Integrin-Mediated Macrophage Adhesion Promotes Lymphovascular Dissemination in Breast Cancer

Graphical Abstract

Highlights

- β4 integrin-expressing macrophages release TGF-β1 near breast cancer lymphovascularure

- TGF-β1 drives β4 integrin clustering on macrophages, enhancing macrophage adhesion

- TGF-β1 signals through RhoA to drive to lymphatic endothelial cell contraction

- Lymphatic remodeling signaling cascade facilitates breast cancer metastasis

Authors

Rachel Evans, Fabian Flores-Borja, Sina Nassiri, ..., Frederic Festy, Michele De Palma, Tony Ng

Correspondence

rachel.evans@ucl.ac.uk (R.E.), tony.ng@kcl.ac.uk (T.N.)

In Brief

Breast cancer metastasis through lymphatic vessels is associated with poor prognosis. Evans et al. describe β4 integrin-expressing macrophages that regulate lymphatic vessel structure in breast cancer. Macrophage-released TGF-β1 drives lymphatic cell contraction via RhoA activation, culminating in lymphatic hyperpermeability. This study defines a signaling cascade that could be targeted therapeutically.
Integrin-Mediated Macrophage Adhesion Promotes Lymphovascular Dissemination in Breast Cancer

Rachel Evans,1,12,4 Fabian Flores-Borja,2,13 Sina Nassiri,3 Elena Miranda,4 Katherine Lawler,1,5 Anita Grigoriadis,2 James Monypenny,1 Cheryl Gillet,6,7 Julie Owen,8,9 Peter Gordon,2 Victoria Male,1,14 Anthony Cheung,2 Farzana Noor,2 Paul Barber,1,11 Rebecca Marlow,2 Erika Francesch-Domenec,2 Gilbert Fruhwirth,8 Mario Squadrito,2 Borivoj Vojnovic,9 Andrew Tutt,3 Frederic Festy,10 Michele De Palma,3 and Tony Ng1,2,11,15,*

1Richard Dimbleby Department of Cancer Research, Randall Division & Division of Cancer Studies, Kings College London, London, UK
2Breast Cancer Now Research Unit, King’s College London, Guy’s Hospital, London, UK
3Swiss Institute for Experimental Cancer Research (ISREC), School of Life Sciences, École Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland
4Pathology Core Facility, University College London Cancer Institute, London, UK
5Institute for Mathematical and Molecular Biomedicine, King’s College London, London, UK
6King’s Health Partners Cancer Biobank, King’s College London, London, UK
7Research Oncology, Division of Cancer Studies, Guy’s Hospital, King’s College London, London, UK
8Division of Imaging Sciences and Biomedical Engineering, King’s College London, London, UK
9Department of Oncology, Cancer Research UK and Medical Research Council, Oxford Institute for Radiation Oncology, University of Oxford, UK
10Tissue Engineering and Biophotonics, King’s College London, London, UK
11UCL Cancer Institute, University College London, London, UK
12Present address: Cancer Institute, University College London, London, UK
13Present address: Centre for Immunobiology and Regenerative Medicine, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London, UK
14Present address: Division of Infection and Immunity, Institute of Immunity and Transplantation, University College London, London, UK
15Lead Contact
*Correspondence: rachel.evans@ucl.ac.uk (R.E.), tony.ng@kcl.ac.uk (T.N.)
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SUMMARY

Lymphatic vasculature is crucial for metastasis in triple-negative breast cancer (TNBC); however, cellular and molecular drivers controlling lymphovascular metastasis are poorly understood. We define a macrophage-dependent signaling cascade that facilitates metastasis through lymphovascular remodeling. TNBC cells instigate mRNA changes in macrophages, resulting in β4 integrin-dependent adhesion to the lymphovascular. β4 integrin retains macrophages proximal to lymphatic endothelial cells (LECs), where release of TGF-β1 drives LEC contraction via RhoA activation. Macrophages promote astrocyte-like changes to lymphovascularization by increasing dilation, hyperpermeability, and disorganization. TGF-β1 drives β4 integrin clustering at the macrophage plasma membrane, further promoting macrophage adhesion and demonstrating the dual functionality of TGF-β1 signaling in this context. β4 integrin-expressing macrophages were identified in human breast tumors, and a combination of vascular-remodeling macrophage gene signature and TGF-β signaling scores correlates with metastasis. We postulate that future clinical strategies for patients with TNBC should target crosstalk between β4 integrin and TGF-β1.

INTRODUCTION

Tumor cells establish complex interactions with cells within their microenvironment that determine malignancy progression (Balkwill et al., 2012). Tumor cell dissemination can occur through blood or lymphovascularization; however, targeting blood vasculature has limited clinical efficacy when lymphatic dissemination is prevalent (Wong and Hynes, 2006).

Breast cancer is divided into subtypes based on histopathological features and gene signatures (Gazinska et al., 2013). Triple-negative breast cancer (TNBC) is characterized by a lack of druggable targets, is highly metastatic, and is associated with dismal prognosis (Gazinska et al., 2013; Dent et al., 2007). The prognostic significance of lymphangiogenesis in TNBC is under debate. However, invasion into lymphatic vessels correlates with poor prognosis, suggesting that targeting an existing lymphatic vessel network could provide an effective treatment strategy (Choi et al., 2005; Mohammed et al., 2007, 2011; Liu et al., 2009).

The relationship between tumor and immune cells is often bidirectional and involves both tumor-promoting and -antagonizing mechanisms (Pollard, 2004; Quail and Joyce, 2013). Among innate immune cells, macrophages have been implicated in the promotion of tumor progression and, in particular, breast cancer metastasis (Condeelis and Pollard, 2006; Kitamura et al., 2015; Pollard, 2004; Harney et al., 2015). However, it remains unclear how certain subsets of tumor-associated macrophages (TAMs) influence breast cancer metastasis spatially, temporally, and at a molecular level.
The integrin family are adhesion receptors of paramount importance for immune cell adhesion and migration during inflammatory processes (Evans et al., 2009). Their ability to form adhesive contacts is regulated by soluble factors, as part of the chemotactic-adhesion crosstalk that causes a combination of changes in integrin conformation and clustering on the plasma membrane (PM) that regulate downstream signaling (Hynes, 2002). In malignancy, many integrins common in epithelial cells are also present in solid tumors, and some, such as αvβ3 and α5β1, are specifically upregulated in cancer (Desgrozeller and Cheresh, 2010). Tumor-expressed integrins affect tumor cell migration, proliferation, survival, and anchorage to the extracellular matrix. Endothelial-cell-expressed integrins are implicated in angiogenesis, lymphangiogenesis, and vascular remodeling (Avraamides et al., 2008).

