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Host endothelial S1PR₁ regulation of vascular permeability modulates tumor growth

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Sarkisyan G, Gay LJ, Nguyen N, Felding BH, Rosen H. Host endothelial S1PR₁ regulation of vascular permeability modulates tumor growth. Am J Physiol Cell Physiol 307: C14–C24, 2014. First published April 16, 2014; doi:10.1152/ajpcell.00043.2014.—Understanding vascular growth and maturation in developing tumors has important implications for tumor progression, spread, and ultimately host survival. Modulating the signaling of endothelial G protein-coupled receptors (GPCRs) in blood and lymphatic vessels can enhance or limit tumor progression. Sphingosine 1-phosphate receptor 1 (S1PR₁) is a GPCR for circulating lysophospholipid S1P that is highly expressed in blood and lymphatic vessels. Using the S1PR₁-enhanced green fluorescent protein (eGFP) mouse model in combination with intravital imaging and pharmacologic modulation of S1PR₁ signaling, we show that boundary conditions of high and low S1PR₁ signaling retard tumor progression by enhancing or destabilizing neovascularization integrity, respectively. In contrast, midrange S1PR₁ signaling, achieved by receptor antagonist titration, promotes abundant growth of small, organized vessels and thereby enhances tumor progression. Furthermore, in vivo S1PR₁ antagonism supports lung colonization by circulating tumor cells. Regulation of endothelial S1PR₁ dynamically controls vascular integrity and maturation and thus modulates angiogenesis, tumor growth, and hematogenous metastasis.

breast cancer; PyMT; angiogenesis; metastasis; vasculature; S1PR₁

GROWTH AND PROGRESSION OF solid tumors require angiogenesis: delivery of blood-derived nutrients and oxygen as well as removal of waste products by forming an active host-tumor interface characterized by new blood and lymphatic vessel ingrowth, maturation, and organization (1). Angiogenesis is well recognized as a developmental hallmark of solid tumor growth and metastatic progression (11, 12, 15, 16, 20, 21, 42–44).

The process of angiogenesis is complex but generally involves pericyte detachment; increased vascular permeability; extravasation of plasma proteins, such as fibronectin, which set the scaffold for further angiogenesis (33) followed by vessel maturation; recruitment of pericytes; and finally, tightening of vessel walls. Currently, antiangiogenic therapies are used for cancer treatment and affect cancer progression with variable outcomes (7, 10, 17, 25). These therapies target several proangiogenic signaling molecules but predominantly function by blocking signaling of a prominent initiator of tumor angiogenesis, vascular endothelial growth factor (VEGF). Variation in the efficacy of antiangiogenic therapies in part arises from the highly heterogeneous nature of tumors with areas of increased vascular permeability and areas with well-established and intact vascular networks (2—4, 26, 28, 32, 37, 38, 46). In addition to VEGF, several other proangiogenic signaling molecules, including platelet-derived growth factor B (PDGF-B), angiopoietin-1 (Ang-1), transforming growth factor-β (TGF-β), and sphingosine-1-phosphate (SIP), control vascular growth (7, 24, 27). S1P, in particular, exerts its angiogenic effects through S1PR₁, S1PR₂, and S1PR₃ and the balanced signaling among these receptors (8, 23, 30, 31, 44, 47). Among these, S1PR₁ is abundantly expressed by both blood and lymphatic endothelia and plays an important role in vasculogenesis (34). A possible role of S1PR₁ in tumor angiogenesis was suggested by distinct cross talk between VEGF signaling and induction of S1PR₁ expression on endothelial cells (22).

Here we show in a polyoma middle T-antigen (PyMT) model of breast cancer that tumor growth exhibits a bell-shaped dependence on S1PR₁ signaling. The tumor growth rate can be downregulated by strongly enhancing or reducing S1PR₁ signaling, which stabilizes or significantly destabilizes tumor blood vessels, respectively. In contrast, midrange modulation of S1PR₁ signaling enhances tumor growth by promoting development of microvessels in the tumor. These findings establish the dynamic range by which the S1PR₁ signaling efficacy controls angiogenesis and regulates tumor growth.

MATERIALS AND METHODS

All animal work complied with National Institutes of Health and institutional guidelines (The Scripps Research Institute is Association for Assessment and Accreditation of Laboratory Animal Care International accredited) and was conducted in accordance with Institutional Animal Care and Use Committee-approved protocols. To accurately characterize the role of S1PR₁ in tumor growth and metastasis, experiments on tumor-bearing mice were performed four times with four mice per treatment group in analysis of primary tumor growth, and three times with four mice per treatment group in analysis of metastasis. All data shown represent an average ± SE of four mice. Tumor growth data were obtained by both caliper measurements and by weighing freshly resected tumors.

