

Preexisting Lymphatic Endothelium but not Endothelial Progenitor Cells Are Essential for Tumor Lymphangiogenesis and Lymphatic Metastasis

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Abstract

Endothelial progenitor cells have been shown to contribute to angiogenesis in various tumor models. Here, we have studied the relative contributions of bone marrow (BM)-derived endothelial progenitors and pre-existing lymphatic vessels to tumor lymphangiogenesis. We did not find significant incorporation of genetically marked BM-derived cells in lymphatic vessels during tumor- or vascular endothelial growth factor C-induced lymphangiogenesis. The degree of tumor lymphangiogenesis correlated with lymphatic vessel density in the peritumoral area, and despite tumor lymphangiogenesis, lymphatic metastasis failed to occur in gene-targeted vascular endothelial growth factor C^{+/-} mice that have hypoplasia of the lymphatic network. Our data demonstrate that during tumor lymphangiogenesis and cancer cell dissemination via the lymphatics, the newly formed lymphatic vessels sprout from the pre-existing local lymphatic network with little if any incorporation of BM-derived endothelial progenitor cells.

Introduction

Cancer cell spread to regional lymph nodes is an early event in the progression of many solid tumors, and the lymphatic vasculature serves as the primary route for metastatic spread (1). Intensive research during the last few years has provided a better understanding of the molecular mechanisms underlying the development and maintenance of lymphatic vessels, and the role of lymphangiogenesis in health and disease (1–3). Two lymphangiogenic growth factors named vascular endothelial growth factor C (VEGF-C) and VEGF-D that signal through VEGF receptor 3 (VEGFR-3) have been discovered, and at least VEGF-C has been shown to be essential for the development of lymphatic vessels in embryos (4). Peritumoral and occasionally intratumoral lymphatic vessels have been detected in some human cancers and in various tumor xenografts in mice, and VEGF-C and VEGF-D expression in tumor tissue has been shown to correlate with lymphatic metastasis (5–10). Furthermore, inhibition of VEGF-C and VEGF-D binding to VEGFR-3 suppressed lymphatic metastasis (6, 8). However, many questions remain to be addressed about the mechanisms of lymphatic metastasis. For example, can tumor-associated lymphatic vessels be generated from bone marrow (BM)-derived cells as has been shown for tumor blood vessels (11)? Lymphatic endothelial progenitor cells occur in avian embryos, and putative VEGFR-3⁺

CD34⁺ endothelial progenitor cells have also recently been identified in human fetal liver and cord blood (12, 13), although a commonly held view is that at least the first lymphatic vessels are formed by sprouting from embryonic veins (4, 14, 15). If circulating lymphatic endothelial progenitor cells are present in adults, they could be involved in tumor-associated lymphangiogenesis. This study shows that tumor-associated lymphatics are formed from the pre-existing lymphatic network, and BM-derived endothelial progenitor cells do not appear to contribute to tumor lymphangiogenesis.

Materials and Methods

Cell Lines. The murine Lewis lung carcinoma (LLC) and B16-F1 melanoma cell lines were obtained from American Type Culture Collection.³ Both cell lines were maintained in DMEM supplemented with 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml), and 10% fetal bovine serum (Autogen Bioclear).

BM Transplantation. The Provincial State Office of Southern Finland approved all animal experiments, which were performed in accordance with institutional guidelines. Chimeric mice reconstituted with enhanced green fluorescent protein (GFP)-positive BM cells were created to study the behavior of BM cells *in vivo* (11). In brief, BM cells were collected by flushing femurs of C57BL/6-TgN(ACTbEGFP)10sb mice (16) obtained from The Jackson Laboratory. Unselected BM cells (2 × 10⁶) from GFP-transgenic mice were transplanted into C57BL/6JO1aHsd wild-type (WT) recipient mice via tail vein injection. The recipient mice were irradiated a day before transplantation by a sublethal dose of 4.0 Gy. Microscopic examination and flow cytometry showed that BM cells as well as peripheral blood cells were almost completely (80–95%) reconstituted with GFP⁺ cells 5 weeks after the transplantation.

Lymphangiogenesis Induced by Adeno-Associated Virus (AAV) Encoding VEGF-C. AAV encoding human VEGF-C (Ref. 17; 5 × 10⁹ viral particles) was injected into the s.c. tissue of the ears of the mice reconstituted with GFP⁺ BM cells. The ears were analyzed 5 weeks after the AAV injections.

