JAM-A is a multifaceted regulator in hepatic fibrogenesis, supporting LSEC integrity and stellate cell quiescence

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Abstract

Background and Aims: Leukocyte infiltration is a hallmark of hepatic inflammation. The Junctional Adhesion Molecule A (JAM-A) is a crucial regulator of leukocyte extravasation and is upregulated in human viral fibrosis. Reduced shear stress within hepatic sinusoids and the specific phenotype of liver sinusoidal endothelial cells (LSEC) cumulate in differing adhesion characteristics during liver fibrosis. The aim of this study was to define the functional role of cell-specific adhesion molecule JAM-A during hepatic fibrogenesis.

Methods: Complete, conditional (intestinal epithelial; endothelial) and bone marrow chimeric Jam-a knockout animals and corresponding C57Bl/6 wild-type animals were treated with carbon tetrachloride (CCI4, 6 weeks). For functional analyses of JAM-A, comprehensive in vivo studies, co-culture models and flow-based adhesion assays were performed.

Results: Complete and bone marrow-derived Jam-a−/− animals showed aggravated fibrosis with increased non-sinusoidal, perivascular accumulation of CD11b+F4/80+ monocyte-derived macrophages in contrast to wild-type mice. Despite being associated with disturbed epithelial barrier function, an intestinal epithelial Jam-a knockout did not affect fibrogenesis. In endothelial-specific Jam-a−/− animals, liver fibrosis was aggravated alongside sinusoid capillarization and hepatic stellate cell (HSC) activation. HSC activation is induced via Jam-a−/− LSEC-derived secretion of soluble factors. Sinusoid CD31 expression and hedgehog gene signalling were increased, but leukocyte infiltration and adhesion to LSECs remained unaffected.

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Conclusions: Our models decipher cell-specific JAM-A to exert crucial functions during hepatic fibrogenesis. JAM-A on bone marrow-derived cells regulates non-sinusoidal vascular immune cell recruitment, while endothelial JAM-A controls liver sinusoid capillarization and HSC quiescence.

KEYWORDS
cell migration, endothelial cells, HSC activation, junctional adhesion molecules, liver fibrosis, sinusoidal capillarization

1 | INTRODUCTION

Parenchymal immune cell infiltration is a crucial step during acute and chronic inflammatory responses. Thus, modulating extravasation across the endothelial barrier has been identified as a potential strategy to treat chronic inflammation. Such capillary extravasation is a well-controlled physiological process facilitated by selectin-mediated endothelial rolling, prior to firm adhesion via endothelial Intercellular Adhesion Molecule 1 (ICAM-1). ICAM-1 signalling induces the redistribution of endothelial surface proteins via the translocation of adhesion receptors such as Junctional Adhesion Molecule A (JAM-A) to the vicinity of leukocytes, thus, facilitating transendothelial migration. JAM-A, the founding member of the JAM immunoglobulin superfamily subgroup, a structurally highly conserved receptor in vertebrates, is predominantly found at endothelial and epithelial tight junctions, but also on circulating leukocytes and platelets. As such, it exerts a wide spectrum of functions, including the regulation of cell polarity, cell migration, vascular integrity and paracellular permeability. Two main forms of intercellular interactions have been identified: homodimerization, as well as heterodimerization with the α1 subunit of β2-integrin lymphocyte function-associated antigen 1 (LFA)-1 (α4β2, CD11a/CD18).11

Chronic liver diseases are characterized by increased immune cell infiltration, initiating and perpetuating hepatic inflammation. Innate immune cells foster the activation of previously quiescent hepatic stellate cells (HSCs) into myofibroblasts, thereby triggering extracellular matrix production and consequently liver fibrogenesis.12 Despite pioneering research efforts, leukocyte migration across hepatic endothelium is not yet fully understood. Owing to the binary hepatic blood supply from arterial branches and portal sinusoids, extravasation in the liver differs from capillary extravasation. This modification is attributed to the fact that liver sinusoid endothelial cells (LSECs)—in contrast to other endothelial cells (ECs)—have fenestrae, no basal membranes or tight junctions and exhibit low shear stress. LSECs are the most abundant non-parenchymal cells in the liver. Contrary to selectin-mediated extravasation, transendothelial migration of immune cells from LSEC-lined sinusoids relies increasingly on direct firm adhesion.13,15 Interestingly, LSECs express only a reduced set of surface molecules involved in adhesion, excluding platelet endothelial cell adhesion molecule (PECAM/CD31), but including ICAM-1 and JAM-A. In human viral fibrosis/cirrhosis, JAM-A expression is significantly increased and consequently might play a crucial role in immune cell extravasation.19

Key points
- Immune cell infiltration is crucially involved in directing liver fibrosis initiation and progression.
- The role of leukocyte and endothelial interaction is not entirely deciphered.
- This study defines JAM-A as a key regulator of extravasation, LSEC capillarization and HSC activation.

FIGURE 1 Jam-a deletion triggers pro-inflammation and liver fibrosis after CCl4 treatment. (A) Immunofluorescence staining for hepatic Jam-A in CCl4-treated wt mice (lower panel) and controls (upper panel). White arrowhead indices perivasculaa increase of JAM-A expression. Alexa Fluor® 590, goat anti-mouse, DAPI counterstaining (left), scale bar: 100 μm. (B) Intrahepatic Jam-a mRNA fold induction change as assessed by qRT-PCR (normalized to mean of control animals). (C) ALAT serum analyses in chronic toxic injury in Jam-a−/− mice and controls. (D) Sirius red (SR) (50x, upper and middle panel, control left, scale bar: 40 μm) and immunofluorescence αSMA (red, Alexa Fluor® 590, goat anti-mouse, DAPI counterstaining, lower panel, control left, scale bar: 20 μm) stainings in Jam-a−/− mice and controls. White arrowhead indices actin deposition. (E) Double-blinded morphometric analysis of SR (upper panel, left)- and αSMA-positive area (upper panel, right, percentage of the total field) in liver tissue of chronic toxic injury in Jam-a−/− mice and controls. Intrahepatic mRNA fold changes in αsma (lower panel, left), Col1α1 (lower panel, right) expression as assessed by qRT-PCR. Fold induction normalized to the mean of control animals. (F) Collagen1α1 (upper panel), α-smooth muscle actin (middle panel) Western blotting of liver protein in Jam-a−/− mice confronted with chronic toxic injury (right) and wt controls (left). β-actin as a loading control (lower panel). *p < 0.05 and **p < 0.01 indicate significant differences between untreated and CCl4-treated (6 weeks ip.) wt mice (n = 4–9/group). Asterisks indicate significant differences between CCl4-treated (6 weeks ip.) Jam-a−/− mice and respective wt controls (n = 3–8/group). Data are given as mean with standard deviation.
As JAM-A is implicated in both immune cell recruitment to liver parenchyma and sinusoid composure, the aim of this current study was to define how JAM-A affects fibrogenesis. In order to elucidate cell-specific function, a knockout strategy using global and tissue-specific deletion in endothelial or intestinal cells for JAM-A, as well as bone marrow (BM) transplantation was applied.

