RESEARCH ARTICLE

A function-blocking CD47 antibody modulates extracellular vesicle-mediated intercellular signaling between breast carcinoma cells and endothelial cells

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Abstract Tumor cells release extracellular vesicles (EVs) into the tumor microenvironment that may facilitate malignant progression and metastasis. Breast carcinoma EVs express high levels of the thrombospondin-1 and signal regulatory protein- α receptor CD47, which is the target of several experimental therapeutics currently in clinical trials. We analyzed changes in gene expression and function in human umbilical vein endothelial cells (HUVEC) induced by treatment with EVs derived from breast carcinoma cells and the effects of the function-blocking CD47 antibody B6H12 on the resulting intercellular communication. CD47⁺ EVs exhibited greater uptake by HUVEC compared to CD47⁻ EVs, but the CD47 antibody did not inhibit their uptake. Global and targeted analyses of transcripts demonstrated that treatment of HUVEC with EVs derived from MDA-MB-231 breast carcinomas cells altered pathways associated with tumor necrosis

factor- α signaling, angiogenesis, lymphangiogenesis, endothelial-mesenchymal transition, and extracellular matrix. EVs from triple-negative MDA-MB-231 cells were more active than EVs from less metastatic breast carcinoma cell lines. Treatment with MDA-MB-231 EVs down-regulated VEGFR2 mRNA expression and tyrosine phosphorylation while enhancing phosphorylation of the tyrosine phosphatase SHP2. VEGFR2 expression and phosphorylation in HUVEC was further inhibited by the CD47 antibody. Consistent with the observed changes in endothelial-mesenchymal transition genes and SHP2, treatment with MDA-MB-231-derived EVs decreased Zeb1 protein levels in HUVEC, whereas the CD47 antibody increased Zeb1 levels. The induction of E-selectin and other known targets of tumor necrosis factor- α signaling by EVs was also enhanced by the CD47 antibody, and E-selectin was the most up-regulated transcript following CD47 antibody treatment alone. These studies reveal several mechanisms by which therapeutics targeting CD47 could modulate tumor growth by altering the cross talk between cancer-derived EVs and nonmalignant cells in the tumor stroma.

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Keywords Extracellular vesicles · Tumor microenvironment · Endothelial-mesenchymal transition · CD47 · Angiogenesis · Tumor necrosis factor- α

Abbreviations

bCSC	Breast cancer stem cells
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
EnMT	Endothelial-mesenchymal transition
EVs	Extracellular vesicles
HUVEC	Human umbilical vein endothelial cells

LYVE1	Lymphatic vessel endothelial hyaluronan receptor 1
SHP2	Tyrosine-protein phosphatase non-receptor type 11
SIRP α	Signal regulatory protein- α
TNF α	Tumor necrosis factor- α
TSP1/ <i>THBS1</i>	Thrombospondin-1 protein/gene
TXNIP	Thioredoxin-interacting protein
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor-2

Introduction

Intercellular communication is essential for the regulating development and physiological functions of multicellular organisms. Communications can be mediated by soluble ligands that signal through cell surface receptors on distant cells and by cytoskeleton proteins that transport metabolites and signaling molecules between cells via gap junctions or tunneling nanotubes (Grimmelikhuijzen and Hauser 2012; Abounit and Zurzolo 2012; Austefjord et al. 2014). Extracellular vesicles (EVs), which include exosomes, ectosomes, and microvesicles (MVs), are increasingly recognized for mediating intracellular communication for essential physiology processes including development, angiogenesis, and inflammation. Recent reports have found that signaling receptors and their soluble ligands are present on some EVs, which can enter target cells and alter their fate via releasing their protein and RNA contents (Pan and Johnstone 1983; Thery et al. 2002; Tkach and Thery 2016). EVs also contain transcripts encoding tumor promoter/suppressor genes, transcription factors, RNA binding proteins, microRNAs and other noncoding RNAs that are delivered to target cells (Valadi et al. 2007). Tumor cells also shed larger EVs that contain enzymes involved in glucose, glutamine and amino acid metabolism (Minciacchi et al. 2015) and proteins that modulate signaling of neighboring stroma cells (Di Vizio et al. 2012).

Cancer cells are highly plastic and can alter their epigenetic regulation to control cell cycle progression, undergo epithelial-mesenchymal transition (EMT), acquire stem cell characteristics, and acquire resistance to cytotoxic drugs and radiation therapy (Holzel et al. 2013). EVs, including oncosomes, shed by tumor cells can deliver their contents into nonmalignant cells and confer drug resistance (Pilzer et al. 2005). Similarly, EVs derived from cancer cells can induce angiogenesis to create a premetastatic niche (Grange et al. 2011; Atala 2012).

