Supplemental Information

Macrophage-Induced Lymphangiogenesis and Metastasis following Paclitaxel Chemotherapy Is Regulated by VEGFR3

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SUPPLEMENTAL INFORMATION ONLINE

Supplemental Experimental Procedures

Bone marrow transplantation

Bone marrow transplantation was performed as previously described (Voloshin et al., 2015). Briefly, BMDCs were obtained by flushing femurs and tibias of 8-week-old donor C57Bl/6 WT and C57Bl/6 heparanase-/- mice. The BMDCs (5x10⁶ per recipient) were transplanted by tail vein injection into lethally irradiated C57Bl/6 WT or heparanase-/- recipients. Irradiation (1000 cGy total body irradiation) was performed at 250cGy/min using Elekta Precise (Elekta Oncology Systems) linear accelerator 6MeV photon beam radiation (Department of Radiation Therapy, Rambam Medical center, Haifa, Israel). After bone marrow cell reconstitution (usually 4-6 weeks), recipient mice were bled from the orbital sinus to verify bone marrow transplantation efficiency by evaluating the levels of heparanase in platelets, as described below.

Evaluation of murine and human VEGF-C protein levels

Blood, BMDCs, organs and macrophages were obtained from 8-10 week old Balb/c mice, 24 hours after they were treated with PTX, 31C1, FOLFOX, gemcitabine or cisplatinum. Blood and tumor tissue samples were also collected from patients with localized breast carcinoma. Patients were treated with neoadjuvant chemotherapy including PTX (before tumor removal) at the HaEmek Medical Center, Afula, Israel or at the Rambam Health Care Campus, Haifa, Israel. Blood was collected at baseline and 24 hours after the first cycle of PTX therapy (n=12). Plasma was separated and stored at -80°C before analysis. Biopsies of breast tumors obtained at baseline before initiation of chemotherapy and tumor specimens
obtained from resected tumors after neoadjuvant therapy (n=6) were obtained from the Department of Pathology at the Rambam Health Care Campus. The study was approved by the ethic committees both at Haemek and at Rambam medical centers, and all patients signed an informed consent. VEGF-C levels in mouse plasma, conditioned media, and organs (normalized to protein concentration) as well as in human patient plasma were determined by ELISA (Cusabio Biotech, China and R&D systems, Minneapolis, respectively), in accordance with the manufacturer’s instructions.

Invasion and migration assays

The invasion and migration properties of lymphatic endothelial cells (LECs) were assessed using the Boyden chamber assay as previously described for other cell types (Gingis-Velitski et al., 2011). Briefly, 2x10^5 LECs were placed in the upper compartment of a Transwell coated with either Matrigel (50µl, for invasion) or fibronectin (10 µg/ml, for migration) and incubated in serum-free DMEM overnight. Serum free DMEM supplemented with 10% plasma from mice treated with PTX or vehicle control was placed in the bottom compartment of the chamber. In some experiments, VEGF-C was depleted from the plasma by immunoprecipitation using anti-VEGF-C neutralizing antibodies (Abcam, United Kingdom ; 10 µg/ml) followed by separation using protein A/G coated sepharose beads (GE Healthcare, United Kingdom). After 24 hours, cells that invaded the Transwell were fixed with 4% paraformaldehyde (PFA) and stained with crystal violet solution (0.5% in DDW). Images were acquired with a camera attached to an inverted microscope (Leica CTR 6000) using Leica Application suite Version 3.4.0 software. Cells were counted and plotted. The experiments were performed in triplicate and results provided are an average of 15 fields/group.

Tube forming assay

Tube formation of LECs on Matrigel was carried out as previously described (Varshavsky et al., 2008). Briefly, LECs were seeded in Matrigel coated 48-well tissue culture plates (1.6x10^4 cells/well) and incubated with EGM growth medium (Lonza) supplemented with 10% plasma collected from mice, 24 hours after they were treated with PTX or vehicle control. Phase-contrast images of tubes were acquired at a ×100 magnification and the number of bifurcations per microscopic field (n=5/group) was counted. Experiments were performed in triplicate.

