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RESEARCH PAPER

Murine junctional adhesion molecules JAM-B and JAM-C mediate endothelial and stellate cell interactions during hepatic fibrosis

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ABSTRACT

Classical junctional adhesion molecules JAM-A, JAM-B and JAM-C influence vascular permeability, cell polarity as well as leukocyte recruitment and immigration into inflamed tissue. As the vasculature becomes remodelled in chronically injured, fibrotic livers we aimed to determine distribution and role of junctional adhesion molecules during this pathological process. Therefore, livers of naïve or carbon tetrachloride-treated mice were analyzed by immunohistochemistry to localize all 3 classical junctional adhesion molecules. Hepatic stellate cells and endothelial cells were isolated and subjected to immunocytochemistry and flow cytometry to determine localization and functionality of JAM-B and JAM-C. Cells were further used to perform contractility and migration assays and to study endothelial tubulogenesis and pericytic coverage by hepatic stellate cells. We found that in healthy tissue, JAM-A was ubiquitously expressed whereas JAM-B and JAM-C were restricted to the vasculature. During fibrosis, JAM-B and JAM-C levels increased in endothelial cells and JAM-C was *de novo* generated in myofibroblastic hepatic stellate cells. Soluble JAM-C blocked contractility but increased motility in hepatic stellate cells. Furthermore, soluble JAM-C reduced endothelial tubulogenesis and endothelial cell/stellate cell interaction. Thus, during liver fibrogenesis, JAM-B and JAM-C expression increase on the vascular endothelium. More importantly, JAM-C appears on myofibroblastic hepatic stellate cells linking them as pericytes to JAM-B positive endothelial cells. This JAM-B/JAM-C mediated interaction between endothelial cells and stellate cells stabilizes vessel walls and may control the sinusoidal diameter. Increased hepatic stellate cell contraction mediated by JAM-C/JAM-C interaction may cause intrahepatic vasoconstriction, which is a major complication in liver cirrhosis.

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Introduction

Junctional adhesion molecules (JAMs) are components of tight junctions (TJ) in epithelial and endothelial cells (ECs) and as such play a role in the regulation of cell permeability, cell polarity and leukocyte transmigration. In addition, murine JAM-C was detected on fibroblasts and smooth muscle cells. In humans JAMs are also expressed by platelets and lymphocytes. They are members of an immunoglobulin super family and consist of 3 classical JAMs (JAM-A, JAM-B, JAM-C) as well as 4 related proteins (JAM-4, JAM-L, CAR, ESAM).[1-3] The extracellular domains form homophilic (all JAMs) and heterophilic (JAM-B with JAM-C) interactions but bind also integrins: JAM-A interacts with integrin $\alpha L\beta 2$, JAM-B binds to integrin $\alpha 4\beta 1$, and JAM-C associates with integrins $\alpha M\beta 2$, $\alpha X\beta 2$ and $\alpha V\beta 3$.[3] Such interactions allow heterotypic cell associations between ECs and platelets, mural cells, cancer cells or leukocytes, the latter playing an important role during tissue homeostasis and immune cell infiltration of inflamed tissue. In fact, tissue inflammation can induce the redistribution of JAMs to the luminal side of ECs, resulting in increased arrest of leukocytes on the endothelial layer, followed by enhanced transendothelial migration.[1-6]

ECs of the hepatic vasculature form large blood vessels like the hepatic artery or the portal vein but also microvessels, the liver-specific sinusoidal channels, which are lined by unique liver sinusoidal endothelial cells (LSECs).[7] LSECs form a discontinuous barrier without classical TJs and their open fenestrations allow

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transvascular exchange between blood and the perisinusoidal space of Dissé, which is further populated by Kupffer cells and hepatic stellate cells (HSCs). The latter are considered liver-specific pericytes since several ultrastructural and physiological features of HSCs are similar to those of pericytes in other organs[7,8]: HSCs have long cytoplasmic processes, which embrace the abluminal surface of the endothelium wall surrounding the sinusoids; when activated, they differentiate into myofibroblasts, which express α-smooth muscle actin (α-SMA); and they show vasomotor activity in response to endothelin, angiotensin II or transforming growth factor-β.[9-11] Thus, HSCs influence the sinusoid diameter and stabilize vessel walls. The close proximity between LSECs and HSCs allows for paracine signaling via endothelial heparin-binding EGF-like growth factor or platelet derived growth factor-BB (PDGF-BB), which attract HSCs to vessels or via HSC-secreted vascular endothelial growth factor supporting angiogenesis.[8,11,12] These observations indicate that HSCs contribute to sinusoidal remodeling and stability, playing a role during organogenesis, regeneration, tumor angiogenesis and fibrosis.[8]

Hepatic fibrosis occurs due to chronic liver injury triggered by toxins, pathogens, metabolic- or autoimmune diseases leading to the progressive replacement of healthy hepatic parenchyma by scar-specific, collagen-rich extracellular matrix, which hinders normal tissue function.[13-16] Main producers of such fibrotic tissue are myofibroblasts, which are mostly derived from resident mesenchymal cells (HSCs and portal fibroblasts) and get activated by inflammatory mediators released during tissue damage.[13-16] Besides their immunomodulatory and phagocytic properties, myofibroblasts are highly proliferative, migratory and contractile. Due to the previously mentioned function of HSCs as mural cells, upregulated contraction in HSC-derived myofibroblasts may contribute to the increase in sinusoidal resistance and the generation of fibrosis/cirrhosis-induced portal hypertension.[8,17,18] Hepatic microcirculation in chronically damaged tissue is further impaired by fibrosis-induced closure of LSEC fenestrations, a process called capillarization.[7]

Whether JAMs play a role in the hepatic vasculature during liver fibrogenesis has not been investigated to date. Here, we report that in a mouse model of chemically-induced hepatic fibrosis, ECs upregulate JAM-B and JAM-C expression and myofibroblastic HSCs show *de novo* synthesis of JAM-C. These proteins are localized to cell-cell junctions and influence HSC motility and contractility as well as EC tube formation *in vitro*.

Results

Classical JAMs show distinct expression patterns in naïve mouse livers

To determine the expression pattern of the 3 classical JAMs, we performed immunohistochemical staining in naïve murine liver tissues. As depicted in Figure 1A, JAM-A showed a ubiquitous distribution, whereas JAM-B and JAM-C seemed restricted to cells of the vascular system. Co-staining experiments with an antibody to cytokeratin 19 (CK19, bile duct marker) revealed JAM-B and JAM-C expression in structures of portal triads (Fig. 1B) and central veins (Suppl. Fig. 1A). The anti-JAM-B antibody decorated only vascular cells, whereas the anti-JAM-C antibody stained also other cells. Due to their proximity to bile ducts and the previous finding that fibroblasts produce JAM-C, these cells likely constitute portal fibroblasts.[3,19] Since there was no co-staining of JAMs and podoplanin, a marker for ECs of lymphatic vessels, we can exclude JAM-B and JAM-C expression in the lymphatic system (Suppl. Fig. 1B). In contrast, co-staining of the endothelial marker platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31) and JAM-B or JAM-C revealed localization of both JAMs at EC borders (Fig. 1C). In fact, ECs of arteries and veins co-expressed JAM-B and JAM-C at cell-cell junctions (Fig. 2). The presence of mRNAs encoding all 3 classical JAMs in naïve rat liver ECs has been published by Géraud et al.[20]

JAM-B and JAM-C expression is upregulated during CCl_4-induced liver fibrosis

To study JAM expression in fibrotic liver tissue, we analyzed the well-established fibrosis model of chronic carbon tetrachloride (CCl_4) treatment.[21] Whereas JAM-A localization and expression were similar in fibrotic and naïve tissue (Suppl. Fig. 2), JAM-B and JAM-C staining showed remarkable features in fibrotic livers. In addition to stronger JAM-B signals on larger blood vessels, JAM-B was also detected in sinusoidal channels (Fig. 3A), where it co-localized with CD146, a protein highly expressed by LSECs.[22] Furthermore, JAM-C staining was no longer restricted to blood vessels, but dispersed deeper into the parenchyma in areas, which stained positive for α-SMA (Fig. 3B) and desmin (data not shown). Desmin and α-SMA are proteins synthesized by HSC-derived myofibroblasts.[19] Therefore, JAM-B/JAM-C-positive large blood vessels (Fig. 3C) and small channels (Fig. 3D), appeared coated by JAM-C-positive myofibroblastic HSCs, likely to act as pericytes in this situation. These findings demonstrate that in the mouse liver the

