PI3Kα activates integrin α4β1 to establish a metastatic niche in lymph nodes

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Lymph nodes are initial sites of tumor metastasis, yet whether the lymph node microenvironment actively promotes tumor metastasis remains unknown. We show here that VEGF-C/PI3Kα-driven remodeling of lymph nodes promotes tumor metastasis by activating integrin α4β1 on lymph node lymphatic endothelium. Activated integrin α4β1 promotes expansion of the lymphatic endothelium in lymph nodes and serves as an adhesive ligand that captures vascular cell adhesion molecule 1 (VCAM-1\textsuperscript{4}) metastatic tumor cells, thereby promoting lymph node metastasis. Experimental induction of α4β1 expression in lymph nodes is sufficient to promote tumor cell adhesion to lymphatic endothelium and lymph node metastasis in vivo, whereas genetic or pharmacological blockade of integrin α4β1 or VCAM-1 inhibits it. As lymph node metastases accurately predict poor disease outcome, and integrin α4β1 is a biomarker of lymphatic endothelium in tumor-draining lymph nodes from animals and patients, these results indicate that targeting integrin α4β1 or VCAM to inhibit the interactions of tumor cells with the lymph node microenvironment may be an effective strategy to suppress tumor metastasis.

Tumor metastases are a leading cause of cancer-related mortality and morbidity, and both tumor cell intrinsic and extrinsic factors promote metastasis (1–4). Metastatic spread occurs primarily via lymphatic and hematogenous routes, and the presence of metastases in tumor draining lymph nodes is an accurate predictor of poor outcome in many types of tumors (5, 6). To further refine therapy for cancer patients, studies that define the mechanisms that promote tumor metastasis to lymph nodes could lead to novel therapeutic regimens that could improve clinical outcomes for cancer patients.

In primary tumors, lymphangiogenesis, the growth of new lymphatic vessels, is strongly correlated with lymph node and distant metastasis. Increased expression of the lymphangiogenic factors VEGF-A, VEGF-C, or VEGF-D in tumors correlates closely with increased incidence of regional lymph node metastases in both humans and animals (7–9). Accordingly, systemic administration of antagonists of the VEGF-C receptor, VEGF-R3, blocked primary tumor lymphangiogenesis and metastasis (10–12).

VEGF-C stimulates the expression of integrin α4β1, which promotes lymphatic endothelial cell (LEC) adhesion and invasion, leading to tumor-associated lymphangiogenesis (13). VEGF-C–mediated signaling stimulates LEC invasion and survival during lymphangiogenesis, as VEGF-R3 activates PI3Kα/akt murine thymoma viral oncogene homolog 1 (Akt) and mammalian target of rapamycin (mTOR) signaling pathways (14, 15). VEGF–VEGF-R3 signaling thus plays an important role in tumor lymphangiogenesis.

Lymphangiogenesis occurs not only with primary tumors but also in tumor draining lymph nodes, where it is associated with increased tumor metastasis (16–18). However, it is unclear whether lymph node lymphangiogenesis plays an independent role in promoting tumor metastasis. Here we present the unique findings that integrin α4β1 is a biomarker of tumor-draining lymph nodes in animals and patients and that lymph node metastases depend on PI3Kα-mediated α4β1 activation in lymph node lymphatic endothelium. Once activated, α4β1 promotes lymph node lymphangiogenesis and facilitates adhesion of VCAM-1\textsuperscript{4} metastatic tumor cells within lymph nodes, thereby promoting tumor spread.