Transgenic Models and Xenotransplantation. The K14-VEGFR-3-immunoglobulin transgenic mice and the heterozygous VEGF-C gene-targeted mice (VEGF-C^{+/-}) were generated as described previously (4, 18). Approximately 3.0 × 10⁶ LLC cells in 100 µl of PBS were implanted in the s.c. tissue of the back flank region of WT C57BL/6JO1aHsd mice (*n* = 38) and the K14-VEGFR-3-immunoglobulin transgenic (*n* = 7) as well as the VEGF-C^{+/-} gene-targeted mice (*n* = 5) of the same genetic background (9–12 weeks old). The mice were sacrificed after 3 weeks, and tissues were processed for histological analysis. In separate experiments, LLC or B16-F1 cells (1–5 × 10⁵ in 30 µl) were injected s.c. into the ears of the above mice (*n* = 12, 6, and 4 for WT, K14-VEGFR-3-immunoglobulin, and VEGF-C^{+/-} mice, respectively) and GFP⁺ BM-reconstituted mice (*n* = 4 for B16-F1 and 6 for LLC tumor). Tumor-transplanted ears were analyzed after 2 weeks.

Fluorescent Microlymphography. The functional lymphatic network surrounding the tumors in the ears was visualized by fluorescent microlymphography using dextran conjugated with FITC (*M_r* 2,000,000; Sigma) that was injected intradermally into the ears. The lymphatic vessels were then examined using a dissection microscope (Leica MZFLIII).

³ <http://www.atcc.org>.

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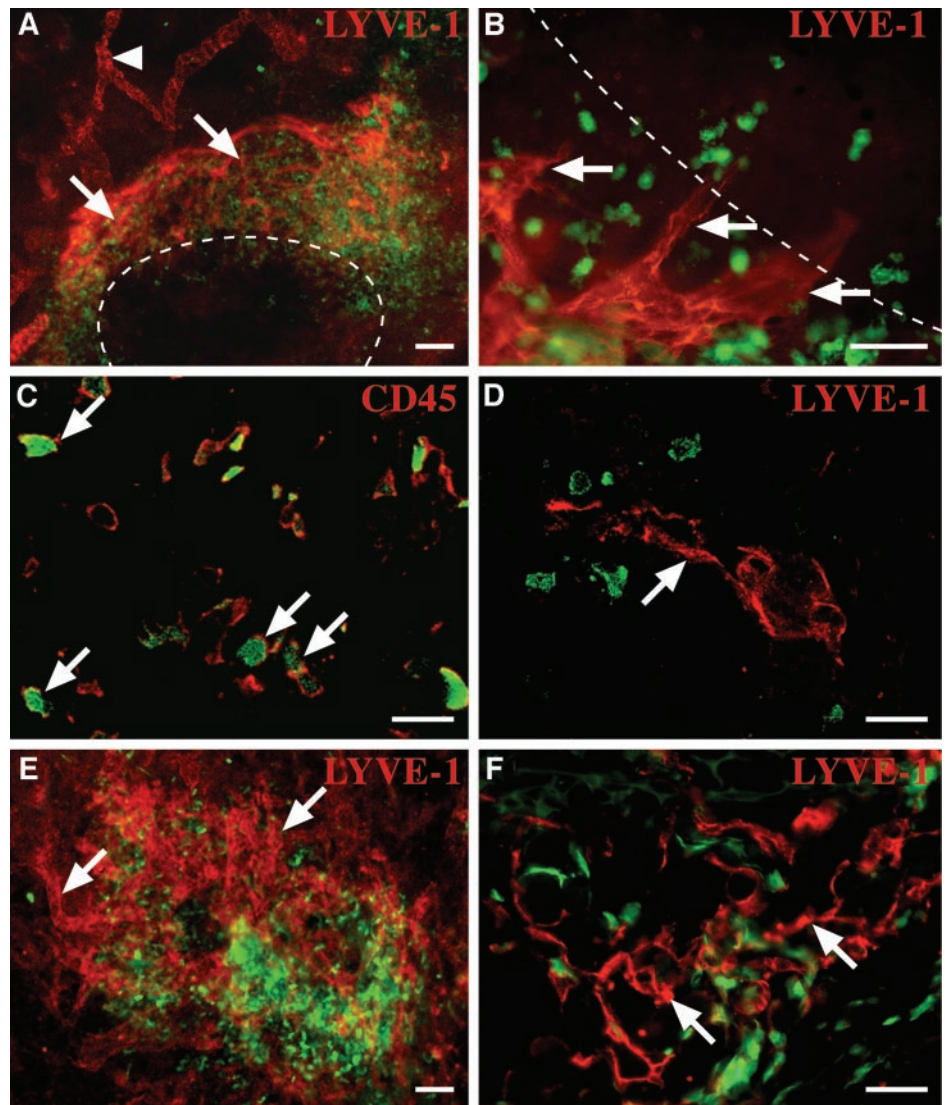
Immunohistochemistry. For whole-mount staining, tissues were fixed in 4% paraformaldehyde, blocked with 3% milk in PBS, and incubated with polyclonal antibodies against LYVE-1 (19) overnight at 4°C. Alexa 594-conjugated secondary antibodies (Molecular Probes) were used for staining, and samples were then mounted with Vectashield (Vector Laboratories) and analyzed with a Zeiss LSM510 confocal microscope. For staining of tissue sections, tissues were fixed in 4% paraformaldehyde overnight at 4°C and paraffin sections (6 μm) were immunostained with anti-LYVE-1 and monoclonal antibodies against PECAM-1 (PharMingen). For staining of cryosections, tissues were fixed in 2% paraformaldehyde for 1 h, incubated in 20% sucrose/PBS overnight and embedded in O.C.T. compound (Tissue-Tek). Sections (6 μm) were immunostained with rat antimouse antibodies against the pan-leukocyte marker CD45 (PharMingen) and anti-LYVE-1. Alexa 594-conjugated antirabbit and Alexa 647-conjugated antirat antibodies were used as secondary antibodies (Molecular Probes).

