Europe PMC Funders Group
Author Manuscript
Cancer Res. Author manuscript; available in PMC 2019 January 07.

Published in final edited form as:
Cancer Res. 2016 September 15; 76(18): 5302–5312. doi:10.1158/0008-5472.CAN-16-0784.

Monocyte induction of E-selectin-mediated endothelial activation releases VE-cadherin junctions to promote tumor cell extravasation in the metastasis cascade

Irina Häuselmann[a], **Marko Roblek**[a], **Darya Protsyuk**[a], **Volker Huck**[b], **Lucia Knopfova**[c], **Sandra Grässle**[b], **Alexander T. Bauer**[b], **Stefan W. Schneider**[b], and **Lubor Borsig**[a]

[a]Institute of Physiology, University of Zürich and Zürich Center for Integrative Human Physiology, CH-8057 Zurich, Switzerland [b]Department of Dermatology, Experimental Dermatology, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany [c]International Clinical Research Center, Center for Biological and Cellular Engineering, St. Anne's University Hospital and Institute of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic

Abstract

Tumor cells interact with blood constituents and these interactions promote metastasis. Selectins are vascular receptors facilitating interactions of tumor cells with platelets, leukocytes and endothelium but the role of endothelial E-selectin remains unclear. Here we show that E-selectin is a major receptor for monocyte recruitment to tumor cell-activated endothelium. Experimental and spontaneous lung metastasis using murine tumor cells, without E-selectin ligands, were attenuated in E-selectin-deficient mice. Tumor cell-derived CCL2 promoted endothelial activation resulting in enhanced endothelial E-selectin expression. The recruitment of inflammatory monocytes to metastasizing tumor cells was dependent on the local endothelial activation and the presence of E-selectin. Monocytes promoted trans-endothelial migration of tumor cells through the induction of E-selectin-dependent endothelial retractions and a subsequent modulation of tight junctions through dephosphorylation of VE-cadherin. Thus, endothelial E-selectin shapes the tumor microenvironment through the recruitment, adhesion and activation of monocytes that facilitate tumor cell extravasation and thereby metastasis. These findings provide evidence that endothelial E-selectin is a novel factor contributing to endothelial retraction required for efficient lung metastasis.

Introduction

Hematogenous metastasis is a multistep process in which diverse interactions between tumor cells and their microenvironment allow the malignant cells to cross physical boundaries, disseminate and colonize distant organs. Specifically, cell-cell interactions between tumor

Corresponding author: Lubor Borsig, Institute of Physiology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland; Phone: +41 44 635-5134; Fax: +41 44 635-6814; lborsig@access.uzh.ch.

The authors disclose no potential conflict of interest.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

cells and blood constituents, such as platelets, leukocytes and endothelial cells, are initially mediated by selectins at different steps of the metastatic cascade (1–3).

Selectins are vascular cell adhesion receptors which are responsible for initial rolling and attachment of leukocytes to the endothelium; enabling leukocyte homeostasis (2, 4). Selectins bind to sialylated and fucosylated lactosamine terminal glycan structures displayed on leukocytes, platelets, and endothelium, or tumor cells. It is accepted that malignant transformation is associated with altered carbohydrate structure presentation on tumor cells, which are potential ligands for selectins, and correlates with poor prognosis due to metastasis (5, 6). E-selectin is the major leukocyte adhesion receptor that is present only on endothelial cells upon endothelial activation and requires *de novo* expression. E-selectin has been investigated as the primary receptor mediating tumor cell metastasis through facilitating adhesion of tumor cells on the endothelium shown *in vitro* (1, 7, 8). In addition, E-selectin upregulation was observed during metastatic liver colonization (9, 10). E-selectin was detected in the tumor cell microenvironment several hours after their arrest, indicating an inflammatory-like endothelial activation (11–13). E-selectin expression in the pre-metastatic lungs correlated with increased tumor cell homing to these tissues and with enhanced recruitment of myeloid cells (14). Although there is accumulating evidence that selectins contribute to the metastatic microenvironment (12, 15), the mechanism of E-selectin contribution to cancer progression requires further *in vivo* studies.

The endothelium in blood vessels controls the extravasation of cells, e.g. leukocytes, and the egress of soluble factors from the plasma. Most leukocytes extravasate from circulation through the paracellular route by opening endothelial junctions at sites of inflammation (16, 17). The maintenance of the vascular barrier function is dependent on the stability of endothelial adherence junctions mediated by vascular endothelial cadherin; VE-cadherin (18). Phosphorylation of Tyr residues of VE-cadherin regulates vascular permeability and the capacity of leukocytes to transmigrate through the endothelium (19, 20). Breast cancer cells were shown to induce disruption of endothelial adherence junctions by inducing phosphorylation of VE-cadherin (21). Yet, tumor cell extravasation is significantly promoted by myeloid cells recruited to the metastatic sites through chemokines such as CCL2 and CCL5 (11, 15, 22–26), albeit the cellular and molecular mechanism of this process remains unclear.

Since E-selectin expression upon tumor cell injection has been frequently observed, the involvement of E-selectin in metastasis could be anticipated. The present study describes the mechanism of E-selectin dependent recruitment and activation of monocytes, which drives the dissociation of VE-cadherin junctions and thereby promotes tumor cell extravasation required for metastasis.

Material and Methods

Cell culture

Mouse colon carcinoma cell line, MC-38 was originally provided by Dr. J. Schlom, NIH Bethesda. MC-38 cells stably expressing GFP (MC-38GFP) were characterized as described (27). B16-BL6 melanoma cells provided by Dr. I. Vlodavsky, Technion Haifa Israel, were

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

grown in DMEM/10% FCS as described (24). Lewis lung carcinoma cells (3LL) were grown in RPMI/10% FCS (12). All cells were kept at low passages and were not further authenticated. Lewis lung carcinoma-LLC1 cells (ATCC) and grown in DMEM/10% FCS. Endothelial cells bEnd.3 (ATCC) were grown in DMEM/10%FCS.

Mice

Animal experiments were performed according to the guidelines of the Swiss Animal Protection Law, and approved by Veterinary Office of Kanton Zurich. C57BL/6, Ccl2 deficient (*Ccl2*$^{-/-}$) and E-selectin deficient mice (*E-sel*$^{-/-}$) were purchased from The Jackson Laboratory. Fucosyltransferase 7 deficient mice (*Fuc-TVII*$^{-/-}$) and Fucosyltransferase 4 and 7 double deficient mice (*Fuc-TIV/VII*$^{-/-}$) were from previous studies (15).

Metastatic mouse models

MC-38GFP cells (300’000 cells) were intravenously injected (i.v.) into the tail vein and metastatic foci analyzed on day 28. 3LL and B16-BL6 cells (150’000 cells) were i.v. injected and lungs analyzed on day 14. LLC1 cells (200’000 cells) were subcutaneously injected into the right flank and primary tumors were removed 18 days later. Mice were terminated at day 30 and lung metastasis was analyzed.

Flow cytometry analysis

Mouse lungs perfused with PBS were minced and digested with Collagenase D and A (1 mg/mL each, Roche) in 2 mL for 1 hour at 37°C. Single cell suspension using 40μm cell strainers was prepared; erythrocytes were lysed with PharmLyse (BD Biosciences) (15). Cells were incubated with anti-mouse CD16/32 mAb and stained with directly-labeled antibodies against CD45, CD11b, F4/80, Ly6G, Ly6C and CD31 (BD Biosciences). Blood samples were treated with PharmLyse and stained as described above. Data were acquired on a FACS Canto II (BD Biosciences) and analyzed using Flow Jo software (Tree Star).

Analysis of leukocytes, tumor cells and selectins in lungs

Cryosections (6 μm) were stained with following antibodies: CD11b-biotin; Ly6G; CD62E (BD Biosciences); and F4/80 (AbD Serotec). Goat anti-rat-Alexa568 Ab or Streptavidin-Alexa647 (Life Technologies) were used for detection using fluorescence microscope (Zeiss). The percentage of tumor cells associated with leukocytes was determined. The analysis of selectin and myeloid cell detection in lung sections was performed with a SP5 confocal microscope (Leica). Images were acquired of a total of 5 μm stacks and analyzed with Imaris Software (Bitplane).

Isolation of primary pulmonary endothelial cells and bone marrow monocytes

Pulmonary endothelial cells were isolated using a positive immuno-magnetic selection as described (24). Femur and tibia were crushed in PBS containing 2% FCS and 2.5mM EDTA. After red blood cell lysis, monocytes were enriched by magnetic-activated cell sorting using biotinylated anti-CD115 (M-CSFR) antibody (Biolegend) and streptavidin-conjugated magnetic beads (Miltenyi Biotec).

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Monocyte recruitment in a microfluidic channel system

Primary lung microvascular cells (175'000 cells) were plated on gelatin-coated µ-Slide $I^{0.2}$ Luer (ibidi GmbH) and grown to confluence for 2 days. The endothelial monolayer was manually perfused five times with $2x10^5$ of MC-38GFP cells in 100µl media. After 4 hours slides were perfused with CellTrace calcein-red-orange, AM (Life Technologies) stained bone marrow monocytes ($2x10^6$ cells/ml) in HEPES/Ringer solution supplemented with 25% washed red blood cells. Using air pressure pump system (ibidi) we applied a flow rate of 2 dyne/cm^2. Mosaic images of slides were acquired using an inverted fluorescence microscope (Zeiss Axio Observer Z.1). Pictures were analyzed with ZEN software (Zeiss).