While the importance of integrins with respect to maintaining a pro-tumoral immune microenvironment in solid tumors is not well defined, in chronic lymphocytic leukemia, impaired integrin signaling in non-leukemic T cells changes the immune microenvironment to be more immunosuppressive, which may facilitate malignancy progression (Ramsay et al., 2013).

We seek to identify the role of TAMs in regulating existing lymphovascularization in TNBC, where lymphatic dissemination is not a direct result of lymphangiogenesis.

We propose that macrophages have an important role in controlling established tumoral lymphatic networks in TNBC and that lymphatic dissemination of cancer cells is facilitated by a cascade of signaling events initiated by integrin-mediated adhesion of macrophages at the sites of lymphatic vessels.

RESULTS

Lymphovascular Macrophages in TNBC Mouse Models Are Retained through Binding of β4 Integrin to Laminin-5

To identify endogenous macrophages with respect to lymphatic vasculature in murine TNBC tumors, we scored F4/80+Tie2+ macrophages within podoplanin+ lymphovascular structures across multiple fields of view (FOVs) from 4T1.2 and BLG-Cre;Brca1<sup>1<sup>7<sup>7<sup> , p53<sup>−/−</sup></sup></sup></sup>/C0 TNBC models (Molyneux et al., 2010; Melchor et al., 2014; Figures 1A and 1B). The Tie2-expressing macrophage (TEM) subset is associated with angiogenesis and lymphatic development (De Palma et al., 2005, 2007; Gordon et al., 2010). Lymphovascular-associated macrophages expressing Tie2 have recently been reported in a small breast cancer cohort (Bron et al., 2015).

In 4T1.2 tumors, we found a mean value of 6.3 F4/80+Tie2+ macrophages within podoplanin+ vasculature (versus 1.7 in podoplanin– regions) per FOV. In BLG-Cre;Brca1<sup>1<sup>7<sup>7<sup> , p53<sup>−/−</sup></sup></sup></sup>/C0 tumors, we observed 8.8 F4/80+Tie2+ macrophages in podoplanin+ vasculature (versus 2.0 in podoplanin– regions) per FOV. Therefore, F4/80+Tie2+ macrophages are enriched in lymphovascular regions in murine TNBC models.

The β4 integrin subunit is a transmembrane glycoprotein associating exclusively with the α6 integrin subunit. α6β4 integrin is expressed predominantly on epithelial and endothelial cells and binds to laminins to form adhesion complexes, hemidesmosomes (Stewart and O’Connor, 2015). Microarray analysis of endogenous macrophages co-cultured with 4T1.2 tumor cells showed a mean 1.8-fold upregulation of β4 integrin at the RNA level, compared with non-educated endogenous macrophages, and that the RAW264.7 macrophage cell line similarly exhibited a mean 1.58-fold increase in β4 integrin levels, compared with endogenous macrophages (Figure 1C; see also data published in ArrayExpress: MTAB-4064).

4T1.2 tumors were excised and disaggregated at day 10. Within 4T1.2 tumors, we defined a population of macrophages as CD45<sup>+</sup>Ly6G<sup>−</sup>Ly6C<sup>−</sup>CD11b<sup>+</sup>Tie2<sup>+</sup>β4 integrin<sup>+</sup> (Figure 1D).

The influence of tumor education on macrophage adhesion to β4 integrin ligand, laminin-5, was investigated. Tumor-educated endogenous macrophages displayed increased adhesion to laminin-5 (30.76±7.2% to 81.7±13.2% adherent cells on 0.5 μM laminin-5; Figure 1E). As laminin-5 is reportedly localized in areas with high blood vessel density, we investigated whether laminin-5 was also in areas of lymphovascularization. 4T1.2 tumor tissue analysis showed laminin-5 furnished around podoplanin+ lymphovascularization (Figure 1F). In addition we observed macrophages expressing α6β4 integrin in lymphovascular regions (Figure 1G).

To study β4 expression in vivo, we used primary 4T1.2 tumor sections stained with Lyve1-Cy3 and β4 integrin-Cy5. Tissues were imaged using a protocol involving laser photobleaching to remove autofluorescence. Our methodology reveals β4 integrin throughout the tumor; however, within lymphatic vessels, there is differential distribution of β4 integrin with relative increases in β4 accumulation observed in lymphovascular areas proximal to Lyve1+ lymphatic endothelial cells (LECs) (Figure 1H, white arrow). Additionally, there were lymphovascular areas with an increased localized Pearson coefficient, suggesting that LECs and β4-integrin-expressing macrophages were in close contact (Figure 1H, blue arrow) (mean colocalization coefficient, 4.094±0.8146).

Figure 1. Lymphovascular Macrophages in TNBC Mouse Models Are Retained through Binding of β4 Integrin to Laminin-5.

(A and B) Tumor sections from 4T1.2 (A) and BLG-Cre;Brca1<sup>1<sup>7<sup>7<sup> , p53<sup>−/−</sup></sup></sup></sup>/C0 (B) were stained with F4/80-FITC, podoplanin-ASF555, and Tie2-Cy5-conjugated antibody. F4/80+Tie2+ macrophages within podoplanin+ areas versus those in other areas were quantified per field of view (FOV). Vessel lumen is outlined; arrow indicates a macrophage within a podoplanin+ area. Images were acquired with a x40 air objective. Scale bars, 100 μm (main image) and 25 μm (zoomed inset).

(C) Array-derived expression profile of β4-integrin (Itgb4) across samples. Barplot shows log2 fold change of normalized expression value for β4 integrin (ratio of the median value of probe in BMMM samples).

(D) Day-12 4T1.2 tumors were disaggregated. Tie2 and β4 integrin FMO controls are indicated in 2 left panels. Right dot plot and histogram depict β4-integrin-expressing macrophages from representative 4T1.2 tumor (n = 8).

(E) BMMS co-cultured alone or with 4T1.2-GFP cells plated on laminin-5. The percentage of adherent cells were quantified in triplicate (n = 2).

(F and G) 4T1.2 tumor sections were stained with laminin-5-DyLight488 and podoplanin-ASF555 (F), and Lyve1-Cy3, F4/80-FITC, and β4 integrin-Cy5 (G); inset shows F4/80+β4 integrin+ macrophages around lymphatic endothelium.