2 | RESULTS

JAM-A expression is increased in murine liver fibrosis

Previous studies demonstrated that JAM-A expression is increased in mice in different acute and chronic hepatic injury settings. To transfer these findings to murine fibrosis, C57Bl/6 wild-type
(wt) mice (m/f) were treated with intraperitoneal CCl₄ injections for 6 weeks. Immunofluorescence (IF) staining revealed enhanced JAM-A expression in murine liver fibrosis (Figure 1A). Interestingly, JAM-A expression was especially enhanced in zones surrounding central venules, suggesting increased importance in sinusoidal areas. Jam-a expression was significantly higher in fibrotic livers compared to controls (Figure 1B, p = 0.005), suggesting increased JAM-A levels in human and murine liver fibrosis alike.

**Jam-a deletion triggers increased liver inflammation and fibrosis**

To functionally investigate the relevance of JAM-A in murine liver fibrosis, Jam-a⁻/⁻ animals and wt mice (Jam-a⁺/⁺) were treated with CCl₄. Untreated litterrmates served as controls. Successful deletion of Jam-a was verified (Figure S1A). Jam-a⁻/⁻ mice subjected to CCl₄ developed significantly higher serum parameters of liver injury as reflected by alanine aminotransferase (ALAT) serum levels (p = 0.038; Figure 1C), accompanied by enhanced fibrosis levels (Sirius Red [SR]-positive areas, p < 0.001, Figure 1D,E). The tissue of Jam-a⁻/⁻ mice displayed an increase in pericellular and -vascular SR fibres and α-smooth-muscle-actin (αSMA, Figure 1D,E, p = 0.016), a key marker of hepatic stellate cell (HSC) activation and myofibroblast transformation. Additionally, mRNA (Figure 1F) and protein (Figure 1F) levels for asma/αSMA and Collagen1α1 (Col1α1)/COL1α1 were significantly increased in Jam-a⁻/⁻ compared to Jam-a⁺/⁺ livers after CCl₄ treatment. CCl₄-treated Jam-a⁻/⁻ mice displayed a significantly increased expression of pro-inflammatory and fibrosis-associated genes, evidenced by transforming growth factor β (Tgfβ), interleukin 1β (Il1β), tissue inhibitor of metalloproteinase 1 (Timp1), tumor-necrosis factor α (Tnfa) and matrix metalloproteinase 9 (Mmp9) compared to CCl₄-treated Jam-a⁻/⁻ mice as well as untreated controls (Figure 2A).

Treatment-naïve Jam-a⁺/⁺ and Jam-a⁻/⁻ livers showed no sign of spontaneous inflammation or fibrosis.

**JAM-A deficiency induces intestinal tight junction alterations but does not influence liver fibrosis**

To differentiate organ-specific mechanisms contributing to the pro-fibrogenic phenotype of Jam-a⁻/⁻ animals, mice with a targeted deletion of intestinal Jam-a (VillinCreERT²F11rTaqRlox/lox or Jam-aavl) were generated. Cre-negative Jam-a⁺/⁺ mice were used as controls. Animals with intestinal epithelial deletion of Jam-a develop a basal intestinal barrier defect, resulting in increased intestinal permeability and translocation of microbiota-derived factors when feeding a high-fat diet. These factors, e.g. bacterial lipopolysaccharides (LPS) and other pathogen-associated molecular patterns (PAMPs), have been shown to play an important role in triggering hepatic fibrosis progression.

In contrast to Jam-a⁻/⁻ mice, Jam-aavl mice displayed no alterations of liver fibrogenesis compared to wt controls as evidenced by SR staining (Figure 2B, p = 0.994). Additionally, markers of liver injury were not significantly altered, compared to wt controls (Figure 2D, both p > 0.05). These results indicate that the pro-fibrogenic phenotype in Jam-a⁻/⁻ animals is not deriving from a lack in endoluminal intestinal JAM-A expression.

The data are in contrast to the aggravated inflammation in Jam-a⁻/⁻ mice after a high-fat diet as described above. To further elucidate the discrepancy, tight junction (TJ) composition upon intestinal epithelial JAM-A deficiency was investigated by immunofluorescence staining (IF) for occludin (Figure 2C). An increase in occludin expression was witnessed in both CCl₄-treated and -naïve Jam-aavl intestine compared to wt mice. These compensatory effects seem to reduce TJ disruption and prevent enhanced liver damage during toxic hepatic injury in the absence of further insults challenging intestinal barrier integrity, such as a high-fat diet. Highlighting these findings, the intestine of mice with global Jam-a deletion, although remaining insignificant, demonstrated a trend of occludin (Ocln) upregulation compared to wt mice (Figure 2E, p = 0.052). Furthermore, mRNA expression of claudins 3 and 7 (Cldn3, 7), crucial compounds to intestinal TJ, were increased (Figure 2E). In line with previous findings, claudin 2 (Cldn2) expression lacked a difference between the conditions. Cldn15 expression only demonstrated a trend towards upregulation, ultimately remaining insignificant (Figure S2A,B).

**CCl₄-treated Jam-a⁻/⁻ mice show perivascular apoptotic cell death and monocyte-derived macrophage accumulation**

To investigate whether the higher inflammatory response in fibrotic Jam-a⁻/⁻ mice is associated with increased cell death, livers were examined using TdT-mediated-dUTP-biotin nick end labelling (TUNEL) staining. CCl₄-treated Jam-a⁻/⁻ mice showed an increase of TUNEL* nuclei compared to liver tissue of Jam-a⁺/⁺ mice (Figure 3A, p < 0.001). Patterns of TUNEL* cells in Jam-a⁺/⁺ livers were almost exclusively evident in close proximity to larger, non-sinusoidal blood vessels. TUNEL* cells specifically centred around vessels of macrovasculature as well as prevenular sinusoids (microvasculature) within the centrilobular region and portal triad (Figure 3A). TUNEL staining is an unspecific marker of cell death, insufficiently discriminating apoptotic from necrotic cell death. Therefore, cleaved cysteine-aspartic-acid-protease 3 (cleaved-caspase-3) was analysed, representing the central executive protein in apoptosis. Levels of intrahepatic cleaved-caspase-3 levels were elevated in CCl₄-treated Jam-a⁻/⁻ animals compared to wt controls (Figure 3B), suggesting higher apoptotic cell death in knockout livers. IF staining of cleaved-caspase-3 revealed a perivascular distribution as well (Figure 3B).