Results

Integrin α4β1 is a Biomarker of Lymphatic Endothelium in Lymph Nodes from Tumor-Bearing Hosts. Tumor-induced changes in the lymph node microenvironment may promote tumor metastasis by establishing a niche within the lymph node that is favorable for the deposition, survival, and growth of metastases. We evaluated lymph nodes of tumor-bearing animals for pathologic changes. In mice implanted s.c. with syngeneic Lewis lung carcinoma (LLC) tumor cells, draining lymph nodes increased substantially in size (SI Appendix, Fig. S1A). Immunostaining of lymph nodes to detect lymphatic vessel endothelial hyaluronan receptor 1 (Lyve-1) (19), a cell surface protein, and prospero homeo box 1 (Prox-1) (20), a transcription factor, and CD31, which forms point contacts between LECs (21), three well-established biomarkers of lymphatic endothelium, identified networks of Prox-1\textsuperscript{−}/Lyve-1\textsuperscript{−} lymphatic vessels and channels that expanded from cortex inward, beginning as early as 7 d after tumor cell implantation; these networks preceded the appearance of lymph node and lung metastases (Fig. 1 A–D and SI Appendix, Fig. S1 A–D). These changes were accompanied by increases in Lyve-1 mRNA expression before appearance of metastases in lymph nodes (SI Appendix, Fig. S1C). We also observed increases in size and Prox-1\textsuperscript{−}/Lyve-1\textsuperscript{−} lymphatic vessel density in lymph nodes of polyoma middle T (PyMT) transgenic mice with spontaneous breast tumors (22) that preceded metastasis to lymph nodes and distant sites (SI Appendix, Fig. S2 A–D). Together these results indicate that significant lymphangiogenesis in lymph node precursors precedes the appearance of metastases. Premetastatic increases in lymphangiogenesis were also detected in regional and distal lymph nodes of tumor-bearing animals (SI Appendix, Fig. S1D). Together, these studies demonstrate that lymph node lymphangiogenesis generally precedes the appearance of tumor metastases in lymph nodes and could play a role in directly promoting lymphatic as well as distant metastases.

To determine whether lymph node lymphangiogenesis plays a functional role in tumor metastasis, we evaluated the expression of key receptors that regulate the formation of lymphatic vessels in premetastatic lymph nodes. We found that integrin α4β1, a cell surface adhesion receptor for VCAM-1 and fibronectin, which promotes tumor lymphangiogenesis (13), was strongly expressed on Lyve-1\textsuperscript{−} vessels in tumor-draining, regional, and distal lymph nodes of mice with LLC and PyMT tumors (Fig. 1 E and F and SI Appendix, Figs. S3 and S4).


The authors declare no conflict of interest.

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tumors versus normal patients (Fig. 2). CD18

Additionally, Lyve-1 staining did not overlap with any immune cell populations in the lymph node, including CD11b+ myeloid cells, CD18+ leukocytes, EGF-like module containing, mucin-like, hormone receptor-like sequence 1 (Emr1) reacting with the F4/80 antibody (F4/80)+ macrophages, or B cell CD45 isofrom of 220 kDa (B220)+ B cells (SI Appendix, Fig. S3 A and B) but did overlap with another LEC marker, Prox-1 (Fig. 1 A and E). Taken together, these studies indicate that integrin α4β1 is a biomarker of proliferative lymph node lymphatic endothelium in tumor-bearing animals.

To determine whether similar changes in lymph node structure can be observed in lymph nodes from patients with breast cancer, we evaluated lymphatic vessel density in human axillary lymph nodes. (A) Lymph nodes from 0, 7, 14, and 21 d after s.c. LLC inoculation immunostained to detect Lyve-1/Prox-1 lymphatic vessels (arrowheads), counterstained with DAPI (blue). Dotted lines show lymph node edge. (B) Mean ± SEM Lyve-1+ pixels per field (n = 10, *P < 0.01, **P < 0.001). (C) Mean ± SEM Prox-1+ pixels per field (n = 10, *P < 0.01, **P < 0.001). (D) Percentage of mice with lymph node (blue) and lung (red) metastases (n = 10, *P < 0.01). (E) Integri

Fig. S3A). Although α4β1 is also expressed on lymphocytes (23, 24), we only observed increased α4β1 expression on lymph node LECs. Additionally, Lyve-1 staining did not overlap with any immune cell populations in the lymph node, including CD11b+ myeloid cells, CD18+ leukocytes, EGF-like module containing, mucin-like, hormone receptor-like sequence 1 (Emr1) reacting with the F4/80 antibody (F4/80)+ macrophages, or B cell CD45 isofrom of 220 kDa (B220)+ B cells (SI Appendix, Fig. S3 B and C) but did overlap with another LEC marker, Prox-1 (Fig. 1 A and E). Taken together, these studies indicate that integrin α4β1 is a biomarker of proliferative lymph node lymphatic endothelium in tumor-bearing animals.