(H) Stained sections (Lyve1-Cy3 and β4 integrin-Cy5) were imaged using a custom-built microscope (x20 air objective). Area of distinct β4 integrin and Lyve1 within lymphatic vessel (white arrow) and area of close contact between β4 integrin and Lyve1 (blue arrow) are indicated. Scale bars, 50 μm (main panels) and 25 μm (inset).
Ai

Dextran-FITC s.c. at tailbase

Peritumoral
Distal to tumour

Peritumoral
Leaky lymphatics
Intact lymphatics

B

Absorbance (630nm)
per g tumour

4T1.2 + PBS
4T1.2 + RAW264.7

![Graph](legend on next page)

Ci

Score=0
Score=1
Score=2
Score=3

Lyve-1
Podoplanin

Di

4T1.2 tumor inoculation
Day 0

endogenous BMM/eBMM l.V
Day 12

Day 4
Day 6
Day 8
Day 10
Day 14

Clodronate treatment every two days
Mice culled and primary tumours excised

TREATMENT STOPPED

ii

4T1.2 + PBS
4T1.2 + BMM
4T1.2 + eBMM

Podoplanin

iii

Lymphatic
disorganisation (A.U.)

**

Ei

LEC Alone
LEC + BMM
LEC + eBMM
LEC + TAM

Area (um^2)

***

Fi

SV-LEC alone
SV-LEC + RAW264.7

Area (um^2)

***

Gi

SV-LEC + BMM
Intensity
Tau

SV-LEC + eBMM
Intensity
Tau

ii

1.5
2.0

3.0

4.0

5.0

6.0

7.0

RhoA-GFP
Raichu R/G

**

(legend on next page)
TAMs Drive Disorganized and Hyperpermeable Lymphatic Architecture, and Contact between Macrophages and LECs Results in RhoA-Dependent Contraction

We used a mammary image window (MIW) subcutaneously implanted over a 4T1.2-mCherry tumor (Kedrin et al., 2008; Figure 2A). Injection of 76 kDa dextran-FITC (fluorescein isothiocyanate) allowed visualization of lymphatic vasculature. Using multiphoton microscopy, we observed that, within the tumor, lymphatic vessels leaked dextran dye across the FOV (Figure 2Aii, left panel), suggesting high levels of vessel permeability; however, in more distal regions, lymphatic vessels had a distinct structure and 4T1.2-mCherry intra-lymphatic tumor cells could be seen within vessels, suggesting ongoing metastasis (Figure 2Aii, middle and right panels, respectively). To understand how increasing TAMs could phenotypically influence lymphatic vasculature, we studied the permeability of lymphatic vessels from 4T1.2 tumor-bearing mice given an intermittent bolus of RAW264.7 macrophages during tumor development. Both RAW264.7 macrophages and the 4T1.2 tumor line are derived from a BALB/c genetic background, allowing us to investigate the effects of elevated macrophage numbers on tumor progression in vivo using a syngeneic model of TNBC.

To quantify lymphatic vessel permeability in vivo, we adapted a protocol previously used in angiogenesis studies (Finsterbusch et al., 2014). Using a subcutaneous injection of Evans Blue dye, we quantified the permeability of the tumoral lymphatics. Tumors with elevated macrophages contained hyperpermeable lymphatic vessels with an increase in mean optical density (OD) per gram from 0.7812 ± 0.2956 to 2.290 ± 0.5160 when compared with PBS-treated control, suggesting a facilitated pathway between the primary tumor and lymphatic vasculature (Figure 2B).

To understand the effects of elevated macrophages on tumor lymphatic vessel architecture, we stained tumor sections from mice treated with PBS or RAW264.7 macrophages with the lymphatic vessel markers, Lyve1 and podoplanin (Figure 2C; Figures S1A and S1B), demonstrating that both lymphatic markers gave a similar staining distribution. Typical sections from PBS-treated mice showed small, well-formed vessels toward the tumor periphery or within the peri-tumoral areas with a mean diameter of 13.66 μm ± 1.295 μm. This was in contrast to RAW264.7-treated mice that had larger vessels with a mean diameter of 48.00 μm ± 6.065 μm, indicating increased vessel dilation (Figure S1C).

To quantify changes in lymphatic architecture in tumors with elevated levels of macrophages, we blindly scored lymphovasculature for disorganization based on the following criteria. Smaller vessels with a clear lumen were given low scores (0 and 1) compared with larger disorganized vessels with unclear borders (2 and 3). PBS-treated tumors had a mean disorganization score of 0.25 ± 0.16 and 1.6 ± 0.33, compared with 1.8 ± 0.29 and 2.5 ± 0.17 for tumors treated with RAW264.7 macrophages (Figure 2C).

To further investigate whether macrophages were sufficient to induce a disorganized lymphatic phenotype, we ablated endogenous macrophages using clodronate-containing liposomes post-establishment of 4T1.2 tumors. Endogenous macrophages were reconstituted post-clodronate treatment with non-educated bone marrow macrophages (BMMs) or tumor-educated BMMS for 48 h (Figure 2Di). The extent of lymphatic disorganization in the 4T1.2 primary tumors was greater after reconstitution with endogenous tumor-educated BMMS, compared with non-educated BMMS (0.333 ± 0.3 to 2 ± 0.29; Figure 2D, ii and iii). These results demonstrate that the presence of TAMs results in a disorganized lymphatic vasculature around the primary tumor, that the extent of disorganization is related to overall macrophage levels, and that this occurs at an early time point in tumor development (days 10–14).

To investigate how TAMs affect lymphatic endothelia, we added endogenous macrophages to monolayers of primary LECs isolated from BALB/c mice (Figure 2E). Primary LECs had a mean spread area of 1,132 μm² ± 247.9 μm², which reduced slightly to 806.6 μm² ± 185.9 μm² after the addition of endogenous uneducated macrophages but dramatically reduced to 324.1 μm² ± 76.43 μm² with tumor-educated macrophages and 473.7 μm² ± 92.8 μm² with eX vivo TAMs (CD45⁺Ly6G⁺CD31⁺CD11b⁺). Similar LEC contraction occurred when the murine LEC line, SV-LEC (Ando et al., 2005), was grown as a monolayer and endogenous macrophages (Figure S1D) or RAW264.7 macrophages added (Figure 2Fii), SV-LEC contraction...
occurred with areas reducing from 835.9 μm² ± 72.32 μm² to 380.5 μm² ± 40.82 μm² and from 632.5 μm² ± 83.0 μm² to 82.67 μm² ± 14.38 μm². In addition, the area of SV-LECs was quantified with and without contact with RAW264.7 macrophages. SV-LEC contraction was only observed when direct contact between the 2 cell types occurred (436.4 μm² ± 63.3 μm² to 116.2 μm² ± 34.6 μm²) (Figure 2Fii). Collectively, our evidence suggests that direct contact between TAMs and LECs is required for contraction events to occur.

