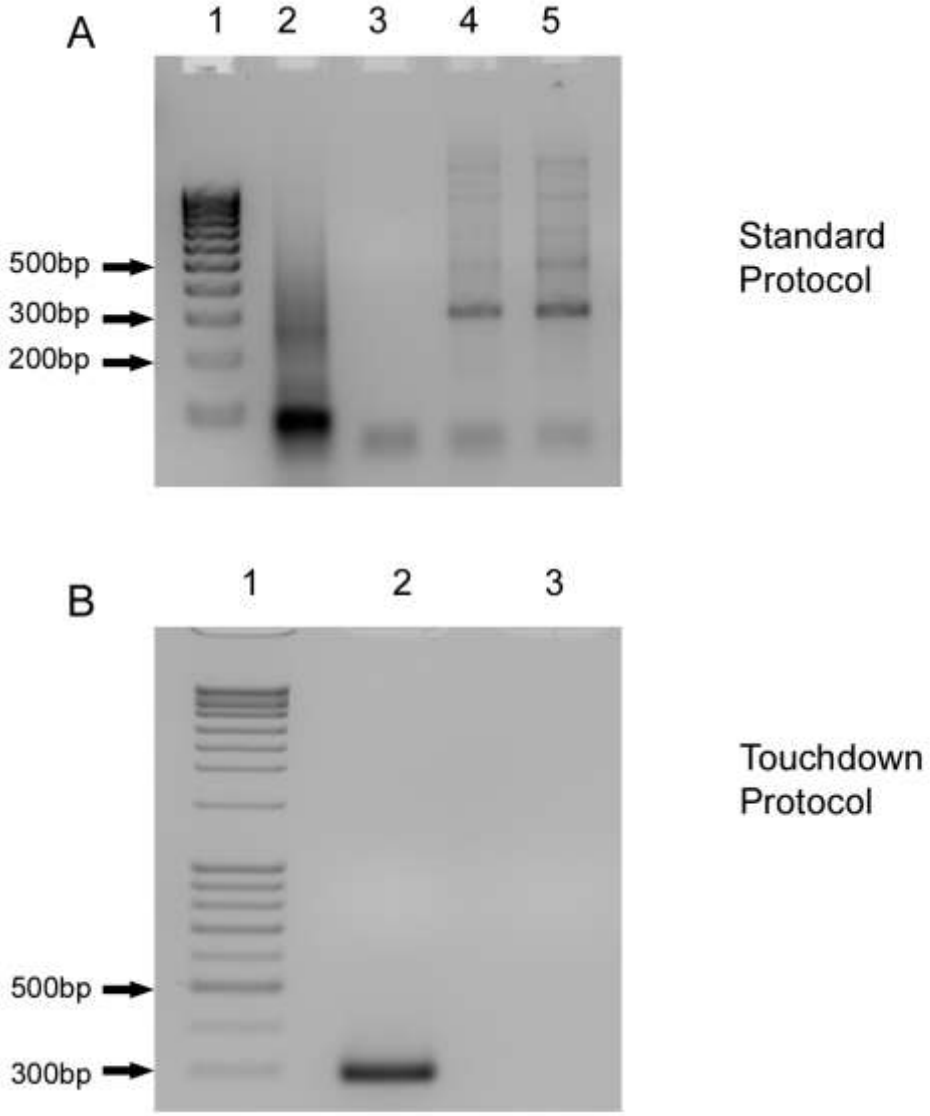

Figure 3-14 **Detection of CLEVER-1 m RNA in HSEC**

RT-PCR was performed by a standard protocol and a representative agarose gel of HSEC isolates is shown (Panel A). Lane 1) Ladder; Lane 2) Positive control; Lane 3) no sample; Lane 4) HSEC passage 3;Lane 5) HSEC passage 6. RT-PCR was performed by a touchdown protocol and a representative agarose gel of a HSEC isolate is shown (Panel B). Lane 1) Ladder; Lane 2) HSEC passage 5; Lane 3) no sample.

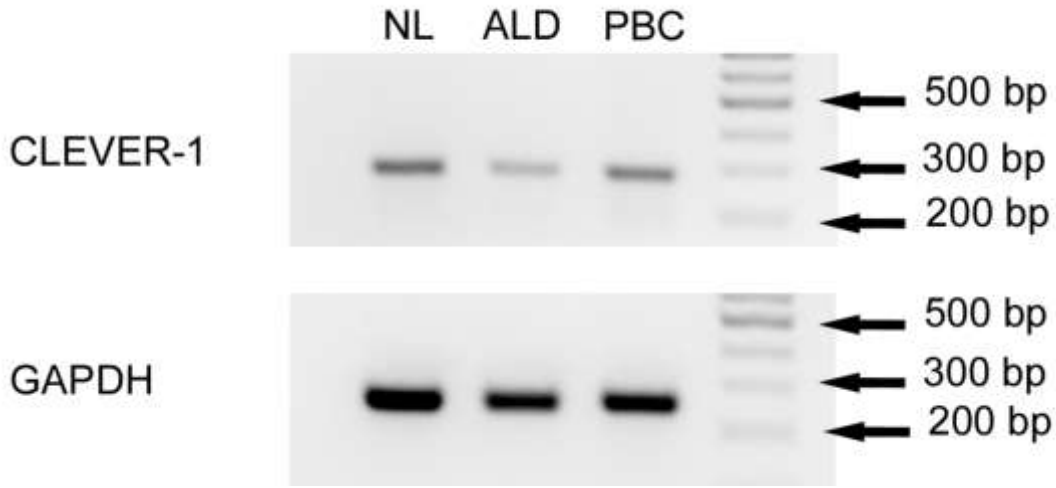

Figure 3-15 **Detection of CLEVER-1 mRNA in HSEC from normal and diseased livers**

A representative agarose gel of HSEC isolates from normal liver (NL), alcoholic liver disease (ALD), primary biliary cirrhosis (PBC).

Isolated HSEC were cultured on coverslips as described in section2.4.2. Immunofluorescent staining confirmed the expression of CLEVER-1 protein in isolated HSEC. As seen in Figure 3-16 CLEVER-1 protein was present in isolated HSEC. CLEVER-1 expression in cultured HSEC was predominantly intracellular with a diffuse perinuclear localisation.

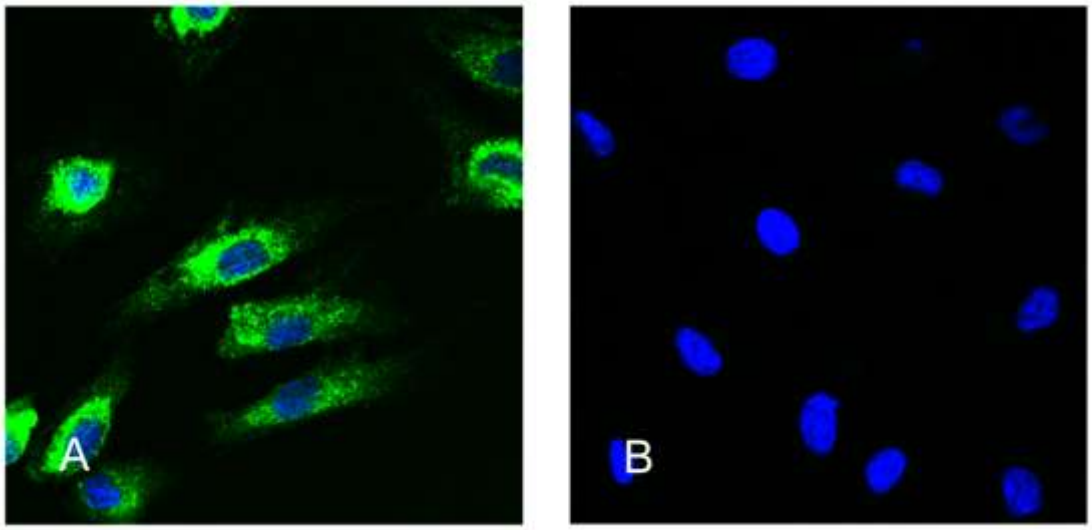

Figure 3-16 **CLEVER-1 expression by immunofluorescnce in primary HSEC**

Representative images of isolated human hepatic sinusoidal cells (HSEC) stained for CLEVER-1 (FITC secondary) (Panel A) and isotype matched control (Panel B). Nucleii were stained with DAPI (Blue). Fields captured with x63 objective.

3.4 Summary of CLEVER-1 expression studies.

The immunohistochemical studies in this chapter have detailed the expression of CLEVER-1 in normal and diseased human liver. Within the normal liver CLEVER-1 was restricted to the hepatic sinusoids, specifically restricted to HSEC and absent from Kupffer cells. To our knowledge studies of CLEVER-1 expression in chronic liver disease or hepatocellular carcinomas have not been published. Examination of tissue sections from a variety of chronic liver diseases confirmed that CLEVER-1 expression was maintained on HSEC during disease with evidence that expression increased compared to normal liver. CLEVER-1 was also found at other sites in the diseased liver. Whereas CLEVER-1 was restricted to sinusoidal endothelium in normal liver tissue in diseased livers its expression was upregulated on neovessels within fibrous septa and vessels supplying portal associated lymphoid tissue. Furthermore, studies of hepatocellular carcinoma samples demonstrated CLEVER-1 within tumour sinusoids and tumour associated vessels. CLEVER-1 protein expression in liver tissue from normal and diseased livers was confirmed by western Blotting. Finally, CLEVER-1 expression in isolated HSEC was confirmed by immunofluorescence and RT-PCR.

The results of CLEVER-1 expression in normal human liver tissue are in keeping with previous studies (Martens et al., 2006). Investigation of diseased liver samples demonstrated changes in CLEVER-1 expression in response to chronic inflammation which is associated with morphological

changes in HSEC. During ongoing inflammation the hepatic sinusoidal vascular bed undergoes a phenomenon termed ‘capillarisation’. Studies using animal models have demonstrated that capillarisation involves the loss of fenestrae from HSEC and the formation of a subendothelial basement membrane (Bhunchet and Fujieda, 1993; Ishak et al., 1991; Martinez-Hernandez and Martinez, 1991; Mori et al., 1993). There is evidence that this change in structure is also associated with a change in receptor expression, including increased expression of vascular markers such as CD31 and CD34 (Couvelard et al., 1993). Examination of tissue sections from a variety of chronic liver diseases confirmed that CLEVER-1 expression was maintained on HSEC during disease and there was evidence that the distribution and intensity of staining actually increased compared to normal livers although this was not confirmed by western blotting. A range of chronic liver diseases were studied and compared. The changes in CLEVER-1 expression in parenchymal and biliary diseases were very similar and this suggested that there were no disease specific differences (Figure 3-3 and Figure 3-4). Other C-type lectins such as LYVE-1 have been shown to be down regulated in liver disease (Arimoto et al., 2009). This suggests that the regulation of CLEVER-1 expression on HSEC differs from other C-type Lectins.

Vascular structures within the fibrous septa found in chronically inflamed livers were also CLEVER-1 positive (Figure 3-6). These neovessels are thought to be vascular in nature and they express markers such as CD34. They may also be sites of lymphocyte recruitment in chronic inflammation

(Freemont, 1988; Grant et al., 2001). Another example of vascular endothelium is found in vessels which supply lymphoid follicles that develop in chronic inflammation. These vessels are similar to high endothelial venules (HEVs) seen in lymph nodes. These HEV-like vessels were also positive for CLEVER-1 (Figure 3-6). The fibrous septum and lymphoid follicles are thought to be important sites of lymphocyte recruitment and retention in chronic inflammation and therefore CLEVER-1 expression in these structures supports the hypothesis that CLEVER-1 plays a role in lymphocyte recruitment to the chronically inflamed liver.

The progression of chronic inflammation to cirrhosis leads to an increased risk of tumour formation, specifically the development of hepatocellular carcinomas. These tumours are dependent on angiogenic factors and develop on the background of dysplastic nodules (Kimura et al., 1998). They can also contain sinusoidal channels as seen in normal tissue but the endothelium is more vascular in nature (Nascimento et al., 2009). Our group and others have also demonstrated that these tumours are infiltrated by a mix of effector and regulatory T cells (Unitt et al., 2005; Yoong et al., 1999). The finding of CLEVER-1 within tumour sinusoids and tumour associated vessels suggests that CLEVER-1 may also play a role in lymphocyte recruitment to hepatocellular carcinomas.

The increased expression of CLEVER-1 at sites of chronic inflammation suggests that inflammatory stimuli may contribute to the regulation of this receptor. The presence in neovessels and hepatocellular carcinomas also

suggest that angiogenic factors may play a role in the induction of CLEVER-1 expression.

Western blotting allowed total protein quantification of CLEVER-1 in normal and diseased liver samples. Whilst the immunohistochemistry results suggested increased CLEVER-1 expression within the sinusoids of diseased livers compared to normal liver, these differences were not demonstrated in the blotting studies in which no differences were seen in the total amounts of CLEVER-1 protein between normal and diseased livers, Figure 3-11. A possible explanation is that the increased expression of CLEVER-1 in the sinusoids is balanced out by the changes in liver tissue during chronic inflammation such as architectural distortion and deposition of extracellular matrix. The immunohistochemistry studies combined with the western blotting data suggests that there is a change in the distribution of CLEVER-1 expression rather than in the total protein levels.

Confirmation of CLEVER-1 expression on isolated HSEC was successful by immunofluorescence and conventional PCR using both high and low passage cells and cells from normal and diseased liver tissue (Figure 3-15). But, whereas immunohistochemistry suggested stronger staining of CLEVER-1 in HSEC from diseased livers, this was not seen with mRNA expression (Figure 3-15). Significant variation in CLEVER-1 expression between batches of isolated endothelial cells has been noted previously in the context of human lymphatic endothelial cells (Salmi et al., 2004). To our knowledge, the expression of CLEVER-1 in endothelial cells from normal and

diseased organs has not been compared previously. A likely explanation for these contrasting results is that HSEC from normal and diseased livers revert to a common phenotype once they are isolated and cultured *in vitro,* as they no longer receive signals from the hepatic microenvironment.

These studies have demonstrated the expression of CLEVER-1 in human hepatic tissue and isolated human HSEC but the signals which influence CLEVER-1 expression *in vivo* have not been identified. In the next chapter we will try to identify these factors by investigating the regulation of CLEVER-1 expression on HSEC.

CHAPTER 4

THE REGULATION OF CLEVER-1 IN HUMAN HEPATIC SINUSOIDAL ENDOTHELIUM

4.1 Response to cytokine stimulation

The regulatory factors which influence CLEVER-1 expression in sinusoidal endothelial cells *in vitro* are unclear. Its expression on cultured macrophages is strongly induced by a combination of IL-4 and dexamethasone, while interferon γ has inhibitory effects (Goerdt et al., 1993). Stimulation by IL-4 and dexamathasone also led to alteration in CLEVER-1 localisation with an increase in cell surface expression (Kzhyshkowska et al., 2004). However, CLEVER-1 expression in endothelial cells appears to be differently regulated; studies in human lymphatic endothelium showed no induction by IL-4 or dexamethasone whereas LPS led to a modest increase (Salmi et al., 2004). In addition, cytokine stimulation has not been shown to alter CLEVER-1 localisation in lymphatic endothelial cells (Prevo et al., 2004). Since there is limited published data relating to the regulation of CLEVER-1 in hepatic sinusoidal endothelial cells, the aims in this chapter were to

1. Study the protein expression of CLEVER-1 in HSEC by cell-based ELISA under basal conditions and in response to various stimuli.
2. Study factors which may influence CLEVER-1 mRNA expression in HSEC by quantitative real time PCR.

4.2 Protein expression of CLEVER-1 in HSEC and its response to cytokine stimulation.

Cell based ELISA was used to measure basal CLEVER-1 protein levels in HSEC and to determine response to cytokine stimulation. We used cell based ELISA rather that flow cytometry because for the latter endothelial cells must be lifted off culture plates and analysed in suspension whereas cell based ELISA keeps them as an intact monolayer which is more physiological. Initial pilot ELISA experiments with untreated or cytokine treated HSEC suggested that levels of CLEVER-1 expression were extremely low or that the protein was absent (Figure 4-1 Panel A). However, later experiments were modified to include an additional goat serum blocking step and this led to consistent detection of CLEVER-1 (Figure 4-1 Panel B). This suggests that adequate blocking of non-specific antibody binding was necessary to allow discrimination of CLEVER-1 antibody binding above that of isotype control signals.

Our group has previously shown that the classical endothelial adhesion molecules are upregulated on HSEC by inflammatory stimuli such as recombinant TNFα and IFNγ (Lalor et al., 2002a). Experiments were performed to investigate if 24 hour stimulation with TNFα, LPS and IFNγ alone or in combination had similar effects on CLEVER-1 expression. I used concentrations of the three cytokines that had been shown to induce optimal levels of ICAM-1 in previous studies from our group Figure 4-2 shows the results of these experiments demonstrating that these cytokines had no

significant effect on CLEVER-1 protein expression when compared to resting conditions.

In view of these findings, alternative cytokine stimuli were studied: TGFβ which is known to have a significant profibrogenic role in chronic liver disease (discussed in section1.6); IL-10 which, as discussed in section 1.5, can be secreted by a variety of liver resident cells and IL-4 which has been shown to induce CLEVER-1 expression in macrophages (discussed in section 4.1). Interestingly these cytokines also had little effect on CLEVER-1 expression by HSEC as shown in, Figure 4-3.

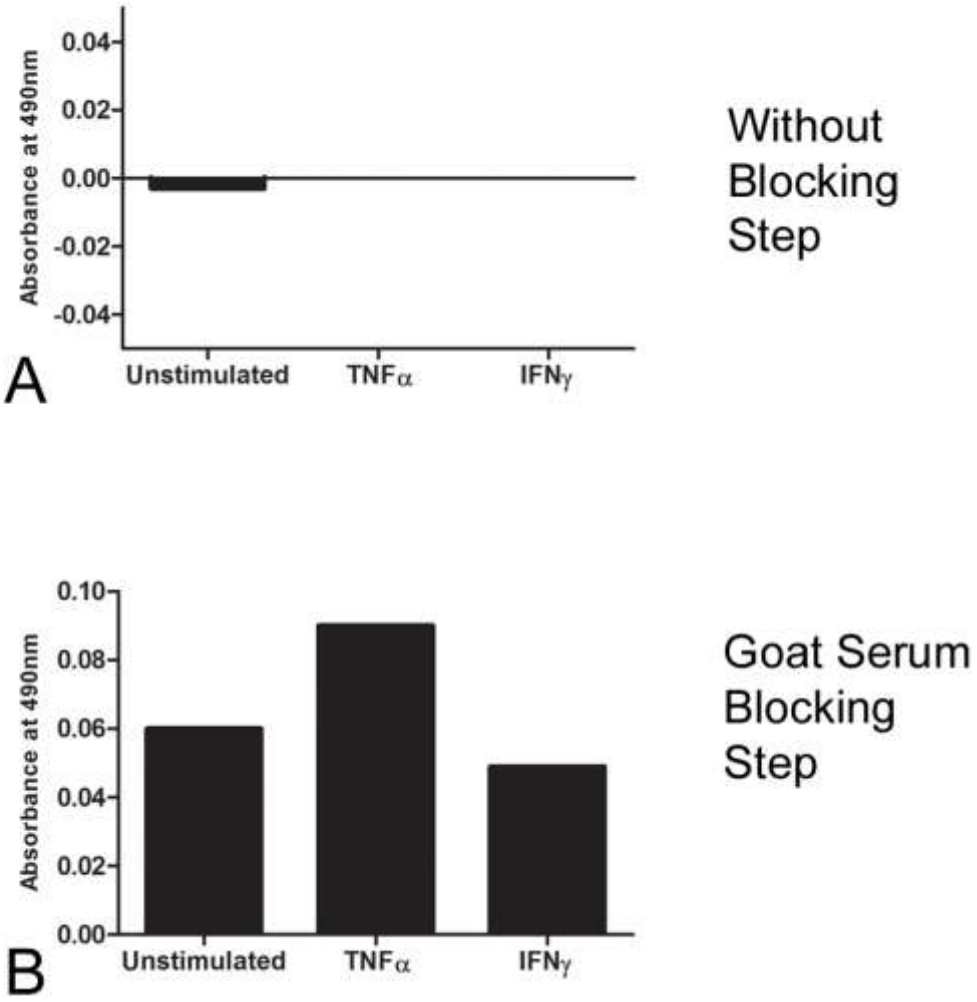

Figure 4-1 **Measurement of CLEVER-1 expression by ELISA in HSEC**

The figure demonstrates representative results of ELISAs for CLEVER-1 detection in unstimulated and cytokine stimulated HSEC without goat serum blocking step (Panel A) and with blocking step (Panel B). The values represent the mean absorbance of three replicate wells minus the absorbance of an isotype matched control antibody. Data are shown for one experiment. Cells were stimulated with cytokines for 24 hours.

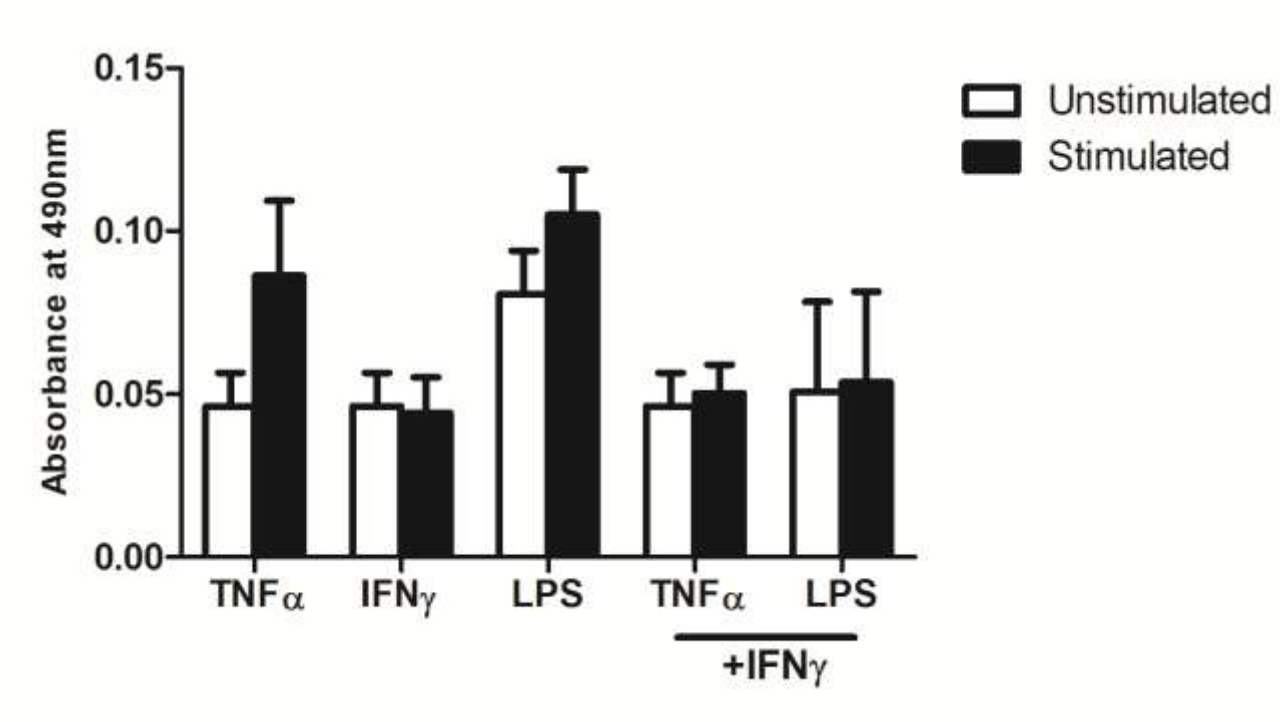

Figure 4-2 **Measurement of CLEVER-1 expression by HSEC in response to Inflammatory Stimuli**

ELISA data comparing the levels of CLEVER-1 protein in unstimulated (white bars) and stimulated (black bars, 24hours) HSEC. Data are the mean and SEM of three independent experiments and values are the mean absorbance of three replicate wells minus the absorbance of an isotype matched control antibody.