How these cancer-derived EVs interact with normal cells in the tumor stroma and alter their fate remains largely unknown (Hosseini-Beheshti et al. 2012). The potential for cancer therapeutics to modulate this crosstalk in

the tumor microenvironment also must be considered. In this study, we examined the mode of action of breast cancer EVs focusing on their functional effects on endothelial cells. The thrombospondin-1 and SIRP α receptor CD47 is highly expressed on many cancer cells, is associated with a poor prognosis, and is known to be present on EVs (Kaur et al. 2014b; Kibria et al. 2016; Chauhan et al. 2017; Sadallah et al. 2011; Roberts et al. 2017). Because biologics targeting CD47 have recently entered human clinical trials (Petrova et al. 2017; Matlung et al. 2017; Liu et al. 2015), we examined how a CD47 blocking antibody alters the communication between EVs derived from MDA-MB-231 breast carcinoma cells and normal endothelial cells. Our data indicate that breast cancer EVs alter the transcriptional profile in recipient endothelial cells, including altering VEGF and TNF α signaling and the endothelial-mesenchymal transition (EnMT). We further show that CD47 on these EVs alters their cellular uptake by endothelial cells. The CD47 blocking antibody also modulates the functional effects of cancer cell-derived EVs in endothelial cells, which may enhance the cancer promoting activity of EVs in the tumor microenvironment.

Material and methods

Cell culture and isolation of EVs HUVEC were purchased from Lonza and cultured using EGM2 medium containing penicillin, streptomycin, and glutamine. THP1 cells were differentiated to a macrophage phenotype using 50 ng/ml of phorbol 12-myristate 13-acetate for 5-days in RPMI 1640 complete medium containing 10% fetal calf serum, penicillin, streptomycin, and glutamine. MDA-MB-231, T47D1 and MCF7 breast carcinoma cells were cultured using RPMI 1640 complete medium. Cancer stem cells were cultured using Mammocult medium from StemCell Technologies. Before EV isolation, the cells were cultured using low human AB serum in the respective media for one passage. The cells were cultured in 75 cm² flasks, and conditioned medium (100 ml) was collected and concentrated to 1 ml using Centricon filters (EMD Millipore) with a cutoff of 10 kDa. A 250 μ l volume of concentrated conditioned medium from the respective cells was used to isolate EVs. The EVs were isolated using the ExoQuick kit according to the manufacturer's instructions (SBI Biosciences). The isolated EVs were washed with PBS and analyzed using flow cytometry as described earlier (Kaur et al. 2014b).

Western blot-immunoprecipitation analysis HUVEC and MDA-MB-231 breast cancer cells (1×10^6 /50 ml) were incubated in 75 cm² flasks at 37 °C. Harvested cells were centrifuged, and conditioned media was separated. The cells were washed with cold PBS, and the cell lysates were prepared using NP-40 lysis buffer. Immunoprecipitation was performed

using a Dynabeads protocol (Kaur et al. 2010). VEGFR2 immunoprecipitation was analyzed using SDS-PAGE (Bis-tris 4–12% gels). Primary antibodies for western blotting were diluted 1:1000, and secondary 1:5000 horseradish peroxidase-conjugated antibodies were used with chemiluminescent or infrared fluorescent imaging.

EV uptake EVs were isolated from HUVEC, and MDA-MB-231 cells (Kaur et al. 2014b) and labeled with PkH26 dye (Sigma). HUVEC were labeled with PkH67 green dye (Sigma). Then 10,000 cells per well were added to 8-well Lab-Tek chambers. The cells were treated with B6H12 antibody (1 and 2 µg/ml), and the cells and EVs were co-cultured for 6 h. The EV uptake was evaluated using confocal microscopy as described (Kaur et al. 2014b). To assess expression of differentiation markers, the 6-day HUVEC cultures were treated with EVs and stained using an antibody sampler kit for EMT from Cell Signaling Technology as described (Kaur et al. 2010).

Phagocytosis assay EVs were extracted using an Exo-spin kit and incubated with CD47-biotin antibody (CC2C6) for 1 h at 4 °C. Then, EVs were labelled with pHrodo Red dye at 37 °C for 15 min. Streptavidin beads were added, and CD47⁺ EVs were captured using magnetic columns and washed with PBS. CD47[−] EVs were subjected to further filtration using spin columns to remove unbound pHrodo Dye and antibody. CD47⁺ or CD47[−] EVs were added to THP1 cells and HUVEC for ~4 h. Flow cytometry analysis and confocal analysis were performed. CD47⁺ EVs were also treated with B6H12 antibody, and flow cytometry analysis was performed.

Tube formation assay HUVEC tube formation assay in Matrigel was performed according to the manufacturer's instructions using triplicates for each treatment as described (Kaur et al. 2014b) using EVs derived from MDA-MB-231 cells.

Microarrays and real time PCR HUVEC were treated with MDA-MB-231 EVs for 3 days alone or in the presence of 1 µg/ml of B6H12 or isotype-matched control IgG. Total RNA from HUVEC treated with EVs from bCSC and differentiated breast cancer cells was extracted, and quality was analyzed using a RNA Bioanalyzer. The microarray analyses were performed using Agilent st1.0 chips as described earlier (Kaur et al. 2014b). The raw data is deposited in NCBI Gene Expression Omnibus (GEO): GSE105457. Real time q-PCR was performed as described (Kaur et al. 2014a, b), and the primers used are listed in Table S1.