RhoA regulates many events in blood-vessel-specific endothelial cells during angiogenesis, such as motility, proliferation, and permeability (Bryan et al., 2010). We sought to test whether RhoA regulates contraction events observed in LECs. SV-LECs were transiently transfected with the GFP- and monomeric red fluorescent protein (mRFP)-expressing Rhoa RAICHU biosensor (Heasman et al., 2010; Makrogianneli et al., 2009; Yoshizaki et al., 2003), which allows measurement of the fluorescence lifetime decay (Tau) when fluorescence resonance energy transfer (FRET) occurs between the GFP and mRFP upon RhoA activation. After SV-LEC transfection, non-educated or tumor-educated endogenous macrophages were added to SV-LECs for 24 h. The fluorescence lifetime of the RAICHU probe (expressed exclusively in the SV-LECs) was measured using multiphoton microscopy. SV-LEC co-culture with tumor-educated macrophages led to a reduction in Tau of the biosensor from 1.797 ns ± 0.0252 ns to 1.622 ns ± 0.0338 ns, indicating an increase in FRET between the GFP- and RFP-terminal fluorophores and, consequently, an increase in RhoA activity (Figure 2G). No change in Tau was observed when SV-LECs were co-cultured with non-educated endogenous macrophages (Figure 2Gii). These results demonstrate that RhoA activity increases during LEC contraction and that this only occurs in the presence of tumor-educated macrophages in contact with lymphatic endothelia.

**LEC Contraction Is Dependent on TGF-β1 Release from Tumor-Educated Macrophages**

Transforming growth factor (TGF)-β receptor ligation in fibroblasts results in RhoA activation (Fleming et al., 2009). We investigated the release of active TGF-β1 and TGF-β2 isoforms from non-educated and tumor-educated macrophages by ELISA (Figure 3A). TGF-β1 levels increased from 2,600 pg to 4,400 pg in tumor-educated endogenous macrophages (increase in optical absorbance at 450 nm from 1.286 ± 0.07119 to 2.585 ± 0.1077). In contrast, TGF-β2 levels were not significantly changed. While TGF-β1 is present throughout the tumor microenvironment, membrane-bound TGF-β can have a potent effect on downstream signaling through increasing the concentration gradient of this molecule (Savage et al., 2008). Our data showed that 4T1.2 education of endogenous macrophages significantly increased the levels of plasma-membrane-bound TGF-β1 (Figure S2A), allowing stringent spatial control of downstream signaling events.

To test the hypothesis that macrophage-released TGF-β1 was responsible for LEC contraction, we investigated the effect of a TGF-β receptor inhibitor, SB-431542 (Inman et al., 2002; Figure S2B). As expected, RAW264.7 macrophages alone induced LEC contraction (950.6 μm² ± 129.9 μm² to 335.8 μm² ± 38.23 μm²); however, this did not occur in the presence of SB-431542 or when TGF-β1 or β4 integrin were transiently knocked down in RAW264.7 macrophages, demonstrating that the presence of β4 integrin and TGF-β1 in macrophages or TGF-β1 receptor ligation on LECs was sufficient to prevent contraction (Figures 3B, S2C, and S2D).

The role of macrophage-released TGF-β1 on lymphovascular disorganization was investigated in vivo. A stable knockdown of TGF-β1 was generated in RAW264.7 macrophages using lentiviral short hairpin RNA (shRNA) (Figure S2E). Similar to our previous in vivo studies, macrophages were administered intravenously throughout tumor development. After 2 weeks’ growth, tissue sections were stained for Lyve1 and podoplanin. The extent of lymphatic disorganization in tumors with RAW264.7-TGFβ1 knockdown, compared with that in RAW264.7-NTC, was blindly scored in Lyve1-podoplanin-stained tissues as described earlier. Our results show that absence of TGF-β1 in RAW264.7 macrophages was sufficient to significantly decrease the extent of lymphatic disorganization observed, compared with that in RAW264.7-NTC macrophages (1.8 ± 0.16 to 1.1 ± 0.18) (Figure 3C) and that these changes were evident at an early time point.

To functionally associate macrophage-released TGF-β1 to structural changes in the lymphatic endothelium in vivo, we...
TNBC patients with distant metastasis
TNBC patients without distant metastasis
Enrichment of TNBC with distant metastasis
Hypergeometric test
P-value = 1.19e-05
quantified levels of phospho-myosin light chain (pMLC) in LECs adjacent to macrophages. Since RhoA activity is high in contracting LECs, and since active RhoA phosphorylates MLC, pMLC can be used as a readout of LEC contractility in cells proximal to lymphatic-associated macrophages. We observed that, when mice were injected with RAW264.7-TGFβ1 knockdown, compared with RAW264.7-NTC, there was a significant reduction in pMLC levels in lymphatic vasculature adjacent to RAW264.7 macrophages when TGF-β1 was absent (1.97 ± 10^5 ± 401,151 to 6.56 × 10^3 × 187,133) (Figure 3D).

**TGF-β1 Controls ITGB4 Clustering at the Macrophage Plasma Membrane**

We studied the effect of TGF-β1 on the phenotypic functionality of macrophages by quantifying the spreading response of macrophages. There was clear reduction in cell spreading when TGF-β1 was knocked down in RAW264.7 macrophages, compared with the non-targeted control counterpart (235.2 μm^2 ± 41.06 μm^2 to 91.91 μm^2 ± 11.62 μm^2) (Figure 3E). To understand how TGF-β1 could control macrophage spreading, we investigated the effect of TGF-β1 on ITGB4 expression. Since integrins can be constitutively expressed on the cell surface, we sought to study the plasma membrane distribution of ITGB4 integrin using structured illumination microscopy in RAW264.7-TGFβ1 shRNA versus RAW264.7-NTC. Our results clearly show that, while there may be small differences in the overall amount of ITGB4 integrin expressed on the cell surface (Figures S3A and S3B), the size of integrin clusters that can form firm adhesive contact with integrin ligand are significantly reduced when TGF-β1 is absent (1.97 μm^2 ± 0.12 μm^2 to 1.559 μm^2 ± 0.0.07 μm^2; Figure 3F, i and ii). These results collectively indicate that TGF-β1 has both a paracrine role in controlling the lymphatic endothelium and an autocrine role in regulating ITGB4 activity in tumor-educated macrophages.

