Common Lymphatic Endothelial and Vascular Endothelial Receptor-1 Mediates the Transmigration of Regulatory T Cells across Human Hepatic Sinusoidal Endothelium

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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. In this study, we demonstrate increased endothelial expression of CLEVER-1/stabilin-1 at sites of leukocyte recruitment to the inflamed human liver including sinusoids, septal vessels, and lymphoid follicles in inflammatory liver disease and tumor-associated vessels in hepatocellular carcinoma. We used primary cultures of human hepatic 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 (Tregs). 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 >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.

Inflammatory liver diseases are characterized by a lymphocyte-predominant infiltrate that 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 multistep adhesion cascade, but there are important differences when compared with lymphocyte extravasation at other sites (2).

The online version of this article contains supplemental material.

Abbreviations used in this article: CLEVER-1, common lymphatic endothelial and vascular endothelial receptor; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; HSEC, hepatic sinusoidal endothelial cells; PALT, portal-associated lymphoid tissue; TEM, transendothelial cell migration; Treg, regulatory T cell; VAP-1, vascular adhesion receptor; VEGF, vascular endothelial growth factor.
regulatory T cells (Tregs) through human hepatic sinusoidal endothelial cells (HSEC) under conditions of shear stress. Detailed analysis revealed that Tregs migrated through transcellular pores in HSEC in close association with CLEVER-1/stabilin-1.

Materials and Methods

Isolation and culture of human HSEC

Liver endothelial cells were isolated from ~30 g 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 from patients in the Liver Unit at Queen Elizabeth Hospital in Birmingham with informed consent and under local ethics committee approval. Briefly, the digested tissue was placed over a 33/77% Percoll (Amersham Biosciences, GE Healthcare, Little Chalfont, U.K.) 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, U.K.). The cells were grown in collagen-coated culture endothelial basal growth medium (Invitrogen), 10% AB human serum (HD Endothelial cells were then cultured in medium composed of human endothelial basal growth medium (Invitrogen), 10% AB human serum (HD Supplies, Glasgow, U.K.), 10 ng/ml vascular endothelial growth factor (VEGF), and 10 ng/ml hepatocyte growth factor (HGF) (PeproTech, Peterborough, U.K.). The cells were grown in collagen-coated culture flasks and were maintained at 37°C in a humidified incubator with 5% CO₂ 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 HSEC 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.

Abs and immunostaining

A mAb against CLEVER-1/stabilin-1 (3-372) has been described (10) and was used at 10 μg/ml. Sinusoidal endothelial cells were identified using Abs against L-SIGN 5 μg/ml (R&D Systems, Minneapolis, MN) and Kupffer cells with CD68 5 μg/ml (BD Biosciences, Oxford, U.K.). Neo-vessels were identified with biotinylated Ab against CD34 2 μg/ml (AbD Serotec, Kidlington, U.K.). Trans-Golgí network was identified by Abs against TGN-46 2 μg/ml (Abcam, Cambridge, U.K.). Mouse IgG1 (DAKO, Ely, U.K.), Ig G2a, IgG2b (R&D Systems), biotinylated IgG1 (AbD Serotec), and polyclonal rabbit IgG (DAKO) were used as controls. FITC-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, and FITC-conjugated goat anti-rabbit IgG were from Southern Biotech (Cambridge, U.K.). Alexa Fluor 488-conjugated goat anti-mouse IgG1 were from Invitrogen.

Sections were taken from normal liver tissue and from a variety of chronic inflammatory liver diseases and from hepatocellular carcinomas (HCCs), with at least three different cases studied from each disease. For standard immunohistochemistry, representative 5-μm cryosections were aceton-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 mAb at optimal concentration in TBS/0.1% Tween for 60 min at room temperature. Control sections were incubated with relevant isotype-matched control. Sections were then incubated for 30 min with 1 μm PRESS Universal anti-mouse/rabbit IgG reagent (Vector Labs, Burlingame, CA) at room temperature and washed with TBS/0.1% Tween prior to color development using the Vector VIP substrate kit (Vector Labs). Finally, sections were washed in water, counterstained with hematoxylin, and mounted. The staining on the sections was scored semiquantitatively for intensity of CLEVER-1 staining (7) using a validated scoring system on the following structures: sinusoids, portal vessels, central veins, and vessels in the fibrous septum.