Vascular permeability assay

Vascular permeability in the lungs was determined with Evans blue assay (24). Briefly, 24 hours after i.v. injection of tumor cells, 2 mg of Evans blue was i.v. injected and mice were terminated 30 min later. Monocyte depletion with i.v. injected clodronate liposomes (1.8 mg) (28) 24 hours prior to tumor cell i.v. injection was followed by Evans blue injection.

RNA isolation and quantitative real-time PCR

Total RNA from perfused and snap frozen lungs was isolated using TRI Reagent (Sigma-Aldrich) and reversely transcribed into cDNA using Omniscript RT Kit (Qiagen). Real-time PCR was performed with the SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) using the MX300P light cycler (Agilent). Intron-spanning primers were used (Table S1) and data normalized to GAPDH.

RNA isolation from sorted pulmonary monocytes and endothelial cells

PBS-perfused lungs were minced and digested in Collagenase D and A (1 mg/mL each, Roche) for 1 hour at 37°C. A single-cell suspension was prepared by passing the digested lungs through 40µm cell strainers (BD Biosciences). Cells were incubated with antibodies against CD31, CD45, CD11b, Ly6C and Ly6G (eBiosciences). Endothelial cells ($CD45^-CD11b^-CD31^+$) and inflammatory monocytes ($CD45^+CD11b^+Ly6C^{high}Ly6G^-$) were sorted with a FACSAria III sorter (BD Biosciences). Total RNA was isolated using the RNeasy Mini Kit (Qiagen).

Cytokine assay

Perfused lungs were homogenized and concentration of the chemokine CCL2 was assessed in the supernatant using the cytometric bead array for mouse CCL2 (BD Biosciences). CCL2 levels were normalized to the total protein amount.

Trans-endothelial migration assay

Trans-endothelial migration assay was performed as described previously (24). Briefly, primary lung endothelial cells (25'000) were seeded on gelatin coated 24-well transwell inserts (8µm pores; BD Biosciences) and were grown to confluence. MC-38GFP cells (25'000) were seeded into transwell inserts with/without monocytes (100'000) purified from bone marrow (untreated or PFA-fixed) in 3% FCS/RPMI and 10% FCS/RPMI was added into the lower chamber.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Staining of endothelial cytoskeleton

Endothelial F-actin was detected with Phalloidin staining (29). Briefly, 40'000 pulmonary endothelial cells grown on gelatin-coated chamber slides for 36 hours were co-incubated with MC-38GFP cells (40'000) stained with a PKH26 red fluorescent dye (Sigma-Aldrich) and bone marrow monocytes (200'000 cells) for 8 hours. Cells were fixed with 2% PFA, permeabilized (0.1% saponin), and stained with Phalloidin-FITC (6.67 mg/ml, Sigma-Aldrich). Nuclei were stained with DAPI and mounted in ProLong Gold (Life technologies).

Immunoprecipitation and immunoblotting of VE-cadherin

Confluent bEnd.3 cells were stimulated with 40 ng/ml rmIL-1β (R&D Systems) for 2 hours. Freshly isolated primary $CD115^+$ monocytes from C57BL/6 or *FucTIV/VII*$^{-/-}$ double deficient mice bone marrow ($10x10^6$) with or without MC-38GFP tumor cells ($5x10^6$) in a T75 flask were co-cultured with activated bEnd.3 cells for 1 hour at 37 °C. Monocytes were removed by washing with pre-warmed PBS (5x). Alternatively, the cells were incubated with 10 μg/ml anti-E-selectin antibody (BD) for 1 hour at 37 °C. After washing with pre-warmed PBS, anti-E-selectin Ab crosslinking was induced with an addition of goat-anti-rat polyclonal IgG for 15 min at 37 °C. bEnd.3 cells were lysed with 20 mM Tris pH 7.5, 150 mM NaCl, 1mM EDTA, 1 % Triton X-100, 2% phosphatase inhibitors and 20x Complete EDTA-free protease inhibitors (Roche). Lysates were pre-cleared with Protein G Mag beads (GE Healthcare) for 1 h at 4 °C and incubated overnight at 4 °C with Protein G Mag beads pre-complexed with 5 μg/sample VE-cadherin antibody (Abcam). Beads were washed with PBS (3x) and boiled in Laemmli buffer and supernatants separated on 7.5 % SDS-PAGE gel. After transfer to a nitrocellulose membrane and blocking with 5% milk in Tris-buffered saline (TBS, pH 7.5), the membrane was incubated with mAb to phosphorylated Tyr731 of VE-cadherin (19) (a generous gift from Dietmar Vestweber, Germany) diluted in 5% BSA in TBS overnight or with VE-cadherin antibody. After incubation with secondary antibody, the proteins were detected with ECL method (GE Healthcare).

Live cell imaging

A glass bottom dish (Mattek) was treated with 0.01% poly-L-lysin for 20 min at room-temperature, washed with water and treated with 0.25% glutaraldehyde for 15 min at room-temperature. The dish was washed with DMEM/20% FCS (3x) and put at 4°C for 30 min. Ice-cold collagen type I (Sigma) was mixed with EC medium and PBS, loaded on the glass dish and incubated for 1 hour at 37°C. Primary lung endothelial cells ($3.7x10^5$) were seeded on the collagen matrix and stained with CellTracker Red CMTPX Dye (Life Technologies) 24 hours later. MC-38GFP cells ($2.5x10^5$) and CellTracker Deep Red Dye stained $CD115^+$ bone-marrow derived monocytes ($1x10^6$) were added and the imaging was performed on a Leica SP5 confocal microscope. Data were analyzed using the Imaris 7.3.1 software (Bitplane).

Statistical analysis

Statistical analysis was performed with the GraphPad Prism software (version 5.01). All data were analyzed by ANOVA with the post-hoc Bonferroni multiple comparison test and are

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

presented as mean ±SEM. Analysis of two samples was performed with Mann-Whitney test unless stated otherwise.

Results

E-selectin facilitates experimental metastasis of tumor cells with no E-selectin ligands

It has been postulated that E-selectin binding to tumor cells facilitates tumor cell lodging in the microvasculature and thereby metastasis (1, 30). To assess whether a direct interaction of tumor cells with E-selectin is required for metastasis, we tested mouse colon carcinoma cells (MC-38GFP) in an experimental metastasis model. MC-38GFP cells express no E-selectin ligands while having P- and L-selectin ligands (Fig. S1A). Intravenous injection (i.v.) of MC-38GFP into C57BL/6 and E-selectin deficient (*E-sel*$^{-/-}$) mice revealed a significant reduction in the number of pulmonary metastatic foci and total tumor burden in *E-sel*$^{-/-}$ mice compared to C57BL/6 mice (Fig. 1A and B). The i.v. injection of Lewis lung carcinoma cells (3LL) and melanoma cells (B16-BL6), both without E-selectin ligands (Fig. S1A); also resulted in reduced metastasis in *E-sel*$^{-/-}$ mice (Fig. S1B-E). Since in the experimental metastasis model tumor cells are present in the lungs prior to endothelial activation (12), and the fact that E-selectin is expressed only on activated endothelium, these findings indicate that E-selectin promotes metastasis without directly interacting with tumor cells.

Next, we tested the role of E-selectin in a spontaneous lung metastasis model using Lewis Lung carcinoma cells (LLC1) that were subcutaneously injected. LLC1 cells do not have any E-selectin ligands (Fig. S1A). Lung metastasis was significantly decreased in *E-sel*$^{-/-}$ mice compared to C57BL/6 mice (Fig. 1C and D).

E-selectin-dependent leukocyte infiltration of the metastatic lungs

To assess whether E-selectin facilitates metastasis through recruitment of leukocytes, we analyzed lungs of mice injected with tumor cells i.v. that were terminated 24 and 48 hours post-tumor cell injection (p.i.) by flow cytometry (Fig. S2A-C). Total leukocyte infiltration was significantly increased in C57BL/6 mice 48 hours p.i. but remained unchanged in *E-sel*$^{-/-}$ mice. Specifically, a significant increase in the number of inflammatory monocytes ($CD11b^+F4/80^-Ly6G^-Ly6C^{hi}$) and macrophages ($CD11b^+F4/80^+$) in C57BL/6 mice compared to *E-sel*$^{-/-}$ mice (Fig. 2A and B) was observed. No differences in granulocyte levels ($CD11b^+F4/80^-Ly6C^{med}Ly6G^+$) were detected (Fig 2C). The reduced numbers of macrophages at 16 and 24 hours p.i. in *E-sel*$^{-/-}$ mice compared to C57BL/6 mice (Fig. 2D and Fig. S2D) was further confirmed by immunohistochemistry. We detected a transient increase (16 hours p.i.) of granulocytes in C57BL/6 compared to *E-sel*$^{-/-}$ mice (Fig. 2E). The analysis of peripheral blood cells from naïve mice showed no difference in numbers of $CD11b^+$, $F4/80^+$, and $Ly6G^+$ cells between *E-sel*$^{-/-}$ and C57BL/6 mice (Fig. S2E).