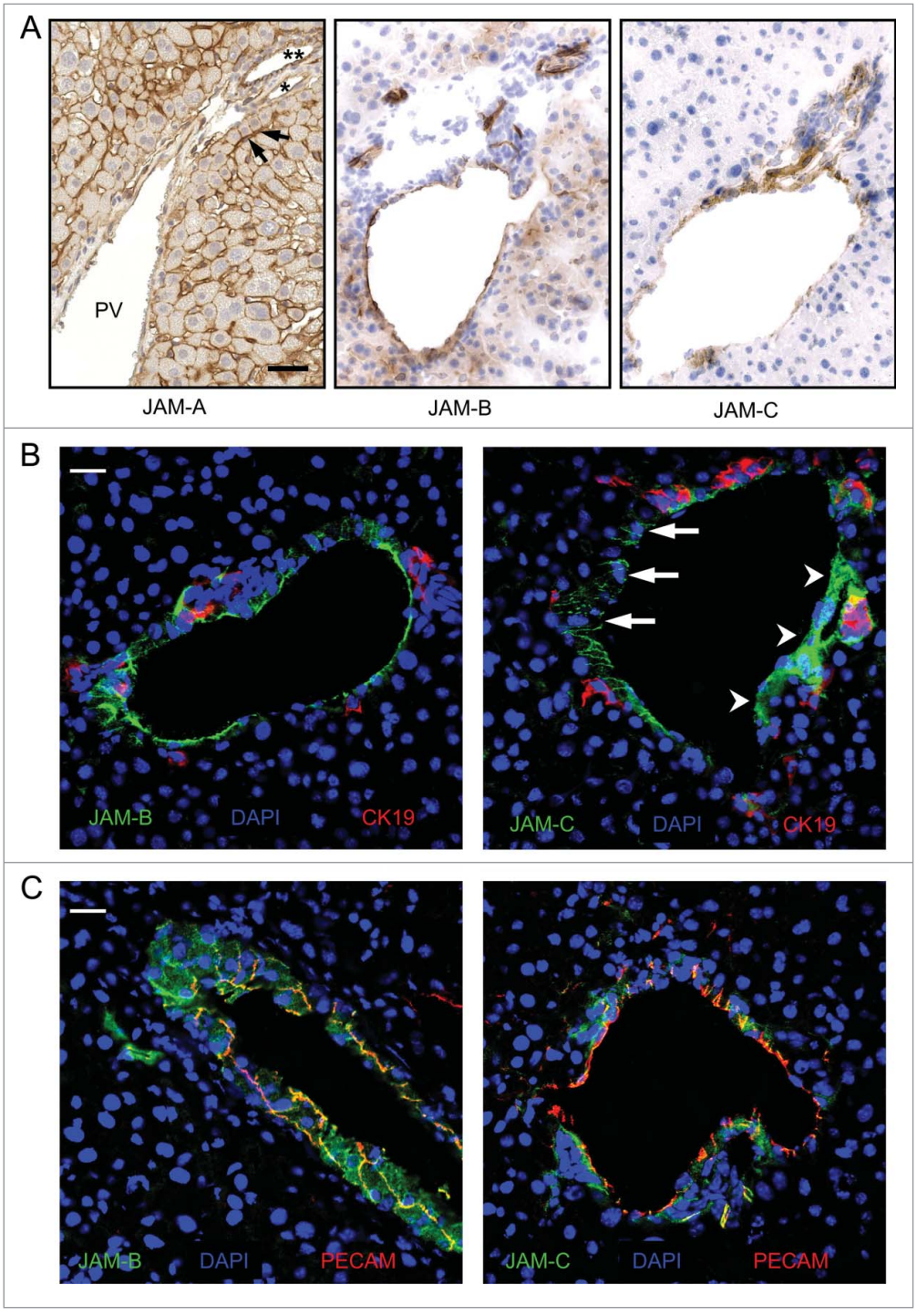

Figure 1. Expression of classical JAM proteins in the naive liver. (A) JAM-A is ubiquitously expressed in naïve mouse livers. Depicted is a portal triad with portal vein (PV), hepatic artery (*) and bile duct (**). Arrows mark a sinusoidal channel. JAM-B and JAM-C showed more restricted expression patterns than JAM-A. JAM-A was stained in HOPE fixed and paraffin embedded tissues. JAM-B and JAM-C were stained in cryosections. Bar, 50 μm. (B) JAM-B and JAM-C were visible on naive ECs of the portal triad, which was identified by the presence of CK19-positive bile ducts. Note that weaker signals at cell-cell junctions represent JAM-C-positive ECs (arrows), whereas strong cytosolic signals (arrow heads) may result from a different cell type, probably portal fibroblasts. (C) Co-localization of the endothelial marker PECAM-1 with JAM-B or JAM-C confirmed the presence of both JAMs on ECs. Bars, 20 μm. Representative pictures of liver sections of 4 mice.

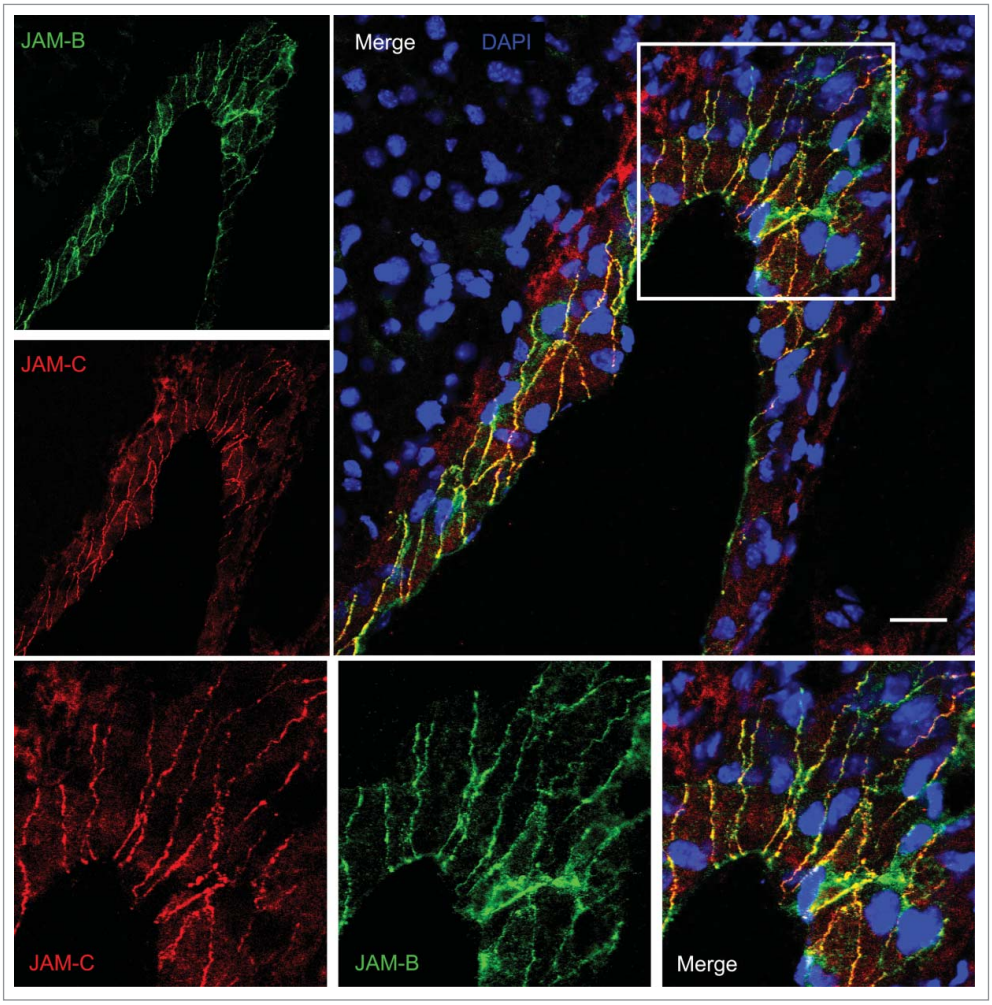

Figure 2. JAM-B and JAM-C co-localize in endothelial cell-cell junctions. Co-staining of both JAMs demonstrated that ECs express JAM-B as well as JAM-C at cell-cell junctions. Higher magnification pictures showed that JAM-B and JAM-C were present both at the cell border and in the cytosol. Bar, 20 μm. Representative pictures of liver sections of 4 mice.

development of fibrotic tissue is associated with upregulated expression of JAM-B and JAM-C in ECs/LSECs and *de novo* synthesis of JAM-C in HSC-derived myofibroblasts.