For immunofluorescent staining, aceton-fixed cryosections were initially incubated with 10% normal goat serum in TBS buffer for 30 min before the addition of primary mouse anti-human mAbs in TBS for 60 min 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 30 min in TBS. Sections were washed in TBS/Tween and mounted with fluorescent mounting medium (DAKO).

![Image](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)

FIGURE 1. CLEVER-1/stabilin-1 is expressed on hepatic endothelial cells in vivo in normal and chronically inflamed human liver, as well as in HCCs and inflammatory neovessels. Immunohistochemical staining of representative examples of normal liver (A, B), autoimmune hepatitis (C, D) demonstrating expression of CLEVER-1/stabilin-1 within the hepatic sinusoids. CLEVER-1/stabilin-1 was also expressed on sinusoids within HCCs (E) and tumor-associated vessels (F), and on high endothelial venule-like vessels within PALT (G, H). The asterisk denotes a germinal center within a lymphoid follicle, and the arrow demonstrates positive vessels within the lymphoid follicle. B–H are high power views of the sections shown in A–G. 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 it is absent from Kupffer cells (L, CLEVER-1/stabilin-1 in red; M, CD68 in green; N, merged image). It is also present on neovessels 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 colocalization indicated by arrows. Control sections did not have detectable staining. A–Q, Scale bars, 50 μm; R–T, scale bars, 10 μm.
Sections were imaged by an Axioskop 40 microscope (Carl Zeiss, Welwyn Garden City, U.K.) with an Axiocam MRc5 camera (Carl Zeiss) and analyzed 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 PBS and permeabilized with 0.3% Triton. After incubation with 10% normal goat serum in TBS buffer for 30 min, cells were incubated with primary mAb or isotype-matched control for 1 h at room temperature. Cells were then incubated with fluorescent secondary Abs for one-half hour before being mounted with fluorescence mounting medium (DAKO). Cells were imaged by confocal microscopy on an LSM 510 microscope equipped with a 63×1.32 objective. Images were acquired and analyzed by LSM software.

### Isolation of RNA

RNA was isolated from HSEC by using the RNeasy Mini Kit (Qiagen, Crawley, U.K.) 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 PCR

Conventional 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-3′, 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

### Table I. Semiquantitative analysis of CLEVER-1 staining of liver tissue

<table>
<thead>
<tr>
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<th>Normal Liver</th>
<th>Primary Biliary Cirrhosis</th>
<th>Primary Sclerosing Cholangitis</th>
<th>Autoimmune Liver Disease</th>
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<td>Vessels in fibrous septa</td>
<td>Not applicable</td>
<td>+/++</td>
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Scoring from three different cases for each condition.

−, negative; +, weak positive; ++, positive; ++++, strong positive.
ATG GAC ACC-3'). The CLEVER-1/stabilin-1 primers amplified a 289-bp product, the reaction conditions were 3 min denaturation (95°C) and then 35 cycles of 1 min (95°C), 1 min annealing (50°C), and 2 min (72°C). Final extension was at 72°C for 4 min. The GAPDH primers amplified a 259-bp product; 35 cycles were run at 94°C for 30 s, 55°C for 30 s, 72°C for 45 s, and a final extension at 72°C for 10 min.