Statistics. Statistical analyses were carried out using the unpaired Student's *t* test or the Fisher's exact test. All *P* values are two-tailed.

Results and Discussion

BM-Derived Endothelial Progenitor Cells Contribute Little If Any to Tumor-Associated Lymphangiogenesis. In this study, the contribution of BM-derived cells to tumor lymphatic vessels was studied by injecting B16-F1 or LLC tumor cells s.c. into syngenic C57BL/6JO1aHsd mice that had been transplanted with a GFP⁺ BM. In immunostaining for the lymphatic endothelial marker LYVE-1, a strong lymphangiogenic effect was seen in the peritumoral area 2 weeks after the tumor cell injection (Fig. 1, *A* and *B*, arrows). In the tumor periphery, intermingled with the newly formed lymphatic vessels, we observed extensive recruitment of GFP⁺ BM cells (green cells in Fig. 1). The majority of the GFP⁺ cells expressed the CD45 pan-leukocyte marker (Fig. 1*C*, arrows). When frozen sections were studied, GFP⁺ cells could frequently be seen to be closely associated with the LYVE-1-positive lymphatic vessels (data not shown). Because it was difficult to distinguish clearly whether some of the GFP⁺ cells were endothelial or peri-endothelial cells by conventional fluorescence microscopy, the possible colocalization of GFP with LYVE-1 in the lymphatic endothelium was analyzed by confocal microscopy of whole-mount samples or frozen sections. No GFP⁺ LYVE-1⁺ lymphatic endothelial cells were detected in B16-F1 or in LLC tumors (Fig. 1*D*; data not shown), indicating that BM-derived endothelial progenitor cells did not significantly contribute to the formation of the tumor lymphatic vessels. Lymphangiogenesis was also induced by using AAV encoding human VEGF-C in mice reconstituted with GFP⁺ BM. When the tissues were analyzed 5 weeks after the AAV injection, an increased number of LYVE-1-positive lymphatic vessels could be seen in comparison with untreated mice (data not shown). Similar to the tumor-bearing mice, no GFP⁺ lymphatic endothelial cells were observed when lymphangiogenesis was induced by VEGF-C (Fig. 1, *E* and *F*).

