In vivo Imaging of Inflammation- and Tumor-Induced Lymph Node Lymphangiogenesis by Immuno–Positron Emission Tomography

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Abstract

Metastasis to regional lymph nodes (LN) is a prognostic indicator for cancer progression. There is a great demand for sensitive and noninvasive methods to detect metastasis to LNs. Whereas conventional in vivo imaging approaches have focused on the detection of cancer cells, lymphangiogenesis within tumor-draining LNs might be the earliest sign of metastasis. In mouse models of LN lymphangiogenesis, we found that systemically injected antibodies to lymphatic epitopes accumulated in the lymphatic vasculature in tissues and LNs. Using a 124I-labeled antibody against the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), we imaged, for the first time, inflammation- and tumor-draining LNs with expanded lymphatic networks in vivo by positron emission tomography (PET). Anti-LYVE-1 immuno-PET enabled visualization of lymphatic vessel expansion in LNs bearing metastases that were not detected by [18F]fluorodeoxyglucose-PET, which is clinically applied to detect cancer metastases. Immuno-PET with lymphatic-specific antibodies may open up new avenues for the early detection of metastasis, and the images obtained might be used as biomarkers for the progression of diseases associated with lymphangiogenesis. Cancer Res; 70(21): 8842–51. © 2010 AACR.

Introduction

Metastasis is a characteristic trait of most tumor types and is the cause for the majority of cancer deaths. In many human cancers, metastasis to regional lymph nodes (LN) represents the first step of tumor dissemination and often serves as a prognostic indicator for the progression of the disease (1). Currently, regional LNs, or in patients with breast cancer or melanoma only the tumor-draining LNs, are dissected and sections are analyzed for metastases. However, this procedure is elaborate and associated with significant morbidity and costs (2–4). Thus, there is a great demand for sensitive, noninvasive, and preferentially simpler methods to detect metastasis to LNs, in particular at the very early stages of dissemination. Thus far, conventional in vivo imaging approaches for cancer metastases in patients have focused on the detection of cancer cells themselves (5–7). These methods have limited sensitivity, because a large number of tumor cells are required for reliable detection (5). In contrast, there is increasing evidence that tumor cells induce changes of the surrounding extracellular matrix and stromal cells at very early stages of metastasis (6, 8, 9). In particular, we showed that tumors induce the expansion of the lymphatic vasculature (lymphangiogenesis) in tumor-draining LNs in different mouse models of cancer metastasis (10, 11). Importantly, this process even starts before the onset of metastasis and is associated with distant metastasis to distant LNs and organs. Tumor-induced LN lymphangiogenesis has also been observed in other experimental models of cancer (12, 13) and in LNs of patients with metastatic melanoma and breast cancer (14, 15).

Based on these findings, we proposed that LN lymphangiogenesis might serve as a novel target to image the very early stages of the metastatic process. We established a method to image LN lymphangiogenesis noninvasively in vivo using positron emission tomography (PET) with radiolabeled antibodies to lymphatic-specific epitopes (immuno-PET). PET is a noninvasive, highly sensitive, and quantitative imaging method that is not limited by tissue depth (9).

To develop our method, we used a well-established experimental model of inflammation-induced LN lymphangiogenesis [K14/vascular endothelial growth factor (VEGF) transgenic mice; refs. 16–18]. In this model, the induction of LN lymphangiogenesis occurs rapidly in all of the mice, with less variability and discomfort for the animals than in metastasis models. We then applied the methodology to image expanded lymphatic networks in tumor-draining LNs in an established mouse model of melanoma-induced LN lymphangiogenesis (13).

Our results reveal that lymphatic vessels can indeed be targeted and imaged by systemically injected radiolabeled...
antibodies. They also represent the first proof of principle for the noninvasive imaging of inflammation- and tumor-induced LN lymphangiogenesis in vivo. This novel method could be used to develop new strategies for the early detection of cancer metastases.

Materials and Methods

Mouse models of LN lymphangiogenesis

Inflammation-induced LN lymphangiogenesis. Delayed-type hypersensitivity reactions were induced in the ear skin of female hemizygous transgenic FVB mice that overexpress VEGF-A164 in the epidermis under control of the human keratin 14 promoter (K14/VEGF mice) as described (11, 17–19). For all studies, age-matched 9- to 21-week-old mice were used.

