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ORIGINAL ARTICLE

Enhanced L1CAM expression on pancreatic tumor endothelium mediates selective tumor cell transmigration

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Abstract L1 cell adhesion molecule (L1CAM) is a transmembrane cell adhesion molecule initially defined as a promigratory molecule in the developing nervous system that appears to be also expressed in some endothelial cells. However, little is known about the functional role of L1CAM on endothelial cells. We observed that L1CAM expression was selectively enhanced on endothelium associated with pancreatic adenocarcinoma in situ and on cultured pancreatic tumor-derived endothelial cells in vitro. L1CAM expression of endothelial cells could be augmented by incubation with immunomodulatory cytokines such as tumor necrosis factor alpha, interferon gamma, or transforming growth factor beta 1. Antibodies to L1CAM and the respective ligand neuropilin-1 blocked tube formation and stromal cell-derived factor 1β induced transmigration of tumor endothelial cells in vitro. L1CAM expression on tumor-derived-endothelial cells enhanced Panc1 carcinoma cell adhesion to endothelial cell monolayers and transendothelial migration. Our data demonstrate a functional role of L1CAM expression on tumor endothelium that could favor metastasis and angiogenesis during tumor progression.

Keywords Cell migration · Pancreatic tumor · Signaling · Integrins

Abbreviations

CFSE	Carboxyfluoresceine diacete succinimidyl-ester
EC	endothelial cells
HUVEC	human umbilical vein endothelial cells
HPMEC	human pulmonary microvascular endothelial cells
L1CAM	L1 cell adhesion molecule
NRP-1	neuropilin-1
Tu PAMEC	tumor-derived pancreatic microvascular endothelial cells

Introduction

Pancreatic cancer is a highly aggressive, treatment-refractory disease and the fourth leading cause of cancer death in the USA [1]. The median survival time is less than 6 months [2, 3]. Potential curative resection of the tumor is still the only option that offers a chance for cure but can only be

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performed in about 10% to 15% of pancreatic cancer patients [4]. The prognosis for patients is still poor, and more than 80% die within 5 years after surgery. Due to its exocrine functions, primary pancreatic cancer often develops multiple metastases [5]. Metastasizing tumor cells can be detected in 60–80% of pancreatic cancer [6]. For metastasis, the attachment of carcinoma cells to endothelial cells (ECs) is crucial. The endothelium is activated by inflammatory cytokines facilitating the extravasation of lymphocytes and tumor cells across the endothelium into adjacent tissue. Recent results have shown that cell adhesion molecules on ECs play an essential role in this process [7].

L1CAM is a 200–220 kDa transmembrane glycoprotein of the immunoglobulin (Ig) superfamily composed of six Ig-like domains and five fibronectin type III repeats followed by a transmembrane region and a highly conserved cytoplasmic tail [8]. L1CAM was first described in the nervous system, where it is important for cell migration and axon outgrowth. Recently, it was reported that overexpression of L1CAM in ovarian and endometrial carcinomas is correlated with bad prognosis and is associated with metastases formation in melanoma and colorectal carcinoma [9–13]. L1CAM expression was also described in other carcinomas such as neuroblastomas and pancreatic adenocarcinoma [14, 15]. It is weakly expressed by hematopoietic cells and was also noted on certain ECs [16–18]. Functionally, L1CAM can interact not only with itself (homophilic) but also with a variety of heterophilic ligands such as integrins, CD24, neurocan, neuropilin-1 (NRP-1), and other members of the neural cell adhesion family [19, 20]. L1CAM associates with NRP-1 to form a semaphorin3A (Sema3A) receptor complex important for axon guidance responses [21]. NRP-1 is a single spanning transmembrane glycoprotein, initially characterized as a neuronal receptor for specific secreted members of the semaphorin family [22]. On ECs, NRP-1 serves as a receptor for some members of the vascular endothelial growth factor (VEGF) family and forms complexes with VEGFR-1 and VEGFR-2 [23]. Thus, neuropilins play an important role not only as axon guidance receptors but also in blood vessel development [24, 25].

Although L1CAM expression on ECs was noted before by immunohistochemical methods, the biological significance of this has not been addressed. L1CAM is cleaved into a soluble form by ectodomain shedding, and soluble L1CAM is able to bind to cells via integrins [26, 27]. For ECs, it remained unclear whether L1CAM was synthesized or passively acquired in a soluble form from other cells. We now observed that pancreatic tumor-derived ECs show strongly enhanced and selective expression of L1CAM. Starting with this observation, we hypothesized that the enhanced vascular expression of L1CAM might play a role in vessel formation. We also examined the role of L1CAM and its ligand NRP-1 in adhesion to and transendothelial migration through the tumor vasculature that is known to be crucial for metastasis formation.

Materials and methods

Tissue samples

Pancreatic tissue samples from 24 patients with histologically confirmed primary pancreatic carcinomas were collected during primary tumor resection (pancreatectomy). Nonmalignant pancreatic tissue was also obtained during pancreatectomy and used as control tissue after pathologic exclusion of tumor infiltration. Tissue samples were either immediately processed or shock frozen in liquid nitrogen for immunohistology. Written informed consent was obtained from all participants, and the protocol was approved by the Ethical Committee of the University of Heidelberg.

Cells and cell culture

Microvascular ECs were isolated from pancreatic tumor tissues. Tissues were washed in phosphate-buffered saline (PBS, Invitrogen, Karlsruhe, Germany), mechanically dissected into small pieces (approximately 1 mm^2) and intensely resuspended with endothelial cell growth medium MV (ECGM) with supplement [5% fetal calf serum (FCS), 0.4% endothelial cell growth supplement/heparin, 10 ng/mL epidermal growth factor, 1 μg/mL hydrocortisone, 1% penicillin/streptomycin; PromoCell, Heidelberg, Germany]. Single cells were obtained from the suspension after filtering through 40-μm cell strainers (Falcon BD, Heidelberg, Germany) and washed with PBS. ECs were magnetically isolated using anti-CD31-Dynabeads (Dynal Biotech, Hamburg, Germany). Isolated ECs were transferred into gelatin-coated (2%) cell culture flasks (TPP, Trasadingen, Switzerland) and cultured in supplemented ECGM until passage 4. Human macrovascular umbilical vein endothelial cells (HUVEC) and human pulmonary microvascular endothelial cells (HMVEC-L; in this paper named HPMEC; Provitro, Berlin, Germany) were cultured in endothelial cell growth medium MV (PromoCell, Heidelberg, Germany) until passage 4. Panc1 tumor cell line was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Biochemical analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions and transfer of proteins to an

Immobilon membrane using semi-dry blotting has been described [11]. After blocking with 5% skim milk in Tris-buffered saline, the blots were developed with the respective primary antibody followed by peroxidase-conjugated secondary antibody and electrochemiluminescence detection.

