Real-Time Imaging Reveals Local, Transient Vascular Permeability, and Tumor Cell Intravasation Stimulated by TIE2 hi Macrophage–Derived VEGFA

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ABSTRACT

Dissemination of tumor cells is an essential step in metastasis. Direct contact between a macrophage, mammalian-enabled (MENA)–overexpressing tumor cell, and endothelial cell [Tumor MicroEnvironment of Metastasis (TMEM)] correlates with metastasis in breast cancer patients. Here we show, using intravital high-resolution two-photon microscopy, that transient vascular permeability and tumor cell intravasation occur simultaneously and exclusively at TMEM. The hyperpermeable nature of tumor vasculature is described as spatially and temporally heterogeneous. Using real-time imaging, we observed that vascular permeability is transient, restricted to the TMEM, and required for tumor cell dissemination. VEGFA signaling from TIE2 hi TMEM macrophages causes local loss of vascular junctions, transient vascular permeability, and tumor cell intravasation, demonstrating a role for the TMEM within the primary mammary tumor. These data provide insight into the mechanism of tumor cell intravasation and vascular permeability in breast cancer, explaining the value of TMEM density as a predictor of distant metastatic recurrence in patients.

SIGNIFICANCE: Tumor vasculature is abnormal with increased permeability. Here, we show that VEGFA signaling from TIE2 hi TMEM macrophages results in local, transient vascular permeability and tumor cell intravasation. These data provide evidence for the mechanism underlying the association of TMEM with distant metastatic recurrence, offering a rationale for therapies targeting TMEM.

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See related commentary by Kadioglu and De Palma, p. 906.
INTRODUCTION

For almost two decades, tumor vasculature has been described as abnormal with increased vascular permeability (1, 2). VEGFA is known to promote vascular permeability, and inhibition of VEGFA results in the normalization of tumor vasculature and a decrease in permeability (3, 4). Due to the significant effects of VEGFA on tumor angiogenesis and vascular permeability, inhibitors of VEGF signaling have become an important research focus in the development of antitumor therapies.

Tumor-associated macrophages (TAM) have been implicated in tumor progression, angiogenesis, and metastasis (5, 6). A subpopulation of perivascular TAMs that have features of protumorigenic macrophages, promoting tumor angiogenesis and metastasis, have been identified as TIE2-expressing macrophages (TEM; ref. 7). Perivascular macrophages are also an essential component of the microanatomical sites termed “Tumor MicroEnvironment of Metastasis” (TMEM) that consist of a TAM in direct contact with a mammalian-enabled (MENA) overexpressing tumor cell and endothelial cell (8). TMEMs have been associated with tumor cell intravasation (9, 10), and TMEM density predicts distant metastatic recurrence in breast cancer patients independently of other clinical prognostic indicators (8, 11). However, the mechanistic link between perivascular macrophages and tumor cell intravasation has remained unclear. Further, hyperpermeability in tumor vasculature is not uniform, but rather is spatially and temporally heterogeneous (12). In a VEGFA overexpression model inducing vascular permeability, the presence of macrophages at vascular branch points was observed at hotspots of vascular permeability (4). Although hyperpermeability of tumor vasculature is widely accepted, a mechanistic understanding of the heterogeneity of vascular permeability, the contribution of TAMs, and the link with tumor cell intravasation have not been described.

Here we show, using intravitral high-resolution two-photon microscopy, that transient vascular permeability events are restricted to TMEM sites of TIE2hi/VEGFAhi perivascular macrophages. Local loss of vascular junctions at the TMEM results in transient vascular permeability and tumor cell intravasation in the spontaneous autochthonous mouse mammary cancer model where the mouse mammary tumor virus long terminal repeat drives the polyoma middle T antigen (MMTV-PyMT), the human patient-derived xenograft model TN1, and human metastatic breast cancer.

RESULTS

TMEM-Associated Tumor Cells and Macrophages Are Stationary in TMEM Structures

To examine the functional role of the TMEM in tumor cell dissemination, we used the spontaneous autochthonous mouse mammary cancer model where the mouse mammary tumor virus long terminal repeat drives the polyoma middle T antigen (MMTV-PyMT), in which tumors exhibit histology similar to human luminal breast cancer and progress to
metastasis (13). IHC revealed that TMEM structures in mouse tumors have the same microanatomical structure as identified in humans (Supplementary Fig. S1A; ref. 11). TMEM density and circulating tumor cells (CTC) increase with tumor progression with elevated TMEM scores in late carcinoma as compared with early carcinoma as seen by IHC (Supplementary Fig. S1A–S1C), though total perivascular macrophage (including macrophages not associated with tumor cells) density is not significantly different (13). High-resolution imaging demonstrates that in TMEM structures tumor cells and macrophages extend protrusions but are relatively nonmigratory and stay in direct contact over time (Supplementary Fig. S1D).