LSECs increase JAM-B and JAM-C expression during CCl_4-induced liver fibrosis

To investigate sinusoidal JAMs in more detail, we isolated LSECs, using anti-CD146 antibody-coated magnetic beads. Immunocytochemical staining (Fig. 4A and B) and flow cytometry experiments (Fig. 4C) confirmed increased expression of both JAMs in LSECs originating from fibrotic tissue in comparison to naïve LSECs. In addition, expression of PECAM-1, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) was also higher in capillarized LSECs than in naïve cells (Fig. 4C), confirming earlier observations.[7] In fact, the upregulation was similar in magnitude for all tested adhesion molecules. Furthermore, naïve and capillarized LSECs expressed more JAM-B than JAM-C. This result was observed with 2 different polyclonal anti-JAM-B or anti-JAM-C antibodies, reducing the possibility that differences in staining intensity were due to differences in antibody affinity. To test whether cell surface expressed JAM proteins indeed bind to their counter-receptors, we used JAM-B-Fc (JB-Fc) and JAM-C-Fc (JC-Fc) fusion proteins as binding partners in flow cytometry experiments. Figure 4D shows that freshly isolated LSECs bound both JB-Fc and JC-Fc. Binding was higher in cells originating from fibrotic than from naïve livers. This indicates that the fraction of JAM proteins which was produced during fibrosis is indeed functional. Using polyclonal anti-JAM-B or anti-JAM-C antibodies, we then tried to block heterophilic JAM interactions. In fact, the anti-JAM-C antibody reduced binding of JB-Fc (Fig. 4E), whereas the

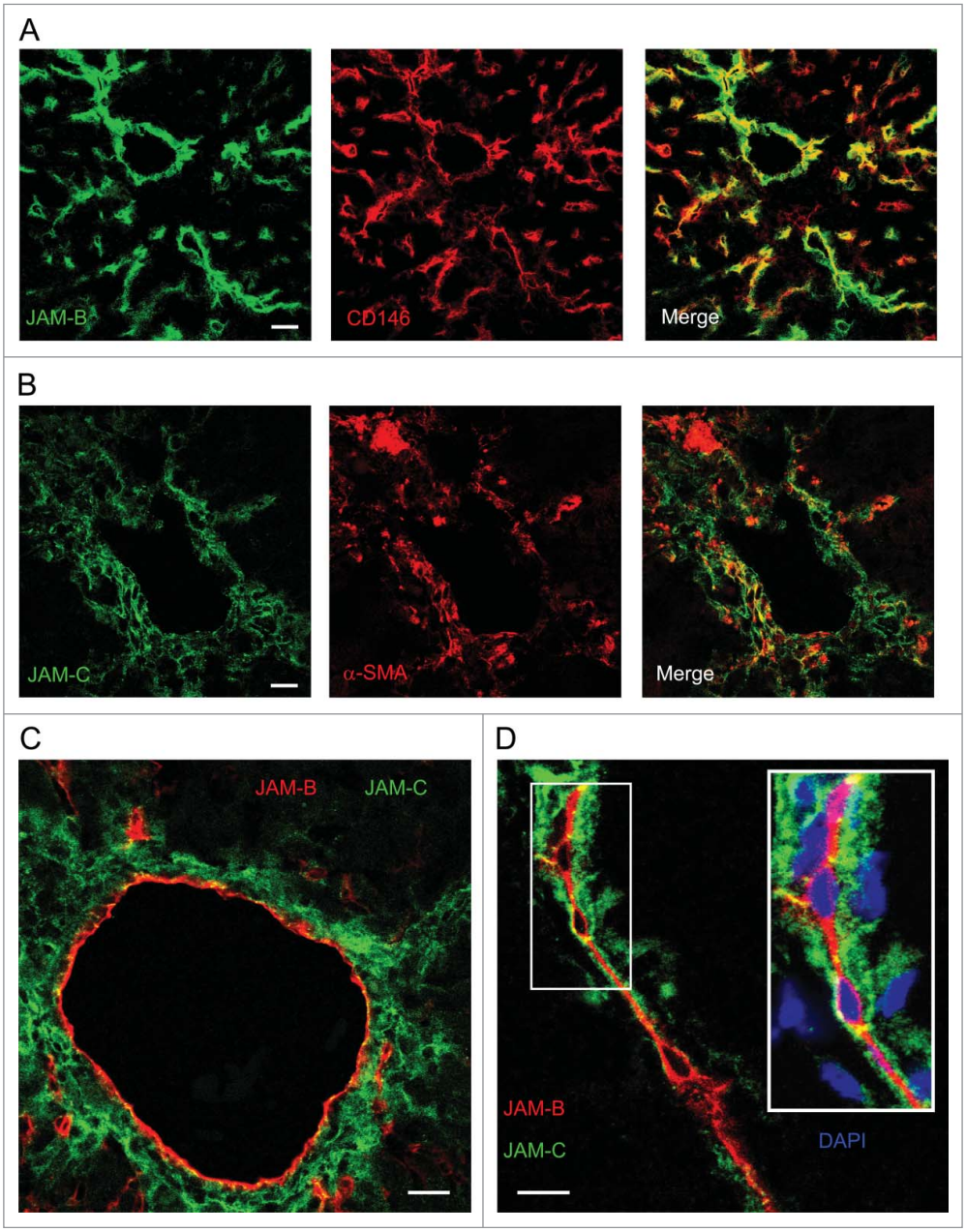

Figure 3. JAM-B and JAM-C levels increase in the fibrotic mouse liver. Livers were collected after 4 weeks of CCl_4 treatment. (A) In fibrotic tissue JAM-B staining was strong on large blood vessels and was highly upregulated on sinusoidal channels. ECs and LSECs were visualized with an antibody to CD146. (B) Fibrosis induction led to *de novo* synthesis of JAM-C by α-SMA-positive myofibroblastic HSCs. (C) Such activated HSCs acted as pericytes covering both (C) large and (D) small blood vessels. Bars, 20 μm. Representative pictures of liver sections of 5 mice.

anti-JAM-B antibody interfered with JC-Fc binding (Fig. 4E). These results suggest that LSEC-expressed JAM-B and JAM-C interact heterophilically.

Upon activation hepatic stellate cells differentiate into JAM-C-positive myofibroblasts

Fibrosis-triggered JAM-C expression by cells co-expressing α-SMA (Fig. 3B) suggested the involvement of myofibroblasts. To test this, we isolated HSCs from livers of CCl_4-exposed mice and kept them in culture for one week to get fully activated myofibroblasts. Indeed, these cells expressed JAM-C at cell-cell junctions (Fig. 5A) but were negative for JAM-B (data not shown). Using JAM-Fc fusion proteins and flow cytometry, we then analyzed whether JAM-C expressed by myofibroblasts is functional. To exclude contaminating cells, we gated myofibroblasts for PDGF receptor β (PDGF-Rβ) expression.[15,23] As depicted in Figure 5B, PDGF-Rβ-positive myofibroblasts were able to bind JB-Fc strongly and showed also a weak