In vitro transmigration assay

Transwell membranes (8-μm pore sizes; Costar, Corning, NY) were coated for 60 min at 4°C with 0.2% gelatin (Sigma-Aldrich, Munich, Germany). The medium in the lower chamber was supplemented with recombinant human stromal cell-derived factor 1β (rhSDF-1β, 100 ng/mL, PromoKine, Heidelberg, Germany) to establish a gradient for transmigration. ECs were added in the upper chamber (1×10^5 per well). For blocking experiments, 5–10 μg/mL mouse anti-human L1CAM (L1-11A), mouse anti-human neuropilin-1 (Miltenyi Biotec, Bergisch Gladbach, Germany), or mouse IgG1 antibodies (as specificity control) (Santa Cruz Biotechnology, Heidelberg, Germany) were supplemented. Transmigrated cells were quantified 24 h later by using a Casy® Cell Counter (Innovatis AG, Reutlingen, Germany). For quantification, cells that adhered to the bottom of the membrane were detached with 10% trypsin and pooled with those cells that had transmigrated into the lower chamber. For transendothelial migration assay ECs (2×10^5 per well) were added on a gelatin-coated membrane (5-μm pore sizes) and cultured for 2 days on the membranes in supplemented ECGM until they reached confluency. EC monolayers were then washed twice with PBS and activated with recombinant human tumor necrosis factor alpha (rhTNF-α, 400 U/mL, PromoKine) for 4 h. Tumor cells (1×10^5 per well) were then added to the upper chamber. The medium in the lower chamber was supplemented with rhSDF-1β (100 ng/mL, PromoKine) to establish a gradient for transendothelial cell transmigration. For blocking experiments, tumor cells or ECs were additionally incubated for 4 h with the respective blocking monoclonal antibodies. All experiments were performed in triplicates ($n=3$ independent experiments).

Flow cytometry

Cells were washed with ice-cold PBS and detached with 5 mM ethylenediaminetetraacetic acid/PBS. Single cell suspension of ECs or Panc1 cells ($1.0x10^5$–$1x10^7$ cells per well) were blocked with polyclonal human Igs (Endobulin, 2.5 mg/ml; Baxter Oncology, Frankfurt, Germany) and stained with the following antibodies: anti-human-neuropilin-1-PE (1:20, Miltenyi Biotec, Bergisch Gladbach, Germany) or anti-human-L1CAM (1 mg/ml, 1:50, L1-11A) in PBS/3% FCS for 30 min on ice. L1CAM antibody was detected by a goat anti-mouse phycoerythrine secondary antibody (1:400, Dianova, Hamburg, Germany). Dead cells, which were labeled with 1 μg/ml propidium iodide (Abcam, Cambridge, UK) immediately before flow cytometry, were excluded from analysis. Recordings were made from at least 1×10^5 cells on a FACSCanto II flow cytometer (Becton Dickinson, Heidelberg, Germany) and analyzed using FlowJo 6.4 software (TreeStar, San Carlos, CA).

Immunohistochemistry

Pieces of freshly isolated tumor and control tissues were embedded in Tissue Tek embedding medium, snap frozen in liquid nitrogen, and stored at −80°C until use. Cryosections (5 μm) were prepared from frozen tissue, fixed in ice-cold acetone, blocked with 4% goat serum (Invitrogen), and incubated with the following primary antibodies: mouse anti-human-L1CAM (1 mg/ml, 1:50, L1-11A), rabbit anti-human-CD31 (1:50, Spring bioscience, Freemont, CA) followed by detection with the following secondary antibodies: goat-anti-mouse-Cy3 (red; Dianova, Hamburg, Germany) and goat-anti-rabbit-AlexaFluor-488 (green; Invitrogen) (all diluted 1:500). Slides were washed three times with PBS and 4′,6-diamidino-2-phenylindol (DAPI staining solution, Hoechst, Darmstadt, Germany) was added in a dilution of 1:3,000 to detect nuclei. After antibody staining, tissue auto-fluorescence was blocked with $CuSO_4$ solution (1–10 mM $CuSO_4$ in 50 mM ammonium acetate buffer, pH 5.0; Sigma, Deisenhofen, Germany), and slides were covered with glycerin-gelatin (Merck, Darmstadt, Germany). Slides were evaluated by automatic determination of stained areas using AnalySIS Software® (Olympus Soft Imaging Solutions, Muenster, Germany). Quantitative analysis of slides was always based on a minimum of triplicate sections per sample ($n=20$ different donors).

Immunocytochemistry

ECs were added on fibronectin (100 μg/mL) coated Lab-Tek™ chamber slides (Nunc, Wiesbaden, Germany) and cultured in supplemented ECGM until they reached confluence. For activation, ECs were stimulated with rhTNF-α (400 U/mL), rhIFN-γ (1,000 U/ml), or TGF-β1 (10 ng/ml) for 24 h, respectively. Cells were fixed with 4% paraformaldehyde (Merck), permeabilized with 0.1% Triton-X 100 (AppliChem, Darmstadt, Germany) and stained with anti-human-L1CAM (1 mg/ml, 1:50, L1-11A) followed by goat anti-mouse-Cy3 (1:500, Dianova) secondary antibody. Slides were evaluated by counting labeled cells ($n=3$ independent experiments).

In vitro tube formation assay

ECs (2×10^4 per well) were added on 96-well plate coated with 100 μl matrigel (BD Pharmingen, Heidelberg, Germany) and incubated for 24 h. For blocking experiments, 10 μg/mL anti-human-L1CAM (L1-11A), anti-human-neuropilin-1 (Miltenyi Biotec, Bergisch Gladbach, Germany), or mouse IgG1 antibodies (as specificity control) (Santa Cruz Biotechnology) were supplemented. Tube formation was quantified by counting the number of vascular joints in two non-overlapping fields (each field defined as the area visualized by a ×10 magnification lens). All experiments were performed in triplicates (n=3 independent experiments).

In vitro adhesion assay

ECs were seeded on fibronectin (100 μg/mL) coated Lab-Tek™ chamber slides (Nunc) and cultured in supplemented ECGM until they reached confluency. ECs were activated with rhTNF-α (400 U/mL) for 4 h, and endothelial and tumor cells were preincubated with inhibiting antibodies for 4 h, respectively. For quantification of tumor cell adhesion capacity, tumor cells were labeled with 25 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) and added to the EC monolayer for 60 min. Non-adherent tumor cells were removed by three washing steps with PBS, and slides were covered with glycerin-gelatin (Merck). Tumor cell adhesion was evaluated by counting five non-overlapping fields (each field defined as the area visualized by a ×10 magnification lens). All experiment were performed in triplicates (n=3 independent experiments).

Statistical analysis

P values were calculated by using two-sided Student's t test. A P value lower than 0.05 was considered statistically significant.

Results

Enhanced endothelial L1CAM expression in pancreatic carcinoma tissue

We analyzed L1CAM expression on tumor infiltrating and peritumoral vessels of pancreatic carcinoma in comparison to non-malignant pancreatic tissue samples using immunohistochemical staining. For this, the pancreatic tumor tissue (Tu) or corresponding non-malignant pancreatic tissue (Con) were stained with mAbs to L1CAM (red) and CD31 (green) to address co-localization on ECs (Fig. 1a). L1CAM showed enhanced expression on pancreatic carcinoma tissues (n=24) compared to non-malignant pancreatic tissue (n=20; Fig. 1b). To demonstrate that the staining detected L1CAM in a full-length form and not as a soluble molecule (devoid of the cytoplasmic tail), we used the mAb 74-5H7 to the cytoplasmic portion. The detection of the cytoplasmic portion of endothelial L1CAM on non-malignant and tumor tissue confirmed the endogenous expression of the full-length molecule (data not shown).