Tumor cell-induced E-selectin expression facilitates leukocyte recruitment

The observed difference in leukocyte infiltration to the lungs indicates that E-selectin promotes the early phase of metastasis. We detected maximal E-selectin expression in the lungs 6 hours p.i. which was reduced by 12 hours p.i. (Fig. 3A). E-selectin was detected only in the vicinity of tumor cells 6 and 14 hours p.i. in lungs of C57BL/6 mice as determined by

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

immunohistochemistry (Fig. 3B). Importantly, we detected myeloid cells ($CD11b^+$) mostly in the E-selectin-positive areas (Fig. 3C). To assess whether E-selectin facilitates leukocyte recruitment to metastatic tumor cells we analyzed the tumor cell-leukocyte association. We observed a significant reduction in both $F4/80^+$ and $Ly6G^+$ cell interactions at 16 hours p.i. in *E-sel*$^{-/-}$ mice compared to C57BL/6 mice, and only $F4/80^+$ cell interactions remained significantly different also 24 hours p.i. (Fig. 3D). To confirm that E-selectin contributes to the leukocyte recruitment, we analyzed this process using a microfluidic system *in vitro* (Fig. S3A). We determined the monocyte recruitment to MC-38GFP cells adherent on primary lung endothelial monolayers derived from C57BL/6 and *E-sel*$^{-/-}$ mice under physiological post-capillary flow conditions. Less monocytes were adherent to endothelial cells around tumor cells on *E-sel*$^{-/-}$-derived compared to C57BL/6-derived endothelial monolayers (Fig. 3E and F). Additionally, the number of adherent monocytes in the vicinity of tumor cells was also reduced on *E-sel*$^{-/-}$-endothelial monolayers (Fig. S3B). These findings indicate that E-selectin promotes the recruitment of monocytes to metastasizing tumor cells.

Intravascular tumor cells induce endothelial activation causing E-selectin-dependent CCL2 expression

E-selectin is an established marker of endothelial activation that is observed both in inflammatory and cancer-related situations (2, 4). Hence, we analyzed the activation status of the lung endothelium in response to tumor cell injection. Expression levels of the activation markers vascular adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) were increased 6 and 12 hours p.i. in lungs of C57BL/6 mice but remained unchanged in lungs of *E-sel*$^{-/-}$ mice compared to untreated mice (Fig. 4A and S4A). In addition, we observed significant increase in expression of a CCL2 chemokine, and its receptor CCR2 in the lungs of C57BL/6 mice 6, 12 and 24 hours p.i., but no changes were detected in *E-sel*$^{-/-}$ mice. CCL2 is produced both by MC-38GFP cells and by stromal cells and the CCL2/CCR2 axis has been associated with promotion of metastasis (22–24). Of note, neither LLC1 nor MC-38GFP cells express CCR2 receptor (31) thereby excluding any direct effect of CCL2 on tumor cells.

Next, we assessed the role of tumor cell-derived CCL2 on the metastatic microenvironment using MC-38GFP cells expressing reduced amounts of CCL2 (MC-38GFP CCL2KD) and compared with parental MC-38GFP cells 6 hours p.i. (Fig. S4B). Significantly reduced endothelial activation was observed in mice injected with MC-38GFP CCL2KD as measured by E-selectin and VCAM-1 expression; and reduced expression of CCL2 and CCR2 (Fig. 4B and Fig. S4B), indicating a direct involvement of CCL2 in endothelial activation.

To test how tumor cell injection changes the CCL2 levels within the metastatic lungs, we analyzed CCL2 protein concentrations in the lungs of C57BL/6, *E-sel*$^{-/-}$, and *Ccl2*-deficient mice (*Ccl2*$^{-/-}$) 6 and 12 hours p.i. Compared to untreated mice (3 pg/mg), CCL2 protein levels were significantly elevated in C57BL/6 mice (27 pg/mg) while a reduced increase in lungs of *E-sel*$^{-/-}$ mice (11 pg/mg) was detected (Fig. 4C). The concentration of CCL2 remained high in the lungs of C57BL/6 mice (8 pg/mg) also 12 hours p.i. (Fig. S4C). In lungs of *Ccl2*$^{-/-}$ mice we observed minimal amounts of CCL2 (2 pg/mg), which likely

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

corresponded to the tumor cell-derived CCL2. These findings suggest that the majority of CCL2 in metastatic lungs is derived from the local microenvironment. To address the source of CCL2 in the metastatic tissue, we sorted endothelial ($CD31^+$) and myeloid ($CD11b^+$/$Ly6C^{high}$) cells from lungs of mice 12 hours p.i. and analyzed CCL2 expression. We observed significant increase in CCL2 expression in endothelial cells from C57BL/6 compared to untreated mice or *E-sel*$^{-/-}$ mice (Fig. 4D). Interestingly, CCL2 expression was also increased in sorted monocytes only from C57BL/6 mice, indicating that E-selectin-mediated activation of monocytes induces CCL2 expression. Indeed, the absolute CCL2 expression levels were mostly derived from monocytes (Fig. S4D). These data show that E-selectin-mediated activation of both endothelial cells and monocytes induces CCL2 expression and thereby contribute to the chemokine pool in the metastatic lungs.

E-selectin-dependent activation of endothelial cells through monocyte binding promotes tumor cell trans-endothelial migration

Increased levels of CCL2 correlate with metastatic progression in various mouse models, and contribute to tumor cell extravasation (23–25, 32). To test whether E-selectin expression is required for tumor cell extravasation, we analyzed lung vascular permeability using the Evans blue assay (24). Tumor cell injection induced lung vascular permeability in C57BL/6 mice which was almost absent in *E-sel*$^{-/-}$ mice (Fig. 5A). We hypothesized that the E-selectin-mediated recruitment and activation of monocytes contribute to vascular permeability. Depletion of circulating monocytes with clodronate liposomes 24 hours prior to MC-38GFP injection resulted in significantly decreased vascular permeability (Fig. 5B) suggesting that monocyte recruitment facilitates tumor cell extravasation by E-selectin-mediated leukocyte-endothelial activation.

Next we analyzed the capacity of monocytes to promote trans-endothelial migration of tumor cells (Fig. S5A). We used lung microvascular endothelial cell monolayers derived from C57BL/6 and *E-sel*$^{-/-}$ mice and studied the migration of MC-38GFP cells (Fig. 5C and S5B). While tumor cells have an intrinsic ability to migrate through endothelial cells, the presence of monocytes significantly promoted this process. However, monocytes did not increase tumor cell migration through E-selectin-deficient endothelial cells suggesting that E-selectin binding to monocytes is essential for trans-endothelial migration. To test this hypothesis we used fixed monocytes, which only present ligands on their surfaces (Fig. 5D). Notably, fixed monocytes increased the tumor cell migration albeit not to the same level as unfixed cells. Next, we tested monocytes derived from Fucosyltransferase-7 deficient mice (*Fuc-TVII*$^{-/-}$) lacking most of E-selectin ligands (15). While *Fuc-TVII*$^{-/-}$ monocytes only partially promoted tumor cell migration, fixed *Fuc-TVII*$^{-/-}$ monocytes showed no effect (Fig. 5D). Thus, the interaction between endothelial E-selectin and selectin ligands on monocytes supports tumor cell transmigration, while soluble factors from monocytes further promote this process. To analyze the role of monocytes, we used a live imaging microscopy to follow the trans-endothelial migration of tumor cells *in vitro* (Fig. 5E, Fig. S5C and Video S1). MC-38GFP cells migrated through the endothelial cell layer in a close contact with monocytes.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

Tumor cell- and monocyte-induced endothelial cell retraction is E-selectin dependent

Activation of selectins by ligand-binding is known to trigger “outside-in” signaling in endothelial cells and leukocytes, which induces migration of leukocytes (33, 34). To elucidate the mechanism how monocyte-E-selectin interaction contributes to tumor cell migration, we analyzed the cytoskeletal retraction of endothelial cells co-cultured with tumor cells and monocytes. While the combination of tumor cells and leukocytes strongly increased the retraction of lung endothelial cells, the individual cells alone only slightly affected retraction (Fig. 6A and S6A). However, we observed no changes in E-selectin deficient endothelial cells after co-culture with tumor cells or monocytes alone; nor with the combination of both cells (Fig. 6A and S6B).

Monocyte-induced disassembly of VE-cadherin junctions is E-selectin dependent

During inflammation, the induction of vascular permeability is dependent on dephosphorylation of Tyr731 on vascular endothelial adhesion molecule VE-cadherin (19). We tested whether enhanced endothelial retraction induced by tumor cell-monocytes is dependent on VE-cadherin modification. Monolayers of endothelioma bEnd.3 cells expressing E-selectin were incubated with either tumor cells, monocytes or both cell types combined. Immunoprecipitated VE-cadherin was analyzed with an antibody specific for phosphorylated Tyr731 (p-Tyr731). While incubation of wt monocytes significantly reduced the p-Tyr731 signal, the addition of MC-38GFP cells alone had no effect (Fig. 6C). The combination of wt monocytes with MC-38GFP cells induced Tyr731 dephosphorylation of VE-cadherin to a similar extent as wt monocytes alone. Next, we used monocytes from Fucosyltransferase-4 and -7 double deficient mice (*Fuc-TIV/VII*$^{-/-}$, DKO), which contain no selectin ligands (35). DKO monocytes induced only partial dephosphorylation of Tyr731 either alone or with MC-38GFP cells, indicating that selectin ligands on monocytes contribute to the E-selectin-mediated signaling. To prove that the presence of selectin ligands on monocytes is sufficient to induce Tyr731 dephosphorylation of VE-cadherin, we used wt monocytes which were fixed prior to addition to bEnd3 cells. Indeed, reduced p-Tyr731 phosphorylation was observed, albeit not to the same level as unfixed cells (Figure 6C), indicating a contribution of monocyte-secreted factors to permeability induction. To test whether the activation of E-selectin is sufficient to induce Tyr731 dephosphorylation we applied anti-E-selectin antibody that was previously shown to activate E-selectin and thereby mimic monocyte adhesion (36, 37). Indeed, E-selectin crosslinking resulted in reduction of Tyr731 phosphorylation albeit not to the same extent as the addition of monocytes (Figure 6D). Taken together, monocyte-assisted trans-endothelial migration of tumor cells involves E-selectin-induced loosening of VE-cadherin tight junctions.