To determine whether similar changes in lymph node structure can be observed in lymph nodes from patients with breast cancer, we evaluated lymphatic vessel density in human axillary lymph nodes from normal patients (n = 35), patients with breast carcinoma in situ without lymph node metastases (stages IB–IIA, n = 24), and patients with breast carcinoma with lymph node metastases (stages II A–IIIA, n = 33). Seventy-five percent of lymph nodes were from patients with ductal carcinomas, whereas the remaining nodes were from patients with lobular or mixed ductal/lobular carcinoma.

Lymph nodes were immunostained with antibodies directed against podoplanin, a well-characterized biomarker of human lymphatic vessels (25). The density of podoplanin+ lymphatic vessels increased significantly in lymph nodes from patients with breast tumors versus normal patients (Fig. 2 A and SI Appendix, Fig. S4 A and B). Overlapping expression of Lyve-1, CD31, and podoplanin was also detected in lymph node lymphatic vessels (Fig. 2 B and C and SI Appendix, Fig. S4 A and B). Lyve-1 and podoplanin were uniformly distributed on the LEC membrane, whereas CD31 expression was generally detected in point contacts between LECs (Fig. 2 C and SI Appendix, Fig. S4 A and B). In lymph nodes from patients with metastases, networks of Lyve-1+ and CD31+ vessels appeared to feed into large-diameter podoplanin+/Lyve-1+/CD31+ vessels or channels. Metastases were readily detected within the lumens of podoplanin+ lymphatic vessels in lymph nodes (SI Appendix, Fig. S4C). The numbers of podoplanin+, Lyve-1+, and CD31+ lymphatic vessels increased significantly in lymph nodes from cancer patients versus normal lymph nodes (Fig. 2 D). We observed that integrin α4β1 was poorly expressed on lymphatic vessels in normal lymph nodes but was expressed on 50% of lymphatic vessels in lymph nodes from patients with nonmetastatic breast carcinoma and on 75% of such vessels in metastasis positive lymph nodes (Fig. 2 C and E and SI Appendix, Fig. S4 D and E). These studies indicate that extensive expansion of integrin α4β1+ lymphatic endothelium characterizes lymph nodes from patients...
Vegf-C and Pi3Kα Activate Integrin α4β1 to Promote Lymph Node Lymphangiogenesis. Our studies indicate that tumors can induce widespread lymph node lymphangiogenesis that is associated with integrin α4β1 expression in draining, regional, and distant lymph nodes of animals with tumors, but it is not clear whether tumor-produced lymphangiogenic factors enter the systemic circulation or locally drain into lymph nodes to promote a systemic lymphatic response. Vegf-C and Vegf-A mRNAs are strongly expressed in LLC tumors, but not in lymph nodes of either normal or tumor-bearing animals (SI Appendix, Fig. S5A). As systemic Vegf-C has been detected in the serum of patients with tumors (26), our studies suggest that systemic Vegfs could stimulate widespread lymph node lymphangiogenesis. To test this hypothesis, we treated mice with i.v. tail vein injections of saline or Vegf-C daily for 7 d. Widespread lymph node lymphangiogenesis resulted from systemic, daily i.v. Vegf-C injections (SI Appendix, Fig. S5 B and C). In contrast, localized but not widespread, lymph node lymphangiogenesis was induced when single inguinal lymph nodes were stimulated by intradermal injections of Vegf-C proximal to the inguinal lymph node. Local stimulation promoted lymphangiogenesis in only the treated inguinal lymph node and not more distal lymph nodes such as the brachial lymph node (SI Appendix, Fig. S5 D and E). These studies indicate that lymph node lymphangiogenesis may occur through either local or systemic release of growth factors.