Immunomodulatory cytokine stimulation augments L1CAM expression of ECs in vitro

We examined whether immunmodulatory cytokines might enhance L1CAM expression on ECs. To address this question in vitro, we analyzed treatment of non-malignant macrovascular HUVECs (Con HUVEC) and microvascular HPMECs (Con HPMEC) as well as tumor-derived microvascular ECs (Tu PAMEC) isolated from pancreatic tumor tissues [7] with TNF-α, interferon gamma (IFN-γ), or transforming growth factor beta 1 (TGF-β1). Significantly enhanced L1CAM expression was observed after TNF-α, IFN-γ, or TGF-β1 stimulation on all three EC lineages (Fig. 2a–c). Non-activated Tu PAMEC already showed a higher expression level of L1CAM than non-malignant ECs (Con HUVEC and Con HPMEC). Activation with TNF-α, IFN-γ, or TGF-β1 further enhanced L1CAM expression on Tu PAMEC (Fig. 2a, b). Western blot analysis with a pAb to the cytoplasmic portion (pcytL1) demonstrated that the detected endothelial L1CAM was expressed in a full-length form and was not attached as a soluble molecule to the cell surface (Fig. 2c). Furthermore, soluble endothelial L1CAM was not detected in Con HUVEC-conditioned medium by enzyme-linked immunosorbent assay confirming the expression of the full-length molecule (data not shown).

We also evaluated the expression level of the L1CAM ligand NRP-1 on ECs. All three ECs lineages showed high NRP-1 expression levels that were not further increased by stimulation with TNF-α, IFN-γ, or TGF-β1 (Suppl. Fig. 1).

L1CAM enhances tumor EC selective angiogenic and migratory capacities in vitro

As L1CAM overexpression on tumor cells augments the haptotactic motility on extracellular matrix proteins [26, 28, 29], we evaluated whether its expression on ECs could affect the migratory capacity using an in vitro SDF-1β stimulated transmigration assays. Results revealed a significantly higher transmigratory capacity of Tu PAMEC than non-malignant ECs (Fig. 3a). Inhibition of transmigration by specific mAb to L1CAM or NRP-1, respectively, showed Tu PAMEC specific inhibition of transmigration in comparison to non-malignant ECs or isotype control,

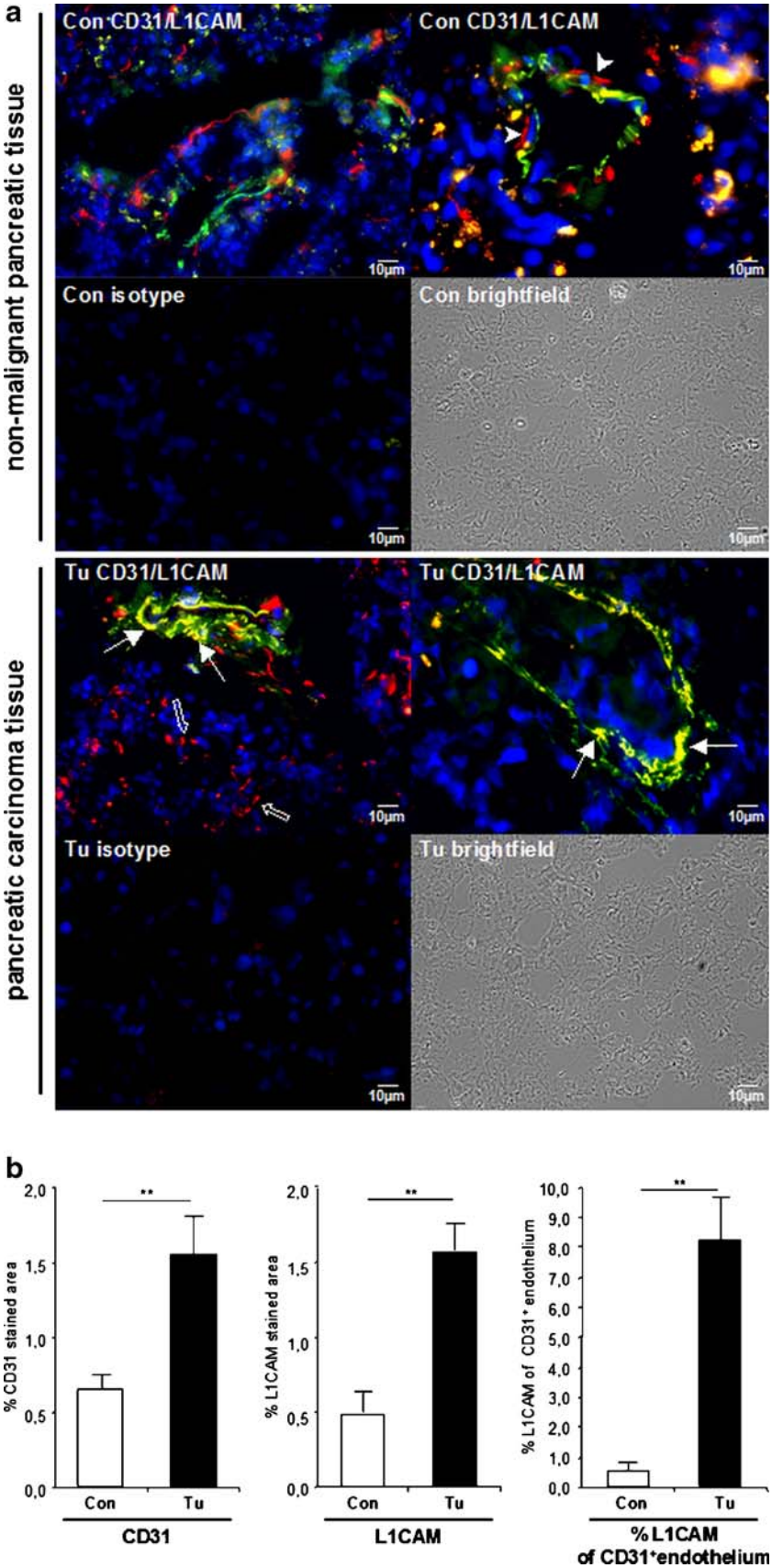


Fig. 1 Enhanced L1CAM expression in non-malignant pancreatic tissue and pancreatic carcinomas. **a** Cryosections (5 μm) of pancreatic tumor tissue (*Tu*) or corresponding non-malignant pancreatic tissue (*Con*) were stained with monoclonal antibodies to L1CAM (*red*) and CD31 (*green*) or with respective isotype antibodies (*bottom left*). Nuclei were counterstained with 4′,6-diamidino-2-phenylindol (*blue*). *Arrowheads* indicate basolateral L1CAM localization, *open arrows* indicate increased L1CAM localization in the tumor tissue, and *filled arrows* indicate L1CAM-CD31 co-localization. **b** Quantification of total L1CAM expression and L1CAM expressing endothelium in primary pancreatic carcinoma tissue (*Tu*, *gray bars*) and nonmalignant pancreas tissue (*Con*, *black bars*). Total percentage of CD31- and L1CAM-expressing endothelium was determined by immunohistology on frozen tissue sections. Means (±SD) of eight to ten tissue samples from 24 independent donors (pancreatic tumor tissue) or 20 independent donors (non-malignant pancreatic tissue), respectively, with evaluation of three to five sections per sample are shown. $^{**}P<0.05$ (two-sided Student's *t* test)

respectively (Fig. 3b). Inhibition of transmigration in the presence of both mAbs did not enhance the blocking capacity of individual mAbs (Fig. 3b).

