

CANCER

Lymph node blood vessels provide exit routes for metastatic tumor cell dissemination in mice

M. Brown,^{1,2*} F. P. Assen,² A. Leithner,² J. Abe,³ H. Schachner,¹ G. Asfour,¹ Z. Bago-Horvath,¹ J. V. Stein,³ P. Uhrin,⁴ M. Sixt,^{2,*†} D. Kerjaschki^{1,*†}

During metastasis, malignant cells escape the primary tumor, intravasate lymphatic vessels, and reach draining sentinel lymph nodes before they colonize distant organs via the blood circulation. Although lymph node metastasis in cancer patients correlates with poor prognosis, evidence is lacking as to whether and how tumor cells enter the bloodstream via lymph nodes. To investigate this question, we delivered carcinoma cells into the lymph nodes of mice by microinfusing the cells into afferent lymphatic vessels. We found that tumor cells rapidly infiltrated the lymph node parenchyma, invaded blood vessels, and seeded lung metastases without involvement of the thoracic duct. These results suggest that the lymph node blood vessels can serve as an exit route for systemic dissemination of cancer cells in experimental mouse models. Whether this form of tumor cell spreading occurs in cancer patients remains to be determined.

Lymph nodes (LNs) are central trafficking hubs for recirculating immune cells (1). Conceivably, tumor cells could use this gateway function of LNs for metastatic colonization of peripheral organs (2). Although this route of tumor dissemination is supported by correlative evidence from mouse models (3–5) and patients (6–17), a causal link between LN colonization (18–21) and peripheral metastasis has not been established (22). A scenario in which LNs act as gateways to systemic dissemination raises the question of whether tumor cells reach the blood circulation via efferent lymphatic vessels, via LNs of higher echelons and the thoracic duct, or directly via the LN blood vasculature.

To investigate this question, we used intralymphatic microinfusion (23) to directly deposit defined numbers of murine 4T1 mammary carcinoma cells into the subcapsular sinus of popliteal LNs of mice (fig. S1A). These experimental LN metastases (ELMs) allow precise analysis of the earliest steps in the development of LN and successive pulmonary metastases in the absence of a primary tumor. Microinfusion was carefully adjusted to preserve the delicate microarchitecture of the LN. It did not affect the physiological solute filter function of the LN stromal backbone (fig. S1, B and C), and it did not alter the conventional migratory paths of infused leukocytes (fig. S6, B and C) (1). Infused particles did not leak into the downstream medial iliac LN or into the lung (fig. S1, D and E).

LNs harvested immediately after intralymphatic infusion with 4T1 tumor cells revealed that these cells initially accumulated in the subcapsular sinus (Fig. 1A and fig. S1F). Within 1 day, tumor cells crossed the floor of the subcapsular sinus, and during the following 3 days, they progressed along the LN stromal network toward the center of the LN (Fig. 1 and fig. S1G). A comparison with tumor-bearing sentinel LN samples from human breast cancer patients (fig. S1H) shows that the histological picture of ELMs closely resembles human pathology. Invasiveness of mammary carcinoma has been linked to the expression of basal cell marker molecules (24), such as p63 and cytokeratin 14 (CK14). Because tumor cells arriving in the avascular subcapsular sinus need rapid access to the parenchymal blood supply for survival, we hypothesized that upon intralymphatic infusion the tumor cells would show early up-regulation of invasion markers. Indeed, 2 days after infusion, CK14 expression in the 4T1 tumor cells was substantially increased in comparison with expression in orthotopically injected or cultured 4T1 cells (fig. S1, I and J). At 3 days after infusion, when tumor cells had infiltrated the deep LN parenchyma, the average CK14 levels had renormalized and the remaining CK14-expressing cells preferentially localized to the invasive margins of the ELM (fig. S1, K and L). Together, these data indicate that ELMs are a valid model of LN metastasis.