Tumor-induced LN lymphangiogenesis. B16-F1 murine melanoma cells (kindly provided by Dr. S. Hemmi, University of Zurich, tested for microbial contaminations before the experiment) were transfected by Lipofectamine (Invitrogen) with full-length human VEGF-C subcloned into the pcDNA3.1 vector (Invitrogen). B16-F1-VEGF-C cells (2 × 10⁵) were injected into the left footpads of female C57BL/6N mice (Charles River Laboratories) as described (13). For bioluminescence imaging experiments, firefly-expressing B16-F10-luc2 cells (Caliper Life Sciences, purchased before the experiment) were transfected with VEGF-C and injected into female C57BL/6j-TyrR-/- (albino) mice (The Jackson Laboratory) as described above. All animal experiments were approved by the cantonal veterinarian office in Zurich, Switzerland (protocols 123/2005, 149/2008, and 128/2008).

Ex vivo fluorescence experiments

Eighty-five micrograms of rat anti-mouse lymphatic vessel endothelial hyaluronan receptor-1 antibody (LYVE-1) antibody (clone 223322, R&D Systems, <0.1 EU endotoxin/μg) or isotype-matched rat control IgG (AbD Serotec, <0.01 EU endotoxin/μg) or endotoxin/μg) were injected into the tail veins of K14/VEGF mice (one mouse per treatment) 1 day after challenging one ear with oxazolone. Twenty-four hours after injection, the animals were sacrificed, and organs were frozen in optimal cutting temperature (OCT) compound (Sakura Finetec). Seven-micrometer sections were fixed with 4% paraformaldehyde in PBS, incubated with an Alexa Fluor 594–conjugated donkey anti-rat IgG antibody (Invitrogen), and costained with a rabbit anti-mouse LYVE-1 antibody (AngioBio) detected by an Alexa Fluor 488 donkey anti-rabbit IgG antibody (Invitrogen) or costained with a biotinylated rat Meca32 antibody (BD Pharmingen) detected by Alexa Fluor 488 streptavidin (Invitrogen). Sections were counterstained with Hoechst 33342 (Invitrogen) and analyzed with an AxioSkop2 mot plus microscope (Zeiss). Images were captured with an AxioCam MRc camera (Zeiss) using the Axio-Vision 4.7 software.

Radioiodination

Anti-mouse LYVE-1 antibody or isotype-matched rat control IgG (AbD Serotec) was radiolabeled with Na¹²⁵I (Perkin-Elmer; radiochemical purity 99.0%, radionuclide purity 99.95% with <0.04% contamination by ¹²⁴I) or with Na¹²⁴I (IBA Molecular, radiochemical purity >95%, radio-nuclide purity >99% with <0.5% contamination by ¹²³I and <0.1% contamination by ¹²⁵I by adapting the standard chloramine T method (20). Briefly, 70 to 380 μCi Na¹²⁵I and 5 μL of 5 mg/mL chloramine T (Sigma-Aldrich) were added per 100 μg antibody in PBS (1 mg/mL), or 2 to 2.7 mCi Na¹²⁴I and 9 μL of 5 mg/mL chloramine T were added to 180 μg antibody in PBS (1 mg/mL). After 2 minutes, the radiolabeled antibodies were separated from free ¹²³I using PD10 columns (GE Healthcare). The radioactivity of the samples was determined using a γ-counter (Cobra Autogamma, Packard Instrument Company). Normal uptake of radiolabeled iodine by the thyroid glands and the intestine of the mice was blocked by administration of potassium iodide in the drinking water starting 4 days before an experiment and oral administration of sodium perchlorate 1 hour before antibody injection.