Targeted RNA sequencing analysis HUVEC were treated in triplicate with EVs derived from differentiated from MDA-MB-231 cells for 3 days. EVs derived from MDA-MB-231

bCSCs were incubated with HUVEC in the presence or absence of 1 µg/ml B6H12 antibody for 24 h in triplicates. Total RNA was extracted using the Trizol method, and RNA sequencing was performed using the QIAseq Targeted RNA Panel and analysis performed using <http://ngsdataanalysis.sabiosciences.com/NGSRNA> according to their instructions. Quality control was measured and one replicate of HUVEC (UT) and B6H12 antibody was discarded due to low reads. Scatter graphs were used to identify gene expression differences >2-fold with $p < 0.05$.

Nanosight analysis of EVs derived from breast cancer cell lines EV concentration and size measurements were performed on NanoSight NS 300 (Salisbury, UK) equipped with 405 nm laser and analyzed by NTA 3.0 software (NanoSight NS300, Malvern Instruments, UK). The measurements were performed with constant sample flow using a syringe pump for 60 s at camera level 13.

Data and statistical analysis Microarray data analysis was performed using the Partek Genomics Suite. Gene expression differences >2-fold with $p < 0.05$ were considered significant. GSEA gene enrichment graphs were generated using the Broad Institute software (<http://software.broadinstitute.org/gsea>). Statistics were analyzed using t-test and ANOVA as indicated: Two factor with replicate used for real time PCR and confocal analysis. ANOVA: Two factor without replicates was used for ZEB1 confocal total intensity analysis. Unpaired t-test was used for assessing changes in tube length and tube area with $P < 0.05$ indicating significance.

Results

Breast cancer cell-derived EVs alter mRNA expression in target endothelial cells

EVs isolated from the conditioned medium of adherent differentiated MDA-MB-231 breast carcinoma cells and a nonadherent subpopulation enriched in bCSC had similar size distributions based on Nanosight analysis (Fig. S1A). To assess uptake by HUVEC, red labeled EVs derived from MDA-MB-231 cells were co-cultured for 6 h with green labeled HUVEC and analyzed using confocal microscopy. Surface rendering and planing analysis indicated that EVs derived from MDA-MB-231 cells were incorporated into HUVEC (Fig. 1a). Treatment of HUVEC with EVs resulted in gradual loss of their typical cobblestone morphology, and after 3-weeks most exhibited an elongated and spindly phenotype (Fig. 1b).

HUVEC were treated for 3-days with EVs derived from MDA-MB-231 cells, and mRNA was isolated to examine

Fig. 1 Breast cancer cell-derived EVs alter mRNA expression in target endothelial cells. **a** HUVEC (Green) were treated with MDA-MB-231 derived EVs (Red) for 6 h, and uptake of EVs was quantified using confocal image analysis. **b** Morphological changes induced by long term exposure of HUVEC to MDA-MB-231 derived EVs. **c** HUVEC were treated in triplicate with MDA-MB-231 EVs for 3 days, mRNA was isolated from the treated HUVEC, and global microarray analysis were performed. Hierarchical clustering of expression data is presented for HUVEC treated with EVs versus untreated cells. **d** Scatter graph comparing transcripts expression for untreated HUVEC versus HUVEC treated with EVs derived from MDA-MB-231 cells. **e** & **f** HUVEC were treated with EVs derived from MDA-MB-231 cells for 3 days, and targeted ECM and Adhesion Molecules RNA sequencing analysis was performed. A scatter graph is presented with a table showing the most differentially expressed genes

global changes in HUVEC gene expression by microarray analysis (Fig. 1c,d, Supplemental Excel Table 1) and targeted RNAseq for extracellular matrix (ECM) and adhesion molecules (Fig. 1e,f, Supplemental Excel Table 2). Global microarray analysis identified 287 differential expressed transcripts, with 181 up-regulated greater than 2-fold with $p < 0.05$ and 106 significantly down-regulated (Fig. 1c and Excel Table 1). Several interferon response genes, LYVE1 and TXNIP, a known CD47-regulated gene in endothelial cells and breast and liver cancer cells (Kaur et al. 2013, 2016; Lee et al. 2014), were among the most highly upregulated, while the integrin subunit ITGA4 and Sulfl were the most downregulated (Fig. 1d and Fig. S1C,D).