Haematoxylin–Eosin (HE) staining was performed to correlate apoptotic patterns to cell morphology. Perivascular cells showed features of the myeloid lineage with non-granular, non-polymorph nuclei (Figure 4A). To better characterize these infiltrating cells, IF studies of liver sections were performed, revealing CD11b* (Integrin α₅) cell accumulation in CCl₄-treated Jam-a⁻/⁻ livers in comparison...
Accumulation was most prominent in such areas of increased apoptotic cells. A significant difference was found in CD11b$^+$ cells between Jam-a$^{-/-}$ and Jam-a$^{+/+}$ livers (Figure 4B, $p = 0.003$).

As JAM-A is deeply involved in cell-to-cell adhesion, induction of cell polarity and migration, clusters of CD11b$^+$ cells were further investigated for deep migration into the liver parenchyma. Herein, CD11b$^+$ immune cells were quantified in correlation to their location in relation to the endothelial border in relation to total CD11b$^+$ cells per centrilobular field was significantly higher in the livers of Jam-a$^{-/-}$ mice, compared to CD11b$^+$ cells in wt controls ($p = 0.005$ and $p = 0.002$, Figure 4B). These results indicate that impaired cell migration might contribute to the pro-fibrogenic phenotype in Jam-a$^{-/-}$ mice.
Multiparametric flow cytometry was used to further characterize CD11b+ immune cells. CCl₄-treated Jam-a−/− livers displayed an accumulation of cells from the myeloid lineage (gated as single cells, viable, CD45+ Ly6G− B220− CD11b+ CD11c+/−), compared to Jam-a+/+ mice (Figure 4C). In detail, a specific subset was identified as CD11b+F4/80+ monocyte-derived macrophages (MDM). Other CD11b+ subsets, like neutrophils (p = 0.701) or dendritic cells (p = 0.824) showed no significant differences. Furthermore, resident liver Kupffer macrophages remained unaltered as well. Conclusively, IF for F4/80 showed similar perivascular immune cell clusters in fibrotic Jam-a−/− livers compared to wt livers (Figure 4D; Figure S3, p < 0.001). Hence, Jam-a deletion specifically triggered the accumulation of monocyte-derived macrophages after chronic CCl₄ treatment.

Deletion of BM-derived Jam-a leads to hepatic MDM accumulation

Global, in contrast to intestinal-specific Jam-a−/− animals showed increased fibrosis in the CCl₄ model. Intrahepatic accumulation of BM-derived MDM was significantly higher in Jam-a−/− livers compared to controls. To investigate the specific contribution of BM-derived cells, experiments were repeated with BM chimeric mice. γ-irradiated C57Bl/6 wt mice were reconstituted with BM from either Jam-a−/− or Jam-a+/+ animals and subsequently analysed. Hepatic histopathology (areas of SR⁺ fibres, Figure 5A,B, p < 0.001) in mice transplanted Jam-a−/− BM showed the same aggravated phenotype as CCl₄-treated Jam-a−/− mice compared to transplanted wt controls (Figure 5A, αSMA immunohistochemistry). Quantification of the intrahepatic content of the collagen-specific amino acid hydroxyproline was performed to validate this observation. Livers of CCl₄-treated Jam-a−/− BM chimaera contained higher hydroxyproline levels than those of CCl₄-treated wt controls (p = 0.004, Figure 5C). mRNA levels of molecular markers of inflammation, HSC activation and fibrogenesis (Figure 5D) were significantly higher in CCl₄-treated Jam-a−/− BM chimaera, compared to wt chimaeras.

Compared to BM-transplanted control mice, CCl₄-treated Jam-a−/− BM chimaera showed histological characteristics of vessel-proximal accumulation of CD11b+ cells (Figure 5E, left panel) in the centrilobular region and portal triad. The number of CD11b+ immune cells that had managed to deeply infiltrate parenchyma was significantly reduced (64.1% ± 6.3% vs. 21.5% ± 3.0%, p < 0.001, Figure 5E).

Flow cytometry showed increased percentages of myeloid. Intrahepatic bone marrow-derived MDMs were similarly increased (Figure 5F). These results suggested that BM-derived Jam-a−/− MDMs might significantly contribute to the pro-fibrotic phenotype, as similarly
**Figure 4**  Jam-a deletion triggers perivascular CD11b⁺F4/80⁺ monocyte-derived macrophage accumulation and impaired cell migration. (A) H&E staining of liver tissue sections of Jam-a⁺/⁺ (right) and Jam-a⁻/⁻ (left) animals. Scale bar: 20 μm. Black arrowheads indicate endothelial cells, blue arrowheads indicate leukocytes. (B) Photomicrographs (left panel) of CD11b⁺ cells in liver tissue of CCl₄-treated wt (left) and knockout animals (right, red, scale bar: 20 μm), DAPI counterstaining (blue, * for the centrilobular vein). Double-blinded analysis (lower panel) of CD11b⁺ accumulation relative to DAPI⁺ nuclei (percentage, per centrilobular field, left graph), total number [n] of accumulated CD11b⁺ cells (all = per field; dm = deep migration per field, middle graph) and percentage of deeply migrated CD11b⁺ cells of total CD11b⁺, right graph). All cells counted in 50x magnification. CD11b⁺ cells with a distance of at least 100 μm to vessels of the hepatic macrovasculature were regarded as deeply migrated. (C) Multicolour flow cytometry analysis of cells of the myeloid lineage (gated as CD 45⁺Ly6G⁻B220⁻CD11c⁻/⁺), MDM (CD11b⁺F4/80⁺), PMN (CD45⁺Ly6G⁺B220⁻CD11c⁻CD11b⁺F4/80⁻/⁺), DC (CD45⁺Ly6G⁻CD11chighF4/80⁻/⁺MHCIhigh) in the percentage of absolute CD45⁺ cells (left panel) and absolute numbers (right panel, MDM for monocyte-derived macrophages, PMN for polymorph nuclear neutrophils, DC for dendritic cells) in livers of CCl₄-treated Jam-a⁻/⁻ and Jam-a⁺/⁺ mice. (D) Photomicrographs of F4/80⁺ cells in liver tissue of CCl₄-treated wt (upper panel) and knockout animals (lower panel, green, scale bar: 100 μm), DAPI counterstaining (blue). For the quantification of F4/80⁺ cells, please refer to supp. Figure 3. *p < 0.05 and **p < 0.01. Asterisks indicate significant differences between CCl₄-treated (6 weeks ip.) Jam-a⁻/⁻ and Jam-a⁺/⁺ mice (n = 3–8/group). Data are given as mean with standard deviation.
found in global Jam-a knockout mice. Interestingly, IF for JAM-A (Figure 6A) revealed prominently JAM-A-deficient areas adjacent to the macrovasculature. Such areas, despite intact endothelial JAM-A lining, were characterized by perivascular accumulation of JAM-A-deficient cells, suggesting architectural destruction of liver tissue.