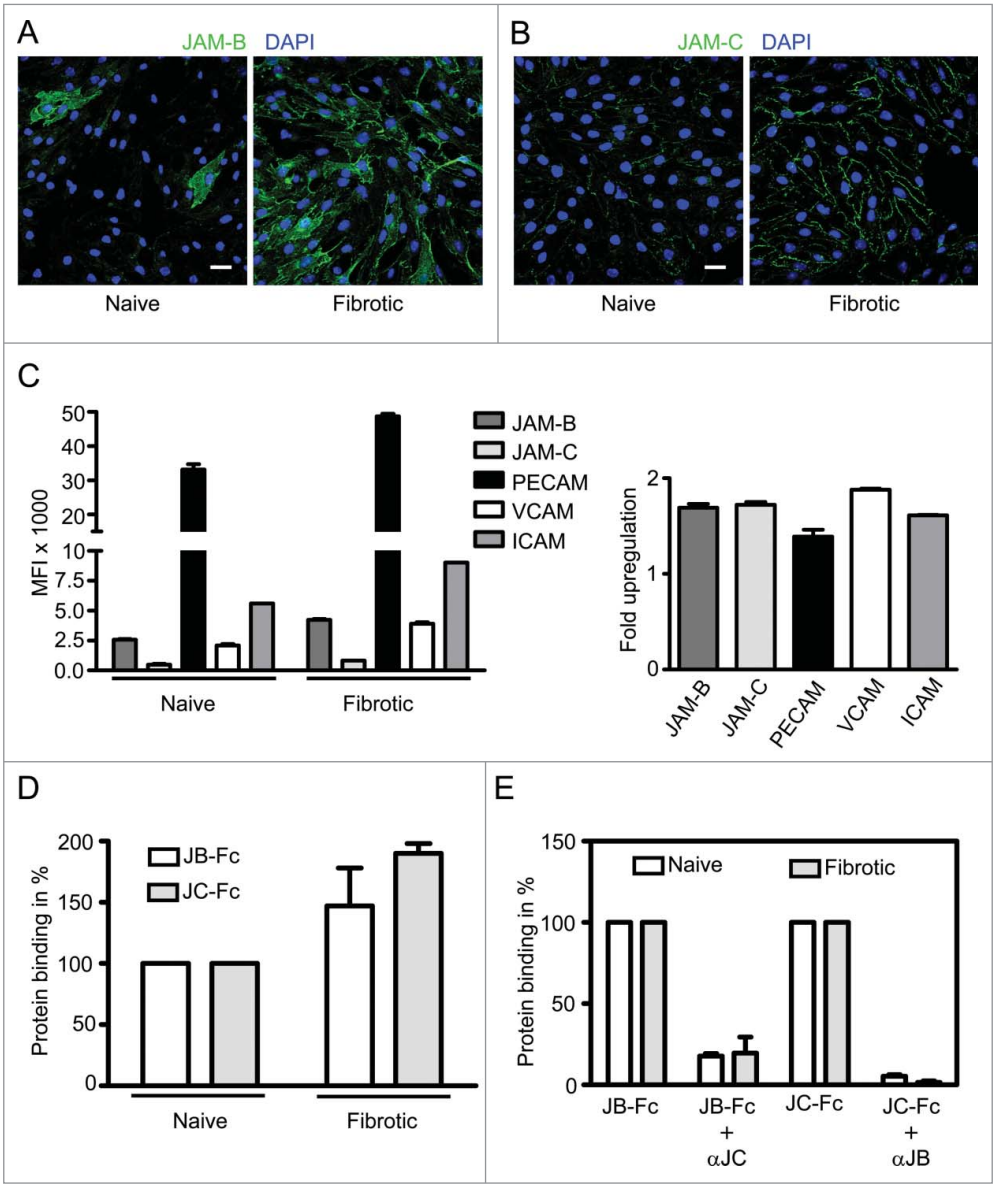

Figure 4. Endothelial JAM proteins are functional and interact heterophilically. (A, B) CD146 affinity-purified LSECs isolated from up to 6 naïve or fibrotic livers were kept in culture for 40 hours before analysis. Immunocytochemical staining revealed upregulation of JAM-B (A) and JAM-C (B) in fibrosis-activated cells. Bar, 20 μm. (C) Fibrosis-triggered increased cell surface expression was confirmed by flow cytometry with LSECs freshly isolated from up to 12 mice. Depicted is mean ± range of mean fluorescence intensity (MFI) after staining of 2 independently purified cell pools for different adhesion molecules. Fibrosis-driven upregulation was similar for all tested proteins. (D) JAMs present on naïve and activated LSECs are functional as the capacity of binding soluble JAM-Fc fusion proteins (JB-Fc or JC-Fc) parallels JAM expression levels shown in (C). Results are mean ± SD, n = 3. (E) Furthermore, binding of JB-Fc was blocked by a polyclonal rabbit antibody to JAM-C (αJC) and binding of JC-Fc was inhibited by a polyclonal rabbit antibody to JAM-B (αJB), indicating heterophilic interactions between JAM-B and JAM-C. Results are mean ± SD, n = 3.

interaction with JC-Fc. JB-Fc binding was blocked with a polyclonal anti-JAM-C antibody, whereas low affinity monoclonal blocking antibodies to JAM-C (H33) or integrin β1 had no such effect. Immunocytochemistry data revealed that JAM-C co-localized with the TJ protein ZO-1 (Fig. 5C) and the adherens junction protein α-catenin (data not shown). Analyzing M1-4HSCs, a cell line derived from activated murine HSCs,[24] we got similar findings: M1–4HSCs expressed JAM-C at cell-cell junctions (Fig. 5D) where it co-localized with α-catenin (Suppl. Fig. 3A) and ZO-1 (data not shown) and was able to interact specifically with JB-Fc (Fig. 5E). Furthermore, LX2, a human HSC-derived myofibroblast cell line, was also JAM-C-positive, both in immunocytochemical staining (Suppl. Fig. 3B) and in flow cytometry (data not shown). Taken together, our results show that HSC-