To evaluate the putative relevance of L1CAM in the angiogenic outgrowth of the tumor vasculature, we performed tube formation assays in vitro. Quantification of tube structures demonstrated enhanced formation of tubes of Tu PAMEC in comparison to non-malignant ECs (Fig. 4a, insert). Blocking with anti-L1CAM or anti-NRP-1 mAbs showed enhanced inhibition of Tu PAMEC tube formation in comparison to non-malignant ECs and respective isotype control (Fig. 4a, b) correlating with the amount of L1CAM expression. Again, inhibition in the presence of both mAbs was not additive.

Fig. 2 Regulation of L1CAM expression of cultured EC by proinflammatory cytokines. **a** Expression of L1CAM (*red*) on cultured non-malignant ECs (Con HUVEC, *left*, Con HPMEC, *middle*) and tumor-derived ECs (Tu PAMEC, *right*) after incubation with immunomodulatory cytokines TNF-α (400 U/ml), IFN-γ (1,000 U/ml) and TGF-β1 (10 ng/ml) for 24 h. Nuclei were counterstained with 4′,6-diamidino-2-phenylindol (*blue*). *Bottom panel* shows staining with respective isotype antibodies. **b** Flow cytometric analysis of L1CAM expression on cultured non-malignant ECs (Con HUVEC, *left*, Con HPMEC, *middle*) and Tu PAMEC (*right*) untreated (*dark gray histograms*) or stimulated for 24 h with immunmodulatory cytokines TNF-α (400 U/ml, *green*), IFN-γ (1,000 U/ml, *red*), and TGF-β1 (10 ng/ml, *black*). *Light gray histograms* represent negative control staining with respective secondary antibodies. **c** Western blot analysis of L1CAM expression on cultured non-malignant ECs (Con HUVEC) untreated or stimulated for 24 h with TNF-α (400 U/ml) or IFN-γ (1,000 U/ml). Full-length L1CAM was detected by an antibody to the cytoplasmic domain of L1CAM (pcytL1). Relative protein amount was calculated in relation to β-actin

Tumor EC L1CAM expression supports cell adhesion and transmigration of carcinoma cells in vitro

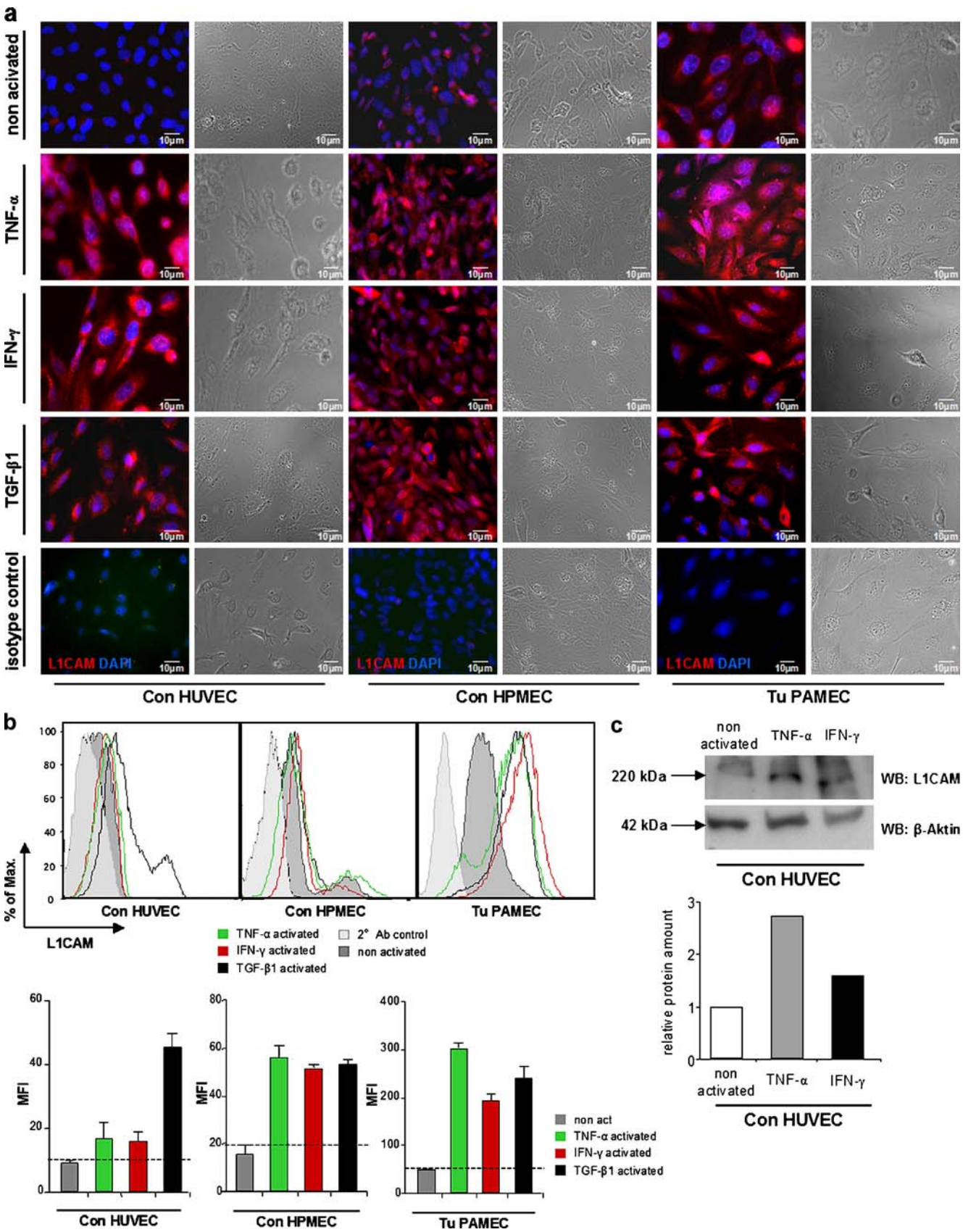
Previous work has shown that L1CAM is involved in the transendothelial migration of melanoma cells [30]. To investigate a putative role of L1CAM for the metastatic spread of pancreatic carcinoma, we performed in vitro cell adhesion assays using Panc1 tumor cells. CFSE-labeled Panc1 cells were incubated for 60 min on TNF-α pre-stimulated EC monolayers. Quantification of adhering cells showed increased adhesion of Panc1 cells to Tu PAMEC in comparison to non-malignant EC monolayers (Fig. 5a). Blocking with anti-L1CAM or anti-NRP-1 mAbs showed enhanced inhibition of Panc1 cell adhesion to Tu PAMEC monolayer in comparison to non-malignant EC monolayer and respective isotype control (Fig. 5b, c). Interestingly, pre-incubation of ECs with L1CAM or NRP-1 mAbs followed by the removal of mAbs showed enhanced inhibition of Panc1 cell adhesion to Tu PAMEC compared to non-malignant ECs (Fig. 5c, left). Pre-incubation of Panc1 cells with L1CAM mAb did not significantly inhibit Panc1 cell adhesion to EC monolayers. However, pre-incubation of Panc1 cells with respective NRP-1 mAb showed increased inhibition of Panc1 cell adhesion to non-malignant EC monolayer, suggesting that L1CAM expression on ECs interacted preferentially with NRP-1 on carcinoma cells (Fig. 5c, right).