As angiogenesis depends on Vegf-A–mediated activation of endothelial cell migration by the phosphatidyl inositol 3,4,5 kinase PI3Kγ (29, 30) and in animals expressing Cre recombinase driven by the Tie2 endothelial-specific promoter and α4 integrin flanked by lox p sites (Tie2Creα4fl/−), which fail to express α4β1 in LECs (13) (SI Appendix, Fig. S7 B and C). We also found that Vegf-C–induced lymph node lymphangiogenesis was inhibited by antibody (SI Appendix, Fig. S7 D and E) and small molecule antagonists of α4β1 (SI Appendix, Fig. S7 F). Together, these results indicate that Vegf-C stimulates lymph node lymphangiogenesis by promoting Pi3Kα-mediated integrin α4β1 activation.

To determine whether integrin α4β1 could play a functional role in lymph node lymphangiogenesis and metastasis, we first stimulated inguinal lymph nodes of mice with local, intradermal injections of saline or Vegf-C and found that Vegf-C–treatment stimulated integrin α4β1 expression on Lyve-1+ lymph node lymphatic vessels (SI Appendix, Fig. S7A). We then found that Vegf-C stimulated lymph node lymphangiogenesis in WT mice but not in mice with an integrin α4 tyrosine to alanine (Y991A) point mutation (Fig. 4 A and B), which impair α4β1 activation (29, 30) and in animals expressing Cre recombinase driven by the Tie2 endothelial-specific promoter and integrin flanked by lox p sites (Tie2Creα4fl/−), which fail to express α4β1 in LECs (13) (SI Appendix, Fig. S7 B and C). We also found that Vegf-C–induced lymph node lymphangiogenesis was inhibited by antibody (SI Appendix, Fig. S7 D and E) and small molecule antagonists of α4β1 (SI Appendix, Fig. S7 F). Together, these results indicate that integrin α4β1 expression and activity are required to promote lymph node lymphangiogenesis.

Tumor Cell Lymphatic Endothelial Cell Adhesion Promotes Lymph Node Metastasis. To determine whether Vegf-C–mediated activation of integrin α4β1 during lymph node lymphangiogenesis plays an independent role in promoting tumor metastasis, we developed a unique model of experimental lymph node metastasis, which is similar to the tail vein injection model of lung metastasis. Inguinal lymph nodes of normal, nontumor-bearing mice were stimulated for 5 d with intradermal injections of saline or Vegf-C proximal to the inguinal lymph node to induce lymphangiogenesis. Fluorescently labeled tumor cells were then injected into the lymphatic vessel-rich footpads of stimulated mice (SI Appendix, Fig. S8A). Vegf-C treatment strongly promoted both lymphangiogenesis and tumor cell retention in inguinal nodes, whereas few tumor cells were retained in unstimulated (saline-treated) lymph nodes or regional (brachial) or distal (mesenteric) lymph nodes, which were not affected by local Vegf-C treatment of inguinal lymph nodes (Fig. 4 C and D and SI Appendix, Fig. S8 B and C). Importantly, little tumor cell arrest was observed in lymph nodes from α4Y991A animals (Fig. 4 E and F) or in animals treated with inhibitors of lymphangiogenesis, such as antagonists of Vegf-CR3 or α4β1 (SI Appendix, Fig. S8 D and E). These results demonstrate that lymph node lymphangiogenesis directly facilitates initial lymph node metastasis.