Real-time PCR
CLEVER-1/stabilin-1 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 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. CLEVER-1/stabilin-1 gene expression was analyzed from cDNA/antigen 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 (10 ng/ml recombinant human TNF-α, 10 ng/ml recombinant human IFN-γ, 10 ng/ml recombinant human IFN-α, 10 ng/ml recombinant human IL-10, 10 ng/ml recombinant human IL-4, 10 ng/ml recombinant human IL-10, all from PeproTech) or VEGF and 10 ng/ml recombinant HGF, both from PeproTech). All stimulations were for 24 h. Cells were preincubated with 2% goat serum (Sigma-Aldrich) for 1 h. This was followed by incubation with mouse anti-human primary Ab (3-372, 10 µg/ml) and control Ab (IgG1, 10 µg/ml; DAKO) for 45 min at room temperature. The cells were then washed and incubated with a peroxidase-conjugated goat anti-mouse secondary Ab (PO447, 1/500; DAKO) for 45 min 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 Ab.

Flow cytometric analysis of HSEC
HSEC (untreated or treated with a combination of TNF-α [10 ng/ml] and IFN-γ [10 ng/ml] for 24 h) were resuspended in cold FACS media (PBS and 10% FCS) and labeled with primary mAb against CLEVER-1/stabilin-1 (Southern Biotech) and analyzed on a Dako Cyan Flow Cytometer using Summit 4.3 software (DakoCytomation, Ely, U.K.).

Bafilomycin treatment
Bafilomycin A1 was purchased from Sigma-Aldrich. Bafilomycin was added directly to cell culture medium at 10 nM and cells incubated for 24 h at 37°C. After treatment, the cells were analyzed by immunofluorescence as described earlier.

Isolation of PBLs and subsets
PBLs were isolated as previously described (24) by density gradient centrifugation over Lympholyte (VH Bio, Gateshead, U.K.) for 25 min at 800 × g. Harvested lymphocytes were washed and resuspended in RPMI 1640/10% FCS. CD4 and CD8 cells were isolated using a negative im-

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 PBLs binding to sinusoidal vessels in human liver tissue sections treated with 3-372 (anti-CLEVER-1/stabilin-1) or appropriate class-matched control Ab. Arrows indicate lymphocytes adherent to vessels. The results of nine independent experiments are shown as mean percentage of maximal binding ± SEM. Scale bar, 50 µm. 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 PBLs to HSEC under flow are shown. CLEVER-1/stabilin-1 blockade increased the number of rolling cells at low shear stress but had no effect at high shear stress (B). *p < 0.01. 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, D). The results are expressed as percentage of binding 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 Ab versus CLEVER-1/stabilin-1 blockade.
munomagnetic selection kit (Invitrogen), and similarly, Tregs 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^+CD25^2 fraction generated by these experiments was kept and used as an effector population in functional experiments. All kits were used according to the manufacturers' instructions.

**Adhesion assays**

**Static adhesion assays.** Tissue sections were first incubated with Ab 3-372 (against CLEVER-1/stabilin-1) or control Ab. They were then overlaid with PBLs (1 × 10^6/ml, 100 μl), which were allowed to adhere in static conditions for 30 min at room temperature. The nonadherent cells were removed, and the adherent cells were fixed in acetone. The number of lymphocytes present in a minimum of 10 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 (TNF-α and IFN-γ for 24 h at 10 ng/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 (1 × 10^6 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 to increase the proportion of rolling cells. Phase contrast video recordings made during lymphocyte perfusion were analyzed offline to determine percentage of rolling cells and adherent and transmigrated cells (see Supplemental Videos 1, 2). Cells appearing phase bright were above the endothelial monolayer, whereas those that were phase dark had migrated through the monolayer. HSEC monolayers were incubated with blocking Abs against CLEVER-1/stabilin-1 (3-372, 20 μg/ml), ICAM-1 (10 μg/ml; R&D Systems), VAP-1 (TK8-14, 10 μg/ml; Biotie, Turku, Finland), or isotype-matched control (IgG1 [DAKO] and IgG2a [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 earlier. HSEC were prelabeled with CellTracker Green CMFDA (Invitrogen) per the manufacturer’s instructions followed by flow assays with CD4 lymphocytes, as described earlier, for 10 min. Cells were then fixed in 4% paraformaldehyde and permeabilized with 0.3% Triton. Cells were stained with mAb for ICAM-1 (10 μg/ml; R&D Systems) and VAP-1 (TK8-14, 10 μg/ml; Biotie, Turku, Finland) followed by Alexa