The intravital studies of pharmacological modulation of S1PR₁ signaling were performed in S1PR₁-enhanced green fluorescent protein (eGFP) knockin mice. This knockin mouse strain was chosen because it is well characterized and eGFP fusion S1PR₁ was shown to function as a wild-type S1PR₁ receptor (6). In addition, in intravital two-photon excitation microscopy (TPEM), the vasculature can be directly visualized due to abundant expression of S1PR₁-eGFP by endothelial cells (41).
**Effects of Endothelial S1PR<sub>1</sub> Signaling on Tumor Growth**

*Cells.* Breast cancer cells derived from PyMT-induced breast tumors (MMTV-PyMT mouse model on a C57BL background) were cultured in L-15 media (Sigma) supplemented with 10% FBS, 2 mM glutamine, and 10 μg/ml insulin, which was replenished with 80% fresh media daily. The cells were passaged at least four times before mammary fat pad implantation.

**Microdialysis pump implantation.** Sustained drug delivery for 28 days was achieved by subcutaneous implantation of microdialysis pumps (Alzet, Cupertino, CA) loaded with the S1PR<sub>1</sub>-specific agonist RP-001 (6) or antagonist W146 (40). All procedures were performed under aseptic conditions. The release rate of the 28-day microdialysis pumps is 0.1 μl/h, and steady-state plasma levels were measured by liquid-chromatography-mass spectrometry (LC-MS).

While the animals were under isoflurane anesthesia, the area between the shoulder blades was shaved and sterilized with 10% betadine solution. Microscopio pumps were carefully inserted under the skin through a small cutaneous incision and securely sutured in place. The suture was further treated with Neosporin. Postsurgery, mice were injected with 1.0 mg/kg flunixin to prevent inflammation and with 5.0 mg/kg enrofloxacin to prevent infection. Sutures were removed 10 days after surgery.

The efficiency of long-term continuous delivery of the implanted microdialysis pumps in each mouse was validated at the end of each experiment by determining drug serum levels using LC-MS as described previously (19). Mice were excluded from the study when LC-MS drug plasma levels were more than 2 SD less than the mean, which was interpreted as pump failure.

**PyMT tumor cell grafts.** Three days after micro-osmotic pump implantation, 2 × 10<sup>5</sup> PyMT breast cancer cells were injected into the fourth surgically exposed mammary fat pad of 8-wk-old female mice.

**Mouse preparation and imaging.** All intravital imaging was performed on a Leica SP5 two-photon microscope using the differential vascular labeling (DVL) method. Briefly, DVL is based on intravenous injection of a two-component cocktail. The cocktail is formulated to extravasate from the blood vessels and accumulate in the lymphatic system, functionally labeling it. The blood vessel impermeable large-molecular-weight particles (qd0705) remain within the constraint of the blood system and hence function as the blood system. The TPEM imaging was conducted on surgically exposed tumors in the inguinal area. Mice were anesthetized by a Ti:Sapphire femtosecond oscillator (Newport, Irvine, CA) was placed over a temperature-controlled microscopy stage kept at 37°C. The imaging chamber was supplied with constant superfusion of oxygenated (95% O<sub>2</sub>-5% CO<sub>2</sub>) RPMI media maintained at 36–37°C. An average tumor volume of 400 × 400 × 50 (X × Y × Z) was imaged by time-lapse microscopy at 512 × 512 pixels (8 bit per pixel) with a Z resolution of 2.5 μm. A typical single time point 3D imaging volume was 800 × 800 × 350 μm (X × Y × Z). The femtosecond laser pulse generated by a Ti:Sapphire femtosecond oscillator (Newport, Irving, CA) was directed into the SP5 scan head through an auxiliary optical input. An Olympus water immersion objective lens with 2-mm working distance and 0.95-numerical apertures was mounted onto a Physik Instrumente piezoelectric actuator for real-time, computer-controlled precision adjustments to compensate for tissue deformation and related drift, keeping the imaging window relatively constant. The backscattered fluorescence signal spectrum was subdivided into four regions with three long-pass dielectric mirrors at 560-, 593-, and 665-nm wavelengths and further filtered and detected registered by four photomultiplier tubes in nondescanned configuration. Postimaging data processing, 3D-4D rendering, and data analysis were performed using Velocity (PerkinElmer) and MatLab (Mathworks) software.