Biodistribution studies

K14/VEGF mice were injected i.v. with either 7 μg (16 μCi, i.e., 2.29 μCi/μg antibody; n = 5), 35 μg (80 μCi, i.e., 2.29 μCi/μg antibody; n = 5), or 90 μg (55 μCi, i.e., 0.61 μCi/μg antibody; n = 3) of ¹²⁴I-anti-LYVE-1 antibody or with equal amounts of ¹²³I-rat control IgG at 6 or 8 days after challenging one ear with oxazolone. Mice were sacrificed 24 hours after injection. Organs were weighed, and radioactivity was measured. The radioactivity content of representative organs was expressed as the percentage of the injected dose per gram of tissue (%ID/g). For time course experiments, K14/VEGF mice were given i.v. injections of 37 μg (52 μCi, i.e., 1.4 μCi/μg antibody) of ¹²⁴I-anti-LYVE-1 antibody 13 days after oxazolone challenge. Four animals each were analyzed at 2 and 3 days after injection. To assess the metabolic stability of ¹²⁴I-anti-LYVE-1, serum was collected (three mice) and applied to PD MiniTrap G-25 gel filtration units (GE Healthcare). Fractions of 300 μL were collected, and radioactivity was measured.

Microradiography

¹²⁴I-anti-LYVE-1 antibody (35 μg, 150 μCi, i.e., 4.3 μCi/μg antibody) or ¹²⁵I-rat control IgG (35 μg, 120 μCi, i.e., 3.4 μCi/μg antibody) was injected i.v. into K14/VEGF mice at 10 days after the challenge (n = 2 per group). In an additional experiment, mice (n = 3) were preinjected with 625 μg unlabeled antibody 1 day before injection of ¹²⁴I-anti-LYVE-1 antibody (three mice with preinjection and two without preinjection). Twenty-four hours after injection, mice were sacrificed, and organs were frozen in OCT compound. Seven-micrometer sections were fixed with 4% paraformaldehyde in PBS. Air-dried sections were coated with KODAK autoradiography emulsion type NTB (Carestream Health, Inc.) and developed according to the manufacturer’s instructions after 2 weeks exposure time.

PET

¹²⁴I-anti-LYVE-1 (38 μg, 0.37–0.42 mCi, i.e., 9.7–11 μCi/μg antibody) or ¹²⁴I-labeled rat control IgG (38 μg, 0.34–0.36 μCi/μg antibody) was injected i.v. into K14/VEGF mice at 10 days after the challenge (n = 2 per group). In an additional experiment, mice (n = 3) were preinjected with 625 μg unlabeled antibody 1 day before injection of ¹²⁴I-anti-LYVE-1 antibody (three mice with preinjection and two without preinjection). Twenty-four hours after injection, mice were sacrificed, and organs were frozen in OCT compound. Seven-micrometer sections were fixed with 4% paraformaldehyde in PBS. Air-dried sections were coated with KODAK autoradiography emulsion type NTB (Carestream Health, Inc.) and developed according to the manufacturer’s instructions after 2 weeks exposure time.
mCi, i.e., 8.9–9.4 μCi/μg antibody) were injected i.v. into K14/VEGF mice after oxazolone treatment of the ear skin (three mice per treatment). PET scans were performed ∼24 hours (two mice per group) or 48 hours (one mouse per group) after i.v. radiotracer injection using the GE Vista/CT camera (GE Healthcare) as described previously (21). For in vivo PET scanning, mice were anesthetized with isoflurane (Abbott Laboratories) in an air/oxygen mixture as described previously (22). Auricular LNs were dissected, and the mice were rescanned ex vivo, with the dissected auricular LNs in agar blocks. For the in vivo and ex vivo approaches, whole-body PET data were acquired in two bed positions (30 minutes of acquisition time per position) and were reconstructed in a single time frame, with pixel sizes of 0.3875 and 0.775 mm in the transverse and axial directions, respectively. Series of coronal image slices and maximum intensity projections (MIP; ref. 23), as well as MIP movies, were generated using the software PMOD (PMOD Technologies Ltd.). Coronal PET sections were displayed with a fixed gray scale for comparison between different mice. Data were corrected for the body weight of the mice and variations of the injected dose.