Flow-cytometry acquisition and analysis
Single cell suspensions of tumor tissue or peritoneal macrophages were analyzed by flow cytometry in order to evaluate the percentage of LECs, macrophages, and the expression of VEGFR3. Briefly, macrophages were stained with anti-F4/80-PE antibodies (Biolegend, San Diego, USA). VEGFR3 expression was evaluated using 31C1 antibody conjugated with FITC or APC (Innova Biosciences, United Kingdom) in accordance with the manufacturer’s protocol. LECs were stained with rabbit polyclonal anti-LYVE-1 antibodies (Abcam, United Kingdom) followed by secondary anti-rabbit Cy-3-conjugated antibody (Jackson Immunoresearch Laboratories, PA, USA) or hamster anti-mouse podoplanin, biotin-conjugated antibodies (Biolegend) followed by FITC-conjugated avidin (Jackson ImmunoResearch). Appropriate controls were used. In some experiments 7-aminoactinomycin D (7AAD) was used to distinguish apoptotic and dead cells from viable cells (Phippott et al., 1996). An acquisition of at least 100,000 events was performed using either the Cyan ADP (Dako, Denmark) or BD LSRSortessa (BD Biosciences, NJ, USA) flow-cytometers. Data analysis was performed with Summit v4.3 software (Beckman Coulter, CA, USA).

**Immunostaining**

Lungs or tumors were embedded in O.C.T. (Sakura, Japan) and subsequently frozen at -80°C. Tissue sections (12 μm thick) were prepared using Leica CM 1950 microtome (Leica, Germany). Sections were fixed in cold acetone for 15 minutes. Fixed samples were stained for lymphatic vessels using anti-LYVE-1 antibodies (Abcam, United Kingdom) followed by secondary Cy-3-conjugated antibodies (Jackson Immunoresearch Laboratories, PA, USA) or hamster anti-mouse podoplanin, biotin-conjugated antibodies (Biolegend) followed by Cy3-conjugated avidin (Jackson ImmunoResearch). Nuclei were stained with DAPI (Electron Microscopy Sciences, PA, USA). Tumor sections were immunostained with anti-cathepsin B antibodies (Abcam, United Kingdom) followed by secondary Dylight 488-conjugated antibodies (Jackson Immunoresearch Laboratories, PA, USA). For the detection of angiogenesis in tumors, tumor sections were immunostained with anti-CD31 antibodies, a specific endothelial cell marker (1:200, BD Biosciences) followed by a Cy3-conjugated secondary antibodies (1:500, Jackson immunoresearch laboratories). For the detection of metastasis, fixed lung samples were stained with hematoxylin and eosin (H&E). Human tumor specimens were embedded in paraffin and sectioned (6-10 μm thick). Sections were stained with mouse anti-human VEGF-C antibodies (R&D systems) and secondary anti-mouse peroxidase-conjugated antibodies (Jackson ImmunoResearch) followed by AEC staining (Nichirei Biosciences Inc., Japan). Images were acquired with a camera attached to an inverted microscope (Leica CTR 6000) using Leica Application suite Version 3.4.0 software. Positive staining was
determined by Adobe Photoshop 6 (San Jose, CA), and the number or percentage of positive pixels were plotted.

**Conditioned medium of macrophage cultures**

Eight-to-ten week old, female, Balb/c mice were induced with thioglycolate (4% in saline). Seventy two hours later, mice were treated with PTX and/or 31C1, MF1, or DC101. After 24 hours, macrophages were collected by peritoneal lavage. Collected macrophages were cultured in serum-free RPMI-1640 media at a concentration of $5 \times 10^6$/ml, and conditioned medium was collected 48 hours later. To obtain conditioned medium from human macrophages, human peripheral blood (5ml, n=4) was cultured in the presence of human recombinant M-CSF (50ng/ml, R&D systems) replaced every other day for a week. Floating cells were removed, and attached cells were cultured in serum-free medium with or without recombinant human VEGF-C (1μg/ml, PeproTech) for 3 sequential days. The conditioned medium was evaluated for heparanase and protease activity as described below. The in vitro experiments were performed in triplicate of three biological repeats.