**Western blot.** After transfer, the blot was blocked for 1 h in 5% nonfat dry milk in TBS-Tween and then incubated with primary antibody in blocking solution at 4°C overnight. Primary antibodies were anti-S1PR<sub>1</sub>, H60 (Santa Cruz cat no. SC-25489) used at 1:500 dilution and anti-GFP (Abcam cat no. Ab6556) used at 1:2,000 dilution. After incubation and three washes, the blot was incubated with secondary antibody for 1 h at room temperature. Anti-S1PR<sub>1</sub> antibody H60 was detected with anti-rabbit horseradish peroxidase (HRP) conjugated at 1:10,000 dilution. Anti-GFP was detected with anti-rabbit HRP conjugate at 1:1,000 dilution. After being washed, the signal was visualized based on chemiluminescence using ECL reagent.

**DAPI staining.** Excised tumor nodules from DVL-injected mice were incubated in DAPI solution for 10–15 min followed by a wash in fresh PBS for 30 min before imaging.

**Immunohistochemistry.** Standard frozen section immunohistochemistry (IHC) was performed on 4% paraformaldehyde (PFA)-preserved tumor sections cut at 10 μm. Sections were blocked for 2 h with goat serum and then incubated with primary antibodies overnight at 4°C. The antibodies used were anti-GFP to detect S1PR<sub>1</sub> expression and CD31 anti-lymphatic vessel endothelial hyaluronan receptor 1 (anti-Lyve-1), all at 1:500 dilution. After incubation, sections were thoroughly washed with PBS and then incubated with goat-anti-rabbit Alexa Fluor 488, goat anti-mouse Lyve-1 Alexa Fluor 546, and rat anti-CD31 Alexa Fluor 594 secondary antibodies at 1:100 dilution for 2–3 h at room temperature, washed, cover slipped, and analyzed by confocal microscopy.

**Metastasis and PCR.** Twenty-one days after tail vein injection of 2 × 10<sup>5</sup> PyMT breast cancer cells, lungs were weighed and analyzed for tumor cells by quantitative Taqman PCR based on amplification of the PyMT transgene using forward primer 5′-CTGACGCGATGACAGCATA-3′ and reverse primer 5′-TCT-TGGTCGCTTCTCTGATACATA-3′ and Taqman probe 5′-[6-FAM]-CCCGGAGACCTCCCGGAGACT[tmra-Q]-3′.

**Statistical analysis.** All statistical analyses were performed using GraphPad Prism by two-tailed, unpaired Student’s t-test to compare different conditions. Primary tumor growth parameters are represented as percent variation from control. The extent of blood vessel leakage is presented as the ratio of qdot705 (qdots are semiconductor nanocrystals small enough to display quantum mechanical properties; Refs. 13, 36) signal intensity inside and outside blood vessels. Microvessel formations were calculated as ratio between fine vs. large blood vessels.

**RESULTS**

**Modulation of S1PR<sub>1</sub> receptor signaling impacts tumor growth.** To ensure proper drug delivery, the function of implanted microdialysis pumps was analyzed at the end of each experiment. Analyses showed sustained serum levels of W146 at 45.1 ± 3.59 nM in mice receiving pumps loaded with 3 mg/ml W146 (low-level treatment) or of 78.39 ± 12.08 nM when pumps were loaded with 8 mg/ml W146 (high-level treatment). Stable RP-001 serum levels of 1.42 ± 0.09 nM were found when pumps were loaded with 0.1 mg/ml RP-001. These results confirm sustained drug delivery at the desired levels.

To modulate S1PR<sub>1</sub> signaling, mice that received breast cancer cell implants into the mammary fat pad were treated with S1PR<sub>1</sub> agonists or antagonists, delivered continuously from osmotic pumps. The functional assessment of S1PR<sub>1</sub>
vascular effects was directly measured in S1PR1-eGFP knockin mice by receptor upregulation or degradation and alterations in vascular permeability. Low-dose treatment with the S1PR1-selective antagonist W146 (40) continuously delivered at 0.3 μg·μl−1·h−1 significantly augmented primary tumor growth, measured 21 days post-PyMT tumor cell allograft. Conversely, high levels of W146 continuously delivered at 0.8 μg·μl−1·h−1 moderately, but significantly, retarded primary tumor growth. Treatment with the S1PR1-selective agonist RP-001 (6) continuously delivered at 0.01 μg·μl−1·h−1 also tended to reduce tumor growth but without statistical significance (Fig. 1A). Tumor mass correlated directly with the S1PR1 agonist-antagonist balance in a bell-shaped manner with significant tumor growth retardation at supraphysiological S1PR1 signaling and in response to S1PR1 antagonism.