Gene set enrichment analysis of the microarray data identified a set of genes with altered expression in EV-treated HUVEC that negatively correlate with angiogenesis signaling (Fig. 1d, Supplemental Excel Table 1, Fig. S1E, Fig. S2A-J). The up-regulated *TXNIP* is required for VEGF-mediated angiogenesis (Abdelsaid et al. 2013; Park et al. 2013), suggesting a pro-angiogenic activity of the EVs. On the other hand, TGF- β 2 was the strongest (3.9-fold) down-regulated response to EVs in the query gene set (Fig. S1E). A recent study found that down-regulation of TGF- β 2 stabilizes endothelial tube formation (DiMaio et al. 2014). The up-regulated *MME* encodes the membrane metalloendopeptidase/CD10, which inhibits angiogenesis by degrading several angiogenic factors (Kajiyama et al. 2005; Horiguchi et al. 2008). Expression of some members of the VEGF pathway were down-regulated. EVs derived from the basal type triple-negative MDA-MB-231 breast carcinoma cell line more profoundly decreased mRNA expression of VEGFR2, AKT3, AKT2 and HIF as compared to EVs derived from the ER+/PR+ luminal breast carcinoma cell line T47D1 (Fig. S2A-C). In contrast, EVs derived from HUVEC did not alter expression of the above genes (Fig. S2D-G,I). Therefore, these changes are specific for highly malignant breast cancer-derived EVs and not a general response to EVs. We used endothelial tube formation in Matrigel to assess functional effects of the same EVs. The MDA-MB-231-derived EVs significantly stimulated

HUVEC tube area and length on Matrigel (Fig. S2 K, L), consistent with a net angiogenic activity and possibly a dominant role of the up-regulation of TXNIP.

Gene set enrichment analysis identified inflammatory signaling mediated by TNF α as another major response pathway in EV-treated HUVEC (normalized enrichment score = 1.21, Fig. S1F). Up-regulated genes in this pathway included the highly ranked *IFIT1*, *IFIT3*, *RSAD2*, *CXCL10*, *HERC6*, *IFI35*, *IFI44*, and *DDX60*. Some of these genes may be regulated through cross-talk between the VEGF and TNF α pathways because the up-regulated *CXCL10*, *CXCL11*, and *CASP1* and the down-regulated *EMCN* and *FLT4* were identified previously as genes that were correspondingly up-regulated or down-regulated in endothelial cells treated with TNF α , and regulated in the opposite direction by TNF α + VEGF (Huang et al. 2015; Zahr et al. 2016). Therefore, increased TNF α signaling and decreased VEGF signaling are consistent with the changes we observed in expression of these genes.

Targeted RNA sequencing for ECM and cell adhesion molecules confirmed that LYVE1 mRNA was upregulated and ITGA4 mRNA was downregulated in EV-treated HUVEC (Fig. 1e,f). LYVE1 is a known lymphatic endothelial marker (Baluk and McDonald 2008) and also implicated in adhesion of cancer cells (Du et al. 2013). Realtime qPCR analysis of HUVEC treated with T47D1 and MDA-MB-231 bCSC-derived EVs confirmed increased mRNA expression of LYVE1 and TXNIP, but down regulated ITGA9, which is involved in tumor-induced lymphangiogenesis (Ou et al. 2011), and PROX1, a major transcription factor controlling physiological lymphangiogenesis (Hirakawa 2011) (Fig. S3B-D). These data suggest that induction of LYVE1 may have a function other than lymphangiogenesis in the cross-talk between HUVEC and breast cancer derived EVs.

CD47 antibody B6H12 alters EV uptake in a cell type-dependent manner

Previously, we found that treatment of MDA-MB-231 bCSCs with the CD47 antibody B6H12 downregulated mRNA expression of epidermal growth factor and its receptor and inhibited asymmetric cell division (Kaur et al. 2016). Consistent with those data, EVs released by MDA-MB-231 cells express a higher level of CD47 than HUVEC derived EVs (Fig. 2a,b). A recent study concluded that CD47 on EVs inhibits their uptake by phagocytic cells in a SIRP α -dependent manner (Kamerkar et al. 2017). In contrast, analysis by flow cytometry indicated that CD47⁺ EVs were taken up more by HUVEC than CD47⁻ EVs (Fig. 2c). However, treatment of the EVs with B6H12 antibody did not inhibit bCSC EV uptake by HUVECs (Fig. 2c). Because HUVEC may take up EVs by non-phagocytic mechanisms (Tian

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Gene Symbol	Fold Regulation		
	B6H12	B6H12+EVs	EVs
MFAP5	3.0073	14.0973	3.5797
PCDHGA7	-1.256	5.2285	3.0457
COL23A1	-1.0704	8.1078	2.9745
PCDHA6	4.9195	28.2857	2.8029
SELE	8.8846	38.9741	2.7707
ITGB6	5.3149	38.9865	2.763
ADAM18	1.9107	13.1757	2.615
RELN	1.7287	19.1877	2.3832
COL4A3	1.4656	4.4726	2.3009
THBS1	1.7142	7.7772	-4.9519
ITGB2	-5.7662	2.0276	-5.8735
SERPINA10	-1.5605	4.2008	-7.1592
CLEC4E	-1.0243	3.0447	-7.2054
MMP9	-4.0401	3.4204	-8.7105

HUVEC+bCSCs_EVs/
(HUVEC (UT))