Vascular Permeability and Tumor Cell Intravasation Occur Concurrently at the TMEM

To directly observe TMEM function in vivo, we used extended time-lapse intravital microscopy (IVM) with high spatial and temporal resolution. To visualize blood flow, vessels were labeled with a high–molecular weight compound (155-kDa dextran or quantum dots; refs. 1, 14; Figs. 1, 2, 3; and Supplementary Fig. S2). In PyMT late carcinoma, migratory tumor cells and macrophages stream toward TMEM at sites with vascular permeability, whereupon tumor cells undergo transendothelial migration at the TMEM (Fig. 1A–E; Supplementary Fig. S2A–S2E). In late carcinoma, transient, local blood vessel permeability was observed at TMEM sites by the extravasation of quantum dots (Supplementary Fig. S2A and S2B) or 155-kDa dextran–tetramethylrhodamine (TMR; Figs. 2A and B; 3C; Supplementary Fig. S2C–S2E; and Supplementary Movie S1). Further, tumor cell intravasation occurs at TMEM sites concurrently with transient permeability (Fig. 2A–H and Supplementary Fig. S2C–S2E). Transient vascular permeability at the TMEM is spatially and temporally heterogeneous (Supplementary Fig. S2F), with events of permeability and tumor cell intravasation at the TMEM occurring predominantly at vascular branch points (Supplementary Fig. S2G). Transendothelial crossing of tumor cells is visualized by the hourglass shape of tumor cells, as they are partially in the vessel lumen and partially in the tissue (Fig. 1C, 2A, C–E; Supplementary Fig. S2E). During transendothelial migration of tumor cells, the TMEM tumor cell and macrophage neither migrate nor intravasate, indicating that tumor cells entering the blood vessel at the TMEM are supplied by the migratory stream of cells (Fig. 1A, B, and D). The stationary phenotype of these cells is consistent with previous results showing macrophage contact–initiated invadopodium formation uniquely in the TMEM tumor cell (9) and that perivascular invadopodium-containing tumor cells are relatively nonmotile in vivo (15).

The peak of extravascular dextran intensity and the appearance of circulating tumor cells coincide temporally and spatially (Fig. 2A–E, H; Supplementary Fig. S2; Supplementary Movie S1), demonstrating a direct link between localized blood vessel permeability and tumor cell intravasation at the TMEM. The coincidence of spontaneous, transient vascular permeability with tumor cell intravasation at the TMEM also has been observed in a patient-derived xenograft model of triple-negative breast cancer, TN1 (Supplementary Fig. S3).

To confirm that the TMEM is associated with transient vascular permeability and tumor cell intravasation, a 100-μm
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Figure 2. Transient, local blood vessel permeability events accompany intravasation at the TMEM. A, IVM time lapse of 155-kDa dextran–TMR extravasation and tumor cell intravasation. TMEM, white box. Blood vessel permeability sites (white arrowheads) and intravasating tumor cell (TC; yellow dashed line, 9’). Clearance of dextran and decrease of CTCs at 30’. Scale bar, 50 μm. At 9’ and 30’, TMEM tumor cells and macrophages are added in false color to increase visibility after bleaching. B, isolated 155-kDa dextran–TMR channel from A. Red arrowheads mark dextran extravasation (white). Dashed red line, luminal side of the endothelium. C, isolated tumor cell channel from A. Yellow arrow marks site of intravasating tumor cell (yellow dashed line) at the TMEM. White dashed line marks the luminal surface of the endothelium. Red box, region adjacent to the TMEM with elevated CTCs. D, single time point of tumor cell intravasation (yellow dashed line) by time-lapse IVM. Scale bar, 50 μm. E, 3D reconstruction of time-lapse IVM from D of tumor cell intravasation at the TMEM. Transmigrating tumor cells (individually numbered, dashed white lines) are isolated from other cell types for clarity with time in minutes from start (J0’) to end of transmigration (J3’). The luminal endothelial surface is outlined in a pink dashed line. Extravascular dextran (red) at the TMEM indicated with a yellow arrowhead and outlined in a yellow dashed line. F, frequency of blood vessel permeability per hour in the presence or absence of the TMEM (Supplementary Fig. S4; n = 16; **, P = 0.0034). G, frequency of tumor cell intravasation per hour in the presence or absence of the TMEM (n = 16; **, P = 0.0012). H, quantification of extravascular dextran intensity and CTC area at the TMEM over time from A. •, extravascular dextran; ■, CTC.