**Cathepsin and heparanase activity assay**

Macrophages obtained from vehicle or PTX-treated Balb/c mice were harvested and cultured in serum-free medium at a concentration of $0.5 \times 10^6$ cells/ml. Cultured macrophages were treated with VEGF-C (1μg/ml) or VEGF-C and 31C1 (10μg/ml). Cathepsin inhibitor GB111-NH$_2$ (1μM) (Salpeter et al., 2015) was added to relevant controls 5 hours prior to medium collection. GB111-NH$_2$ is a cysteine cathepsin inhibitor that blocks the activity of cathepsins B, L and S (Salpeter et al., 2015). Cathepsin probe GB123 was added to all samples 4 hours prior to medium collection, as previously described (Salpeter et al., 2015). Conditioned medium was precipitated in 70% acetone for 4 hours. After centrifugation, pellets were resuspended in RIPA and filtered through an Amicon Ultra centrifugal filter unit Ultra-15. Protein content was quantified by Bradford and an equal amount of protein was resolved by SDS PAGE. Fluorescent bands were analyzed by a Typhoon flatbed scanner (GE Healthcare: Excitation/Emission 630/670 nm). Intensities of the relevant bands were quantified by densitometry, using TotalLab Quant software (TotalLab, UK). Experiments were performed in triplicate, and the average of densitometry analyses was calculated.

**Heparanase activity assay**
Preparation of Na$_2^{35}$SO$_4$-labeled extracellular matrix (ECM)-coated 35-mm dishes and determination of heparanase activity were performed essentially as described in detail elsewhere (Bar-Ner et al., 1985; Vlodavsky et al., 1999). Briefly, macrophage conditioned medium was incubated with $^{35}$S-labeled ECM for 18 hours at 37°C. The incubation medium (1 ml) containing sulfate-labeled degradation fragments, was subjected to gel filtration on a Sepharose CL-6B column. Fractions (0.2 ml) were eluted with PBS and radioactivity was counted in a β-scintillation counter, Tri-Carb 2100TR (Packard Bioscience, CT). Degradation fragments of heparan sulfate (HS) side chains produced by heparanase are eluted at 0.5 < Kav < 0.8 (peak II, fractions 18–25). Nearly intact HS proteoglycans (HSPGs) released from the ECM are eluted just after the V0 (Kav < 0.2, peak I, fractions 3–15). These high molecular weight products are released by proteases that cleave the HSPG core protein (Bar-Ner et al., 1985; Vlodavsky et al., 1999). The results of the different fractions were plotted, and a representative graph from three biological replicates is provided.

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Supplemental Figures

Figure S1 (related to Figure 1)

Figure S1 legend

VEGF-C expression in plasma, bone marrow conditioned medium, and organs following treatment with chemotherapy. Non-tumor bearing, 8-10 week old Balb/c mice (n=4 mice/group) were treated with FOLFOX (folinic acid [30mg/kg], 5-FU [50mg/kg] and oxaliplatin [14 mg/kg]), cisplatinum (6mg/kg), or gemcitabine (500mg/kg). Control mice were treated with PBS. After 24 hours, blood was drawn by cardiac puncture and plasma was separated; bone marrow (BM) cells were flushed from the bone and conditioned medium was generated; and lungs and liver were harvested and lysates were prepared. VEGF-C levels were assessed using ELISA. *p<0.05; **, 0.01≥p>0.001; ***, p≤0.001 using one way ANOVA followed by Tukey post-hoc test.
Figure S2 (related to Figure 3)
Figure S2 legend