Invading 4T1 tumor cells were found in close proximity and in direct contact with blood vessels 2 days after infusion (Fig. 2A and movie S1). On day 3, the cells had wrapped around (movie S2) and intravasated (Fig. 2, B to D, and fig. S2, A to C) the blood vessel lumen. This association between tumor cells and blood vessels was not due to local induction of angiogenesis, as we found similar vessel densities within and outside infiltrated areas (fig. S2D). The LN blood vasculature is charac-

terized by special postcapillary segments termed “high endothelial venules” (HEVs), which serve as entry ports for incoming lymphocytes (1). Two- and three-dimensional morphometrical analyses of ELM specimens showed that upon their progression toward the LN center, tumor cells gradually became associated with HEVs and frequently localized in their lumen (Fig. 2, C to E; fig. S2, E and F; and movies S3 to S6). These data indicate that HEVs are the main exit route by which tumor cells gain access to the blood circulation. Similar tumor cell–blood vessel associations and intravasations were observed in samples of LN micrometastases from human breast cancer patients (fig. S2G). Furthermore, in *ex vivo* adhesion assays (fig. S2H) in which 4T1 cells were incubated on slices of native mouse LNs, we likewise observed colocalization of tumor cells and blood vessels (fig. S2I).

To explore whether the associations between tumor cells and LN blood vessels correlate with the formation of systemic metastasis, we intralymphatically infused mice with mCherry- and luciferase-expressing (mCherry⁺ luciferase⁺) 4T1 cells and then performed whole-animal *in vivo* bioluminescence imaging. Genetic labeling with fluorescent proteins and firefly luciferase did not alter tumor cell proliferation or migration (fig. S3). As late as 35 days after intralymphatic infusion with the tumor cells, the lung was the only organ showing macrometastasis (Fig. 3A and fig. S4A). This metastatic seeding pattern was similar to that seen when 4T1 tumor cells were orthotopically injected into syngeneic mice (fig. S4B) and to that seen when MDA-MB-231 human mammary carcinoma cells were intralymphatically infused into immunodeficient mice (fig. S4C). Bioluminescence imaging of isolated lungs (Fig. 3B and fig. S4D) showed luciferase-expressing multifocal metastases 11 and 21 days after intralymphatic infusion with 4T1 tumor cells, and microscopic examination revealed tumor cell clusters as early as 6 and 11 days after infusion (fig. S4, E and F). These data demonstrate that ELMs rapidly disseminate to peripheral tissues.

To determine the earliest time point at which tumor cells colonized the lungs, we intralymphatically infused luciferase⁺ mCherry⁺ 4T1 cells into LNs and removed the LNs after 2 or 3 days (Fig. 3C and fig. S4G). The 2-day versus 3-day treatment groups were examined 11 days after infusion. In the 3-day cohort, 33.3% of the mice displayed lung metastases, whereas none were detected in the 2-day cohort (Fig. 3D and fig. S4H). These results supported our finding that tumor cells invaded LN blood vessels 3 days after intralymphatic infusion (Fig. 2, B to E, and fig. S2, A to F). Accordingly, using flow cytometry (FC), we detected circulating tumor cells in the blood as early as 3 days after intralymphatic infusion with fluorescent 4T1 cells (Fig. 3, E and F).

These results were at odds with the conventional view that tumor spreading from LNs occurs via passage through efferent lymphatic vessels, LNs of higher echelons, the thoracic duct, and the subclavian vein (25). Hence, we used quantitative

¹Clinical Institute of Pathology, Medical University of Vienna, 1090 Vienna, Austria. ²Institute of Science and Technology Austria (IST Austria), 3400 Klosterneuburg, Austria.

³Theodor Kocher Institute, University of Bern, 3012 Bern, Switzerland. ⁴Institute of Vascular Biology, Medical University of Vienna, 1090 Vienna, Austria.

*These authors contributed equally to this work.

†Corresponding author. Email: dontscho.kerjaschki@meduniwien.ac.at (D.K.); michael.sixt@ist.ac.at (M.S.)