To allow the analysis of transmigration through EC monolayers, we first established conditions to achieve a confluent EC monolayer on a gelatin-coated membrane of the transmigration chamber. A confluent EC monolayer was obtained by culturing 2×10^5 cells for 48 h (Suppl. Fig. 2). These conditions were used for all transendothelial migration assays. Evaluation of Panc1 cells demonstrated increased transendothelial migration through Tu PAMEC in comparison to non-malignant EC monolayer (Fig. 6a). Pre-incubation of ECs with L1CAM or NRP-1 mAbs showed enhanced inhibition of transendothelial migration through Tu PAMEC compared to non-malignant EC monolayer (Fig. 6b, left). In contrast, pre-incubation of Panc1 cells with L1CAM or NRP-1 mAbs did not inhibit transendothelial migration (Fig. 6b, right).

Discussion

In this paper, we demonstrate that tumor endothelium of pancreatic carcinoma, but not endothelium in non-malignant pancreatic tissue of the same patients, showed increased L1CAM expression in situ. This was confirmed on isolated Tu PAMEC kept in short-term culture in vitro. We observed a significantly higher L1CAM expression level on Tu PAMEC compared to non-malignant ECs. Furthermore, L1CAM expression could be augmented by incubation of non-malignant ECs (Con HUVEC and Con HPMEC) with immunmodulatory cytokines such as TNF-α, IFN-γ, or TGF-β1. Antibodies to L1CAM and NRP-1 blocked tube formation and transmigration of tumor-derived ECs in vitro. Enhanced L1CAM expression increased cell adhesion of Panc1 cells to EC monolayer and supported Panc1 cell transendothelial migration. Our data demonstrate a hitherto unrecognized role for L1CAM selectively on tumor endothelium that could be important for the angiogenic and metastastatic processes.

Our study was initiated by the analysis of primary human pancreatic adenocarcinoma tissues by immunohistochemical methods. In agreement with a previous study [15], but contrasting to others [31], we observed that the pancreatic tumor mass expressed L1CAM. Surprisingly, we observed that higher L1CAM expression in the carcinoma tissue was positively correlated to an increased vascularization in comparison to non-malignant pancreatic tissue of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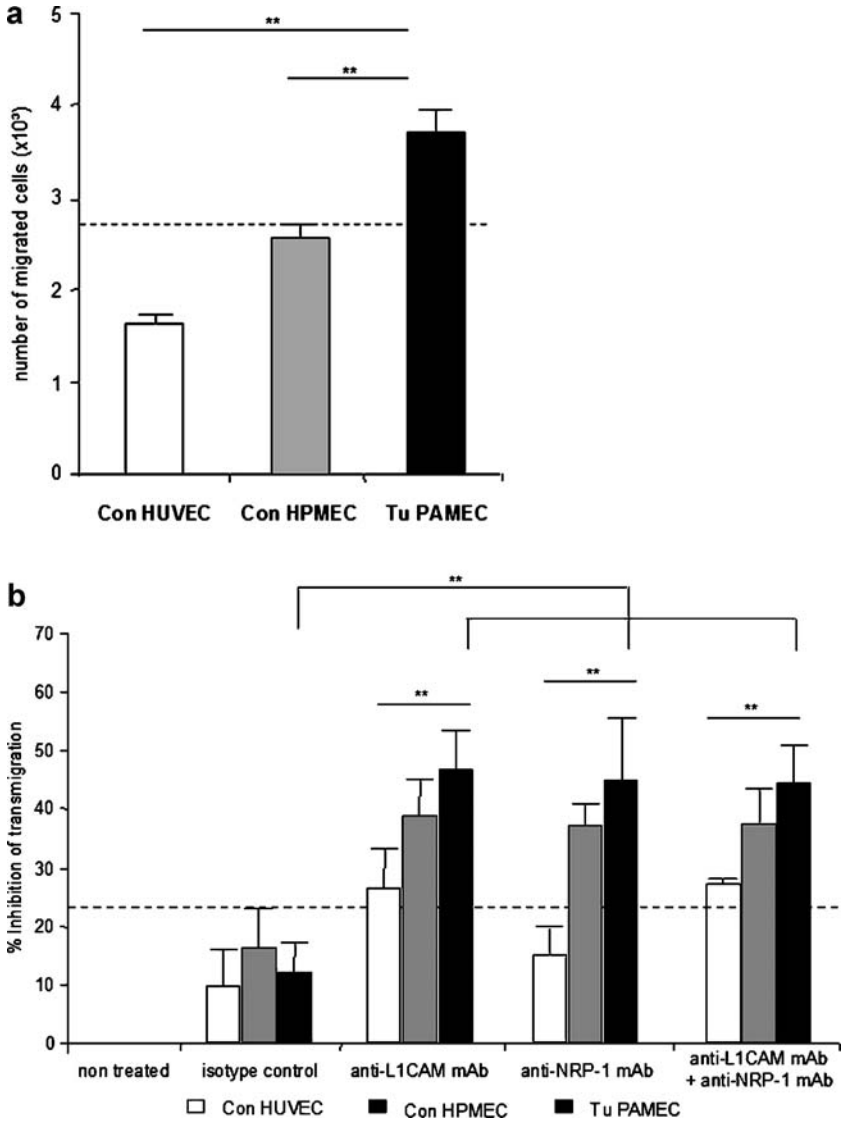


Fig. 3 Contribution of L1CAM and binding partner NRP-1 to transmigration of EC. **a** Basic transmigration of non-malignant ECs (Con HUVEC, *white bars*; Con HPMEC *gray bars*) and tumor-derived ECs (Tu PAMEC, *black bars*). ECs were added to transmigration chambers (8-μm pores), and after 24 h, transmigrated cells in the lower chamber were counted. Mean cell numbers (±SD) of three independent experiments each performed in triplicates are shown. $^{**}P<0.05$ (two-sided Student's *t* test). **b** Inhibition of SDF-1β stimulated transmigration of non-malignant ECs (Con HUVEC, *white bars*, Con HPMEC, *gray bars*, 10^5/100 μl cells per well) and tumor-derived ECs (Tu PAMEC, *black bars*, 10^5/100 μl cells per well) after blocking of L1CAM and NRP-1 by respective mAbs. Mean relative inhibition (±SD) of transmigration compared to blocking by respective isotype control. $^{**}P<0.05$ (two-sided Student's *t* test)

the same patients (Fig. 1a, b). It was reported before that a high microvessel density in pancreatic tumor tissue is related to liver metastasis that is associated with the poor prognosis of pancreatic ductal adenocarcinoma patients [32, 33]. We found that L1CAM significantly co-localized with the endothelial marker PECAM1 in pancreatic carcinoma tissue that was not the case in non-malignant pancreatic tissue. The co-localization of L1CAM and PECAM1 was most prominent on the luminal side of vessels, suggesting a role of L1CAM in cell adhesion and extravasation (Fig. 1a). Indeed, many studies have shown that L1CAM can mediate cell–cell adhesion between different cell types, including leukocytes to ECs [34] and adhesion of tumor cells to platelets and mesothelial cells [23, 35]. L1CAM was also shown to promote cell motility of neuronal and tumor cells [26, 28, 29, 36, 37], suggesting that enhanced L1CAM expression on tumor ECs could lead to increased EC migration, tube formation, and therefore enhanced vascularization of the pancreatic tumor tissue.