**ITGB4 Integrin+ Macrophages and Lymphatic Remodeling Are Associated with TGF-β Signaling and Adverse Outcome in TNBC Patients**

To establish that human macrophages express ITGB4 RNA (ITGB4 integrin), we performed an analysis of a compendium of data on ITGB4 expression in human macrophages. The y axis indicates normalized expression on log 2 scale. Red line indicates median expression of all genes. Raw gene counts were obtained from the ARCHS4 database. (A) ITGB4 expression in human macrophages. The y axis indicates normalized expression on log 2 scale. Red line indicates median expression of all genes. Raw gene counts were obtained from the ARCHS4 database. (B) Correlation between ITGB4 expression and enrichment of TGF-β1 signaling in human macrophages (Spearman rho = 0.26; p < 0.001). The x axis indicates normalized expression on the log 2 scale. The y axis indicates single sample gene set enrichment analysis (ssGSEA) enrichment scores computed for the TGF-β1 hallmark gene set obtained from the molecular signatures database (MSigDB). Red curve indicates loess fit. Association strength was quantified using Spearman correlation coefficient. Raw gene counts were obtained from the ARCHS4 database. (C) Expression of ITGB4 in single cell RNA sequencing (scRNAseq) data of primary breast cancer (GEO: GSE75688). Data are reported as log2(TPM+1). TPM, transcripts per million. (D) Activation score of TEM gene signature and TGF-β1 signaling. Red and green dots indicate TNBC with or without distant metastasis, respectively. Enrichment of TNBC with distant metastasis in the top right quadrant, established by hypergeometric testing. (E) Kaplan-Meier survival curves showing distant metastasis-free survival in TNBC. Stratification based on samples with high TGF-β1 signaling and TEM gene signature activation score classified as “High TEM-TGFβ1 signature” versus the remainder (“Low TEM-TGFβ1 signature”). (F) Representative breast cancer section (from n = 20) stained with CD14 (red) and podoplanin (brown). Scale bars, 100 μm. Zoomed inset demonstrates CD14+ macrophages associated with podoplanin+ lymphatic vasculature (black arrows). Tissues were selected from 8 patients with or without lymph node positivity. Consecutive sections were stained singly for podoplanin lymphovasculature or doubly using pan-macroage marker, CD68, and anti-b4 integrin antibody. (G) Double-stained macrophages per square millimeter shown with patient clinical details (LVI and lymph node positivity). (H) CD68+ITGB4+ macrophages are indicated in upper right panels (red arrows). CD68 and ITGB4 stainings are indicated below as 2 single panels; CD68+ITGB4+ macrophages are indicated with red arrows. Podoplanin+ vessels shown in upper left images (black arrows). Scale bars, 20 μm.
In breast cancer samples previously characterized for LVI, we identified a population of endogenous macrophages that express \( \alpha_6 \beta_4 \) integrin and are adherent to laminin-5 in lymphovascular areas. Collectively, our data suggest that \( \beta_4 \) integrin acts to ensure that tumor-infiltrating macrophages are in a prime location for sustained interaction with LECs.

We have defined dual functionality of TGF-\( \beta_1 \) where it can affect signaling within TAMs and LECs. First, we show that TGF-\( \beta_1 \) is required for \( \beta_4 \) integrin clustering at the macrophage plasma membrane. Integrin clustering can positively regulate levels of cell adhesion rapidly in response to soluble stimuli (Hyynes, 2002). TGF-\( \beta \) has previously been demonstrated to control \( \alpha_6 \beta_1 \) and \( \alpha_6 \beta_4 \) integrin clustering in HER2-overexpressing mammary tumor cells (Wang et al., 2009). Here, we describe TGF-\( \beta_1 \)-dependent \( \beta_4 \) integrin clustering in macrophages that control the macrophage-spreading response necessary for TAM adhesion at the site of lymphatic vasculature.

Second, TGF-\( \beta_1 \) acts in a paracrine manner to activate RhoA in LECs lining the lymphatic vessel, as demonstrated through RAICHAU-fluorescent lifetime imaging microscopy (FLIM) technology (Heasman et al., 2010; Makrogianneli et al., 2009; Vega et al., 2011). Our study shows that signaling within LECs in contact with TAMs drives LEC contraction, which correlates to gross architectural changes and hyperpermeability of the lymphatic vessel network that could actively facilitate metastasis. We have previously demonstrated the activation of RhoGTPases by integrin signaling in cis (on the immune cells that are triggered by adhesion processes) (Makrogianneli et al., 2009; Carlin et al., 2011; Heasman et al., 2010; Ramsay et al., 2013). Our present study indicates that this phenomenon can also occur in trans, i.e., activation of RhoGTPases in the endothelial cells that are contacted by the adherent macrophages, through the expression of factors such as TGF-\( \beta_1 \). The role of macrophage-released TGF-\( \beta_1 \) in vivo is shown to have an effect on the RhoA pathway in proximal LECs and a concomitant role in lymphovascular disorganization.

In summary, this study identifies an alternative macrophage-mediated signaling pathway involved in the promotion of lymphatic metastasis. Our work emphasizes the importance in considering crosstalk between macrophages and the lymphatic vessel network in TNBC, where aggressive tumor growth and rapid metastasis often mean a poor outcome. We hope this study will guide future endeavors to focus on therapeutically targeting the lymphatic remodeling signaling cascade in TNBC disease progression.

**STAR METHODS**

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cellrep.2019.04.076.

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REFERENCES

Ando, T., Joh, T., Wang, Y., Jennings, M.H., Houghton, J., and Alexander, J.S. (2005). Isolation and characterization of a novel mouse lymphatic endothelial cell line: SV-LEC. Lymphat. Res. Biol. 3, 105–115.

Avraamides, C.J., Garny-Suain, B., and Vamer, J.A. (2008). Integrins in angiogenesis and lymphangiogenesis. Nat. Rev. Cancer 8, 604–617.

Baikwell, F.R., Capasso, M., and Hagemann, T. (2012). The tumor microenviroment at a glance. J. Cell Sci. 125, 5591–5596.

Barber, P.R., Ameer-Beg, S.M., Gilbey, J., Carlin, L.M., Keppler, M., Ng, T.C., and Vojnovic, B. (2009). Multiphoton time-domain fluorescence lifetime imaging microscopy: practical application to protein-protein interactions using global analysis. J. R. Soc. Interface 6, S93–S105.

Barber, P.R., Tullis, I.D., Pierce, G.P., Newman, R.G., Prentice, J., Rowley, M.I., Matthews, D.R., Ameer-Beg, S.M., and Vojnovic, B. (2013). The Gray Institute ‘open’ high-content, fluorescence lifetime microscopes. J. Microsc. 257, 154–167.