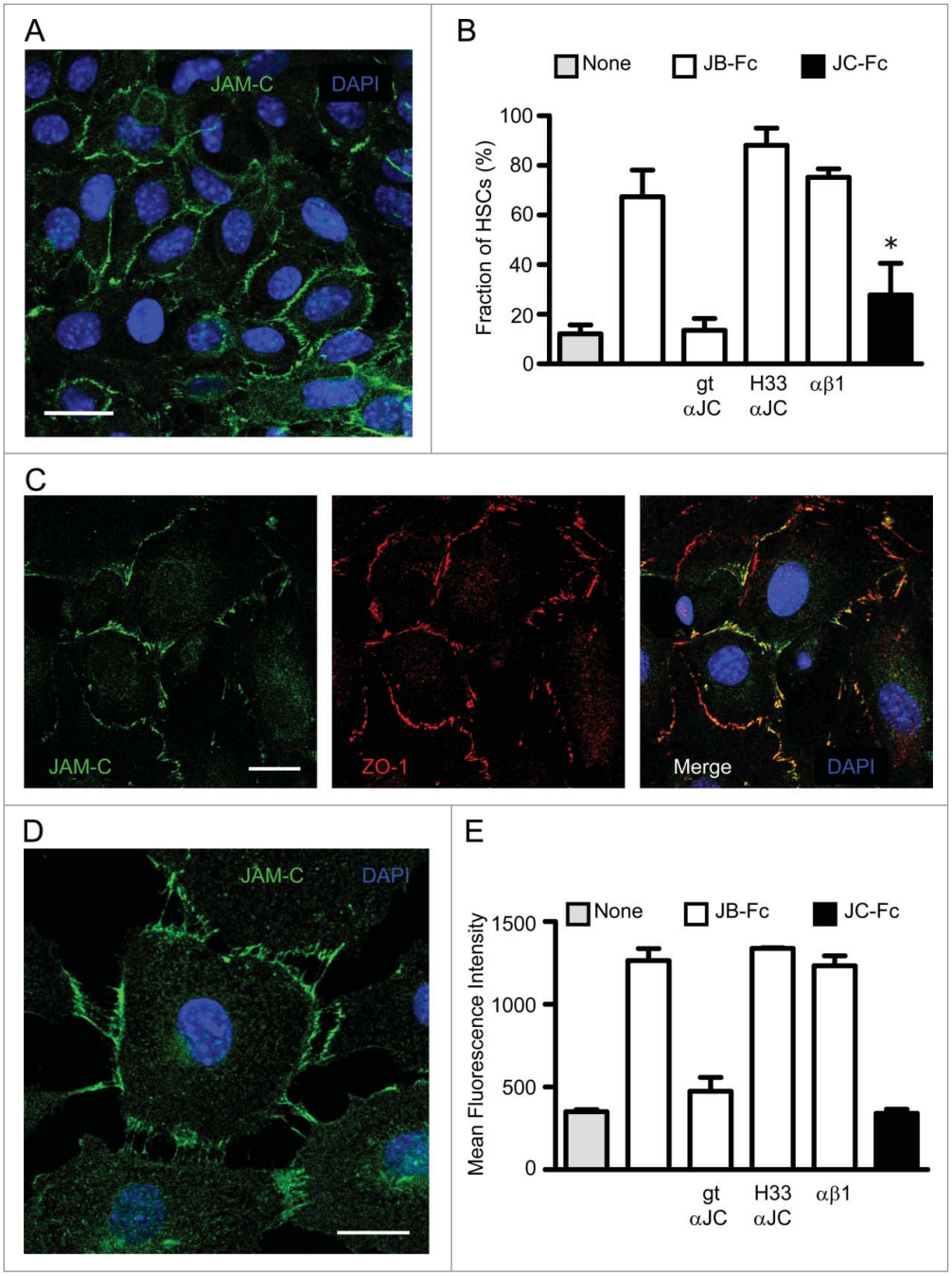

Figure 5. JAM-C expressed by activated HSCs is present in cell junctions and binds soluble JAM-B-Fc fusion protein. (A) HSCs isolated from livers of 6 CCl_4-exposed mice were kept in culture for 7 d to become fully activated myofibroblasts. Immunocytochemical staining revealed JAM-C expression at HSC cell-cell contact sites. Bar, 20 μm. (B) *In vitro* activated PDGF-Rβ-positive HSCs were tested for their capacity to bind soluble JAM-Fc proteins by flow cytometry. Depicted is the fraction of PDGF-Rβ-positive HSCs interacting with JAM fusion proteins. JB-Fc was bound specifically by JAM-C since this interaction was blocked by a polyclonal goat anti-JAM-C antibody (gt αJC). Monoclonal antibodies to JAM-C (H33) and β1 integrin ($\alpha\beta$1) had no inhibitory effect. Homophilic interaction between JAM-C and JC-Fc was too weak to show significant binding (*, 2-tailed Mann-Whitney test, p = 0.108). Results are mean $\pm$ SD, n = 3. (C) JAM-C co-localized with the TJ protein ZO-1. Depicted are primary HSCs as in (A). Bar, 20 μm. (D) In the mouse cell line M1–4HSC JAM-C staining appeared at cell-cell contact sites. Bar, 20 μm. (E) Binding of PDGF-Rβ-positive M1–4HSCs to JB-Fc and JC-Fc in presence or absence of anti-JAM-C antibodies (αJC) was analyzed by flow cytometry. Note that binding of JB-Fc was only blocked by the polyclonal anti-JAM-C antibody (gt αJC). JC-Fc binding was not detectable. Shown is mean fluorescence intensity of cells that have bound JAM fusion protein. Results are mean $\pm$ SD, n = 3.

derived myofibroblasts from fibrotic livers of CCl_4 exposed mice express functional JAM-C at the cell surface. These JAM-C molecules may bind to JB-Fc fusion protein and therefore might be involved in mediating heterotypic or homotypic cellular interactions.

Hepatic myofibroblast motility and contractility are influenced by JAM-C

Next we sought to identify the role(s) JAM-C might play in myofibroblastic HSCs. Immunocytochemical analyses of M1-HSCs and activated HSCs exposed to JAM-Fc fusion proteins revealed that JAM-C localization at cell-cell contact sites was reduced. In fact, JAM-C staining at cell junctions was weaker in M1–4HSCs and HSCs treated with JC-Fc compared to untreated cells (Fig. 6A). In HSCs, also JB-Fc treatment decreased JAM-C levels at the cell border (Fig. 6A). Thus, JAM-Fc fusion proteins impair the formation of JAM-C homodimers between adjacent cells. When adhesion between neighboring cells is reduced it is likely that the motility of individual cells increases and that the contractile force of the cellular collective subsides. Indeed, in scratch migration assays, M1–4HSCs showed increased motility when they were treated with JC-Fc (Fig. 6B) in comparison to untreated cells. Furthermore, we performed contractility assays with activated HSCs or M1–4HSCs cultured on collagen I gel blocks in the presence or absence of JAM-Fc fusion proteins. When JC-Fc was added contractility was significantly reduced in both cell types (Fig. 6C). JB-Fc blocked contractility in activated HSCs only (Fig. 6C). The generation of tensile force in myofibroblasts depends on α-SMA-containing stress fibers, which develop also during HSC activation.[14] Co-staining experiments in myofibroblastic HSCs showed that JAM-C was located at the terminal portion of such α-SMA-positive stress fibers (Suppl. Fig. 3C). Finally, we tested whether homophilic or heterophilic binding of JAM-C has an impact on *de novo* α-SMA expression. To this end, we cultured freshly isolated HSCs in the presence of JAM-Fc fusion proteins to mimic formation of cell-cell contacts when cell density was still low. After one week *in vitro* activation, α-SMA protein levels were determined proportional to the expression of glial fibrillary acidic protein (GFAP), which is a marker for quiescent and activated HSCs.[19] We observed a slight increase in α-SMA expression when cells were treated with JC-Fc (Fig. 6D), suggesting that JAM-C mediated cell-cell adhesion may potentiate stress fiber formation. Using the same conditions, M1–4HSCs cells showed no difference in α-SMA protein levels (data not shown). Based on these results we conclude that JAM-C is involved in mediating homotypic HSC cell-cell adhesion and in coordinating HSC contractility.

JAM-B and JAM-C participate in LSEC/HSC interactions during the formation of LSEC vascular structures in 3D cultures

Due to the pericyte function of HSCs in hepatic sinusoids, we next wanted to investigate whether JAMs mediate LSEC/HSC interactions. Therefore, we analyzed the impact of myofibroblastic HSCs on LSEC tube formation. To this end, LSECs expressing green-fluorescent protein (GFP) and HSCs expressing *Discosoma* red-fluorescent protein (DsRed) were cultured in 3-dimensional (3D) matrigel matrices either separately or as a 1:1 mixture. Under the chosen conditions, LSECs attracted HSCs, as published for other EC/pericyte systems.[8,25] HSCs cultured alone showed a uniform distribution, whereas in co-cultures HSCs were observed at sites where LSECs were located (Fig. 7A). Furthermore, LSECs appeared to form longer and more branching tube networks when HSCs were present (Fig. 7B), demonstrating that activated HSCs indeed act as pericytes, stabilizing sinusoidal channels. However, stabilization worked only temporarily, as tube networks started to collapse over time (Suppl. Fig. 4). Nevertheless, the experimental set-up allowed us to determine the total length of the LSEC tube network formed within 5hrs and to count LSEC branches covered by HSCs (Fig. 7C). The collected data showed that the tube network was indeed longer when LSECs were co-cultured with HSCs (Fig. 7D). In the presence of JAM-FLAG fusion proteins, JC-FLAG but not JB-FLAG reduced network length in LSEC monocultures and in LSEC/HSC co-cultures (Fig. 7D). Furthermore, JC-FLAG reduced the number of LSEC tubes which showed associated HSCs (Fig. 7E), suggesting that LSEC/HSC adhesion depends partially on JAM-B/JAM-C binding. Taken together, our data demonstrate that JAM-B and JAM-C are involved in mediating LSEC-HSC cell-cell interactions.

