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CLEVER-1 Mediates the Transmigration of T Regulatory Cells Across Human Hepatic Sinusoidal Endothelium

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Abstract

The common lymphatic endothelial and vascular endothelial receptor (CLEVER-1; also known as FEEL-1 and stabilin-1) is a recycling and intracellular trafficking receptor with multifunctional properties. Here we demonstrate for the first time increased endothelial expression of CLEVER-1/stabilin-1 at sites of leucocyte recruitment to the inflamed human liver including sinusoids, septal vessels and lymphoid follicles in inflammatory liver disease and tumour-associated vessels in hepatocellular carcinoma. We used primary cultures of human sinusoidal endothelial cells (HSEC) to demonstrate that CLEVER-1/stabilin-1 expression is enhanced by hepatocyte growth factor but not by classical proinflammatory cytokines. We then showed that CLEVER-1/stabilin-1 supports T cell transendothelial migration across HSEC under conditions of flow with strong preferential activity for CD4 FoxP3+ regulatory T cells. CLEVER-1/stabilin-1 inhibition reduced Treg transendothelial migration by 40% and when combined with blockade of ICAM-1 and vascular adhesion protein-1 (VAP-1) reduced it by more than 80%. Confocal microscopy demonstrated that 60% of transmigrating Tregs underwent transcellular migration through HSEC via ICAM-1 and VAP-1 rich transcellular pores in close association with CLEVER-1/stabilin-1. Thus CLEVER-1/stabilin-1 and VAP-1 may provide an organ-specific signal for Treg recruitment to the inflamed liver and to hepatocellular carcinoma.

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Abbreviations

CLEVER-1- Common Lymphatic Endothelial and Vascular Endothelial Receptor-1
VAP-1- Vascular Adhesion Receptor-1
HEV- High Endothelial Venule
HCC- Hepatocellular Carcinoma
HGF- Hepatocyte Growth Factor

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Introduction

Inflammatory liver diseases are characterised by a lymphocyte-predominant infiltrate which drives the development of fibrosis and cirrhosis. Most lymphocytes are recruited to the liver via the hepatic sinusoids with subsequent redistribution to the hepatic parenchyma in lobular hepatitis or to the portal tracts in portal and interface hepatitis (1). The hepatic sinusoids are a low-flow microvascular bed lined by morphologically and functionally unique sinusoidal endothelial cells to which lymphocytes must bind and then cross to enter the parenchyma. Lymphocyte-endothelial interactions within the hepatic sinusoids follow a multi-step adhesion cascade but there are important differences when compared with lymphocyte extravasation at other sites (2). The low shear environment requires only a brief, selectin-independent, tethering rather than a rolling step prior to firm adhesion and extravasation (3–5) and the lack of classical tight junctions results in non-classical molecules being involved in diapedesis. One such molecule is vascular adhesion protein-1 (VAP-1) which contributes to sialic acid dependent tethering and transendothelial migration across hepatic sinusoids (6–9). This led us to look for other endothelial adhesion receptors that might contribute to lymphocyte recruitment via the sinusoids.

The common lymphatic endothelial and vascular endothelial receptor (CLEVER-1) is expressed on lymphatic vessels and high endothelial venules (HEV) (10) where it supports lymphocyte adhesion and transmigration (11). It consists of 7 fasciclin domains, a proteoglycan-link protein-like sequence, 22 epidermal growth factor-like repeats, and 2 RGD motifs. Other groups identified this molecule as stabilin-1 and FEEL-1(12;13) and have shown it to be expressed on non-continuous endothelium and alternatively activated macrophages (12). In macrophages it acts as a scavenger receptor for acetylated low density lipoprotein (acLDL) and SPARC (secreted protein acidic and rich in cysteine), and as an intracellular cargo carrier for stabilin-1 chitinase like protein (SI-CLP) (14). It can bind bacteria and has angiogenic properties *in vitro* (13). Studies on rat liver sinusoidal endothelial cells failed to demonstrate a clear scavenging profile for stabilin-1 (15). Its close homologue stabilin-2 was recently shown to support lymphocyte adhesion to hepatic endothelium via interactions with αMβ2 integrin (16).

Here we demonstrate the presence of CLEVER-1/stabilin-1 on endothelium within the sinusoids, neovessels in fibrous septa and portal associated lymphoid tissue (PALT) in patients with chronic inflammatory liver disease and on tumour vessels in hepatocellular carcinoma. We show *in vitro* CLEVER-1/stabilin-1 preferentially promotes the recruitment of CD4 FoxP3+ regulatory T cells through HSEC under conditions of shear stress. Detailed analysis revealed that T regulatory cells migrated through transcellular pores in HSEC in close association with CLEVER-1/stabilin-1. This is the first demonstration of an adhesion molecule that preferentially recruits Treg to tumours and sites of inflammation.

Materials and Methods

Isolation and culture of human hepatic sinusoidal endothelial cells (HSEC)

Liver endothelial cells were isolated from approximately 30g of human liver tissue obtained from explanted livers or donor tissue surplus to surgical requirements using a collagenase

digestion (0.2% collagenase type Ia Sigma Aldrich, St Louis, MO) as described previously (6). All tissue was collected with informed consent and under the local ethics committee approval from patients in the Liver Unit at Queen Elizabeth Hospital in Birmingham. Briefly, the digested tissue was placed over a 33/77% Percoll™ (Amersham Biosciences, GE Healthcare, Little Chalfont, UK) density gradient. The non-parenchymal cell band was then removed and the endothelial cells were isolated by immunomagnetic selection using Abs against CD31 conjugated to Dynabeads (Invitrogen, Paisley, UK). The endothelial cells were then cultured in medium composed of human endothelial basal growth medium (Invitrogen), 10% AB human serum (HD supplies, Glasgow UK), 10ng/ml of vascular endothelial growth factor (VEGF) and 10ng/ml hepatocyte growth factor (HGF) (PeproTech, Peterborough, UK). The cells were grown in collagen-coated culture flasks and were maintained at 37°C in a humidified incubator with 5% CO_2 until confluent. This protocol was developed to isolate sufficient cells from either normal or diseased human liver for use in functional assays. In rats it has been suggested that CD31 should not be used to isolate sinusoidal cells because cell-surface CD31 is absent from quiescent sinusoidal endothelium and its use generates cells with low frequencies of fenestrae (17). However we find that human sinusoidal endothelial cells express cell surface CD31, albeit at lower levels than vascular endothelium, a finding consistent with other published reports (18). To confirm that CD31-selected cells from human liver have a sinusoidal phenotype we demonstrated expression of several receptors that are present on sinusoidal but not vascular endothelium including the hyaluronan receptor LYVE-1(19), the C-type lectins L-SIGN(20), L-SECtin, and the mannose receptor (21–23). These cells thus have a unique sinusoidal phenotype.

Antibodies and Immunostaining

Monoclonal antibody against CLEVER-1/stabilin-1 (3-372) has been described (10) and was used at 10μg/ml. Sinusoidal endothelial cells were identified using Abs against L-SIGN 5μg/ml (R&D Systems, Minneapolis, MN), kupffer cells with CD68 5μg/ml (BD Biosciences, Oxford, UK). Neovessels were identified with biotinylated Ab against CD34 2μg/ml (AbD Serotec, Kidlington, UK). Trans-Golgi network were identified by Abs against TGN-46 2μg/ml (Abcam, Cambridge, UK). Mouse IgG1 (DAKO, Ely, UK), Ig G2a, IgG2b (R&D sytems), biotinylated IgG1 (AbD Serotec), Polyclonal Rabbit IgG (DAKO) were used as controls. Fluorescein isothiocyanate (FITC)-conjugated goat anti mouse IgG1, IgG2a, IgG2b and FITC-conjugated Goat anti Rabbit IgG were from Southern Biotech, Cambridge UK, Alexa Fluor 546 conjugated goat anti mouse IgG, Alexa and Streptavidin 488 conjugated goat anti mouse IgG1 were from Invitrogen.

Sections were taken from normal liver tissue as well from a variety of chronic inflammatory liver diseases and sections from hepatocellular carcinomas (HCC), with at least three different cases studied from each disease. For standard immunohistochemistry representative 5μm cryosections were acetone-fixed and an endogenous peroxidase block was performed with 0.3% hydrogen peroxide in methanol prior to staining. Sections were incubated with primary anti-human monoclonal antibody at optimal concentration in TRIS buffered saline/ 0.1% Tween (TBS/Tween) for 60 minutes at room temperature. Control sections were incubated with relevant isotype-matched control. Sections were then incubated for 30 minutes with ImmPRESS ™ Universal anti-mouse/rabbit IgG reagent (Vector Labs,

Burlingame CA) at room temperature and washed with TBS/0.1%Tween prior to colour development using the Vector VIP substrate kit (Vector Labs). Finally, sections were washed in water, counterstained with haematoxylin, and mounted. The staining on the sections was scored semiquantitatively using a validated scoring system on the following structures: sinusoids, portal vessels, central veins and vessels in the fibrous septum for intensity of CLEVER-1 staining (7).

For immunofluorescent staining, acetone fixed cryosections were initially incubated with 10% normal goat serum in TBS buffer for 30 minutes before the addition of primary mouse anti-human monoclonal antibodies in TBS for 60 minutes at room temperature in a humidified container. After washing in TBS/Tween the sections were incubated with the relevant fluorescent conjugated goat anti-mouse secondary for 30minutes in TBS. Sections were washed in TBS/Tween and mounted with fluorescence mounting medium (DAKO). Sections were imaged by an Axioskop 40 microscope (Carl Zeiss, Welwyn Garden City, UK) with an Axiocam MRc5 camera (Carl Zeiss) and analysed using Axiovision software (Carl Zeiss).