Barbie, D.A., Tamayo, P., Boehm, J.S., Kim, S.Y., Moody, S.E., Dunn, I.F., Schinzel, A.C., Sandy, P., Meylan, E., Scholl, C., et al. (2009). Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. Nature 462, 108–112.

Bran, S., Henry, L., Faes-Van’t Hull, E., Turini, R., Vancheek, D., Guex, N., Iliticene-Trebourx, A., Martinac, E., Semilitoef, A., Rifer, N., et al. (2015). TIE-2-expressing monocytes are lymphangiogenic and associate specifically with lymphatics of human breast cancer. OncolImmunology 5, e1073882.

Bryan, B.A., Dennstedt, E., Mitchell, D.C., Walshe, T.E., Noma, K., Loureiro, R., Saint-Geniez, M., Campaingnac, J.P., Liao, J.K., and D’Amore, P.A. (2010). RhoA/ROCK signaling is essential for multiple aspects of VEGF-mediated angiogenesis. FASEB J. 24, 3186–3195.

Carlin, L.M., Evans, R., Milewicz, H., Fernandes, L., Matthews, D.R., Perani, M., Levitt, J., Keppler, M.D., Monypenny, J., Coolen, T., et al. (2011). A targeted siRNA screen identifies regulators of Cdc42 activity at the natural killer cell immunological synapse. Sci. Signal. 4, ra81.

Choi, W.W., Lewis, M.M., Lawson, D., Yin-Goen, Q., Birdsong, G.G., Cotsonis, G.A., Cohen, C., and Young, A.N. (2005). Angiogenic and lymphangiogenic microvessel density in breast carcinoma: correlation with clinicopathologic parameters and VEGF-family gene expression. Mod. Pathol. 18, 143–152.

Condeelis, J., and Pollard, J.W. (2006). Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell 124, 253–266.

De Palma, M., Venneri, M.A., Galli, R., Sergi Sergi, L., Politi, L.S., Sampaolesi, M., and Naldini, L. (2005). Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. Cancer Cell 8, 211–226.

De Palma, M., Murdoch, C., Venneri, M.A., Naldini, L., and Lewis, C.E. (2007). Tie2-expressing monocytes: regulation of tumor angiogenesis and therapeutic implications. Trends Immunol. 26, 519–524.

Dent, R., Trudeau, M., Pritchard, K.L., Hanna, W.M., Kahn, H.K., Sawka, C.A., Lickley, L.A., Ravlinson, E., Sun, P., and Narod, S.A. (2007). Triple-negative breast cancer: clinical features and patterns of recurrence. Clin. Cancer Res. 13, 4429–4434.

Desgrosellier, J.S., and Cheres, D.A. (2010). Integrins in cancer: biological implications and therapeutic opportunities. Nat. Rev. Cancer 10, 9–22.

Diaz, L.K., Cristofanilli, M., Zhou, X., Welch, K.L., Smith, T.L., Yang, Y., Snejega, N., Sahin, A.A., and Gilcrease, M.Z. (2005). Beta4 integrin subunit gene expression correlates with tumor size and nuclear grade in early breast cancer. Cell Reports 27, 2017–2027.

Finsterbusch, M., Voisin, M.B., Beyrau, M., Williams, T.J., and Nourshargh, S. (2014). Neutrophils recruited by chemoattractants in vivo induce microvascular plasma protein leakage through secretion of TNF. J. Exp. Med. 217, 1307–1314.

Fleming, Y.M., Ferguson, G.J., Spender, L.C., Larsson, J., Karlsson, S., Ozanne, B.W., Grosse, R., and Inman, G.J. (2009). TGF-beta-mediated activation of RhoA signalling is required for efficient (V12)HaRas and (V600E)BRAF transformation. Oncogene 28, 983–993.

Gazinska, P., Grigoriadis, A., Brown, J.P., Millis, R.R., Mera, A., Gillett, C.E., Holmberg, L.H., Tutt, A.N., and Pinder, S.E. (2013). Comparison of basal-like triple-negative breast cancer defined by morphology, immunohistochemistry and transcriptional profiles. Mod. Pathol. 26, 955–966.
Gordon, E.J., Rao, S., Pollard, J.W., Nutt, S.L., Lang, R.A., and Harvey, N.L. (2010). Macrophages define dermal lymphatic vessel calibre during development by regulating lymphatic endothelial cell proliferation. Development 137, 3899–3910.

Harney, A.S., Arwert, E.N., Enntenberg, D., Wang, Y., Guo, P., Qian, B.Z., Oktay, M.H., Pollard, J.W., Jones, J.N., and Condeelis, J.S. (2015). Real-time imaging reveals local, transient vascular permeability, and tumor cell intravasation stimulated by TIE2hi macrophage-derived VEGFA. Cancer Discov. 5, 932–943.

Heasman, S.J., Carlin, L.M., Cox, S., Ng, T., and Ridley, A.J. (2010). Coordinated RhoA signaling at the leading edge and uropod is required for T cell transendothelial migration. J. Cell Biol.

Hynes, R.O. (2002). Integrins: bidirectional, allosteric signaling machines. Cell 110, 673–687.

Inman, G.J., Nicolás, F.J., Callahan, J.F., Harling, J.D., Gaster, L.M., Reith, A.D., Laping, N.J., and Hill, C.S. (2002). SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol. Pharmacol.

Inman, G.J., Nicola ´ s, F.J., Callahan, J.F., Harling, J.D., Gaster, L.M., Reith, A.D., Laping, N.J., and Hill, C.S. (2002). SB-431542 is a potent and specific in-

Heasman, S.J., Carlin, L.M., Cox, S., Ng, T., and Ridley, A.J. (2010). Coordinated RhoA signaling at the leading edge and uropod is required for T cell transendothelial migration. J. Cell Biol.

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Inman, G.J., Nicolás, F.J., Callahan, J.F., Harling, J.D., Gaster, L.M., Reith, A.D., Laping, N.J., and Hill, C.S. (2002). SB-431542 is a potent and specific in-

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Inman, G.J., Nicolás, F.J., Callahan, J.F., Harling, J.D., Gaster, L.M., Reith, A.D., Laping, N.J., and Hill, C.S. (2002). SB-431542 is a potent and specific in-

Heasman, S.J., Carlin, L.M., Cox, S., Ng, T., and Ridley, A.J. (2010). Coordinated RhoA signaling at the leading edge and uropod is required for T cell transendothelial migration. J. Cell Biol.