To determine the extent of tumor and/or host intrinsic effects of S1PR1 signaling perturbations, the expression levels of S1PR1 in tumors were analyzed by Western blot. The blot labeled with anti-S1PR1 antibody showed no tumor-derived S1PR1 in tumors. Tumor tissue only expressed an ~55-kDa

Fig. 1. Effects of sphingosine 1-phosphate receptor 1 (S1PR1) signaling modulation on tumor growth in the mammary fat pad. Data points represent average ± SE of 4 mice per group. One of 4 independent experiments is shown. Measurements were performed 2 wk after polyoma middle T-antigen (PyMT) cell implant. A: low-dose (3 mg/ml) W146 (S1PR1 antagonist) treatment significantly increases tumor growth to ~200% of the control group. High-dose (8 mg/ml) W146 treatment reduces tumor growth to 80% of the control group. *P < 0.05. Treatment with RP-001 (0.1 mg/ml; S1PR1 agonist) marginally reduces tumor growth without statistical significance. Bell-shaped dependence of tumor growth on the S1PR1 signaling rheostat is shown with decreased tumor growth at high concentrations of both agonist and antagonist, with increased growth in the midrange. B: Western blot analysis of S1PR1 and S1PR1-enhanced green fluorescent protein (eGFP) expression levels in tumor. Top: detection of S1PR1-eGFP fusion protein; neither PyMT cells alone nor wild-type mouse lungs express S1PR1-eGFP. Two bands from the tumors are seen in close proximity to each other, with the high-molecular-weight band corresponding to the expected ~55-kDa S1PR1-GFP fusion protein. Bottom: detection of S1PR1 with H60 polyclonal antibody, as a positive control; lysate from wild-type (WT) mouse lung was used to show specificity of the antibody. The direct labeling of S1PR1 with H60 polyclonal antibody detected high levels of S1PR1 in WT lungs at ~40 kDa and showed no detectable levels of S1PR1 in tumors. NSB, nonspecific band. C: microvessels that accumulate dextran from the differential vascular labeling (DVL) cocktail are lymphatic microtubular: S1PR1-eGFP (green), dextran 10K Alexa Fluor 594 (blue), qdot705 (red), DAPI (orange), and second harmonic generation (gray). Cell nuclei in an explanted tumor nodule are labeled with DAPI. C, right: high-magnification image of lymphatic microvessel invasion into developing tumor. Lateral optical cross section through single lymphatic microtubule in explanted, DAPI-stained tumor nodules shows formation of tubular lumen structures by cell membrane wrapping around. Each tubule is S1PR1-eGFP positive (green) and contains dextran (blue). The nucleus of the cells (orange) is located on the periphery of the vessel, consistent with single cell tubule formation.
S1PR$_1$-eGFP band. Lung tissue used as a positive control showed extended S1PR$_1$ labeling at $\sim$40 kDa, which was completely absent in PyMT tumor tissue (Fig. 1B). Based on these findings, we hypothesize that disruption of physiological host S1PR$_1$ signaling at high and low extremes exerts mechanistically distinct effects on vascular growth and maturation that inhibit tumor growth.

**Tumor-initiated vascular development.** To analyze vascular changes in response to S1PR$_1$ signaling pharmacological modulation, we employed the DVL method of intravital vasculature labeling (41). This method was successfully used for intravital characterization and differentiation between blood and lymphatic systems in lymph nodes (41). We imaged both lymphatic and blood vessels infiltration into the tumor nodule. Blood vessels appeared as highly branched S1PR$_1$-eGFP-positive structures. The lymphatic microvessels were imaged most often in lateral cross section; these structures readily accumulated dextran 10K Alexa Fluor 594 and were impermeable to qdot705, consistent with lymphatic system. To demonstrate complete structures of these ductal microvessels, the whole tumor preparation was stained with DAPI for visualization of individual cells forming the microvessel ducts (Fig. 1C). With the use of DVL staining, the lymphatic vessel can be visualized intravitably at high magnification (Fig. 1C, right). These features together define the fine lymphatic structures as single cell endothelial microvessels with plasma membrane expression of S1PR$_1$ with peripheral nucleus and dextran-containing lymphatic lumen.