Quantification of tumor growth, lymphangiogenesis and pulmonary metastasis lesions in mice bearing tumors treated with paclitaxel and/or 31C1. (A) Mice bearing 4T1 tumors were treated with PTX and/or 31C1 (n=5 mice/group). When tumors in each group reached endpoint, lungs were removed and sectioned. Lung sections were stained for H&E (Figure 3A), and the number of metastatic lesions in the lungs were counted (n=15 fields/group). (B) CB.17 SCID mice bearing LM2-4 human metastatic breast carcinomas (n=5 mice from the 6-7 mice/group) were let grow until they reached 100-150mm³. Subsequently, treatment with PTX and/or 31C1 was initiated, and three 3 days later, the primary tumors were harvested. Tumor sections were stained for podoplanin (red). DAPI was used to stain nuclei (blue). Scale bar = 200µm. The number of lymphatic vessels in the tumors was counted per field (n=20 fields/group). (C) CB.17 SCID mice bearing LM2-4 human metastatic breast carcinoma (n=5 mice/group) were let grow until they reached 300mm³. Subsequently, treatment with PTX and/or 31C1 was initiated and mice were sacrificed when primary tumors from control group reached endpoint (~1000mm³). Lungs were harvested and lung sections were stained for H&E. Scale bar = 200µm. The number of metastatic lesions per field was counted and plotted (n=20 fields/group). (D-F) Eight-to-ten week old CB.17 SCID mice (n=5 mice/group) were subcutaneously injected with A549 human lung carcinoma cells (5x10⁶). (D) When tumors reached 150mm³, treatment with PTX and/or 31C1 was initiated and tumor growth was monitored until endpoint. Control mice were treated with rat IgG isotype control. (E) In a parallel experiment, when tumors reached 500mm³ (n=5 mice/group) treatment with PTX and/or 31C1 was initiated, and three days later tumors were harvested. Tumor sections were stained with anti-podoplanin (red). DAPI was used to stain nuclei (blue). Scale bar = 200µm. The number of lymphatic vessels in the tumors was counted per field for each staining (n=20/ group). (F) In another experiment, mice bearing A549 were treated with vehicle or IgG isotype control until tumor reached end-point. Tumors were harvested and sectioned. Tumor sections were stained with anti-podoplanin (red). DAPI was used to stain nuclei (blue). Scale bar = 200µm. The number of lymphatic vessels in the tumors was counted per field for each staining (n=10/ group). ns, non-significant; *p<0.05; **, 0.01≥p>0.001; ***, p≤0.001 using one way ANOVA followed by Tukey post-hoc test.
Figure S3 (related to Figure 4)
**Figure S3 legend**

**VEGF-C and VEGFR3 expression in macrophages.** (A) Peritoneal macrophages were obtained from control mice. The macrophages were cultured in serum-free medium in the presence of 200nM PTX, 1µg/ml 31C1, or the combination of the two drugs. After 48 hours, the conditioned medium was assessed for levels of VEGF-C by ELISA. (B-C) Peritoneal macrophages were obtained from mice treated with vehicle, PTX, 31C1 or the combination of the two drugs and further assessed by flow cytometry. A representative dot-plot of VEGFR3+ F4/80+ cells of macrophages from vehicle group is shown (B), and the percentage of macrophages (F4/80+) expressing VEGFR3 was plotted (C). Results were obtained in triplicates, and no significant differences between groups were observed. (D) Flow cytometry representative dot-plots of VEGFR3-expressing macrophages and lymphatic endothelial cells from the following experiment: A549 tumors from Figure S2D were prepared as single cell suspensions. Cells were immunostained for VEGFR3+, macrophages (F4/80+), and lymphatic endothelial cells (LYVE-1+). Viable cells were determined as 7AAD negative. Representative dot-plots are shown in the figure, and the quantifications of viable VEGFR3-expressing macrophages or lymphatic endothelial cells from a total of 10,000 cells are shown in Figure 4D.
Figure S4 (related to Figure 5)
Figure S4 legend