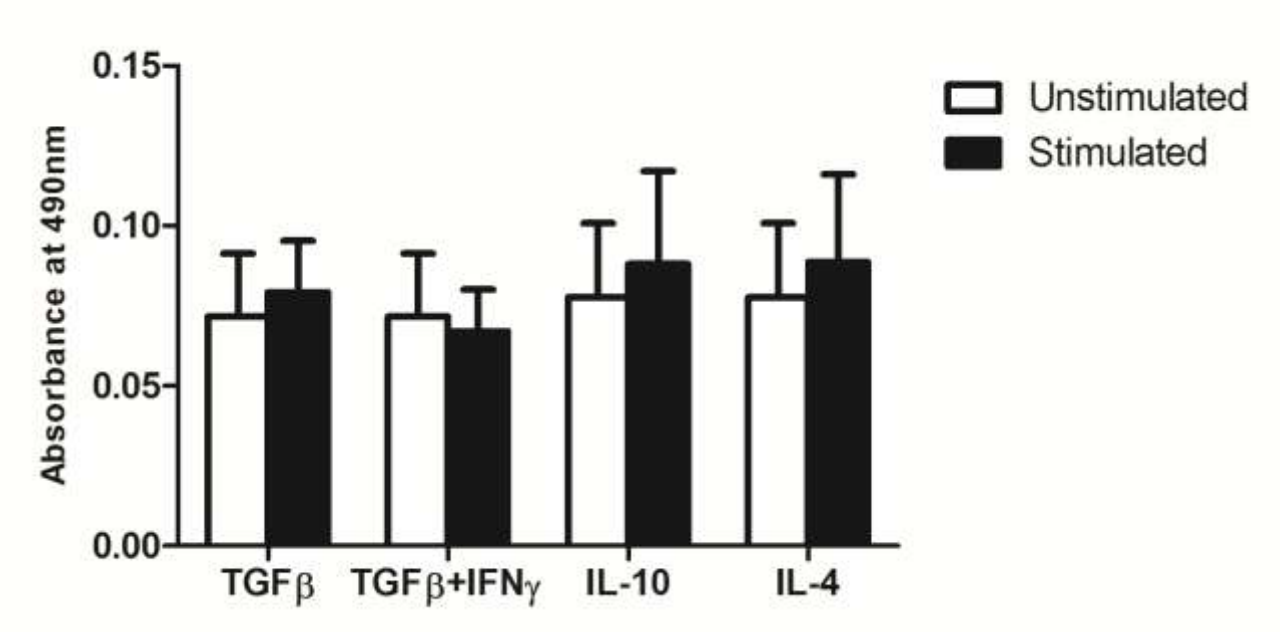

Figure 4-3 **Measurement of CLEVER-1 expression by HSEC in response to cytokine stimulation**

ELISA data comparing the levels of CLEVER-1 protein in unstimulated (white bars) and stimulated (black bars, 24hours) HSEC. Data are the mean and SEM of three independent experiments and values are the mean absorbance of three replicate wells minus the absorbance of an isotype matched control antibody.

4.3 Expression of CLEVER-1 in HSEC and its response to growth factor stimulation.

The growth factors VEGF and HGF are known to be important in the crosstalk between sinusoidal endothelial cells and hepatocytes within the liver (LeCouter et al., 2003) and we use them to maintain the phenotype of HSEC *in vitro*. Since these factors would be present in the *in vivo* environment in which HSEC reside we investigated whether they contribute to CLEVER-1 expression in vitro by ELISA. Figure 4-4, demonstrates that treatment of HSEC for 24 hrs with 10ng/ml HGF significantly increased the expression of CLEVER-1. Although VEGF on its own had no discernible effect the combination of HGF plus VEGF appeared more potent than HGF alone at upregulating CLEVER-1 expression.

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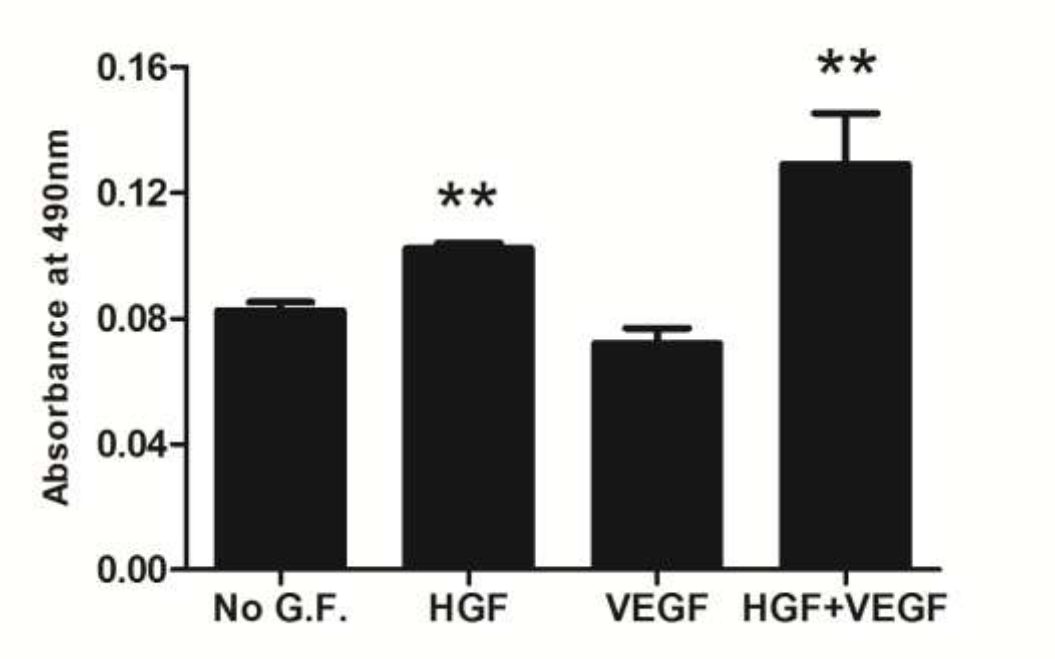

Figure 4-4 **Hepatocyte growth factor Increases CLEVER-1 protein expression**

ELISA data comparing the levels of CLEVER-1 protein in unstimulated to hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) stimulated HSEC. Data are the mean of three independent experiments and values are the mean absorbance of three replicate wells minus the absorbance of an isotype matched control antibody : **$p<0.005$ (Student's T test). Cells were stimulated with 10ng/ml of growth factor for 24 hrs.

4.4 Quantitative analysis of CLEVER-1 mRNA levels in HSEC.

Having demonstrated CLEVER-1 protein expression in HSEC we next studied the regulation of CLEVER-1 at the messenger RNA level using quantitative real time PCR.

4.4.1 Response to cytokine stimulation

HSEC were stimulated with the same panel of cytokines for 24 hours TNFα, IFNγ, IL-4, LPS, and IL-10). In support of our ELISA data, initial analysis of mRNA levels did not demonstrate any consistent responses to cytokine stimulation.

However the HSEC used for these experiments had been isolated from two broad groups of liver tissue: HSEC isolated from 'normal livers' (donor livers, resection margins) and HSEC isolated from explanted livers from patients with end-stage liver disease. Upon analysis of these two groups separately it appeared that the cells responded differently to cytokine stimulation. The trend in HSEC from 'normal' livers was for TNFα stimulation to increase CLEVER-1 expression, in contrast IFNγ treatment led to a downregulation in expression whereas the opposite responses were seen in HSEC from diseased livers (Figure 4-5). A possible explanation for these differential responses could be that 'diseased' HSEC were primed to respond because of their exposure to inflammatory signals *in vivo* thus requiring shorter periods of stimulation. I therefore compared 4 and 24 hour stimulations with

TNFα. The response to cytokine stimulation showed the same pattern when 4 hours was compared to 24 hour incubation (Figure 4-6), suggesting that the differences in ‘normal’ and ‘diseased’ HSEC were unlikely due to priming *in vivo*.

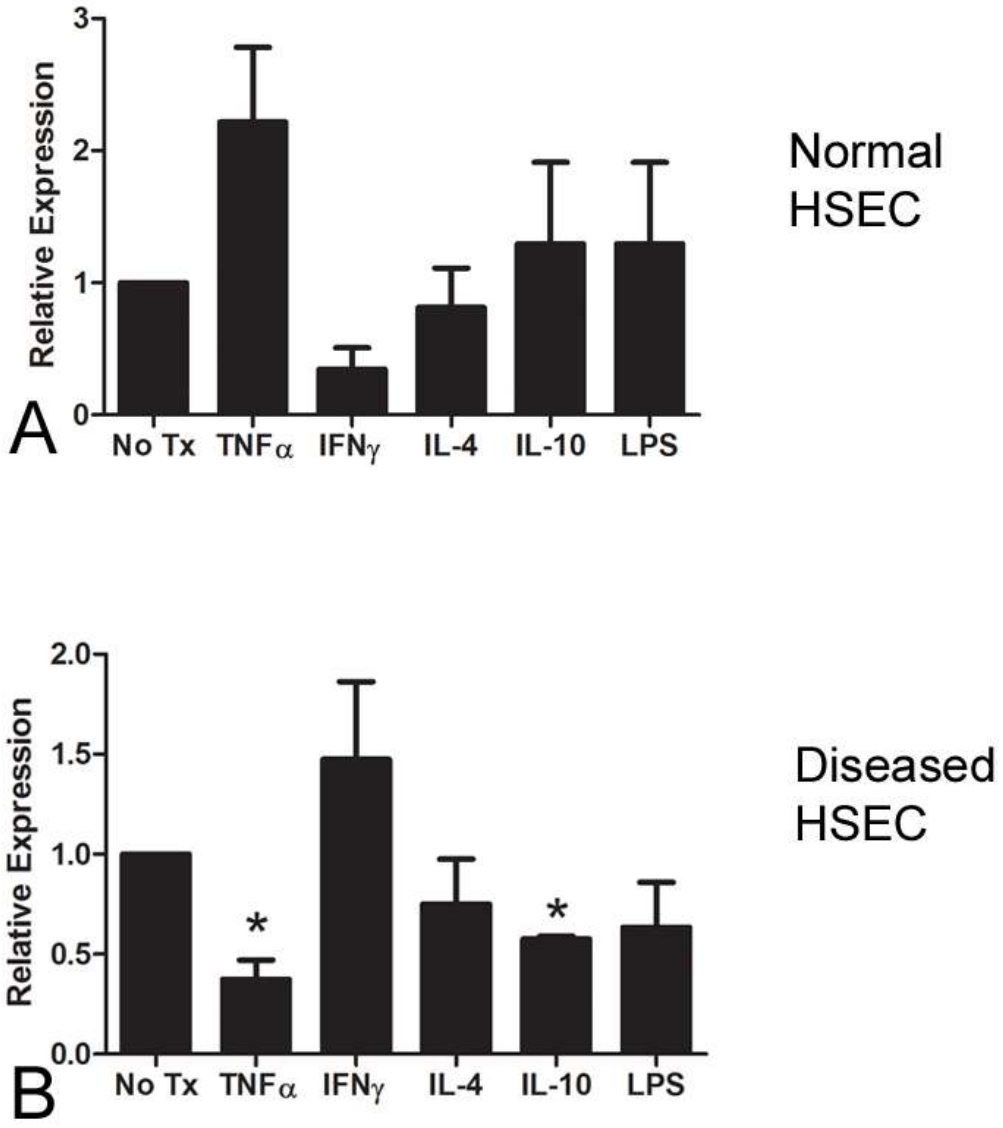

Figure 4-5 **CLEVER-1 mRNA Expression in HSEC under cytokine stimulation**

Relative expression of CLEVER-1 mRNA in cytokine treated HSEC compared to unstimulated HSEC. HSEC in these experiments were isolated from (A) Normal liver tissue or (B) explanted livers from patients chronic liver disease. The data are the mean of at three independent experiments. : *p<0.05 (Students T test). Cells were stimulated with cytokines for 24 hours.

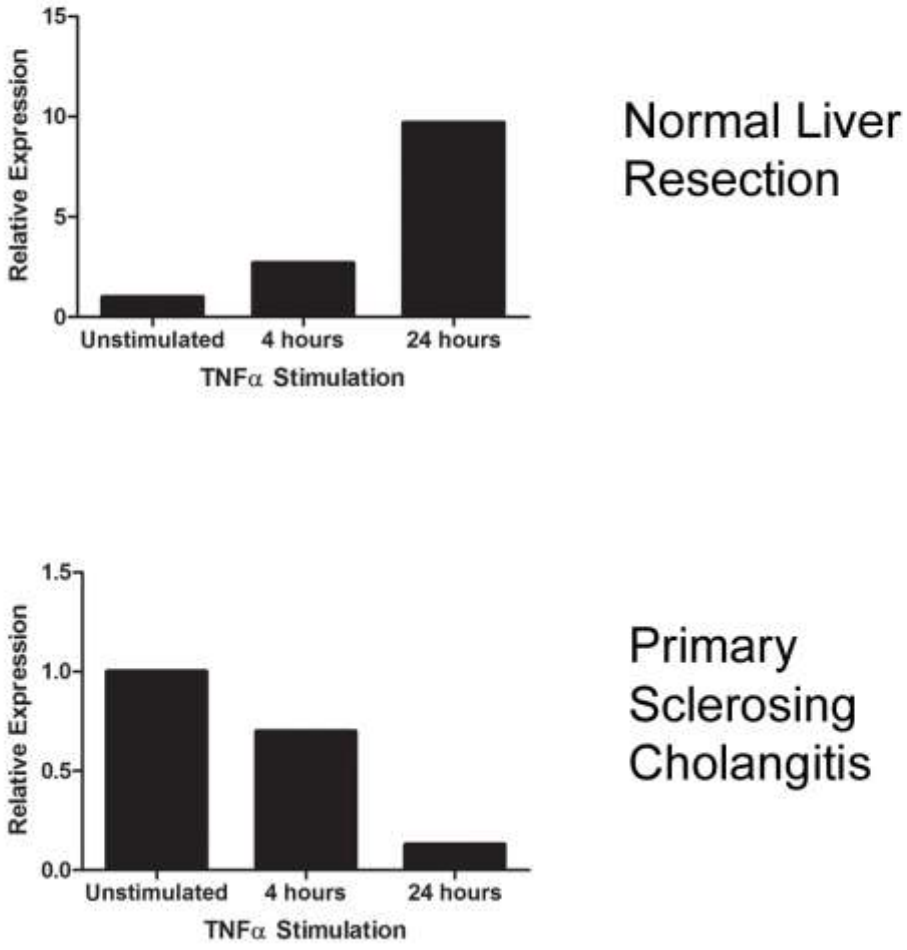

Figure 4-6 **Comparison of CLEVER-1 mRNA expression in HSEC from normal and diseased livers**

Representative experiments comparing CLEVER-1 expression in response to TNFα stimulation over time in HSEC from normal and diseased livers. Data shown are results of one isolation from each condition.

4.4.2 Response to hypoxia, bile acids and growth factors.

Our data so far suggested that unlike conventional adhesion molecules, CLEVER-1 expression was not increased by cytokine stimulation of HSEC. Therefore other factors which are characteristic of the hepatic microenvironment were studied for their influence on CLEVER-1 expression. These factors included the impact of hypoxia, since a significant proportion of hepatic blood supply arises from the portal vein and Zone 3 of the hepatic sinusoids is more 'hypoxic' than the portal areas where blood enters the sinusoids; and stimulation by bile acids.

We exposed cultures of HSEC from different livers to 0.1% oxygen for 24 hours and saw no consistent changes in CLEVER-1 mRNA. This is illustrated in Figure 4-7 where CLEVER-1 expression was downregulated in HSEC isolates from a normal resection and an autoimmune hepatitis liver but upregulated in HSEC isolates from primary bilary cirrhosis and primary sclerosing cholangitis. It is possible that HSEC from biliary diseases show different responses to hypoxia compared with other aetiologies and given more time I would have explored this further.

Similarly stimulation of HSEC with bile acids for 24 hours also had variable results on CLEVER-1 mRNA expression. As seen with hypoxia, the responses in HSEC from biliary diseases were different with suppressed CLEVER-1 mRNA in response to bile stimulation (Figure 4-8). Interestingly the cells isolated from a PBC liver showed complete downregulation of CLEVER-1 after treatment.

In contrast to hypoxia and bile acids however, growth factor stimulation had a consistent and reproducible effect on CLEVER-1 mRNA expression regardless of the origin of the HSEC. As discussed in section 2.2.4 isolated HSEC are cultured in a combination of VEGF and HEGF. Comparison was made of the expression of CLEVER-1 in HSEC cultured in media supplemented with growth factors and cells cultured for 24 hours in media without growth factors. A consistent reduction of CLEVER-1 expression in HSEC from diseased and normal livers was detected in the absence of growth factors (Figure 4-9 A). Further expression analysis of HSEC stimulated by HGF or VEGF demonstrated that, in agreement with our ELISA data, HGF had the dominant effect on CLEVER-1 expression (Figure 4-9 B).

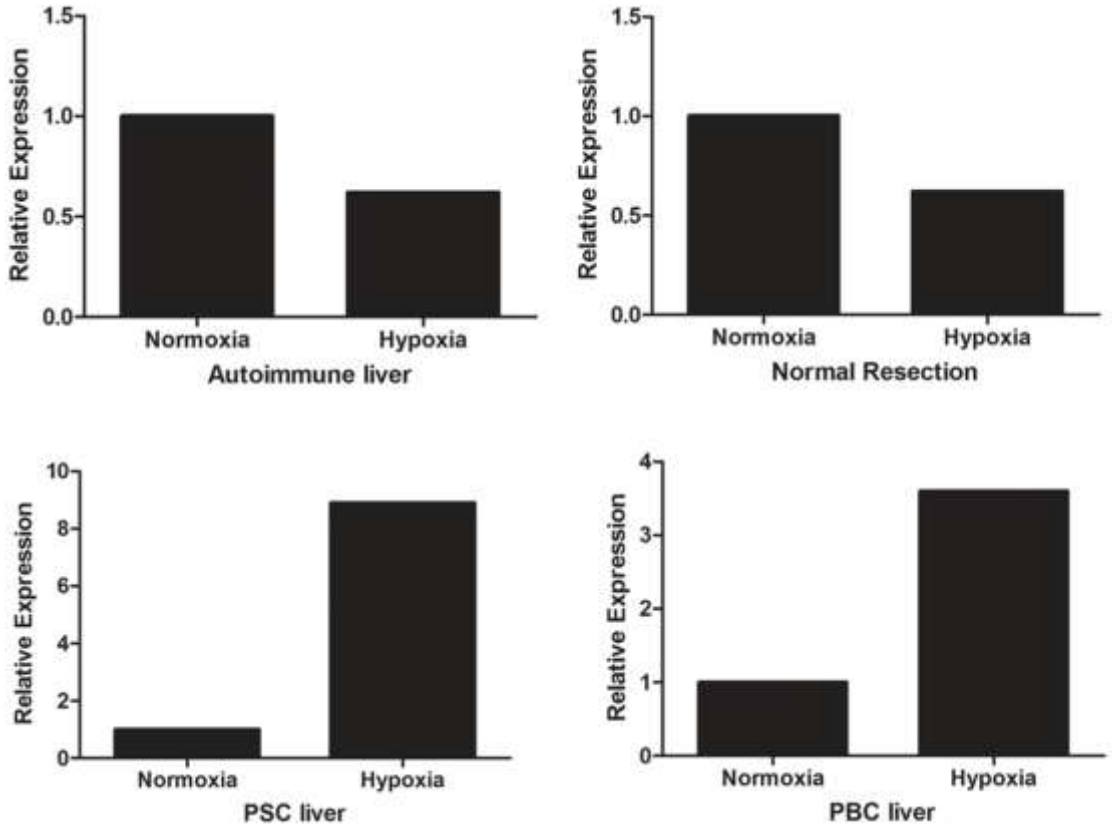

Figure 4-7 **CLEVER-1 expression in HSEC in response to Hypoxia**

Relative expression of CLEVER-1 mRNA in HSEC, in each experiment HSEC cultured in normoxic conditions were compared to HSEC cultured for 24 hours in a hypoxic chamber. Data shown are HSEC from one isolation from each condition. PSC – Primary Sclerosing Cholangitis, PBC- Primary Biliary Cirrhosis

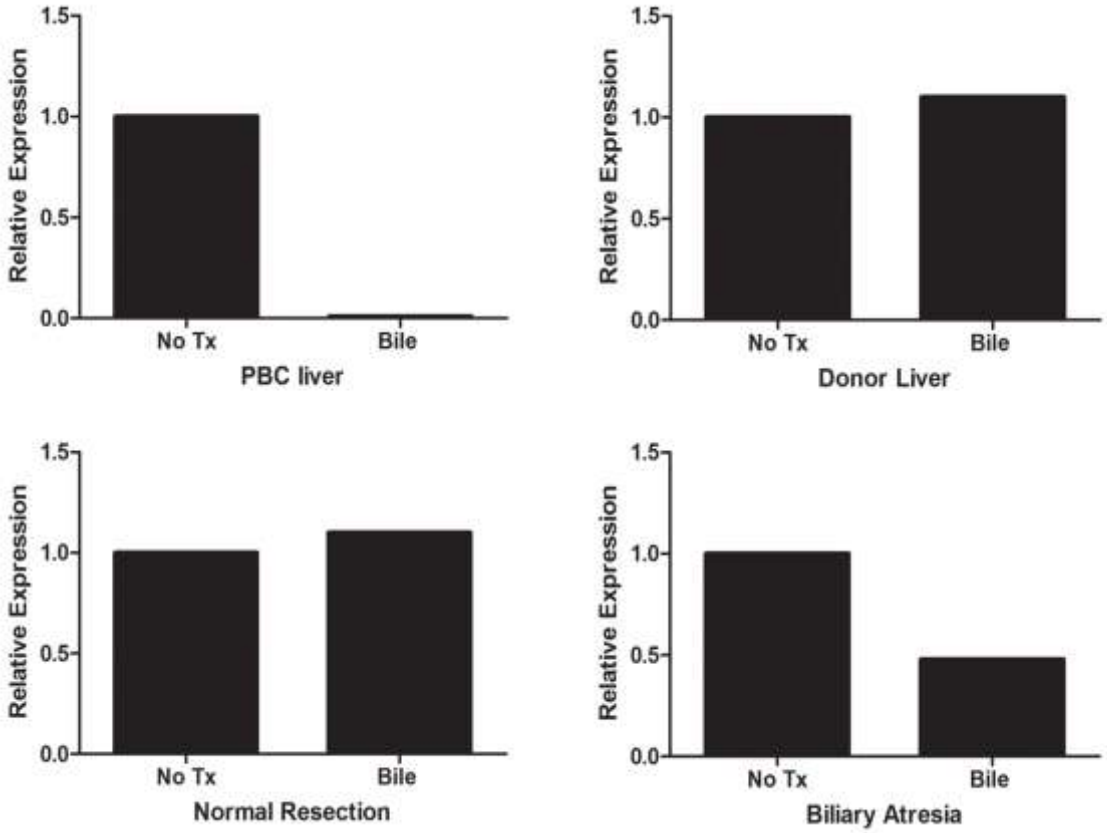

Figure 4-8 **CLEVER-1 expression in HSEC in response to bile**

Relative expression of CLEVER-1 mRNA in HSEC, in each experiment HSEC were cultured in media were compared to HSEC cultured in media supplemented with Bile for 24 hours. Data shown are HSEC from one isolation from each condition PBC- Primary Biliary Cirrhosis

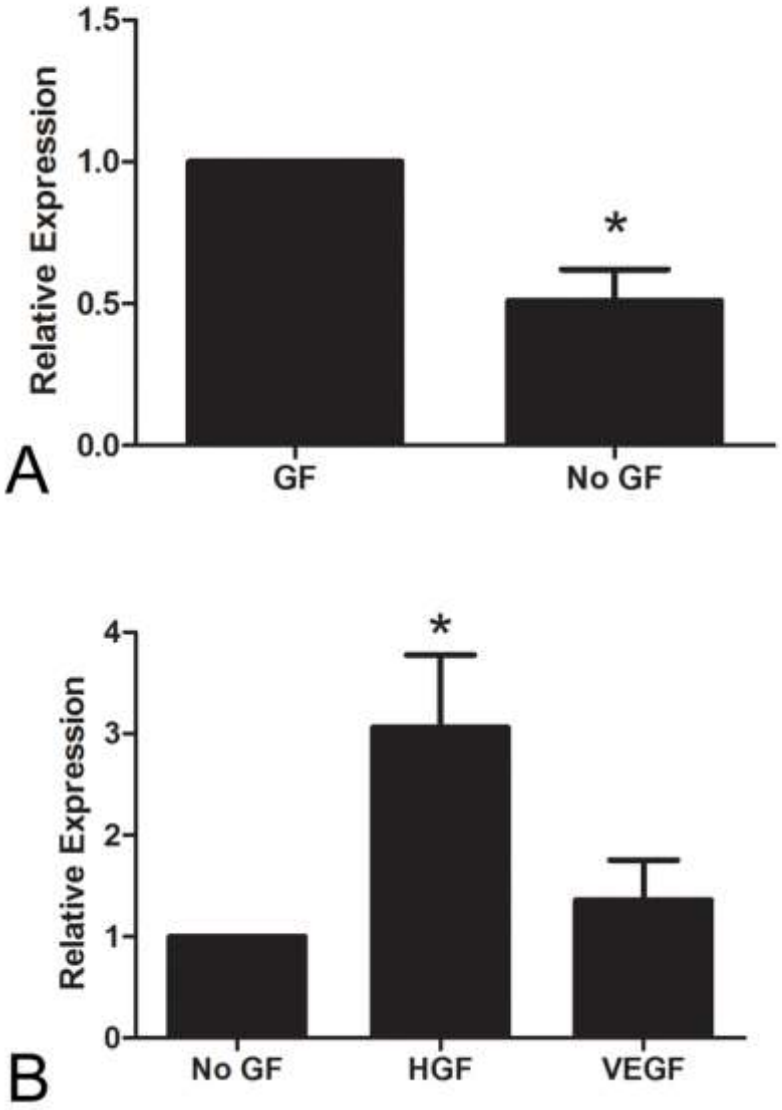

Figure 4-9 **Hepatocyte growth factor upregulates CLEVER-1 mRNA expression**

A- Relative expression of CLEVER-1 mRNA in HSEC cultured in media with or without growth factor supplementation. The mean and SEM of three independent experiments are shown:* $p<0.05$ (Student's T test). B- Relative expression of CLEVER-1 mRNA in HSEC cultured in media without growth factor supplementation or with either hepatocyte growth factor (HGF) or vascular epidermal growth factor (VEGF). The mean and SEM of four experiments are shown :* $p<0.05$ (Student's T test) Growth Factor (GF). Cells were stimulated with growth factors for 24 hours.