The initial tumor development and vascularization defined two distinct stages. The first stage was characterized as avascular (Fig. 2A) in which only dextran 10K Alexa Fluor 594 component was able to permeate the tumor stroma. The second stage was characterized by development of fine lymphatic ducts alongside leaky blood vessels (Fig. 2, B and C). Within this development stage, the diffuse stromal dextran 10K Alexa Fluor 594 levels drop by accumulating within S1PR$_1$-eGFP-positive lymphatic microvessels. The ability of these ducts to exclude qdot705 defused into the tumor further stresses their lymphatic origin (41). Throughout the progression of tumor development and vascularization, functional visualization of vascular and interstitial spaces using DVL: S1PR$_1$-eGFP (green); dextran 10K Alexa Fluor 594 (blue); qdot705 (red); and collagen (second harmonic generation; gray). **Fig. 2.** A: during the early stage of development, the tumor mass is composed primarily of tumor cells without host blood or lymphatic vessels. At this stage, only low-molecular-weight dextran 10K (blue) effectively diffuses into the tumor. Preexisting blood and lymphatic S1PR$_1$-positive vessels (green) are seen in the periphery of the tumor. B: development of fine, functional primary lymphatic vessel infiltrates that accumulate dextran 10K from the surrounding tumor stroma. At this stage, microvessels already express S1PR$_1$-eGFP (see Supplemental Video S1). C: development of blood and primitive lymphatic vessels in the tumor occurs on day 6 posttumor cell implant. At this stage, blood vessels (identified by qdot705 containment, red) begin to grow into the tumor, and a functional lymphatic system begins to develop (dextran 10K containment, blue), while immature S1PR$_1$-bright endothelial structures (green) are seen that connect to neither blood nor lymph at this time, suggesting developing endothelial structures have yet to make vascular connections. D: compartmentalization of primary lymphatic vessels and formation of lymphatic sinuses. Lymphatic vessels are recognized in vivo by their exclusion of qdot705 signal and were confirmed by immunohistochemistry (IHC) based on lymphatic vessel endothelial hyaluronan receptor 1 (Lyve-1) expression (Supplemental Video S4). E: development of new lymphatic vessels into organized lymphatic structures. By day 11, blood-containing vessels (qdot705-positive) show integrity and the absence of interstitial leakage. The new lymphatics have become functionally competent, filling with dextran 10K and defining an organized lattice-work of lymph carriage. F: formation of lymphatic sinuses. This organization of the lymphatic latticework, providing contained transport of dextran 10K, is confirmed by imaging tumors at day 12 posttumor cell implantation.
angiogenesis, regardless of the state of the blood vessel development, lymphatic vessels remained largely intact and formed anatomically discrete and organized structures (Fig. 2, D–F). On the day of intravitral imaging (8–13 days post-PyMT cell implantation), tumor lesion sizes ranged from 5 to 8 mm with distinctly different consistency and appearance compared with the surrounding fat tissue (Fig. 3).

Effects of S1PR₁ signaling on tumor vessel growth and integrity. Having identified basal neovascular growth parameters and established host-derived S1PR₁ expression in tumor growth, we intravitally analyzed the impact of pharmacologic S1PR₁ perturbation on tumor vessel growth and maturation. Changes observed in tumor growth in response to S1PR₁ signaling were correlated with vascular integrity and growth changes between treatment groups (Fig. 4A). The extent of vascular leakage in tumors was assessed by the ratio of qdot705 signal inside blood vessels vs. that in surrounding stroma. This is a reliable measure of vessel integrity because qdot705 have been shown to be completely retained within intact, normal blood vessels (29, 41). In tumor-bearing mice treated with low-dose W146, a diffuse stromal qdot705 signal was similar to that seen in controls and mainly detected in the proximity of S1PR₁-eGFP-positive vessels (Fig. 4D). In contrast, tumor-bearing mice treated with high-dose W146 showed increased leakage from blood vessels and significantly higher ratios of stromal vs. blood vessel-contained qdot705 signals (Fig. 4E). Mice treated with the S1PR₁ agonist RP-001 showed reduced S1PR₁-eGFP expression in blood vessel endothelia and definitive confinement of the qdot705 signal to the blood vessel lumen, without leakage into the stroma (Fig. 4F). These results show a bell-shaped response where tumor growth is inhibited by agonism or full antagonism of S1PR₁ but by different mechanisms. The precedent for this (39) and significance of this bell-shaped S1PR₁ response are discussed below. The effect of S1PR₁ modulator treatment on blood microvessel growth within tumors was further assessed by the ratio of the number of established, qdot705-containing blood vessels vs. qdot705-containing blood microvessels in the area (Fig. 4B). This analysis revealed that treatment with low-dose W146 caused a significant increase in blood microvessel development within tumors (Fig. 4D). Assessment of blood microvessel development with RP-001 treatment was difficult because of abnormal vessel appearance, high heterogeneity in vessel diameters, reduced numbers of well-established fine vessels, and an increased number of blunt ended vessel buds (Fig. 4F).