Vascular Permeability at the TMEM Is a Highly Localized and Transient Event

Tumor vasculature has been previously described as abnormal with increased permeability, which has been attributed to larger vascular intercellular openings (1, 12, 16). However, vascular permeability is not spatially or temporally uniform, with hotspots at vascular branch points (4, 12). Here, we demonstrate that vascular permeability is transient, occurs exclusively at TMEM sites, and is temporally heterogeneous, explaining the previously unresolved heterogeneity in vascular permeability (Fig. 2F and Supplementary Fig. S2E). Events of spontaneous,
Figure 3. TMEM-mediated vascular permeability is transient and localized. A, time-lapse imaging demonstrates that laser-induced damage to the endothelium creates a hole allowing for extravasation of 155-kDa dextran–TMR. The location of the hole is marked by a white dot (2 μm) and a yellow arrowhead. 155-kDa dextran–TMR extravasates and increases over time up to 60′ filling the field of view and not clearing from the tissue (n = 4). Scale bar, 50 μm. B, 155-kDa dextran–TMR is injected by tail vein i.v. catheter followed by 8 μg of VEGF-A 165 at 0′. VEGF-A 165 induces blood vessel permeability in all of the blood vessels in the field of view. Peak extravascular dextran is observed at 20′ followed by clearance by 60′ after resealing of vascular junctions (n = 4). Scale bar, 50 μm. C, spontaneous vascular permeability at the TMEM is both transient and local. Local peak extravasation of 155-kDa dextran–TMR occurs after 20′ (yellow arrowhead) and clears within 60′ (n = 11). Scale bar, 50 μm. D, quantification of average relative intensity of extravascular 155-kDa dextran–TMR is observed at 20′ followed by clearance by 60′ after resealing of vascular junctions (n = 4). Scale bar, 50 μm. E, table of parameters from curve fitting to an Exponentially Modified Gaussian function using data from D. F, quantification of total extravascular 155-kDa dextran–TMR area after laser-induced damage, i.e. injection of VEGF-A 165, or spontaneous permeability at the TMEM from individual animals represented in A, B, and C. Peak of 155-kDa dextran–TMR area in spontaneous permeability at the TMEM indicated with a red arrowhead. •, laser damage; ■, intravenous VEGF-A 165; ▲, spontaneous vascular permeability at the TMEM.
local vascular permeability and tumor cell intravasation at TMEM occur predominantly at vascular branch points, consistent with previous reports of vascular permeability (Supplementary Fig. S2C). If tumor blood vessels were uniformly leaky, high–molecular weight vascular probes would extravasate immediately and continuously after injection. Although the high–molecular weight probe, 155-kDa dextran–TMR, remains in the vasculature in the absence of transient TMEM-associated permeability events for the duration of the time-lapse imaging, a low-molecular weight dextran, 10-kDa dextran–FITC, which is below the molecular-weight cutoff size of the endothelium (1, 14), leaks from blood vessels and clears from the vascular space (Supplementary Fig. S5).

Further, transient permeability events are distinct from mechanical damage to the endothelium. After creating a 2-μm hole in the endothelium with a laser, 155-kDa dextran–TMR extravasates continuously, filling the field of view (Fig. 3A). In contrast, VEGFA-mediated permeability is transient (12). Intravenous injection of VEGFA165, the soluble isoform of VEGFA with properties of native VEGF (17), results in vascular permeability with peak intensity of extravascular dextran at 20 minutes (Fig. 3B). Spontaneous vascular permeability at the TMEM follows similar kinetics to VEGFA165-mediated permeability with peak intensity of extravascular dextran at 20 minutes, but is restricted to individual TMEM sites (Fig. 3C). The curves obtained for average intensity of extravasal 155-kDa dextran–TMR after laser damage, VEGFA165, and spontaneous permeability were fit to an exponentially modified Gaussian function (Fig. 3D and E). Although the curve for laser damage does not have a clearance term as dextran continues to extravasate for the entire time lapse, both the VEGFA165 and spontaneous curves have similar extravasation and clearance rates. A significant difference between VEGFA165 and spontaneous TMEM-mediated permeability is that permeability at the TMEM is highly local, whereas VEGFA165 results in dextran extravasation from all blood vessels within a field of view. Thus, the area of extravascular 155-kDa dextran–TMR from local TMEM-mediated permeability is markedly less than permeability from VEGFA165 or laser-induced damage (Fig. 3F), further emphasizing the local nature of TMEM-mediated vascular permeability.