Quantification of tumor-associated lymphatic vessels and endothelial cells in tumors from mice exposed to different conditions. (A) Peritoneal macrophages obtained from mice treated with vehicle, PTX, 31C1 or the combination of the two drugs were orthotopically co-implanted in mice (n=5 mice/group) in a 1:1 ratio with 4T1 cells (0.5x10^5 cells). At end-point of control group, tumors from all groups were removed and tumor sections were immunostained with podoplanin (red). Nuclei were stained with DAPI. Scale bar = 200µm. The number of lymphatic vessels per field is shown (n=15 fields/group). (B) Peritoneal macrophages from mice treated with PTX and anti-VEGFR1 (MF1) or PTX and anti-VEGFR2 (DC101) were orthotopically co-implanted into mice (n=5 mice/group) in a 1:1 ratio with 4T1 cells. Tumor sections were immunostained for LYVE-1 (red). Nuclei were stained with DAPI (blue). Scale bar=200µm. The number of lymphatic vessels per field is shown (n=15 fields/group). (C) Tumors sections from (A) were stained for endothelial cells using anti-CD31 antibodies (red). Scale bar=200µm. The number of microvessels (MVD) per field (n= 20 fields/group) was counted and plotted. Results did not reach statistical significance as assessed by one way ANOVA followed by Tukey post-hoc test. (D) Non-tumor bearing Balb/c mice (n=3 mice/group) were treated Clodrolip as described in Experimental Procedures. Spleens were removed and prepared as single cell suspension. The percentage of F4/80+ cells in the spleen was assessed by flow cytometry. (E) Eight-to-ten week old Balb/c mice (n=6 mice/group) were implanted with 4T1 tumor cells (0.5x10^6). When tumors reached 300mm³, treatment with Clodrolip, PTX, and VEGF-C or the combination of the drugs was initiated at the schedule and doses indicated in Experimental Procedures. After 3 days, tumors were harvested and sections were prepared and stained for LYVE-1 (red) and DAPI (blue). The number of lymphatic vessels per field were counted (n=20 / group). (F) Plasma VEGF-C levels were evaluated by ELISA in non-tumor bearing Balb/c mice, 72 hours after they were depleted for macrophages, and 24 hours after they were treated with PTX (n=4 mice/group). *p<0.05; **, 0.01≥p>0.001; ***, p≤0.001 using one way ANOVA followed by Tukey post-hoc test.
Figure S5 (related to Figure 7)
Figure S5 legend

Lymphatic vessel phenotype and VEGF-C plasma level in tumors of mice depleted for heparanase or inhibited for cathepsin activity. (A) Assessment of heparanase activity in platelets of heparanase chimeric mice was performed by using platelets obtained from chimeric wild-type mice (n=3 mice/group) which were used as recipients of heparanase-/- bone marrow cells (WT-BM<sup>hep-/-</sup>), or heparanase-/- mice (n=3 mice/group) which were used as recipients of wild-type bone marrow cells (Hep<sup>-/-</sup>BM<sup>wt</sup>). Heparanase enzymatic activity was determined as described in Experimental Procedures. A representative graph is plotted. (B) LLC cells (0.5x10<sup>6</sup>) were implanted into the flanks of chimeric wild-type mice used as recipients of heparanase-/- bone marrow cells (WT-BM<sup>hep-/-</sup>), or chimeric heparanase-/- mice used as recipients of wild-type bone marrow cells (Hep<sup>-/-</sup>BM<sup>wt</sup>). When tumors reached 500mm<sup>3</sup>, treatment with PTX or PTX+31C1 was initiated. After 3 days tumors were removed and tumor sections were stained for LYVE-1 (green) and DAPI (blue). The number of lymphatic vessels in tumor sections were counted per field (n=15 fields/group). (C) Plasma obtained from wild-type and heparanase-/- mice (n=4 mice/group), 24 hours after they were treated with Vehicle (Veh) or PTX was assessed for VEGF-C levels using specific ELISA. (D) LLC cells (0.5x10<sup>6</sup>) were implanted into the flanks of C57Bl/6 mice. When tumors reached 500mm<sup>3</sup>, treatment with Vehicle (Veh), E64, PTX or the combination of PTX+E64 was initiated in the schedule and doses described in Experimental Procedures. After 3 days, tumors were removed and tumor sections were stained for LYVE-1 (red) and DAPI (blue). The number of lymphatic vessels in tumor sections were counted per field (n=15 fields/group). Scale bar = 200µm. *p<0.05; **p≤0.001, using one way ANOVA followed by Tukey post-hoc test. * 0.05<p<0.01, using two-tailed student t-test.