Tumor blood vessels of animals treated with RP-001 exhibited a reduced eGFP signal, indicating a drop in S1PR₁ expression due to sustained S1PR₁ agonist exposure. Nevertheless, the presence of little diffuse stromal qdot705 signal indicated very well-defined vessel boundaries and therefore intact vascular barrier functions. To further characterize the development of blood microvessels, we performed IHC in the same tumor samples from drug-treated mice. To visualize blood vessels, the tissues were immunostained for CD31 and Lyve-1. The blood vessels were defined as CD31⁺ and Lyve-1⁻ vascular structures (Fig. 5A). The IHC further supported that modulation of S1PR₁ signaling profoundly impacts blood vascular growth and integrity.

In contrast to blood vessel development, neither W146 nor RP-001 treatment caused significant alterations in the development of lymphatic vascular structures. The finding that pharmacologic S1PR₁ receptor modulation has negligible effects on the lymphatic system in this model was demonstrated intravitally and confirmed in frozen sections of PFA-perfused tumors analyzed for Lyve-1 expression by IHC (Fig. 5B). The Lyve-1 expression in frozen tumor sections was preferentially upregulated in the rapidly growing tumor perimeter and most prominent within the imaging depth of two-photon microscopy. S1PR₁ modulator treatment (either agonist or antagonist) had a negligible effect on Lyve-1 expression levels. However, agonist treatment led to a reduction of S1PR₁-eGFP expression on all vascular surfaces (Fig. 5, B, IV, Intravital and IHC). Modulating endothelial integrity potentially has both local (primary tumor) and distant (metastasis) effects on tumor biology. The result that S1PR₁ modulation impacts vascular integrity during tumor development prompted the question of whether S1PR₁ signaling might also effect metastatic colonization of target organs by hematogenous tumor cells. To address this question directly, we followed the fate of intravenously injected PyMT breast cancer cells with S1PR₁ signaling modulation in an extravasation assay. Measurement of the PyMT tumor cell signal by quantitative PCR 21 days post-

Fig. 3. Capillary networks in the mammary fat pad compared with the developing tumor at day 11. Green: S1PR₁-eGFP; blue: dextran 10K Alexa Fluor 594; red: qdot705. A: fine capillary networks of blood (red) and lymphatic (blue) vessels in normal host breast tissue. S1PR₁ expression (eGFP, green) is seen on both vessel types. B: vascularized tumor with destabilized, chaotic vessel formation. Some vessels that are not yet integrated into the circulatory system (i) and others are constricted (ii).
tumor cell inoculation revealed that tumor cell lung colonization was significantly enhanced by high-dose W146 treatment. This observation indicates that increased vascular leakage resulting from interference with S1PR1-mediated vascular barrier functions can regulate target organ colonization by circulating tumor cells during metastasis (Fig. 6).

DISCUSSION

Tumor-induced angiogenesis and vascular remodeling are key determinants of primary tumor growth and metastatic progression. Therefore, the focus on mechanisms of tumor vascularization has led to novel therapeutic strategies to control cancer growth. However, the complexity and temporal dynamics of tumor host responses in the angiogenic process have limited the success of major therapeutic antiangiogenic approaches in breast cancer.

In this article, we show that vascular the GPCR S1PR1 is a major contributor to vascular development in breast tumor growth and that balanced signaling of this receptor is important for efficient tumor microvascularization. Importantly,
we found that endothelial S1PR1 dynamically modulates angiogenesis through vascular destabilization and maturation and thereby provides a mechanism of tumor growth control that can be pharmacologically targeted.

Specific vascular development was revealed by intravital two-photon microscopy conducted on S1PR1-eGFP mice using DVL. Throughout tumor development, tumor-associated blood vessels become large and very distinct from the capillary