**FIGURE 4.** CLEVER-1/stabilin-1 mediates the transmigration of CD4 lymphocytes, specifically Tregs, but not CD8 lymphocytes. A and B, Flow adhesion assays using HSEC were carried out with purified peripheral blood CD4 (A) and CD8 (B) lymphocytes and the proportion of adherent cells and 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 cell transmigration (A) whereas it had no effect on CD8 T cell transmigration (B). Flow adhesion assays were then repeated with highly purified peripheral blood CD4 T cells sorted into either CD4^+CD25^− effector cells or CD4^+CD25^+ Tregs. More than 90% of the CD4^+CD25^+ cells were also FOXP3 and expressed low levels of CD127 consistent with a Treg phenotype. C, The proportion of adherent Tregs that transmigrated was reduced significantly by CLEVER-1/stabilin-1 blockade. The results are expressed as percentage of binding 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 Tregs under flow was assessed. E, No Ab alone significantly affected adhesion, but all three were implicated in transendothelial migration. The results are percentage of binding compared with an isotype-matched control shown as the mean ± SEM of four independent experiments using different HSEC and PBLs from different donors.
Fluor 546-conjugated goat anti-mouse IgG1 or Alexa Fluor 488-conjugated goat anti-mouse IgG2a (Invitrogen). To identify T regulatory lymphocytes, cells were stained with FOXP3 (5 μg/ml; eBioscience, Hatfield, U.K.) followed by goat anti-rat Alexa Fluor 633 with prior fixation/permeabilization performed with FOXP3 staining kit (eBioscience); rat IgG2a (eBioscience) was used as control. CLEVER-1/stabilin-1 was stained with 3372 (10 μg/ml), and goat anti-mouse Alexa Fluor 546 was used at the secondary stage. Cells were stained with a DAPI stain (Invitrogen) for nuclear staining. Slides were then examined using an LSM 510 microscope equipped with a 63×1.32 objective. Confocal images and z-stacks were acquired and analyzed 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 that took either the transcellular route or the paracellular route.

For live cell imaging, HSEC were again prelabeled with CellTracker Green (Invitrogen). CD4 lymphocytes were prelabeled with CellTracker Violet BMQC (Invitrogen) per the manufacturer’s guidelines. Flow assays were performed with the prelabeled lymphocytes. After this, the cell boundaries were stained with CellMask Orange plasma membrane stain, per the manufacturer’s guidelines, for 5 min. Slides were then immediately examined using an LSM 510 microscope equipped with a 63×1.32 objective. Confocal images and z-stacks were acquired and analyzed by LSM software.

**Statistical analysis**

Paired two-tailed t tests were performed using the TTEST function in the Excel program of Microsoft Office 2007. Variation between multiple treatments was evaluated using ANOVA, followed by Dunnett test for comparison of control using GraphPad Prism 5 software. A p value <0.05 was considered as statistically significant.

**Results**

**CLEVER-1/stabilin-1 is expressed on hepatic endothelium in sinusoids, vessels supplying PALT, and on tumor endothelium in HCC**

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 that of normal liver tissue and strong expression on endothelium in lymphoid follicles in portal areas (Fig. 1G, 1H, Table I). In HCC, CLEVER-1/stabilin-1 was detected within tumor sinusoids and on tumor-associated vessels (Fig. 1E, 1F). 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 CLEVER-1 has been reported on macrophages, it was important to ascertain which cell type expressed CLEVER-1. We thus carried out dual immunofluorescence staining with anti-CLEVER-1/stabilin-1 and Abs against 1) L-SIGN (a specific marker of liver endothelium), 2) CD68 (a macrophage marker not present on endothelium), and 3) 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+ sinusoidal endothelial cells but not on CD68+ Kupffer cells in the hepatic sinusoids (Fig. 1I–K, IL–N) and on CD34+ endothelium in neovessels at sites of chronic inflammation (Fig. 1O–Q). These findings were confirmed with confocal microscopy (Fig. 1R–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, 2C). RT-PCR confirmed expression of CLEVER-1/stabilin-1 by HSEC isolated from either normal or diseased livers, and semiquantitative analysis demonstrated some variation in CLEVER-1 levels between isolates (Fig. 2D, 2E).