For staining HSEC, cells were seeded at confluence on rat tail collagen coated coverslips and incubated overnight. The cells were fixed with 4%paraformaldehyde solution in phosphate buffered saline (PBS) and permeabilized with 0.3% Triton. Following incubation with 10% normal goat serum in TBS buffer for 30 minutes cells were incubated with primary monoclonal antibody or isotype-matched control for 1 hour at room temperature. Cells were then incubated with fluorescent secondary antibodies for half an hour before being mounted with fluorescence mounting medium (DAKO). Cells were imaged by confocal microscopy on an LSM 510 microscope equipped with 63x1.32 objective. Images were acquired and analysed by LSM software.

Isolation of RNA

RNA was isolated from human HSEC by using the RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. RNA was reverse transcribed using Superscript II RNase reverse transcriptase (Invitrogen) and random hexamers, and then stored at -20°C.

Conventional Polymerase Chain Reaction

Conventional polymerase chain reaction (PCR) was performed using primers designed from the Genbank sequences for GAPDH and CLEVER-1/stabilin-1 (GAPDH forward, 5' –GCC AAG GTC ATC CAT GAC AAC TTT GG and reverse, 5'-GCC TGC TTC ACC ACC TTC TTG ATG TC-3'-: CLEVER-1 forward, 5'-ACT CTG TCC TGG ACA GCG-3' and reverse, 5'-CAG CCG CTC ATG GAC ACC-3'). The CLEVER-1/stabilin-1 primers amplified a 289 bp product, the reaction conditions were 3 minutes denaturation (95°C) and then 35 cycles of 1 minute (95°C), 1 minute annealing (50°C), and 2 minutes (72 °C). Final extension was at 72 °C for 4 minutes. The GAPDH primers amplified a 259 bp product, 35 cycles were run at 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.

Real Time PCR

CLEVER-1/stabilin-1 messenger RNA (mRNA) levels were assessed by real-time PCR using Taqman Gene Expression Assays Stabilin-1, Hs01109068_m1 (Applied Biosystems, Carlsbad, CA) and GAPDH control. The reactions were carried out in MicroAmp Optical 96 well plates using Taqman Universal PCR mastermix and the plates were run in a 7900 Real-time PCR sequence detector system (Applied Biosystems). The reaction mixtures were subjected to the following amplification 50°C for 2minutes, 95°C for ten minutes and then 40 cycles of 95°C for 15 seconds and 60°C for one minute. CLEVER-1/stabilin-1 gene expression was analysed from cytokine/growth factor stimulated and unstimulated cultured HSEC.

Measurement of CLEVER-1/stabilin-1 expression on HSEC by ELISA

Endothelial cells were grown to confluence in collagen-coated 96-well flat bottom plates and fixed with methanol before performing ELISA. Cells were left under basal conditions or stimulated with cytokines (10ng/ml recombinant human TNF-α, 10ng/ml recombinant human interferon-γ, 10ng/ml recombinant humanTGF-β, 10ng/ml recombinant human IL-4, 10 ng/ml recombinant human IL-10, all from PeproTech) or LPS (10 μg/ml,Sigma Aldrich) or stimulated with growth factors (10ng/ml recombinant VEGF and 10ng/ml recombinant HGF both from PeproTech). All stimulations were for 24 hours. Cells were pre-incubated with 2% goat serum (Sigma Aldrich) for 1 hour. This was followed by incubation with mouse anti human primary antibody mAb (3-372, 10μg/ml) and control antibody (IgG1, 10 μg/ml, DAKO) for 45 minutes at room temperature. The cells were then washed and incubated with a peroxidase-conjugated goat anti-mouse secondary Ab (P0447, 1/500, DAKO) for 45 minutes at room temperature. The ELISA was developed using O-phenylenediamine substrate (DAKO) according to the manufacturer's instructions. CLEVER-1/stabilin-1 staining was expressed as the mean absorbance from three replicate wells minus the absorbance of an isotype- matched control antibody.

Flow cytometric analysis of HSEC

HSEC (untreated or treated with a combination of TNF α (10ng/ml) and IFNγ (10ng/ml) for 24 hours) were resuspended in cold fluorescence-activated cell sorting media (PBS and 10% fetal calf serum) and labelled with primary monoclonal antibody against CLEVER-1 (3-372) or isotype matched control (IgG1 DAKO). Following a washing step, cells were labelled with a phycoerythrin conjugated goat anti-mouse IgG1 secondary (Southern Biotech) and analysed on a Dako Cyan Flow Cytometer using Summit 4.3 software (DakoCytomation, Ely, UK).

Bafilomycin treatment

Bafilomycin A1 was purchased from Sigma Aldrich. Bafilomycin was added directly to cell culture medium at 10nanomolar and cells incubated for 24 hours at 37°C. Following treatment the cells were analysed by immunofluorescence as described above.

Isolation of peripheral blood lymphocytes and subsets

Peripheral blood lymphocytes were isolated as previously described (24) by density gradient centrifugation over Lympholyte (VH Bio, Gateshead, UK) for 25 minutes at 800g. Harvested lymphocytes were washed and resuspended in RPMI 1640/10% fetal calf serum. CD4 and CD8 cells were isolated using a negative immunomagnetic selection kit (Invitrogen), and similarly, T regulatory cells were isolated by using an immunomagnetic selection kit for CD4+CD25+ T cells (Invitrogen). The CD4+CD25+ population demonstrated a 90% FoxP3 positivity. The CD4+CD25- fraction generated by these experiments were kept and used as an effector population in functional experiments. All kits were used according to manufacturers' instructions.

Adhesion assays

Static Adhesion Assays—Tissue sections were first incubated with antibody 3-372 (against CLEVER-1/stabilin-1) or control antibody. They were then overlaid with peripheral blood lymphocytes ($1x10^6$/ml, 100 μl) which were allowed to adhere in static conditions for 30 minutes at room temperature. The non-adherent cells were removed and the adherent cells were fixed in acetone. The number of lymphocytes present in a minimum of ten representative high power fields per section were counted.

Flow-Based Adhesion assay—To study the dynamic role of CLEVER-1/stabilin-1 in the adhesion cascade within the hepatic sinusoids, cytokine-stimulated HSEC (TNFα and IFNγ for 24 hours at 10ng/ml) were grown to confluence in capillary tubes and connected to the flow system previously described (6). We have used these assays previously to demonstrate the contribution of ICAM-1, VCAM-1, VAP-1 and CXCR3 in lymphocyte recruitment to HSEC from flow (6;25) Lymphocytes ($1x10^6$ cells/ml) were perfused through the microslide over the endothelial cells at a shear stress of 0.05 Pa. In some experiments the shear stress was increased to 0.1 Pa in order to increase the proportion of rolling cells. Phase contrast video recordings made during lymphocyte perfusion were analyzed offline to determine percentage of rolling cells, adherent and transmigrated cells (see Movie 1 and 2 in supplemental material). Cells appearing phase bright were above the endothelial monolayer whilst those that were phase dark had migrated through the monolayer. HSEC monolayers were incubated with blocking antibodies against CLEVER-1/stabilin-1 (3-372, 20μg/ml), ICAM-1 (R&D Systems, 10μg/ml), VAP-1 (TK8-14, 10μg/ml, Biotie, Turku, Finland) or isotype matched control (Ig G1 DAKO and Ig G2a R&D Systems).

Immunofluorescence staining and confocal microscopy to image lymphocyte transmigration

To study the transmigratory route taken by CD4 lymphocytes and FoxP3+ cells across HSEC we cultured HSEC in capillary tubes for flow adhesion as described above. HSEC were pre-labelled with CellTracker™ Green CMFDA (Invitrogen) as per manufacturer's instructions and followed by flow assays with CD4 lymphocytes as described above for ten minutes. Cells were then fixed in 4% paraformaldehyde and permeabilised with 0.3% Triton. Cells were stained with mAb for ICAM-1 (R&D systems, 10μg/ml) and VAP-1 (TK8-14, 10μg/ml, Biotie, Turku, Finland). Followed by Alexa Fluor 546 conjugated goat anti mouse

IgG1 or Alexa Fluor 488 conjugated goat anti mouse IgG1 and Alexa Fluor 546 conjugated goat anti mouse IgG2a (Invitrogen). To identify T regulatory lymphocytes, cells were stained with FoxP3 (eBioscience, Hatfield UK, 5μg/ml,) followed by Goat anti rat Alexafluor 633 with prior fixation/permeabilisation performed with FoxP3 staining kit (eBioscience), Rat IgG2a (eBioscience) was used as control. CLEVER-1/stabilin-1 was stained with 3-372 (10ug/ml) and Goat anti mouse Alexafluor 546 was used at the secondary stage. Cells were stained with a DAPI stain (Invitrogen) for nuclear staining. Slides were then examined using a LSM 510 microscope equipped with 63x1.32 objective. Confocal Images and z-stacks were acquired and analysed by LSM software. We counted the number of CD4 cells undergoing transcellular migration and the number using paracellular migration at junctions in randomly selected high power fields. The total numbers were then expressed as the proportion of cells which took either the transcellular route or the paracellular route.

For live cell imaging HSEC were again prelabelled with CellTracker™Green, (Invitrogen). CD4 lymphocytes were prelabelled with CellTracker™ Violet BMQC (Invitrogen) as per manufacturers guidelines. Flow assays were performed with the pre-labelled lymphocytes. Following this the cell boundaries were stained with CellMask™ Orange plasma membrane stain as per manufacturer's guidelines for 5 minutes. Slides were then immediately examined using a LSM 510 microscope equipped with 63x1.32 objective. Confocal Images and z-stacks were acquired and analysed by LSM software.