4.5 The trafficking of CLEVER-1

Our preliminary data suggested that HSEC constitutively express CLEVER-1 at protein and RNA level and that expression was regulated by growth factors (HGF), with no consistent response to cytokines. The nature of the response to cytokine stimulation appeared to vary dependent on the origin of the HSEC. We considered it possible that our results may be complicated by altered trafficking of CLEVER-1 in the cells and by the generation of soluble CLEVER-1. Previous studies in sinusoidal endothelial cells have shown that the majority of intracellular CLEVER-1 is membrane anchored with no evidence of secretion of a soluble form. The receptor appears to undergo constitutive recycling between the plasma membrane and early endosomes in cultured human sinusoidal endothelial cells (Prevo et al., 2004). A further study investigated the role of CLEVER-1 and its close homologue, Stabilin-2, in rat liver sinusoidal endothelial cells. Both receptors were found in early endosomes and their association with clathrin and adaptor protein 2 (AP-2) suggested a role in receptor mediated endocytosis (Hansen et al., 2005b).

Thus we decided to use our cultured HSEC to study the intracellular localisation of CLEVER-1 in more detail in human cells. The aims of these studies were to determine the intracellular trafficking and localisation of CLEVER-1 in HSEC using agents that block receptor re-cycling.

4.6 The regulation of intracellular trafficking and surface expression of CLEVER-1.

Localisation of CLEVER-1 was studied by immunofluoresence and confocal microscopy. HSEC stimulated by inflammatory cytokines were studied for any alteration of CLEVER-1 distribution. Our fluorescent micrographs suggest that CLEVER-1 resides in the cytoplasm of unstimulated cells and has a 'granular' distribution, and that stimulation with TNFα had no effect on this distribution pattern (Figure 4-10). Although our ELISA and Quantitative PCR had demonstrated that CLEVER-1 expression was upregulated by growth factors, growth factor stimulation did not appear to alter CLEVER-1 distribution (Figure 4-10).

In order to determine whether there is a rapid recycling of CLEVER-1 from the cell surface to the cytoplasm of HSEC, live cells were incubated with CLEVER-1 antibody followed by fixation and fluorescent staining. The cytoplasmic pattern of staining observed suggests that CLEVER-1 bound to antibody was internalised from the cell surface and that this occurred within 15 minutes (Figure 4-11).

In order to further characterise the localisation of the cytoplasmic pool of CLEVER-1 we treated the cells with bafilomycin A1. This is a macrolide which is a highly specific inhibitor of vacuolar type H+-ATPase and has been shown to block endocytic transport (Bayer et al., 1998). Previous groups have colocalised CLEVER-1 with early endosomes (Hansen et al., 2005b). HSEC were treated with Bafilomycin A1 for 24 hours to asses if it altered

CLEVER-1 localisation and influenced surface expression. After treatment the receptor remained predominantly intracellular (Figure 4-12) but was seen to aggregate in large vesicles. There did not appear to be any increase in surface expression after treatment. Dual colour immunofluoresence and confocal microscopy were then used to colocalise CLEVER-1 with subcellular organelles. CLEVER-1 did not colocalise with early or late endosome markers (Figure 4-13) but further analysis demonstrated colocalisation with the Golgi Apparatus and the Trans Golgi Network of HSEC (Figure 4-14).

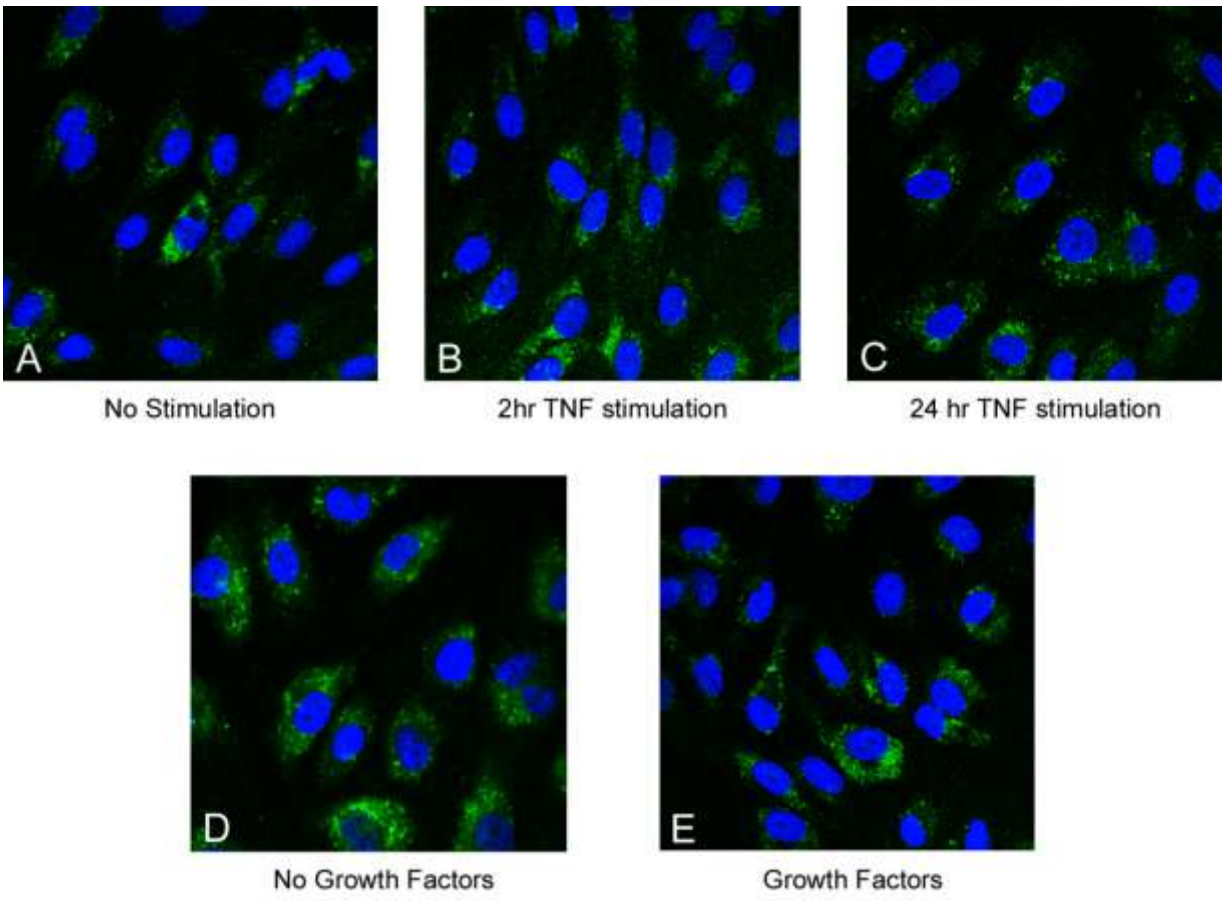

Figure 4-10 **Stimulation of HSEC with cytokines or growth factors does not alter CLEVER-1 localisation**

These panels show representative confocal images of HSEC grown on coverslips and stained for the CLEVER-1 receptor under, resting conditions, TNFα stimulation for 2 and 24 hours and growth factor stimulation. CLEVER-1 is shown with a FITC secondary and the nucleii are stained with dapi (blue) Isotype matched controls were negative. Fields captured with 63x objective.

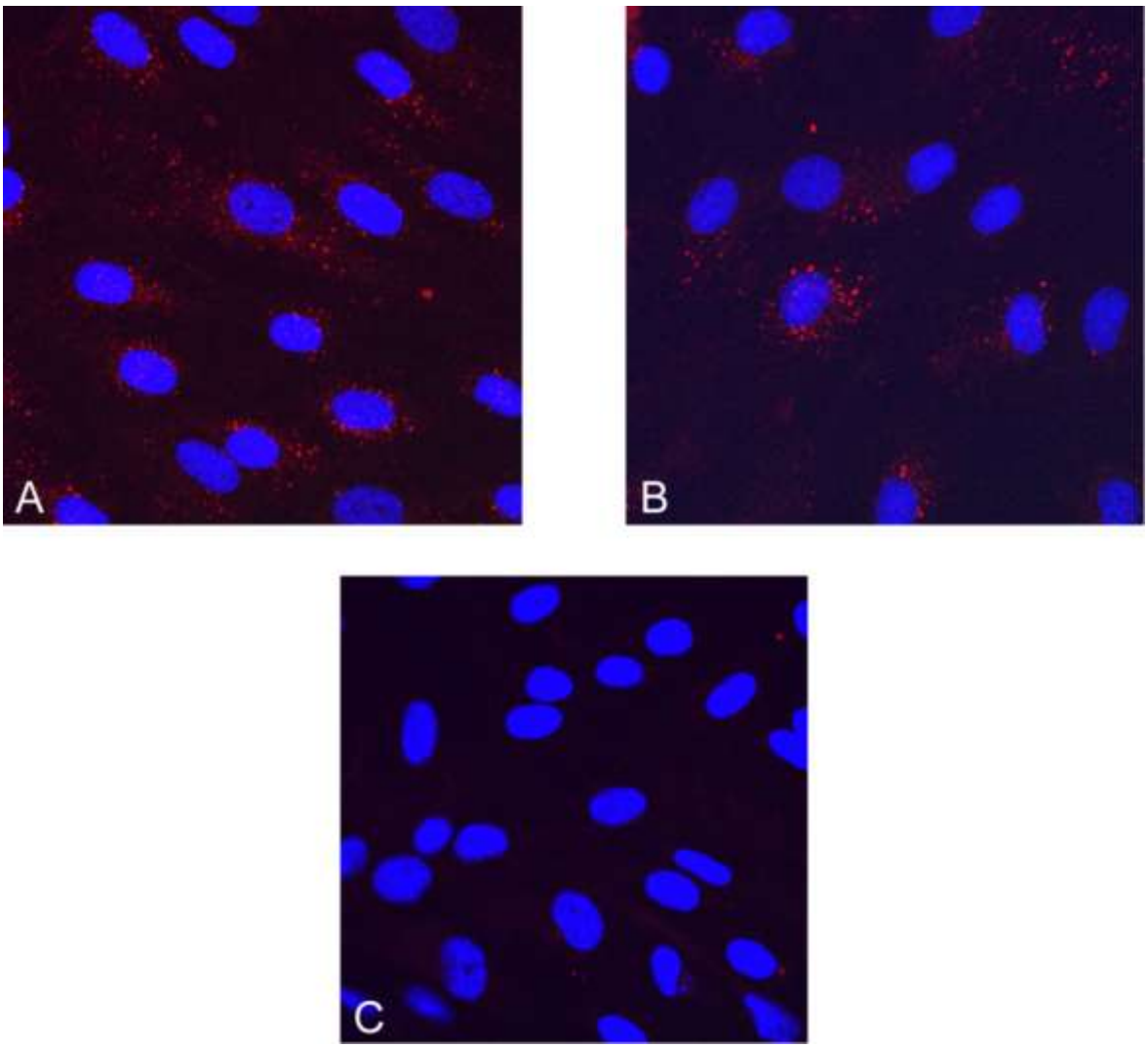

Figure 4-11 **CLEVER-1 internalises antibody from HSEC cell surface within 15 minutes**

HSEC were incubated with CLEVER-1 antibody or isotype matched control for 15 or 30 minutes. Following incubation HSEC were fixed, permeabilised and stained with fluorescent secondary (Alexafluor 546). Confocal images taken of HSEC incubated with 3-372 for 15 minutes (Panel A), 3-372 for 30 minutes (Panel B) and isotype matched control for 30 minutes (Panel C). Nucleii are stained with DAPI (blue). Fields captured with 63xobjective.

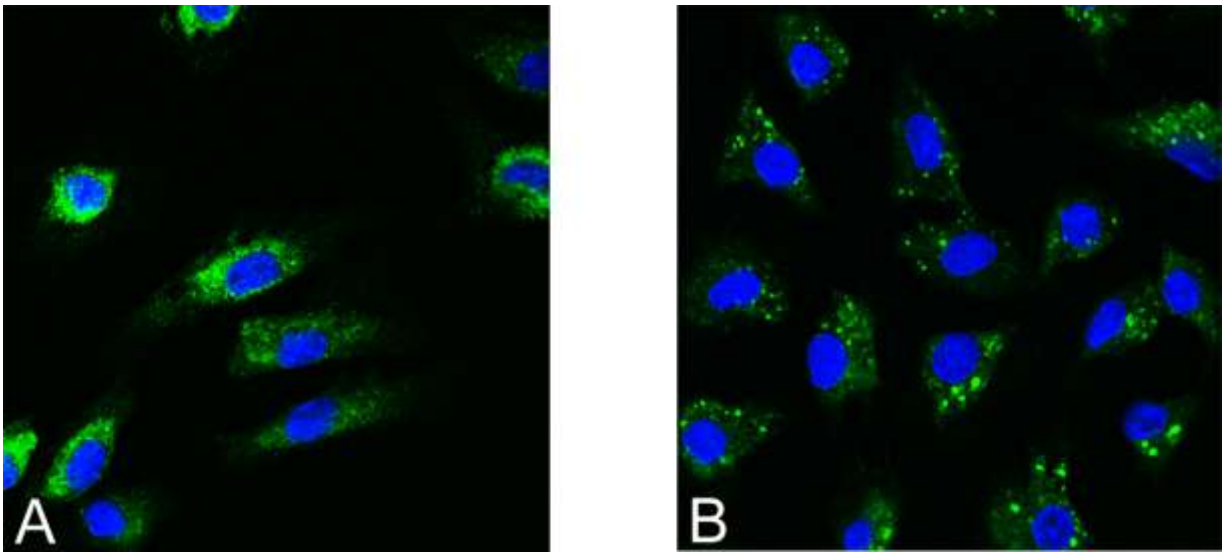

Figure 4-12 **Bafilomycin A1 treatment alters CLEVER-1 trafficking in HSEC**

Representative confocal images of HSEC stained for CLEVER-1. Panel A shows untreated cells. Panel B shows cells treated with 10 nanomolar bafilomycin A1 for 24 hours. Experiments were performed on three separate isolates. CLEVER-1 is shown with a FITC secondary and the nucleii are stained with dapi (blue). Isotype matched controls were negative. Fields captured with 63x objective.

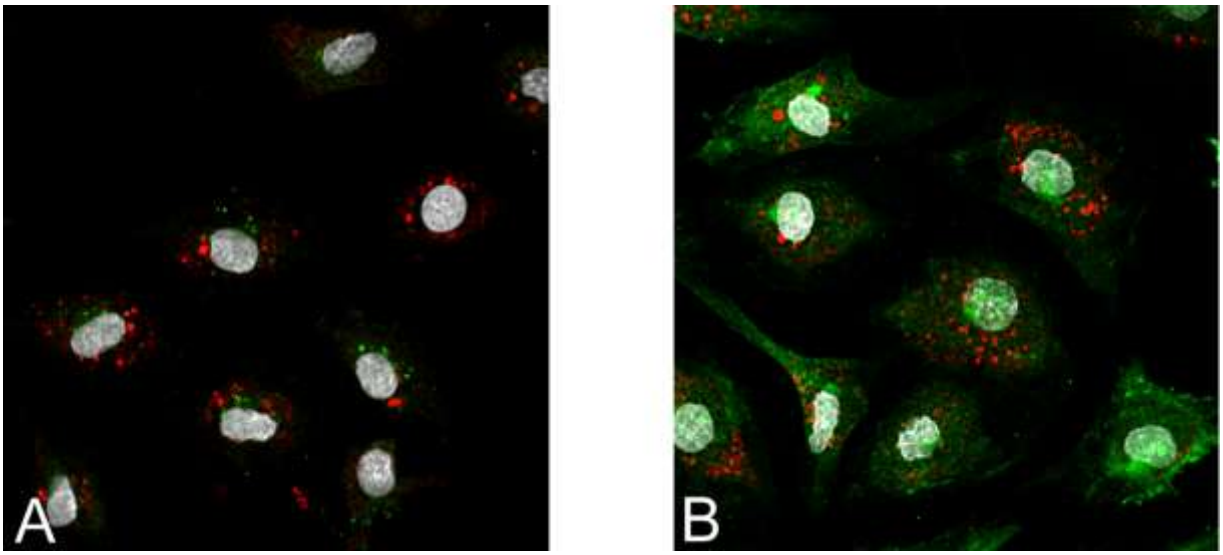

Figure 4-13 **CLEVER-1 does not colocalise with endosomes or lysosomes in Bafilomycin treated HSEC**

Representative confocal images of Bafilomycin A1 treated HSEC. Panel A shows dual colour immunofluorescence for CLEVER-1(red) and the early endosome marker Rab 5 (green). Panel B shows dual colour immunofluorescence for CLEVER-1 (red) and late endosome marker LAMP-1(green). Nuclei are stained with dapi (grey). Isotype matched controls were negative. Fields captured with 63x objective.

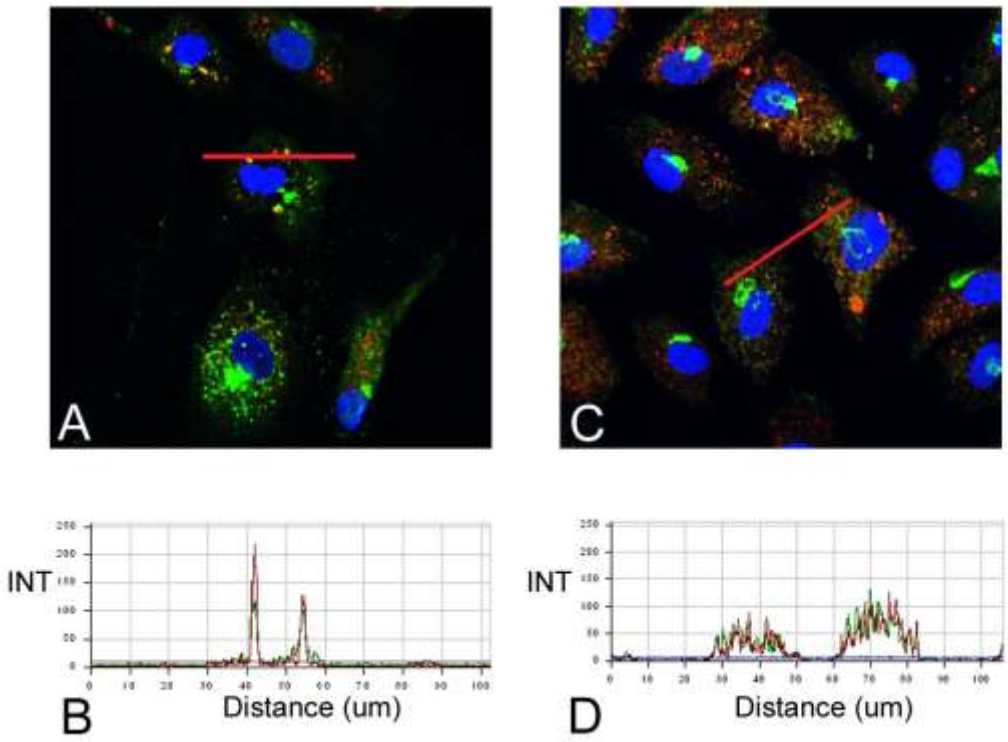

Figure 4-14 **CLEVER-1 colocalises with Golgi and Trans Golgi markers in Bafilomycin treated HSEC**

Representative confocal images of Bafilomycin A1 treated HSEC. Panel A shows dual colour immunofluorescence for CLEVER-1 (red) and Golgi lectin marker(green). Panel B is a profile image of the red line in Panel A . Panel C shows dual colour immunofluorescence for CLEVER-1(red) And Trans Golgi network marker TGN46 (green). Panel D is a profile image of the red line in panel C. Isotype matched controls were negative. INT= Fluorescent intensity. Fields captured with 63x objective.

4.7 Summary of regulation studies

These studies investigated the expression and trafficking of CLEVER-1 in response to cytokine stimulation. It was not possible to increase CLEVER-1 protein expression in HSEC by pro-inflammatory cytokines or by TGFβ, IL-10 or IL-4. Although I saw some intriguing responses in some disease groups to hypoxic conditions or exposure to bile acids these were not consistent and will require further investigation to either confirm or refute. In contrast, we found a consistent upregulation of CLEVER-1 with HGF stimulation which, to our knowledge, has not been described previously. Bafilomycin had a profound effect on CLEVER-1 intracellular localisation leading to aggregation of receptor in large vesicles that colocalised with the trans Golgi network and the Golgi Apparatus. Previous studies in alternatively activated human macrophage populations studies have also detected CLEVER-1 in the trans Golgi network (Kzhyshkowska et al., 2004).

I used cell based ELISA to study CLEVER-1 protein expression and in these assays the HSEC are permeabilised and the assays therefore measure total CLEVER-1 expression (ie membranous and intracellular). We were able to confirm constitutive expression of CLEVER-1 in HSEC but we could not significantly upregulate this basal level of expression. Thus CLEVER-1 does not behave like conventional adhesion molecules such as ICAM-1 or VCAM-1 which are markedly upregulated by pro-inflammatory cytokines. Previous studies in lymphatic endothelium have demonstrated modest increases with LPS treatment (Salmi et al., 2004), but we did not find this with HSEC. This

may be due to the fact that HSEC *in vivo* are continuously exposed to LPS and therefore have a altered responsiveness to LPS challenge (Crispe, 2009).

IL-4, which is known to induce CLEVER-1 expression in macrophages (Goerdt et al., 1993) and increases the expression of other C-type lectins such as L-SIGN on HSEC, had no effect on CLEVER-1 (Lai et al., 2006). This is consistent with studies in lymphatic endothelium where IL-4 has no effect (Prevo et al., 2004)**.** This confirms that the pathways which lead to CLEVER-1 upregulation in macrophages do not operate in endothelial cells and in addition that its regulation differs from other C-type lectins.

HSEC isolated from normal and diseased liver expressed similar levels of CLEVER-1 protein, but difference in response to cytokines were seen using quantitative PCR studies. Whereas there was a trend for TNFα to increase CLEVER-1 mRNA in 'normal' HSEC, the opposite was seen in 'diseased' HSEC. This could reflect the desensitisation of 'diseased' HSEC after prolonged exposure to inflammatory cytokines *in vivo* however the changes were small and were not associated with significant changes in protein casting doubt on their significance.

ELISA studies and quantitative PCR both demonstrated that HGF supplementation led to an induction of CLEVER-1 expression in HSEC. This was a consistent finding in HSEC isolates from normal and diseased livers. HGF was the growth factor which had the dominant effect on CLEVER-1

expression rather than VEGF. HGF potentially contributes to the expression of CLEVER-1 *in vivo*, this is discussed in more detail in section 6.2.

ELISA and PCR studies allow the assessment of total protein and mRNA expression respectively, but they give no information on the cellular localisation of receptors. Confocal microscopy allowed detailed analysis of CLEVER-1 localisation in isolated HSEC. These studies demonstrated that CLEVER-1 was predominantly intracellular in isolated HSEC. A striking feature was the response to Bafilomycin A1 which led to CLEVER-1 densely aggregating in vesicles confirming that CLEVER-1 does have clear trafficking properties within HSEC.

Despite attempted stimulation with various factors we could not increase the surface expression of CLEVER-1 on HSEC, other groups have also found it difficult to consistently upregulate CLEVER-1 on the surface of human lymphatic endothelial cells (Prevo et al., 2004; Salmi et al., 2004). This low surface expression could be explained by CLEVER-1's trafficking behaviour with CLEVER-1 rapidly moving from the plasma membrane back to intracellular organelles. We demonstrated this by showing the rapid uptake of CLEVER-1 antibody by cultured HSEC.

The studies in this chapter have attempted to understand the regulation of CLEVER-1 in human HSEC. In the next chapter we attempt to investigate the functional properties of CLEVER-1 in the context of lymphocyte recruitment to HSEC.

CHAPTER 5

THE FUNCTIONAL ROLE OF CLEVER-1 IN HUMAN HEPATIC SINUSOIDAL ENDOTHELIUM

My expression studies demonstrate that CLEVER-1 is expressed at important sites of lymphocyte recruitment in the normal and chronically inflamed human liver, especially the hepatic sinusoids, neovessels within the fibrous septum and the vessels within portal associated lymphoid tissue. My regulation studies confirm that CLEVER-1 is a trafficking receptor within HSEC moving from the plasma membrane to intracellular compartments and within the cells to the trans golgi network, which is similar to the trafficking of CLEVER-1 reported in macrophages (Kzhyshkowska et al., 2004). In terms of function, CLEVER-1 has been shown to mediate lymphocyte recruitment to lymphatic endothelium and lymphatic vessels in both *in vitro* and *in vivo* studies (Karikoski et al., 2009b; Salmi et al., 2004). Since lymphatic endothelium shares several phenotypic features with hepatic sinusoidal endothelium, including the expression of CLEVER-1, we hypothesised that CLEVER-1 would mediate lymphocyte recruitment to the liver via hepatic sinusoidal endothelium. To study the role of this trafficking receptor in lymphocyte recruitment to the human liver I used static adhesion assays to assay binding to intact vessels in tissue sections and to elucidate its role in the adhesion cascade I used flow-based adhesion assays incorporating HSEC monolayers. The aim of my functional assays were to:

Determine whether CLEVER-1 supports lymphocyte adhesion to hepatic endothelium and if so to determine the lymphocyte subsets involved and the precise role of CLEVER-1 in the adhesion cascade.