FC to determine the tumor cell seeding kinetics of mCherry⁺ 4T1 ELMs into lungs versus into medial iliac LNs, which drain the ELM-bearing popliteal LN (Fig. 3G and fig. S5, A to C). We found that tumor cell numbers in medial iliac

LNs did not predict lung metastatic burden (fig. S5C). At 3 days after infusion, when metastatic tumor cells were detected in the lungs of 80% of all infused mice, medial iliac LNs were invariably free of tumor cells. All medial iliac

LNs became colonized only after 28 days (fig. S5D). ELMs with the mCherry-expressing mouse colon carcinoma cell line CT26 revealed similar results (fig. S5, E to H): 12 days after infusion, CT26 cells had disseminated into the lungs in 83%

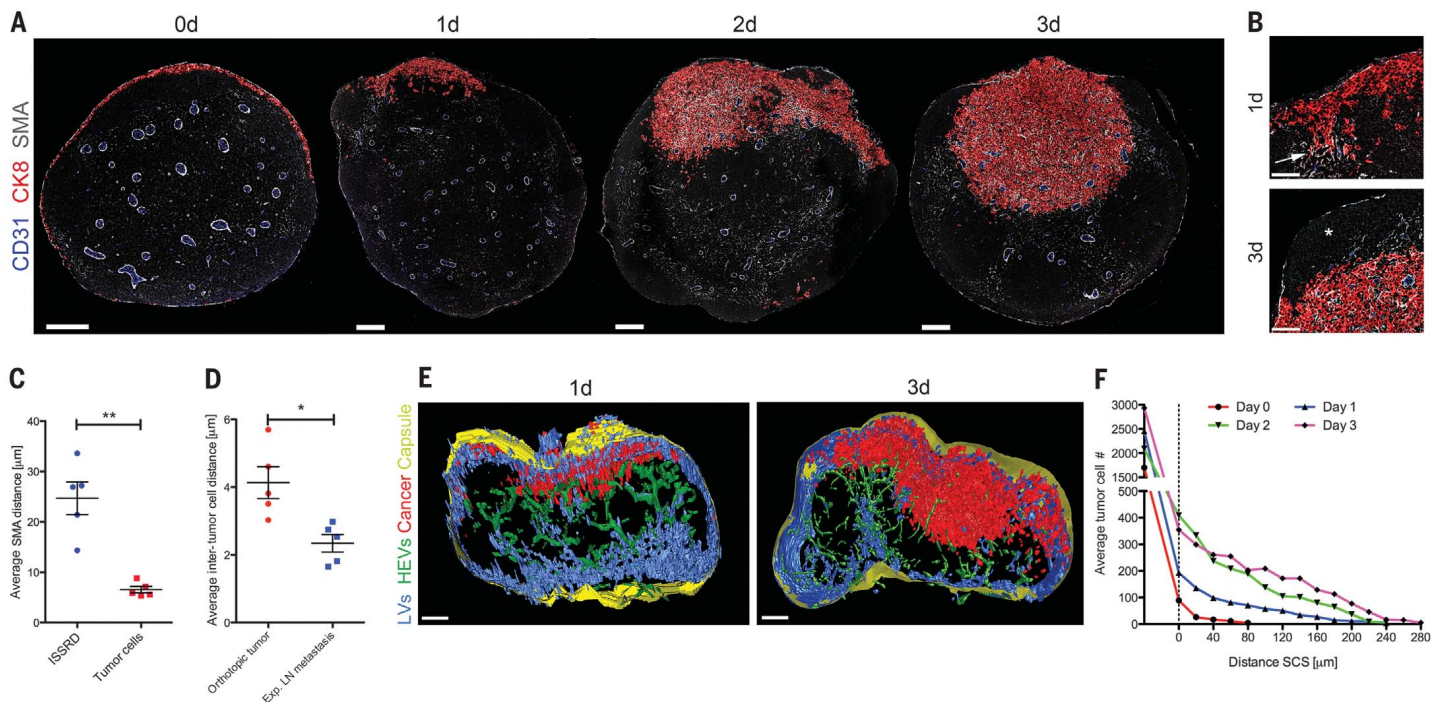


Fig. 1. Intralymphatically infused 4T1 mammary carcinoma cells invade the LNs. (A) Immunofluorescence of 4T1 ELMs 0, 1, 2, and 3 days after intralymphatic infusion. Red, cytokeratin 8 (CK8); blue, CD31; gray, smooth muscle actin (SMA). Scale bars, 200 μm . $n = 5$ ELMs. d, day. (B) Zoom-in of images from (A) for 1 day (top) and 3 days (bottom) after intralymphatic infusion showing the SMA⁺ fibroblastic reticular cell network (arrow) and B cell follicles (asterisk). Scale bars, 100 μm . (C) Distances (means \pm SEM) of tumor cells and in silico-simulated equally distributed isosurfaces (ISSRD) from the SMA⁺ fibroblastic reticular cell network in sections of 4T1 ELMs 3 days after infusion. $n = 5$ ELMs (paired, two-tailed t test). (D) Distances (means \pm SEM)