Discussion

The cross-talk between tumor cells and their environment is essential in all steps of the metastatic cascade (3). E-selectin has been previously studied only as a direct receptor for tumor cell-endothelial adhesion (12, 38). However, initial arrest of tumor cells is also likely caused by physical constrictions in capillaries and therefore independent of active adhesion (39). Since we used tumor cells without endogenous E-selectin ligands, a direct tumor cell binding to E-selectin and thereby endothelial adhesion could be excluded. The observed

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

reduction of monocyte recruitment to tumor cells in the lungs of *E-sel*$^{-/-}$ mice suggest that E-selectin mediates host cell interactions within the tumor microenvironment.

Markers of endothelial activation and inflammation are often up-regulated during the initial hours of intravascular tumor cell arrest in lung and liver experimental metastasis models (12, 38, 40, 41). Conversely reduction of endothelial activation diminished myeloid cell recruitment, tumor cell survival and metastasis (15, 41–43). In line with these observations, we observed up-regulation of E-selectin shortly after tumor cell arrest in the lungs that correlated with increased in the endothelial activation markers VCAM-1 and ICAM-1 where detected in the lung vasculature of C57BL/6 mice, but not of *E-sel*$^{-/-}$ mice. Tumor cell-derived factors together with physical factors like shear forces may trigger endothelial expression of E-selectin (40, 44). Tumor cells with reduced CCL2 expression elicited weaker E-selectin expression and correspondingly lower local endothelial activation. This observation is in line with previous findings that endothelial CCR2 expression in the lungs triggers increased vascular permeability and thereby assists tumor cell extravasation (24). However, whether CCL2-induced endothelial activation is directly linked to E-selectin expression or whether tumor cell-derived factors are involved, requires further analysis. Chemokine production has been linked to E-selectin expression only in few studies using inflammatory models (45, 46). In an atherosclerotic rat model, the expression of CCL2 and adhesion molecules, including E-selectin, were up-regulated on the endothelium and associated with monocyte recruitment (45). The inhibition of p38 MAPKs in TNF-α stimulated-endothelial cells resulted in reduced expression of E-selectin and cytokines including CCL2, IL-8, and IL-6 (46). Our data indicate that tumor cells induce endothelial activation and E-selectin expression (Fig. 6E), which in turn contributes to enhanced presence of CCL2 in the metastatic lungs.

CCL2 is a potent regulator of monocyte recruitment to metastasizing tumor cells; and increased CCL2 levels at metastatic sites strongly correlate with metastasis (23–25, 32). We observed decreased expression of CCL2 in lungs of *E-sel*$^{-/-}$ mice compared to C57BL/6 mice and identified both monocytes and endothelial cells as the major source of CCL2 in the metastatic lungs. Stromal-derived CCL2 was previously linked to metastasis (15, 23, 25, 26). The unchanged CCL2 expression in monocytes isolated from lungs of *E-sel*$^{-/-}$ mice is likely due to the lacking activation. Lukacs *et al* previously reported that monocyte-HUVEC interactions trigger an increased expression of CCL2 and IL-8 in monocytes, suggesting a need for cellular contacts (47). Our findings indicate that E-selectin binding is essential for increased CCL2 production by myeloid cells within the developing metastatic niche. In addition, reduced recruitment of myeloid cells to metastatic sites was observed in lungs of *E-sel*$^{-/-}$ mice. The absence of E-selectin, together with reduced CCL2 levels, is likely impeding efficient capturing and firm adhesion of monocytes.

Leukocyte binding to E-selectin is known to induce signaling through MAPK, Erk/Src or MLC/p38 pathways and linked to endothelial permeability (10, 17, 33). Leukocyte adhesion to endothelial cells induces E-selectin clustering and the attachment of E-selectin to actin cytoskeleton which is partially dependent on Rho GTPase activity (36, 37). We showed that monocytes assist tumor cells to induce endothelial retraction in an E-selectin-dependent manner, while the depletion of monocytes prevented an increase in lung vascular

Europe PMC Funders Author Manuscripts

permeability. Furthermore, we provided the first evidence that a direct E-selectin activation is linked to Tyr731 dephosphorylation of VE-cadherin. These observations are in line with previous findings that a disruption of VE-cadherin/β-catenin complex and/or formation of stress fibers is required for leukocyte migration through the endothelium (10, 17, 33). Thus, we propose that activation of pulmonary endothelial E-selectin shapes the metastatic lungs and effects VE-cadherin junctions (Fig. 6E) thereby promoting metastasis. Although the process of leukocyte crossing through the endothelial barrier is well characterized (17), the mechanism of loosening of VE-cadherin junctions remains to be defined. Accordingly, E-selectin is likely an additional factor to VCAM-1 inducing this process. While selectin ligands on tumor cells might induce efficient trans-endothelial migration through a direct E-selectin engagement (10), the presence of monocytes apparently promotes this process. The presented data suggest that monocyte recruitment and E-selectin activation are important regulators of pulmonary vascular permeability for tumor cell extravasation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors acknowledge the assistance of the Center for Microscopy and Image Analysis, University of Zurich for confocal microscopy experiments.

Financial support: This study was supported by the SNF grant #310030-152901 (L. Borsig). Further support in part by the DFG-SFB/Transregio 23 and SHENC-Research Unit FOR1543 (S.W. Schneider). Further support in part by National Program of Sustainability II LQ1605-MEYS CR; project FNUSA-ICRC (CZ.1.05/1.1.00/02.0123) and ICRC-ERA-HumanBridge/no. 316345 (L. Knopfova).

References

1. Witz IP. The selectin-selectin ligand axis in tumor progression. Cancer Metastasis Rev. 2008; 27:19–30. [PubMed: 18180878]
2. Läubli H, Borsig L. Selectins promote tumor metastasis. Semin Cancer Biol. 2010; 20:169–77. [PubMed: 20452433]
3. Labelle M, Hynes RO. The initial hours of metastasis: the importance of cooperative host-tumor cell interactions during hematogenous dissemination. Cancer Discov. 2012; 2:1091–9. [PubMed: 23166151]
4. Sperandio M, Gleissner CA, Ley K. Glycosylation in immune cell trafficking. Immunol Rev. 2009; 230:97–113. [PubMed: 19594631]
5. Kannagi R. Molecular mechanism for cancer-associated induction of sialyl Lewis X and sialyl Lewis A expression-The Warburg effect revisited. Glycoconj J. 2004; 20:353–64. [PubMed: 15229399]
6. Hauselmann I, Borsig L. Altered tumor-cell glycosylation promotes metastasis. Front Oncol. 2014; 4:28. [PubMed: 24592356]
7. St Hill CA, Bullard KM, Walcheck B. Expression of the high-affinity selectin glycan ligand C2-O-sLeX by colon carcinoma cells. Cancer Lett. 2005; 217:105–13. [PubMed: 15596301]
8. Dimitroff CJ, Descheny L, Trujillo N, Kim R, Nguyen V, Huang W, et al. Identification of leukocyte E-selectin ligands, P-selectin glycoprotein ligand-1 and E-selectin ligand-1, on human metastatic prostate tumor cells. Cancer Res. 2005; 65:5750–60. [PubMed: 15994950]
9. Laferriere J, Houle F, Taher MM, Valerie K, Huot J. Transendothelial migration of colon carcinoma cells requires expression of E-selectin by endothelial cells and activation of stress-activated protein kinase-2 (SAPK2/p38) in the tumor cells. J Biol Chem. 2001; 276:33762–72. [PubMed: 11448946]