To do this I

1. Used modified Stamper Woodruff assays to study lymphocyte adhesion to CLEVER-1 in liver tissue.
2. Used flow based adhesion assays to study the role of CLEVER-1 in peripheral blood lymphocyte recruitment to HSEC.
3. Investigated if CLEVER-1 preferentially recruits subsets of lymphocytes by studying purified populations of CD4+, CD8+ and B lymphocytes under conditions of flow.

5.1 Static adhesion assays

My earlier expression studies had demonstrated CLEVER-1 expression in the sinusoids of normal liver and showed discreet areas of increased endothelial expression in both parenchymal and biliary liver disease. I therefore performed static adhesion assays on chronically inflamed liver tissue and assessed lymphocyte binding to parenchymal regions. Modified Stamper Woodruff assays were used incorporating tissue sections from explanted livers from patients with chronic liver disease (including alcoholic liver disease n=2, autoimmune liver disease n=1, primary sclerosing cholangitis n=4, primary biliary cirrhosis n=2). These assays demonstrated a significant reduction in lymphocyte binding to hepatic tissue with CLEVER-1 antibody blockade. The number of adherent lymphocytes per high power field in control sections was 6.9 ± 2.5 cells compared to 4.6 cells ± 1.9 cells after CLEVER-1 blockade. This translated to a 30% reduction of lymphocyte binding when CLEVER-1 blockade was compared to isotype matched control Figure 5-1.

5.2 Flow-Based Adhesion Assays

The static adhesion assays described above allowed me to study the function of CLEVER-1 within liver tissue and confirm that it could support lymphocyte adhesion to endothelium within the liver parenchyma. However these simple static adhesion assays do not clarify the specific role CLEVER-1 plays in the adhesion cascade. Flow assays allow the study of lymphocyte endothelial interactions under conditions of physiological shear stress. We recapitulate the hepatic sinusoids by culturing HSEC in glass capillaries and then lymphocytes are perfused over this monolayer of cells at a rate of flow calculated to reproduce the shear stress within hepatic sinusoids. The shear stress is a stress that is applied parallel or tangential to a surface as opposed to normal stress which is perpendicular. Any fluid that is moving along a boundary will exert a shear stress on that boundary. Shear stress has been shown to be an essential component of lymphocyte transmigration across endothelium (Cinamon et al., 2001). The flow rate of the assays used for the experiments described here was set to generate a shear stress of 0.5 dyne/cm^2, to reflect the stress within the hepatic sinusoids.

Initial flow assays were carried out with HSEC grown in microslides, these have been used by our group previously to study the role of ICAM-1 and VAP-1 in lymphocyte recruitment (Lalor et al., 2002a) . Additionally, to perform multiple blocking assays HSEC were grown in Ibidi µ slide VI chamber slides where 6 parallel microchannels are mounted in one slide. This allowed assays on the same isolate of HSEC to be performed

consecutively with minimal handling and delay. When I commenced my experiments Ibidi slides had not been routinely used in flow assays by our group and so an initial comparison was made with conventional microslides. Here HSEC were cultured in both types of slides and flow assays were performed using peripheral blood lymphocytes with either unstimulated HSEC or HSEC stimulated with TNFα and IFNγ for 24 hours. These experiments confirmed that lymphocytes did not bind to unstimulated HSEC cultured in either slide type but underwent tethering, firm adhesion and transmigration across cytokine stimulated HSEC in both types of microslides. Essentially the adhesive behaviour was the same in either culture system, although we noted a reproducible but small reduction in the total number of adherent cells binding to HSEC in ibidi slides in comparison to conventional microslides (Figure 5-2)

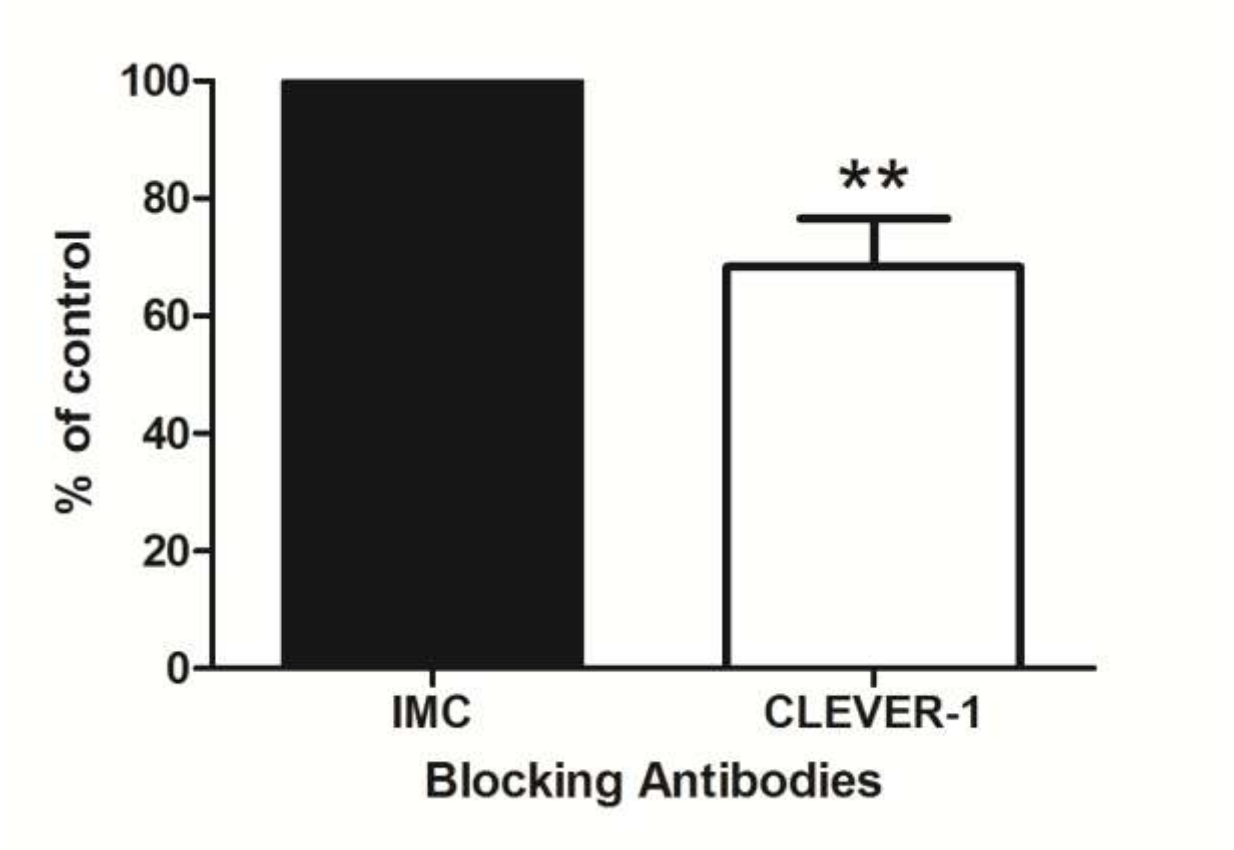

Figure 5-1 **CLEVER-1 mediates the adhesion of lymphocytes to liver tissue**

Peripheral blood lymphocytes (1 x10^6/ml) were adhered to liver sections under static conditions. Prior to adhesion sections were blocked with CLEVER-1 antibody (3-372 20µg/ml or isotype matched control (IMC). A significant reduction in lymphocyte adhesion per high power field was seen with CLEVER-1 blockade compared to IMC. Mean and SEM of 9 experiments are shown : ** $p<0.005$ (Student's T test).

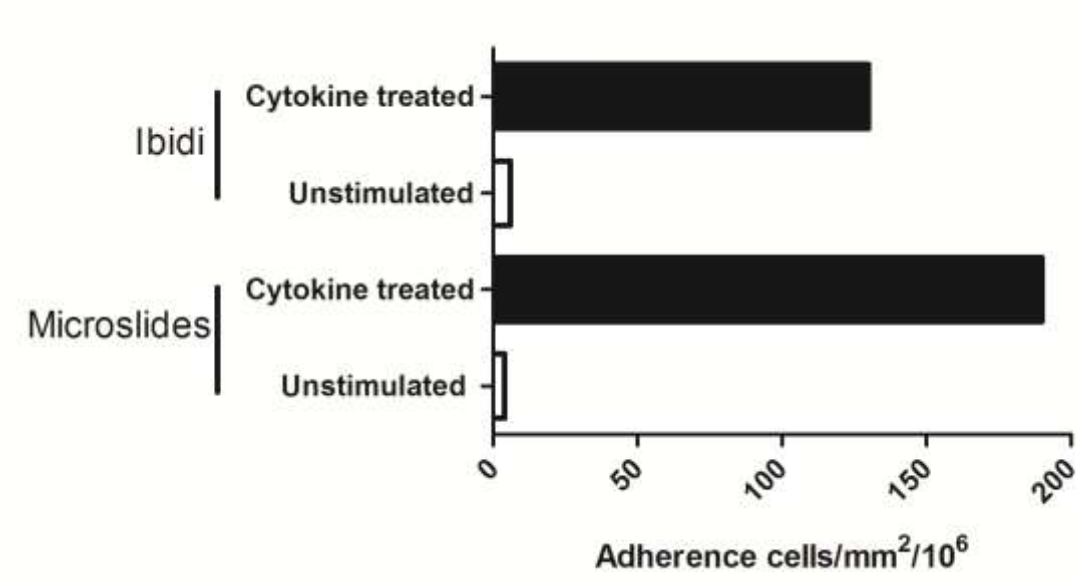

Figure 5-2 **Flow based adhesion assays in microslides and Ibidi chamberslides**

HSEC were cultured in microslides and Ibidi chamberslides and flow adhesion assays were performed with peripheral blood lymphocytes ($1x10^6$/ml) perfused at the same shear stress of 0.05 Pa. The figure compares the adherence of lymphocytes to monolayers of untreated or cytokine stimulated HSEC in both types of slide. Data shown is for one assay for each condition. Cells were treated with interferon γ and TNFα at 10ng/ml for 24 hours.

5.2.1 The role of CLEVER-1 in peripheral blood lymphocyte recruitment via HSEC

Next, I carried out flow based adhesion assays on HSEC to elucidate the exact role CLEVER-1 played in the adhesion cascade. Initial experiments used peripheral blood lymphocytes isolated from whole blood. As before, HSEC under resting conditions did not support lymphocyte recruitment from flow and cytokine stimulation was required prior to lymphocyte perfusion (Lalor et al., 2002a) to allow adhesion. Therefore HSEC monolayers were stimulated with TNFα and interferonγ for 24 hours prior to assays. We have previously shown that <10% of adherent lymphocytes persistently roll over stimulated HSEC in these assays, and the majority of adherent cells at any one time will be arrested on the endothelium with a proportion of these cells then undergoing transendothelial migration. Interestingly, the presence of a blocking antibody against CLEVER-1 led to a significantly larger proportion of lymphocytes exhibiting persistent rolling across the HSEC monolayer (Figure 5-3). CLEVER-1 blockade did not alter the total adherence of lymphocytes to HSEC but it significantly reduced the proportion of adherent lymphocytes undergoing transendothelial migration compared to an isotype matched control (Figure 5-4). Since our previous quantititative PCR experiments had demonstrated differential regulation of CLEVER-1 in HSEC from normal and diseased livers it was important to study CLEVER-1 function in HSEC from these two groups of livers. However, flow assays using diseased and 'normal' HSEC did not show a difference in the contribution of CLEVER-`1 to

transmigration, with approximately 25% of transmigration events being inhibited on both normal and diseased HSEC after blockade (Figure 5-5).

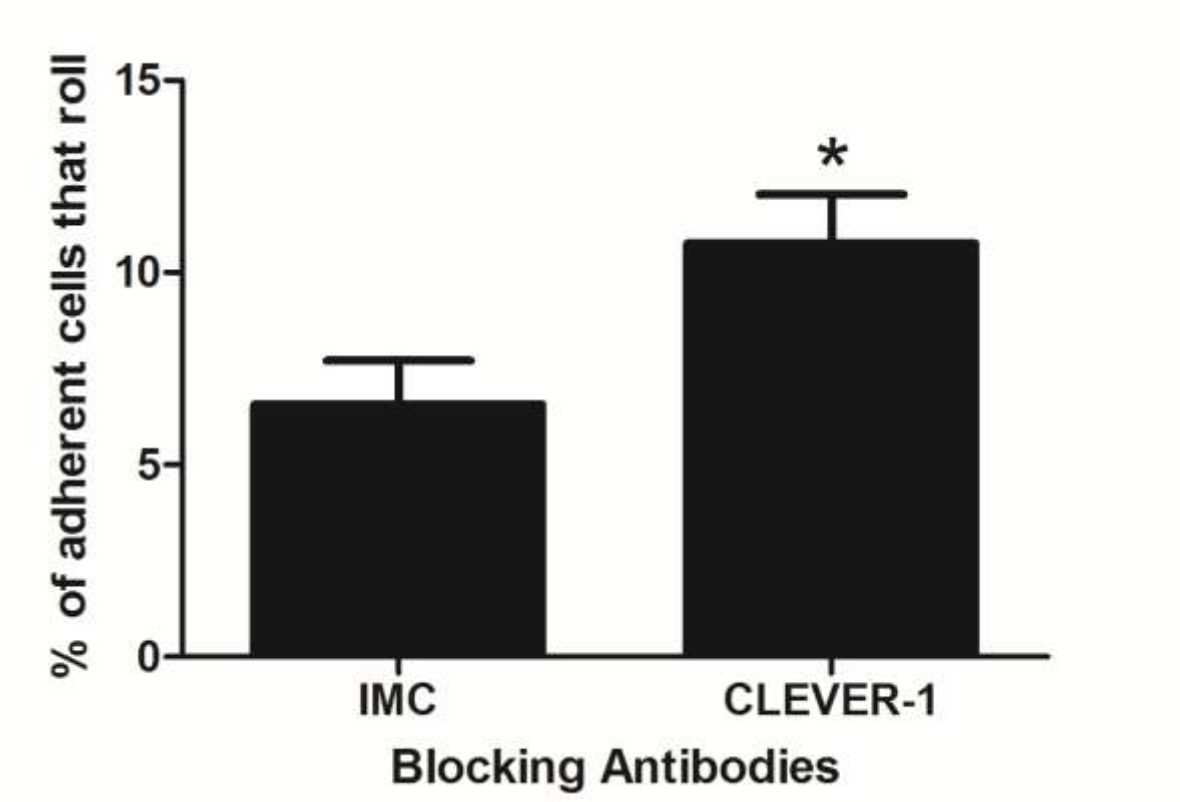

Figure 5-3 **CLEVER-1 blockade increases the proportion of peripheral blood lymphocytes that roll across HSEC**

Peripheral blood lymphocytes ($1x10^6$/ml) were perfused across cytokine treated HSEC monolayers (24 hour stimulation) at a shear stress of 0.05Pa. HSEC monolayers were blocked with CLEVER-1 antibody (3-372 20µg/ml) or an isotype matched control prior to assay. A larger proportion of adherent lymphocytes that were rolling in the presence of CLEVER-1 blockade compared to an isotype matched control. Mean and SEM of 5 experiments are shown (Student's T test).

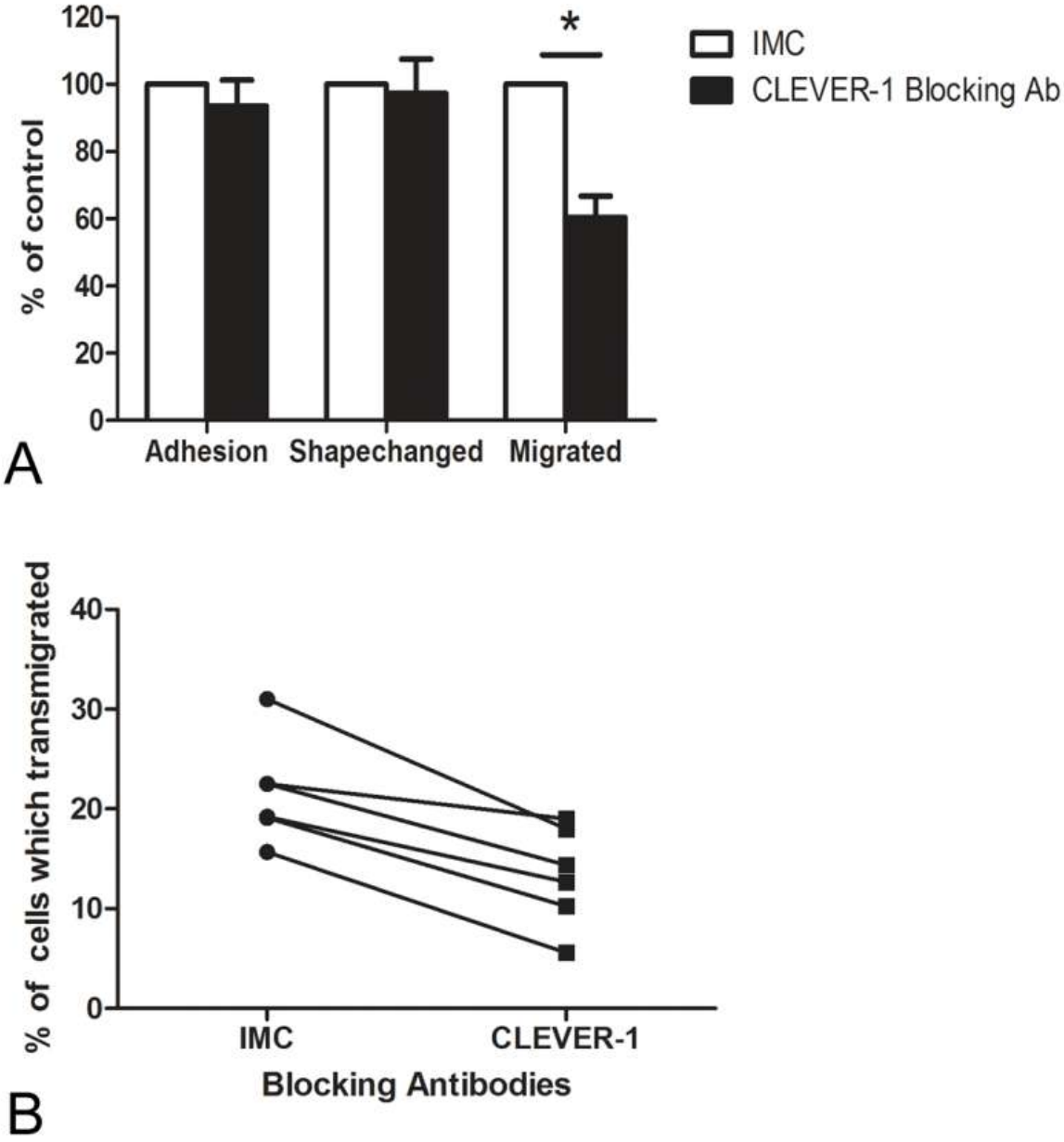

Figure 5-4 **CLEVER-1 mediates the transendothelial migration of peripheral blood lymphocytes across HSEC**

The number of adherent, shapechanged and transmigrated cells were enumerated after flow assays performed at a shear stress of 0.05 Pa. Panel A demonstrates that CLEVER-1 blockade significantly reduced transmigration. The results are expressed as a percentage of binding when compared to isotype matched control (IMC) and are a mean and SEM of six independent experiments: *$p<0.05$ (Student's T test). Panel B shows the percentage of adherent cells that transmigrated across HSEC in each experiment comparing CLEVER-1 to IMC.

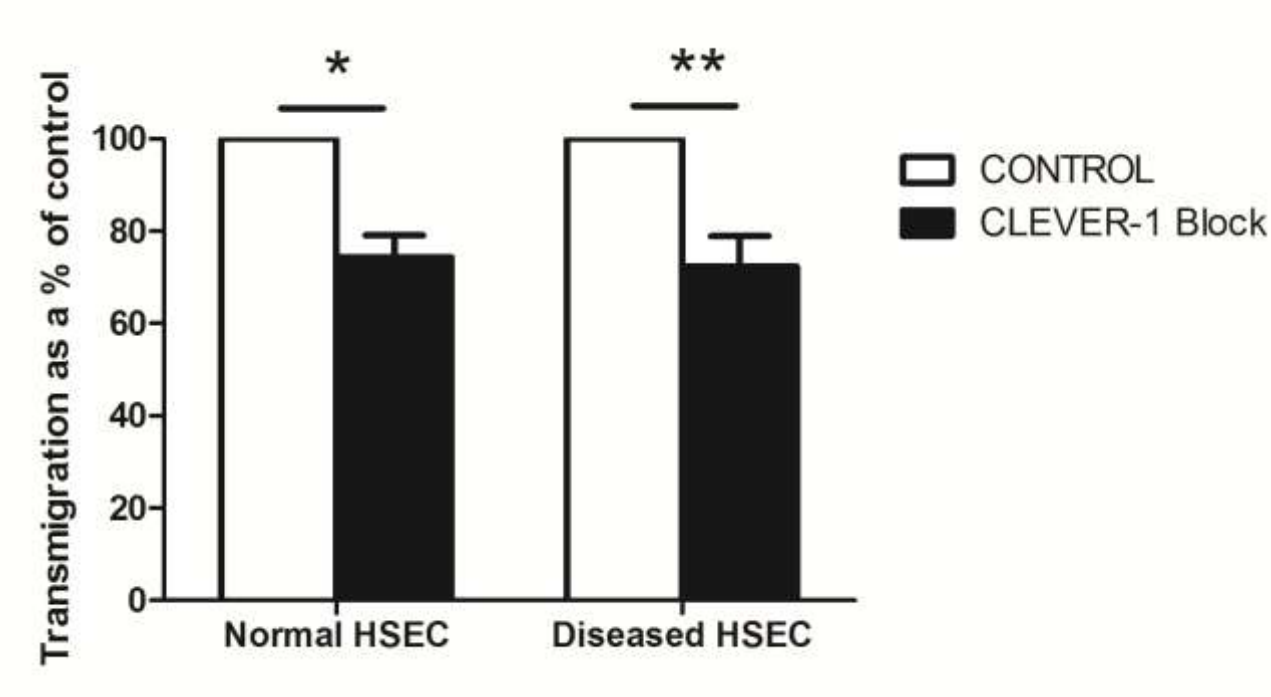

Figure 5-5 **Comparison of CLEVER-1 blockade in normal and diseased HSEC**

The proportion of adherent cells that underwent transmigration were enumerated after flow assays performed at a shear stress of 0.05 Pa. Comparison is made with flow assays performed with cytokine treated (24 stimulation) normal HSEC and diseased HSEC. The results are expressed as a percentage compared to isotype matched control and are a mean and SEM of four independent experiments: *$p<0.05$, **$p<0.005$(Student's T test).

5.2.2 Comparison of CD4 and CD8 Subsets

As discussed in section 1.4, the peripheral blood lymphocyte population is comprised of a range of subsets and there is evidence that these subsets can respond to different signals during recruitment to tissue. To study whether CLEVER-1 preferentially recruited a specific subset I isolated the major subsets of lymphocytes from whole blood and then performed flow based adhesion experiments with each group. I began by purifying CD4+ lymphocytes, and CD8+ lymphocytes from whole blood using magnetic bead selection kits. Typical isolations yielded cells with a purity for CD4+ and CD8+ cells of 95% and 94% respectively (Figure 5-6).

Flow assays with these two subsets demonstrated similar levels of absolute binding: total adhesion of CD4 lymphocytes was 124 ± 32 cells/mm^2/10^6; total adhesion of CD8 lymphocytes was 160 ± 15 cells/ mm^2/10^6. Blockade of CLEVER-1 in flow assays with purified CD4+ lymphocytes demonstrated no effect on the firm adhesion of these cells but led to a significant reduction in the proportion of adherent cells that underwent transendothelial migration (Figure 5-7). In contrast, in flow assays with purified CD8+ lymphocytes CLEVER-1 blockade had no effect transendothelial migration (Figure 5-8), although we noted a modest but statistically insignificant increase in adhesion and the proportion of shape-changed cells after blockade.