Fig. 1. BM-derived cells are not incorporated into lymphatic vessels. *A* and *B*, whole-mount fluorescent staining of lymphatic vessels surrounding a s.c. B16-F1 tumor in ears of mice transplanted with GFP⁺ BM. Shown are whole-mount views from the s.c. side. Note that BM-derived GFP⁺ cells (green) are present in the tumor periphery but do not colocalize with lymphatic endothelial cells stained for LYVE-1 (red). Dotted lines show the tumor position, arrowhead indicates the pre-existing lymphatic network, and arrows point to the lymphatic sprouts toward the tumor. *C*, staining with the pan-leukocyte marker CD45 (red). Note that the majority of the GFP⁺ cells (arrows) are CD45⁺ hematopoietic cells. *D*, confocal microscopic analysis of LYVE-1 stained lymphatic endothelium (arrow) and GFP⁺ cells. *E* and *F*, immunostaining of lymphatic vessels (arrows) in the ears that received injections of AAV-VEGF-C. Note the presence of GFP⁺ cells around lymphatic vessels (*E*; whole mount) and the absence of GFP⁺ lymphatic endothelial cells (*F*; cryosection). Bars in *A* = 100 μm ; in *B* = 40 μm ; in *C*, *D*, and *F* = 20 μm ; and in *E* = 80 μm .



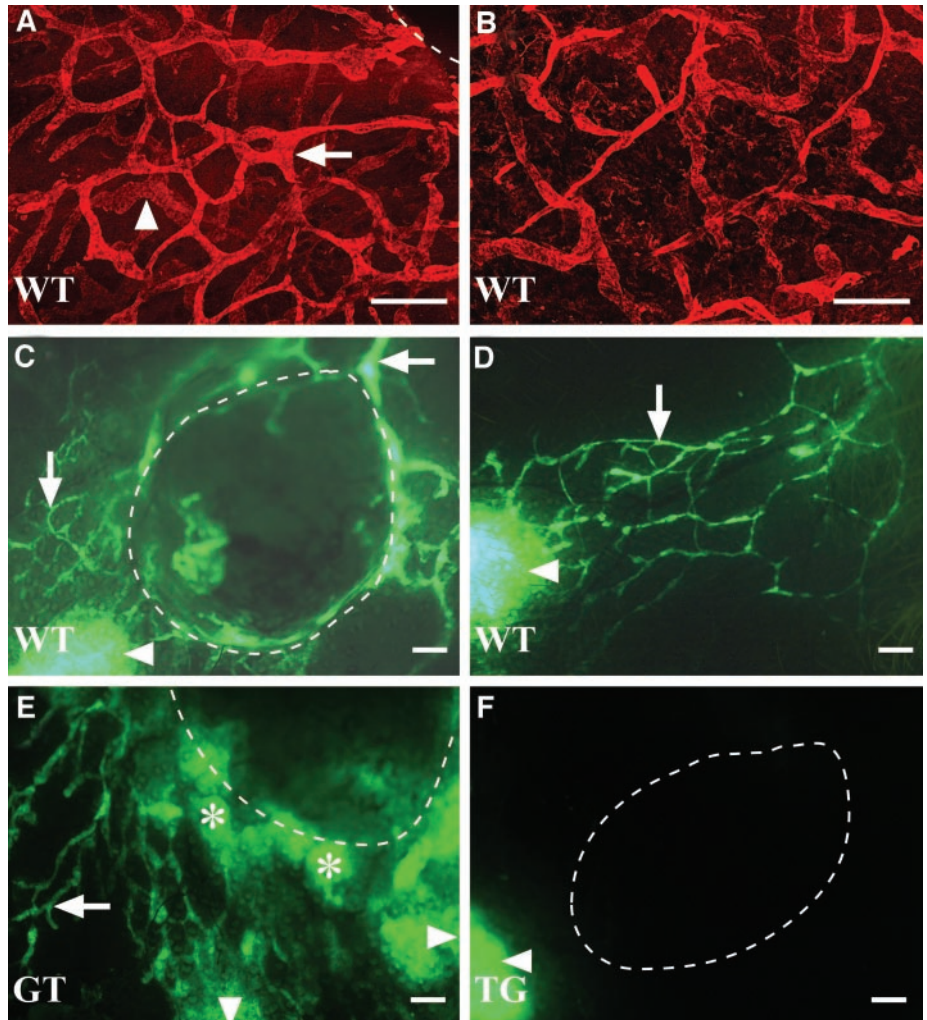


Fig. 2. Whole-mount analysis of tumor-induced lymphatic vessels. *A* and *B*, whole-mount staining for LYVE-1 in the ears of WT mice with (*A*) or without tumors (*B*). Two layers of lymphatic vessels (arrow and arrowhead) are observed in the peritumoral region (*A*). *C–F*, FITC-dextran microlymphography of the lymphatic vessels (arrows) in ears of WT mice with (*C*) or without (*D*) tumors and of the tumor-bearing VEGF-C^{+/-} gene-targeted (*GT*; *E*) and the K14-VEGFR-3-immunoglobulin transgenic mice (*TG*; *F*). *, note the leakage of FITC-dextran in *E*. Dotted lines show the tumor position, and arrowheads in *C–F* mark the injection sites of FITC-dextran. Bars in *A* = 100 μ m.