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

10. Tremblay PL, Auger FA, Huot J. Regulation of transendothelial migration of colon cancer cells by E-selectin-mediated activation of p38 and ERK MAP kinases. Oncogene. 2006; 25:6563–73. [PubMed: 16715142]
11. Läubli H, Spanaus KS, Borsig L. Selectin-mediated activation of endothelial cells induces expression of CCL5 and promotes metastasis through recruitment of monocytes. Blood. 2009; 114:4583–91. [PubMed: 19779041]
12. Läubli H, Borsig L. Selectins as mediators of lung metastasis. Cancer Microenviron. 2010; 3:97–105. [PubMed: 21209777]
13. Auguste P, Fallavollita L, Wang N, Burnier J, Bikfalvi A, Brodt P. The host inflammatory response promotes liver metastasis by increasing tumor cell arrest and extravasation. Am J Pathol. 2007; 170:1781–92. [PubMed: 17456781]
14. Hiratsuka S, Goel S, Kamoun WS, Maru Y, Fukumura D, Duda DG, et al. Endothelial focal adhesion kinase mediates cancer cell homing to discrete regions of the lungs via E-selectin up-regulation. Proc Natl Acad Sci U S A. 2011; 108:3725–30. [PubMed: 21321210]
15. Hoos A, Protsyuk D, Borsig L. Metastatic growth progression caused by PSGL-1-mediated recruitment of monocytes to metastatic sites. Cancer Res. 2014; 74:695–704. [PubMed: 24322980]
16. Mehta D, Malik AB. Signaling mechanisms regulating endothelial permeability. Physiol Rev. 2006; 86:279–367. [PubMed: 16371600]
17. Vestweber D. How leukocytes cross the vascular endothelium. Nat Rev Immunol. 2015; 15:692–704. [PubMed: 26471775]
18. Weber C, Fraemohs L, Dejana E. The role of junctional adhesion molecules in vascular inflammation. Nat Rev Immunol. 2007; 7:467–77. [PubMed: 17525755]
19. Wessel F, Winderlich M, Holm M, Frye M, Rivera-Galdos R, Vockel M, et al. Leukocyte extravasation and vascular permeability are each controlled in vivo by different tyrosine residues of VE-cadherin. Nat Immunol. 2014; 15:223–30. [PubMed: 24487320]
20. Alcaide P, Newton G, Auerbach S, Sehrawat S, Mayadas TN, Golan DE, et al. p120-Catenin regulates leukocyte transmigration through an effect on VE-cadherin phosphorylation. Blood. 2008; 112:2770–9. [PubMed: 18641366]
21. Haidari M, Zhang W, Caivano A, Chen Z, Ganjehei L, Mortazavi A, et al. Integrin alpha2beta1 mediates tyrosine phosphorylation of vascular endothelial cadherin induced by invasive breast cancer cells. J Biol Chem. 2012; 287:32981–92. [PubMed: 22833667]
22. Qian B, Deng Y, Im JH, Muschel RJ, Zou Y, Li J, et al. A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. PLoS One. 2009; 4:e6562. [PubMed: 19668347]
23. Lu X, Kang Y. Chemokine (C-C motif) ligand 2 engages CCR2+ stromal cells of monocytic origin to promote breast cancer metastasis to lung and bone. J Biol Chem. 2009; 284:29087–96. [PubMed: 19720836]
24. Wolf MJ, Hoos A, Bauer J, Boettcher S, Knust M, Weber A, et al. Endothelial CCR2 signaling induced by colon carcinoma cells enables extravasation via the JAK2-Stat5 and p38MAPK pathway. Cancer Cell. 2012; 22:91–105. [PubMed: 22789541]
25. Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature. 2011; 475:222–5. [PubMed: 21654748]
26. Zhao L, Lim SY, Gordon-Weeks AN, Tapmeier TT, Im JH, Cao Y, et al. Recruitment of a myeloid cell subset (CD11b/Gr1(mid))via CCL2/CCR2 promotes thedevelopment of colorectal cancer liver metastasis. Hepatology. 2013; 57:829–39. [PubMed: 23081697]
27. Borsig L, Wong R, Hynes RO, Varki NM, Varki A. Synergistic effects of L- and P-selectin in facilitating tumor metastasis can involve non-mucin ligands and implicate leukocytes as enhancers of metastasis. Proc Natl Acad Sci U S A. 2002; 99:2193–8. [PubMed: 11854515]
28. Zeisberger SM, Odermatt B, Marty C, Zehnder-Fjallman AH, Ballmer-Hofer K, Schwendener RA. Clodronate-liposome-mediated depletion of tumour-associated macrophages: a new and highly effective antiangiogenic therapy approach. Br J Cancer. 2006; 95:272–81. [PubMed: 16832418]
29. Zarbock A, Singbartl K, Ley K. Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation. J Clin Invest. 2006; 116:3211–9. [PubMed: 17143330]

Europe PMC Funders Author Manuscripts

30. St Hill CA. Interactions between endothelial selectins and cancer cells regulate metastasis. Front Biosci. 2011; 17:3233–51.
31. Roblek M, Strutzmann E, Zankl C, Adage T, Heikenwalder M, Atlic A, et al. Targeting of CCL2-CCR2-Glycosaminoglycan Axis Using a CCL2 Decoy Protein Attenuates Metastasis through Inhibition of Tumor Cell Seeding. Neoplasia. 2016; 18:49–59. [PubMed: 26806351]
32. Borsig L, Wolf MJ, Roblek M, Lorentzen A, Heikenwalder M. Inflammatory chemokines and metastasis-tracing the accessory. Oncogene. 2014; 33:3217–24. [PubMed: 23851506]
33. Hu Y, Kiely JM, Szente BE, Rosenzweig A, Gimbrone MA Jr. E-selectin-dependent signaling via the mitogen-activated protein kinase pathway in vascular endothelial cells. J Immunol. 2000; 165:2142–8. [PubMed: 10925300]
34. McEver RP. Selectins: lectins that initiate cell adhesion under flow. Curr Opin Cell Biol. 2002; 14:581–6. [PubMed: 12231353]
35. Homeister JW, Thall AD, Petryniak B, Maly P, Rogers CE, Smith PL, et al. The alpha(1,3)fucosyltransferases FucT-IV and FucT-VII exert collaborative control over selectin-dependent leukocyte recruitment and lymphocyte homing. Immunity. 2001; 15:115–26. [PubMed: 11485743]
36. Wojciak-Stothard B, Williams L, Ridley AJ. Monocyte adhesion and spreading on human endothelial cells is dependent on Rho-regulated receptor clustering. J Cell Biol. 1999; 145:1293–307. [PubMed: 10366600]
37. Yoshida M, Westlin WF, Wang N, Ingber DE, Rosenzweig A, Resnick N, et al. Leukocyte adhesion to vascular endothelium induces E-selectin linkage to the actin cytoskeleton. J Cell Biol. 1996; 133:445–55. [PubMed: 8609175]
38. Khatib AM, Kontogiannea M, Fallavollita L, Jamison B, Meterissian S, Brodt P. Rapid induction of cytokine and E-selectin expression in the liver in response to metastatic tumor cells. Cancer Res. 1999; 59:1356–61. [PubMed: 10096570]
39. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer. 2002; 2:563–72. [PubMed: 12154349]
40. Vidal-Vanaclocha F, Fantuzzi G, Mendoza L, Fuentes AM, Anasagasti MJ, Martin J, et al. IL-18 regulates IL-1beta-dependent hepatic melanoma metastasis via vascular cell adhesion molecule-1. Proc Natl Acad Sci U S A. 2000; 97:734–9. [PubMed: 10639148]
41. Ferjancic S, Gil-Bernabe AM, Hill SA, Allen PD, Richardson P, Sparey T, et al. VCAM-1 and VAP-1 recruit myeloid cells that promote pulmonary metastasis in mice. Blood. 2013; 121:3289–97. [PubMed: 23407548]
42. Matsuo Y, Amano S, Furuya M, Namiki K, Sakurai K, Nishiyama M, et al. Involvement of p38alpha mitogen-activated protein kinase in lung metastasis of tumor cells. J Biol Chem. 2006; 281:36767–75. [PubMed: 17028194]
43. Kobayashi K, Matsumoto S, Morishima T, Kawabe T, Okamoto T. Cimetidine inhibits cancer cell adhesion to endothelial cells and prevents metastasis by blocking E-selectin expression. Cancer Res. 2000; 60:3978–84. [PubMed: 10919677]
44. Desch A, Strozyk EA, Bauer AT, Huck V, Niemeyer V, Wieland T, et al. Highly invasive melanoma cells activate the vascular endothelium via an MMP-2/integrin alphavbeta5-induced secretion of VEGF-A. Am J Pathol. 2012; 181:693–705. [PubMed: 22659470]
45. Wang G, Woo CW, Sung FL, Siow YL, O K. Increased monocyte adhesion to aortic endothelium in rats with hyperhomocysteinemia: role of chemokine and adhesion molecules. Arterioscler Thromb Vasc Biol. 2002; 22:1777–83. [PubMed: 12426204]
46. Westra J, Kuldo JM, van Rijswijk MH, Molema G, Limburg PC. Chemokine production and E-selectin expression in activated endothelial cells are inhibited by p38 MAPK (mitogen activated protein kinase) inhibitor RWJ 67657. Int Immunopharmacol. 2005; 5:1259–69. [PubMed: 15914330]
47. Lukacs NW, Strieter RM, Elner V, Evanoff HL, Burdick MD, Kunkel SL. Production of chemokines, interleukin-8 and monocyte chemoattractant protein-1, during monocyte: endothelial cell interactions. Blood. 1995; 86:2767–73. [PubMed: 7545470]