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**A.**

Green: CD31
Red: Lyve-1
Blue: DAPI

**B. Intravital**
Gray: Collagen
Green: S1PR1-eGFP
Blue: Dextran 10K

**B. IHC**
Green: S1PR1-eGFP
Gray: Lyve-1

Fig. 5. A, IHC: effects of S1PR1 receptor modulation on blood (CD31 positive, Lyve-1 negative) microvessel formation in mammary fat pad tumors. Large vessels are encircled; arrows indicate blood microvessels. Assay was performed 21 days after tumor cell implant. I: blood vessel appearance in tumors from vehicle-treated mice. II: blood vessel appearance in tumors from mice treated with low-dose W146. Such treatment significantly increases blood microvessel formation compared with control (see I). III: tumor vasculature in high-dose W146-treated mice develop blood microvessels but in lesser abundance compared with the low-dose W146 treatment. IV: R-001 treatment leads to increased chaotic appearance of CD31 positive blood vessel. B, Intravital: lack of effect of S1PR1 modulation on lymphatic vessel maturation. Green: S1PR1-eGFP; blue: dextran 10K; gray: collagen (second harmonic generation). Intravital imaging of lymphatic ingrowth 12 days after tumor cell implantation. I: tumor lymphatic vessels in vehicle-treated mice. II: tumor lymphatic vessels in low-dose W146-treated mice. III: tumor lymphatic vessels in high-dose W146-treated mice. IV: tumor lymphatic vessels in RP-001-treated mice. IHC: effects of S1PR1 modulation on S1PR1 expression in the lymphatic tumor vasculature at day 12. Green, S1PR1-eGFP; gray, Lyve-1 Alexa Fluor 546 conjugated. IHC analysis of Lyve-1 and S1PR1-eGFP expression is shown. The staining shows predominant lymphatic ingrowth on the frontal, rapidly growing periphery of the tumor. Vehicle treatment (I) or sustained S1PR1 antagonism by W146 treatment (II and III) exert minimal effects on expression levels of Lyve-1 or S1PR1-eGFP. Sustained agonism by RP-001 treatment (IV) significantly reduces S1PR1-eGFP expression within tumor tissue, without noticeable effects on Lyve-1 expression.
network of the host mammary tissue. These blood vessels were highly permeable, as indicated by extravasation of qdot705 into the tumor stroma. In contrast, development of lymphatic networks exhibited different growth dynamics, in which lymphatic vessels underwent distinct stages of maturation. Lymphatic development was delineated anatomically as microvessels that are S1PR1 positive, with an eccentric nucleus and a lumen filled with dextran 10K Alexa Fluor 594. Initially, fine lymphatic microvessels infiltrated the tumor, followed by integration of these vessels into connected and organized lymphatic domains. The DVL studies clearly revealed effective functional maturation of the developing lymphatic system.

While dextran 10K Alexa Fluor 594 initially accumulated within the tumor stroma, maturation of the nascent lymphatic system led to efficient draining of the dextran 10K Alexa Fluor 594 out of the tumor. In addition, the IHC staining of PFA-fixed tumor sections stained for Lyve-1 showed significant expression of lymphatic structures (Lyve-1 positive) on the periphery of the tumors where tumor growth is rapid. Modulation of S1PR1 signaling by W146 or RP-001 did not result in significant alterations in the development of lymphatic vascular structures.

Differences in vascular endothelial permeability in blood vs. lymphatic vessels in response to S1PR1 modulation could be...
partially due to the passive nature of lymphatic flow. Nevertheless, the lymphatic endothelium remained impermeable to qdot705 with S1PR1 antagonism, indicating minimal changes in lymphatic vessel integrity, while effective drainage of dextran 10K Alexa Fluor 594 from the tumor stroma through developing lymphatic networks indicated their functional maturation. This notion was further confirmed with IHC analyses showing no differences in the Lyve-1 expression between treatment groups.

Our results show that the effects of pharmacologic S1PR1 receptor modulation are both mode and dose dependent. The S1PR1-directed treatment effects are exclusively host tissue mediated, as the tumor cells in our syngeneic tumor graft model do not express S1PR1. Surprisingly, partial antagonism of the S1PR1 receptor with the low-dose small molecule S1PR1 inhibitor W146 increased the rate of tumor growth by marginally destabilizing vessel integrity and modulating the rate of vessel maturation. In response to this treatment, increased in vessel leakage may result in deposition of plasma proteins such as thymosins, fibrin, collagen, and actin into the extravascular matrix, which are known to promote neovascularization and angiogenesis (9, 33). The pronounced increase in vessel leakage from W146 treatment is likely to replicate this mechanism (Fig. 4D and Supplemental Videos S1 and S2; Supplemental Material for this article is available online at the Am J Physiol Cell Physiol website). In conjunction with increased primary tumor growth, low-dose W146 treatment facilitated breast cancer cell colonization of the lungs from the blood stream. In contrast, high-dose antagonism of S1PR1 signaling with W146, while promoting tumor microvascularization, reduced tumor growth by disrupting vascular integrity, rendering it dysfunctional and thus unable to provide sufficient blood supply to the tumor (Fig. 4E and Supplemental Video S3). A more modest effect on tumor growth resulted from treatment with the S1PR1 agonist RP-001, which significantly stabilized neovascularity. The vasculature in tumors of RP-001-treated mice lacked organization and normal appearance (Fig. 4F and Supplemental Video S4) as a result of rapid vessel maturation and condensed angiogenic time course by the vessel-stabilizing effects of RP-001. RP-001 treatment also induced reduction of endothelial S1PR1-eGFP expression levels. Such agonist-dependent changes in endothelial expression levels of GFP-tagged S1PR1 have been previously reported (6) and were confirmed by flow cytometry and Western blot analysis (5, 6, 18).