Discussion

Our study represents the first detailed analysis of classical JAM expression in the vasculature of naïve and fibrotic mouse liver. We identified JAM-A as a broadly distributed protein expressed for example on ECs like LSECs, on hepatocytes or on biliary epithelial cells. The much stronger expression of JAM-A over JAM-B and JAM-C in the naïve murine liver confirms former Northern blot data.[26] Yet, JAM-B and JAM-C

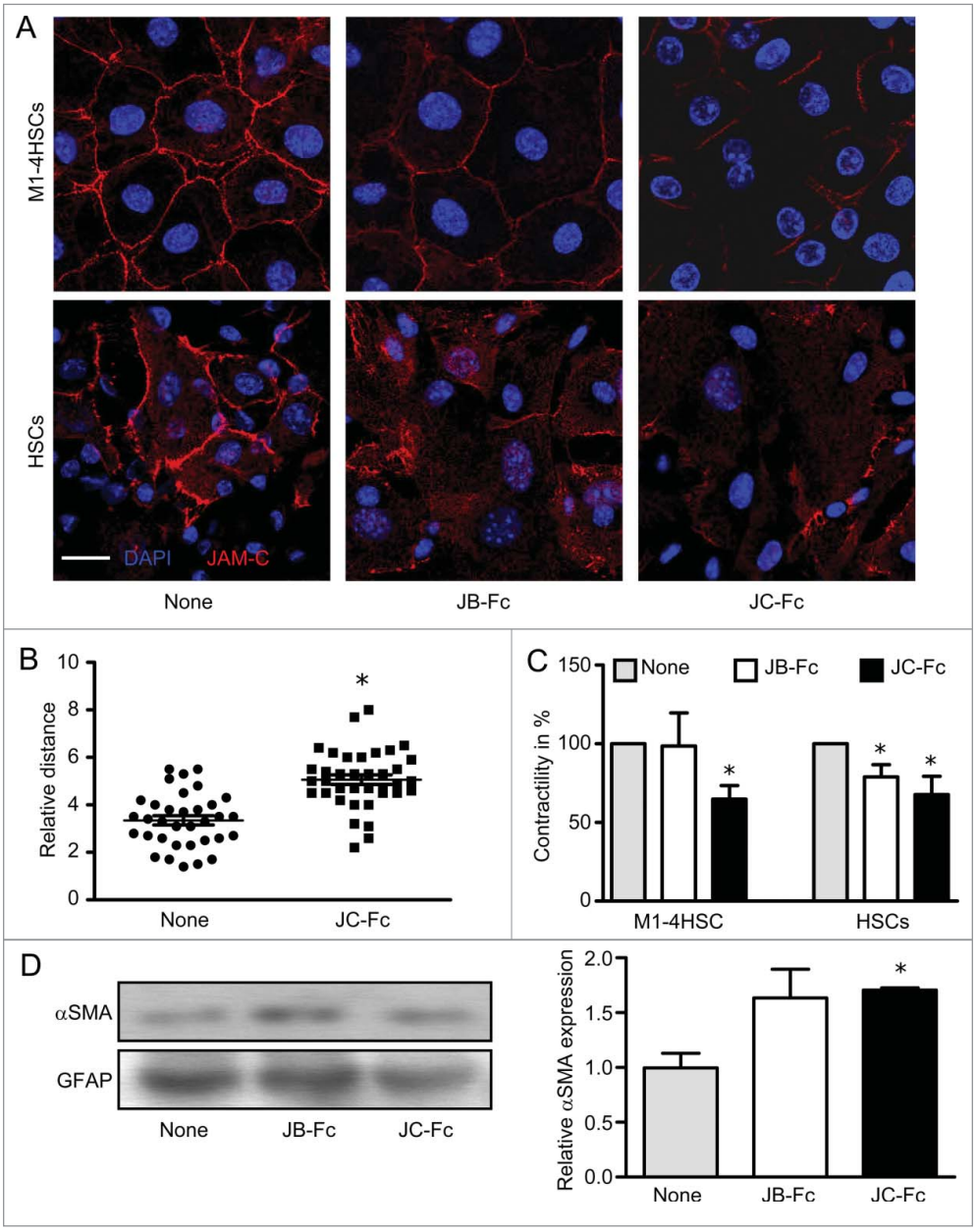

Figure 6. Motility and contractility of myofibroblastic HSCs are influenced by JAM-C. (A) M1–4HSCs or myofibroblastic HSCs were cultured for 20 hrs in the absence (none) or presence of 20 μg/ml JAM-Fc fusion proteins. Treatment with JB-Fc and even more so with JC-Fc reduced JAM-C staining at cell-cell contact sites. Bar, 20 μm. Representative of n = 3. (B) In scratch migration assays, confluent M1–4HSC monolayers were wounded and further incubated in the absence (none) or presence of 20 μg/ml JC-Fc fusion protein. Wound width was determined at several specified locations before and after incubation with JC-Fc. Depicted is mean ± SEM of relative distance migrated in 24 hrs. Cells treated with JC-Fc migrated significantly farther (*, 2-tailed Mann-Whitney test, $p < 0.05$). Representative of n = 3. (C) In contractility assays, M1–4HSCs or activated HSCs were cultured on collagen I gels in the absence (none) or presence of 20 μg/ml JAM-Fc proteins. Area contracted by control cells (none) was set as 100% contractility. Contractility was significantly reduced by JC-Fc in M1–4HSCs and by both JAM-Fc proteins in primary HSCs (*, 2-tailed Mann-Whitney test, $p < 0.05$). Results are mean ± SD, n = 3. (D) Primary HSCs were *in vitro* activated for one week in the absence (none) or presence of 20 μg/ml JAM-Fc fusion proteins. Total protein extracts were analyzed for the expression of α-SMA and GFAP by immunoblotting. Expression levels of α-SMA relative to GFAP levels is shown in the bar graph. Treatment with JC-Fc increased α-SMA levels significantly (*, 2-tailed Mann-Whitney test, $p < 0.05$). Results are mean ± SD of 2 assays done in triplicates.

expression was restricted to the portal tract and the central vein. Both proteins were synthesized by ECs of the vascular - but not the lymphatic system. Upon chronic tissue damage by CCl_4, neither the expression pattern nor level of JAM-A were influenced. This stands in contrast to JAM-B and JAM-C, whose expression on ECs was increased on large blood vessels but also on sinusoidal channels. The latter are major sites of leukocyte