Fig. 3. Vegf-C stimulates Pi3Kα–dependent integrin α4β1 activation during lymph node lymphangiogenesis. (A) pAkt/Akt immunoblotting in Vegf-C–stimulated LECs. (B) Immunoblot of Vegf-C isoforms in cultured LECs and vascular endothelial cells. (C) pAkt/Akt immunoblotting in Vegf-C–stimulated LECs. L = 500 nM Pi3Kα2 or control. (D) Lymph node treated with saline, Vegf-C, the Pi3Kγ inhibitor (TG100–115), or the Pi3Kα inhibitor (PiK2α). (E) Lyve-1+ pixels per field in Vegf-C–treated, TG100–115, LY294002, or PiK2α treated lymph nodes from WT or mutant animals lacking Pi3Kγ (p110γ−/−) animals (n = 10, **P < 0.001). (F–H) Saline- or Vegf-C–stimulated LEC adhesion to VCAM-1. (G) Saline– or Vegf-C–stimulated LEC adhesion to VCAM-1. L = 1 μM Pi3Kα inhibitor PiK2α. (H) LEC adhesion to VCAM-1 and control and p110α (His P123CA, B) sRNA transfected cells ± Vegf-C. (Scale bars, 50 μm.)
Our studies suggest that lymph node lymphangiogenesis may promote metastasis by increasing tumor cell adhesion and arrest in the lymph node microenvironment. As we have shown that integrin α4β1 is a key lymphatic endothelial cell surface receptor, which is activated by VEGF-C and PI3Kα, we tested the possibility that this adhesion protein could capture metastatic tumor cells that pass through lymph node lymphatic vessels. Importantly, fluorescently labeled α4β1 negative LLC cells (Fig. 5A) adhered strongly to monolayers of α4β1-expressing human LECs in vitro (Fig. 5A–C); this adhesion was suppressed by an antibody antagonist specific for human α4β1 (HP2/1), but not by an isotype-matched control antibody (cIgG) (Fig. 5B and C). In addition, adhesion of tumor cells to LECs was suppressed by siRNA-mediated knockdown of α4β1 (Fig. 5D and SI Appendix, Fig. S9A). These studies indicate that integrin α4β1 can facilitate adhesion of tumor cells to lymphatic endothelium.

To determine whether α4β1 promotes the adhesion of tumor cells within lymph nodes in vivo, inguinal lymph nodes of normal mice were stimulated for 5 d with intradermal injections of VEGF-C to promote lymphangiogenesis and induce α4β1 expression. These lymph nodes were then treated with a single intradermal application of function-blocking anti-human α4β1 (HP2/1) or isotype-matched control antibodies proximal to the inguinal lymph node. Two hours later, LLC cells were injected in the footpad and after 4 h, lymph nodes were cryopreserved. Anti-human α4β1, but not control, antibodies blocked the accumulation of tumor cells in VEGF-C-stimulated lymph nodes without affecting lymphatic vessel density in lymph nodes (Fig. 5E and F and SI Appendix, Fig. S9B). These results demonstrate that LEC integrin α4β1 can serve as a receptor to capture metastatic tumor cells from the lymph and may therefore promote tumor metastasis to the lymph node.

As integrin α4β1 serves as a receptor for the Ig superfamily molecule VCAM-1, we evaluated the expression of VCAM-1 in human breast cancer metastasis positive lymph nodes by immunohistochemistry. Six of 33 metastases, or 18%, were VCAM positive at the time of lymph node excision (Fig. 6A–D). No clear correlation was observed between VCAM expression status and estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor 2 (Her2/neu) status, although all of the VCAM+ cases were stages IIB or IIIA. Importantly, VCAM was previously identified as one member of a breast cancer metastasis gene signature in mice and humans (31). Subsequent studies showed that tumor cell VCAM-1 interactions with macrophage lineage cell integrin α4β1 promote lung and bone metastasis (32). We therefore asked whether VCAM-1 could promote adhesion between metastatic murine tumor cells and lymphatic endothelium. Integrin α4β1 negative LLC and PyMT mouse tumor cells strongly expressed VCAM-1, whereas LECs expressed α4β1 but not VCAM-1 (Fig. 6B and C). LLC adhesion to human LECs in vitro was suppressed by antibody antagonists of murine VCAM-1 but not by isotype-matched control antibodies (cIgG) (Fig. 6D and SI Appendix, Fig. S10A). Adhesion of LECs to LECs was also suppressed by stable shRNA-mediated knockdown of VCAM-1 in LLC tumor cells (Fig. 6E and SI Appendix, Fig. S10B). Importantly, in vitro and in vivo LLC proliferation and apoptosis were not affected by VCAM knockdown. Additionally, R40P pancreatic tumor cell variants selected to express high levels of surface VCAM-1 adhered to LEC at a much greater rate than R40P cells selected to express low levels of VCAM-1 (SI Appendix, Fig. S10C). Taken together, these studies indicate that tumor cell VCAM-1 facilitates adhesion of tumor cells to LECs.