between tumor cells of 4T1 tumors 3 days after orthotopic transplantation of tumor spheroids or intralymphatic infusion of tumor cell suspensions. $n = 5$ experimental tumor samples (unpaired, two-tailed t test). Significance: $*P \leq 0.05$; $**P \leq 0.01$. (E) Representative segmentations of 200- μm sections in light sheet fluorescence microscopy images showing LNs at 1 and 3 days after infusion with mCherry⁺ 4T1 tumor cells. Red, mCherry⁺ tumor cells; yellow, autofluorescent capsule; green, peripheral node addressin (PNAd)-positive HEVs; cyan, Prox1⁺ LN sinusoids. Scale bars, 200 μm . $n = 5$ sections. (F) Average 4T1 tumor cell numbers at the indicated distances from the subcapsular sinus (SCS) 0, 1, 2, and 3 days after intralymphatic infusion. Dotted line, sinus border or floor. $n = 5$ ELMs.

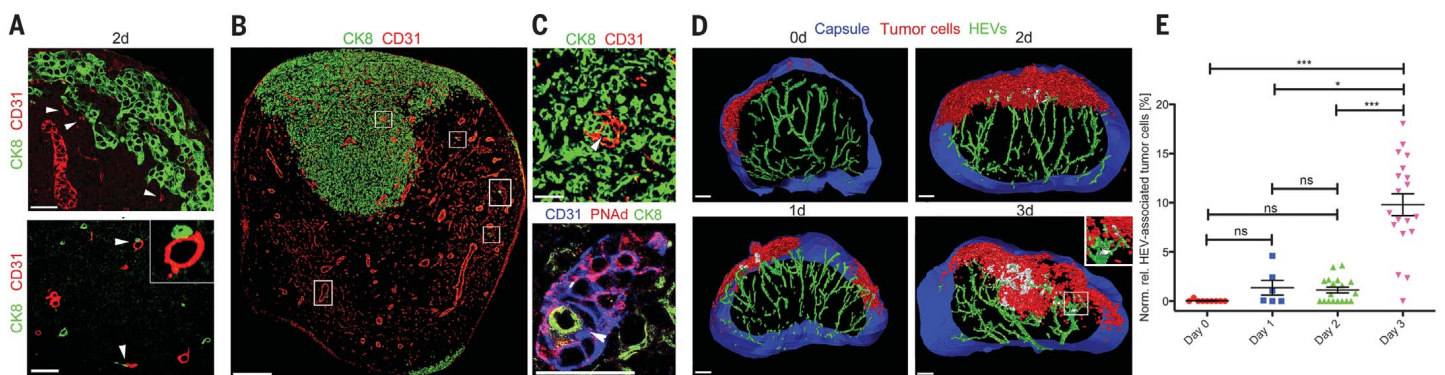


Fig. 2. 4T1 Tumor cells associate with and intravasate LN blood vessels. (A) Immunofluorescence 2 days after intralymphatic infusion with 4T1 tumor cells. Arrowheads indicate CD31⁺ blood vessels. The inset is a zoom-in showing tumor cell–blood vessel interaction. Scale bars, 50 μm . $n = 5$ ELMs. (B) Image of 4T1 ELMs 3 days after infusion. Scale bar, 300 μm . $n = 5$ ELMs. (C) Zoom-in and serial section of a representative boxed area of (B). (Top) Tumor cell (arrowhead) in blood vessel lumen. (Bottom) Tumor cell (arrowhead) in HEV lumen. Scale bars, 50 μm . (D) Representative

segmentations of 200- μm sections in light sheet fluorescence microscopy images showing 4T1 ELMs 0, 1, 2, and 3 days after infusion. Red, mCherry⁺ tumor cells; blue, autofluorescent capsule; green, PNAAd⁺ HEVs. Tumor cell–HEV interactions are marked in white. Scale bars, 100 μm . $n = 5$ ELMs. (E) Tumor cell associations with HEVs [means \pm SEM, normalized relative (norm. rel.) to tumor cell density] 0, 1, 2, and 3 days after infusion. Significance: $*P \leq 0.05$; $***P \leq 0.001$; ns, $P > 0.05$ ($n \geq 6$ ELMs; Kruskal-Wallis test).