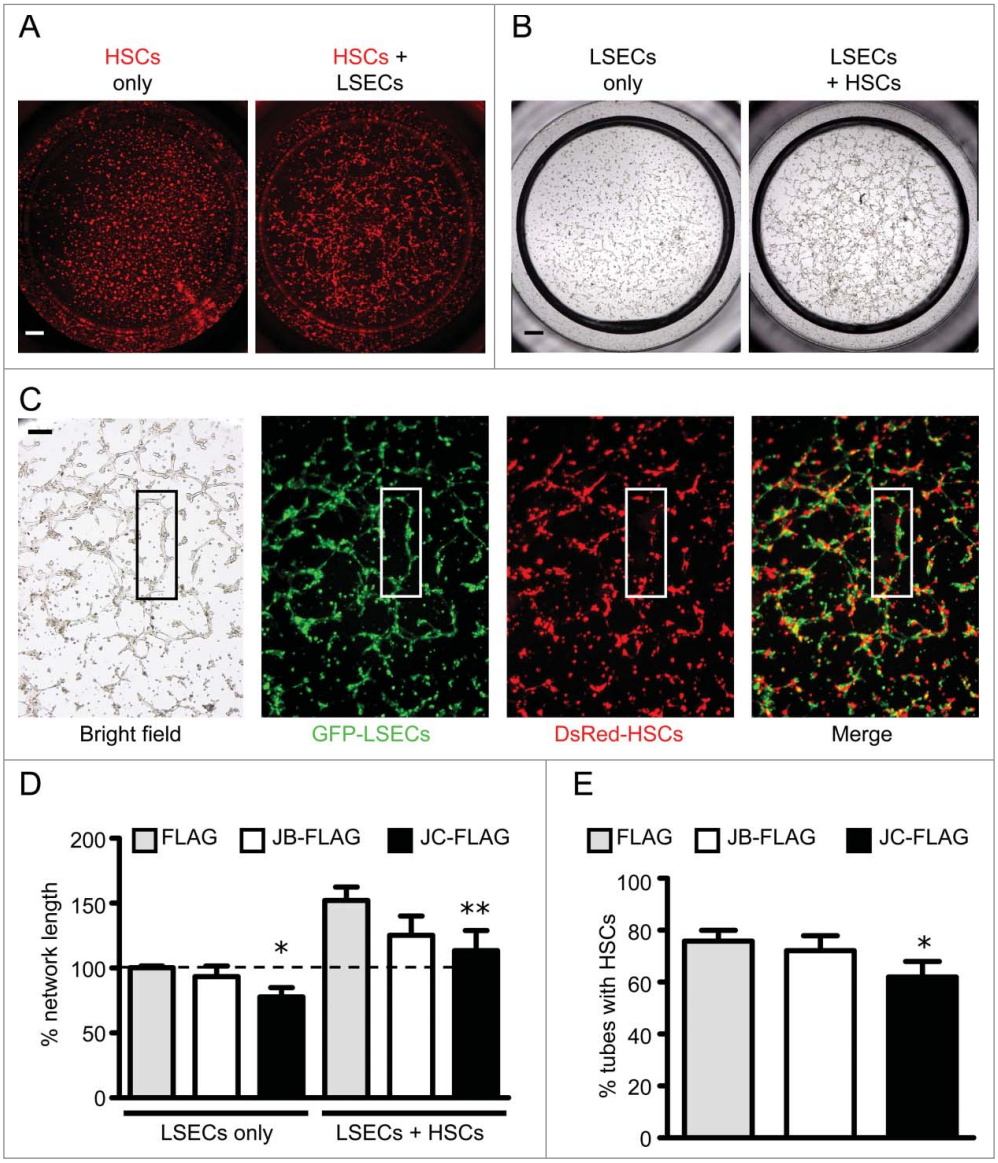

Figure 7. Blockade of JAM-B and JAM-C reduces LSEC/HSC interactions in 3D matrigel cultures. (A- C) HSCs expressing *Discosoma* red-fluorescent protein (DsRed) were kept in 3D matrigel cultures for 5 hrs in the absence or presence of LSECs expressing green-fluorescent protein (GFP). (A) When seeded alone, HSCs were randomly distributed and formed large aggregates. In the presence of LSECs, HSCs showed a targeted orientation. (B) LSECs formed more and longer tube-like structures when HSCs were present. (C) When co-cultured, HSCs (red) acted as pericytes, gathering along LSEC tubes (green). Bars, 400 μm and 200 μm. Representative pictures of 4 experiments done in duplicates. (D) LSECs and HSCs were treated with FLAG peptide or individual JAM-FLAG fusion proteins (JB-FLAG and JC-FLAG) at 40 μg/ml before they were mixed at a 1:1 ratio and used to prepare 3D matrigel cultures. Pictures were taken after 5 hrs incubation. The length of each individual tube was determined. Values were added up to calculate the total length of the tube network per well. Total network length of LSECs treated with FLAG peptide was set at 100%. Results are mean $\pm$ SD, n = 3. JC-FLAG reduced total network length in LSECs alone (*, $p < 0.05$) and when both cells types were mixed (**, $p < 0.1$) (2-tailed Mann-Whitney test). (E) Percentage of individual LSEC tubes which showed attachment of one or more HSC was calculated. Results are mean $\pm$ SD of 2 assays done in triplicates. In the presence of JC-FLAG less HSCs were bound to LSEC tubes. (*, two-tailed Mann-Whitney test, $p < 0.05$).

extravasation in the inflamed liver.[7] Therefore, upregulation of adhesion molecules involved in leukocyte capture and transendothelial migration allows selective recruitment of leukocytes to the damaged tissue. Enhanced JAM-C expression during inflammatory diseases has been reported for the pancreas[27,28] and the involvement of both JAMs in leukocyte recruitment has been well-documented.[6,28-31] Therefore, we suggest that upregulation of JAM-B and JAM-C by ECs/LSECs during fibrogenesis is involved in the recruitment of immune cells to the chronically injured liver.

Novel is the finding that HSCs start to express functional JAM-C during their myofibroblastic differentiation process. PDGF-Rβ, α-SMA, ICAM-1 or VCAM-1

are other proteins known to be upregulated during HSC activation.[15,32] We found JAM-C localized at HSC cell-cell junctions. When JAM-C function was blocked by soluble JAM-Fc fusion proteins, cells were less well incorporated into a cellular collective. Therefore, they migrated farther after scratch wounding but transmitted contractile force less well. During HSC activation, the cells become more motile and increase their contractility.[13-16] These changes may not necessarily occur simultaneously but may happen sequentially. In our hands, soluble JC-Fc fusion protein blocked HSC contractility more efficiently than JB-Fc, suggesting that JAM-C expressed by myofibroblastic HSCs interacts preferentially in a homophilic way. This is surprising since our binding studies showed the opposite result. JB-Fc was easily bound by HSCs and M1-4HSC, whereas binding of JC-Fc was marginal and only detected with HSCs. Furthermore, Lamagna et al. showed that JAM-C/JAM-B interaction is stronger than JAM-C homophilic binding.[33] However, contractility regulation does not simply depend on proper protein interaction at the cell surface but needs also well-orchestrated intracellular signal transduction in line.[11] Thus, only JAM-C homophilic binding may provide the structural requirements for proper downstream control of HSC contractility.

Since naive and activated HSCs act as liver-specific pericytes and as such cover sinusoidal channels, it is tempting to speculate that myofibroblastic HSCs and LSECs could interact via JAM-C/JAM-B binding. Therefore, upregulation of JAM-B and JAM-C in chronically injured tissue may represent a fibrosis-driven mechanism for stronger association between LSECs and their pericytes, in addition to strengthened homotypic binding of HSCs or LSECs and increased LSEC/leukocyte interaction. The fact that capillarized LSECs express more JAM-B than JAM-C guarantees production of enough JAM-B to interact simultaneously with JAM-C expressed on neighboring LSECs, with integrin $\alpha4\beta1$ on leukocytes and with JAM-C present on HSCs. Interestingly, our data suggest that JAM-B/JAM-C binding involves different protein domains in HSC/LSEC interactions than during LSEC/LSEC binding: Inhibition of JB-Fc binding by HSCs required a polyclonal anti-JAM-C antibody and was not possible with H33, a low affinity monoclonal antibody which was reported to block JAM-B/JAM-C interaction in other experimental systems.[33-35] In the fibrotic liver, cell-type specific structural requirements for *trans* JAM-B/JAM-C heterophilic adhesion may trigger distinct intracellular signaling pathways allowing LSECs to discriminate between binding to adjacent LSECs and adhesion to pericytic HSCs.

The crosstalk between ECs and pericytes plays a fundamental role during vascular morphogenesis and involves, additional to direct cell-cell contact, also the release of soluble factors as well as extracellular matrix assembly and remodeling.[36] We demonstrate that capillarized murine LSECs form tube-like structures in 3D matrigel matrices and that the tube network is stabilized in the presence of HSCs, confirming the pericyte function of activated HSCs.[8,37] Furthermore, tube formation was reduced in the presence of soluble JC-FLAG, demonstrating that disruption of JAM-C function interferes with EC assembly during tubulogenesis. This is in line with the finding that soluble JAM-C and anti-JAM-C IgG H33 reduce hypoxia-induced angiogenesis in the retina[38,39] and that H33 interferes with tumor vascularization.[38] Sacharidou et al. demonstrated that JAM-B and JAM-C are needed for human EC lumen and tube formation since both proteins are components of a signaling complex, which controls tubular morphogenesis.[40] Importantly, we show that soluble JC-FLAG interferes with binding between LSECs and HSCs, as the number of HSCs associated with LSEC tubes was reduced in the presence of JC-FLAG. Mural lining of LSECs by HSCs may control sinusoidal blood flow rate, since activated HSCs can respond to vasoactive substances thereby changing the sinusoidal diameter.[7,8] Analysis of JAM-C-deficient mice suggested that JAM-C expressed by smooth muscle cells is involved in esophageal or bronchial contraction control, as in the absence of JAM-C a mega-esophagus developed.[41] Here, the detection of JAM-C expression in differentiating HSCs, which are characterized among other things by increased contractile forces[11] together with our finding that soluble JAMs reduce HSC contractility *in vitro* indicate that JAM-B and JAM-C may play a role in pericyte-mediated contraction of sinusoidal channels. Such sinusoidal remodeling may lead to intrahepatic vasoconstriction, resulting in increased hepatic vascular resistance, causing portal hypertension, a major complication of cirrhosis.[18]

Materials and methods

Mice and CCl_4 treatment

C57BL/6 mice were from Harlan (Horst, Netherlands). C57BL/6 mice expressing GFP or DsRed under the actin promoter were from mfd Diagnostics (Wendelsheim, Germany). Mice were handled in strict accordance with good animal practice. Animal work was approved by the local Ethics Animal Review Board (Darmstadt, Germany). Carbon tetrachloride (CCl_4) was from Sigma (02671). CCl_4 treatment in 6 - 8 weeks old animals was performed twice weekly by intraperitoneal injection of 5μl CCl_4 diluted 1:20 in corn oil for 4 weeks.