Statistical Analysis

Paired two-tailed *t* Tests were performed using the TTEST function in the Excel programme of Microsoft Office 2007. Variation between multiple treatments was evaluated using analysis of variance (ANOVA), followed by Dunnett test for comparison of control using GraphPad Prism 5 software. $P<0.05$ was considered as statistically significant.

Results

CLEVER-1/stabilin-1 is expressed on hepatic endothelium in sinusoids, vessels supplying portal associated lymphoid tissue and on tumour endothelium in hepatocellular carcinoma

Immunohistochemistry demonstrated CLEVER-1/stabilin-1 within the sinusoids of normal and chronically inflamed human liver (Fig 1A-D). In normal liver CLEVER-1/stabilin-1 was restricted to the sinusoids and absent from portal vessels. Staining of diseased tissues demonstrated increased intensity of sinusoidal expression compared with normal liver tissue and strong expression on endothelium in lymphoid follicles in portal areas (Fig 1 G and H and Table I). In hepatocellular carcinoma CLEVER-1/stabilin-1 was detected within tumour sinusoids and on tumour-associated vessels (Fig 1E and F). Isotype-matched controls were negative.

CLEVER-1/stabilin-1 is expressed on human sinusoidal endothelium *in vivo*

Sinusoidal endothelial cells and kupffer cells (liver-resident macrophages) are the major cell populations within hepatic sinusoids. Because it has been reported on macrophages it was important to ascertain which cell-type expressed CLEVER-1. We thus carried out dual immunofluoresence staining with anti-CLEVER-1/stabilin-1 and antibodies against a) L-

SIGN (a specific marker of liver endothelium) b) CD68 (a macrophage marker not present on endothelium) c) CD34 (absent from sinusoidal endothelium in the normal liver but expressed on vascular endothelium and neovessels at sites of inflammation). CLEVER-1/stabilin-1 was expressed on L-SIGN positive sinusoidal endothelial cells but not on CD68+ kupffer cells in the hepatic sinusoids (Fig 1 I-K) (Fig 1 L-N) and on CD34+ endothelium in neo-vessels at sites of chronic inflammation (Fig 1 O-Q). These findings were confirmed with confocal microscopy (Fig 1 R-T).

Isolated HSEC express CLEVER-1/stabilin-1

HSEC in culture expressed CLEVER-1/stabilin-1 as shown by immunofluorescence (Fig 2A). CLEVER-1/stabilin-1 distribution within HSEC showed clear differences after treatment with bafilomycin A1 being localized to the trans Golgi network (Fig 2B,C). RT-PCR confirmed the expression CLEVER-1/stabilin-1 of HSEC from normal and diseased livers and semiquantitative analysis demonstrated some variation in CLEVER-1 levels between isolates (Fig 2D and E).

We attempted to increase CLEVER-1/stabilin-1 expression on HSEC using TNF-α, IFN-γ, TGF-β, IL-4, IL-10 and LPS. None of these cytokines consistently increased CLEVER-1/stabilin-1 expression. However, we detected a consistent and significant increase in CLEVER-1 protein and mRNA in HSEC treated with hepatocyte growth factor (HGF) (Fig 2 F,G).

CLEVER-1 was detected on the surface of HSEC by FACS analysis but at low levels; we were able to increase the amount of detectable CLEVER-1 on the cells surface by treatment with IFNγ and TNFα (Fig 2H). The response was very variable depending on cell batch and passage number.

CLEVER-1/stabilin-1 mediates transmigration of peripheral blood lymphocytes across HSEC

Modified Stamper-Woodruff tissue-binding assays were used to show that antibody blockade of CLEVER-1/stabilin-1 significantly inhibited lymphocyte binding to hepatic vessels in frozen sections of diseased liver tissue (Figure 3A). We then studied lymphocyte binding to primary HSEC from both normal and diseased livers in flow-based adhesion assays under physiologically relevant flow rates. Lymphocyte adhesion to unstimulated HSEC *in vitro* is very low so HSEC were treated with TNFα and IFNγ overnight before carrying out the assays. Very few cells undergo rolling on HSEC but blocking CLEVER-1/stabilin-1 led to an increase in the proportion of lymphocytes rolling from 6% to 10% (Fig 3B). To determine whether CLEVER-1 is a true rolling receptor we increased level of shear stress to 1.0Pa, a level that requires more efficient capture from flow. At this level of shear stress CLEVER-1 blockade had no effect on the proportion of cells rolling compared to control suggesting it is not a classical rolling receptor (Fig 3B). The proportion of cells undergoing stable adhesion was unaffected by CLEVER-1 blockade (Fig 3C). We next quantified the proportion of adherent cells that underwent shape-change to a motile phenotype and those that subsequently underwent transendothelial migration under flow. CLEVER-1/stabilin-1 blockade did not affect the number of shaped-changed cells bound to the endothelium

suggesting that it does not mediate intravascular crawling, however the proportion of cells undergoing transendothelial migration was significantly reduced (Fig 3C,D).

CLEVER-1/stabilin-1 mediates the transmigration of CD4 lymphocytes, in particular T regulatory cells, but not CD8 lymphocytes across HSEC

We next studied the response of highly pure subpopulations of CD4 and CD8 T cells. CLEVER-1/stabilin-1 blockade had no effect on capture and stable adhesion from flow of purified CD4 or CD8 cells. However, the proportion of adherent CD4 lymphocytes undergoing transendothelial migration was significantly reduced whereas CD8 T cell migration was unchanged (Fig 4A,B). We further purified CD4 cells into CD4+CD25+FoxP3+ regulatory T cells and CD4+CD25- cells and found that CLEVER-1/stabilin-1 blockade significantly inhibited transendothelial migration of the former but not the latter subset (Fig 4C). CLEVER-1/stabilin-1 blockade reduced but did not abolish Treg transendothelial cell migration (TEM) and because we have previously reported a role for ICAM-1 and VAP-1 in lymphocyte transendothelial migration through HSEC we blocked these receptors on HSEC as well. Antibodies against ICAM-1 or VAP-1 had no effect on the adherence of Treg (figure 4D) but reduced TEM by approximately 50% and when combined with anti-CLEVER-1/stabilin-1 80% of TEM was inhibited (figure 4E).

CD4 lymphocytes transmigrate through transcellular pores in HSEC closely associated with CLEVER-1/stabilin-1

We used confocal microscopy to analyse CD4 lymphocyte transmigration across HSEC monolayers under flow. A significant proportion of cells used the transcellular route (Figure 5 A-C). We found that 62% of cells (SEM ±6%) underwent transcellular migration and 42% (SEM± 6%) underwent paracellular migration (Data from n=27 fields). Immunofluorescent staining demonstrated that transcellular channels were lined by ICAM-1 and by VAP-1(Figure 5 D-K). FoxP3+CD4 lymphocytes were clearly seen to migrate through channels in the HSEC cytoplasm (Figure 5 L-O) and closely associated with CLEVER-1/stabilin-1 (Fig 5 P-S). We confirmed transcellular migration of CD4 lymphocytes across HSEC with live cell imaging (Figure 6).

Discussion

To our knowledge this is the first report implicating CLEVER-1/stabilin-1 in the recruitment of regulatory T cells to chronically inflamed tissue. We demonstrate that CLEVER-1/stabilin-1 expression is maintained within the sinusoids of diseased human livers, whereas other scavenger type receptors such as LYVE-1 are lost during disease-associated sinusoidal capillarisation (26). CLEVER-1/stabilin-1 was restricted to endothelium within the hepatic sinusoids and also detected on neovessels associated with inflammation in fibrous septa and vessels supplying lymphoid follicles, all potential sites for lymphocyte recruitment in inflammatory liver disease (27–29) Taken together with our findings that CLEVER-1/stabilin-1 can support lymphocyte transendothelial migration these observations implicate CLEVER-1/stabilin-1 in lymphocyte recruitment to the inflamed liver. We also detected CLEVER-1/stabilin-1 on tumour-associated vessels and sinusoids within hepatocellular carcinomas (HCCs) suggesting that it could also play a role in promoting lymphocyte

recruitment to HCC which are infiltrated by lymphocytes, including regulatory T cells (30;31).

We show that HSEC maintain expression of CLEVER-1/stabilin-1 *in vitro*. The factors that regulate the surface expression of CLEVER-1 on alternatively activated macrophages *in vitro* include IL-4 and dexamethasone but little is known about the regulation of CLEVER-1/stabilin-1 in endothelial cells (12). In our studies pro-inflammatory cytokines, including IL-4, had no effect on overall levels of CLEVER-1 expression on HSEC whereas levels were increased by treatment with HGF. HGF is a critical factor in liver growth and regeneration which also promotes both angiogenesis and lymphangiogenesis (32–34). Thus the detection of CLEVER-1/stabilin-1 on neovessels and vessels supplying lymphoid follicles in chronically inflamed liver and on tumour-associated vessels might reflect local secretion of HGF as part of the tissue remodelling that accompanies chronic inflammation and cancer development rather than a response to a classical proinflammatory signal.

Previous studies have shown that CLEVER-1/stabilin-1 on the surface of endothelial cells undergoes rapid cycling between the cell surface and early endosomes (11;19;35). It has been hypothesised that this recycling could facilitate lymphocyte transmigration through lymphatic endothelium (36). We attempted to increase the surface expression of CLEVER-1/stabilin-1 by treating HSEC with bafilomycin A1 which blocks vacuolar-type H+ ATPases thereby altering endosomal pH. We demonstrated a redistribution of intracellular CLEVER-1/stabilin-1 in response to bafilomycin A1 to the trans-Golgi network (TGN) although treatment did not lead to increased cell surface expression (Fig 2A-C). This is consistent with previous studies in human macrophages demonstrating that a large proportion of CLEVER-1/stabilin-1 can be relocalized from endosomal vesicles to TGN (37). We did however find increased detectable cell surface CLEVER-1 in cells treated with TNFα and IFNγ (Fig 2 H) although the response was variable probably as a consequence of dynamic trafficking of the receptor.