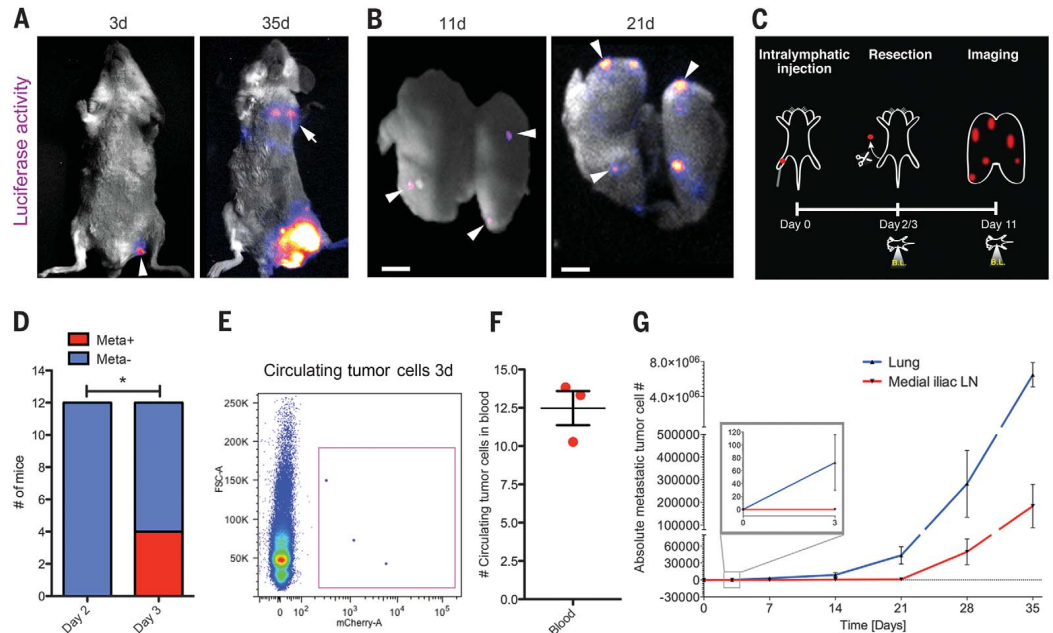
Fig. 3. ELMs seed lung metastases independently of the thoracic duct.

(A and B) Bioluminescence after intralymphatic infusion with mCherry⁺ luciferase⁺ 4T1 tumor cells. (A) Popliteal fossa (arrowhead) and lung (arrow) 3 and 35 days after infusion. *n* = 12 mice. (B) Lung metastases (arrowheads) 11 and 21 days after infusion. Scale bars, 4 mm. *n* = 5 mice.

(C) Experimental design. At 0 days mCherry⁺ luciferase⁺ 4T1 tumor cells were infused (red). At 2 and 3 days, in vivo bioluminescence confirmed tumor growth, and ELMs were resected thereafter. At 11 days, lungs analyzed by in vivo bioluminescence were harvested for ex vivo analysis. (D) Quantitation of metastasis-positive (Meta⁺) and metastasis-negative (Meta⁻) lungs of mice with ELMs represented in (C). *n* = 12 mice (**P* ≤ 0.05; two-sided, chi-square test).

(E) FC analysis of circulating mCherry⁺ 4T1 tumor cells pooled from five mice 3 days after infusion. *x* axis, mCherry fluorescence intensity; *y* axis, forward scatter (FSC). *n* = 3 pooled samples.