For PET of tumor-induced LN lymphangiogenesis, 124I-anti-LYVE-1 (30 μg, 0.26–0.38 mCi, i.e., 8.7–12.7 μCi/μg antibody) or 124I-rat control IgG (30 μg, 0.32–0.33 mCi, i.e., 10.7–11 μCi/μg antibody) were injected i.v. into K14/VEGF mice after oxazolone treatment of the ear skin (three mice per treatment). PET scans were performed ∼24 hours (two mice per group) or 48 hours (one mouse per group) after i.v. radiotracer injection using the GE Vista/CT camera (GE Healthcare) as described previously (21). For in vivo PET scanning, mice were anesthetized with isoflurane (Abbott Laboratories) in an air/oxygen mixture as described previously (22). Auricular LNs were dissected, and the mice were rescanned ex vivo, with the dissected auricular LNs in agar blocks. For the in vivo and ex vivo approaches, whole-body PET data were acquired in two bed positions (30 minutes of acquisition time per position) and were reconstructed in a single time frame, with pixel sizes of 0.3875 and 0.775 mm in the transverse and axial directions, respectively. Series of coronal image slices and maximum intensity projections (MIP; ref. 23), as well as MIP movies, were generated using the software PMOD (PMOD Technologies Ltd.). Coronal PET sections were displayed with a fixed gray scale for comparison between different mice. Data were corrected for the body weight of the mice and variations of the injected dose.

Figure 1. A systemically injected anti-LYVE-1 antibody accumulates in the lymphatic vasculature of the LNs. Differential immunofluorescence analysis of systemically injected anti-LYVE-1 antibody in sections of control (A–H) and inflamed auricular LNs (I–P). The injected antibody to LYVE-1 (B, F, J, and N) colocalized with LYVE-1 stained lymphatic vessels (C and K) but not with strongly Meca32-positive blood vessels (G and O) in control and inflamed auricular LNs. D, merged B and C. H, merged F and G. L, merged J and K. P, merged N and O. The injected control IgG (R) did not colocalize with LYVE-1-positive lymphatic vessels (S). A, E, I, M, and Q show nuclear staining by Hoechst dye (blue). Scale bars, 100 μm.
Figure 2. A systemically injected radiolabeled anti-LYVE-1 antibody accumulates in the lymphatic vasculature. A-U, microradiographs of tissue sections from mice injected with $^{125}$I-anti-LYVE-1 or control IgG. The radioactive signal (black) of the injected $^{125}$I-anti-LYVE-1 antibody (A-F and H-N), but not of control IgG (O-T), was detected in sections of control (A, H, O) and inflamed auricular LNs (B, I, P), control (C, J, Q) and inflamed ears (D, K, R), lung (E, L, S), and intestine (F, M, T). H-N, preinjection of unlabeled anti-LYVE-1 antibody inhibited the binding of $^{125}$I-anti-LYVE-1 antibody in lymphatic vessels. V-Y, serial sections of an inflamed auricular LN (V, W) and ear (X, Y) of a $^{125}$I-anti-LYVE-1–injected mouse. The radioactive signal of the $^{125}$I-anti-LYVE-1 antibody (V, X) overlapped with immunofluorescence staining for LYVE-1–positive lymphatic vessels (W, Y). Scale bars, 100 μm.
antibody) were injected i.v. into tumor-bearing mice 19 or 20 days after tumor cell injection. PET and biodistribution analyses of selected organs were performed ~18 hours after antibody injection (two mice per group) as described above. For [18F]fluorodeoxyglucose ([18F]FDG–PET, 0.3 to 0.6 mCi [18F]FDG was injected i.v. 20 days after B16-F10-luc2-VEGF-C tumor cell injection. PET scans were performed on the GE Vista/CT scanner as described (24).

Bioluminescence imaging

Nineteen days after B16-F10-luc2-VEGF-C tumor cell injection, the legs of the mice were shaved, and mice were anesthetized with an isoflurane/oxygen mixture. Mice were given an i.p. injection of 210 μL d-luciferin substrate in PBS (15 mg/mL, Caliper Life Sciences). Twenty minutes later, the animals were imaged with an IVIS Spectrum imaging system (Caliper Life Sciences). Photons were collected for 180 seconds. Images were analyzed with the Living Image v.3.1 software (Caliper Life Sciences).