K

Gene Symbol	Fold Regulation		
	B6H12	B6H12+EVs	EVs
SELE	8.8846	38.9741	2.7707
MMP26	6.0751	35.8879	-1.0233
ITGB6	5.3149	38.9865	2.763
PCDHA6	4.9195	28.2857	2.8029
CHL1	4.3663	36.7606	1.0322
MUC3A	3.705	29.6536	2.172
ADAM30	3.3802	11.5629	-1.219
LGALS14	3.2838	35.0153	1.0356
MMP20	3.023	16.6163	-1.609
MFAP5	3.0073	14.0973	3.5797
ASTN1	-3.8281	5.5816	-1.1851
MMP9	-4.0401	3.4204	-8.7105
CDH20	-4.106	14.2936	-1.0759
ITGB2	-5.7662	2.0276	-5.8735

HUVEC+B6H12 /
(HUVEC (UT))

L

Gene Symbol	Fold Regulation		
	B6H12	B6H12+EVs	EVs
STAB1	2.313	49.3457	-1.1116
PCDHA7	1.2383	49.3372	-1.903
ITGB6	5.3149	38.9865	2.763
SELE	8.8846	38.9741	2.7707
ADAMDEC1	2.9615	37.3423	-1.3136
CD84	2.1831	37.3423	-1.3136
ADAM7	1.0156	37.3423	1.0322
CHL1	4.3663	36.7606	1.0322
CDH9	1.4047	36.7606	1.5742
SERPINB8	-1.2807	-1.5625	-1.3833
LOXL1	1.1268	-1.7271	-1.3895
ITGA7	-1.3165	-1.781	-2.2913
LGALS8	-2.5092	-1.8864	-2.3215

HUVEC+bCSCs_EVs+B6H12/
(HUVEC (UT))

Fig. 2 Effects of a CD47 function-blocking antibody on uptake of breast cancer cell derived EVs by HUVEC and effects on HUVEC gene expression. **a & b** EVs from HUVEC and MDA-MB-231 cells were extracted and analyzed flow cytometry. **c** CD47+ and CD47- EVs were isolated from MDA-MB-231 bCSCs and labeled using pHrodo Red dye. Their uptake by HUVEC in the presence or absence of the CD47 antibody B6H12 was quantified using by flow cytometry analysis. **d** EVs were extracted from bCSCs of MDA-MB-231 cells, and captured CD47+ and CD47- EVs were labeled with pHrodo Red dye. THP1 cells were induced with 50 ng/ml of phorbol 12-myristate 13-acetate and incubated with CD47+ and CD47- EVs for 3 h. **e–f** HUVEC (Green) were treated with MDA-MB-231 cell-derived EVs (Red) in the presence of B6H12 antibody or control IgG for 6 h, and uptake of EVs was analyzed by confocal image analysis. * = $p < 0.05$. **g–i** HUVEC were treated with EVs derived from MDA-MB-231 bCSCs in the presence or absence of B6H12 antibody for 24 h, and targeted ECM and Adhesion Molecules RNA sequencing analysis was performed. Scatter and volcano graphs represent differential gene expression between untreated HUVEC versus HUVEC treated with EVs, untreated HUVEC versus HUVEC treated with B6H12 antibody alone, and untreated compared with HUVEC treated with EVs + B6H12 antibody. Lines indicate the threshold for $p < 0.05$. **j–l** Lists of the top upregulated (red) and downregulated genes (green)

et al. 2014), we repeated this EV uptake experiment using THP1-derived macrophages (Fig. 2d). Again, CD47⁺ EVs were taken up more than CD47[−] EVs, but in this case B6H12 inhibited uptake of CD47⁺ EVs by THP1 cells. A control incubated at 4 °C confirmed that the signal represented primarily phagocytosis by THP1 cells rather than surface binding of the EVs. In contrast to the flow cytometry data, confocal image analysis indicated that B6H12 antibody treatment enhanced uptake of EVs by HUVEC in a dose-dependent manner relative to untreated or control IgG treated cells (Fig. 2e,f), but this method cannot exclude increased binding of EVs to cells without internalization. VEGF and TSP1 treatments did not alter the uptake of MDA-MB-231 EVs by HUVEC (data not shown). The molecular mechanism for this cell-type dependence for the effects of B6H12 remain to be defined, but possibilities include differences in Fc receptor expression (Raaz-Schrauder et al. 2014).

CD47 antibody alters the effects of bCSC EVs on HUVEC gene expression

Targeted ECM and adhesion molecule RNA sequencing was performed after treating HUVEC with B6H12 or with EVs from MDA-MB-231 bCSCs in the presence and absence of B6H12 antibody for 24 h. We compared untreated HUVEC with HUVEC treated with EVs derived from MDA-MB-231 bCSC in the presence and absence of B6H12 antibody, and selected transcripts with at least 2-fold higher expression and $p < 0.05$ are shown in scatter graphs (Fig. 2g–i). EV treatment alone upregulated 15 genes and downregulated 18 genes at least 2-fold in HUVEC (supplemental Excel Table 2). Genes with the greatest response to B6H12 are listed in Fig. 2j.