TMEM-Associated Macrophages Are Essential for Vascular Permeability and Tumor Cell Intravasation

To determine if TMEM macrophages regulate vascular permeability and tumor cell intravasation, macrophages were depleted in the mammary tumor using the previously characterized mouse model, macrophage fas-induced apoptosis (Mafia; refs. 18, 19), with orthotopic MMTV-PyMT tumor implants. Depletion of macrophages is systemic, including within the mammary tumor, thus resulting in a depletion of TAM and TMEM by 67% and 72%, respectively (Fig. 4A–C). When macrophages are depleted, extravascular dextran decreases, as does the number of circulating tumor cells (Fig. 4D–F). These data demonstrate that macrophages are essential for vascular permeability and tumor cell intravasation at the TMEM. Because blood vessel permeability observed by IVM is restricted to TMEM, we examined whether vascular junction protein localization was altered in the absence of macrophages, reflecting a requirement for macrophage-dependent signaling events to induce vascular permeability. Staining for vascular junction proteins ZO-1 and VE-Cadherin increased in the tumor vasculature after depletion of macrophages in MAFIA mouse tumors (Fig. 4D, G, and H), indicating that macrophages are involved in vascular junction disassembly during vascular permeability events at the TMEM.

TEMs Are Localized in TMEM Structures

In PyMT mammary carcinoma, a subpopulation of TAMs has been identified as TIE2hi/VEGFAhi macrophages (7, 20, 21). TEMs have been shown to upregulate the TIE2 tyrosine kinase receptor by 100-fold after recruitment to the tumor (22). TEMs have features of protumorigenic macrophages and promote tumor angiogenesis (7). TEMs are further characterized as MRC1+/CD11b+/F4/80+/CD11c+ and are associated with CD31+ tumor blood vessels (20). Thus, we sought to determine if TEMs are located in the TMEM. Immunofluorescence of the TMEM markers MENA (tumor cells), CD31 (endothelial cells), and CD68 (macrophages; Fig. 5A) compared with TIE2, VEGFA, and CD31 in sequential tissue sections demonstrates that TIE2hi/VEGFAhi macrophages are enriched in TMEM structures (Fig. 5B and Supplementary Fig. S6A–S6E). VEGFA is elevated in TIE2hi macrophages, as compared with the adjacent endothelial cells and surrounding tumor tissue (Fig. 5C and D). Further, 100% of TIE2hi/ VEGFAhi TEM-associated macrophages express the TEM markers MRC1, CD11b, and F4/80 while lacking CD11c (Fig. 5E and F and Supplementary Fig. S6).

Inhibition of VEGFA Signaling Reduces Vascular Permeability and Tumor Cell Intravasation

To investigate the importance of VEGFA in TMEM function, we blocked VEGFA binding to VEGFRs using a neutralizing antibody (B20-4.1.1), and found a decrease in extravascular dextran and circulating tumor cells (Fig. 6A–C). Binding of VEGFA to VEGFR2 leads to junction disassembly (23). Vascular ZO-1 and VE-Cadherin immunostaining increased during VEGFA inhibition, suggesting an increase in integrity of endothelial adherens and tight junctions from reduced bioavailability of VEGFA, including VEGFA from the TMEM (Fig. 6A, D, and E).

VEGFA Signaling from TIE2hi/VEGFAhi TMEM Macrophages Mediates Vascular Permeability and Tumor Cell Intravasation

To determine if the subpopulation of TIE2hi/VEGFAhi macrophages in the TMEM is an essential source of VEGFA in the tumor microenvironment required for transient vascular permeability and tumor cell intravasation, VEGFA was selectively ablated in monocytes and macrophages using the Vegfaflox/fl ox/Csf1r-Mer-Cre-Mer transgenic mouse deletion model of VEGFA that targets myeloid cells expressing CSF1R, including both Ly6Chi and Ly6Clo populations, and the TEM population (24). Macrophage-specific depletion of VEGFA reduced transient vascular permeability and circulating tumor cells and restored vascular junctions (Fig. 6F–J). Immunofluorescence of sequential sections demonstrates that blood vessels adjacent to CD68/TIE2hi/VEGFAhi TMEM macrophage have significantly reduced vascular VE-Cadherin/CD31 relative intensity.
compared with regions of vasculature away from TMEM sites in Vegfa<sup>fl ox</sup> tumors (Fig. 7A and B). Further, when VEGFA has been ablated in Vegfa<sup>fl ox-<i>Csf1r-Cre</i></sup> tumors, VE-Cadherin/CD31 relative staining intensity is the same along the tumor vasculature as in regions away from the TMEM (Fig. 7C and D). Therefore, vascular junction integrity, as measured by VE-Cadherin/CD31 relative staining intensity, is significantly reduced only in regions of vasculature adjacent to VEGFA<sup>hi</sup> TMEM macrophages in the TMEM (Fig. 7E). Further, pericyte coverage of the vasculature is reduced in regions of VEGFA<sup>hi</sup> TEMs in the TMEM as compared with regions away from VEGFA<sup>hi</sup> TMEM structures (Supplementary Fig. S6F–S6H). A decrease in pericyte coverage of vasculature has been correlated with increased metastasis and vascular permeability (25).