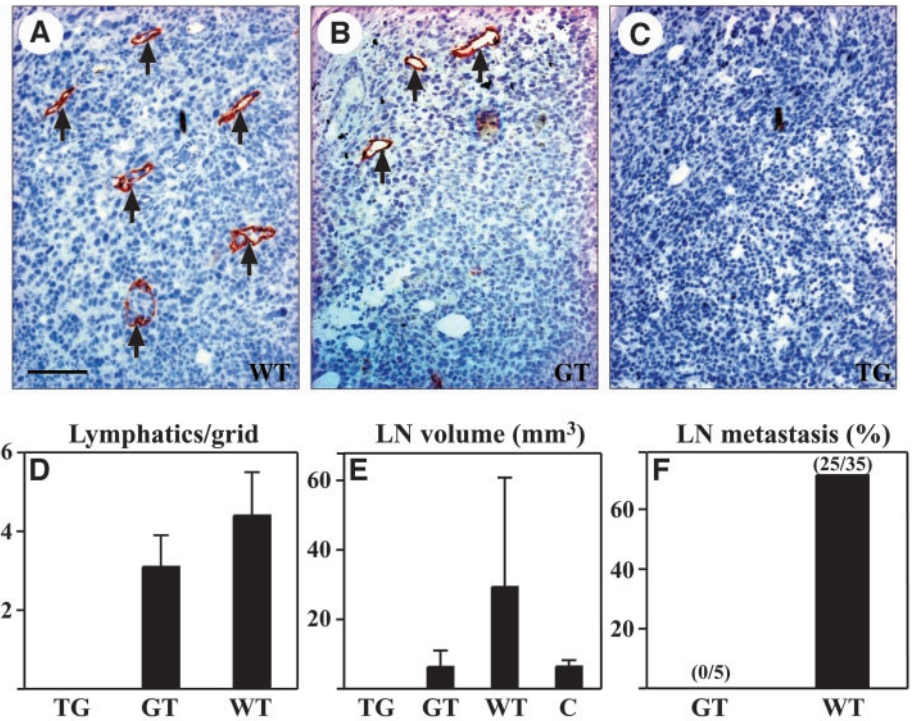
Tumor-Induced Lymphatic Vessels Originate from the Pre-Existing Lymphatic Network. For analysis of tumor-induced lymphangiogenesis, LLC tumor cells were injected s.c. into the ears of the mice. Two layers of lymphatic capillaries were seen in the periphery of the tumor in whole-mount staining of ears for LYVE-1 (Fig. 2*A*), whereas the mice without tumors had only a single lymphatic capillary layer (Fig. 2*B*). The induction of lymphangiogenesis was also evident in microlymphography of the ears with tumors (Fig. 2, *C* and *D*). The LLC tumors also induced some lymphatic vessel growth in the ears of the VEGF-C^{+/-} gene-targeted mice that have hypoplasia of the lymphatic vessels, although fewer peritumoral lymphatic vessels were seen in comparison with the WT mice (data not shown). However, upon microlymphography, the lymphatic vessels generated in the VEGF-C^{+/-} mice were leaky, and unlike in the WT mice, they were not properly connected into a lymphatic capillary network (Fig. 2*E*; data not shown). K14-VEGFR-3-immunoglobulin transgenic mice that lack lymphatic vessels in the skin failed to transport dextran (Fig. 2*F*).