B6H12 treatment without EVs upregulated 32 genes and downregulated 11 genes at least 2-fold (supplemental Excel Table 2), and those with the greatest change are listed in Fig. 2k. B6H12 treatment in the presence of EVs increased 225 genes but decreased no genes more than 2-fold compared to either B6H12 or EVs alone, and those with the greatest change are listed in Fig. 2l.

E-selectin mRNA expression was increased in all treatments and is a well characterized endothelial cell response to activation of the TNF α pathway at the time point used for this analysis (Collins et al. 1995). E-selectin is a key player in cross talk between tumor and endothelium (Witz 2006) and in inflammation and cancer metastasis (St Hill 2011; Borsig et al. 2002; Ley 2003). The EV-induced TNF α pathway activation can account for the increased endothelial cell E-selectin expression. However, CD47 signaling was not previously known to regulate the TNF α pathway, and B6H12 has additive effects on E-selectin induction when combined with EVs (Fig. 2k). Several additional known target genes of TNF α signaling showed increased induction in response to B6H12 + EVs versus EVs alone including *VCAM1*, *ICAM1*, *MMP8*, and *THBS1* (Neish et al. 1992; Awad et al. 2010; Fairaq et al. 2015; Palenski et al. 2013). LYVE1 showed similar B6H12-induced expression in the RNAseq data (supplemental Excel Table 2), and this induction was replicated by qPCR (Fig. S4E). Regulation of LYVE1 protein levels by TNF α has been reported (Johnson et al. 2007), but we are not aware of evidence for mRNA regulation.

SERPINB8 was downregulated in all treatments. Serpins are protease inhibitors through a suicide mechanism (Law et al. 2006) Endothelial serpins are also important for the protection of vasculature (Forsyth et al. 1994). Little is known about the function of SERPINB8 in endothelial cells, and the mechanism by which B6H12 in the presence of EVs further alters expression of SERPINB8 remains to be defined.

EVs and CD47 antibody modulate VEGFR2 and SHP2 phosphorylation

In vascular smooth muscle cells CD47 regulates the activity of tyrosine kinase receptors by interaction with its counter-receptor SIRP α (Maile et al. 2003). CD47 binding regulates phosphorylation of tyrosine residues on the cytoplasmic domain of SIRP α , which controls recruitment of the tyrosine phosphatase SHP2. We found that SIRP α is also expressed on HUVEC but exhibited limited colocalization with its counter-receptor CD47 (Fig. 3a). However, SIRP α coimmunoprecipitated with VEGFR2 basally and after VEGF treatment (Fig. 3a,b). Treatment of HUVEC with the CD47 antibody B6H12 alone enhanced basal VEGFR2 phosphorylation (Fig. 3c lane 4), but the same antibody treatment inhibited VEGFR2 phosphorylation induced by VEGF (Fig. 3c lanes 2–3). Treatment with MDA-MB-231 derived EVs alone or

Fig. 3 SIRP α expression and interaction with VEGFR2 and CD47-dependent regulation of VEGFR2 and SHP2 phosphorylation. **a** Expression and localization of CD47 and SIRP α were analyzed via confocal microscopy. **b** VEGFR2 and SIRP α interaction in HUVEC was assessed by co-immunoprecipitation in the absence or presence of VEGF (30 ng/ml) for the indicated times. **c** HUVEC were treated with EVs derived from MDA-MB-231 bCSCs, VEGF, or B6H12 in the

indicated combinations for 6 h, and VEGFR2, and SHP-2 phosphorylation was analyzed using western blot analysis. **d** HUVEC were treated with MDA-MB-231 cell-derived EVs, VEGF, and B6H12 in the indicated combinations for 6 h and VEGFR2 and SHP-2 phosphorylation was analyzed using western blot analysis. Representative blots are shown

in the presence of B6H12 further downregulated VEGFR2 phosphorylation and enhanced phosphorylation of SHP-2 but not SHP-1 (Fig. 3c, d). Treatment of HUVEC with MDA-MB-231 EVs, and to a lesser extent with T47D EVs but not HUVEC EVs, for 24 h decreased expression of VEGFR2 mRNA (Fig. S2A, D). These data suggest that CD47 can regulate VEGFR2 phosphorylation by a SIRP α - and SHP2-dependent mechanism analogous to that described for regulation of insulin-like growth factor receptor phosphorylation in smooth muscle cells. Messenger RNAs encoding the VEGFR2 signaling targets Akt2 and Akt3 but not Akt1 were also down-regulated specifically by MDA-MB-231 EVs (Fig. S2B, C, E-G, J).