To establish the relevance of TIE2<sup>hi</sup>/VEGFA<sup>hi</sup> macrophages in TMEM structures in mediating vascular permeability and tumor cell dissemination in metastatic breast cancer, vascular junction staining was measured in human breast cancer patient samples. Staining of sequential sections demonstrates that blood vessels adjacent to TIE2<sup>hi</sup>/VEGFA<sup>hi</sup> macrophages in the TMEM have significantly reduced vascular VE-Cadherin fluorescence intensity compared with regions of vasculature away from the TMEM (Fig. 7F and G and Supplementary Fig. S7).

Together, these data establish that the TIE2<sup>hi</sup>/VEGFA<sup>hi</sup> TMEM macrophages interact with endothelial cells through VEGFA signaling to mediate local, transient blood vessel hyperpermeability, demonstrating the mechanism underlying the clinically demonstrated association of TMEM density with metastatic recurrence of breast cancer.

**DISCUSSION**

Although the abnormality and permeability of tumor vasculature have been well characterized, the mechanism leading to spatial and temporal heterogeneity in permeability has not been resolved. The use of high-resolution multiphoton microscopy has allowed for the study of vascular permeability and tumor cell dissemination in mammary carcinoma at unprecedented spatial and temporal resolution. Our data show that in the PyMT autochthonous mouse mammary carcinoma and human patient-derived xenograft TN1 models, vascular permeability is dynamic, localized, and restricted to the TMEM. These data are consistent with previous findings that hyperpermeability of tumor vasculature is heterogeneous and often in the presence of perivascular macrophages (4), but further explain the observed heterogeneity and that tumor cell intravasation occurs at sites of vascular permeability.
The sites of dynamic tumor vascular permeability have been identified at sites of VEGFA<sup>hi</sup> perivascular macrophages at the TMEM. The clinical significance of TMEM density in predicting metastatic risk has been recently expanded to a large cohort of patients, further emphasizing the importance of the TMEM in breast cancer metastasis (11). These data demonstrate that TIE2<sup>hi</sup>/VEGFA<sup>hi</sup> perivascular macrophages in TMEM share the characteristics of the proangiogenic and prometastatic TEMs (7). Thus, we have been able to expand our understanding of the function of this subset of TAMs in the tumor microenvironment in promoting metastasis.

Mechanistically, macrophage/tumor cell streams migrate to TMEM sites through the EGFR/CSF1R paracrine loop (26). Elevated expression of VEGFA in the TIE2<sup>hi</sup> TMEM macrophage results in transient permeability of tumor blood vessels proximal to the TMEM that occurs by disassembling endothelial cell junctions. The simultaneous attraction of migratory tumor cells and transient blood vessel permeability results in a concurrent spike in tumor cell intravasation with vascular permeability at TMEM sites (Fig. 7H). These data, together with the clinical association of the TMEM with distant metastatic tumor recurrence in human breast cancer patients, explain why TMEM density can predict metastasis and argue for the development of therapeutic approaches targeted against both TMEM formation and function.