**Loss of endothelial JAM-A induces hepatic inflammation and fibrosis, but no alterations in myeloid recruitment**

Anti-inflammatory effects of endothelial Jam-a deletion have been reported in atherogenesis. To dissect the specific role of endothelial JAM-A in global Jam-a deletion, endothelial-specific Jam-a knockout mice (Jam-a\(^{endo}\)) were created (VECad\(^{Cre}\), Jam-a\(^{flo/lo}\), Tamoxifen induction). VECad-Cre-negative Jam-a\(^{flo/lo}\) littermates served as controls. Control and Jam-a\(^{endo}\) mice underwent CCl\(_4\) treatment prior to sacrifice. Reduction of endothelial JAM-A expression was ensured (Figure 6B,C). Interestingly, SR staining of Jam-a\(^{endo}\) livers showed a significantly increased fibrosis in CCl\(_4\)-treated Jam-a\(^{endo}\) mice compared to controls (Figure 6D,E, p = 0.026). Likewise, Jam-a\(^{endo}\) animals showed elevated markers of HSC activation and fibrogenesis, as well as pro-inflammatory regulators, evidenced by enhanced mRNA levels of usma, Col1a1, II 6 and Mmp 9 (Figure 6F). There were no significant alterations between CCl\(_4\)-naive strains. TUNEL staining revealed significantly more cell death in CCl\(_4\)-treated Jam-a\(^{endo}\) livers in comparison to corresponding controls (p < 0.001). However, the distribution of TUNEL\(^+\) and CD11b\(^+\) cells showed no perivascular accumulation (Figure S4A,B). Furthermore, infiltration of monocytes was unaltered (Figure S4C).

**Macrophage adhesion to LSECs is Jam-a independent**

JAM-A-deficiency in BM-derived leukocytes leads to increased endothelial adhesion in capillary extravasation, depending on the firm arrest mediated by ICAM-1/LFA1 interaction. Schmitt et al. demonstrated a link between Jam-a deletion in BM-derived cells and increased arrest towards ICAM-1 in vitro as well as decreased detachment after firm adhesion. In this current study, no effect on MDM accumulation was witnessed in Jam-a\(^{endo}\) livers. To further elucidate this mechanism, we investigated adhesion between Jam-a\(^{−/−}\) LSECs and leukocytes in vitro. Murine macrophages (RAW 264.7) were perfused over freshly isolated Jam-a\(^{−/−}\) and Jam-a\(^{+/+}\) LSECs with physiological shear stress (0.2 dyne/cm\(^2\)). Shear stress was increased to evaluate the detachment of adhesive leukocytes (Figure 7A). Neither did leukocyte adhesion differ between Jam-a\(^{−/−}\) and Jam-a\(^{+/+}\) LSECs, nor did leukocytes show signs of decreased detachment from LSECs (Figure 7A, left). To investigate the influence of endothelial ICAM-1 in absence of Jam-a, LSECs were stimulated with TNF\(\alpha\), increasing ICAM-1 expression. This led to enhanced leukocyte adhesion (p = 0.043 and p = 0.005 for 0.2 and 1 dyne/cm\(^2\) for Jam-a\(^{−/−}\) LSECs; p < 0.001 and p = 0.014 for 0.2 and 1 dyne/cm\(^2\) for Jam-a\(^{−/−}\) LSECs, Figure 7A) but demonstrated no alterations between either Jam-a\(^{+/+}\) and Jam-a\(^{−/−}\) LSECs.

To link such a one-sided influence of JAM-A on leukocytes versus endothelial Jam-a on α\(_{\text{L}}\)β\(_{2}\)/LFA-1/ICAM-1-activity, an analysis of Icam-1 expression was performed. Whereas all genetic Jam-a\(^{−/−}\) models exhibited an increase in Icam-1 expression (7B), only BM-derived Jam-a-deficiency orchestrated mononuclear accumulation. Endothelial deletion did not increase leukocyte adhesion.

**Endothelial JAM-A-deficiency induces LSEC capillarization and HSC activation**

In unharmed liver tissue, differentiated (d)LSECs actively maintain HSC quiescence through yet unknown, supposedly paracrine mediators. dLSECs are reported to express low levels of CD31 (PECAM) after embryogenic development. Upon liver damage and dedifferentiation/capillarization, suppressive LSEC communication towards maintaining HSC quiescence is abrogated. usma

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**FIGURE 5** Deletion of BM-derived Jam-a is the driver of perivascular MDM accumulation. (A) Sirius red-stained liver tissue sections of wt > wt and Jam-a\(^{−/−}\) > wt BM chimaera treated with intraperitoneal CCl\(_4\) injections over 6 weeks (upper panel). Scale bar: 20 μm. Exemplary photomicrographs of immunohistochemistry staining for α-smA in liver tissue of either wt > wt (lower panel, left) or Jam-a\(^{−/−}\) > wt (lower panel, right) BM chimaera (scale bar: 20 μm). Black arrowheads indicate actin deposition in Jam-a\(^{−/−}\) > wt animals. (B) Double-blinded morphometric analysis of SR-positive area (percentage of field, measurements taken in magnification 50x) in BM chimeric animals treated with CCl\(_4\). (C) Hydroxyproline assay of liver tissue of wt > wt and Jam-a\(^{−/−}\) > wt BM chimaera (ng/ml). (D) usma mRNA expression and markers of inflammation and fibrosis of wt > wt and Jam-a\(^{−/−}\) > wt BM chimaera, either treated with CCl\(_4\) injections or untreated. Fold change induction to mRNA expression as assessed by qRT-PCR, fold induction normalized to controls. (E) Exemplary photomicrographs of CD11b\(^+\) immunofluorescence staining (red), nuclei counterstaining via DAPI (blue) of wt > wt bm chimaera (left) and Jam-a\(^{−/−}\) > wt. White arrowheads indicate perivascular CD11b\(^+\) cell accumulation in Jam-a\(^{−/−}\) > wt chimaera. Scale bar: 40 μm. Double-blinded analysis (right) of CD11b\(^+\) accumulation in relation to DAPI-positive nuclei (percentage, per centrilobular field) and percentage of deeply migrated CD11b\(^+\) cells in the parenchyma in relation to total CD11b\(^+\) cells per centrilobular field (right). All cells counted in 50x magnification. CD11b\(^+\) cells with a distance of at least 100 μm to vessels of the hepatic macrovasculature were regarded as deeply migrated. (F) Multicolour flow cytometry analysis of cells of the myeloid lineage (as gated on CD45\(^{Ly6G^B220^CD11c^{−/−}}\) and CD11b\(^{F4/80^{+}}\) in percentage (of total CD45\(^+\) cells, left) and absolute numbers (right, MDM = monocyte-derived macrophage). *p < 0.05, **p < 0.01, ***p < 0.001. Asterisks indicate significant differences between CCl\(_4\)-treated (6 weeks ip.) Jam-a\(^{−/−}\) > wt (n = 4) and wt > wt (n = 4) BM chimaera. Data are given as mean with standard deviation.
expression, as a marker of HSC activation, was significantly increased in JAM-A-deficient strains compared to wt controls. Interestingly, specific deletion of Jam-a in endothelial cells resulted in a significant difference even between CCl4 treatment-naive Jam-aΔendo animals and controls. αsma expression was up to fourfold in Jam-aΔendo livers (7C).