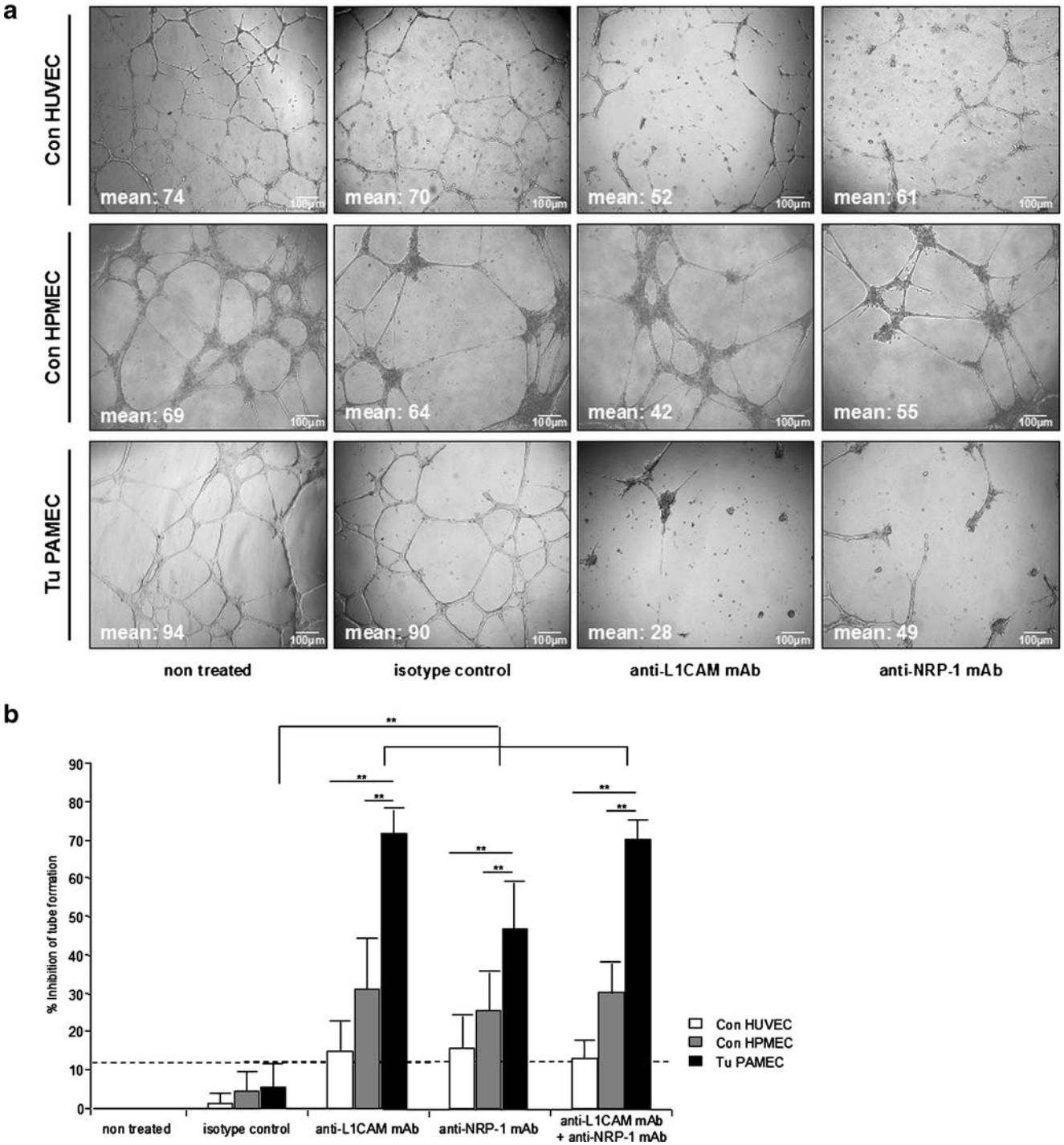


Fig. 4 Contribution of L1CAM and NRP-1 for endothelial cell tube formation. **a** Tube formation on matrigel of non-malignant ECs (Con HUVEC, *upper panel*) and tumor-derived ECs (Tu PAMEC, *bottom panel*). Inhibition of tube formation of non-malignant ECs (Con HUVEC, 2×10^4/100 µl) and tumor-derived ECs (Tu PAMEC, 2×10^4/100 µl) after blocking of L1CAM (anti-L1CAM mAb, *middle right*) and NRP-1 (anti-NRP-1 mAb, *right*) by respective mAbs are shown. Respective isotype antibody was used for specificity control (isotype control, *middle left*). *Numbers in bottom line* indicate quantification of basic tube formation on matrigel after 24 h of tumor-derived ECs (Tu PAMEC) and non-malignant ECs (Con HUVEC). Mean (±SD) of three independent experiments each performed in triplicates are shown. $^{**}P<0.05$ (two-sided Student's *t* test). **b** Relative inhibition of tube formation of tumor-derived ECs (Tu PAMEC, *black bars*) and non-malignant ECs (Con HUVEC, *white bars*, Con HPMEC, *gray bars*) after blocking of L1CAM (anti-L1CAM mAb) and NRP-1 (anti-NRP-1 mAb) by mAbs compared to respective isotype antibody (isotype control). Mean (±SD) of three independent experiments each performed in triplicates are shown. $^{**}P<0.05$ (two-sided Student's *t* test)