EVs and B6H12 antibody modulate the EnMT transcription factor Zeb1

Several of the genes with altered expression in EV-treated HUVEC are involved in the EMT pathway (Fig. 4a, b). The downregulated transgelin is an early transformation marker (Lawson et al. 1997). SNAI2 (Fenouille et al. 2012) and BDNF (Cherubini et al. 2017) are required for EMT in cancer

invasiveness (Abdelsaid et al. 2013; Park et al. 2013), suggesting a transforming activity of the EVs. KEGG pathway analysis of the ECM targeted RNAseq data identified EMT as the top enriched pathway in HUVEC treated with B6H12 in the presence or absence of EVs (Fig. S4D). To confirm change in EnMT phenotype after treatment with B6H12 antibody, HUVEC were treated with EVs for 6-days, and confocal analysis of a panel of established EMT markers was performed. Confocal imaging showed that treatment with EVs decreased Zeb1 protein expression, and B6H12 antibody treatment in the presence of EVs reversed this response and increased Zeb1 protein levels (Fig. 4c, d). Consistent with the known regulation of COL1A1 by Zeb1 and the regulation of COL1A1 by TSP1 (Okumura et al. 2015; Zhou et al. 2006), we found that MDA-MB-231 derived EVs decreased TSP1 and enhanced expression of COL1A1 mRNA (Fig. 4e, f), which is known to downregulate VEGFR2 phosphorylation via enhancing SHP-2 (Fig. 3c). By enhancing ZEB1 expression the B6H12 antibody could induce EnMT (Krebs et al. 2017) and prevent apoptosis (Magenta et al. 2011).

Fig. 4 B6H12 antibody and EV treatment alters EnMT marker expression in HUVEC. **a** & **b** Gene enrichment analysis from Fig. 1C showed enrichment of genes in the EMT pathway. **c** HUVEC were treated with EVs derived from MDA-MB-231 cells for 6 days, and immunostaining of Zeb1 protein was performed and analyzed using confocal microscopy. **d** Quantification of total

Intensity was calculated by (mean intensity) x (total pixels) and subtracting background threshold from each image. **e** & **f** EVs isolated from HUVEC, MDA-MB-231 cells, and T47D1 cells were incubated with HUVEC for 24 h. Total RNAs were purified, and mRNAs for TSP1 and Col1a1 were analyzed via q-PCR analysis. * = $p < 0.05$. ** = $p < 0.01$

Discussion

EVs released into the tumor microenvironment by tumor cells can promote tumor growth and metastatic spread while limiting antitumor immunity and the efficacy of cytotoxic chemotherapy (Naito et al. 2017; Wendler et al. 2017). Endothelial

cells are an important target in the tumor microenvironment for EVs released by tumor cells that enhance tumor angiogenesis (Ohyashiki et al. 2016). The present data demonstrates that EVs released by breast carcinoma cells alter the expression of multiple genes in endothelial cells that control angiogenesis and inflammatory responses and alter the responses of

endothelial cells to angiogenic factors such as VEGF. We also demonstrate that a function-blocking CD47 antibody, which was the prototype for humanized antibodies currently undergoing clinical trials as immune checkpoint inhibitors (Majeti et al. 2009; Liu et al. 2015), alters some responses of endothelial cells to cancer cell EVs. Thus, such experimental therapeutics should be recognized as more promiscuous modulators of the tumor microenvironment, and effects on the tumor vasculature may contribute both to their efficacy and to potential adverse side effects.

Based on the global microarray analysis, genes in the TNF α pathway were highly enriched among those altered following treatment of endothelial cells with breast carcinoma EVs. The mechanism by which EVs regulate this pathway remains to be determined, but here we focused on potential effects of therapeutics designed to block CD47 signaling and identified the TNF α target E-selectin as the top up-regulated transcript in HUVEC treated with the CD47 antibody. Combining the CD47 antibody with EV treatment resulted in an additional >4-fold increase in E-selectin mRNA. Among the other ECM genes examined, known TNF α targets including *VCAM1*, *ICAM1*, *MMP8*, and *THBS1* showed similarly enhanced expression in response to EVs + anti-CD47 relative to either treatment alone. Therefore, increased

TNF α -dependent inflammatory signaling is one potential side effect of therapeutic CD47 blockade in the tumor microenvironment.

Previous studies provide some mechanistic basis for this conclusion and implicate both SIRP α and TSP1 signaling through CD47 (Fig. 5). TNF α treatment induces Tyr phosphorylation of SIRP α in 3 T3 cells and recruitment of SHP2, which in turn increases ERK and AKT phosphorylation (Ruhul Amin et al. 2002). TSP1 was also reported to induce VCAM1 and ICAM1 expression on endothelial cells by a TNF α -dependent pathway, and siRNA knockdown of CD47 blocked this response to TSP1 (Narizhneva et al. 2005). Further studies will be required to determine whether the observed effects of B6H12 on the TNF α pathway are mediated by blocking CD47 binding to either SIRP α or, TSP1 or rather, involve perturbation of CD47 signaling independent of ligand binding.