Cell lines and cell isolation

The mouse cell line M1-4HSC was provided by W. Mikulits (Institute of Cancer Research, Medical University of Vienna, Vienna, Austria) and the human cell line LX-2 was from S.L. Friedman (Division of Liver Disease, Mount Sinai School of Medicine, New York, USA).

HSCs were isolated by the pronase-collagenase method and a 12% Nycodenz (Sigma; D2158) gradient as described[24] and were cultured for 1 week to get fully activated. LSECs were isolated as described[42]: Livers of 6 - 12 mice were digested with collagenase (Serva; 17465.01) and DNase (Sigma; DN-25), non-parenchymal cells were centrifuged in a 17% Optiprep (Sigma; D1556) gradient and LSECs were enriched, using anti-CD146 IgG-coated magnetic beads (Miltenyi Biotec; 130-092-007). LSECs were cultured on collagen I-coated (20 μg/ml) surfaces.

Generation of soluble JAM-FLAG proteins and polyclonal anti-JAM antibodies

Constructs encoding the extracellular domains of JAM-B or JAM-C fused to the FLAG-tag[35] were used to transfect COS7 cells. Conditioned media were collected and fusion proteins JB-FLAG or JC-FLAG were purified with anti-FLAG affinity resin (Sigma; A2220). Rabbit immunizations were performed at Coring Systems Diagnostix (Gernsheim, Germany). Antibodies were affinity purified using Protein G Sepharose (GE Healthcare; 17-0618-01). Alexa488-labeled IgGs were generated using a labeling kit (Life Technologies; A20181).

Flow cytometry and JAM-Fc binding experiments

Freshly isolated LSECs or activated HSCs were stained using the following antibodies: anti-JAM-B-Alexa488 and anti-JAM-C-Alexa488 (see above), anti-ICAM2-eFluor450, anti-PECAM-PerCP-eFluor710 and anti-VCAM-eFluor660 (eBioscience; 48-1021-80, 46-0311-80, 50-1061-80) and anti-PDGF-Rβ-PE (BioLegend; 136005). To test JAM-Fc binding, cells were first incubated with anti-JAM-C IgG H33,[35] goat anti-mouse JAM-C IgG (R&D Systems, AF1213), blocking anti-β1 integrin IgG (BioLegend, 102209) or our own IgGs to JAM-B or JAM-C, all at 20 μg/ml. Then, soluble recombinant mouse JAM-B or JAM-C each fused to human IgG1 Fc (R&D Systems, 988-VJ-050, 1213-J3-050) were added: JB-Fc at 1 μg/ml and JC-Fc at 5 μg/ml. Fusion protein binding was detected with FITC-labeled donkey anti-human IgG (Jackson ImmunoResearch, 709-095-149) and analyzed with a FACSCantoII flow cytometer, using BD-DIVA software (BD Biosciences).

Immunohistochemistry and microscopic analysis

Cryosections (7μm) were stained with the following primary antibodies: goat anti-mouse JAM-A (R&D Systems, AF1077), rabbit anti-cytokeratin 19 (TROMA-III, DSHB), rat anti-mouse CD31 (BD Biosciences, 557355), rat anti-mouse CD146 (Miltenyi Biotec, 130-092-025), rabbit anti-ZO-1 (Life Technologies, 40-2300), goat anti-α-E-catenin (Santa Cruz Biotechnology, sc-1495) and goat anti-mouse podoplanin (R&D Systems, AF3244). For co-staining experiments, secondary IgGs were Alexa594-conjugated or Alexa488-conjugated (Jackson ImmunoResearch, 712-095-153; Life Technologies, A-21206, A11058). Nuclei were identified with DAPI (Sigma, D8417). α-SMA was stained with mAb 1A4 (DakoCytomation, M0851) and the MOM kit (Vector laboratories, BMK-2202). Where indicated, livers were processed for HOPE® fixation and embedding (DCS Innovative Diagnostik-Systeme). Brightfield pictures were taken with an Axiocam camera on a Axioscope microscope (10x and 20x objectives), using Axiovision software (Zeiss). Fluorescent signals were analyzed using a LSM510 META confocal microscope with a 40x oil objective and ZEN software (Zeiss). Photoshop software was used for image processing.

Protein Immunoblotting

Primary HSCs or M1-4HSCs were cultured for 1 week in the presence of 20 μg/ml JAM-Fc proteins (R&D Systems, 988-VJ-050, 1213-J3-050). Total protein extracts were prepared in PBS containing 1 % Triton X-100, 10 % glycerol, 1 % sodiumdeoxycholate, 0.1% SDS, 6 mM EDTA and complete EDTA-free protease inhibitor cocktail (Roche Diagnostics, 11873580001). Equal protein amounts were resolved on a 10% SDS-polyacrylamide gel. Immunoblots were incubated overnight at 4°C with mouse anti-α-SMA antibody (DakoCytomation, M0851). Alkaline phosphatase-conjugated secondary antibody (Bio-Rad, 170-6520) was visualized with ECF substrate Vistra (Amersham Biosciences, RPN5785) and the Pharos FX Plus imaging system (Bio-Rad). Sequentially, blots were incubated with rabbit anti-GFAP antibody (DakoCytomation, Z0334) and alkaline phosphatase-conjugated secondary antibody (Bio-Rad, 170-6518). Signal intensity was quantified using Quantity One software (BioRad).

Contractility assay

Cells were seeded in duplicates in 24-well plates on 0.5ml gel lattices prepared as described.[43] After 2 days, cells were washed 4 times with DMEM/1% BSA and JAM-Fc proteins (R&D Systems, 988-VJ-050, 1213-J3-050) at

20 μg/ml were added. Two hours later, gels were dislodged with a 27 G needle and pictures were taken (Gel Doc, Bio-Rad). Plates were incubated at 37°C for 18 hrs before pictures were taken again. Contraction was monitored as change in lattice area over time, using Quantity One software (BioRad). Area reduction observed in control wells (no JAM-Fc protein added) was used as measure for 100% contraction.

Migration assay

Scratch migration assay: Confluent M1-4HSC layers in 24-well plates in duplicates were wounded with a pipet tip. Monolayers washed 2 times with DMEM/1% BSA were incubated for 30 min at 37°C and were washed again before 8 pictures per wound were taken. Then, JC-Fc (R&D Systems, 1213-J3-050) was added (40 μg/ml) for 24 hrs, before the same 8 locations were photographed again. Scratch width was measured at several positions in each picture and relative distances migrated during 24 hrs were determined.

3D Matrigel tube formation assay

Angiogenesis μ-slides (ibidi) were used to set up a 3D matrigel system. Briefly, 10μl growth factor-reduced, phenol red-free matrigel (Corning, 356231) were added. While matrigel got set, single-cell suspensions of GFP-LSECs and activated DsRed-HSCs were prepared in complete phenol red-free RPMI medium. Cells were treated with JAM-FLAG proteins or FLAG peptide (40 μg/ml; genscript, RP10586-1) at RT for 15min. Then, cells were mixed at a 1:1 ratio and further incubated at RT for 15min before 80,000 cells were seeded. After 1 hour at 37°C, culture medium was removed and settled cells were carefully overlaid with 16 μl matrigel. When matrigel was set, 30 μl culture medium was added. Pictures were taken at indicated times with a Keyence fluorescence microscope (4x objective) and tube length was determined using BZ analyzer software (Keyence). A tube was defined as green LSEC elongation connecting 2 branching points. HSCs associated with LSEC tubes were identified in merged pictures as red cells attached to green tubes.

Statistical analysis

Mann-Whitney tests were performed with GraphPad Prism software (GraphPad Software).

Abbreviations

α-SMA	α-smooth muscle actin
CCl_4	carbon tetrachloride
CK19	cytokeratin 19
EC	endothelial cell
HSC	hepatic stellate cell
ICAM	intercellular adhesion molecule
JAM	junctional adhesion molecule
LSEC	liver sinusoidal endothelial cell
PECAM	platelet-endothelial cell adhesion molecule
PDGF	platelet derived growth factor
TJ	tight junctions
VCAM	vascular cell adhesion molecule

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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