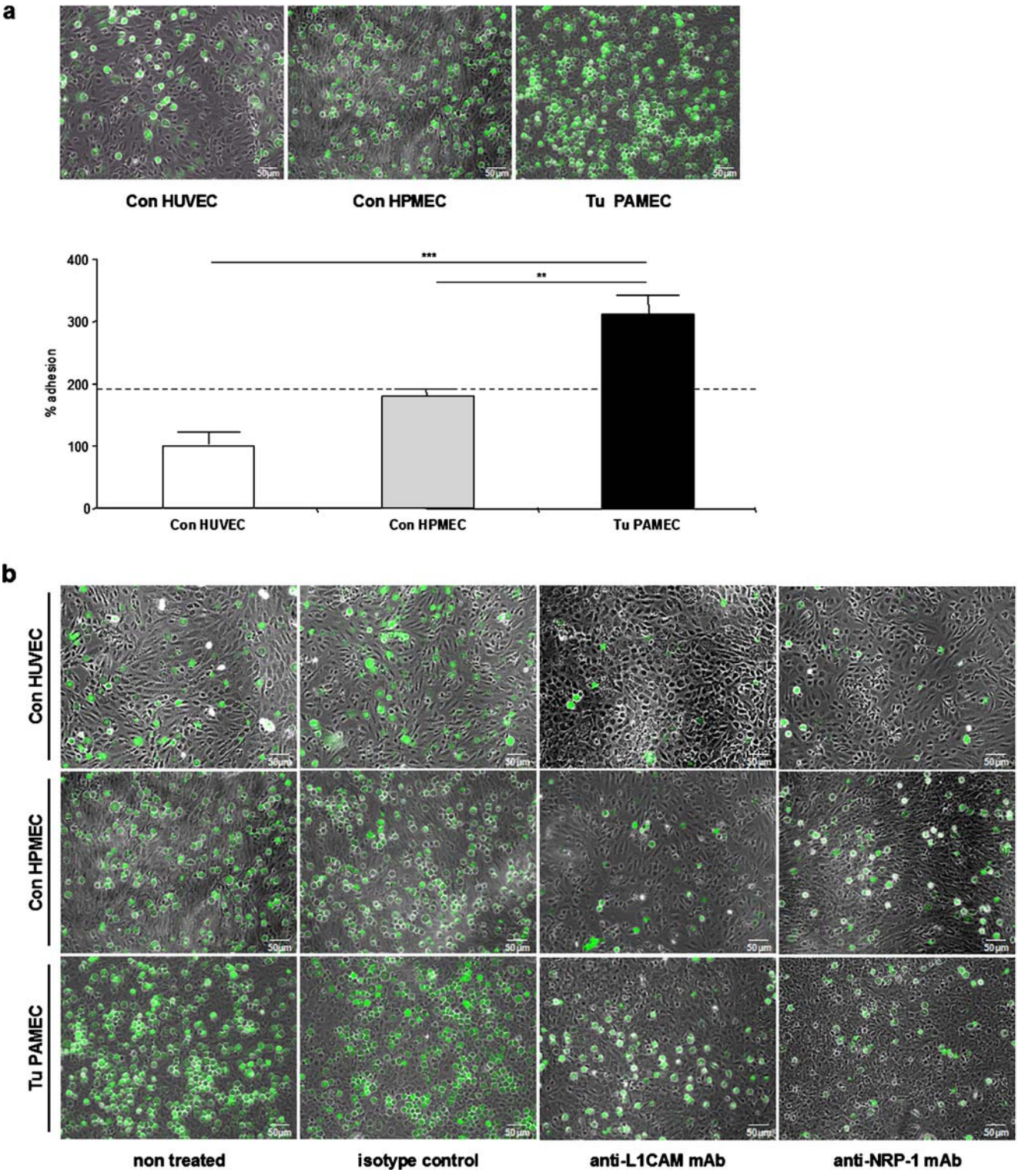
Changes in the expression and localization of L1CAM and that of other adhesion molecules are likely induced by the tumor microenvironment. Indeed, it was reported before that expression of different adhesion molecules on pancreatic tumor ECs was augmented by autologous tumor lysates in vitro [7]. We show in this paper that the immunmodulatory cytokines TNF-α, IFN-γ, and TGF-β1 enhanced L1CAM expression on non-malignant and tumor-derived ECs (Fig. 2). Pancreatic carcinomas or associated pancreatic myofibroblast cells (PMF) can secrete high levels of immunomodulatory cytokines such as TNF-α, IFN-γ, and TGF-β1 [38–40]. A recent report has shown that the cocultivation of PMF with untransformed pancreatic ductal epithelial cells upregulated L1CAM expression of the latter cells, which is regulated by TGF-β1 [41]. Interestingly, cocultivation of PMF with ECs resulted in similar effects (Issa, Y. and Sebens Müerköster, S. unpublished data). It was recently shown that, on ECs, the expression of different adhesion molecules, such as ICAM-1 and VCAM-1, can be induced by pancreatic tumor cells and by cytokines present in pancreatic tumor tissues, including TNF-α, IFN-γ, and TGF-β1 [7, 42, 43]. Enhanced L1CAM expression on tumor-derived ECs after cytokine stimulation might reflect higher sensitivity toward immunomodulatory cytokines (Fig. 2a, b). It can be speculated that the increased cytokine sensitivity could be due to elevated levels of the respective cytokine receptors. Fonsatti et al. [44] demonstrated that Endoglin, the auxiliary cell surface component of the TGF-β receptor complex, is upregulated on proliferating ECs and strongly expressed in the neovasculature of a wide range of human tumors. An upregulation of TNF receptor 2 on tumor-derived ECs was recently shown for human liver carcinoma [45].

NRP-1 is a ligand for L1CAM [21, 23]. NRP-1 also serves as a receptor for some members of the VEGF family and semaphorins [46]. NRP-1 and the axon guidance factor Sem3A are highly overexpressed in pancreatic carcinomas and are associated with bad prognosis [47, 48]. We observed strong NRP-1 expression on ECs that was not increased after stimulation with TNF-α, IFN-γ, or TGF-β1 (Suppl. Fig. 1). MAb to L1CAM and NRP-1 could partially block tube formation and migration of ECs, suggesting a functional role of both molecules in these assays (Figs. 3 and 4). Importantly, the blocking activity in the presence of both antibodies was not higher than in the presence of individual mAbs, suggesting a functional cooperation of both molecules (Figs. 3b and 4b). One possible explanation for these findings could be that L1CAM and NRP-1 act as receptor–ligand pair. However, in view of the complexity of NRP-1 interactions, we do not rule out the possibility that the mAb to NRP-1 blocked other L1CAM-independent effector mechanisms.

Fig. 5 Selective functional properties of L1CAM and NRP-1 on EC for Panc1 tumor cell adhesion to endothelial cell monolayer. **a** Cell adhesion of carboxyfluorescein diacetate succinimidyl ester (*CFSE*) stained Panc1 tumor cells to tumor-derived EC monolayer (Tu PAMEC, *right*) and non-malignant EC monolayer (Con HUVEC, *left*, Con HPMEC, *middle*). Immunocytologic quantification of relative adhesion of Panc1 cells to tumor-derived EC monolayer (Tu PAMEC, *black bars*) and non-malignant EC monolayer (Con HUVEC, *white bars*, Con HPMEC, *gray bars*) is shown. Original magnification ×100. Means (±SD) of three independent experiments performed in triplicates with five to eight sections per sample are shown. $^{**}P<0.05$, $^{***}P<0.001$ (two-sided Student's *t* test). **b** Inhibition of Panc1 cell adhesion to tumor-derived EC monolayer (Tu PAMEC, *bottom panel*) and non-malignant EC monolayer (Con HUVEC, *upper panel*, Con HPMEC, *middle panel*) after blocking of L1CAM (anti-L1CAM mAb) and NRP-1 (anti-NRP-1 mAb) by respective mAbs. Respective isotype antibody was used as specificity control (isotype control). Original magnification ×100. **c** Relative inhibition of Panc1 cell adhesion to tumor-derived EC monolayer (Tu PAMEC, *black bars*) and non-malignant EC monolayer (Con HUVEC, *white bars*, Con HPMEC, *gray bars*) after selective EC blocking (*left panel*) or tumor cell blocking (*right panel*) of L1CAM (anti-L1CAM mAb) and NRP-1 (anti-NRP-1 mAb) by respective mAbs. Isotype antibody (isotype control) was used as specificity control. Means (±SD) of three independent experiments performed in triplicates are shown. $^{**}P<0.05$ (two-sided Student's *t* test)

Earlier work by Voura et al. [30] could demonstrate that L1CAM expressed on ECs plays an important role in transendothelial migration of melanoma cells in vitro. In this work the $\alpha_v\beta_3$ integrin expressed by melanoma cells served as ligand for L1CAM on ECs and transendothelial migration was blocked by anti-L1CAM and anti-$\alpha_v\beta_3$ antibodies [30]. Although Panc1 cells express L1CAM (Suppl. Fig. 3) and NRP-1 similar to ECs, we observed that the blocking effect of L1CAM and NRP-1 specific mAbs was on the EC site rather than on the tumor cell site (Fig. 6b). Interestingly, a recent study of ovarian carcinoma cell transendothelial migration has come to a different conclusion. Using siRNA-mediated depletion of L1CAM in IGROV1 cells, a twofold decrease in the ability to cross the monolayer of lymphatic ECs was observed [49]. However, this study did not address the expression of L1CAM on ECs.