Results

A systemically injected antibody to a lymphatic epitope accumulates in the lymphatic vasculature

We first tested whether the antibody accumulates in the lymphatic vasculature in an established mouse model of chronic skin inflammation following the systemic injection of an antibody against a lymphatic-specific epitope (17, 18). After topical application of the contact sensitizer oxazolone, K14/VEGF mice develop a chronic ear skin inflammation that is associated with vascular hyperpermeability and prominent lymphangiogenesis in the ear skin and in the

Figure 3. Concentration- and time-dependent accumulation of 125I-anti-LYVE-1 antibody in LNs. A, concentration dependent accumulation in the ear-draining LNs of different doses of 125I-anti-LYVE-1 antibody or 125I-control IgG in mice. The results were expressed as cpm ± SD. Data of the 90-μg groups were normalized to the specific activity of the 7-μg and 35-μg groups. B, time-dependent accumulation of 125I-anti-LYVE-1 antibody in the ear-draining LNs; 35-μg antibody injected. Data of the 48-h and 72-h groups were normalized to the specific activity of the 24-h group. C, preinjection of a 17.8-fold excess of unlabeled anti-LYVE-1 antibody inhibits binding of injected 125I-anti-LYVE-1 antibody in the ear-draining LNs.
ear-draining (auricular) LNs (18). K14/VEGF mice were given i.v. injections of a rat antibody to the LYVE-1 (25, 26) or control IgG. In tissue sections of auricular LNs obtained 24 hours after injection, the anti-LYVE-1 antibody was detected by a fluorescently labeled antirat IgG antibody (Fig. 1B, F, J, and N). Importantly, the localization of the injected anti-LYVE-1 antibody overlapped with the localization of LYVE-1 by external costaining with a rabbit antibody to LYVE-1 (Fig. 1C and K). The anti-LYVE-1 antibody did not colocalize with blood vessels that strongly expressed the vascular marker Meca32 (Fig. 1G and O). The injected rat IgG control antibody was not detected on lymphatic vessels (Fig. 1R). Thus, the injected anti-LYVE-1 antibody specifically accumulated in the lymphatic vessels.

**Binding of anti-LYVE-1 antibody to its target is maintained after iodination**

We aimed to use antibodies against lymphatic epitopes that were labeled with the radionuclide $^{125}\text{I}$ in the biodistribution experiments and therefore assessed the quality of $^{125}\text{I}$-anti-LYVE-1 antibody. The immunoreactivity of $^{125}\text{I}$-anti-LYVE-1 antibody to LYVE-1 was 92%. To compare the binding capacities of $^{125}\text{I}$-anti-LYVE-1 and unlabeled antibody, we performed a competitive RIA. Unlabeled anti-LYVE-1 antibody dose-dependently inhibited binding of $^{125}\text{I}$-anti-LYVE-1 antibody to immobilized LYVE-1 (Supplementary Fig. S1). Half-maximal binding of $^{125}\text{I}$-anti-LYVE-1 antibody was reached at equal amounts of labeled and unlabeled antibody, confirming that the affinity of the anti-LYVE-1 antibody was maintained after iodination.

Next, we systemically injected $^{125}\text{I}$-anti-LYVE-1 antibody into K14/VEGF mice with unilateral LN lymphangiogenesis. Microradiography of tissue sections obtained 24 hours after injection revealed localization of the $^{125}\text{I}$-anti-LYVE-1 antibody at vessel-like structures in the draining LNs, ears, lungs, and intestine (Fig. 2A–F and H–M). No signal was detected in samples obtained from mice injected with $^{125}\text{I}$-control IgG (Fig. 2O–U). The localization pattern of the $^{125}\text{I}$-anti-LYVE-1 antibody determined by microautoradiography overlapped with staining for LYVE-1 with a rabbit antibody in serial sections (Fig. 2V–Y). Preinjection of a 17.8-fold excess of unlabeled anti-LYVE-1 antibody inhibited binding of $^{125}\text{I}$-anti-LYVE-1 antibody to lymphatic vessels (Fig. 2H–N) and also reduced the uptake of radiolabeled antibody into LNs (Fig. 3C), confirming that the binding of $^{125}\text{I}$-anti-LYVE-1 antibody was specific.

**Systemically injected anti-VEGF receptor-3 antibody accumulates in the lymphatic vasculature in vivo**

We confirmed the feasibility of directing antibodies to epitopes of lymphatic vessel by systemic administration with an antibody to VEGF receptor-3 (VEGFR-3). Detection of the injected anti-VEGFR-3 antibody in tissue sections by fluorescently labeled secondary antibodies (Supplementary Fig. S2) and microradiographies of tissue sections of mice injected with $^{125}\text{I}$-anti-VEGFR-3 (Supplementary Fig. S3) indicated the specific accumulation of anti-VEGFR-3 antibody in lymphatic vessels. Despite these findings, we used the antibody to LYVE-1 for our further studies because...
the anti-VEGFR-3 antibody strongly inhibits lymphangiogenesis in vivo (27, 28).