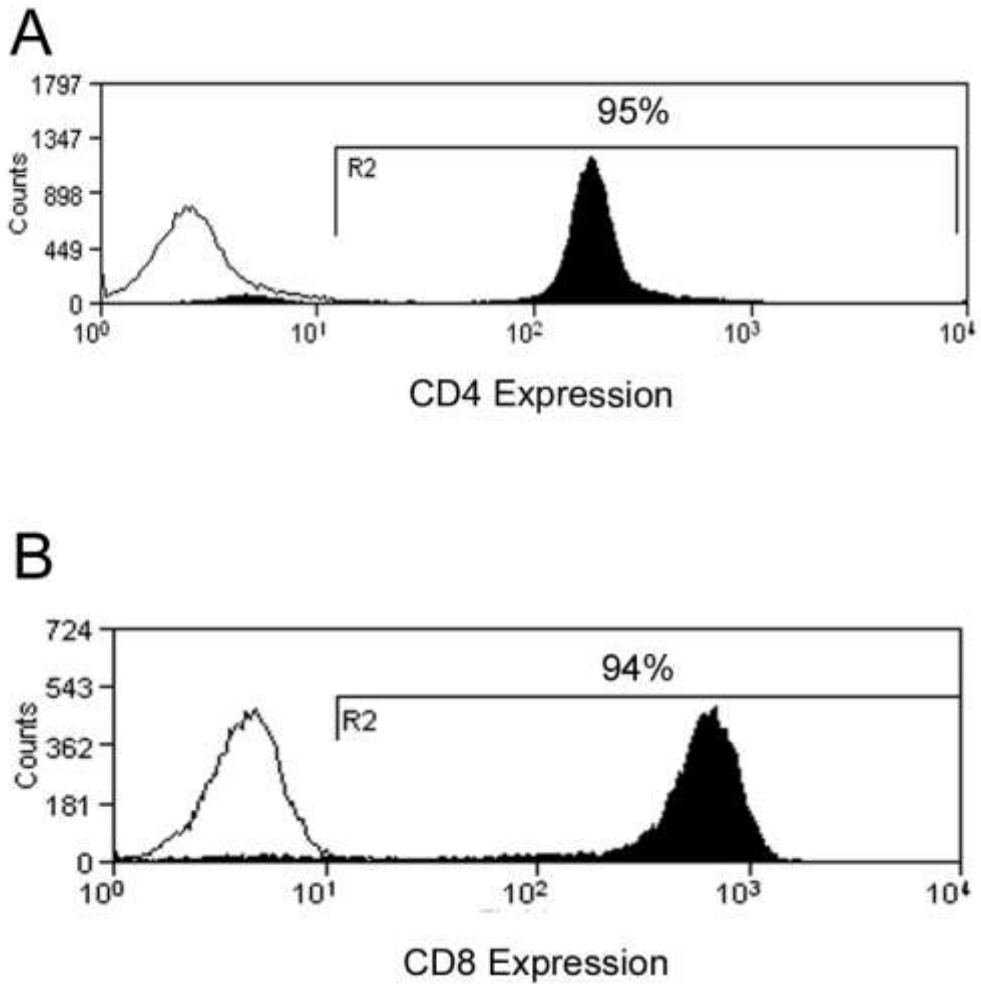

Figure 5-6 **Isolation of CD4 and CD8 lymphocytes from whole blood using negative selection Dynabeads ® kits**

Peripheral blood lymphocytes were purified for CD4 and CD8 lymphocyte fractions for use in flow adhesion assays. Purity was assessed by flow cytometry. The CD4 lymphocyte fractions were 95% pure (Panel A) and CD8 lymphocytes were 94% pure (Panel B).

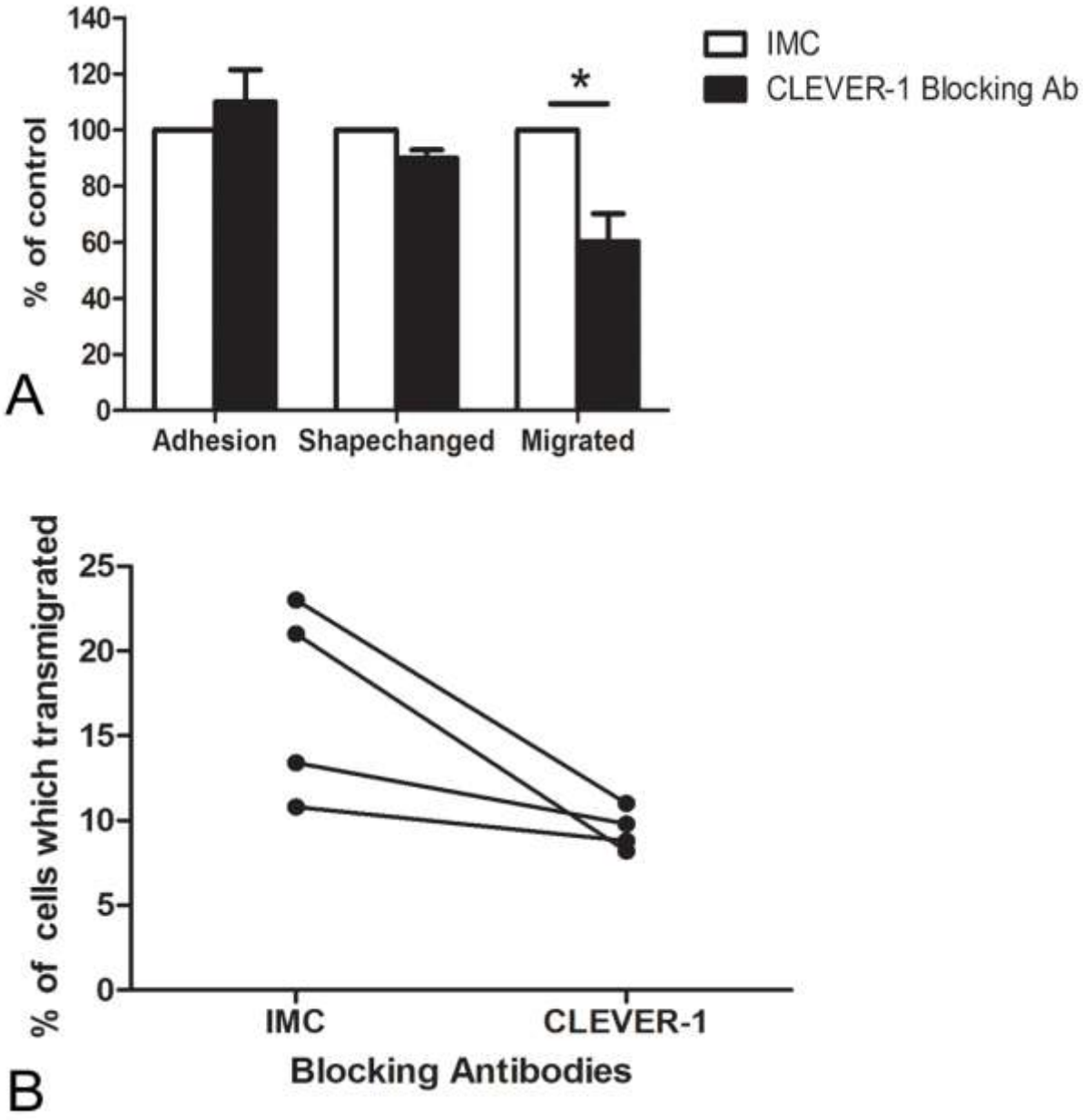

Figure 5-7 **CLEVER-1 mediates CD4 transmigration across HSEC under conditions of flow.**

The number of adherent, shapechanged and transmigrated cells were enumerated after flow assays. Panel A demonstrates that CLEVER-1 blockade using 20µg/ml of 3-372 mAb significantly reduced transmigration. The results are expressed as a percentage of binding when compared to an isotype matched control (IMC) and are a mean and SEM of four independent experiments: *p<0.05(Student's T test). Panel B shows the percentage of adherent cells that transmigrated across HSEC in each experiment comparing CLEVER-1 to IMC. (Shear stress 0.05 Pa).

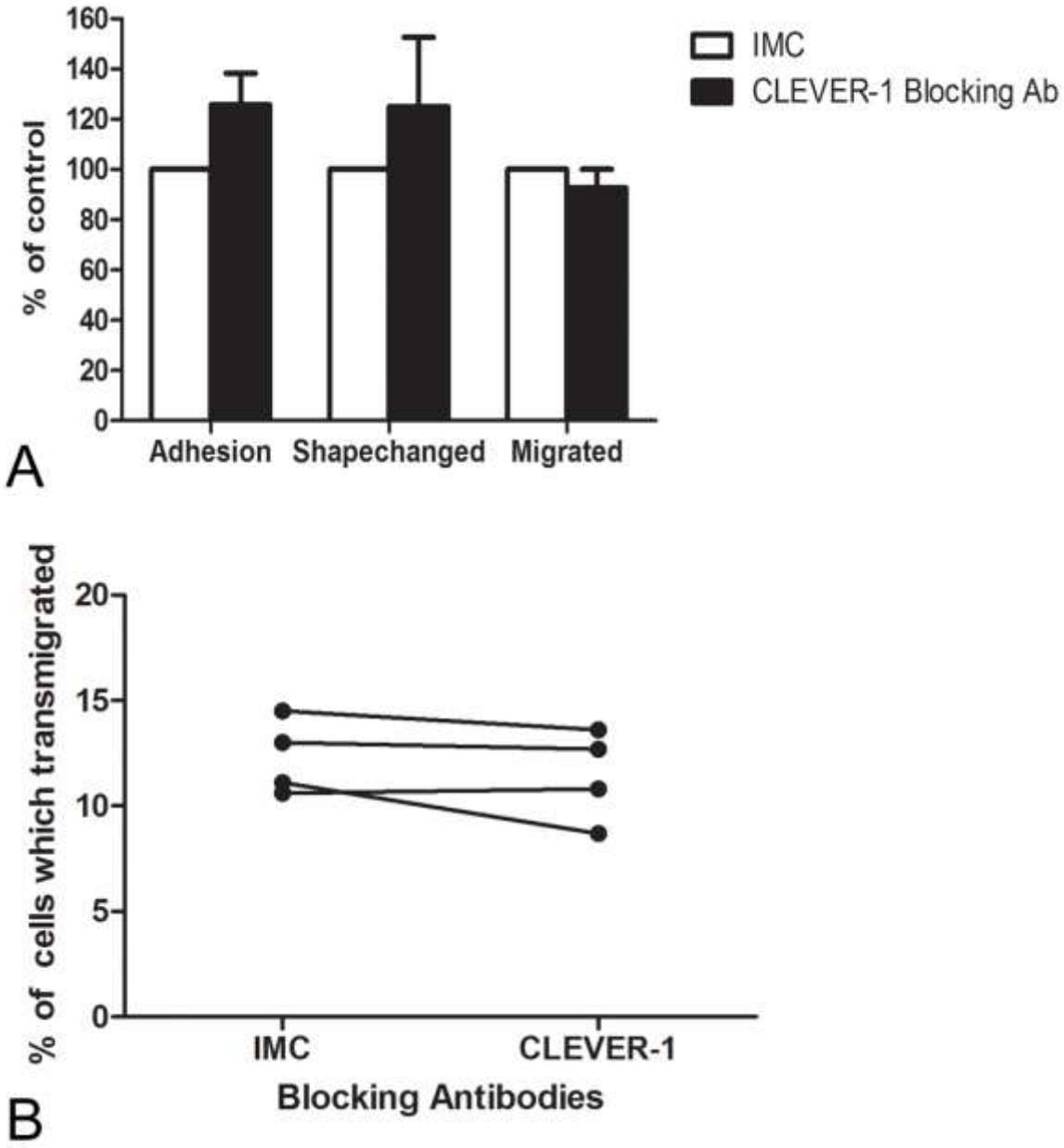

Figure 5-8 **CLEVER-1 does not mediate CD8 transmigration across HSEC under conditions of flow**

The number of adherent, shapechanged and transmigrated cells were enumerated after flow assays. Panel A demonstrates that CLEVER-1 blockade, 20µg/ml of mAb 3-372, had no effect on lymphocyte firm adhesion, shapechange/activation or transmigration. Results expressed as a percentage of binding compared to isotype matched control (IMC) and are a mean and SEM of four independent experiments (Student's T test). Panel B shows the percentage of adherent cells that transmigrated across HSEC in each experiment comparing CLEVER-1 to IMC. (Shear stress 0.05 Pa).

5.2.3 Regulatory T cells and Effector CD4 lymphocytes

In view of the finding that CLEVER-1 preferentially recruited CD4 lymphocytes I analysed CD4 lymphocyte subsets. Section 1.4.2 discusses the separate CD4 subsets and their functions in details, but they can be broadly divided into two groups: the effector group and the suppressor group (T regulatory cells). The effector group is characterised by low levels of cell surface IL-2 receptor (CD25) expression on their surface whereas the T regulatory (Treg) are known to express CD25. These differences in receptor expression were used to isolate the two populations from whole blood using a magnetic bead selection kit. The CD25 positive population expressed FOXP3 at high levels (Figure 5-9) confirming that this was the suppressor population. I performed flow adhesion assays with these two populations and assessed the effect of CLEVER-1 blockade. The two subpopulations demonstrated similar levels of absolute binding: total adhesion of CD4 effector lymphocytes was 148 ± 38 cells/$mm^2/10^6$; total adhesion of Tregs was 138 ± 13 cells/ $mm^2/10^6$. CLEVER-1 blockade significantly reduced the proportion of Treg cells undergoing transendothelial migration (Figure 5-10). In contrast, CLEVER-1 blockade did not significantly reduce the proportion of CD4 effector cells undergoing transendothelial migration, an increase in the proportion of shapechanged cells was noted but this was not significant (Figure 5-11).

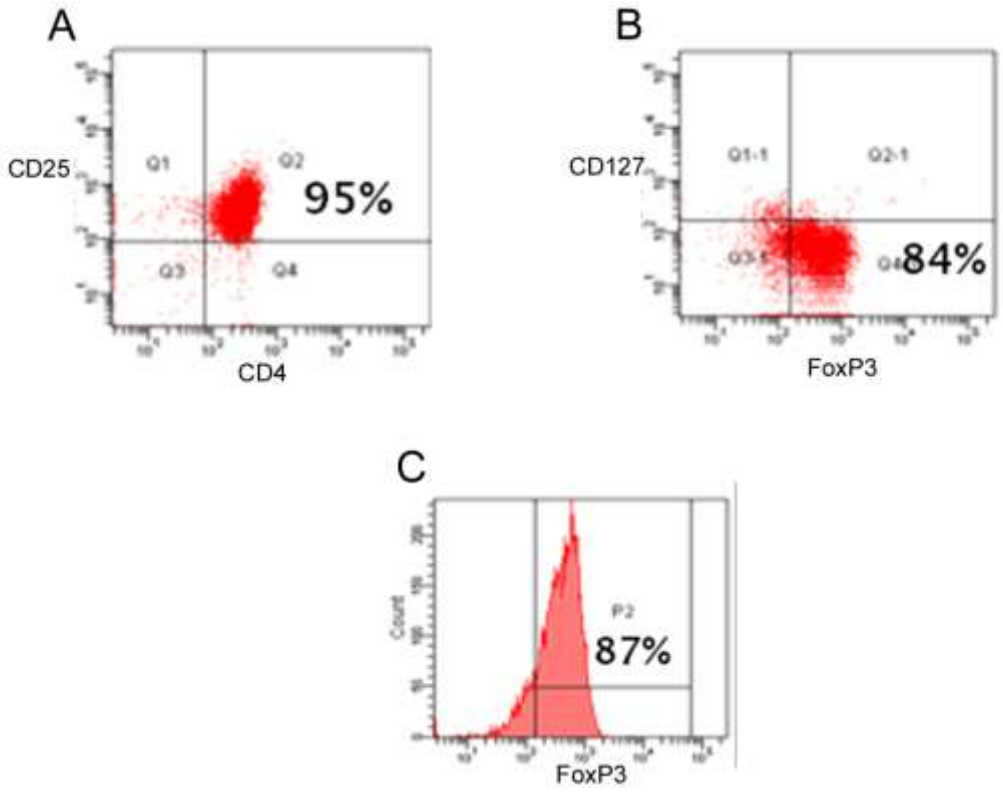

Figure 5-9 **T regulatory cell isolation from whole blood with CD4+CD25+ Dynabead® kit**

Peripheral blood lymphocytes were purified for T regulatory cells. The isolated lymphocytes were 95% pure for CD4+CD25+ cells (Panel A). Within the CD4+CD25+ population, 84% expressed FoxP3 and were negative for CD127 (Panel B). In total, 87% CD4+CD25+ population expressed FoxP3.

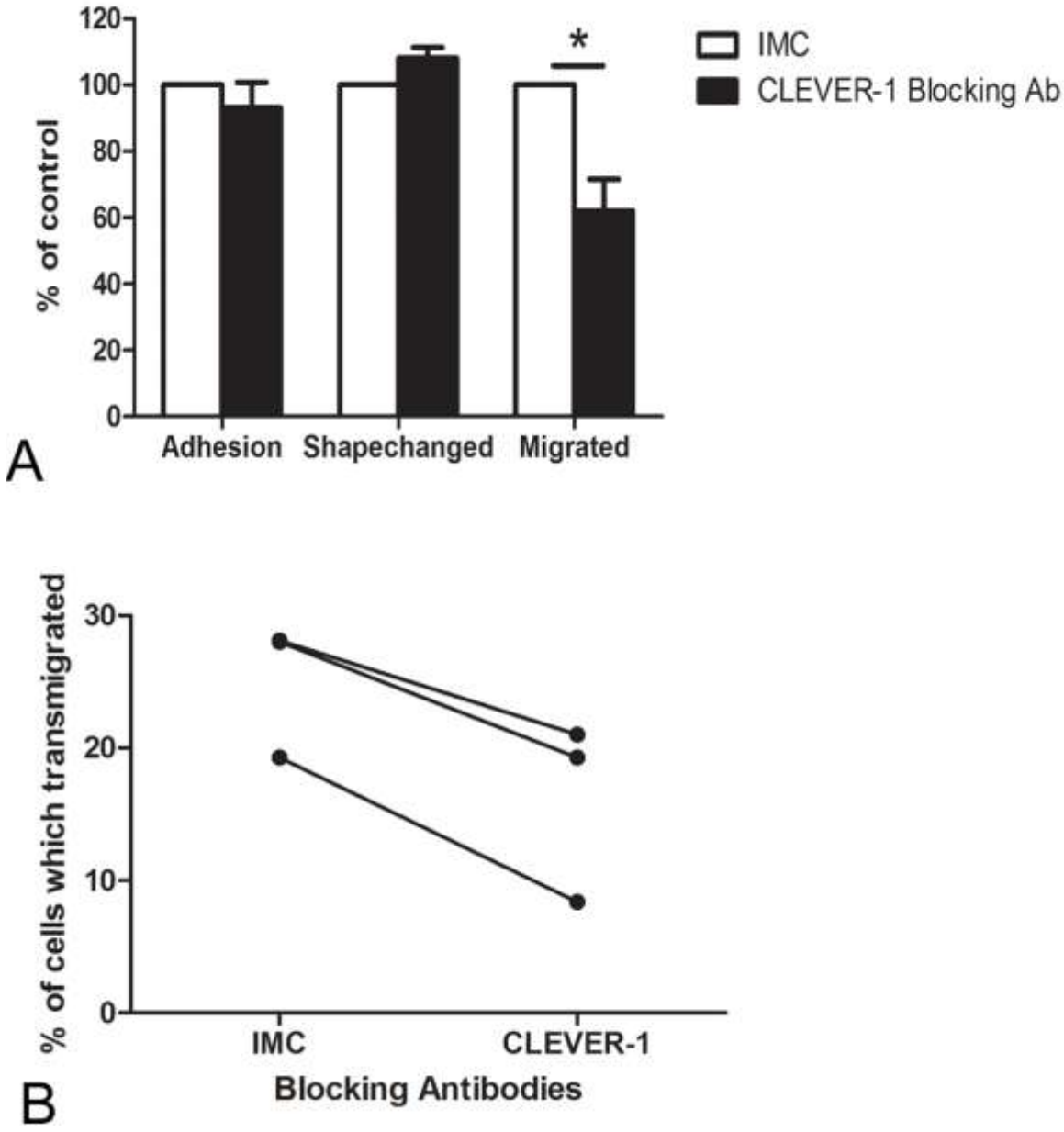

Figure 5-10 **The effect of CLEVER-1 blockade on Treg adhesion to HSEC under conditions of flow**

The number of adherent, shapechanged and transmigrated cells were enumerated after flow assays (shear stress 0.05 Pa). Panel A demonstrates that CLEVER-1 blockade, 20µg/ml of 3-372, significantly reduced transmigration. The results are expressed as a percentage of binding when compared to isotype matched control (IMC) and are a mean and SEM of three independent experiments: $p<0.05$ (Student's T test). Panel B shows the percentage of adherent cells that transmigrated across HSEC in each experiment comparing CLEVER-1 blockade to IMC.

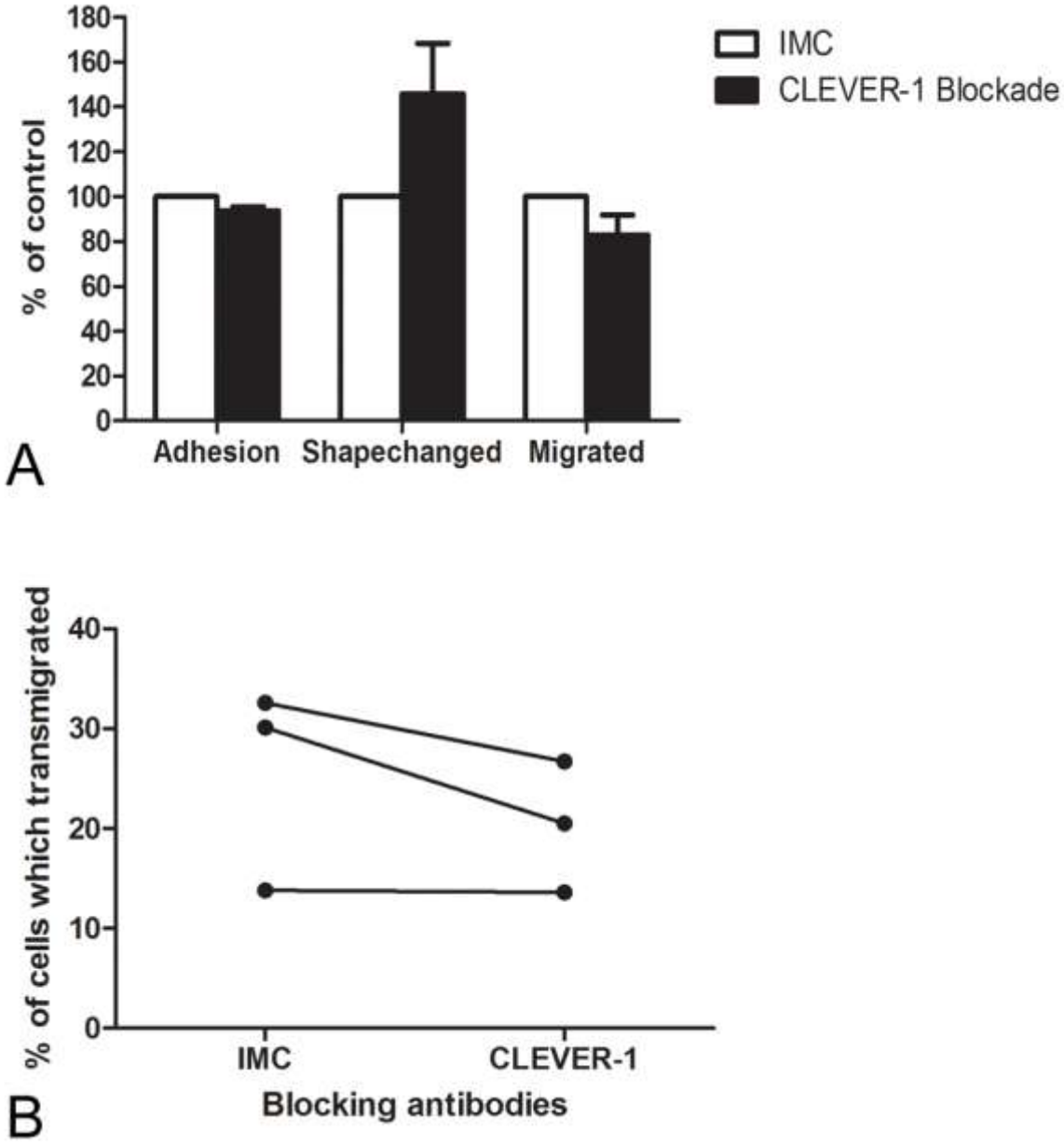

Figure 5-11 **The effect of CLEVER-1 blockade on CD4 effector cell adhesion to HSEC under conditions of flow**

The number of adherent, shapechanged and transmigrated cells were enumerated after flow assays (shear stress 0.05 Pa). Panel A demonstrates that CLEVER-1 blockade, 20µg/ml 3-372, had no effect on lymphocyte firm adhesion or transmigration. Results expressed as a percentage of binding when compared to isotype matched control (IMC) and are a mean and SEM of three independent experiments (Student's T test). Panel B shows the percentage of adherent cells that transmigrated across HSEC in each experiment comparing CLEVER-1 blockade to IMC.

5.2.4 The molecular mechanisms of T regulatory cell transmigration across HSEC

The flow assays with CD4 subsets suggested that CLEVER-1 was involved in the transmigration of T regs across hepatic sinusoidal endothelium. I then proceeded to study the molecular mechanisms of Treg transendothelial migration through hepatic sinusoidal endothelium in more detail and examined the interplay of CLEVER-1 with other molecules. Our group has previously published that VAP-1, ICAM-1 and the chemokine receptor CXCR3 are all involved in lymphocyte transendoethelial migration across HSEC (Curbishley et al., 2005; Lalor et al., 2002a). They have also reported roles for CCR4 and in some cells CCR10 in recruitment of Tregs beyond the endothelium into the liver (Eksteen et al., 2006; Oo et al., 2010). In contrast there is limited information on the adhesion molecules specifically utilised by Tregs. Thus I concentrated on the transmigration of Tregs and studied the effects of CLEVER-1 blockade in combination with ICAM-1 and VAP-1, two well known mediators of T cell transmigration across HSEC (Lalor et al., 2002a). It has been previously published that VCAM-1 predominantly mediates the capture and adhesion of Tregs on HSEC (Oo et al., 2010). In support of this, my experiments involving blockade of ICAM-1, VAP-1 and CLEVER-1 showed that these receptors did not play a significant role in the capture and adhesion of the Tregs (Figure 5-12). However, further analysis demonstrated that the blockade of each receptor led to a decrease in the proportion of adherent cells undergoing transendothelial migration and the

blockade of all three receptors in combination led to a cumulative effect (Figure 5-13).