(F) Average absolute circulating tumor cell numbers ± SEM in FC analyses 3 days after infusion with mCherry⁺ 4T1 tumor cells. Blood was pooled from five mice per experiment, and values were normalized to those for control mice. *n* = 3 pooled samples. (G) Kinetics of average absolute mCherry⁺ 4T1 tumor cell numbers ± SEM in FC analyses of medial iliac LNs and lungs over the 35-day time course after infusion. Tumor cell numbers were normalized to those for control mice. *n* ≥ 5 mice.



of the mice, whereas only 17% of mice showed evidence of tumor cells in medial iliac LNs. Together, these results indicate that early peripheral dissemination of ELMs is unlikely to occur via efferent lymphatics. To experimentally prevent passage via the efferent lymphatics, we surgically ligated the efferent lymphatic vessel before intralymphatic infusion with mCherry⁺ luciferase⁺ 4T1 tumor cells (fig. S6D). Complete blockade of the efferent lymphatic vessel was confirmed by the inability of an intralymphatically injected dye to reach the downstream medial iliac LN (fig. S6A), as well as the inability of intralymphatically injected T cells to recirculate into the spleen (fig. S6, B and C). We found that lymphatic ligation did not compromise the ability of 4T1 tumor cells to seed lung metastasis within 3 days after infusion (fig. S6, E and F). On the basis of these results, we conclude that in our experimental mouse model, LN blood vessels are a gateway for the early transit of nodal tumor cells into the systemic circulation.

Our experimental setup establishes the contribution of an ELM to peripheral metastasis in the absence of a primary tumor. However, there is substantial evidence that the metastatic process is preceded by adaptations both within the tumor and within the host microenvironment (8, 9, 20, 22, 26), which are both bypassed in the ELM model. To model the premetastatic conditions in the host, we implanted mice with a peripheral tumor by inoculating unlabeled 4T1 tumor cells into the lateral tarsal zone above the ankle (fig. S7A). After 8 days of priming, ELMs were induced by intralymphatic infusion with mCherry⁺ luciferase⁺ 4T1 tumor cells (fig. S7, B

and C). These experiments revealed that ELMs in LNs that were previously primed by a peripheral tumor seeded lung metastasis at a frequency similar to that for ELMs in naïve LNs (fig. S7, D and E). Under primed conditions, metastatic dissemination was equally independent of the thoracic duct passage as under unprimed conditions (fig. S7, D and E). To mimic possible adaptations of the tumor cells (27, 28), we allowed orthotopically induced mCherry⁺ luciferase⁺ 4T1 tumors to colonize draining LNs. We recovered tumor cells from these LNs, briefly recultured them, and reinfused them into popliteal LNs of new host animals (fig. S7F). Under these conditions, 83.3% of infused animals developed lung metastases, and this was again independent of the thoracic duct passage (fig. S7, G and H). Collectively, these results demonstrate that the direct metastatic dissemination via the LN blood vasculature is not artificially induced by intralymphatic injection of naïve tumor cells into unprimed LN environments.

We have established a mouse model of sentinel LN metastasis that allows the analysis of early metastatic pathways via the LN. We discovered that early tumor cell attachment to and invasion into the LN blood vasculature coincided with the appearance of circulating tumor cells and the successful metastatic colonization of the lung. Our experimental model revealed that LN blood vessels are effective gateways from lymphatics into the systemic circulation and that this pathway may be more efficient than direct metastatic dissemination from the primary tumor (fig. S8). Our data are similar to results obtained independently by Pereira *et al.* using different

methodologies with mouse models (29). Though our investigations in a reductionist mouse model support the view that LNs are active hubs for systemic tumor cell spreading, future clinical and experimental studies are required to determine whether these findings are relevant to human cancer.

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interests: None declared. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the supplementary materials.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/359/6382/1408/suppl/DC1
Materials and Methods
Figs. S1 to S8
References (30, 31)
Movies S1 to S6

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An alternate route for metastatic cells

Metastatic tumor cells are thought to reach distant organs by traveling through the blood circulation or the lymphatic system. Two studies of mouse models now suggest a hybrid route for tumor cell dissemination. Pereira *et al.* and Brown *et al.* used distinct methodologies to monitor the fate of tumor cells in lymph nodes. They found that tumor cells could invade local blood vessels within a node, exit the node by entering the blood circulation, then go on to colonize the lung. Whether this dissemination route occurs in cancer patients is unknown; the answer could potentially change the way that affected lymph nodes are treated in cancer.

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