Interestingly, L1CAM has structural and functional similarity to PECAM1, an important molecule for paracellular migration of leukocytes through ECs. L1CAM and PECAM1 share similar homophilic binding mechanisms and high affinity for a_vb_3 integrin [50]. The enhanced expression of L1CAM on tumor-derived ECs and the observed co-localization of L1CAM with PECAM1 might suggest also a functionally similar role on ECs. Thus, L1CAM expression on the migrating cell and on ECs could likewise facilitate both adhesion and transendothelial migration. Further work is needed to closer define the role of L1CAM on ECs.


a
Con HUVEC
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400
300
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% adhesion
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Con HUVEC
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isotype control
anti-L1CAM mAb
anti-NRP-1 mAb

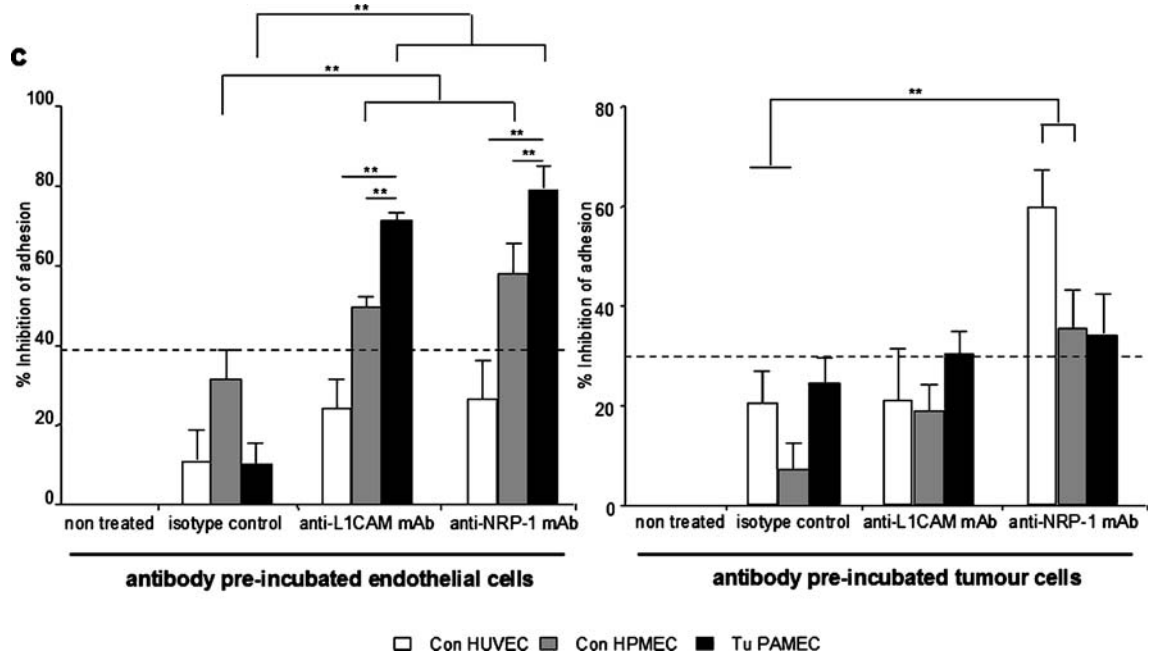

Fig. 5 Continued.

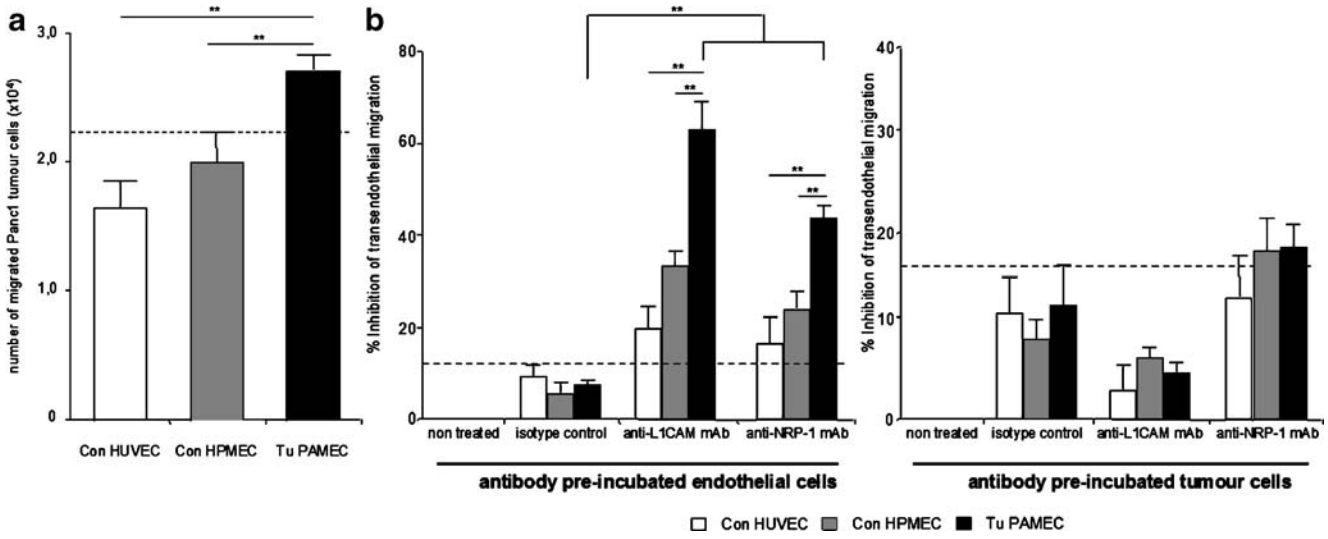

Fig. 6 Analysis of Panc1 tumor cell transmigration through endothelial cell monolayers. Tumor-derived EC (Tu PAMEC, *black bars*, 2×10^5/100 µl) and non-malignant EC monolayer (Con HUVEC, *white bars*, 2×10^5/100 µl, Con HPMEC, gray bars, 2×10^5/100 µl) were cultured for 48 h on gelatin-coated transmigration membranes (5-µm pores) until confluency. ECs were activated for the last 24 h with TNF-α. Subsequently, Panc1 cells were added (1×10^5/100 µl). Transmigrated Panc1 cells were counted after 24 h. Three independent experiments each performed in triplicates are shown. **a** Basic transendothelial migration of Panc1 cells through non-malignant ECs (Con HUVEC, *white bars*; Con HPMEC *gray bars*) and tumor-derived ECs (Tu PAMEC, *black bars*). Mean cell numbers (±SD) of three independent experiments each performed in triplicates are shown. $^{**}P<0.05$ (two-sided Student's *t* test). **b** Relative inhibition of SDF-1β stimulated transmigrated Panc1 cells through tumor-derived EC (Tu PAMEC, *black bars*) and non-malignant EC monolayer (Con HUVEC, *white bars*, Con HPMEC, *gray bars*) after selective EC blocking (*left panel*) or tumor cell blocking (*right panel*) of L1CAM (anti-L1CAM mAb) and NRP-1 (anti-NRP-1 mAb) by respective mAbs. Isotype antibody (isotype control) was used as specificity control. Mean (±SD) of three independent experiments each performed in triplicates is shown. $^{**}P<0.05$ (two-sided Student's *t* test)

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