To investigate the level of LSEC dedifferentiation, the expression of Pecam was quantified. PECAM is reported to serve as a
(A) wt > wt

(B) wt > wt

(C) Jam-a^endo

(D) JAM-A Nucleus

(E) Sirius Red [G]

(F) α-smα mRNA Col1α1 mRNA Il6 mRNA Mmp9 mRNA

wt Jam-a^endo
dedifferentiation marker associated with the capillarized (c)LSEC phenotype, in contrast to its embryonic loss. Pecam expression was significantly increased in CCl₄-treated Jam-a⁻/⁻ and Jam-a⁺endod livers, but not in those of Jam-a⁻/⁻ BM chimaera (Figure 7D). PECAM distribution in Jam-a⁺endod livers extended into the sinusoidal area (7E).

Loss of endothelial JAM-A triggers hedgehog signalling and loss of VEGFR1/2

Recently, Xie et al. linked vascular remodelling and LSEC capillarization during liver damage to increased signalling within the Hedgehog (Hh) pathway. Furthermore, they suggest that LSEC VEGFR1/2 expression is regulated by Hh induction. To dissect Hh signalling in Jam-a⁻/⁻ LSECs, livers of wt and Jam-a⁺endod mice were investigated for the Hh-sensitive transcription factor GLI2 family zinc finger 2 (Gli2) as well as the Hh target gene-secreted phosphoprotein 1 (Spp1). Molecular analysis showed a clear trend towards Hh pathway activation in endothelial JAM-A loss, as evidenced by increased Gli2 and Spp1 mRNA in livers of both CCl₄-treated and untreated Jam-a⁺endod mice (Figure 8A). Simultaneously, mRNA of both receptors for VEGF signalling-induced angiogenesis, kinase insert domain receptor (Kdr) and Fms-related receptor tyrosine kinase 1 (Ft1), while remaining insignificant, showed trends towards reduction in livers of CCl₄-treated and untreated Jam-a⁺endod mice, compared to wt controls (Figure 8B, \( p = 0.054 \) and \( p = 0.051 \)). To further strengthen these findings, LSECs from Jam-a⁻/⁻ and Jam-a⁺/+ mice were isolated, purified (Figure 5A) and analysed via multicolour flow cytometry. Unstimulated, freshly isolated LSECs from Jam-a⁻/⁻ showed a tendency of reduced mean fluorescence intensity of KDR/VEGFR2 (Figure 8C), but ultimately lacked difference between conditions. qRT-PCR revealed either a trend (Kdr) or significantly reduced (Ft1) mRNA expression in total liver tissues as well as isolated LSECs of Jam-a⁻/⁻ mice compared to the corresponding Jam-a⁺/+ counterparts (Figure 8C).

Lastly, as mice with an intestinal epithelial Jam-a deletion demonstrated compensatory TJ alterations, expression of occludin within the hepatic endothelium was further investigated. Ocln in livers of Jam-a⁺endod showed no significant changes in vivo (Figure 5B). Ocln in total liver tissue or freshly isolated LSECs from Jam-a⁻/⁻ mice further underlined these findings (Figure 5B).

HSC activation might be suppressed by LSEC-derived and JAM-A-dependent paracrine mediators

Loss of endothelial JAM-A resulted in increased HSC activation. To fill the emerging knowledge gap, the link between LSECs and HSCs was further investigated. Herein, freshly isolated LSECs from Jam-a⁻/⁻ and Jam-a⁺/+ livers and HSCs (GRX cell line) were co-cultivated and markers of HSC activation were analysed. After 24 h, neither levels of \( \alpha_{\text{sma}}, \text{Mmp9} \) or \( \text{Timp1} \) demonstrated any difference between HSCs that were incubated with Jam-a⁻/⁻ or Jam-a⁺/+ LSECs (Figure 8D). Hence, induction of HSC quiescence does not rely on JAM-A-dependent cell-to-cell contacts. To investigate the role of paracrine mediators, Jam-a⁻/⁻ or Jam-a⁺/+ LSECs were co-cultured through Transwell inserts, with both cell types sharing their medium and no cell-to-cell contact. As the paracrine mechanism require secretion and accumulation of mediators, the experiment was extended to 48 hours. HSCs incubated with Jam-a⁻/⁻ LSECs showed trends of increased activation (\( \alpha_{\text{sma}}, \text{Mmp9} \) and \( \text{Timp1} \) mRNA, Figure 8D) over time, however, results still remain insignificant. Taken together, one might speculate that influences on HSC activation might rely on LSEC-derived paracrine mediators rather than on cell adhesion-dependent effects. However, further evidence is needed.

3 | CONCLUSIONS

During chronic liver injury, several infiltrating immune cells shape the intrahepatic inflammatory microenvironment in a highly dynamic manner. In contrast to other organs, the liver shows unique anatomic variations of venous and arterial blood supply. Consequently, the expression of adhesion molecules in the liver differs from endothelial surfaces of capillaries. An example is a selectin-independent extravasation in the sinusoidal microvasculature: In the liver, endothelial rolling is reduced and firm adhesion increased. The hepatic prevalence of adhesion protein JAM-A, capable of regulating both cell polarity and migration, has led investigators to suggest a
FIGURE 7 Macrophage adhesion to LSECs is independent of endothelial JAM-A, but JAM-A loss leads to LSEC capillarization and HSC activation. (A) In vitro flow adhesion assays with JAM-A-proficient (Jam-a+/+) monocytes and JAM-A-deficient (Jam-a−/−) primary LSECs (left) after fresh isolation. LSEC-adhesive RAW264.7 mouse-monoocytes dyed with nuclear Syto13-dye counted per field and flow condition (each n = 6). Adhesion assay performed with or without prior TNFα stimulation to increase ICAM-1 expression. 1 dyne or 0.2 dyne shear stress to simulate increased flow conditions. Exemplary bright field and photomicrographs of Syto13-dyed RAW264.7 (green) cells (right). Singular bright-field (upper panel), fluorescent (lower panel) and merged photomicrographs (middle panel). Scale bar: 40 μm. X-fold induction changes to qRT-PCR mRNA expression of (B) Icam-1, (C) ₋sma, (D) Pecam (gene for CD31, as capillarized eLSEC marker), in indicated genotypes and wt or wt > wt chimeric controls after 6 weeks of intraperitoneal CCl4 treatment. (E) Photomicrographs of centrilobular and sinusoidal PECAM/CD31 (red) expression in wt > wt, Jam-a−/− > wt (left) BM chimeric mice as well as Jam-a−/− (right) animals and wt controls. White arrowheads indicate an increase in perisinusoidal PECAM/CD31 expression in Jam-a−/−(Delta) animals. Scale bar: 40 μm. *p < 0.05, **p < 0.01, ***p < 0.001. Asterisks indicate significant differences between CCl4-treated (6 weeks intraperitoneal injections) knockouts and wt or wt > wt chimeric mice in the indicated genotypes after intraperitoneal treatment with CCl4 for 6 weeks. Hash indicates statistical significance between untreated controls. Data are given as mean with standard deviation.