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

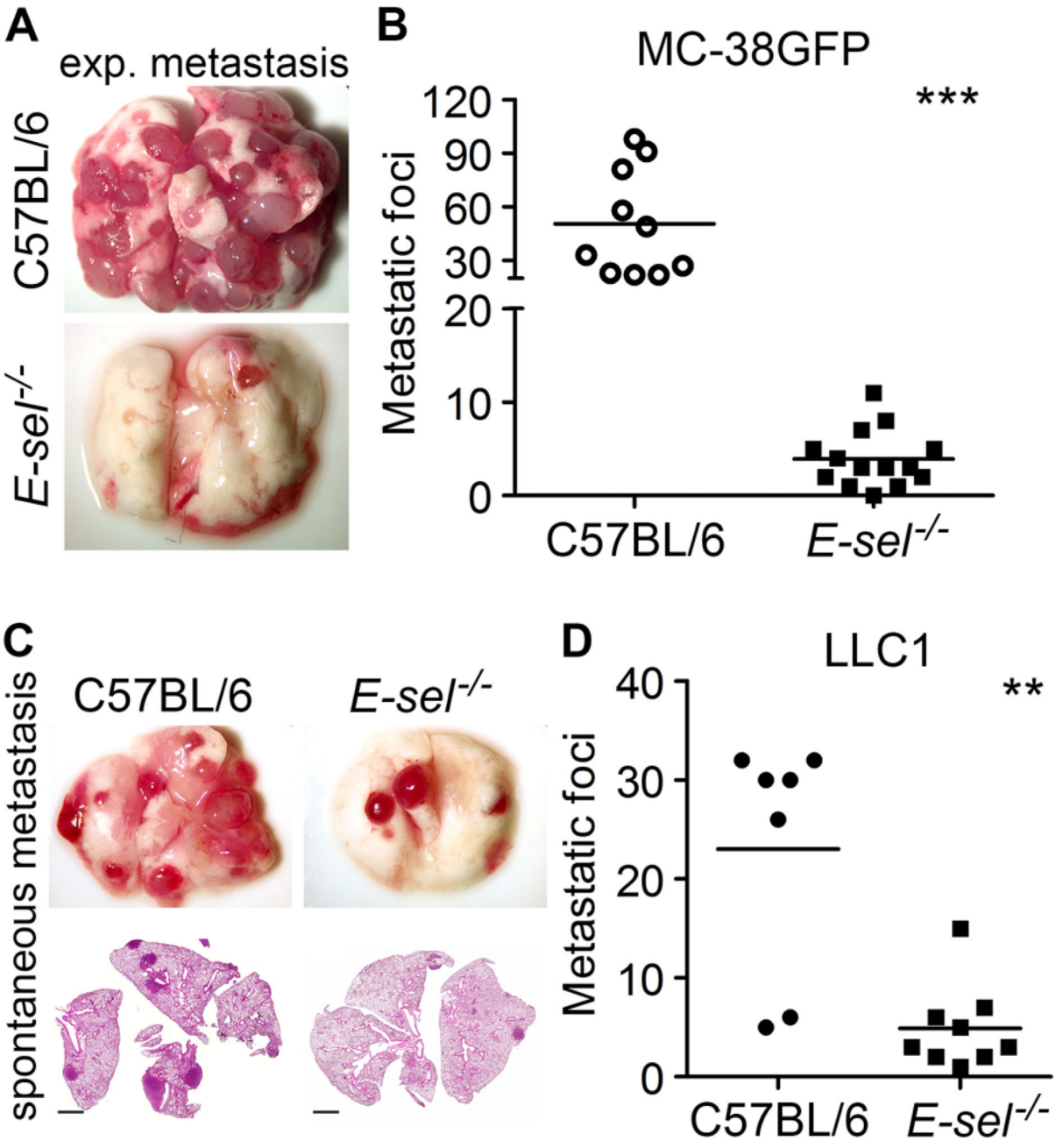

Figure 1. E-selectin facilitates experimental and spontaneous metastasis of tumor cells carrying no E-selectin ligands.

Mice were intravenously injected with tumor cells and lungs analyzed for metastasis. **A)** Representative images of dissected lungs 28 days after MC-38GFP cell injection. **B)** Quantification of metastatic foci (3 independent experiments). **C)** Mice were subcutaneously injected Lewis Lung carcinoma cells (LLC1) and spontaneous lung metastasis was analyzed. Representative images of dissected lungs from C57BL/6 and *E-sel*$^{-/-}$ mice and H&E stained lung sections. Bar = 2 mm. **D)** Number of metastatic foci in lungs of tumor bearing

C57BL/6 and *E-set*$^{-/-}$ mice 30 days after LLC1 cell injection (2 independent experiments). **; p<0.01; ***; p<0.001.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts

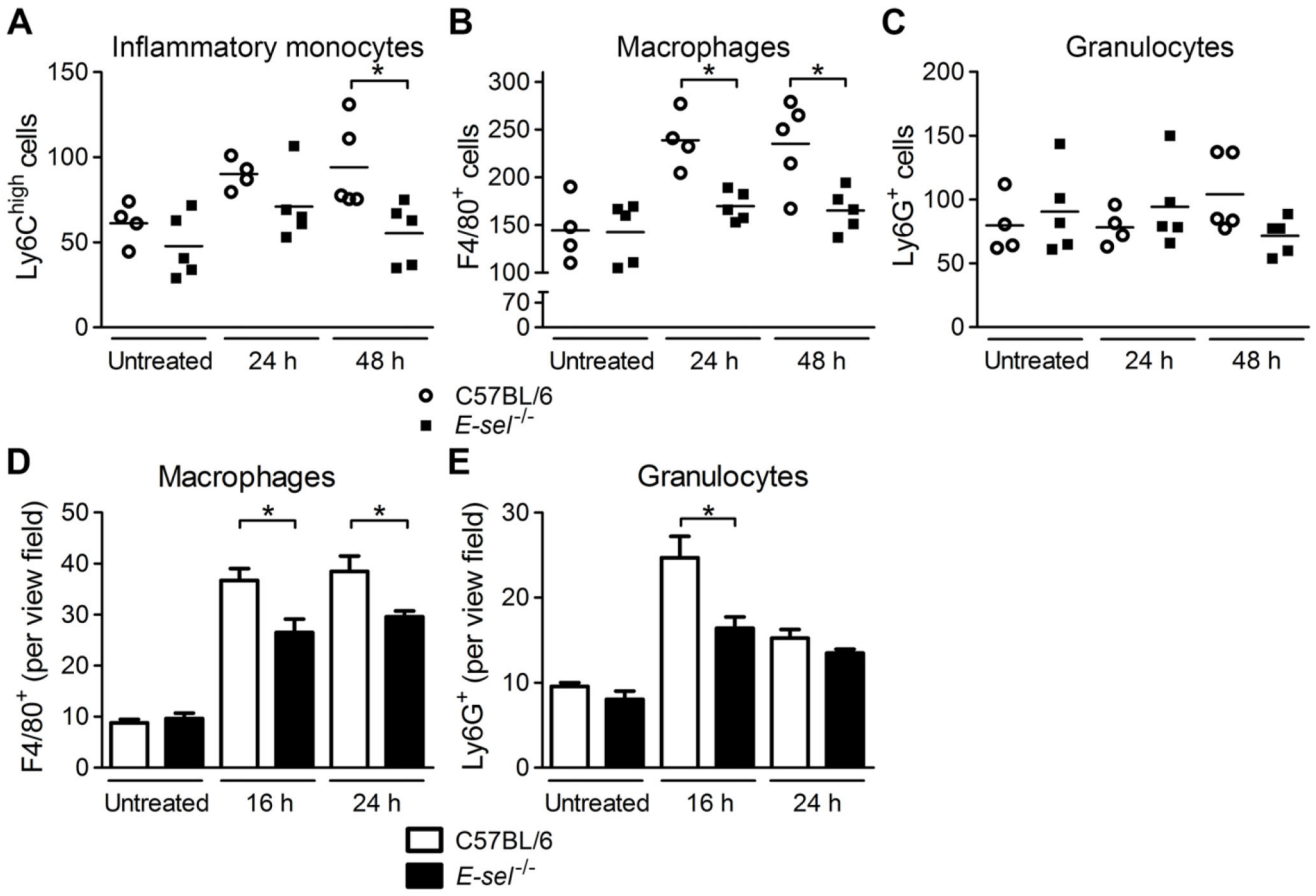

Figure 2. E-selectin-dependent leukocyte recruitment to metastatic lungs.

A-C) Flow cytometry analysis of lungs from C57BL/6 and *E-sel*$^{-/-}$ mice at 24 or 48 hours p.i. were compared to lungs of naïve mice, respectively. The number of inflammatory monocytes (Ly6C^{high}), macrophages (F4/80^{+}) and granulocytes (Ly6G^{+}) was normalized to 1'000 endothelial cells (CD31^{+}). **D-E)** Lung cryosection analysis for macrophages (F4/80^{+}) and granulocytes (Ly6G^{+}) at 16 and 24 hours p.i. were compared to untreated controls. *; $p<0.05$.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