Fig. 4. Lymph node lymphangiogenesis promotes tumor cell adhesion in lymph nodes. (A) Lyve-1+ lymphatic vessels in saline- and VEGF-C-stimulated inguinal lymph nodes from wild-type (WT) and integrin α4Y991A mice (n = 10). (B) Mean ± SEM Lyve-1+ pixels per field from A. (C and D) Inguinal lymph nodes were stimulated for 5 d with VEGF-C or saline, and then mice were inoculated in the footpad with red fluorescent LLC cells. (C) Lyve-1+ lymphatic vessels (green) and tumor cells (red) in stimulated inguinal lymph nodes. (D) Mean ± SEM red fluorescent pixels per field in lymph nodes (n = 10). (E) Red fluorescent tumor cells (arrowheads) in VEGF-C- or saline-stimulated inguinal lymph nodes from WT or α4Y991A animals (n = 8). (F) Mean ± SEM red fluorescent pixels per field. (Scale bar, 50 μm.)

Fig. 5. LEC integrin α4β1 promotes adhesion of tumor cells to lymphatic endothelium. (A) FACs analysis of α4β1 expression in LEC and LLC cells (black); isotype control (gray). (B) Adhesion of 5-(and-6)-[(4-chloromethyl) benzoyl]amino]tetramethylrhodamine] (CMTMR)-labeled LLC cells (red) to LEC (brightfield) with and without anti-α4β1 or isotype control antibodies. (C) Mean ± SEM adherent LLC cells per field. (D) Mean ± SEM LLC cells transfected with siRNA and LLC cells were inoculated in the footpad with or without control or α4β1 siRNA adherent to LEC. (E and F) Inguinal lymph nodes of mice were stimulated 5 d with VEGF-C and treated with anti-α4β1 or control antibodies 2 h before footpad inoculation with red fluorescent LLC cells (n = 10). (E) Mean ± SEM tumor cells (pixels per field). (F) Red fluorescent tumor cells in lymph nodes.
were suppressed in (Fig. 7) of tumor cells with lymphatic endothelium. To determine whether motes spontaneous tumor cell metastasis by enabling interactions with lymphatic endothelium.

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we analyzed lymph node lymphangiogenesis and metastasis in SI Appendix in VEGF-C (9046 VCAM-1 promotes tumor cell adhesion to lymphatic endothelium. (Fig. 6. A) Mean adhesion to human LEC + SEM red fluorescent tumor cells in lymph nodes. These results indicate that VCAM-1 promoting lymph node metastasis. Integrin α4β1-mediated lymph node lymphangiogenesis plays a key role in promoting tumor metastasis to lymph nodes, serving not only as an adhesive ligand for VCAM-1 tumor cells, but also to promote expansion of the lymphatic vessel network in lymph nodes. Selective suppression of integrin α4β1 function not only blocks lymphangiogenic factors VEGF-A, VEGF-C, or VEGF-D can be detected in the serum of patients with tumors (26), our studies indicate that systemic growth factors can induce widespread lymph node lymphangiogenesis. As the lymphangiogenic factors VEGF-A, VEGF-C, or VEGF-D can be detected in the serum of patients with tumors (26), our studies indicate that systemic lymphangiogenic factors might promote widespread lymph node lymphangiogenesis and consequently, widespread dissemination of tumor metastases.

In the studies presented here, we found that integrin α4β1 mediated lymph node lymphangiogenesis plays a key role in promoting lymph node metastasis. Integrin α4β1 plays two functional roles in tumor metastasis to lymph nodes, serving not only as an adhesive ligand for VCAM-1 tumor cells, but also to promote expansion of the lymphatic vessel network in lymph nodes. Selective suppression of integrin α4β1 function not only blocks lymphangiogenic factors VEGF-A, VEGF-C, or VEGF-D can be detected in the serum of patients with tumors (26), our studies indicate that systemic growth factors can induce widespread lymph node lymphangiogenesis. As the lymphangiogenic factors VEGF-A, VEGF-C, or VEGF-D can be detected in the serum of patients with tumors (26), our studies indicate that systemic lymphangiogenic factors might promote widespread lymph node lymphangiogenesis and consequently, widespread dissemination of tumor metastases.