substantial role in hepatic injury.10,20 Previous reports have outlined a wide array of organ-specific and somewhat contradictory effects by using global, leukocyte or endothelial JAM-A-deficiencies in organ-specific inflammation.20,30,31,39 Paradoxically, global JAM-A deficiency showed anti-inflammatory effects, while leukocyte-specific JAM-A has been reported to trigger inflammatory progress. This cell-dependent functional inconsistency of pro- and anti-inflammatory may be because of the heterogeneity of genetic backgrounds or the organ-specific vasculatures investigated. It might furthermore suggest that besides organ-specific mechanisms also injury-dependent pathways might be relevant.

In this current study, we found overexpression of JAM-A in murine liver fibrosis and therefore aimed to better define the functional role of JAM-A. We used global Jam-a knockout mice and generated tissue-specific knockouts for endothelial and intestinal epithelial cells. Additionally, we performed BM experiments to dissect the compartment-specific mechanisms relevant to controlling JAM-A-dependent steps during liver fibrogenesis.

We found that global JAM-A-deficiency promotes inflammation and fibrotic scarring and demonstrates that a major part of this observation might be mediated by JAM-A-deficient BM-derived cells infiltrating the liver. JAM-A-deficient neutrophils and monocytes display reduced extravasation in hepatic ischaemia–reperfusion injury, peritonitis and atherogenesis.30,31 Especially Jam-A-deficient monocytes show defects in de-adhesion and regular migration. Our results appear consistent with JAM-A limiting (αιβ2 (LFA-1)-activity, its deficiency ergo stimulating adhesion to ICAM-1. This effect is reported to rely on either JAM-A-dependent intracellular signalling or JAM-A/JAM-A intercellular crosstalk.31,40 Physiologically, complex and coordinated hetero (JAM-A/LFA-1)- and homophilic (JAM-A/ JAM-A) interactions confer balanced signals of de-adhesion and polarity. Deletion on bone marrow-derived cells inhibits such interactions and thus might intensively reduce polarity-dependent cell migration in murine fibrosis. As a result, infiltrating MDMs might either lose cell polarity—crucial for regular mechanisms of deep parenchymal infiltration—or become entrapped in the vascular lining, possibly facilitating vascular-to-tissue leakage of cytokines and pro-inflammatory damage-associated molecular pattern (DAMP) release.

Notably, in this study, the accumulation of MDMs markedly increased surrounding vessels of the portal triad and in centrilobular venules compared to sinusoids. This focal accumulation may be explained by the expression of ICAM-1 and the existence of base membrane structures in endothelial cells other than LSEC. As ICAM-1 expression in the hepatic microvasculature is highest in vessels of non-sinusoidal origin, MDMs with JAM-A deficiency might only exert polarized motility in areas of increased β2-intensity. Further studies are necessary to elucidate these differential clusters of accumulation. Our data do suggest a profound influence of BM-derived JAM-A on the infiltration of immune cells in liver fibrogenesis. Selectin-independent mechanisms of leukocyte–endothelium interactions in sinusoids, however, may rely on diverging mechanisms. Our data underline the complex diversity of leukocyte–endothelium interactions in the different hepatic vessel compartments.

Finally, our findings imply that JAM-A permits physiological, polarity-dependent infiltration of mononuclear cells.

In Jam-a−/− and BM chimaera mice, this phenotype seems to mainly affect mononuclear lineages. This might be attributable to multiple mechanisms: (1) Infiltrating immune cells, as opposed to resident Kupffer macrophages, need to be homed towards the liver and actively infiltrate the hepatic parenchyma, to which cell polarity is paramount and (2) CD11b itself is part of the β2-integrin αMβ2, additionally responsible for mediating firm cellular interaction via αMβ2/ICAM-1.

Of note, Rahman et al. reported increased liver infiltration of CD11b+F4/80+ cells in Jam-a−/− mice fed with a high-fat diet.26 Similar to the current study, the effects of disturbed mononuclear immune cell migration might have contributed to hepatic inflammation in models of dysbiosis and NASH. As Jam-a−/− animals in NASH were prone to increased liver inflammation, we sought to dissect possible influences of intestinal barrier defects. We demonstrate that the observed pro-fibrotic phenotype after global Jam-a deletion is not mediated by intestinal leakage affecting the gut-liver axis in our model. An increase in hepatic inflammation through intestinal barrier deficiency might therefore be limited to additional nutritive stimuli (e.g. high-fat diet and dysbiosis). Mice carrying an epithelial intestinal specific JAM-A-deficiency demonstrate mechanisms of tight junction alterations, possibly limiting translocation of deleterious compounds from the gut to the liver in the absence of further intestinal stressors. Rahman et al. present evidence on increased colonic occludin expression as a signal of spontaneous TJ disruption.
among intestinal-specific might be the reduced amount of TJs within the liver. Ultimately, further hepatic TJ composition are needed.

Additionally influence this process.

migration through activation of platelet aggregation might addition-

Specifically, the spatial distribution of PECAM in

of adhesion molecules. Similar to the latter, we demonstrate that leukocyte (de)-adhesion from LSECs is not mediated if LSECs support JAM-A-dependent recruitment of leukocytes and/or divergent diets. Interestingly, compensatory mechanisms for the loss of JAM-A were organ-specific. A possible explanation might be the reduced amount of TJs within the liver. Ultimately, further studies on the individual factors that regulate intra- and extrahepatic TJ composition are needed.

Other differences between phenotypes of global, BM-specific and intestinal-specific Jam-a knockout animals in this study might be caused by the effects of Jam-a deletion in other cells. Here, especially platelets could be of further interest because former and very recent studies have detected a dichotomous role in their potential as promoters of inflammatory responses and regeneration. Platelet-leukocyte interaction and JAM-A as a cofactor of leukocyte migration through activation of platelet aggregation might additionally influence this process.