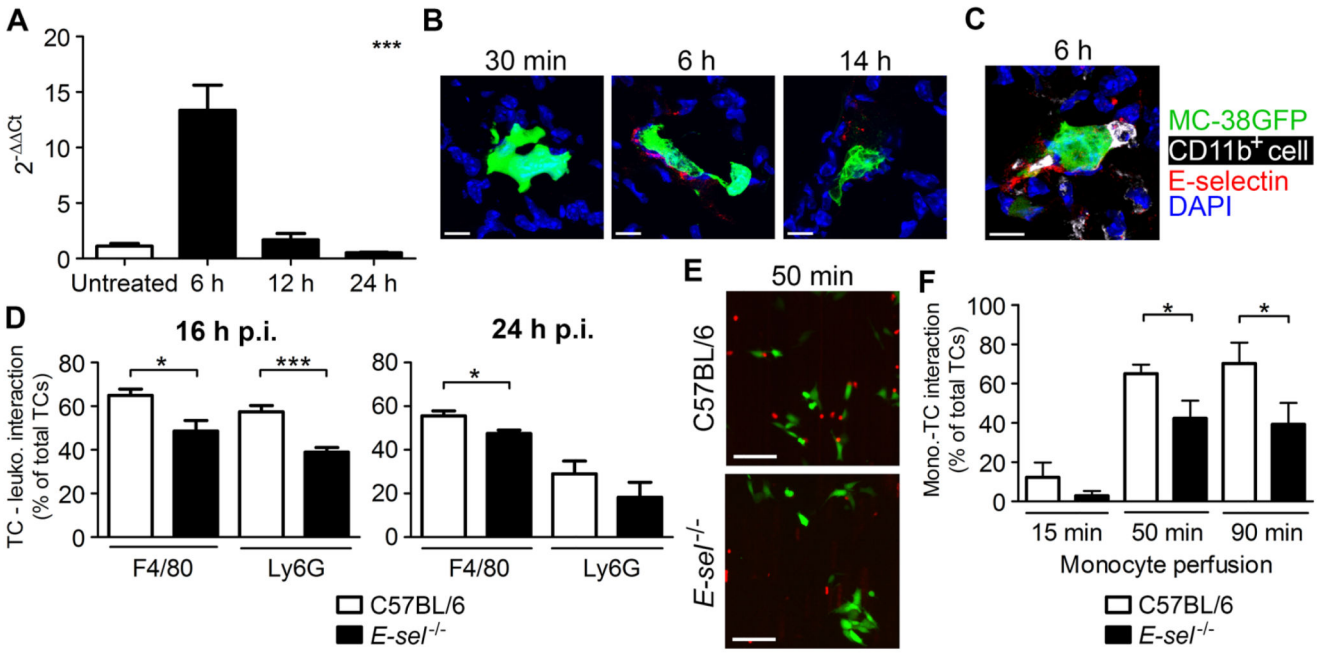

Figure 3. Tumor cell-induced E-selectin expression facilitates specific leukocyte association with tumor cells at metastatic sites.

A) E-selectin expression in the lungs of C57BL/6 mice at 6, 12 and 24 hours p.i. were compared to lungs of untreated mice. Expression levels determined by real-time PCR were normalized to GAPDH expression (n = 4). Data are expressed as mean ± SEM. ***; p<0.001. **B)** Microscopy images of E-selectin expression (red) in the vicinity of tumor cells (green) in the lungs at indicated times p.i. Nuclei (blue) were stained with DAPI. Bar = 10 μm. **C)** Microscopy image of a lung section 6 hours p.i. with a tumor cell (green) associated with $CD11b^+$ cells (white) in the vicinity of $E\text{-selectin}^+$ (red) endothelial cells. Nuclei (blue) were stained with DAPI. **D)** Analysis of tumor cell-leukocyte association in lungs of C57BL/6 and *E-sel*$^{-/-}$ mice 16 and 24 hours p.i. (n = 3). **E)** Representative images of tumor cells (green) adherent on endothelial cells derived from C57BL/6 (middle panel) and *E-sel*$^{-/-}$ mice (right panel) and recruited monocytes (red) 50 minutes after initiation of monocyte perfusion. **F)** Number of monocytes associated with MC-38GFP cells on endothelial cells at 15, 50 and 90 minutes after perfusion with monocytes was induced in a microfluidic slide (n = 3). *; p<0.05; ***; p<0.001.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

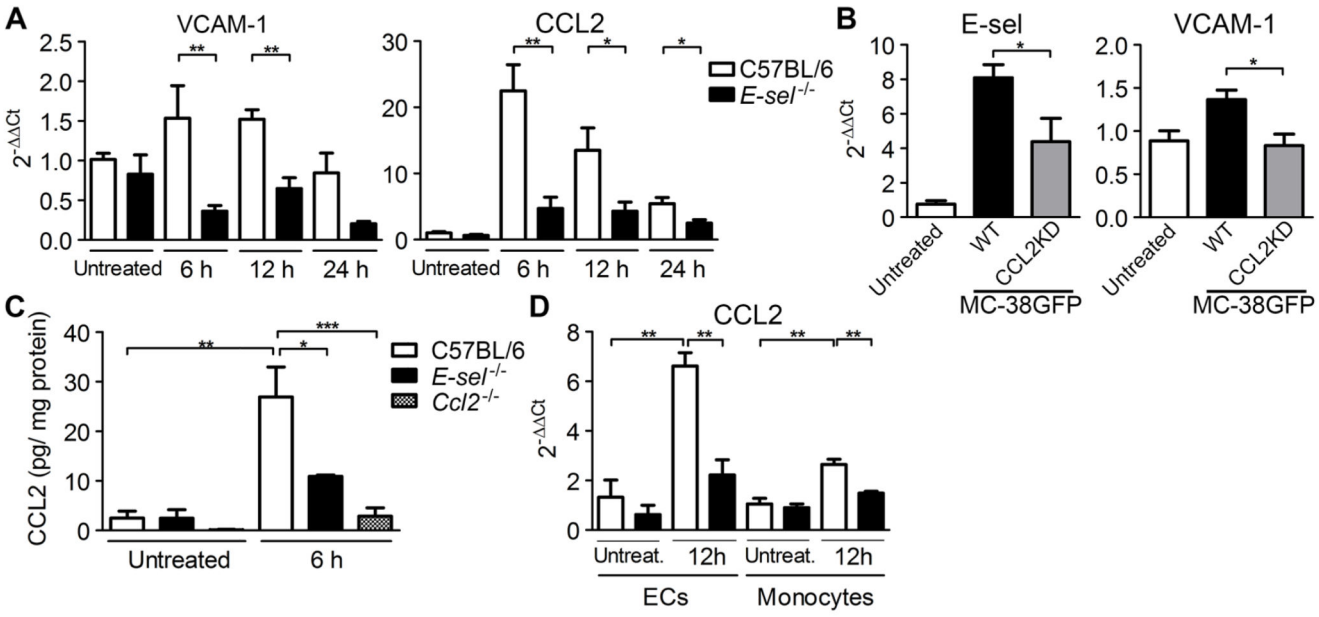

Figure 4. E-selectin expression is induced through tumor cell-derived CCL2 and further endothelial activation facilitates increased CCL2 expression in metastatic lungs.

A) Expression levels of VCAM-1 and CCL2 in the lungs of C57BL/6 and *E-sel*$^{-/-}$ mice 6, 12 and 24 hours p.i. of MC-38GFP cells or of untreated mice were analyzed by real-time PCR, normalized to GAPDH and displayed as relative values to untreated controls (n = 3). **B)** Expression levels of E-selectin and VCAM-1 in the lungs of C57BL/6 mice 6 hours p.i. with MC-38GFP and MC-38GFP CCL2KD cells or of untreated mice were normalized to GAPDH and displayed as relative values to untreated controls (n = 3). *; p<0.05; **; p<0.01. **C)** CCL2 protein levels in the homogenate of perfused lungs from untreated C57BL/6, *E-sel*$^{-/-}$ and *Ccl2*$^{-/-}$ mice 6 hours p.i. (n = 3); *; p<0.05; **; p<0.01; ***; p<0.001. **D)** CCL2 expression levels in pulmonary endothelial cells (CD31^{+}) and inflammatory monocytes (CD11b^{+}/Ly6C^{hi}) sorted from lungs of untreated C57BL/6 and *E-sel*$^{-/-}$ mice and 12 hours p.i. Real-time PCR of CCL2 were normalized to GAPDH and displayed as relative values to untreated controls (n = 3); **; p<0.01 by Student's t-test.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