Hynes, R.O. (2002). Integrins: bidirectional, allosteric signaling machines. Cell 110, 673–687.
## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rat monoclonal anti-Lyve1 | Novus Biologicals | #NB-600-1008 |
| Rabbit polyclonal anti-Tie2 (C-20) | Santa Cruz | #sc-324 |
| Rabbit polyclonal phospho-Smad2/3 (D27F4) | Cell Signaling | #8828 |
| Mouse monoclonal anti-ITGB4 | Abcam | #ab29042 |
| Mouse monoclonal anti-CD68 antibody | Ventana Cell Marque | #168M |
| Mouse monoclonal anti-CD14 (EP3653) | Ventana Cell Marque | #114R |
| Mouse monoclonal anti-podoplanin (D2-40) | Ventana Cell Marque | #332M |
| Rat monoclonal anti-CD45-APC-Cy7 | Biolegend | #103115 |
| Rat monoclonal Ly6G-Biotin | Biolegend | #127603 |
| Streptavidin AF488 | Biolegend | #405235 |
| Rat monoclonal CD11b-eFluor450 | ThermoFisher Scientific | #48-0112-82 |
| Rat monoclonal Tie-2 PE | Biolegend | #124007 |
| Rat monoclonal j4 integrin-BV711 | BD Biosciences | #744154 |
| CD31 PerCPCy5.5 | Biolegend | #102419 |
| Rat monoclonal anti-F4/80-FITC (clone BM8) | Abcam | #Ab60348 |
| Rabbit polyclonal anti-laminin-5 | Abcam | #Ab14509 |
| Rabbit polyclonal Anti-Phospho myson light chain (Ser19) | Cell Signaling | #3671 |
| Mouse monoclonal anti-podoplanin antibody | Santa Cruz | #sc-166906 |
| Rabbit polyclonal anti-TGFβ1 antibody | Proteintech | #11522-1-AP |
| **Biological Samples** |        |            |
| Breast cancer tumor tissues (paraffin-embedded) | King’s College London breast cancer biobank | Team lead – Dr Cheryl Gillet |
| 4T1.2 tumor tissues (frozen) | King’s College London | Dr Rachel Evans |
| LG-cre;Bra11fr:p53+/+ tumor tissues (frozen) | King’s College London | Dr Rebecca Marlow |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| Cell tracker™ red (CMTMR) and Cell tracker™ green (CMFDA) | Life Technologies | #C34552, C2925 |
| Murine CSF1 | Sigma | #M9170 |
| Human recombinant laminin-5 | Novus Biologicals | #H00003911 |
| Clodronate and PBS liposomes | Liposoma Technology | #CP-005-005 |
| 2',7'-bis-(2-carboxyethyl)-5-((and-6)-carboxyfluorescein-acetoxymethyl ester (BCECF) | Thermo Scientific | #B1170 |
| SB-431542 | Sigma | #S4317 |
| Evans Blue dye | Sigma | #E2129 |
| Formamide | Sigma | #F9037 |
| 76kDa dextran Texas Red | Sigma | #R05027 |
| 76kDa dextran fluorescein | Santa Cruz | #sc-263323 |
| **Critical Commercial Assays** |        |            |
| Murine TGFβ1 quantikine ELISA kit | R&D Ltd | #MB100B |
| Murine TGFβ2 quantikine ELISA kit | R&D Ltd | #DB250 |
| **Deposited Data** |        |            |
| Experiment ArrayExpress accession | ArrayExpress | ArrayExpress: E-MTAB-4064. |
| Breast Cancer Gene Expression data | Gene Expression Omnibus | GEO: GSE75688 |
| ARCHS4 database | (Lachmann et al., 2018) | N/A |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to the Lead Contact, Tony Ng (tony.ng@kcl.ac.uk).

For a detailed description of the experimental procedures please see Supplemental Information.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Tissue culture

Bone marrow macrophages

Monocytes were isolated from female BALB/c mice femurs and cultured in mCSF-1 for 5 d.

Cell lines

All cell lines were tested as mycoplasma negative and authenticated by IDEXX Laboratories Ltd, UK.

Tumor-bearing mice

4T1.2

BALB/c immune-competent mice were 6–8 weeks of age and maintained under pathogen-free conditions. Tumors were established by injection of 1x10⁶ 4T1.2 (Lelekakis et al., 1999) cells into the mammary fat pad.

BLG-Cre;Brca1<sup>f/f</sup>,p53<sup>+/−</sup>

Mammary tumor chunks (approximately 0.2cm³) dissected from BLG-Cre;Brca1<sup>f/f</sup>,p53<sup>+/−</sup> mice (Molyneux et al., 2010) were transplanted orthotopically into mammary fat pads of recipient 5-week old C57BL6J mice. Tumors were grown for 4-8 weeks before mice were culled and tumor tissues harvested.

Human breast cancer samples

Paraffin embedded samples (n = 20) (KHP Cancer Biobank Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset cohort) were used. Ten patients were previously characterized as having lymphatic vessel invasion (LVI) and the remaining 10 did not have LVI. Please see SI for details on staining.
Study approval
All experiments were performed in accordance with the local ethical review panel, the UK Home Office Animals Scientific Procedures Act, 1986 and the UKCCCR guidelines.

METHOD DETAILS

RAW264.7 macrophage treatment
Tumor-bearing mice were injected with 100 μL PBS or 1x10⁶ RAW264.7 macrophages starting on the second day after tumor inoculation and repeated every 2 days until the end of the experiment.

Clodronate treatment
Endogenous macrophages were ablated using clodronate-containing liposomes (Weisser et al., 2012).

Immunofluorescence
Tissue sections were fixed with 4% paraformaldehyde (PFA), blocked in 5% BSA followed by staining. Hoechst-33342 (0.1 μg/ml) was used for nuclear staining and samples mounted using Mowiol (with DABCO). Image acquisition by confocal microscopy was performed using a Nikon Eclipse Ni-E Upright. Image acquisition was conducted using NIS Elements C software and analyzed using ImageJ software.

Image acquisition and analysis for colocalization studies in tissue
Cy3 and AF647 dyes were imaged before and after photobleaching using (x20 0.75NA air objective, Nikon) and a cooled CCD detector (Hamamatsu ORCA-03G, 1024 × 1024) with respective integration time of 100 ms and 1000 ms. Dyes were photobleached using a mode-locked Titanium Sapphire Laser (Coherent, Chameleon Ultra 2) tuned at 730 nm with pulse duration of about 200 fs, a repetition rate of 80 MHz and average laser power on the sample of 30 mW. To measure the relative level of β4 integrin expression within the lymphovasculature compared with the rest of the tissue, we measured average AF647 intensity within lymphovascular areas (high Cy3 intensity) normalized by the average AF647 intensity outside lymphovascular areas (low Cy3 intensity).