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 HGF (Fig. 2F, 2G).

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 surfaces of cells by treatment with IFN-γ and TNF-α (Fig. 2H). The response was very variable depending on cell batch and passage number.

CLEVER-1/stabilin-1 mediates transmigration of PBLs across HSEC

Modified Stamper–Woodruff tissue-binding assays were used to show that Ab blockade of CLEVER-1/stabilin-1 significantly inhibited lymphocyte binding to hepatic vessels in frozen sections of diseased liver tissue (Fig. 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 TNF-α and IFN-γ 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 the level of shear stress to 1.0 Pa, 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 with 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 shape-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, 3D).

CLEVER-1/stabilin-1 mediates the transmigration of CD4 lymphocytes, in particular Tregs, 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, 4B). We further purified CD4 cells into CD4+CD25+FOXP3+ Tregs 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. Abs against ICAM-1 or VAP-1 had no effect on the adherence of Treg (Fig. 4D) but reduced TEM by ~50%, and when combined with anti-CLEVER-1/stabilin-1, 80% of TEM was inhibited (Fig. 4E).

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

We used confocal microscopy to analyze CD4 lymphocyte transmigration across HSEC monolayers under flow. A significant proportion of cells used the transcellular route (Fig. 5A–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 (Fig. 5D–K).
FOXP3+CD4 lymphocytes were clearly seen to migrate through channels in the HSEC cytoplasm (Fig. 5L–O) and were closely associated with CLEVER-1/stabilin-1 (Fig. 5P–S). We confirmed transcellular migration of CD4 lymphocytes across HSEC with live cell imaging (Fig. 6).

**Discussion**

To our knowledge, this is the first report implicating CLEVER-1/stabilin-1 in the recruitment of Tregs 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 capillarization (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 tumor-associated vessels and sinusoids within HCCs suggesting that it could also play a role in promoting lymphocyte recruitment to HCCs that are infiltrated by lymphocytes, including Tregs (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, proinflammatory 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 and 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 tumor-associated vessels might reflect local secretion of HGF as part of the tissue remodeling 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, 15, 19). It has been hypothesized that this recycling could facilitate lymphocyte transmigration through lymphatic endothelium (35). 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, 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 the trans-Golgi network (36). We did, however, find increased detectable cell surface CLEVER-1 in cells treated with TNF-α and IFN-γ (Fig. 2H), 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 Tregs. Two other adhesion molecules were implicated in the transendothelial migration of Tregs 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 Tregs use VCAM-1 to bind to HSEC (37).

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 (38). 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 that of transcellular migration.

Staining for FOXP3 confirmed that Tregs also use the transcellular route (Fig. 5A–C, 5L–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 and VAP-1 (Fig. 5D–K). CLEVER-1/stabilin-1 was also enriched in these pores during the process of Treg diapedesis and could be seen closely associated with T cells migrating through the pores (Fig. 5P–S). The transcellular route has been described in detail previously (39, 42), 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 that favors transcellular migration. Other receptors including CD31 and 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 (S.J. and M.S.) recently hypothesized that the epidermal growth factor-like domains of CLEVER-1 on endothelium may interact with phosphatidyl serine on transmigrating lymphocytes, which is consistent with the relocation of CLEVER-1 around transmigrating cells that we observed in the current 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 HCCs. 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.
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DISCLOSURES

The authors have no financial conflicts of interest.

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