**Radiolabeled anti-LYVE-1 antibody accumulates in a dose-dependent manner in the lymphatic vessels of LNs**

We performed biodistribution experiments with the 125I-anti-LYVE-1 antibody and 125I-control rat IgG. Seven, 35, or 90 μg of antibody were injected i.v. into K14/VEGF mice with unilateral, inflammation-induced LN lymphangiogenesis and biodistribution analyses were performed 1 day after antibody injection. The auricular LNs draining the inflamed ear accumulated 1.4- to 1.9-fold more anti-LYVE-1 antibody than the control LN (Fig. 3A). Calculation of the percentage of the injected dose per gram of tissue for different mouse tissues revealed that increasing the antibody dose from 7 to 35 μg led to a 4.6-fold increased uptake in the inflamed LNs (Supplementary Fig. S4A–C). Elevation of the dose to 90 μg did not further increase the antibody concentration in LNs compared with other organs (Supplementary Fig. S4A–C). The uptake of control IgG in LNs was less than in mice injected with the anti-LYVE-1 antibody.

The ratio between the auricular LNs and the neighboring salivary glands did not change significantly over time (Supplementary Fig. S5). Therefore, and because the radioactivity that accumulated within the LNs diminished from day 1 to day 3 (Fig. 3B), we chose day 1 as the best time point for the subsequent in vivo imaging studies.

Analysis of serum at 4, 24, and 52 hours after antibody injection by gel filtration revealed increasing relative amounts of free compared with antibody-bound 125I over time (Supplementary Fig. S6). No major shift to smaller fragments was detected, indicating that the antibody was stable during the observed period.

**In vivo imaging of lymphangiogenesis in inflamed LNs by PET**

Based on the encouraging biodistribution results, we investigated whether it was possible to visualize lymphatic vessels within LNs by in vivo PET of mice. We radiolabeled the anti-LYVE-1 antibody and control rat IgG with the positron emitter 124I. The immunoreactivity of 124I-anti-LYVE-1 antibody to LYVE-1 was 94%. We performed competitive RIAs and found that the affinity of LYVE-1 antibody to LYVE-1 was unchanged after radiolabeling (Supplementary Fig. S7).

K14/VEGF mice bearing unilateral LN lymphangiogenesis were scanned by PET at day 1 after antibody injection. We chose this time point because we found the highest specific accumulation of the radioactively labeled antibody in the LNs at this time point in the biodistribution studies with 125I-anti-LYVE-1 antibody (Fig. 3B). In mice injected with 124I-anti-LYVE-1 antibody, strong radioactive signals were produced at the sites of the auricular, brachial, and axillary LNs (Fig. 4A; Supplementary Video S1). Importantly, the inflamed auricular LNs with ongoing LN lymphangiogenesis produced stronger radioactive signals than the contralateral LNs (Fig. 4A; Supplementary Video S1) that were also imaged at day 2 after antibody injection (Supplementary Video S5). 124I-control IgG had a different distribution pattern, with localization primarily in the blood (Fig. 4B; Supplementary Videos S2 and S6).

There was an increased accumulation of the 124I-anti-LYVE-1 antibody, compared with 124I-control IgG, in the LNs. The 124I-anti-LYVE-1 antibody accumulation was clearly visible in planes of auricular, axillary, and brachial LNs (Fig. 4C, Supplementary Fig. S8) in normalized coronal PET...
sections. Meanwhile, no LNs could be discerned from the corresponding sections of mice injected with 124I-control IgG (Fig. 4D; Supplementary Fig. S9).

For a definitive proof that the 124I-anti-LYVE-1 antibody was localized to the auricular LNs, animals were sacrificed and imaged ex vivo by PET, with the dissected auricular LNs placed next to the heads (Supplementary Video S3). The isolated LNs emitted a radioactive signal, whereas the radioactive signals in the throat region that had been present in the in vivo scans were gone (Fig. 4E and F; Supplementary Fig. S10). In mice injected with control IgG, no LNs could be detected (Supplementary Fig. S11; Supplementary Video S4). This is the first demonstration that LN lymphangiogenesis can be imaged in vivo by PET following systemic injection of a radiolabeled, lymphatic-specific antibody.