In immunostainings of s.c. tumors in the WT mice, intratumoral lymphatic vessels were detected mainly in the tumor periphery (Fig. 3*A*). Similar to the observations by Padera *et al.* (9), most lymphatic vessels were within 1 mm from the tumor margin, and few grew beyond 2 mm into the tumor. Less lymphatic vessels were seen in the tumor sections of the VEGF-C^{+/-} mice (Fig. 3*B*), and none was seen in those from the K14-VEGFR-3-immunoglobulin transgenic mice (Fig. 3*C*). The average number of LYVE-1-positive intratumoral vessels as determined from three microscopic fields of the highest vessel density is shown in Fig. 3*D*

(K14-VEGFR-3-immunoglobulin, 0 vessels/grid, $n = 7$; VEGF-C^{+/-}, 3.1 ± 0.8 vessels/grid, $n = 5$; WT, 4.4 ± 1.1 vessels/grid, $n = 33$). There were significantly fewer tumor-associated lymphatic vessels in the VEGF-C^{+/-} mice than in the WT mice ($P = 0.016$). Furthermore, in the tumors grown s.c., the tumor-associated lymphatic vessels were predominantly on the epidermal side but not on the side facing the abdominal muscle layer into which the tumor cells rarely invaded. These data indicate the critical involvement of the pre-existing skin lymphatic capillary network in the growth of tumor-associated lymphatic vessels.

Lymph Node Metastasis Requires a Functional Lymphatic Network. Consistent with previous observations, the occurrence of lymph node metastasis correlated with the presence of tumor-associated lymphatic vessels (5–8, 20). In WT tumor-bearing mice, the axillary lymph node volume was 29.3 ± 31.5 mm³ ($n = 35$), which was significantly greater than in WT mice without tumors (6.4 ± 1.8 mm³, $n = 12$; $P = 0.0162$). However, the axillary lymph nodes in the tumor-bearing VEGF-C^{+/-} mice were much smaller (6.3 ± 4.7 mm³, $n = 5$) than those in the WT tumor-bearing mice (Fig. 3*E*). More than 70% of the tumor-bearing WT mice developed lymph node metastasis, but no lymph node metastasis was observed in the VEGF-C^{+/-} mice by histological analysis, although intratumoral lymphatic vessels were also detected in these mice (Fig. 3*F*). This may, at least partly, be due to the hypoplastic, poorly organized, and functionally defective lymphatic vessel system of the skin of these mice (Fig. 2*E*; data not shown). Axillary lymph nodes were not found in the K14-VEGFR-3-immunoglobulin transgenic mice, thus precluding an evaluation.

Fig. 3. Analysis of tumor-associated lymphatic vessels and lymphatic metastasis. A-C, sections of the LLC tumors from WT (A), the VEGF-C^{+/-} gene-targeted (GT; B), and the K14-VEGFR-3-immunoglobulin transgenic mice (TG; C) were stained for LYVE-1 to identify lymphatic vessels (arrows). D, the average number of LYVE-1-positive vessels was determined from three microscopic fields of the highest vessel density. E, plotting of lymph node (LN) volume \pm SD. F, average axillary lymph node volume from WT mice without tumor. F, percentage of mice with lymph node metastasis; the number of mice is shown in parentheses. Bars in A-C = 100 μ m.



In contrast to the differences in tumor-associated lymphangiogenesis, there were no significant differences in blood vessel density in tumors from the three groups of mice (data not shown). Furthermore, there was also no significant difference in tumor weight (K14-VEGFR-3-immunoglobulin, 3.9 ± 2.0 g, $n = 7$; VEGF-C^{+/-}, 5.4 ± 2.0 g, $n = 5$; WT, 4.6 ± 2.1 g, $n = 38$) or in the frequency of metastasis to the lungs (K14-VEGFR-3-immunoglobulin, 7 out of 7; VEGF-C^{+/-}, 4 out of 5; and WT, 30 out of 38 mice) among the three groups. These results are consistent with our earlier observations from another tumor model, where inhibition of tumor lymphangiogenesis by interruption of VEGFR-3 activation suppressed lymphatic but not lung metastasis (8), although this result may depend on the tumor type and anatomical site (20).

Taken together, this study indicates that BM-derived endothelial progenitor cells contribute little if any to tumor- or VEGF-C-induced lymphangiogenesis at least in a relatively short-term tumor xenograft assay in which there is no genetic or immunological selection against the pre-existing endothelial cells such as some gene targeting models (21) or organ transplants (22). Consistent with this, the generation of tumor-associated lymphatic vessels was critically dependent on pre-existing peritumoral lymphatic vessels in the host tissues. These results should have significant implications for strategies to block lymph node metastasis of human cancers.

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