CLEVER-1 blockade led to an increase in the proportion of cells undergoing rolling at low shear stress but this was not replicated at high shear stress suggesting that CLEVER-1 does not behave as a classical rolling receptor. We demonstrated that CLEVER-1/stabilin-1 mediates lymphocyte transmigration across hepatic sinusoidal endothelium at levels of shear stress that replicate those found within the hepatic sinusoids *in vivo*. Furthermore, CLEVER-1/stabilin-1 displayed preferential activity for CD4 T cells and particularly CD25+FoxP3+ Tregs. We saw no effect of CLEVER-1 on shape change of adherent lymphocytes under flow suggesting that it promotes diapedesis rather than having a global effect on motility. This is to our knowledge the first report of an adhesion molecule, as opposed to a chemokine, demonstrating preferential activity for Tregs. Thus induction of CLEVER-1/stabilin-1 at sites of chronic inflammation may aid tissue repair and resolution of inflammation by promoting the recruitment of anti-inflammatory regulatory T cells. Two other adhesion molecules were implicated in the transendothelial migration of Treg across HSEC, ICAM-1 and VAP-1. ICAM-1 has been shown in several studies to be an important player in lymphocyte transendothelial migration and we have previously reported that ICAM-1 and VAP-1 support lymphocyte transendothelial migration across HSEC (6).

ICAM-1 did not support Treg arrest on HSEC under flow consistent with our recent report that Treg use VCAM-1 to bind to HSEC (38).

During diapedesis lymphocytes can take two pathways to cross the endothelium; a transcellular route in which they migrate through the endothelial cell itself or a paracellular route in which they migrate through tight junctions between endothelial cells (39). Confocal imaging of lymphocytes transmigrating across monolayers of HSEC under physiological flow demonstrated that a larger proportion of CD4 lymphocytes were undergoing transcellular rather than paracellular migration. It is possible that our analysis underestimates paracellular migration as the velocity of paracellular migration may be greater than transcellular migration.

Staining for FoxP3 confirmed that T regulatory cells also use the transcellular route (Figure 5 A-C and L-O). Analysis of live cells as well as fixed cells demonstrated that cells migrated through clearly defined transcellular pores that were highly enriched for ICAM-1, as has been reported previously in HUVEC (40–42), and VAP-1 (Figure 5 D-K). CLEVER-1/stabilin-1 was also enriched in these pores during the process of T regulatory cell diapedesis and could be seen closely associated with T cells migrating through the pores (Figure 5 P-S). The transcellular route has been described in detail previously (43;44) but, to our knowledge, this is the first description of transcellular migration in HSEC. HSEC lack classical tight junctions and it may be a lack of junctional proteins in this vascular bed which favours transcellular migration. Other receptors including CD31, CD99 have been reported to promote leukocyte transcellular migration though endothelium and we now add VAP-1 and CLEVER-1. The lymphocyte receptor for CLEVER-1 is unknown. Two of us (SJ and MS) recently hypothesised that the EGF-like domains of CLEVER-1 on endothelium may interact with phosphatidyl serine on transmigrating lymphocytes which is consistent with the relocalisation of CLEVER-1 around transmigrating cells that we observed in the present study.

In conclusion, CLEVER-1/stabilin-1 is expressed within the sinusoids of normal human liver, at sites of lymphocyte recruitment in chronically inflamed livers and within hepatocellular carcinomas. We have demonstrated that CLEVER-1/stabilin-1 plays an important role in the extravasation of lymphocytes, particularly CD4 lymphocytes and that together with ICAM-1 and VAP-1 it can support transcellular migration. Thus CLEVER-1/stabilin-1 may provide an important recruitment signal for CD4 T cell entry into the liver and thereby affect pathogenesis of chronic inflammatory liver disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Reference List

1. Xu XD, Ueta H, Zhou S, Shi C, Koga D, Ushiki T, Matsuno K. Trafficking of recirculating lymphocytes in the rat liver: rapid transmigration into the portal area and then to the hepatic lymph. Liver Int. 2008; 28:319–330. [PubMed: 18290774]
2. Lalor PF, Shields P, Grant A, Adams DH. Recruitment of lymphocytes to the human liver. Immunol Cell Biol. 2002; 80:52–64. [PubMed: 11869363]
3. Adams DH, Hubscher SG, Fisher NC, Williams A, Robinson M. Expression of E-selectin and E-selectin ligands in human liver inflammation. Hepatology. 1996; 24:533–538. [PubMed: 8781319]
4. Steinhoff G, Behrend M, Schrader B, Duijvestijn AM, Wonigeit K. Expression patterns of leukocyte adhesion ligand molecules on human liver endothelia - lack of ELAM-1 and CD62 inducibility on sinusoidal endothelia and distinct distribution of VCAM-1, ICAM-1, ICAM-2 and LFA-3. American Journal of Pathology. 1993; 142:481–488. [PubMed: 8434643]
5. Wong J, Johnston B, Lee SS, Bullard DC, Smith CW, Beaudet AL, Kubes P. A minimal role for selectins in the recruitment of leukocytes into the inflamed liver microvasculature. J Clin Invest. 1997; 99:2782–2790. [PubMed: 9169509]
6. Lalor PF, Edwards S, McNab G, Salmi M, Jalkanen S, Adams DH. Vascular adhesion protein-1 mediates adhesion and transmigration of lymphocytes on human hepatic endothelial cells. The Journal of Immunology. 2002; 169:983–92. [PubMed: 12097405]
7. McNab G, Reeves JL, Salmi M, Hubscher S, Jalkanen S, Adams DH. Vascular adhesion protein 1 mediates binding of T cells to human hepatic endothelium. Gastroenterology. 1996; 110:522–528. [PubMed: 8566600]
8. Yoong KF, McNab G, Hubscher SG, Adams DH. Vascular adhesion protein-1 and ICAM-1 support the adhesion of tumor-infiltrating lymphocytes to tumor endothelium in human hepatocellular carcinoma. The Journal of Immunology. 1998; 160:3978–88. [PubMed: 9558106]
9. Bonder CS, Norman MU, Swain MG, Zbytnuik LD, Yamanouchi J, Santamaria P, Ajuebor M, Salmi M, Jalkanen S, Kubes P. Rules of recruitment for Th1 and th2 lymphocytes in inflamed liver: a role for alpha-4 integrin and vascular adhesion protein-1. Immunity. 2005; 23:153–163. [PubMed: 16111634]
10. Irjala H, Elima K, Johansson EL, Merinen M, Kontula K, Alanen K, Grenman R, Salmi M, Jalkanen S. The same endothelial receptor controls lymphocyte traffic both in vascular and lymphatic vessels. Eur J Immunol. 2003; 33:815–824. [PubMed: 12616502]
11. Salmi M, Koskinen K, Henttinen T, Elima K, Jalkanen S. CLEVER-1 mediates lymphocyte transmigration through vascular and lymphatic endothelium. Blood. 2004; 104:3849–3857. [PubMed: 15297319]
12. Politz O, Gratchev A, McCourt PA, Schledzewski K, Guillot P, Johansson S, Svineng G, Franke P, Kannicht C, Kzhyshkowska J, Longati P, et al. Stabilin-1 and -2 constitute a novel family of fasciclin-like hyaluronan receptor homologues. Biochem J. 2002; 362:155–164. [PubMed: 11829752]
13. Adachi H, Tsujimoto M. FEEL-1, a novel scavenger receptor with in vitro bacteria-binding and angiogenesis-modulating activities. J Biol Chem. 2002; 277:34264–34270. [PubMed: 12077138]
14. Kzhyshkowska J, Gratchev A, Goerdt S. Stabilin-1, a homeostatic scavenger receptor with multiple functions. J Cell Mol Med. 2006; 10:635–649. [PubMed: 16989725]
15. Hansen B, Longati P, Elvevold K, Nedredal GI, Schledzewski K, Olsen R, Falkowski M, Kzhyshkowska J, Carlsson F, Johansson S, Smedsrod B, et al. Stabilin-1 and stabilin-2 are both directed into the early endocytic pathway in hepatic sinusoidal endothelium via interactions with clathrin/AP-2, independent of ligand binding. Exp Cell Res. 2005; 303:160–173. [PubMed: 15572036]
16. Jung MY, Park SY, Kim IS. Stabilin-2 is involved in lymphocyte adhesion to the hepatic sinusoidal endothelium via the interaction with alphaMbeta2 integrin. J Leukoc Biol. 2007; 82:1156–1165. [PubMed: 17675564]
17. DeLeve LD, Wang X, McCuskey MK, Mccuskey RS. Rat liver endothelial cells isolated by anti-CD31 immunomagnetic separation lack fenestrae and sieve plates. Am J Physiol Gastrointest Liver Physiol. 2006; 291:G1187–G1189. [PubMed: 16782698]