**In vivo imaging of lymphangiogenesis in tumor-draining LNs by PET**

We next injected 124I-anti-LYVE-1 antibody or 124I-control IgG into C37BL/6N mice bearing B16-F1-VEGF-C tumors in the footpads that were ∼90 mm³ in size. Strikingly, in 124I-anti-LYVE-1 antibody–injected mice, tumor-draining popliteal LNs were clearly visible by PET, in contrast to contralateral control LNs (Fig. 5A; Supplementary Video S7). In comparison, 124I-control IgG was mainly localized in the blood (Fig. 5B; Supplementary Video S8). Normalized serial sections confirmed that 124I-anti-LYVE-1 antibody, but not 124I-control IgG, accumulated in the tumor-draining LNs (Fig. 5C and D; Supplementary Figs. S12 and S13). After the in vivo scan, the popliteal LNs were removed, and the animals and the dissected popliteal LNs were rescanned by PET. The isolated LNs emitted a radioactive signal, whereas the radioactive signal in the knee region that had been present in the in vivo scan was gone (data not shown). In agreement, immunofluorescence analysis of popliteal LN sections showed expansion of LYVE-1–positive lymphatic vessels in tumor-draining LNs compared with control LNs (Fig. 5E and F). Tissue distributions of 124I-anti-LYVE-1 antibody and 124I-control IgG were quantified directly after PET (Supplementary Fig. S14A), with a 4.1-fold to 5.5-fold enhanced 124I-anti-LYVE-1 antibody accumulation in tumor-draining LNs compared with control LNs (Supplementary Fig. S14B).

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Figure 6. LYVE-1 immuno-PET detects metastasis-associated lymphangiogenesis in LNs that are not detected by conventional 18F-FDG-PET. A–D, bioluminescence imaging of luciferase expressing metastatic melanoma cells in the tumor-draining popliteal LNs. The color bars indicate photons/second/cm²/surface radiance (p/sec/cm²/sr). E–H, 18F-FDG-PET signal in the popliteal LN (arrow head) of mouse 1 (E), but of none of the other mice (F–H). I–L, 124I-anti-LYVE-1 immuno-PET visualizing lymphatic vessel expansion in the tumor-draining LNs (arrows) of all mice.
Detection of expanded lymphatic vessels in metastatic LNs that are not detected by $^{18}$F-FDG-PET

We next compared the $^{124}$I-anti-LYVE-1-PET method with $^{18}$F-FDG-PET imaging, which is currently used to detect cancer metastases in human patients (29). To investigate whether the mice harbored metastases in their tumor-draining LNs, bioluminescence imaging was performed 19 days after the injection of B16-F10-luc2-VEGFR-C tumor cells into the footpads of 16 C57BL/6J-Tyr c −/− mice. Three mice showed a strong bioluminescence signal and one mouse showed a weak bioluminescence signal in their tumor-draining LNs (Fig. 6A–D), indicating the presence of metastases in all four mice. $^{18}$F-FDG-PET on the following day detected a radioactive signal in the region of the popliteal LN of only one mouse (Fig. 6E–H). In contrast, $^{124}$I-anti-LYVE-1-PET performed 1 day later revealed lymphatic vessel expansion in the LNs of all four mice (Fig. 6I–L). These data suggest that imaging of tumor-induced stromal changes by immuno-PET might be more sensitive for the detection of metastasis than conventional $^{18}$F-FDG-PET.

Discussion

We show for the first time that lymphatic vessels can be targeted and imaged by specific antibodies and provide the proof-of-principle for the noninvasive in vivo imaging of lymphangiogenesis in inflammation and tumor-draining LNs by PET. To determine whether a systemically injected antibody against a lymphatic epitope could localize to and be imaged in the lymphatic vessels, we chose previously described rat anti-mouse LYVE-1 and VEGFR-3 antibodies, because LYVE-1 and VEGFR-3 are almost exclusively expressed by lymphatic vessels (25, 26, 30–35). Our combined microautoradiography and immunofluorescence studies revealed that these antibodies specifically accumulated in LYVE-1–positive lymphatic vessels of the skin and the LNs after i.v. administration. The accumulation of the antibodies in lymphatic vessels was specific, because injection of equal amounts of control IgG did not lead to any detectable accumulation in lymphatic vessels. The LN targeting performance of $^{125}$I-anti-LYVE-1 antibody increased when the antibody dose was increased, probably because at higher doses the LYVE-1 molecules in the lung were saturated. Increased targeting performance with increased levels of ligand have been described previously (36).