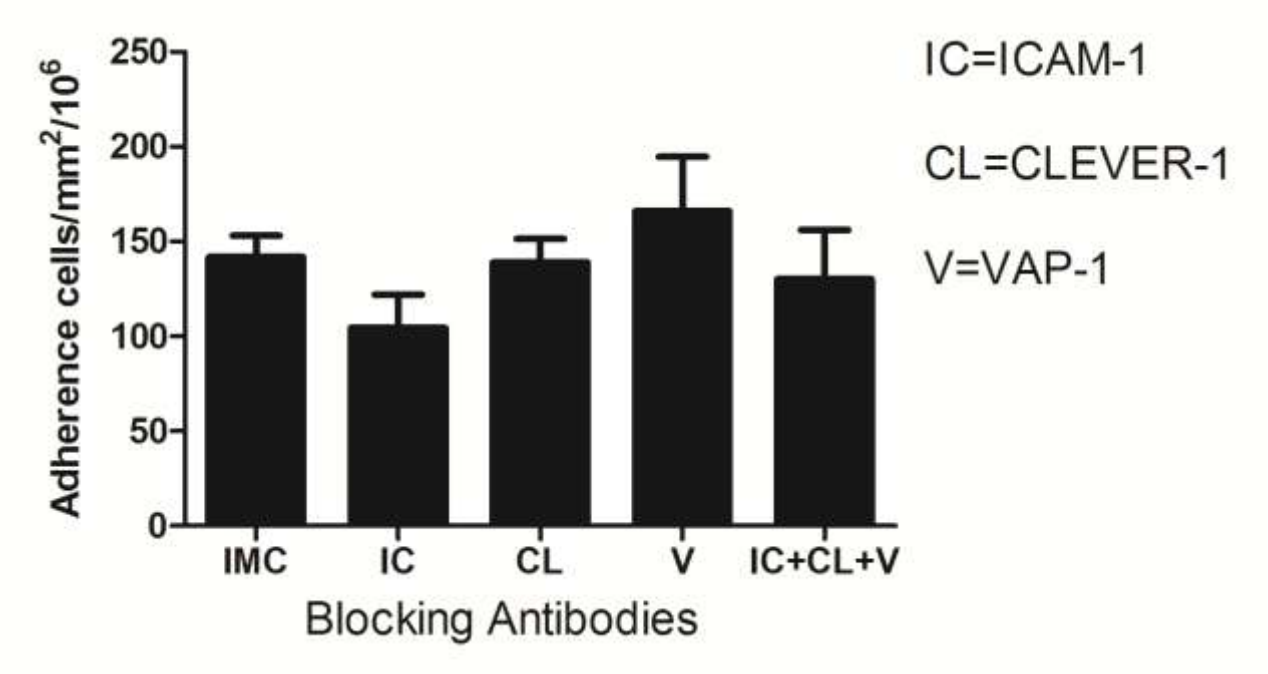

Figure 5-12 **ICAM-1, CLEVER-1 and VAP-1 do not mediate the adhesion of T regulatory cells to HSEC under conditions of flow**

The number of adherent cells on cytokine treated HSEC were enumerated from flow assays (shear stress of 0.05 Pa) after pretreatment with blocking antibodies to ICAM-1, CLEVER-1 and VAP-1 and the three receptors in combination. Results were normalized to adherent cells per mm^2 per 10^6 cells. Results were compared to an isotype matched control (IMC) and are the mean of three independent experiments and SEM.

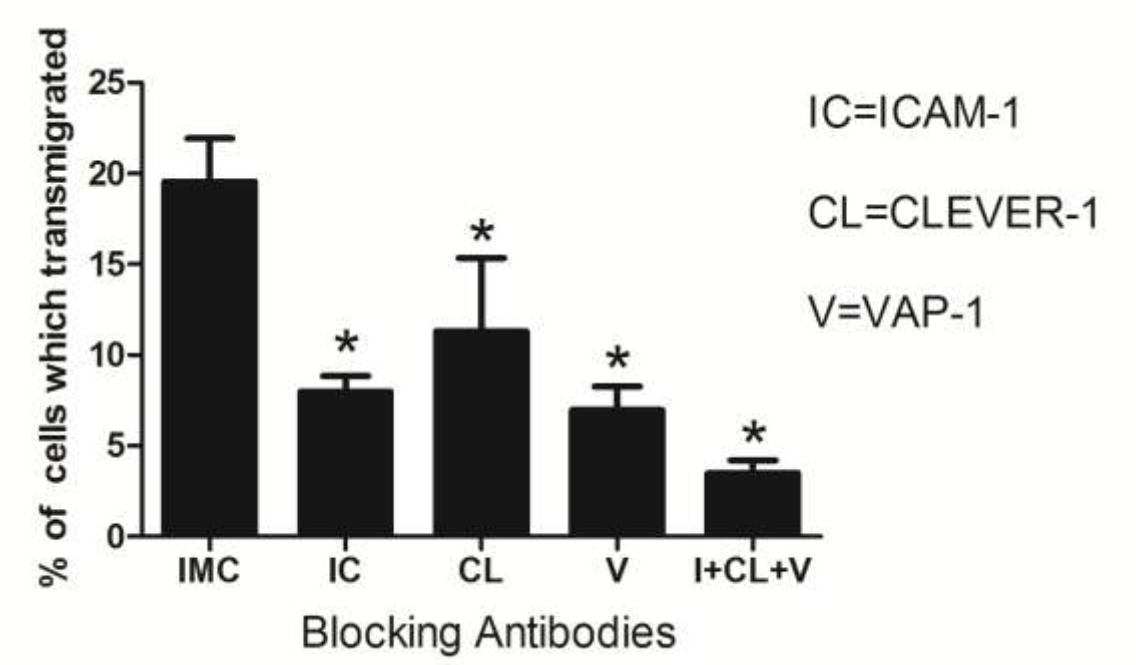

Figure 5-13 **ICAM-1, CLEVER-1 and VAP-1 mediate the transendothelial migration of T regulatory cells across HSEC under conditions of flow**

The transmigration of Tregs across cytokine treated HSEC under conditions of flow (shear stress of 0.05 Pa) is shown with prior blockade of ICAM-1, CLEVER-1, VAP-1 and all three receptors in combination. Adherence is compared to an isotype matched control and data is expressed as the proportion of firmly adherent lymphocytes which underwent transmigration. Mean and SEM are shown of three independent experiments. *$p<0.05$ (Student's T test).

5.2.5 The recruitment of B cells

We also investigated the role of CLEVER-1 in the recruitment of B lymphocytes. B cells are implicated in the pathogenesis of liver disease (discussed in section1.7.3) but there is a paucity of literature regarding the recruitment of B cells to the liver via hepatic sinusoidal endothelium. I used the flow assays to systematically determine the molecules involved in B cell adhesion to and migration across HSEC. B cells were isolated from whole blood using magnetic bead selection with a purity of 98% (Figure 5-14). Experiments were carried out in which antibodies were used to block the conventional adhesion receptors VCAM-1 and ICAM-1 and the unconventional adhesion molecules VAP-1 and CLEVER-1 blockade. The contribution of chemokines was assessed by using pertussis toxin treatment of B cells to inhibit G-protein coupled chemokine receptors. Flow assays were performed under the conditions described above with TNFα and IFNγ treated HSEC and a shear stress of 0.05Pa.

In contrast to T cells, B cells did not undergo even a brief rolling/tethering step prior to firm adhesion on HSEC. The primary mediator of B cell firm adhesion was VCAM-1, with no significant role for the other molecules (Figure 5-15). Analysis of the transmigration step demonstrated that a smaller proportion of adherent B cells underwent this step in comparison to T cells (proportion of B cells undergoing transmigration 7.6± 0.5% vs CD4 cells 25 ± 5%). Additionally, transmigration of B cells was reduced by ICAM-1,

CLEVER-1, VAP-1 and pertussis blockade (Figure 5-16). Interestingly, VCAM-1 did not play a role in the transmigration step for B cells.

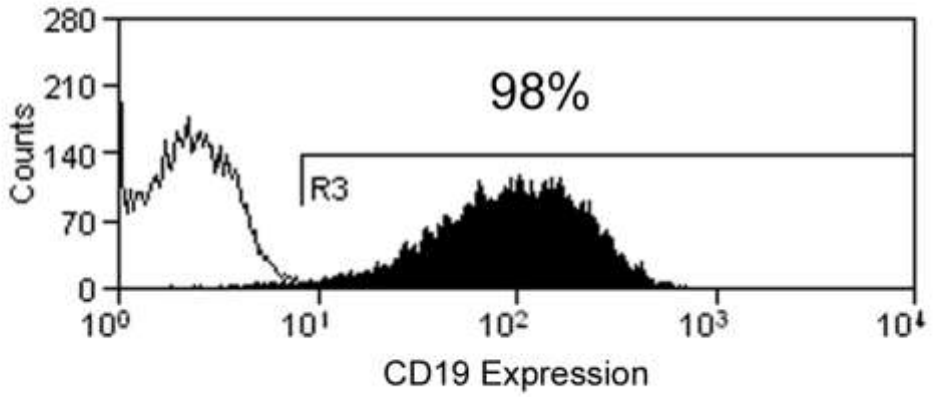

Figure 5-14 **Isolation of B lymphocyte population from whole blood using a CD19+ Dynabead ® kit**

Peripheral blood lymphocytes were purified for B cell fractions for use in flow adhesion assays. Purity was assessed by flow cytometry and demonstrated a purity of 98%.

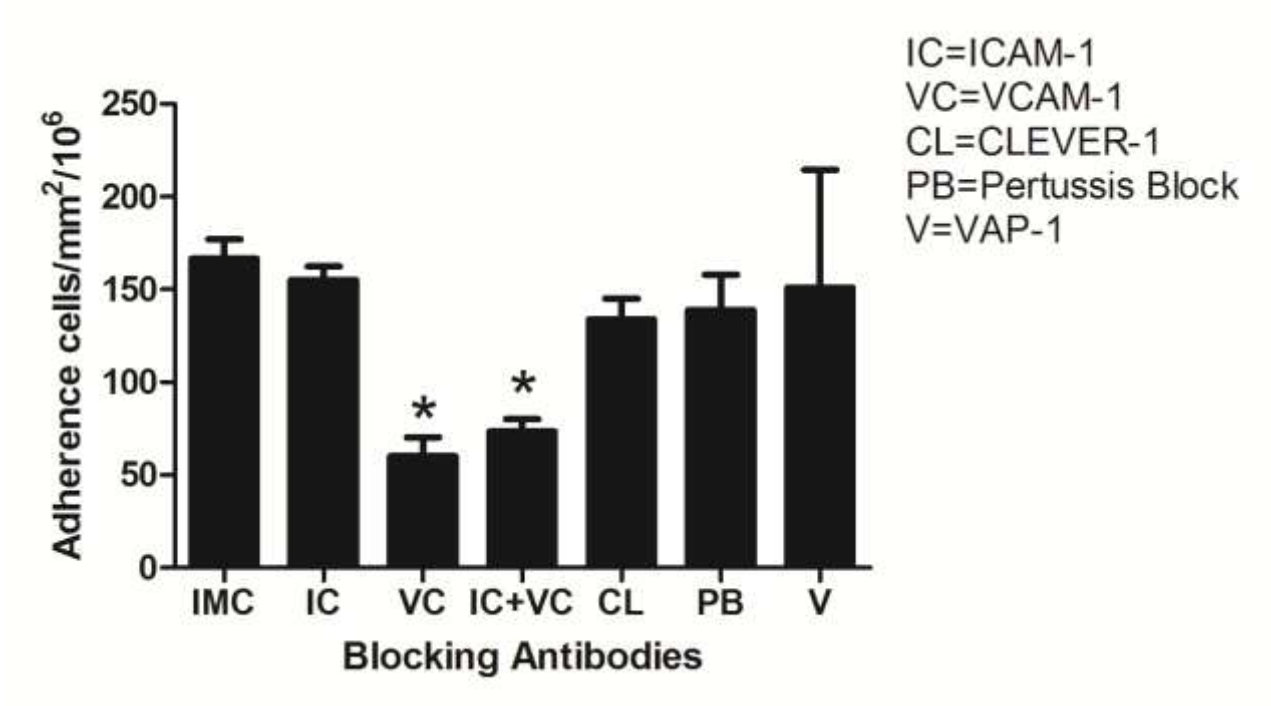

Figure 5-15 **B cell adherence to HSEC under conditions of flow**

The number of adherent cells on cytokine treated HSEC were enumerated from flow assays (shear stress of 0.05 Pa) after pretreatment with various blocking antibodies. Results were normalized to adherent cells per mm^2 per 10^6 cells. Results were compared to an isotype matched control (IMC) and are the mean of three independent experiments and SEM: *$p<0.05$ (Students T test).

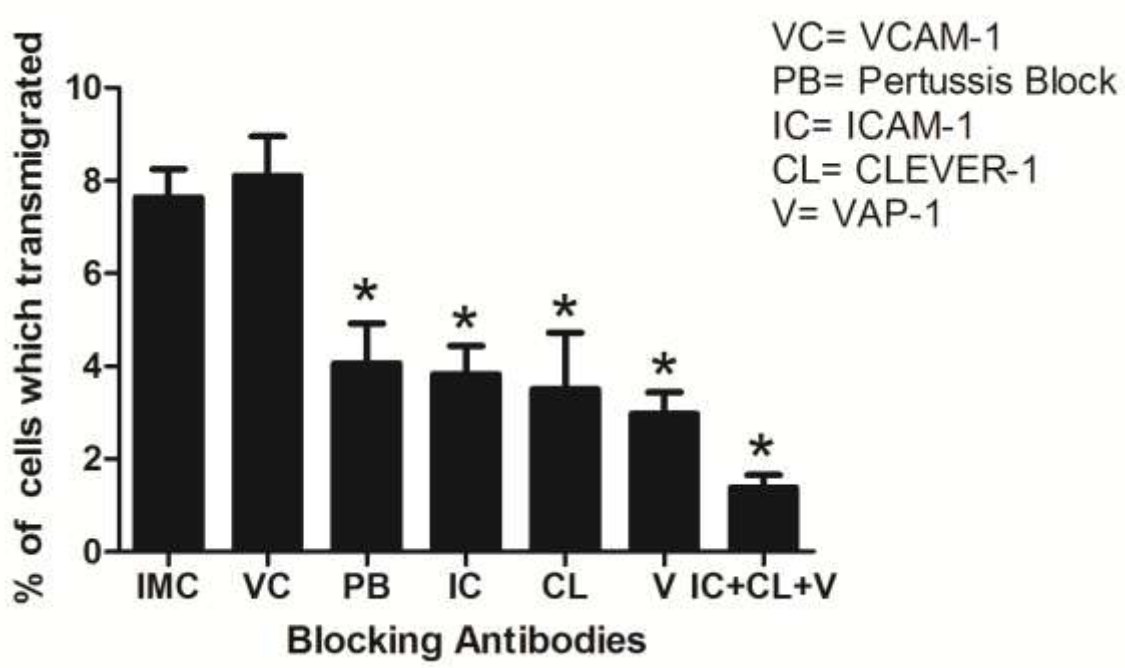

Figure 5-16 **B cell transmigration across HSEC under conditions of flow**

The transmigration of B cells across cytokine treated HSEC under conditions of flow (shear stress of 0.05 Pa) is shown with prior blockade of various receptors. Adherence is compared to an isotype matched control and data is expressed as the proportion of firmly adherent lymphocytes which underwent transmigration. Mean and SEM are shown of three independent experiments: *$p<0.05$ (Student's T test).

5.3 Comparison of B and T cell motility

Flow adhesion assays had demonstrated differences in B cell recruitment when compared to T cells in terms of rolling behaviour, firm adhesion and transendothelial migration. As described in section 1.9.3 the 'typical' adhesion cascade comprises an additional step of 'crawling', which occurs once leucocytes have undergone firm adhesion to the endothelial surface. Leucocytes can crawl across the apical surface of the endothelial monolayer and, if the necessary signals are present, migrate into the subcellular space. To investigate this phenomenon we compared the crawling of B cells and T cells by using Image J software to manually track each lymphocyte in a field of view over a set period of time. A chemotaxis tool allowed this tracking to be plotted graphically. B cell and T cell behaviour were markedly different: while T cells clearly demonstrated the ability to crawl across the endothelial surface, B cells demonstrated minimal motility under conditions of flow (Figure 5-17). These data were concordant with the transendothelial migration data where T cells exhibited a greater propensity to migrate across the endothelial monolayer than B cells under conditions of flow.

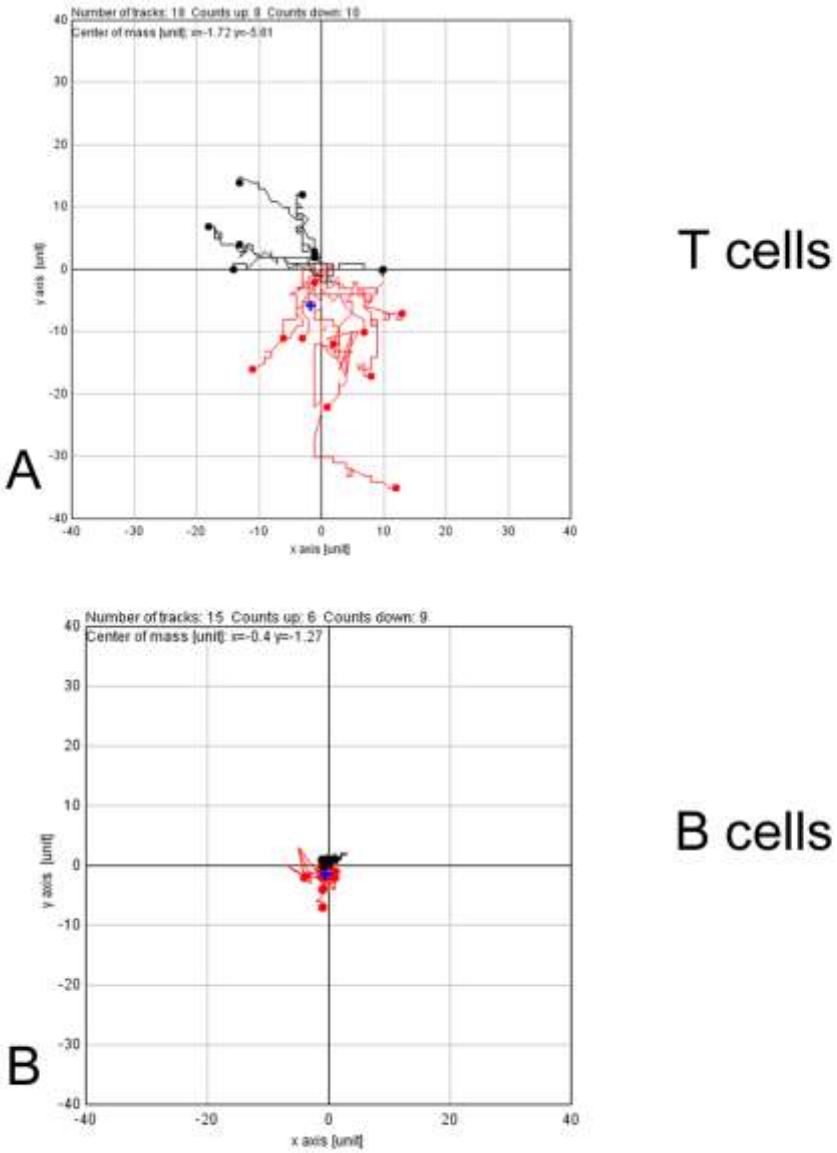

Figure 5-17 **The motility of T and B cells on cytokine treated HSEC under conditions of flow.**

B cells and T cells were perfused separately over monolayers of cytokine treated HSEC at a shear stress of 0.05 Pa . Adherent cells within a single field were manually tracked over a period of 5minutes using Image J software. Tracking data was analysed using a chemotaxis tool which plotted the movement of each T cell (Panel A) and each B cell (Panel B) from a common point. Each dot is an individual lymphocyte, black labelling demonstrates overall movement against the direction of flow and red labelling demonstrates overall movement in the direction of flow.

5.4 The transmigration of CD4 lymphocytes across HSEC

Thus far my flow based adhesion assays had demonstrated for the first time that CLEVER-1 is involved in the transmigration step of lymphocyte recruitment through hepatic sinusoidal endothelium. Moreover I show a marked subset preference for CD4 lymphocytes and B cells. Transmigration is the least well understood step of lymphocyte diapedesis, and so to elucidate the role of CLEVER-1 in this process I decided to examine transmigration in more detail. Assays with B cells had shown that only a small proportion of adherent cells underwent transendothelial migration. I therefore decided to concentrate on CD4 lymphocyte transmigration across HSEC under conditions of flow as an experimental system to investigate transendothelial migration. Initial experiments involved perfusing CD4 lymphocytes over HSEC monolayers in ibidi chamber slides. The cells were immediately fixed in paraformaldehyde followed by staining with a nuclear DAPI stain. The ibidi slides with adherent cells *in situ* were then imaged with confocal microscopy. Lymphocyte nuclei could be clearly distinguished from HSEC nuclei and there were several examples where lymphocyte nuclei were deforming HSEC nuclei suggesting that the attached lymphocytes were transmigrating through the endothelial cytoplasm rather than across cell junctions distal to the nuclei (Figure 5-18).

To confirm that CD4 lymphocytes were transmigrating through a transcellular route flow assays were repeated using HSEC and CD4 lymphocytes which were fluorescently labelled prior to the experiment. Following perfusion and

adhesion of lymphocytes a ‘cell mask’ membrane stain was applied and live cell imaging was carried out. ‘Cell Mask’ probes specifically bind to plasma membranes. They are comprised of amphipatic molecules which have a lipophilic moiety and a negatively charged hydrophilic dye. These properties allow membrane loading and anchoring of the probes to the membrane with stability for up to 60 minutes. These probes can be used with live cells and therefore were ideal for studying the integrity of cellular boundaries during transendothelial migration. Imaging of these fluorescently labelled cells clearly demonstrated transcellular pores within the HSEC surrounding transmigrating lymphocytes (Figure 5-19).