Discussion
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We established unique experimental approaches to separate the roles of primary tumor and lymph node lymphangiogenesis in tumor metastasis, demonstrating that systemic growth factors can induce widespread lymph node lymphangiogenesis. As the lymphangiogenic factors VEGF-A, VEGF-C, or VEGF-D can be detected in the serum of patients with tumors (26), our studies indicate that systemic lymphangiogenic factors might promote widespread lymph node lymphangiogenesis and consequently, widespread dissemination of tumor metastases.

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lymphangiogenesis in lymph nodes but also prevents the adhesion of tumor cells to lymphatic endothelium, thereby disrupting metastasis in vivo without affecting primary tumor lymphangiogenesis or growth. These studies demonstrate that selective suppression of lymphangiogenesis in lymph nodes prevents tumor metastasis without affecting the primary tumor.

The work presented here demonstrates that lymphangiogenesis in lymph nodes facilitates metastasis by promoting direct adhesion of tumor cells to LECs in lymph nodes, in part through tumor cell VCAM-1 binding to LEC integrin α4β1. Previous studies showed VCAM-1 is abnormally expressed on metastatic breast, renal, and gastric carcinoma cells and is a component of a breast tumor metastasis gene signature (31, 32). Our studies demonstrate that lung and breast tumor cell VCAM-1 can act as an adhesive ligand for integrin α4β1 expressed on LEC and can play a key role in establishing tumor metastases.

Our studies have demonstrated that PI3Kα- or β-mediated integrin α4β1 activation promotes lymphangiogenesis in lymph nodes and independently promotes metastasis by providing novel adhesion sites in the lymph node for metastatic tumor cells (SI Appendix, Fig. S13). These studies show that antagonists of molecules that promote lymph node lymphangiogenesis, such as integrin α4β1 or VEGF-R3, or of molecules that promote tumor cell adhesion to lymphatic endothelium, such as VCAM-1, may form the basis for new therapies to suppress tumor metastasis.

**Experimental Procedures**

Additional experimental procedures can be found in *SI Appendix*.

**Human Tissue Immunohistochemistry.** All studies with human tissues were approved by the Institutional Review Board for human subjects research of the University of California, San Diego (UCSD). Informed consent was obtained from all patients prior to surgery. All animal studies were approved by the Institutional Animal Care and Use Committee of UCSD. Patients at the Moores Cancer Center (UCSD) underwent planned procedures for breast surgical treatment. All surgeries were performed at the UCSD, and standard techniques were used for resection of breast tissue. Normal tissue was also obtained from patients undergoing breast reduction or prophylactic mastectomy. Specimens were removed, sent to the UCSD Medical Center pathology laboratory for analysis, and reviewed by a pathologist to assess the surgical margin tissue. Tissues not needed for diagnosis were embedded in paraffin. Tissues were evaluated for the presence of integrin α4−lymphatic vessels by immunostaining using paraffin or frozen sections.

Formalin-fixed, paraffin-embedded human lymph nodes were sectioned at 4 μm thickness and dewaxed according to standard protocols. Antigen retrieval was performed in Dako Target Retrieval Solution in a steamer for 20 min or by proteinase K digestion for 10 min (for integrin α4). Slides were blocked for 2 h in 8% (vol/vol) normal goat serum in a humidified chamber. Slides were incubated in primary antibodies overnight at 4 °C in a humidified chamber as follows: 4 μg/mL anti-VCAM-1 (E-10) from Santa Cruz Biotechnology, 2 μg/mL PECAM-1 (sc-1505-R) from Santa Cruz Biotechnology, 2 μg/mL anti-podoplanin (D2-40) from Covance, 2 μg/mL anti-LYVE-1 (70R-LR006) from Fitzgerald Industries International, and 2 μg/mL integrin α4 (sc-14008) from Santa Cruz. Slides were then incubated in biotinylated cross-absorbed donkey anti-rabbit or mouse Ig from Jackson Immunoresearch and developed using Vectastain ABC kit (Vector Laboratories) with 3,3′-diaminobenzidine (DAB) as a color substrate.

**Statistical Analyses.** Data were tested for significance with one-way ANOVA (in vivo studies), Student t test (in vitro studies) or Fisher’s exact test (inclusion of metastasis). All experiments were performed three times, and the data from a single representative experiment is presented.

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