18. Neubauer K, Lindhorst A, Tron K, Ramadori G, Saile B. Decrease of PECAM-1-gene-expression induced by proinflammatory cytokines IFN-gamma and IFN-alpha is reversed by TGF-beta in sinusoidal endothelial cells and hepatic mononuclear phagocytes. BMC Physiol. 2008; 8:9. [PubMed: 18466611]
19. Prevo R, Banerji S, Ni J, Jackson DG. Rapid plasma membrane-endosomal trafficking of the lymph node sinus and high endothelial venule scavenger receptor/homing receptor stabilin-1 (FEEL-1/CLEVER-1). J Biol Chem. 2004; 279:52580–52592. [PubMed: 15345716]
20. Lai WK, Sun PJ, Zhang J, Jennings A, Lalor PF, Hubscher S, McKeating JA, Adams DH. Expression of DC-SIGN and DC-SIGNR on Human Sinusoidal Endothelium: A Role for Capturing Hepatitis C Virus Particles. Am J Pathol. 2006; 169:200–208. [PubMed: 16816373]
21. Gramberg T, Hofmann H, Moller P, Lalor PF, Marzi A, Geier M, Krumbiegel M, Winkler T, Kirchhoff F, Adams DH, Becker S, et al. LSECtin interacts with filovirus glycoproteins and the spike protein of SARS coronavirus. Virology. 2005; 340:224–236. [PubMed: 16051304]
22. Gramberg T, Soilleux E, Fisch T, Lalor PF, Hofmann H, Wheeldon S, Cotterill A, Wegele A, Winkler T, Adams DH, Pohlmann S. Interactions of LSECtin and DC-SIGN/DC-SIGNR with viral ligands: Differential pH dependence, internalization and virion binding. Virology. 2007
23. Shetty S, Lalor PF, Adams DH. Lymphocyte recruitment to the liver: Molecular insights into the pathogenesis of liver injury and hepatitis. Toxicology. 2008; 254:136–146. [PubMed: 18775762]
24. Lalor PF, Clements JM, Pigott R, Humphries MJ, Spragg JH, Nash GB. Association between receptor density, cellular activation, and transformation of adhesive behavior of flowing lymphocytes binding to VCAM-1. Eur J Immunol. 1997; 27:1422–1426. [PubMed: 9209494]
25. Curbishley SM, Eksteen B, Gladue RP, Lalor P, Adams DH. CXCR3 Activation Promotes Lymphocyte Transendothelial Migration across Human Hepatic Endothelium under Fluid Flow. Am J Pathol. 2005; 167:887–899. [PubMed: 16127166]
26. Arimoto J, Ikura Y, Suekane T, Nakagawa M, Kitabayashi C, Iwasa Y, Sugioka K, Naruko T, Arakawa T, Ueda M. Expression of LYVE-1 in sinusoidal endothelium is reduced in chronically inflamed human livers. J Gastroenterol. 2009
27. Grant AJ, Goddard S, Ahmed-Choudhury J, Reynolds G, Jackson DG, Briskin M, Wu L, Hubscher SG, Adams DH. Hepatic expression of secondary lymphoid chemokine (CCL21) promotes the development of portal-associated lymphoid tissue in chronic inflammatory liver disease. Am J Pathol. 2002; 160:1445–1455. [PubMed: 11943728]
28. Murakami J, Shimizu Y, Kashii Y, Kato T, Minemura M, Okada K, Nambu S, Takahara T, Higuchi K, Maeda Y, Kumada T, et al. Functional B-cell response in intrahepatic lymphoid follicles in chronic hepatitis C. Hepatology. 1999; 30:143–150. [PubMed: 10385650]
29. Lee WY, Kubes P. Leukocyte adhesion in the liver: distinct adhesion paradigm from other organs. J Hepatol. 2008; 48:504–512. [PubMed: 18192055]
30. Yoong KF, Afford SC, Jones R, Aujla P, Qin S, Price K, Hubscher SG, Adams DH. Expression and function of CXC and CC chemokines in human malignant liver tumors: a role for human monokine induced by gamma-interferon in lymphocyte recruitment to hepatocellular carcinoma. Hepatology. 1999; 30:100–111. [PubMed: 10385645]
31. Fu J, Xu D, Liu Z, Shi M, Zhao P, Fu B, Zhang Z, Yang H, Zhang H, Zhou C, Yao J, et al. Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. Gastroenterology. 2007; 132:2328–39. [PubMed: 17570208]
32. Bussolino F, Di Renzo MF, Ziche M, Bocchietto E, Olivero M, Naldini L, Gaudino G, Tamagnone L, Coffer A, Comoglio PM. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J Cell Biol. 1992; 119:629–641. [PubMed: 1383237]
33. Grant DS, Kleinman HK, Goldberg ID, Bhargava MM, Nickoloff BJ, Kinsella JL, Polverini P, Rosen EM. Scatter factor induces blood vessel formation in vivo. Proc Natl Acad Sci U S A. 1993; 90:1937–1941. [PubMed: 7680481]
34. Martin TA, Jiang WG. Hepatocyte growth factor and its receptor signalling complex as targets in cancer therapy. Anticancer Agents Med Chem. 2010; 10:2–6. [PubMed: 20015008]
35. Hansen B, Longati P, Elvevold K, Nedredal GI, Schledzewski K, Olsen R, Falkowski M, Kzhyshkowska J, Carlsson F, Johansson S, Smedsrod B, et al. Stabilin-1 and stabilin-2 are both

directed into the early endocytic pathway in hepatic sinusoidal endothelium via interactions with clathrin/AP-2, independent of ligand binding. Exp Cell Res. 2005; 303:160–173. [PubMed: 15572036]

36. Karikoski M, Irjala H, Maksimow M, Miiluniemi M, Granfors K, Hernesniemi S, Elima K, Moldenhauer G, Schledzewski K, Kzhyshkowska J, Goerdt S, et al. Clever-1/Stabilin-1 regulates lymphocyte migration within lymphatics and leukocyte entrance to sites of inflammation. Eur J Immunol. 2009; 39:3477–3487. [PubMed: 19830743]
37. Kzhyshkowska J, Gratchev A, Martens JH, Pervushina O, Mamidi S, Johansson S, Schledzewski K, Hansen B, He X, Tang J, Nakayama K, et al. Stabilin-1 localizes to endosomes and the trans-Golgi network in human macrophages and interacts with GGA adaptors. J Leukoc Biol. 2004; 76:1151–1161. [PubMed: 15345724]
38. Oo YH, Weston CJ, Lalor PF, Curbishley SM, Withers DR, Reynolds GM, Shetty S, Harki J, Shaw JC, Eksteen B, Hubscher SG, et al. Distinct roles for CCR4 and CXCR3 in the recruitment and positioning of regulatory T cells in the inflamed human liver. J Immunol. 2010; 184:2886–2898. [PubMed: 20164417]
39. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol. 2007; 7:678–689. [PubMed: 17717539]
40. Carman CV, Springer TA. A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. J Cell Biol. 2004; 167:377–388. [PubMed: 15504916]
41. Carman CV, Sage PT, Sciuto TE, de la Fuente MA, Geha RS, Ochs HD, Dvorak HF, Dvorak AM, Springer TA. Transcellular diapedesis is initiated by invasive podosomes. Immunity. 2007; 26:784–797. [PubMed: 17570692]
42. Yang L, Froio RM, Sciuto TE, Dvorak AM, Alon R, Luscinskas FW. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. Blood. 2005; 106:584–592. [PubMed: 15811956]
43. Mamdouh Z, Mikhailov A, Muller WA. Transcellular migration of leukocytes is mediated by the endothelial lateral border recycling compartment. J Exp Med. 2009; 206:2795–2808. [PubMed: 19887395]
44. Carman CV, Springer TA. A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. J Cell Biol. 2004; 167:377–388. [PubMed: 15504916]

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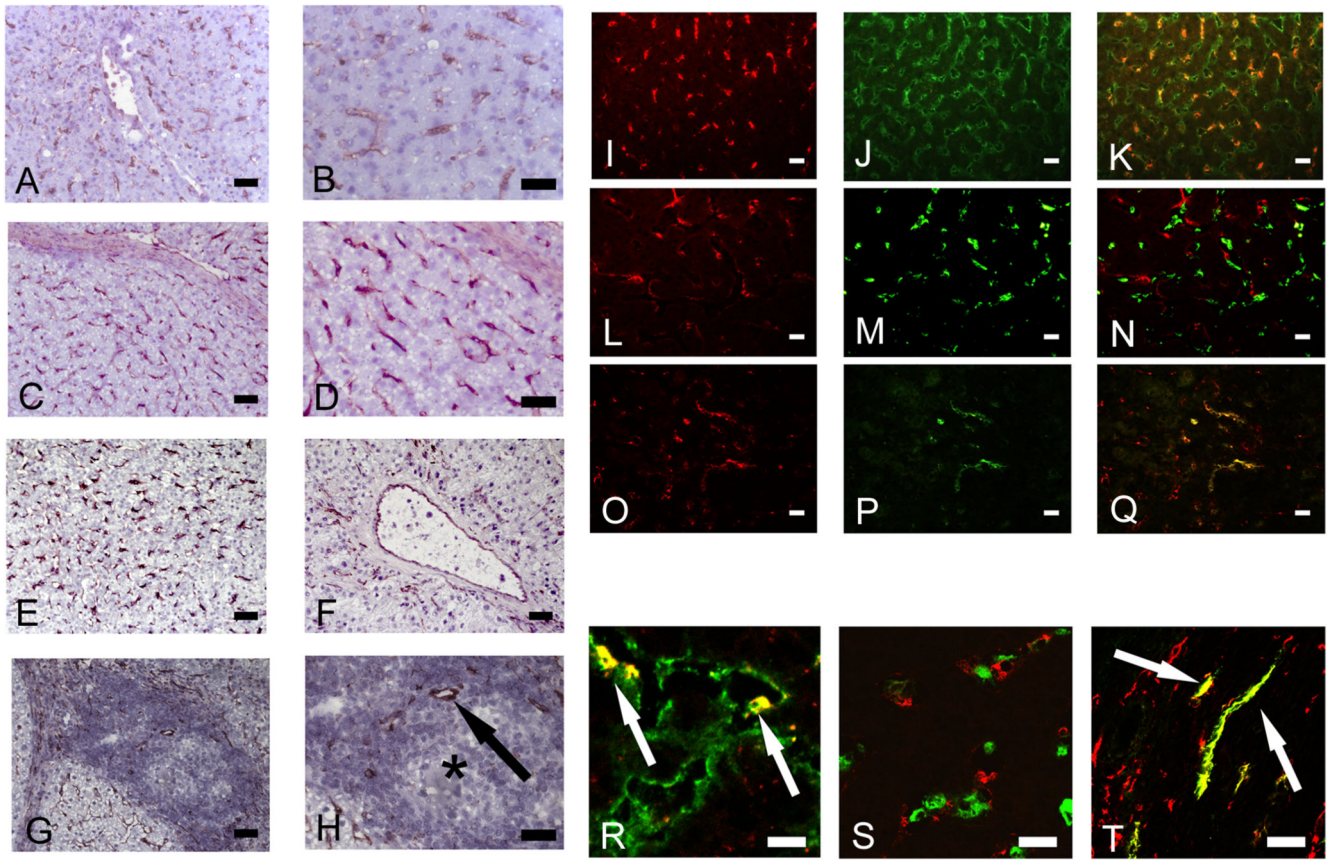