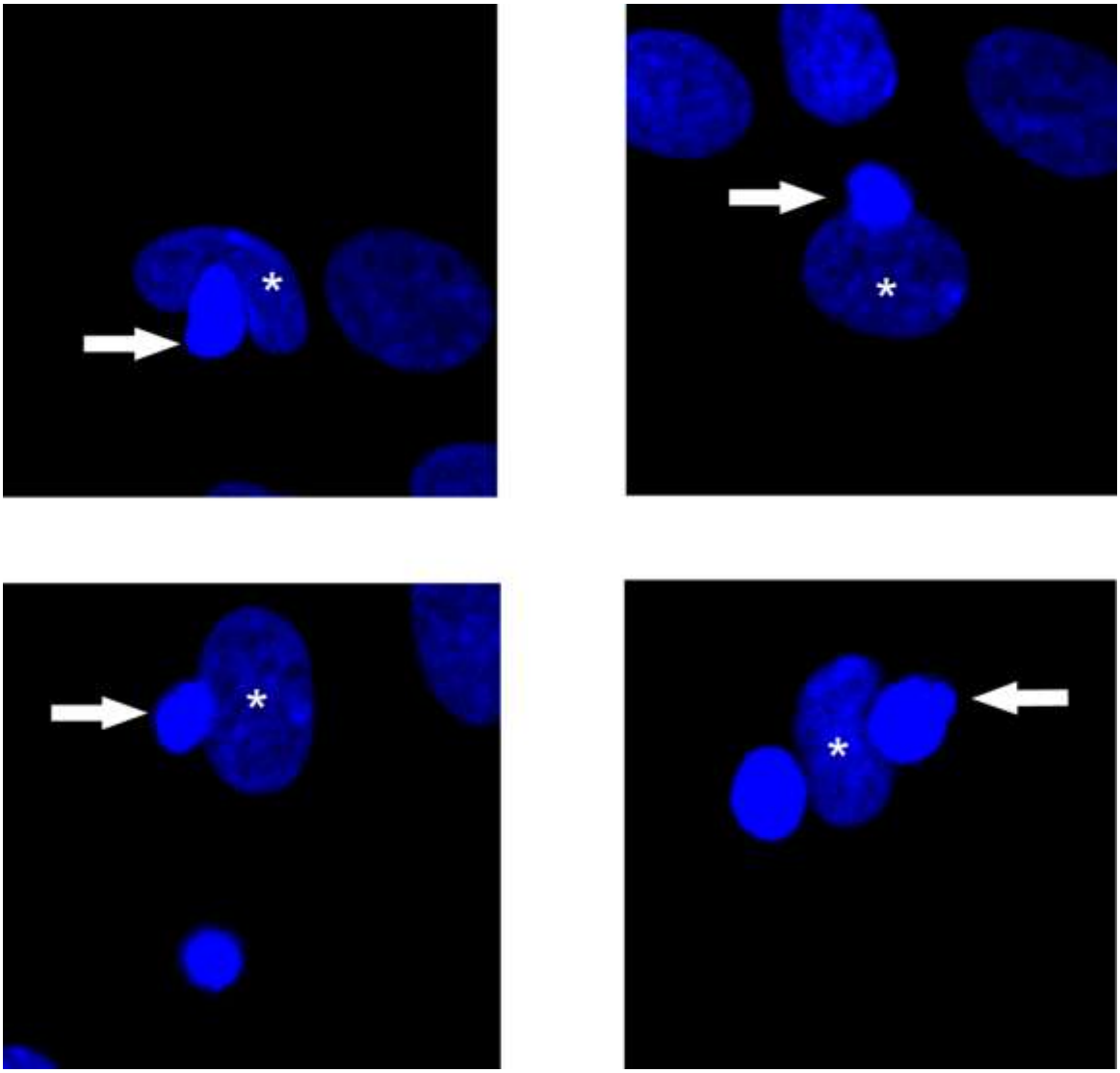

Figure 5-18 **CD4 lymphocyte interaction with HSEC under conditions of flow**

Representative confocal images of fixed cells after flow assays (shear stress 0.05 Pa) performed with CD4 lymphocytes with HSEC monolayers in Ibidi chamber slides. HSEC nucleii (*) can be seen being deformed by lymphocyte nucleii (arrows). Cell nucleii stained with DAPI. Fields captured with 63x objective

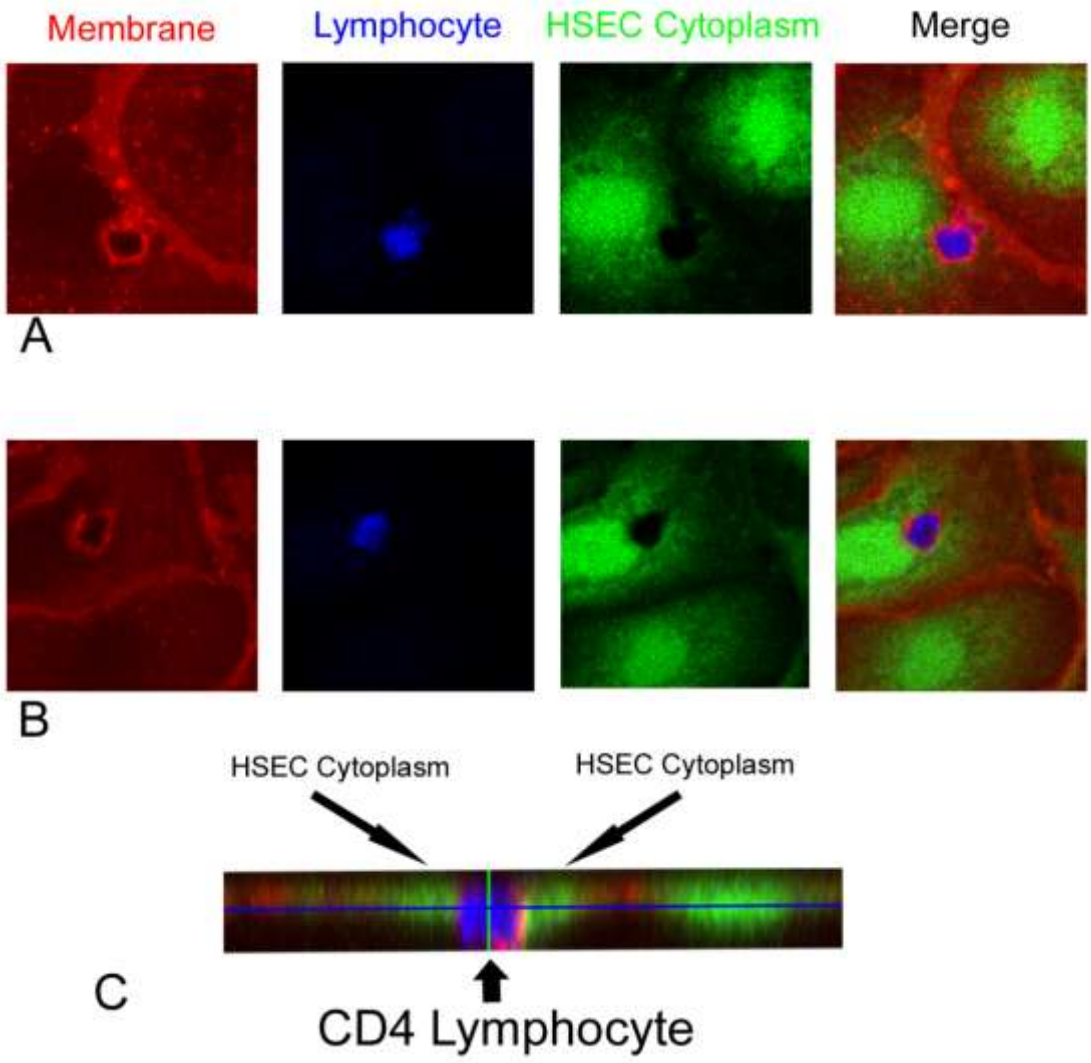

Figure 5-19 **Transcellular migration of CD4 lymphocytes through HSEC**

Live cell imaging of CD4 lymphocytes adhering to HSEC monolayers after flow based adhesion assays (shear stress 0.05 Pa) in Ibidi chamberslides. Panel A and B demonstrate CD4 lymphocytes forming transcellular pores through HSEC monolayers . Panel C is a representative orthogonal (z stack) image of a CD4 lymphocyte transmigrating through the HSEC monolayer. HSEC were stained with CFMDA (green), Lymphocytes were stained with cell tracker violet (blue) and plasma membranes were stained with cell mask (red). Fields captured with 63x objective.

5.4.1 Transcellular migration of CD4 lymphocytes and the role of ICAM-1 and VAP-1

It was not possible to ascertain from these initial experiments the proportion of CD4 lymphocytes which were transmigrating via the transcellular route. To maximise the number of lymphocyte adhesion events to permit more detailed characterisation of transmigration we increased the duration of perfusion of lymphocytes in flow assays from 5 minutes to 10 minutes. This led to increased numbers of adherent and therefore transmigrating cells. It was possible to image more cells transmigrating and from these experiments (Figure 5-20). We counted the number of CD4 cells undergoing transcellular migration and the number which were migrating at junctions in each field using z-stack imaging to confirm transendothelial migration. The total numbers could then be expressed as the proportion of cells which took either the transcellular route or the paracellular route. We found that 62% of cells (SEM ±6%) underwent transcellular migration and 42% (SEM± 6%) of cells underwent paracellular migration. Data from n=27 fields.

ICAM-1 is known to play a central role in leucocyte transmigration. Our group has shown previously that ICAM-1 mediates lymphocyte transmigration across HSEC, and it has also been shown to mediate transcellular leucocyte migration in other endothelial cells (Carman et al., 2007; Lalor et al., 2002a; Yang et al., 2005). In view of these findings I proceeded to characterise the localisation of ICAM-1 expression in relation to the transcellular pores in HSEC. Flow based assays were repeated with

CD4 lymphocytes and, following fixation, immunofluorecent staining for ICAM-1was performed as described in section 2.12. These experiments demonstrated an enrichment of ICAM-1 which clearly demarcated the borders of the transcellular pores as the CD4 lymphocyte underwent transmigration (Figure 5-21).

As discussed in section 1.10.5, studies have shown that VAP-1 mediates transmigration of lymphocytes across HSEC (Lalor et al., 2002a) I therefore studied VAP-1 localisation during transcellular migration using the method described above. These experiments demonstrated colocalisation of VAP-1 with ICAM-1 during lymphocyte transcellular migration (Figure 5-22).

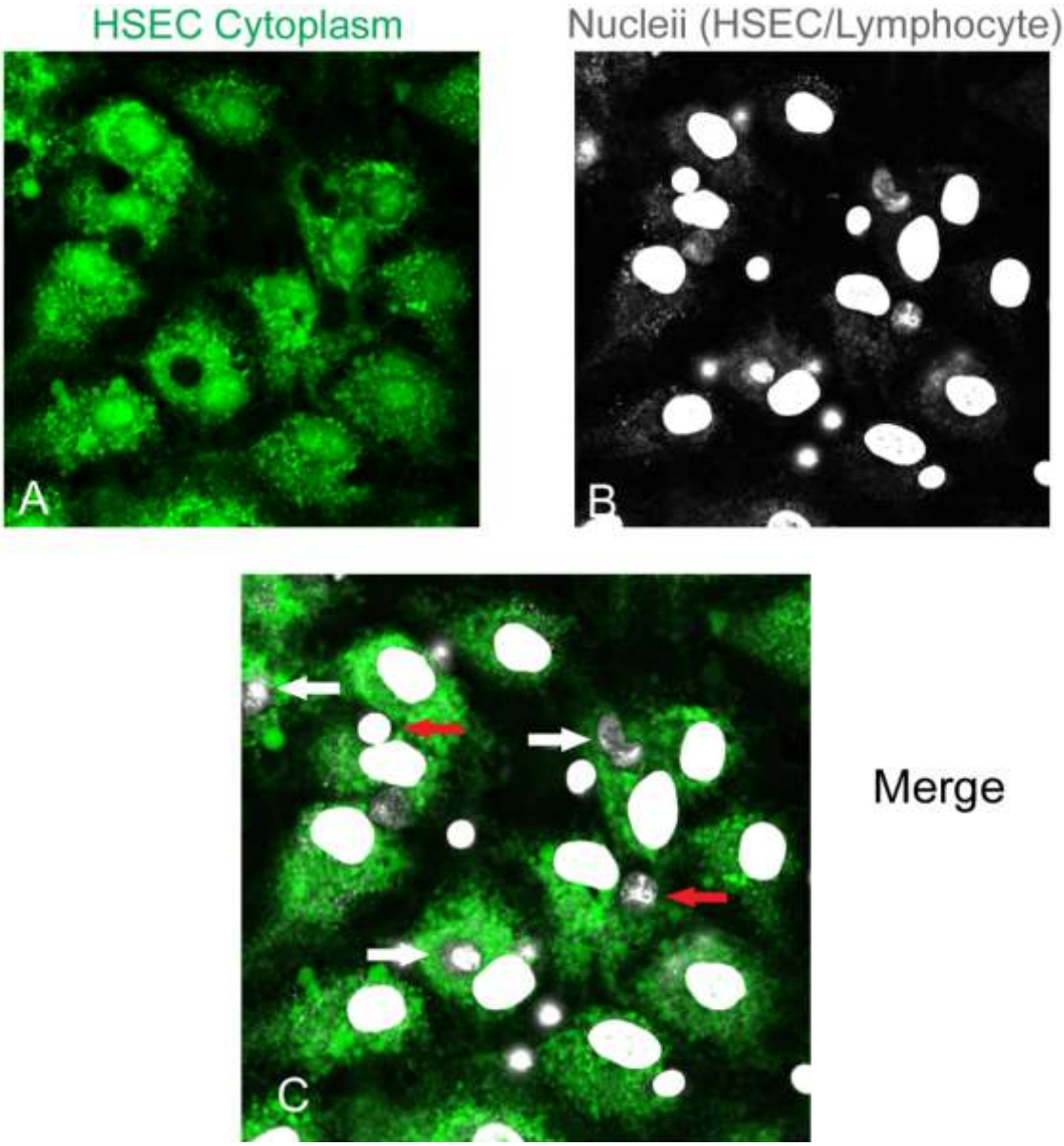

Figure 5-20 **CD4 Lymphocytes transmigrate via the paracellular route and the transcellular route**

Representative confocal image of CD4 lymphocytes transmigrating across HSEC monolayers after flow adhesion assay. Lymphocytes were perfused over a monolayer of HSEC at a shear stress of 0.05 Pa before fixation with paraformaldehyde. HSEC were pre-labelled with cell tracker green (Panel A), lymphocyte and HSEC nucleii were stained with DAPI (Panel B). Merged image is shown in Panel C, white arrows denoting areas of CD4 transcellular migration and red arrows denoting areas of CD4 paracellular migration. Fields captured with a 63x objective.

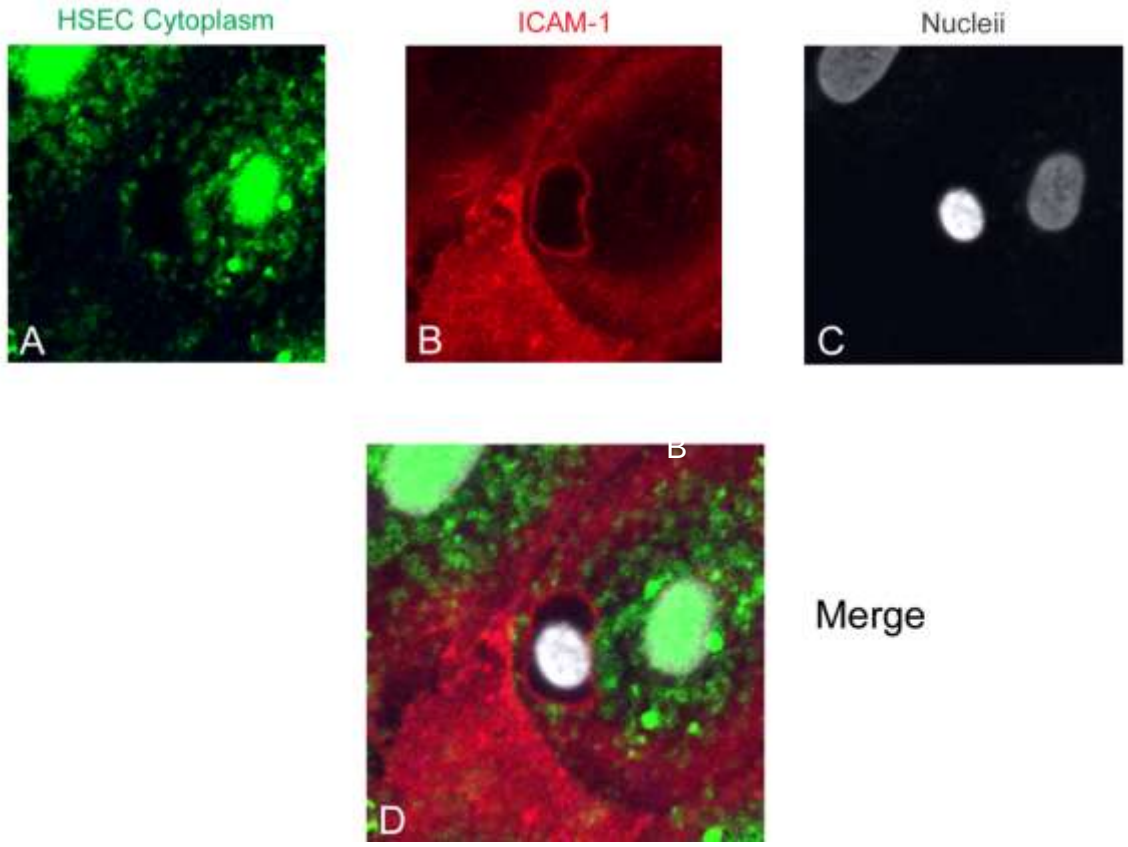

Figure 5-21 **ICAM-1 enriches around transmigrating CD4 lymphocytes**

Representative confocal image of CD4 lymphocytes transmigrating across HSEC monolayers after flow adhesion assay. Lymphocytes were perfused over a monolayer of HSEC at a shear stress of 0.05 Pa before fixation with paraformaldehyde. HSEC were pre-labelled with cell tracker green (Panel A). Following fixation cells were stained with ICAM-1 (Panel B) and nucleii were stained with DAPI (Panel C). Merged image is shown in Panel D. Fields captured with 63x objective.

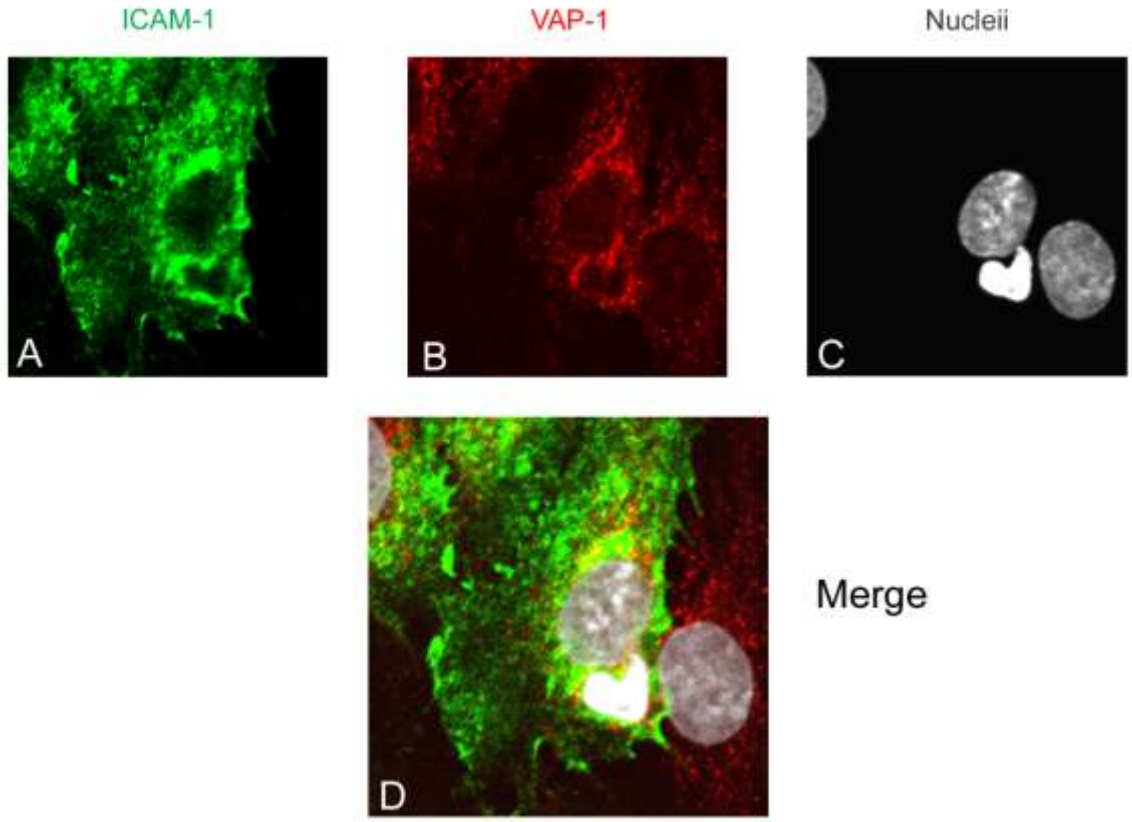

Figure 5-22 **ICAM-1 and VAP-1 colocalise around transmigrating CD4 lymphocytes.**

Representative confocal image of CD4 lymphocytes transmigrating across HSEC monolayers after flow adhesion assay. Lymphocytes were perfused over a monolayer of HSEC at a shear stress of 0.05 Pa before fixation with paraformaldehyde. Following fixation cells were stained with ICAM-1 (Panel A), VAP-1 (Panel B) and nucleii were stained with DAPI (Panel C). Merged image is shown in Panel D. Fields captured with 63x objective.

5.5 Transcellular migration of T regulatory cells and the role of CLEVER-1

Our functional assays confirmed that CLEVER-1 preferentially mediated the recruitment of the Treg subset of CD4 lymphocytes (see 5.2.3), but we wanted to determine whether Tregs followed the transcellular or paracellular pathway of migration, and if CLEVER-1 was involved in either process. Flow based adhesion assays were repeated with T regulatory cells isolated from whole blood and after fixation and permeabilisation a FOXP3 immunofluorescent stain was used to confirm the T regulatory cell phenotype of adherent cells (see section 2.12). Subsequent confocal imaging suggested that T regulatory cells transmigrated using the transcellular route (Figure 5-23).

Following our observation of T reg transcellular migration, the next step was to study the distribution of CLEVER-1 in HSEC during T reg extravasation. This was done using the same immunofluorescent staining described for ICAM-1 detection (see 2.12). Our experiments confirmed that CLEVER-1 was visible on HSEC as T regs underwent diapedesis (Figure 5-24) and z-stack image analysis confirmed the enrichment of CLEVER-1 around transmigrating T regs (Figure 5-24).

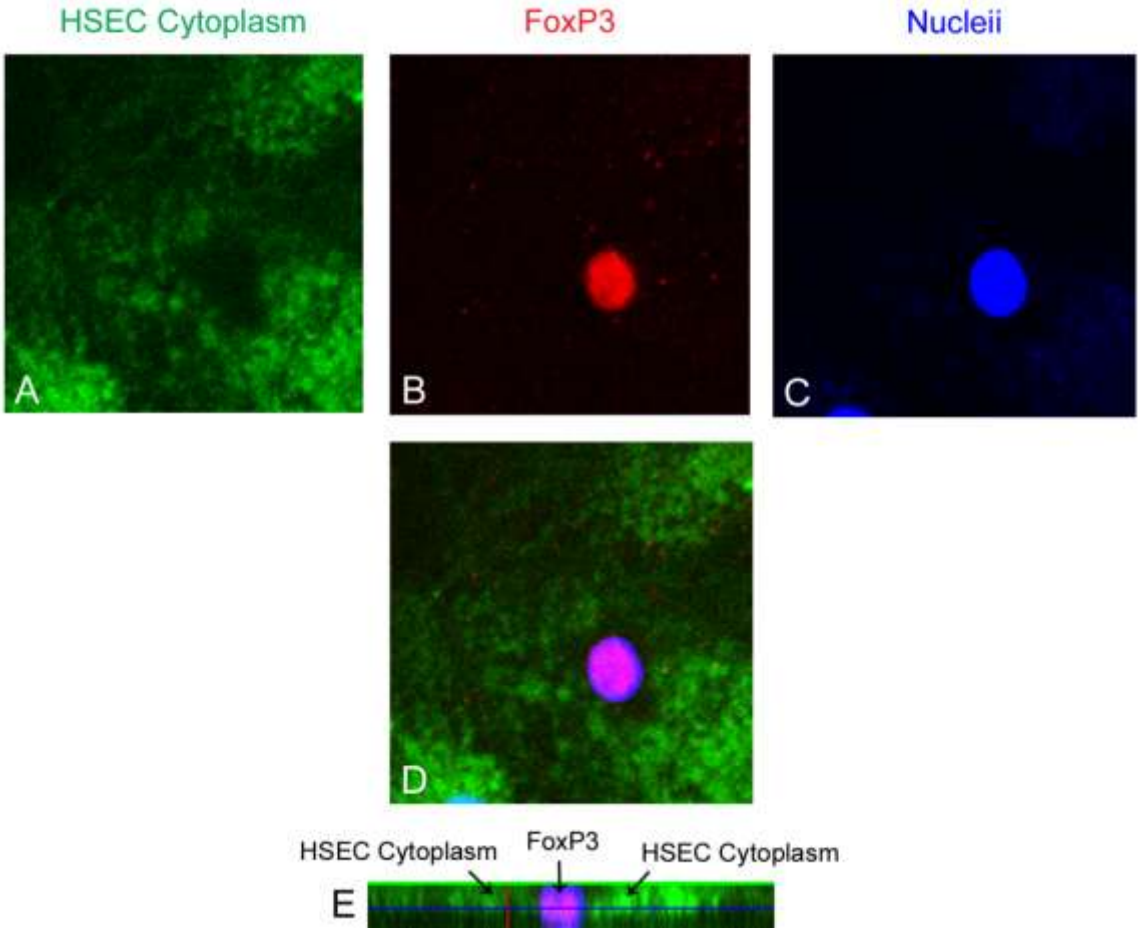

Figure 5-23 **Tregs transmigrate via the transcellular route**

Representative confocal image of T reg transmigrating across HSEC monolayers after flow adhesion assay. T regs were perfused over a monolayer of HSEC at a shear stress of 0.05 Pa before fixation. HSEC were pre-labelled with cell tracker green (Panel A). Following fixation cells were stained for FoxP3 (Panel B) and nucleii were stained with DAPI (Panel C). Merged image is shown in Panel D. Z stack image is shown in Panel E. Fields captured with 63x objective.