**METHODS**

**Mice**

All studies involving mice were carried out in accordance with the NIH regulation concerning the care and use of experimental animals and approved by the Albert Einstein College of Medicine Animal Care and Use Committee. PyMT (MMTV-PyMT) transgenic mice were bred in house. MAFIA mice [C57BL/6-Tg(CSF1R-EGFP-FKBP1A/TNFRSF6)2Bck/J] were obtained from The Jackson Laboratory and were implanted with tumor pieces (2 mm<sup>2</sup>× 2 mm) into the fourth mammary fat pad on the left side. For multiphoton microscopy, transgenic mice were generated to label the myeloid lineage and mammary tumor cells by crossing MacBlue mice [Csf1r–GAL4-VP16/UAS-enhanced cyan fluorescent protein (ECFP); ref. 27] in a C57BL/6 background with Tg(MMTV-iCre)-Tg(loxP-stop-loxP-PDendra2)jwp (28) mice of FVB background.
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Inhibition of VEGFA or macrophage-specific ablation of Vegfa from TIE2+/VEGFA+ TMEM macrophages reduces vascular permeability and tumor cell intravasation. A, immunofluorescence imaging of tumor sections after blocking VEGFA with anti-VEGFA blocking antibody (B20-4.1.1). Tumors are stained for vasculature (CD31; green), 155-kDa dextran–TMR (red) and DAPI (blue), ZO-1 (magenta), or VE-Cadherin (magenta) as indicated, demonstrating changes in vascular permeability by extravascular dextran and vascular junction staining. Scale bar, 50 μm. B, quantification of extravascular 155-kDa dextran–TMR from A (n = 10, ***P = 0.0015). C, circulating tumor cells (*, P = 0.0497). D, vascular ZO-1 from A (***, P = 0.005), and E, vascular VE-Cadherin from A (*, P = 0.0465). F, immunofluorescence of tumor sections stained for vasculature (CD31; green), 155-kDa dextran–TMR (red) and DAPI (blue), ZO-1 (magenta), or VE-Cadherin (magenta) as indicated, demonstrating changes in vascular permeability after ablation of Vegfa by extravascular dextran and vascular junction staining. Scale bar, 50 μm. G, quantification of extravascular 155-kDa dextran–TMR from F and (Vegfafl ox ; Vegfafl ox ; Csf1r-Cre)1jwp from A (n = 3, ***P = 0.0029). H, circulating tumor cells (*, P = 0.0177); I, vascular ZO-1 from F (**, P = 0.0054); and J, vascular VE-Cadherin from F (*, P = 0.0457).

Animals were administered 5 mg/kg B20-4.1.1 VEGFA-neutralizing antibody (Genentech) or antibody isotype control by intravenous injection 6 hours before termination of the experiment.

Macrophage Depletion with B/B Homodimerizer in MAFIA Mice

Animals were administered 10 mg/kg B/B homodimerizer (AP20187; Clontech) diluted in 4% ethanol, 10% PEG-400, and 1.7% Tween-20 or vehicle control by intraperitoneal injection daily for 5 days. Twenty-four hours after the last injection of B/B homodimerizer, animals were administered 3 mg of 155-kDa dextran–TMR for 1 hour and 100 μL of anti-CD31 antibody for 10 minutes.

Labeling of Tumor Vasculature and Extravasation of 155-kDa Dextran–TMR

One hour before the termination of the experiments with inhibitors in MAFIA mice, 3 mg of 155-kDa TMR–dextran was administered by i.v. tail vein to label sites of vascular permeability. In tumor tissue, several transient permeability events may occur at any given time due to the spatial and temporal heterogeneity of vascular permeability, thus quantitation of extravascular dextran over the course of