The cell biology of S1PR1 signaling has been well characterized using the specific pharmacological tools used in the current study. At midrange receptor occupancy, the receptor has a significant caveolar reserve and thus signals without desensitization (6). Higher levels of agonist on endothelium do not fully downmodulate the receptor (as seen in the eGFP knockin mice); hence, the tonic S1PR1 signal is maintained, protecting from VEGF-mediated capillary leakage (40). This modulation of the VEGF effect slows neovascularization and tumor growth. High receptor occupancy by antagonist enhances capillary leakage (6, 40) and inhibits receptor signaling, disrupting the development of organized vasculature and thus tumor growth. Similar mechanisms have been demonstrated for poles of signaling agonism and antagonism in the inhibition of lymphocyte egress, where tonic S1PR1 signaling is required for lymphocyte movement and disruption of that signal towards either boundary is sufficient to disrupt function (39).

Conclusion. Together, our results establish a new concept that S1PR1 receptor signaling tone modulates tumor growth in the following ways: regulation of S1PR1 signaling efficiency at boundary conditions results in reduced tumor growth by destabilizing (agonism with high-dose W146 treatment) or by rapidly stabilizing (agonism with RP-001) blood vessels. Midrange antagonism (low-dose W146 treatment) of S1PR1 promotes development of microvessels in the tumor and thereby increases efficiency of nutrient delivery leading to accelerated tumor growth. Enhancement of lung colonization by blood-borne breast cancer cells in response to S1PR1 antagonism may involve enhanced plasma protein egress into the lung that supports tumor cell extravasation and provides a favorable environment for tumor cell growth. In addition, the S1PR1 may trigger changes in endothelial integrin expression that are involved in cancer cell arrest necessary for metastatic ingress into the target organ (14).

The mechanism of S1PR1 antagonism in enhancing metastatic lung colonization reflects vascular effects of S1PR1 activity and not immunosuppression. RP-001 is immunomodulatory and triggers lymphopenia (35, 48), but enhanced vascular integrity does not promote hematogenous metastasis. The W146 enhances vascular leakage and promotes hematogenous spread of tumor at maximal plasma concentrations of 90 nM. In contrast, peak plasma levels of ~20 μM of W146 are required for short lasting lymphopenic effects (W146 physiologic half life is ~73 min) (45). These highly significant differences in plasma concentrations are adequate for vascular leakage effects but significantly low for immunomodulatory effects.

There is a delicate relationship between the rates of tumor growth and associated angiogenesis. These rates have to be synchronous for optimal tumor development. Vessel development is slightly lagging because angiogenesis is an intrinsic response to the tumor itself. Consequently, significant drug-induced dissociation of the rate of angiogenesis from the rate of tumor growth will retard tumor growth. Tumor growth retardation in the case of increased vessel maturation (treatment with RP-001) as opposed to extreme vessel destabilization (treatment with high-dose W146) is mechanistically very different. Highly destabilized tumor vessels that do not mature at the rate of tumor-initiated angiogenesis render the tumor hypoxic and nutrient deficient due to the failure to form functional vascular network. Slight destabilization of tumor vessels increases vascularization efficiency, as observed in the case of low-dose S1PR1 antagonism. Interestingly, S1PR1 agonism causes an increased rate of angiogenic vessel maturation and shortening of the time in which the vessels become functionally sessile. This mechanism also reduces tumor growth by preventing adequate vascularization. S1PR1-dependent modulation of angiogenesis in and around tumors thus has the capacity to be advantageous to the host if properly controlled. Evidence for tumor nodule inhibition at the poles of high and low S1PR1-signaling tone can be potentially useful, whereas the midrange potentiation is a serious risk to the host. Furthermore, the rate at which circulating tumor cells may seed in a distant organ is affected by levels of S1PR1 signaling, as these affect the vascular interface. Understanding both host and tumor contributions in this process is essential to defining...
tumor subsets and timing that may provide benefit to patients through modulation of angiogenic signaling. Thus it is important to recognize that therapies directed towards appropriate patient groups at distinct phases of their tumor development may be successful when prospectively identified and well controlled.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


REFERENCES