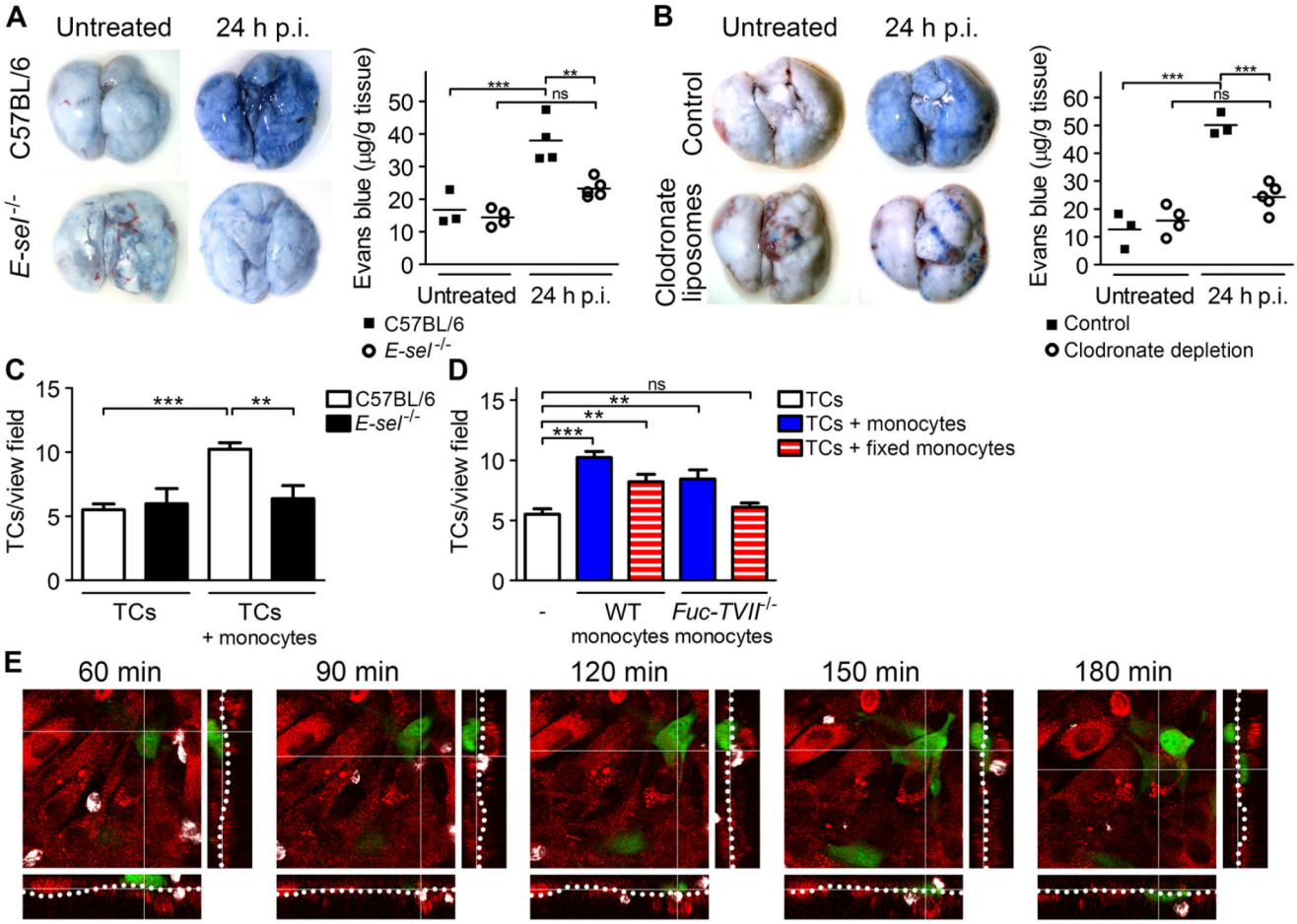

Figure 5. E-selectin expression promotes increased lung vascular permeability and facilitates tumor cell transmigration in monocyte-derived selectin ligand-dependent manner

A) Macroscopic images of lungs from untreated C57BL/6 and *E-sel*$^{-/-}$ mice and 24 hours p.i. injected with Evans blue and quantification of the dye extracted from lungs (n = 2). **B)** Macroscopic images of lungs from untreated or clodronate liposome-treated C57BL/6 mice 24 hours p.i. injected with Evans blue and quantification of the dye extracted from lungs (n = 2). **C)** Transmigrated MC-38GFP cells through lung endothelial cells derived from C57BL/6 and *E-sel*$^{-/-}$ mice in the absence or presence of monocytes after 16 hours of co-culture (n = 3). **D)** Number of MC-38GFP cells transmigrated through lung endothelial cells derived from C57BL/6 mice in the presence of monocytes. Normal (unfixed) monocytes were compared to fixed monocytes isolated from C57BL/6 or *Fuc-TVII*$^{-/-}$ mice after 16 hours of co-culture (n = 3, 4 independent experiments). $p<0.01$; ***; $p<0.001$; ns = not significant. **E)** Live imaging of MC-38GFP (green) trans-endothelial (red) migration assisted by monocytes (white) captured at different time points. Dotted line indicates the endothelial monolayer (2 independent experiments). Bar = 30 µm.

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

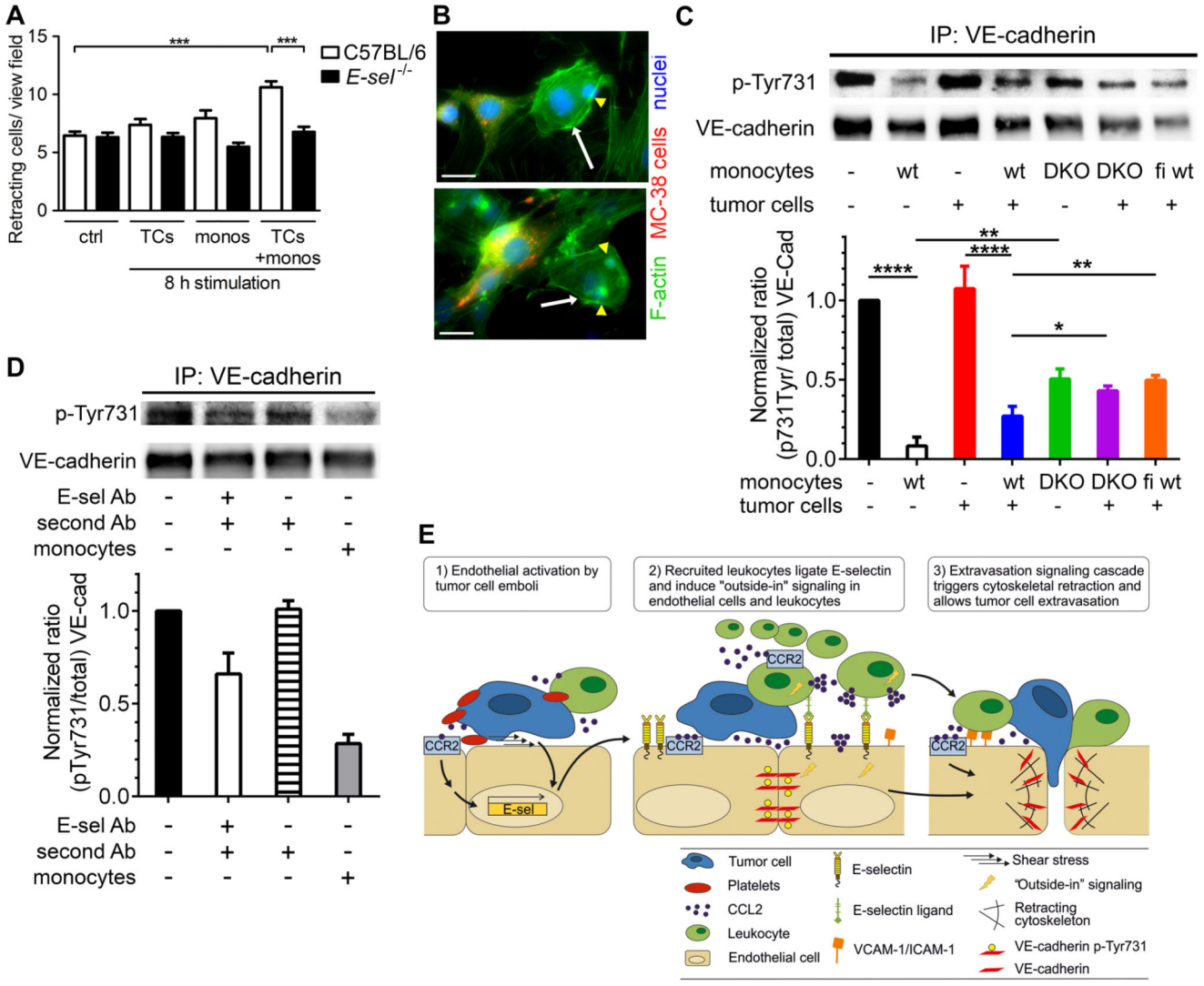

Figure 6. E-selectin-dependent endothelial cell retraction is induced by tumor cells together with monocytes and depends on VE-cadherin Tyr731 dephosphorylation.

A) Quantification of endothelial cell retraction stained with Phalloidin-FITC of untreated endothelial cells from C57BL/6 or *E-sel*$^{-/-}$ mice (ctrl); or after co-culture with MC-38 cells (TCs) and/or monocytes (monos) (n = 5). ***; p<0.001. **B)** High power images of endothelial cells from C57BL/6 mice with MC-38 cells (red) and monocytes, showing endothelial cell retraction stained by Phalloidin-FITC (arrows) and the adjacent monocytes (arrowheads) after co-culture for 8 hours. Nuclei (blue) are stained with DAPI. Bar = 20 μm. **C)** Immunoblot analysis of VE-cadherin immunoprecipitated from IL-1β-activated bEnd.3 cells upon stimulation with monocytes (wt = C57BL/6; DKO = *FucTIV/VII*$^{-/-}$ double deficient; or fi wt = fixed wt), MC-38GFP cells (tumor cells) or both for 1 h. We used either mAb mp731 (upper panel) or anti VE-cadherin (lower panel). Quantification of results are presented as a ratio of pTyr731/total VE-cadherin ratio (n = 3); *; p<0.05; **; p<0.01; ***; p<0.001. **D)** Immunoblot analysis of VE-cadherin from activated bEnd.3 cells stimulated by crosslinking with an E-selectin antibody or with wt monocytes (upper panel). After anti p-

Europe PMC Funders Author Manuscripts
Europe PMC Funders Author Manuscripts

Tyr731 antibody staining, the membrane was developed with anti-VE-cadherin antibody. Quantification of results (lower panel) are presented as a ratio of pTyr731/total VE-cadherin ratio (n = 2). **E**) Model of the E-selectin involvement in metastatic niche formation. 1) Circulating tumor cells interact with platelets and leukocytes and arrest in the microvasculature. Shear stresses together with tumor- and blood cell-derived factors, e.g. CCL2, activate the endothelium resulting in E-selectin expression. 2) Endothelial E-selectin mediates adhesion of leukocytes, which are recruited by chemokines. Upon ligation of E-selectin by leukocytes “outside-in” signaling is induced both in leukocytes and in endothelial cells starting the extravasation cascade. 3) This includes firm adhesion of leukocytes to endothelium via integrins and cytoskeletal remodeling and retraction in endothelial cells. Ultimately, dephosphorylation of VE-cadherin results in increased vascular permeability, enabling tumor cells extravasation.

Europe PMC Funders Author Manuscripts

Europe PMC Funders Author Manuscripts