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Animals were administered 5 mg/kg B20-4.1.1 VEGFA-neutralizing antibody (Genentech) or antibody isotype control by intravenous injection 6 hours before termination of the experiment.
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Figure 7. Macrophage-specific ablation of Vegfa in PyMT implant tumors blocks blood vessel permeability and tumor cell intravasation at the TMEM. A and C, immunofluorescence staining for the presence of vascular junction proteins at TMEM macrophages. Tumor sections are stained for VE-Cadherin (red), CD31 (green), and VEGFA (gray). Sequential sections are stained for CD31 (green), TIE2 (red), and CD68 (gray). A, control tumors (Vegfa<sup>flox</sup>; Csf1r-Cre<sup>+</sup>), or C after ablation of Vegfa (Vegfa<sup>flox</sup>; Csf1r-Cre<sup>−</sup>). CD68<sup>+</sup> macrophage in the TMEM outlined in white box, and adjacent endothelium in the TMEM in pink box. Yellow indicates merged signal of CD31 and TIE2 (left) or CD31 and VE-Cadherin (right). Decreased VE-Cadherin at the TMEM (F, right) seen as decreased VE-Cadherin, resulting in green (CD31) at vascular junction. Scale bar, 15 μm. B and D, quantification of the relative intensity of VEGFA or vascular junction proteins (ratio of VE-cadherin to CD31 in blood vessels) in A and C at TMEM or away from the TMEM in (B) control tumors (Vegfa<sup>flox</sup>) or (D) after ablation of Vegfa in Vegfa<sup>flox</sup>; Csf1r-Cre tumors along at TMEM. B and D, quantification of the relative intensity of VEGFA or vascular junction proteins (ratio of VE-cadherin to CD31 in blood vessels) in A and C at TMEM or away from the TMEM in (B) control tumors (Vegfa<sup>flox</sup>) or (D) after ablation of Vegfa in Vegfa<sup>flox</sup>; Csf1r-Cre tumors along 25 μm lengths of blood vessel (n = 3). •, relative fluorescence intensity of VE-Cadherin/CD31; ▲, relative VEGFA intensity. Red dashed line, presence of a CD68<sup>+</sup> macrophage. E, quantification of average pixel intensity of VE-Cadherin/CDS1 immuno-fluorescence staining in 25-μm lengths of blood vessel at TMEM or away from the TMEM in the presence of VEGFA<sup>−</sup> macrophages (Vegfa<sup>flox</sup>−, n = 3) or after macrophage-specific ablation of Vegfa (Vegfa<sup>flox</sup>; Csf1r-Cre<sup>−</sup>) from data in B and D. Asterisks indicate post-ANOVA comparisons with significant difference. F, Human breast cancer tumor sections stained for the presence of vascular junction proteins at TMEM macrophages. Tumor sections are stained for TMEM, MENA (red), CD68 (brown), and CD31 (blue). RBC in aqua by IHC. Sequential sections are stained for CD31 (green), TIE2 (gray), and VEGFA (red) by immunofluorescence. TMEM outlined in black box in IHC and white box in immunofluorescence. Scale bar, 15 μm. G, quantification of normalized average pixel intensity of VE-Cadherin staining in vasculature at TIE2<sup>+</sup>/VEGFA<sup>−</sup> macrophages of the TMEM or away from the TMEM (n = 23 at the TMEM, n = 24 away from the TMEM in 5 individual patient samples; ***, P = 0.0001). H, diagram summarizing TMEM macrophage-mediated induction of blood vessel permeability promotes tumor cell intravasation. TMEM assembly with close association between the normoglycemic TMEM tumor cell (TC, green, T1) and TIE2<sup>+</sup>/VEGFA<sup>−</sup> macrophage (blue, M1) on blood vessels. VEGFA destabilizes vascular junctions, resulting in vascular permeability and tumor cell (T2) intravasation.

1 hour will capture these dynamic events. Anti-mouse CD31-biotin was administered by i.v. tail vein for 10 minutes to label flowing blood vessels. Tumors were fixed in 4% paraformaldehyde and cryo-protected in 30% sucrose in PBS before freezing in optimal cutting temperature. Sections (5 μm) of tumors were cut, and immunofluorescence was performed.

CTCs for PyMT

CTCs were isolated from anesthetized mice from blood drawn from the right ventricle of the heart. Blood burden experiments obtained by cardiac puncture were used for endpoint experiments to capture all of the CTCs in the animal blood at the experiment endpoint. Red blood cells were lysed using RBC lysis buffer (multispecies;
were cut into 5-μm sections, deparaffinized, and stained for H&E or
ribonucleic acid (H&E) or TMEM IHC
Mouse tumor sections were fixed overnight in 10% neutral buffered saline prior to embedding in paraffin. Tumor sections (5 μm) were deparaffinized and stained for hematoxylin and eosin (H&E) or TMEM using anti–IBA-1 (macrophages), anti-endomucin (blood vessels), and anti-MENA (tumor cells; ref. 36) and the TMEM was quantified as previously described (8).

Human IHC and Immunofluorescence
Formalin-fixed paraffin-embedded patient tissue from 5 invasive ductal carcinomas was collected under the Montefiore-Einstein Institutional Review Board approval. Paraffin-embedded human breast cancer tumors were cut into 5-μm sections, deparaffinized, and stained for H&E or TMEM. The sequence was anti-CD31 (clone JC70A; DAKO) and Vector Blue chromogen (for endothelial cells); anti-CD68 (clone PG-M1; DAKO) with and J3’3-diaminobenzidine (DAB) chromogen (for macrophages); and anti-pan-MENA with Fast Red chromogen (for carcinoma cells; refs. 8, 11). Sequential sections were cut for tyramide signal amplification (TSA) for quantitative immunofluorescence using the Opal 3-plex kit (Perkin Elmer) according to the manufacturer’s directions. The sequence was anti-VEGF (1:2,000; Rb 9031-P0-A; Thermo), rat anti-F4/80 (1:50, clone BM8; eBioscience), goat anti–VE-Cadherin (1:200, clone C-19), rabbit anti-VEGFA (1:200, clone A-20; Santa Cruz Biotechnology), hamster anti-CD11c (1:100, clone HL-3; BD Bioscience), rat anti-CD11b (1:2,000, clone iCSP; BD Biosystems), rat anti-F4/80 (1:150, clone BM8; ebioscience), goat anti–MBC1/CD206 (1,200, R&D Systems), rat anti–TIE2 (1:50, clone TEK4; ebioscience), and rabbit anti–NG2 (Millipore). Sections were washed with PBS, and the primary antibodies were detected with AlexaFluor488, 555, or 647 secondary antibody conjugates (Molecular Probes/Invitrogen) and nuclei stained with 4,6-diamidino-2-phenylindole (DAPI). All fluorescently labeled samples were mounted with Prolong Gold antifade reagent (Molecular Probes/Invitrogen) and analyzed with a compound fluorescent microscope (Zeiss Axio Observer; 40x objective with numerical aperture 1.3). Images of tumor sections were acquired using mosaic tiling of 40x images in AxioRel version 4.8. All images were acquired as 16-bit TIFF images, and all quantitative analyses were performed on the raw 16-bit TIFF images in ImageJ.