LSECs and non-sinusoidal liver ECs express ubiquitous JAM-A and especially the vascular peculiarity of the liver directed our interest to generate endothelial-specific Jam-a-/- mice. Previous work suggested that endothelial Jam-A–LFA-1 interaction in the liver might play an important role in sinusoidal transmigration, as LSECs lack other subsets of adhesion molecules. In contrast to our findings in global Jam-a-/- mice or after Jam-a-/- BM transplantation, mice with a specifically endothelial deficiency were not prone to accumulation of mononuclear cells. We thus performed in vitro adhesion assays to investigate if LSECs support Jam-A-dependent recruitment of leukocytes and demonstrate that leukocyte (de)-adhesion from LSECs is not mediated by Jam-A on LSECs. Our results suggest that endothelial Jam-A is not a cofactor of sinusoidal extravasation in vivo.

However, we demonstrate that endothelial Jam-A exerts a key function in liver fibrosis. There is evidence that the surface integrity of LSECs contributes to HSC quiescence, while changes lead to HSC activation. We found disturbed LSEC surface integrity and capillarization in global and endothelial-specific Jam-a-/- mice. This observation was not evident in animals after Jam-a-deleted BM transplantation. Specifically, the spatial distribution of PECAM in Jam-a-/- mice extended into sinusoidal areas, while it remained centrilobular in Jam-a-/- BM chimaera. Most intriguingly, LSECs with loss of Jam-A displayed not only an increase in hedgehog pathway induction but also trends of VEGFR1/2 loss. Further research is necessary to investigate, whether Jam-A is able to directly influence Hh signalling or might even link Hedgehog induction to the reduction of VEGFR1/2.

In turn, in Transwell experiments with LSECs, HSCs exhibited a tendency towards enhanced activation in the absence of endothelial Jam-A, which might point to a role of paracrine LSEC-derived mediators in the activation process rather than adhesion-mediated effects. Yet, perhaps relating to incubation time, we cannot present significant differences between the conditions. As the accumulation of paracrine mediators increases over time, these effects may become stronger in chronic settings in vivo. However, further evidence is needed to confirm this hypothesis.

In conclusion, Jam-A has a dual role in the prevention of liver fibrosis. On BM-derived immune cells, Jam-A crucially contributes to infiltration from non-sinusoidal intrahepatic vessels. Endothelial Jam-A expression, in contrast, displays no influence on cell migration from sinusoids or adhesion to LSECs. However, our results suggest that Jam-A plays an important role in protecting LSEC surface integrity from capillarization and might trigger LSEC-dependent signals for maintaining the quiescence of hepatic stellate cells. Hence, our findings demonstrate the complexity of leukocyte-endothelia interaction in facilitating liver fibrosis and underline the clinical relevance of protecting LSEC composure to suppress fibrogenesis as a possible therapeutic target.

4 | METHODS

Murine in vivo experiments

Mice with a somatic deletion of the Jam-a/F11r gene (Jam-a-/-) were a generous gift from Prof. E. Dejana and backcrossed onto a C57BL/6 background for more than 10 generations. Knockout mice with bone marrow (BM)-specific deletion of Jam-A were created as chimaeras, transplanting freshly isolated BM from F11r-/- donors into wt mice with a regular C57BL/6 background. These mice had previously undergone fractionated whole-body γ-irradiation with two doses of 6 Gray (Gy) in 4–6 hours at
0.8 Gy/min, as previously described.46 Mice with an inducible, endothelium-specific Jam-a knockout (Jam-a^{end}) were generated by crossing Vascular endothelial Cadherin Cre/ERT2 (Tg(Cdh5-Cre/ERT2)^CIVE23(Mlia)) mice with F11r^{lox/lox}. Upon reaching 3 weeks of age, VE-Cad Cre/ERT2 F11r^{lox/lox} mice were administered tamoxifen (0.04 mg/g body weight in Miglyol® 810, intraperitoneal ip.) to induce ErtCre nuclei translocation and F11r-cut-out at the loxP-flanked site as previously described.30,47 An inducible knockout was chosen because the constitutive activity of Cre-recombinase can lead to F11r/Jam-a knockout in haematopoietic stem cells.48 An endoluminal, intestine-specific knock- out was created with Villin Cre and F11r^{lox/lox} mice (Jam-a^{vill}). Animals were cared for in a specific pathogen-free environment in the experimental animal housing facilities of the Institute for Animal Research at RWTH Aachen University Hospital. We confirm that all animal experiments and experimental pro- tocols were performed and approved in accordance with the guidelines of the animal welfare review board (LANUV) of the federal government of North-Rhine Westfalia and conforming to EU Directive 2010/63. All mouse strains and feedings were approved by the animal welfare review board (LANUV) of the federal government of North-Rhine Westfalia. To all lines, either wt, wt > wt BM chimaera, Villin-Cre or VECad-Cre nega- tive Jam-a^{lox/lox} littermates with a C57Bl/6 background served as controls. To induce liver fibrosis, 6–8-week-old animals received two intraperitoneal carbon tetrachloride injections weekly (0.6 μl/g body weight in a 50 μl corn oil dilution) over for 6 weeks. Mice were sacrificed 3 days following the last injection via cardiotensis.

Following the guidelines of the International Committee for Standardized Genetic Nomenclature for Mice, gene symbols are given with a capital letter and italicized (e.g. TIMP1 and Jam-a), protein symbols are in capital letters (e.g. TIMP1 and JAM-A).

Liver enzymes

Blood samples were taken during the sacrifice of mice through cardioticensis, immediately centrifuged and stored away at ~80°C. Serum aspartate aminotransferase (ASAT) and alanine aminotrans- ferase (ALAT) serum concentrations were measured using standard- ized test systems.

Histological staining of murine tissue

Three micrometres paraffin cuts were dewaxed and dehydrated following a standard xylene/ethanol protocol. The tissue was stained with haematoxylin–eosin (0.5%) and Sirius Red solution respectively. After washing, slides were fixed using synthetic his- tology kits (Carl Roth; Roti Histokit). SR stock was created by diluting 0.1 g SR dye (Sirius Rot F3BA; Chromo Muenster) in 100 ml picric acid.

Immunofluorescence staining of murine liver and intestinal tissue

Frozen 5 μm liver and intestinal tissue cuts were fixed according to standard operating procedures with 4% paraformaldehyde (polyoxy- methylene) in phosphate buffered saline solution (PBS; PAN Biotech GmbH) and thereafter stained using monoclonal antibodies specific to αSMA (rabbit; Abcam), JAM-A (goat, R&D Systems Inc.), CD11b (rat; eBioscience Inc.), PECAM/CD31 (rabbit; Abcam), cleaved canyonase-3 (rabbit; Abcam) and F4/80 (rat; Abcam). Nuclei counter- stained was performed using 4’,6-diamidino-2-phenyline (DAPI, Vectorashield, Vector Laboratories, Burlingame, USA). As fluorescent secondary antibodies, suitable Alexa fluor® 488 or Cy3 was used (both Thermo Fisher Scientific). Non-specific staining was blocked with 5% bovine serum albumin (BSA; Blomol GmbH), diluted in PBS.