Figure 1. CLEVER-1/stabilin-1 is expressed on hepatic endothelial cells *in vivo* in normal and chronically inflamed human liver as well as hepatocellular carcinomas and inflammatory neovessels.

Immunohistochemical staining of representative examples of normal liver (A and B (inset of previous image)), autoimmune hepatitis (C and D (inset of previous image) demonstrating expression of CLEVER-1/stabilin-1 within the hepatic sinusoids. CLEVER-1/stabilin-1 was also expressed on sinusoids within hepatocellular carcinomas (E) and tumour associated vessels (F), and HEV-like vessels within portal associated lymphoid tissue (G and H (inset of previous image)). Asterisk denotes a germinal centre within a lymphoid follicle and arrow demonstrates positive vessels within the lymphoid follicle. Immunofluorescent staining demonstrates that CLEVER-1/stabilin-1 is expressed by HSEC *in vivo* (I,CLEVER-1/stabilin-1 in red; J, L-SIGN in green; K, merged image) whereas is absent from kupffer cells (L, CLEVER-1/stabilin-1 in red; M,CD68 in GREEN; N,merged image). Also present on neo-vessels in chronically inflamed tissue (O,CLEVER-1 in red; P, CD34 in green; Q, merged image). Confocal microscopy confirmed these findings, merged images are shown of CLEVER-1 and L-SIGN staining (R), CLEVER-1 and CD68 staining (S) and CLEVER-1 and CD34 staining (T) where yellow staining demonstrates colocalisation. Control sections did not have detectable staining. Bar 50μm apart from R-T where Bar 10μm.

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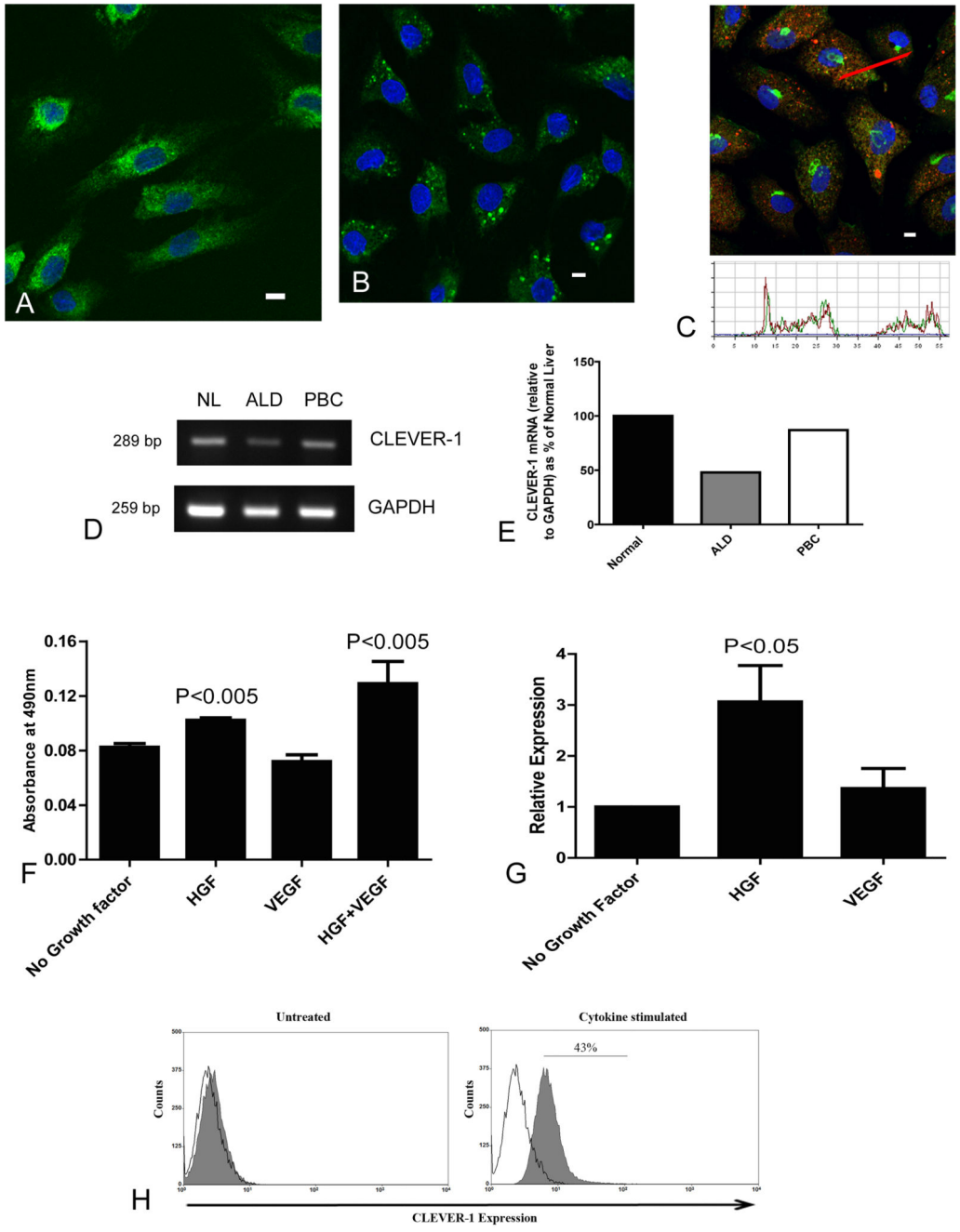

Figure 2. CLEVER-1/stabilin-1 is expressed on hepatic endothelial cells *in vitro* and undergoes trafficking with colocalisation to Trans Golgi Network.

(A) Representative image of immunofluorescent staining of HSEC with CLEVER-1/stabilin-1 (green) and nuclear counter stain(blue). (B) Representative image of immunofluorescent staining of HSEC after bafilomycin treatment showing CLEVER-1 localisation to intracellular vesicles (C) Confirmation of CLEVER-1/stabilin-1 in trans-Golgi network (TGN) after bafilomycin treatment; CLEVER-1/stabilin-1 (red) and TGN marker TGN-46 (green), with graphical representation below of fluorescent intensities to demonstrate areas of overlap. Bar 10μm.

D) RT-PCR confirming CLEVER-1/stabilin-1 mRNA in HSEC isolated from liver tissue from normal subjects and those with liver disease (NL- normal liver, ALD-alcoholic liver disease, PBC- primary biliary cirrhosis) and (E) semiquantitative measurement of CLEVER-1 mRNA levels in these samples.

(F) Cell based ELISA of HSEC stained with anti-CLEVER-1/stabilin-1 antibodies unstimulated and HGF or VEGF stimulated HSEC. Data are the mean of 3 replicate experiments and values represent the mean absorbance of three replicate wells minus the absorbance of an isotype- matched control Ab.

(G) Quantitatve PCR showing relative expression of CLEVER-1 mRNA in HSEC from unstimulated HSEC and growth factor stimulated HSEC. Data are the mean of 3 independent experiments.

(H) FACS analysis demonstrating surface expression CLEVER-1 on untreated and cytokine (TNFα and IFNγ) treated HSEC.