Structured Illumination Microscopy (SIM)
RAW264.7-NTC or RAW264.7-TGFβ1 KD were stained with rat anti-β4 integrin antibody and anti-rat AF647 antibody. Image acquisition by SIM was performed using Nikon N-SIM microscope equipped with a 640nm laser, a Andor iXon Ultra 897 EMCCD camera and a 100x 1.49NA oil immersion objective. Images were analyzed using ImageJ software.

Mammary imaging window implantation and intravital microscopy
Mammary Imaging Window (MIW) surgery was performed 10-14 days after tumor inoculation (Kedrin et al., 2008). Images shown are representative of a minimum of 5 independent experiments. For imaging lymphatic vasculature, mice were injected subcutaneously at the tail base with 50 μL 76kDa dextran-fluorescein or dextran-Texas red 15 min prior to imaging. Mice were imaged for a maximum period of 4 h per day using a x20 air objective. All post hoc image processing and image reconstructions were done using ImageJ software.

Lymphatic vessel permeability
Tumor-bearing mice were injected subcutaneously at the tail base with 1% Evans Blue dye. After 30 min the mice were culled and the tumors incubated in formamide overnight at 55°C. Optical density of formamide was read at 620nm and quantification of lymphatic permeability was given as OD per g tumor.

Adhesion assay
Laminin-5 was plated onto 96 well plates overnight at 4°C and non-specific interactions blocked with BSA. Macrophages (5 × 10⁶/ml) were labeled with 1 μM 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxymethyl ester (BCECF) for 30 min at room temperature. 100 μL of cells (1 × 10⁶/ml) were added at 37°C, plates washed, and adhering macrophages quantified using a fluorescence microtiter plate reader.

Lymphatic endothelial cell contraction
SV-LEC cells or primary lymphatic endothelial cells were grown as a monolayer. On day 3 LECs and macrophages were stained for 30 min at 37°C using 1 μg/ml CMTMR or CMFDA respectively. Macrophages were added to SV-LEC monolayers overnight. Confocal images of the co-culture and the area around individual SV-LECs was calculated using ImageJ software.

RhoA biosensor
SV-LECs were transiently transfected with the RAICHU RhoA biosensor (Yoshizaki et al., 2003). The biosensor was modified to express GFP and mRFP (Makrogianneli et al., 2009). Multiphoton time-correlated single photon counting FLIM was performed to
quantify RhoA biosensor FRET Fluorescence excitation was provided by a Fianium laser, which generates optical pulses with a duration of 40 ps at a repetition rate of 80 MHz. For the imaging of RAICHU-transfected SV-LECs, multi-photon excitation was employed using a solid-state pumped (8-W Verdi; Coherent), femtosecond self-mode locked Ti:Sapphire (Mira; Coherent) laser system (Peter et al., 2005; Barber et al., 2009). Imaging data comprised of 256 × 256 pixel resolution and 256 time channels. The fluorescence lifetime was calculated as described (Barber et al., 2013).

TGFβ1 stable knockdown in RAW264.7 macrophages
Stable TGFβ1 knockdown RAW 264.7 macrophage lines were generated by lentiviral transduction using the pGIPZ system (Open Biosystems). Viral packaging was performed by transiently transfecting HEK293T cells with the pGIPZ shRNA transfer vector and the accessory plasmids pCMV-dR8.91 and pMD2G. Stable cell lines were established using three different shRNA lentiviral vectors. RAW 264.7 macrophages were cultured in puromycin (1 μg/ml) to enable the selection of successfully transduced cells and efficacy of knockdown was assessed by western blotting.

FACS analysis
RAW264.7 cell lines (TGFβ1-knockdown or NTC) were stained with a Live-Dead Yellow dye followed by staining with a primary rat anti-β4 integrin antibody and anti-rat AF647-conjugated secondary antibody.

Human tissue staining
Sections were stained using anti-CD14/anti-podoplanin using Ventana Benchmark Ultra and Ultra view DAB and Alkaline Phosphatase detection systems. Sections were assessed independently by two histopathologists and scored for CD14+ macrophages within or proximal to lymphatic vasculature.

Alternatively, using consecutive sections the first section was stained with anti-podoplanin and the second section stained with anti-ITGB4 anti-CD68. All sections were stained with DAB+ substrate/chromagen. All incubations were at room temperature.

The slides were scanned in the Hamamatsu NanoZoomer S210 Digital slide scanner. The image analysis was performed on the whole section with the color deconvolution module and the positive pixel algorithm from QuPath image analysis software.

QUANTIFICATION AND STATISTICAL ANALYSIS

Gene expression microarray analysis
RNA was extracted from macrophage cell cultures and profiled using Affymetrix Mouse Gene 1.0 ST arrays. Differential expression between conditions was estimated by fitting a linear model and performing empirical Bayes moderated t tests using the package ‘limma’ (v3.22.4) (Ritchie et al., 2015). The expression score for a specific gene in each sample is defined as the weighted sum of gene-standardized (Z-score) expression values, with weights +1/-1 according to relative increase or decrease in BMM + 4T1.2 compared with BMM.

Analysis of gene signatures
To establish ITGB4 expression and assess association between ITGB4 expression and activation of the TGFβ signaling in macrophages, processed gene counts were obtained from the ARCHS4 database (Lachmann et al., 2018) and further normalized for downstream analyses. Enrichment of TGFβ signaling was computed using the ssGSEA method (Barbie et al., 2009) as implemented in the GSVA package from Bioconductor.

False zero expression due to dropout events in scRNA-seq data was corrected using the scImpute algorithm as previously described (Li and Li, 2018). scRNAseq data is reported as log2(TPM+1).

Macrophage-mediated vascular remodeling pathway signature (Pucci et al., 2009) was converted to a human gene list using BioMart ID conversion (Ensembl Genes 84// Mus musculus genes GRChm38.p4). TGFβ (KEGG) gene signature was derived from (MSigDB). Gene signature activity was calculated using a weighted average sum over all genes for each tumor. Pearson’s correlation between the activation scores was reported. Hypergeometric testing was used to establish the significance of overlap between TNBC with distant metastasis (DM) on those of dual high activation scores. Kaplan-Meier plots were generated for each dataset to provide a visualization of survival stratification.

All other statistical analysis is described in the text and legends and was performed using Prism software (GraphPad). P values less than 0.05 were considered significant. The statistical test used is indicated in the figure legends and the significance of findings is indicated in the figures.

DATA AND SOFTWARE AVAILABILITY

The accession number for the microRNA experimental data reported in this paper is ArrayExpress: E-MTAB-4064.