Analyses were performed using a Zeiss light microscope (Carl Zeiss AG) and ImageJ (NIH, LOCI) software. Experiments and analyses with an anti-CD11b antibody were carried out as described in the figures.

Cell death analysis

To detect cell death, a FITC-conjugated TdT-mediated-dUTP-biotin nick end labelling (TUNEL) assay (Roche Holding AG) was used on slides of the snap-frozen murine liver (5 μm) and analysed using light microscopy. The arithmetic mean of single-cell death per nuclei per field was taken from three individual fields after quantification using ImageJ software.

RNA isolation and gene expression analysis

RNA from the snap-frozen liver and intestinal tissue was isolated using peqGold Tri-Fast (VWR International), then centrifuged and re- suspended in RNAse-free water. Concentration was analysed using a NanoDrop system (ThermoFisher Scientific). Quantitative real-time PCR (qRT-PCR) was carried out following RNA-to-cDNA transcription. qRT-PCR was done by a 7300 System (Applied Biosystems) with a SYBR Green PCR kit (Invitrogen) and analysed with the corresponding software. 18s served as a reference gene. For sequences of the primers applied, see Supporting Information (Appendix S1; Table S1).

LSECs isolation and analyses

Following mechanical and enzymatic digestion (Pronase E, Collagenase D and DNase I, Roche Holding AG), liver tissue from Jam-a^{+/−} and wt mice was filtered (70 μm nylon cell strainer, Falcon®, Corning Inc., Corning, USA) and centrifuged at 500 rpm for 1 min at 4°C. Supernatants, containing the LSECs, were again centrifuged (500 g for 8 min at 4°C) and resuspended using GBSS/B, containing
DNAse I. Nycodenz solutions were used for density-gradient separation. Purification was carried out using magnetic-activated cell sorting (MACS®, Miltenyi Biotec, Bergisch Gladbach, Germany) with CD146 (mouse LSEC, order no. 130-092-007, Miltenyi Biotec) microbeads. LSEC isolation for co-culture/Transwell experiments was performed using the gentleMACS™ dissociator (liver dissociation kit 130-105-807, m_liver_01 and m_liver02, combined protocol, Miltenyi Biotec) for semi-automated tissue dissociation and fresh liver tissue from Jam-a−/− and wt mice. Isolation was carried out adhering to the manufacturers' instruction. Dead cell removal was carried out using the designated removal kit (130-090-101; Miltenyi Biotec). Purification was again carried out using CD146 microbeads. Purification was verified through multicolour flow cytometry for CD146 and KDR/VEGFR2. Additionally, LSEC phenotyping was investigated using qRT-PCR and flow cytometry for KDR/VEGFR2.

Co-culture/Transwell assays

Following LSEC isolation, immortalized HSCs (GRX cell lines) were plated on 24-well plates. LSECs were then plated either cultured together with HSCs or separately in collagen-coated Transwell inserts (3 μm pore size, Corning® Transwell® inserts; Sigma Aldrich) and placed within the wells. Within the Transwell assays, both cell lines were able to communicate through the shared culture medium (Prigrow Medium I, Applied Biological Materials Inc.). Following incubation for either 24 (co-culture) or 48 h (Transwells), cells were removed, RNA was isolated, and activation of HSCs was investigated as described above.

Flow chamber adhesion assay

Petri dishes of 35 mm (Falcon®, Corning Inc.) were coated with collagen I (30 μg/ml) and seeded with freshly isolated murine LSECs from Jam-a−/− and wt mice (EBM2; Lonza Group Ltd., Gentamicin/Amphotericin-B supplement), forming a confluent monolayer. Layers were confirmed using phase-contrast microscopy. Commercially acquired RAW 264.7 murine macrophages (ATCC® TIB-71™; LGC Standards GmbH) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Thermo Fisher Scientific, Penicillin/Streptomycin supplement) and labelled with SYTO13™ nucleic acid fluorescent staining (Thermo Fisher Scientific, 1 μM). Cells were thereafter resuspended to a concentration of 0.5 × 10⁶ cells/mL in several tubes containing 3 mM CaCl₂, 2 mM MgCl₂, HBSS with 10 mM HEPES (Gibco, Thermo Fisher Scientific) and 0.2% naïve HHBSA buffer at pH 7.4 for test conditions.

LSECs were activated with TNFα (25 ng/ml) for at least 4 h prior to administering dishes to a continuous flow of RAW 264.7 macrophages as previously described for platelets.⁴⁹ For optimal conditions, an initial 0.15 ml/min flow rate was chosen. Differential shear stress was measured in dynes/cm² and calculated using algorithms according to Son et al.⁵⁰ Adherent cells were manually counted in three fields per dish and condition. Measurements were taken in four different settings: with or without prior TNFα stimulation³² and with 0.2 dyne shear stress or 1 dyne shear stress. Increasing shear stress was undertaken to evaluate the detachment of adhesive leukocytes. A fluorescent microscope with a 20x objective and CCD camera cell imaging system (Evos-FL, now Thermo Fisher Scientific) was used to take pictures.

Access

All authors had full access to the study data and foregoing publication, have reviewed and approved the final manuscript.

Statistics

Data are given as mean with standard deviation and numbers (absolute and percentage). Means of two groups of continuous variables were compared by a two-sided t test, with Welch’s correction in the event of unequal variances. When comparing means of more than two groups, a one-way ANOVA (analysis of variance) test with Tukey’s multiple post-hoc comparison was performed. Variances were screened for heteroscedasticity using Levene’s test. *p < 0.05, **p < 0.01 and ***p < 0.001 were considered significant. Statistical tests were performed using SPSS® (IBM) and GraphPad Prism 8® (GraphPad Software). Significant outliers were identified using Grubb’s test (http://graphpad.com/quickcalcs/Grubbs1.cfm, as accessed on September 10, 2020).

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DISCLOSURES

No conflicts of interest exist.

AUTHOR CONTRIBUTIONS

Administration: JFB, RRK, HS, CT, MLB. Study concept and design: JFB, HS, CT, MLB. Acquisition of data: JFB, EFB, MS, PF, THW, AF, ANR, TV, ACAH, AAR. Statistical analyses: JFB, MS. Analyses and interpretation: JFB, EFB, MS, MLB. Drafting of the manuscript: JFB. Critical revision of the manuscript for important intellectual content:
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**SUPPORTING INFORMATION**

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