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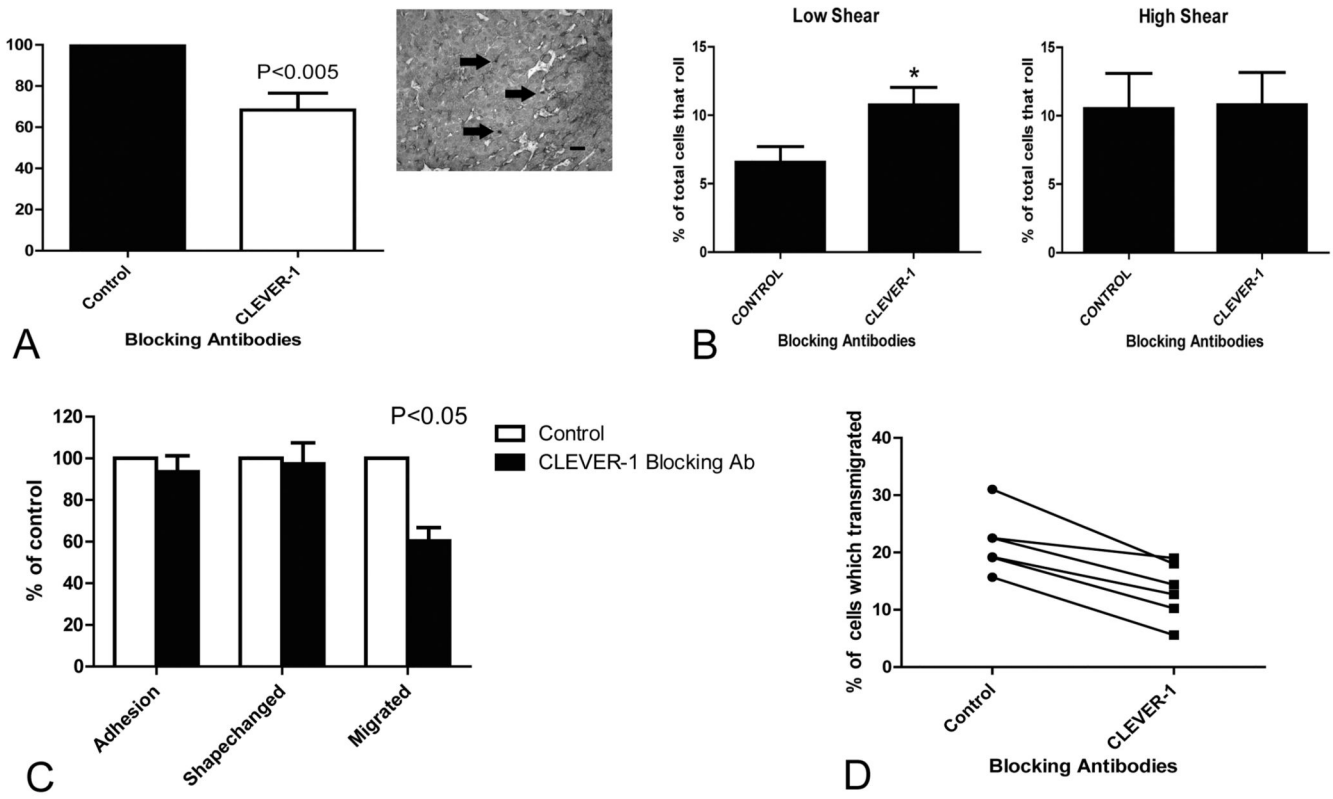

Figure 3. CLEVER-1/stabilin-1 mediates lymphocyte binding to hepatic sinusoids and the transmigration of lymphocytes across cytokine treated HSEC under shear flow.

(A) Static Stamper-Woodruff adhesion assays of peripheral blood lymphocyte binding to sinusoidal vessels in human liver tissue sections treated with 3-372 (anti CLEVER-1/stabilin-1) or appropriate class matched control antibody. The results of 9 independent experiments are shown as mean percentage of maximal binding +/-SEM. Bar 50μm. Blocking CLEVER-1 significantly reduced binding compared with control mAb.

(B) – (D) the effect of blocking CLEVER-1/stabilin-1 on the adhesion of unfractionated peripheral blood lymphocytes to HSEC under flow are shown. (B) CLEVER-1/stabilin-1 blockade increased the number of rolling cells at low shear stress but had no effect at high shear stress. There was no alteration of cells undergoing firm adhesion or the proportion undergoing activation/shape change (C). However the proportion of adherent cells that underwent transendothelial migration through HSEC was significantly reduced by CLEVER-1/stabilin-1 blockade (C and D).

The results are expressed as percentage of binding when compared with an isotype matched control. The results are mean+/- SEM of six independent experiments using different HSEC and PBLs from different donors except for experiments at high shear stress which are the mean +/- SEM of three independent experiments. The reduction in the percentage of adherent cells that transmigrated across HSEC (D) is shown for each experiment, direct comparison is made with control antibody vs CLEVER-1/stabilin-1 blockade.

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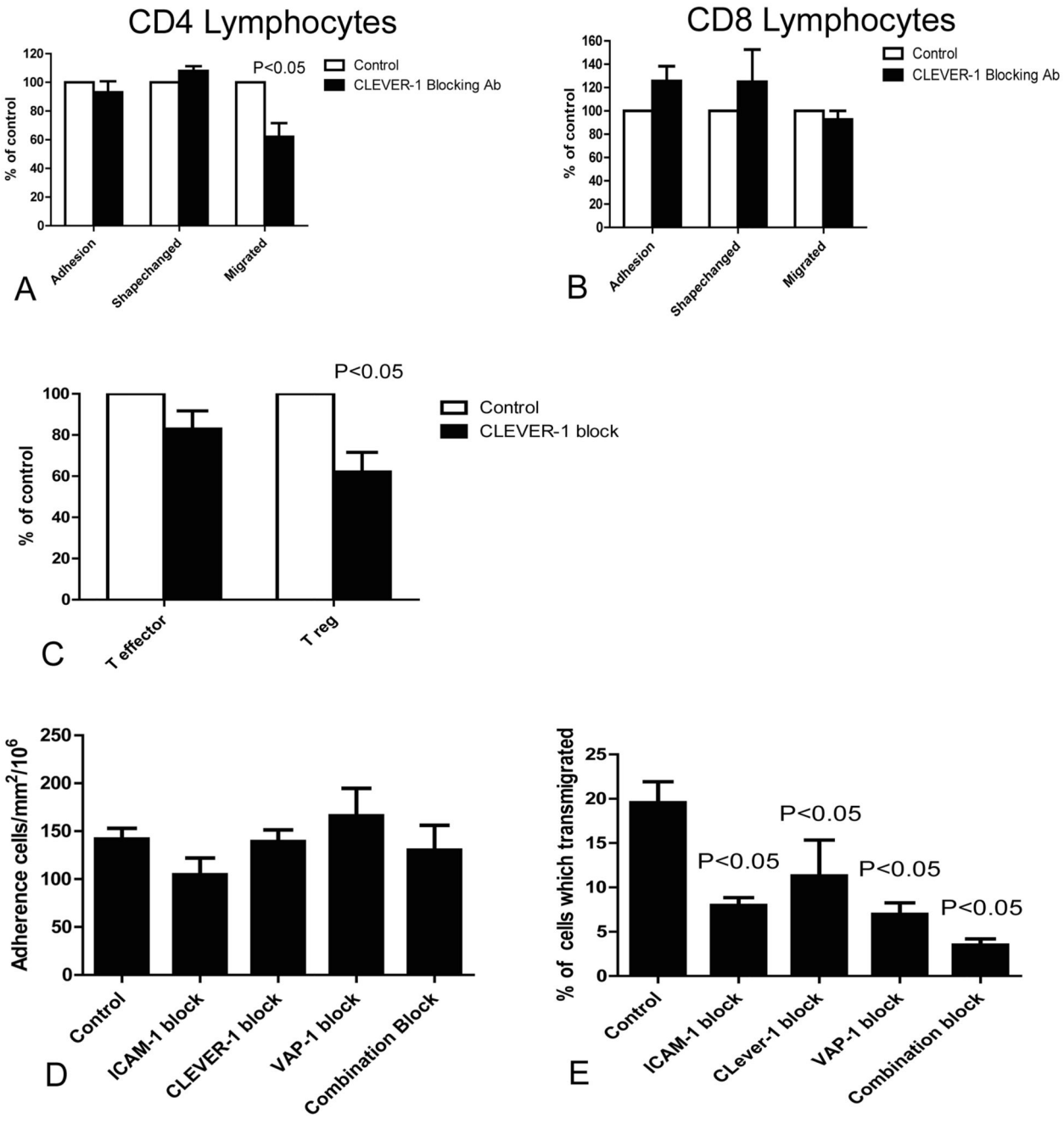

Figure 4. CLEVER-1/stabilin-1 mediates the transmigration of CD4 lymphocytes, specifically T regulatory cells, but not CD8 lymphocytes.

Flow adhesion assays using HSEC were carried out with purified peripheral blood A) CD4 and B) CD8 lymphocytes and the proportion of adherent cells, shape changed and migrated cells quantified. CLEVER-1/stabilin-1 blockade had no effect on static adhesion or shape change but significantly reduced CD4 T cells transmigration (A) but had no effect on CD8 T cells transmigration (B).

Flow adhesion assays were then repeated with highly purified peripheral blood CD4 T cells sorted into either effector cells CD4+CD25- or regulatory T cells CD4+CD25+ cells. More

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than 90% of the CD4+CD25+ cells were also FoxP3+ and expressed low levels of CD127 consistent with a T regulatory cell phenotype. (C) The proportion of adherent T regulatory cells that transmigrated was reduced significantly by CLEVER-1/stabilin-1 blockade. The results are expressed as percentage of binding when compared with an isotype matched control. The results are mean+/- SEM of four independent experiments using different HSEC and PBLs from different donors.

(D) The contribution of ICAM-1 and VAP-1 together with CLEVER-1/stabilin-1 blockade to the transendothelial migration of T regulatory cells under flow was assessed. (E) No antibody alone significantly affected adhesion but all three were implicated in transendothelial migration. The results are percentage of binding when compared with an isotype matched control shown as the mean+/- SEM of four independent experiments using different HSEC and PBLs from different donors.