Angiogenesis-related genes were also modulated by the EVs, and among these integrin $\alpha 4$ is known to associate with CD47 (Barazi et al. 2002) and to mediate some proangiogenic activities of the N-terminal domain of TSP1 (Calzada et al. 2004). The lymphangiogenesis marker LYVE1 was among the most significantly regulated HUVEC genes. Recent reports suggest that LYVE1 is not exclusively a lymphatic

Fig. 5 Proposed pathway for regulation of endothelial cell signaling by breast cancer cell-derived EVs and a function-blocking CD47 antibody. CD47 is expressed on tumor cell-derived EVs and their target endothelial cells, and the function-blocking CD47 antibody B6H12 could act at both locations. The right panel depicts previously identified CD47-dependent signaling pathways and the newly identified interaction between SIRP α

and VEGFR2, which may be direct or mediated by the previously identified direct lateral interaction between CD47 and VEGFR2 (Kaur et al. 2010). Green arrows indicate targets regulated in their expression or phosphorylation by EVs, and red arrows indicate targets regulated by the CD47 function-blocking antibody

vessel marker (Mouta Carreira et al. 2001). CD47 and SIRP α interact via their extracellular domains (Jiang et al. 1999), and SIRP α has four YXX(L/V/I) tyrosine phosphorylation motifs sites that bind to the SH2 domains of SHP-2 (Fujioka et al. 1996) (Fig. 5). Down-regulation of VEGFR2 phosphorylation and enhanced SHP-2 phosphorylation may be mediated in part through a TSP1-regulated induction of type I collagen (Zhou et al. 2006; Mitola et al. 2006). Induction of SHP-2 also can increase expression of Zeb1 transcription factor, which induces EMT and invasion in mice and humans (Zhang et al. 2016). Our data suggests that this pathway is conserved in endothelial cells.

Several of the genes that were differentially regulated by breast carcinoma cell EVs have established linkages to CD47 signaling. LYVE1 expression was 2.2-fold lower in CD47 null endothelial cells (Kaur et al. 2014b) and increased 4-fold in HUVEC treated with CD47⁺ EVs from Jurkat T cells (Kaur et al. 2014b). Similarly, B6H12 antibody treatment upregulated TXNIP expression breast cancer stem cells derived from triple negative MDA-MB-231 cell lines (Kaur et al. 2016). TSP1 limits expression of type I collagen in endothelial cells (Zhou et al. 2006). This may be controlled through nitric oxide, which thrombospondin-1 controls through CD47 (Isenberg et al. 2008). Consistent with this previous data, the present study shows down-regulation of thrombospondin-1 mRNA and up-regulation of collagen 1A1 mRNA in endothelial cells treated with MDA-MB-231 EVs.

Although some of the effects of the CD47 antibody on endothelial cell responses to the breast carcinoma cell EVs are consistent with known TSP1 signaling, we cannot exclude a role of the CD47-SIRP α interaction (Fig. 5). Endothelial cells express both SIRP α and CD47, and the present data demonstrate that SIRP α is closely associated with VEGFR2. It remains unclear whether SIRP α and VEGFR2 interact directly or are part of a larger lateral interaction network in the plasma membrane. CD47 is one possible mediator given that it directly interacts with both SIRP α and VEGFR2 (Kaur et al. 2010).

Because B6H12 inhibits binding of SIRP α to CD47 (Isenberg et al. 2009), this antibody increases the phagocytosis of CD47-expressing cells by macrophages (Matozaki et al. 2009; Burger et al. 2012). Although one study has concluded that CD47 has a similar ‘don’t eat me’ function on EVs (Kamerkar et al. 2017), our data demonstrating increased phagocytosis of CD47⁺ EVs by macrophages and inhibition of that uptake in the presence of B6H12 does not support that hypothesis. One reason why the ‘don’t eat me’ function of CD47 may not extend to EVs is that the inhibitory signal in macrophages, generated by touching a potential target cell results from low affinity multivalent engagement of CD47 by SIRP α , and this signal is known to require a critical density of CD47 (Tsai et al. 2010). Because EVs are much smaller than cells, a macrophage encountering an EV may not engage the minimal number of SIRP α receptors to generate a

functional inhibitory signal. Conversely, monovalent or oligovalent binding of CD47 on EVs to SIRP α on a phagocyte may increase the probability of the cell recognizing other pro-phagocytic molecules presented on cancer cell EVs, which is consistent with our data where CD47⁺ EVs had increased uptake compared to CD47⁻ EVs by HUVEC and THP1-derived macrophages. Further studies will be required to determine whether engaging SIRP α on HUVEC can inhibit any phagocytic activity of those cells.

In summary, the present results demonstrate that a well characterized function-blocking CD47 antibody can modulate multiple EV-mediated signals between tumor cells and stromal cells that are important for supporting tumor growth and metastasis. It remains to be determined whether this activity will generalize to the humanized CD47 antibodies currently in clinical trials or to related biologics in clinical testing such as the SIRP α decoy TTI-621 (Petrova et al. 2017). Additional studies will also be required to determine whether CD47 modulates effects of tumor cell-derived EV on the pro- and antitumor activities of other classes of stromal cells in the tumor microenvironment.

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Author contributions DDR and SK designed the research, SK, AG, SPS, AA performed experiments. SK, DDR, SPS and AG analyzed and interpreted the data. SK and DDR wrote the manuscript.

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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