TMEM IHC
Mouse tumor sections were fixed overnight in 10% neutral buffered saline prior to embedding in paraffin. Tumor sections (5 μm) were deparaffinized and stained for hematoxylin and eosin (H&E) or TMEM using anti–IBA-1 (macrophages), anti-endomucin (blood vessels), and anti-MENA (tumor cells; ref. 36) and the TMEM was quantified as previously described (8).

Immunofluorescence
Tumor sections were fixed and permeabilized with cold acetone, washed with PBS, and blocked with block solution (1% BSA, 10% FBS, and 0.0025% fish skin gelatin in PBS/Tween). The following primary antibodies were used: rat anti-mouse CD68 (1:200, clone FA-11; Sero- tec), AlexaFluor647-conjugated CD68 (1:100, ebioscience), mouse anti-MENA (1:100, from F. Gertler), rat anti-ZO-1 (1:100, clone R40.76; Millipore), goat anti–VE-Cadherin (1:200, clone C-19), rabbit anti-VEGFA (1:200, clone A-20; Santa Cruz Biotechnology), hamster anti-CD11c (1:100, clone HL-3; BD Bioscience), rat anti-CD11b (1:2,000, clone iCSP; BD Biosystems), rat anti-F4/80 (1:150, clone BM8; ebioscience), goat anti–MBC1/CD206 (1,200, R&D Systems), rat anti–TIE2 (1:50, clone TEK4; ebioscience), and rabbit anti–NG2 (Millipore). Sections were washed with PBS, and the primary antibodies were detected with AlexaFluor488, 555, or 647 secondary antibody conjugates (Molecular Probes/Invitrogen) and nuclei stained with 4,6-diamidino-2-phenylindole (DAPI). All fluorescently labeled samples were mounted with Prolong Gold antifade reagent (Molecular Probes/Invitrogen) and analyzed with a compound fluorescent microscope (Zeiss Axio Observer; 40x objective with numerical aperture 1.3). Images of tumor sections were acquired using mosaic tiling of 40x images in AxioRel version 4.8. All images were acquired as 16-bit TIFF images, and all quantitative analyses were performed on the raw 16-bit TIFF images in ImageJ.

Exponentially Modified Gaussian Function Fitting
Vascular leakage is composed of two competing processes: an increase in extravascular signal due to leakage from the vasculature and a diffusive clearance. The Exponentially Modified Gaussian asymmetric function (37) is composed of the product of a sigmoidal error function with an exponential decay. Least squares curve fitting was performed utilizing a nonlinear Generalized Reduced Gradient (GRG2) solver and values were directly compared (38).

Statistical Analysis
Individual animals are presented as individual points, a horizontal line indicates the mean, and error bars represent the SEM. One-way or two-way ANOVA analysis was performed for data sets with more than two groups to determine significance. Statistical significance was determined by the comparison of the means of two groups using an unpaired, two-sided t test using Prism (Graph Pad Inc.). Data sets were checked for normality (D’Agostino & Pearson omnibus normality test or Shapiro–Wilk normality test) and unequal variance using Prism (Graph Pad Inc.). Welch’s correction was applied to t tests as needed. P-values of less than 0.05 were deemed significant.

Disclosure of Potential Conflicts of Interest
A.S. Harney reports receiving commercial research support from Deciphera Pharmaceuticals, LLC. J.G. Jones has ownership interest (including patents) in MetaStat and is a consultant/advisory board member for the same. J.S. Condeelis reports receiving a commercial research grant from Deciphera; has ownership interest (including patents) in MetaStat; and is a consultant/advisory board member for Deciphera and MetaStat. No potential conflicts of interest were disclosed by the other authors.

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