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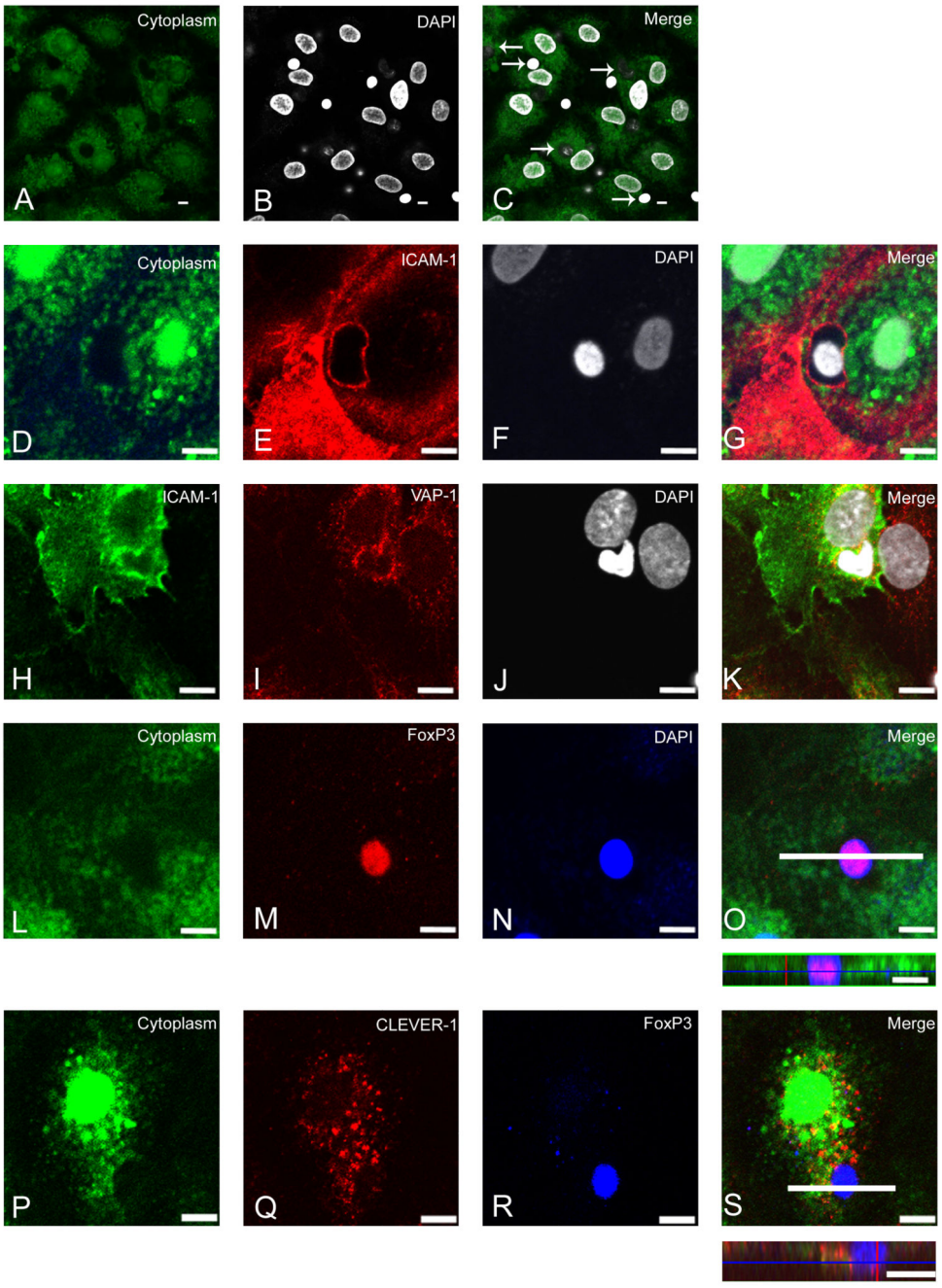

Figure 5. T regulatory cells transmigrate via the transcellular route across HSEC with enrichment of CLEVER-1/stabilin-1 during diapedesis.

Representative confocal image of CD4 lymphocytes undergoing transcellular migration. HSEC were stained with cell tracker green (A) and HSEC and lymphocyte nucleii were stained with DAPI (B). Merged image (C) demonstrates lymphocytes (arrows) clearly transmigrating through channels formed within the HSEC cytoplasm (green). Transcellular pores were enriched with a ring of ICAM-1 around transmigrating lymphocytes (D-G). VAP-1 was also detected around lymphocytes in close association with ICAM-1 (H-K). We confirmed that T regulatory cells underwent transcellular migration (L-O) with FoxP3

staining and orthogonal (XZ) projections across the plane indicated by the white line in panel O demonstrating that the T regulatory cell was crossing the endothelial cell. Staining with cell tracker green and mAbs demonstrate that CLEVER-1/stabilin-1 colocalises around FoxP3 positive cell (P-S). Orthogonal projection across the plane indicated by the white line in panel S confirms that CLEVER-1/stabilin-1 enriches around T regulatory cell during transmigration. Bar 10μm

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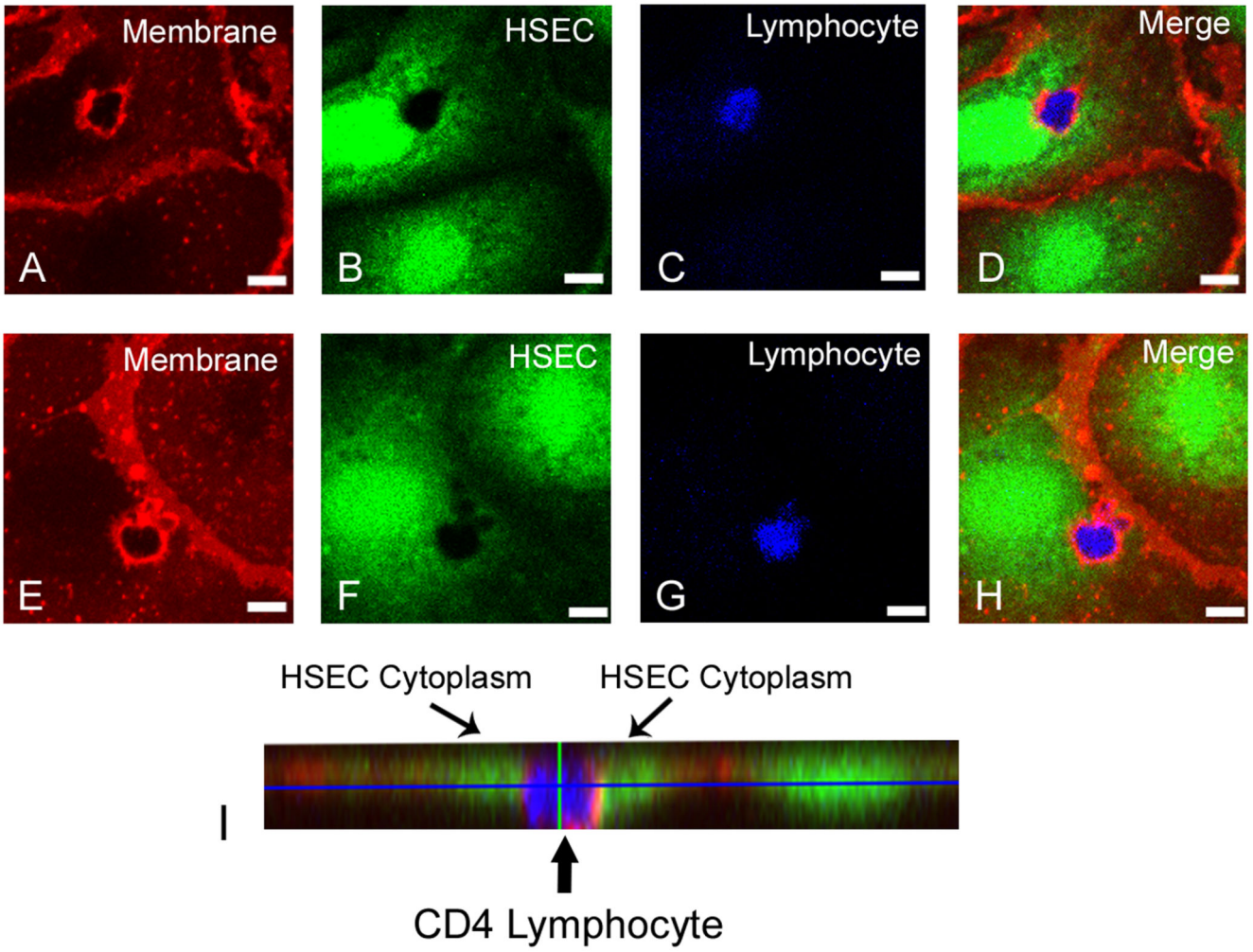

Figure 6. Live cell imaging of CD4 lymphocytes undergoing transcellular migration across HSEC

To confirm that the above results were not affected by fixation artefacts we carried out live cell imaging of CD4 T cells migrating through monolayers of HSEC under flow. Imaging with confocal microscopy was able to capture lymphocytes undergoing transmigration through HSEC cytoplasm. HSEC and lymphocyte cell membranes can be seen labelled with a red dye (A, E) whilst HSEC cytoplasm was prelabelled with a green dye (B, F), lymphocytes were prelabelled with blue dye (C,G). Lymphocytes within transcellular pores were clearly seen in HSEC (D, H). Representative Z stack imaging (I) confirms lymphocytes undergoing transcellular migration. Bar 10μm

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Table I

Semiquantitative analysis of CLEVER-1 staining of liver tissue.

CLEVER-1	Normal Liver	Primary Biliary Cirrhosis	Primary Sclerosing Cholangitis	Autoimmune liver disease	Alcoholic Liver Disease
Sinusoids	(+)	(+/++)	(++)	(++)	(++)
Portal vessels	(-)	(-)	(-)	(-)	(-)
Central Vein	(-)	(-)	(-)	(-)	(-)
Vessels in fibrous septa	Not applicable	(+/++)	(+)	(+)	(+)

Scoring : (-) NEGATIVE, (+) WEAK POSITIVE, (++) POSITIVE, (+++) STRONG POSITIVE
Scoring from three different cases for each condition.