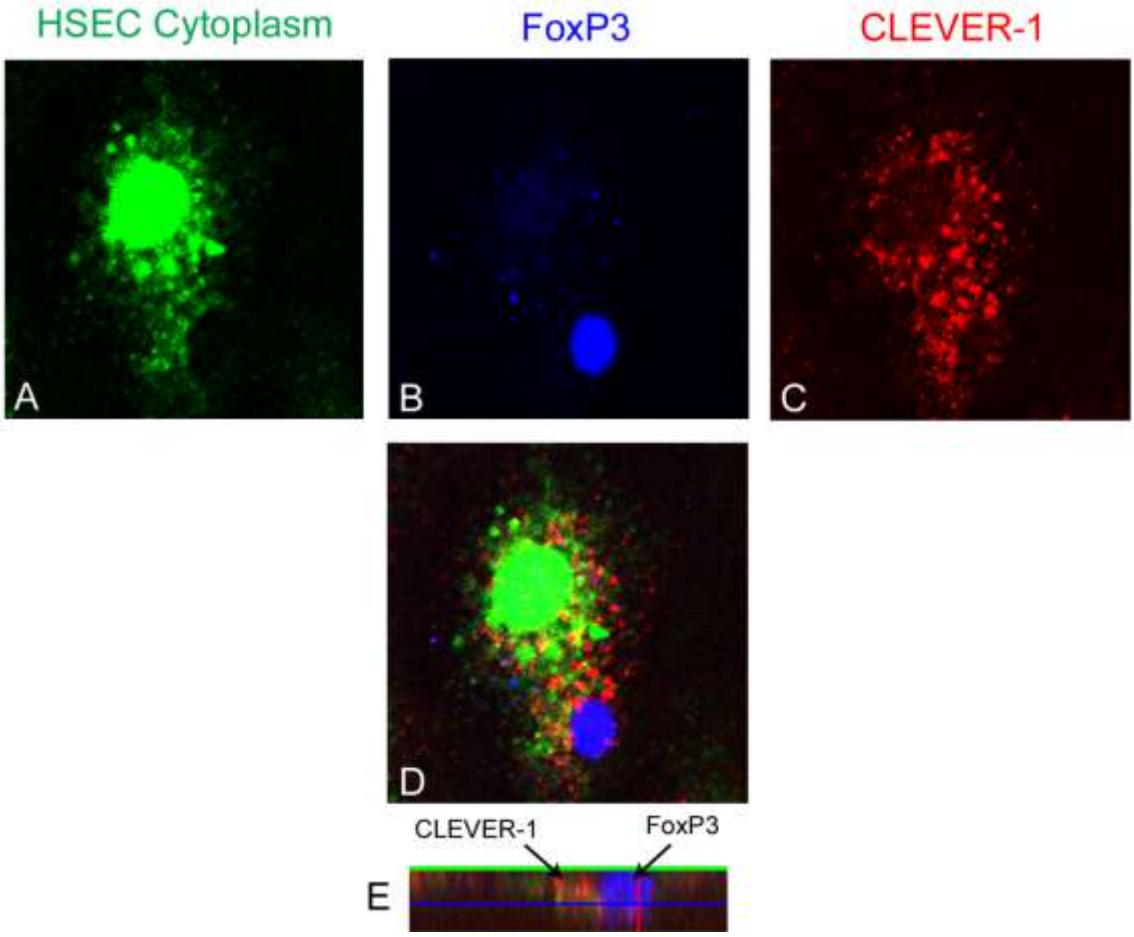

Figure 5-24 **CLEVER-1 colocalises with T regulatory cells during transmigration**

Representative confocal image of T reg transmigrating across HSEC monolayers after flow adhesion assay. T regs were perfused over a monolayer of HSEC at a shear stress of 0.05 Pa before fixation. HSEC were pre-labelled with cell tracker green (Panel A). Following fixation cells were stained for FoxP3 (Panel B) and CLEVER-1 (Panel C). Merged image is shown in Panel D. Z stack image is shown in Panel E. Fields captured with 63x objective.

5.6 Summary of Functional Assays

A combination of static and adhesion assays were used to study the role of CLEVER-1 in lymphocyte trafficking to the liver. Static adhesion assays confirmed that CLEVER-1 mediated the binding of lymphocytes to liver tissue. Flow adhesion assays were then used to assess the role of CLEVER-1 in lymphocyte recruitment across HSEC. Initial assays with peripheral blood lymphocytes demonstrated that CLEVER-1 blockade led to a significant reduction in the proportion of lymphocytes undergoing transendothelial migration.

Lymphocyte subsets were isolated from whole blood to assess if CLEVER-1 preferentially recruited a particular subset. Assays with CD4 and CD8 lymphocytes confirmed that CLEVER-1 mediated the transmigration of CD4 lymphocytes but had no effect on CD8 lymphocytes. More detailed analysis of CD4 subsets demonstrated a preferential activity for T regulatory cells.

The adhesion of B cells to HSEC was also studied under conditions of flow. B cells were captured from flow and underwent firm adhesionmediated by VCAM-1. Only a small proportion of B cells underwent transmigration but this process involved a combination of ICAM-1, VAP-1 and CLEVER-1.

The use of Ibidi chamberslides allowed me to use confocal microscopy to image lymphocytes adhering to HSEC and undergoing transmigration. CD4 lymphocytes were visualised transmigrating through HSEC via the transcellular route as well as the paracellular route. Transmigration took

place through transcellular pores which were surrounded by a ring of ICAM-1 and included VAP-1. Further studies showed that the Treg subpopulation of CD4 lymphocytes also followed the transcellular route during transmigration. Confocal imaging demonstrated that CLEVER-1 colocalised with T regs during their transmigration, confirming the role of this receptor during the extravasation step of T reg recruitment.

I initially used the modified Stamper Woodruff assay to study the role of CLEVER-1 in lymphocyte recruitment. It was important to start with this assay because it allowed me to study CLEVER-1 in the context of whole human liver tissue. The primary drawback of this assay is that it is a static assay and therefore only assesses the adhesive properties of CLEVER-1 but does not give an indication if CLEVER-1 plays a specific role in the adhesion cascade.

It is not possible to study the leucocyte adhesion cascade within the human liver using an *in vivo* technique. Intravital microscopy with animal models could be used but important differences between human and animal cells make human *in vitro* studies essential. The *in vitro* flow based adhesion assays used in this chapter have the advantage of incorporating primary human hepatic sinusoidal endothelial cells. I have already confirmed that these cells express CLEVER-1 in culture and we performed these assays under physiological shear stress relevant to the hepatic sinusoids. Our group has previously used this model to study adhesion molecules such as ICAM-1, VCAM-1 and VAP-1 (Lalor et al., 2002a). The steps of the adhesion cascade

which are demonstrated by these *in vitro* flow assays have been reproduced in hepatic *in vivo* assays using animals (Wong et al., 1997). Importantly, the major molecular mechanisms mediating these steps *in vitro* have also been confirmed *in vivo* (Bonder et al., 2005; Wong et al., 1997).

A further potential confounding factor is the use of lymphocytes that have been isolated from blood and cultured *in vitro* during which time their molecular expression may be altered in comparison to *in vivo* human peripheral blood lymphocytes. We minimised these changes by using freshly isolated lymphocytes without culturing them prior to adhesion assays. We studied highly pure subsets of lymphocytes to determine whether CLEVER-1 had preferential activity for particular subsets. I was careful to use magnetic bead kits and negative immunomagnetic selection to minimise affects of beads or antibody binding on the subsets of CD4, CD8 and B cells. This was not possible with the T regulatory cells as these isolation kits required positive selection using CD25, the benefit of this positive Treg isolation was that it allowed the comparison between a highly pure population of Foxp3 positive CD4 population against an effector population.

The flow assays demonstrated that CLEVER-1 contributed to the transmigration step of lymphocyte recruitment. Blockade of CLEVER-1 could not abolish total lymphocyte transmigration. This led us to investigate whether CLEVER-1 had a preferential effect on a particular subset of lymphocytes. We found CLEVER-1 mediated CD4 and B cell transendothelial migration with preferential activity for T regulatory cells.

Even when subsets were used CLEVER-1 blockade only partially inhibited transendothelial migration suggesting that these processes involve multiple receptors during the transmigration step. Previous studies support the idea that paracellular migration requires sequential molecular interactions (Lou et al., 2007; Schenkel et al., 2002) Our findings suggest that transmigration through HSEC also requires multiple interactions and in the case of B cell and Treg migration these involve a combination of ICAM-1, VAP-1 and CLEVER-1. Blocking all three of these receptors abolished most but not all of the transmigration. Our i*n vitro* findings will have to be confirmed by i*n vivo* experiments. There is *in vivo* evidence that CLEVER- 1mediates lymphocyte recruitment to the lymphatic system but a subset specific activity has yet to be shown (Karikoski et al., 2009a).

Once we had confirmed the role of CLEVER-1 in transmigration we attempted to study the mechanism by which CLEVER-1 mediated this process. By prelabelling our cells with fluorescent dyes prior to flow assays we were able to visualise the transmigration step using a confocal microscope. To our surprise we visualised lymphocytes transmigrating through transcellular pores within endothelial cells without disruption of endothelial junctions. This phenomenon was visualised in live cells and therefore it was not an artefact of fixation. Furthermore, we were able to show that ICAM-1 is enriched around these transcellular pores. This is important since ICAM-1 is a major mediator of lymphocyte transmigration and has been shown in transcellular pores in other types of endothelial cell (Millan et al., 2006).

This technique also allowed us to demonstrate that T regulatory cells could transmigrate via the transcellular route and there was an enrichment of CLEVER-1 around these cells by cross sectional imaging using the z-stack function. Similar techniques have been used previously to demonstrate the role of ICAM-1, CD31 and CD99 in leucocyte transmigration in other systems (Carman & Springer, 2004a; Lou et al., 2007). To our knowledge this is the first demonstration that transcellular migration is implicated in the liver and that Tregulatory cells undergo transcellular migration.

CHAPTER 6

DISCUSSION

6.1 CLEVER-1 expression in the human liver

Endothelial cells line all blood vessels and lymphatics in the human body but the structure and function of these endothelial cells can differ markedly between vascular beds (Aird, 2007a). The endothelial cells of the hepatic sinusoid highlight this heterogeneity by displaying a unique morphology and phenotype (Aird, 2007b). These characteristics could potentially be used to identify liver-specific therapeutic targets.

The aim of this work was to study the scavenger receptor CLEVER-1, a receptor which is restricted to sinusoidal vascular beds (Goerdt et al., 1991), and elucidate if it plays a role in lymphocyte trafficking and thereby identify a potential organ specific target for lymphocyte recruitment.

There are several published studies describing the expression of the CLEVER-1 receptor in normal liver tissue and hepatic sinusoidal endothelial cells. To our knowledge these studies have not gone on to study CLEVER-1 expression in the context of either inflammatory liver diseases or malignant liver disease. Using surgical samples from normal and diseased livers it was possible to compare CLEVER-1 expression in normal and disease states. Chronic inflammatory liver diseases can be broadly divided into parenchymal or biliary liver diseases depending on the site of lymphocyte infiltration and both these groups were studied. In the normal human liver CLEVER-1 was restricted to the sinusoidal vascular bed. On the background of chronic inflammation CLEVER-1 expression changed markedly. The uniform expression of CLEVER-1 was lost with a more patchy expression across the

sinusoids. The intensity of CLEVER-1 staining at these sites was much stronger in comparison to normal liver tissue. Sections of diseased liver also demonstrated the presence of CLEVER-1 in other vascular locations such as neovessels of the fibrous septum and vessels supplying portal lymphoid tissue which are characterised by a 'continuous' vascular phenotype. There were no disease specific differences in CLEVER-1 expression. As discussed in section 1.6 most liver diseases follow a common pathway. The expression of CLEVER-1 is probably driven by tissue injury and chronic inflammation irrespective of aetiology, making it a possible therapeutic target for a wide range of liver diseases.

It was clear that CLEVER-1 expression on vascular endothelial cells and sinusoidal endothelial cells is differentially regulated. CLEVER-1 is constitutively expressed on HSEC *in vivo*. Vessels of 'continuous' vascular origin that develop in response to chronic injury and inflammation are also CLEVER-1 positive. Interestingly, these locations are all potential sites of lymphocyte recruitment or retention.

The presence of CLEVER-1 was also noted in primary liver tumours (hepatocellular carcinomas), within tumour sinusoids and tumour associated vessels both potential sites of lymphocyte trafficking within tumours. Studies by our group and others have demonstrated that lymphocyte infiltration is an important component of tumour development (Fu et al., 2007; Yoong et al., 1999). These findings suggest that the tumour microenvironment influences CLEVER-1 expression at sites of lymphocyte recruitment. CLEVER-1 may

therefore have an important role in tumour development as well as chronic inflammation.

In view of these findings it was important define the factors which were driving CLEVER-1 expression. Chronic inflammation is a common theme in the liver diseases studied suggesting that pro-inflammatory cytokines could be inducers of CLEVER-1 expression. Hepatocellular carcinomas develop on the background of liver cirrhosis and therefore cytokines may influence CLEVER-1 expression within these tumours. Hepatocellular carcinomas are also highly vascularised tumours (Bruix et al., 2001), a consequence of the shift in the secretion of antiangiogenic factors to pro-angiogenic factors (Semela and Dufour, 2004). This shift is another possible factor that drives the expression of CLEVER-1 in tumour sinusoids and tumour vessels. Angiogenesis has also been described as a response to chronic inflammatory liver disease. Thus either a proinflammatory or a proangiogeneic environment could explain the increased expression of CLEVER-1 in tumour vessels and neo-vessels of the fibrous septum and vessels supplying tertiary lymphoid follicles (Medina et al., 2004). Previous studies with FEEL-1/CLEVER-1 have suggested a link with angiogenesis, where blockade of the receptor reduced angiogenesis (Adachi & Tsujimoto, 2002).

6.2 The regulation of CLEVER-1 in HSEC

Using a method developed by our group to isolate hepatic sinusoidal endothelial cells I demonstrated that these cells maintained their expression

of CLEVER-1 *in vitro*. This allowed me to study the changes in CLEVER-1 expression in response to various stimuli. Conventional endothelial adhesion molecules such as ICAM-1 and VCAM-1 are upregulated by proinflammatory cytokines but I was unable to consistently increase the expression of CLEVER-1 with proinflammatory cytokines, suggesting that its regulation was more complex. The response of CLEVER-1 expression to factors such as hypoxia or bile acids was extremely variable and the only consistent effect I observed was detected with hepatocyte growth factor which led to increased CLEVER-1 expression.

Hepatocyte growth factor (HGF) could influence the expression of CLEVER-1 in normal and diseased liver tissue. It is a cytokine that is described as a motogen, mitogen and morphogen and which has been implicated in liver development, growth, regeneration chronic inflammation and carcinogenesis (Jiang et al., 2005). It was initially identified as a potent mitogen of rat hepatocytes (Michalopoulos et al., 1984). HGF was also separately identified and named scatter factor because of its ability to promote epithelial cell dissociation and increase cell motility (Weidner et al., 1991). In the liver its main source is stellate cells (Hu et al., 1993). The induction of CLEVER-1 expression may relate to its ability to promote angiogenesis *in vitro* and *in vivo* (Bussolino et al., 1992; Grant et al., 1993) and lymphangiogenesis (Martin and Jiang, 2010) Abnormalities in HGF signalling have also been implicated in carcinogenesis including the development of HCCs, although the exact mechanism is not clear (Comoglio et al., 2008; Villanueva et al., 2007). HGF may therefore contribute to the maintenance of CLEVER-1

expression on sinusoidal endothelium and its induction on neovessels and vessels supplying lymphoid follicles in chronically inflamed tissue as well as tumour associated vessels in HCCs.

Having investigated levels of CLEVER-1 protein and mRNA I went on to study its intracellular and surface localisation in HSEC as CLEVER-1 is known to undergo intracellular trafficking in other cell types. CLEVER-1 was predominantly intracellular, this finding was consistent with previous studies in lymphatic endothelium and alternatively activated macrophages (Kzhyshkowska et al., 2004; Prevo et al., 2004). Stimulation with growth factors and cytokines had no effect on the cellular localisation of CLEVER-1. The trafficking properties were confirmed by treatment with Bafilomycin A1, this led to accumulation of CLEVER-1 in large vesicles. These vesicles were not early endosomes but were found to colocalise with the trans-Golgi network and Golgi apparatus. This supports previous findings that CLEVER-1 is involved in two pathways 1) trafficking from the plasma membrane to early endosomes and 2) intracellular sorting pathway involving trafficking between the endosomal compartment and the biosynthetic compartment (trans Golgi network)(Kzhyshkowska et al., 2004).

It is clear from these regulation studies that CLEVER-1 does not behave as a conventional adhesion molecule. Its mRNA expression and surface expression are unaltered by inflammatory cytokines and it does not localise to inter-cellular junctions. Other features are consistent with a scavenger receptor profile: primarily its trafficking behaviour from the plasma membrane

to intracellular compartments. The regulation of CLEVER-1 is probably a complex process *in vivo* with hepatocyte growth factor contributing to an increase of CLEVER-1 expression in sinusoidal endothelial cells and an induction in vascular endothelial cells that develop in response to chronic inflammation.

6.3 The role of CLEVER-1 in lymphocyte trafficking to the Liver

I used modified Stamper-Woodruff assays to confirm that CLEVER-1 on hepatic sinusoidal endothelium in *ex vivo* tissues supports lymphocyte adhesion. This assay allows the study of CLEVER-1 in the context of whole liver tissue but does not clarify the exact role of CLEVER-1 within the adhesion cascade.

This was studied using flow based adhesion assays which recapitulate the hepatic sinusoids, the channels where lymphocyte recruitment takes place *in vivo*. There is evidence that the hepatic sinusoids are a unique environment for lymphocyte recruitment: 1) Recruitment occurs in a low shear environment, 2) The endothelial cells that line the hepatic sinusoids have a unique phenotype, 3) Conventional adhesion molecules, such as selectins are absent from this vascular bed, 4) Unconventional adhesion molecules such as VAP-1 play a role in the recruitment of lymphocytes in this vascular bed.

Lymphocyte recruitment involves a multistep adhesion process which is mediated by several receptors. Flow based adhesion assays have been used to elucidate the exact role of several receptors in the adhesion cascade. Our group has previously used flow based adhesion assays to study the role of conventional adhesion molecules (ICAM-1 and VCAM-1) and unconventional adhesion molecules (VAP-1) in lymphocyte recruitment to HSEC.

By pre-blocking HSEC monolayers with an antibody to CLEVER-1 it was clear that CLEVER-1 played a role in the transmigration of peripheral blood lymphocytes. These findings were similar to those detected in lymphatic endothelium (Salmi et al., 2004). There was also an increase in the rolling of lymphocytes across the endothelial layer, but the overall proportion of lymphocytes rolling in our model is small (<10%) which reflects the low shear environment. Blockade of CLEVER-1 had no effect on firm adhesion or activation of lymphocytes.

The transmigration step of leucocyte extravasation is a complex process and appears to involve several receptors depending on both the leucocyte and endothelial type involved (Vestweber, 2007). Neutrophils, monocytes and lymphocytes use different receptors and appear to follow different routes during this process. We hypothesised that lymphocyte subsets may also use different receptors during extravasation across endothelium. . Two of the major populations of lymphocytes, CD4 and CD8 lymphocytes, were studied. Flow assays with these two subsets demonstrated that CLEVER-1 played a

specific role in CD4 lymphocyte transmigration across HSEC rather than CD8 lymphocytes. Further analysis of CD4 subsets demonstrated that CLEVER-1 promoted the transmigration of FoxP3 positive T regulatory cells.

Another subset which is recruited to the liver and contributes to liver immunopathology are B cells. B cells have been shown to be present within normal and diseased liver tissue and must therefore migrate from the peripheral circulation across the endothelial barrier. Despite this, there is little understanding of B cell recruitment to tissue and so I proceeded to study their behaviour in flow assays. There were clear differences in B cell recruitment compared with T cells. B cells were captured from flow and underwent firm adhesion similar to other subsets but there was no prior tethering step. VCAM-1 was the primary capture receptor for B cells but only small proportion underwent transmigration. It was clear from the flow assays that B cells did not have the same level of motility seen in T cells. This lack of motility probably explains the reduced efficiency in transmigration. As in other leucocytes, B cell transmigration was a multistep process with ICAM-1, VAP-1 and CLEVER-1 all contributing to diapedesis.

These functional assays suggest that CLEVER-1 is a recruitment signal for T regulatory cells and B cells by promoting the transmigration of these subsets. It was important to try and understand the mechanism by which CLEVER-1 promoted transmigration. As discussed in section 1.9.3 there have been two pathways described for transmigration: between endothelial junctions (paracellular); and directly through endothelial cells (transcellular). The

pathway chosen is dependent on the type of endothelium, as well as the leucocyte subset. (Carman et al., 2007; Vestweber, 2007). To our knowledge, the route taken by transmigrating lymphocytes has not been studied in the context of HSEC. Using confocal imaging it was clear that a large proportion of CD4 lymphocytes transmigrated across HSEC using the transcellular route. This supports the finding that CLEVER-1 mediates transmigration across HSEC. Receptors that support paracellular migration are found on the endothelial cell surface and are enriched at intercellular junctions. Transcellular migration is less well understood but probably requires a combination of receptors that are surface expressed (ICAM-1) and intracellular receptors (CLEVER-1). I confirmed this by demonstrating ICAM-1 enriched around transcellular pores as well as CLEVER-1 enriching close to transmigrating T regulatory cells.

These studies identify CLEVER-1 as a potential target for therapy of liver disease. CLEVER-1 is a non-conventional adhesion molecule which in normal conditions is restricted to sinusoidal endothelium. In inflammatory liver diseases it is also induced on inflammatory neo vessels and tumour vasculature. This restricted expression profile makes CLEVER-1 an attractive target for therapy in comparison to conventional adhesion molecules which are more widely expressed on vascular endothelium.

The preferential recruitment of Tregulatory cells by CLEVER-1 is a possible tissue-based response to chronic inflammation, to prevent excessive tissue damage. Induction of CLEVER-1 expression may therefore be beneficial in

controlling immune-mediated liver diseases (eg autoimmune liver disease, primary biliary cirrhosis) to enhance T regulatory cell recruitment. This may suppress inflammation and inhibit self reactive T cells. Alternatively, CLEVER-1 blockade may be beneficial in other liver diseases such as chronic viral hepatitis. In these conditions Tregulatory cells may prevent virus-specific immune responses and blocking their recruitment may enhance viral eradication. Similarly, blocking CLEVER-1 in the context of hepatocellular carcinomas may prevent T reg infiltration and thereby promote tumour specific cytotoxicity.

CLEVER-1 also appears to be a recruitment signal for B cells. There is gathering interest in the contribution of B cells to liver disease. They have been implicated in the development of liver fibrosis and the transmission of viruses such as Hepatitis C. Therefore CLEVER-1 blockade may influence the development of fibrosis in chronic hepatitis as well as viral infection of the liver.

The ligand on Tregulatory cells and B cells which binds to CLEVER-1 is unknown. As discussed in 1.13.1 CLEVER-1 has been shown to bind to phosphatidylserine (PS). PS is a molecule which is expressed on the cytosolic side of cell membranes and becomes exposed on the surface of the cell during apoptosis. During early apoptosis the exposure of PS on the surface allows phagocytosis by macrophages (Verhoven et al., 1995). CLEVER-1 has been shown to recognise phosphatidylserine via its epidermal growth factor repeats (Park et al., 2009). Interestingly, there are

studies which show that PS is not only exposed on the cell surface during apoptosis. It has been detected on the surface of viable B cells as well as a subset of CD4+ memory T cells (Dillon et al., 2000; Elliott et al., 2005). In view of these findings PS is a candidate for the CLEVER-1 ligand on lymphocytes.

In conclusion, these studies have identified CLEVER-1 as a new lymphocyte adhesion molecule within the hepatic sinusoids. It does not play a role in the capture or activation of lymphocytes but mediates the downstream step of lymphocyte diapedesis via the transcellular route. Moreover, CLEVER-1 appears to be a subset specific adhesion molecule which mediates the recruitment of regulatory T cells and B cells.

CHAPTER 7

FUTURE WORK

Within the human liver, CLEVER-1 is expressed constitutively by sinusoidal endothelium but only on vascular endothelium that develops in response to inflammation and malignancy. Further work is required to understand why CLEVER-1 is differentially regulated on these two types of endothelium. CLEVER-1 expression may be influenced by other hepatic resident cells. Therefore co-culture experiments with stellate cells, kupffer cells and hepatocytes would be an important step to gain further understanding of CLEVER-1 regulation.

In functional assays CLEVER-1 was found to promote CD4 lymphocytes and B cell transmigration. These studies concentrated on lymphocyte populations but CLEVER-1 may mediate the recruitment of other leucocyte populations. Future flow assays with neutrophils and monocytes would answer these questions, as well as studies with NK and NKT cells.

CLEVER-1 appears to play a role in transcellular migration across HSEC. I and others (Karikoski et al., 2009a) have hypothesised that phosphatidylserine could be a possible ligand on transmigrating lymphocytes but this requires confirmation

To my knowledge this is the first demonstration of transcellular migration across HSEC. Further work is required to understand this process and the contribution of ultrastructural components of HSEC such as fenestrae, actin cytoskeleton and microtubules. Other scavenger receptors, the mannose receptor and stabilin 2, may also contribute to this phenomenon. .

As discussed earlier, *in vivo* studies have demonstrated a role for CLEVER-1 in lymphocyte recruitment via lymphatics. Animal models would be an important step in further understanding the importance of CLEVER-1 in liver injury. Function blocking antibodies or CLEVER-1 knockout models could be used to investigate the role of CLEVER-1 in the development of fibrosis and cirrhosis.

These studies have concentrated on the role of CLEVER-1 in lymphocyte recruitment. There is evidence that tumour cells metastasise using mechanisms similar to lymphocyte trafficking. Furthermore, scavenger receptors such as CLEVER-1 and mannose receptor have been implicated in tumour cell adhesion to endothelium (Irjala et al., 2003a). *In vitro* flow assays with tumour cells may elucidate the mechanism by which tumours, such as colonic carcinomas, metastasise to the liver and may implicate CLEVER-1 in this process.

The findings of CLEVER-1 on neo vessels, up regulation by HGF and studies by other groups suggest that CLEVER-1 may also have a role in angiogenesis. Other transmigration molecules such as VAP-1 and JAM-C have also been implicated in angiogenesis (Marttila-Ichihara et al., 2009; Rabquer et al., 2010). Studying the angiogenic properties of CLEVER-1 in HSEC may uncover a further contribution of CLEVER-1 to the pathogenesis of chronic liver disease and hepatocellular carcinomas.

This thesis has described the presence of the CLEVER-1 receptor at sites of lymphocyte homing to the inflamed human liver and demonstrated a role for

this receptor in transcellular migration of lymphocytes. Further *in vivo* and *in vitro* studies are required to see if CLEVER-1 is a potential therapeutic target for inflammatory liver diseases

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