The signal in PET produced by the inflamed auricular LN, which had ongoing LN lymphangiogenesis, was stronger than that of the uninflamed control LN. The increased accumulation of the antibody at this site was likely to result from an increased number of LYVE-1 molecules in areas of expanded lymphatic networks, because immunoblot analyses of LN lysates also revealed an increased amount of LYVE-1 protein in inflammation-draining LNs compared with normal LNs (data not shown).

Most importantly, $^{124}$I-anti-LYVE-1 antibody–based PET enabled detection of lymphatic vessel expansion within melanoma-draining LNs. This novel method of imaging LN lymphangiogenesis provides a new strategy for the early detection of metastases in LNs and has several advantages over currently used methods. LN lymphangiogenesis has been identified as an early marker of metastasis to LNs in experimental models (10–13) and has also been observed in patients with melanoma or breast cancer and found to be a significant predictor of distant metastasis (14, 15). Thus, the use of PET with radiolabeled anti-LYVE-1 antibodies to detect LN lymphangiogenesis may represent a less invasive, simpler, and potentially more sensitive method to identify patients with LN metastases than current approaches, including sentinel LN dissection, which is associated with significant side effects such as lymphedema (2–4). The method also avoids the need to inject dyes around tumors; this technique does not always lead to the detection of all draining LNs due to the location of the injection.

Our results suggest that LYVE-1 immuno-PET might be more sensitive in detecting metastatic LNs than conventional $^{18}$F-FDG-PET. However, inflammation and possibly also autoimmune responses or infections might also cause lymphatic vessel expansion in LNs (18, 37), and these conditions could coexist in some patients. At present, the method is not specific enough to discern the etiology of expanded lymphatic vessels in LNs. Still, it could be applied to indicate potential (pre-)metastatic LNs and therefore avoid unnecessary dissection of unaffected sentinel LNs for prognostic purposes and the related side effects.

The biodistribution data revealed that anti-LYVE-1 did not strongly accumulate in organs besides LNs or lung, suggesting potential low tissue toxicity of radiolabeled LYVE-1 antibodies in most tissues. However, dosimetry will be indispensable to evaluate the applicability of radiolabeled anti-LYVE-1 antibodies in human cancer patients. Potential issues with high uptake in the lungs might be avoided by a preinjection of unlabeled anti-LYVE-1 antibody to block the LYVE-1 molecules in the lung, because our biodistribution data showed preferential binding of anti-LYVE-1 antibody in the lung. A second injection with radioactively labeled antibody might then bind more specifically to LYVE-1 molecules in the LNs.

Immuno-PET with lymphatic specific antibodies could be applied to medical fields beyond oncology, because many pathologic conditions (e.g., chronic inflammatory diseases including rheumatoid arthritis) are associated with lymphangiogenesis (38–40). Thus, lymphangiogenesis could be imaged and used as a biomarker for disease progression or response to therapy.

Disclosure of Potential Conflicts of Interest

V. Mumprecht and M. Detmar have filed for a patent (International Patent Number WO 2010/097182A1) for a method of lymphangiogenesis by immune-positron emission. This potential patent has not caused any minor or major conflict to date, but it could bring some financial gain in the future.

Acknowledgments

We thank Claudia Keller, Dr. Cornelia Halin-Winter, Dr. Christoph Wissmann, and Martin Badertscher for technical support.
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Received 03/12/2010; revised 09/01/2010; accepted 09/02/2010; published OnlineFirst 10/26/2010.

Noninvasive Imaging of Lymph Node Lymphangiogenesis

Grant Support
NIH grant CA69184, Swiss National Science Foundation grant 3100A0-108207, Austrian Science Foundation grant S9408-B11, and Cancer League Zurich, Oncosuisse and Commission of the European Communities grant LSHC-CT-2005-518178 (M. Detmar).

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In vivo Imaging of Inflammation- and Tumor-Induced Lymph Node Lymphangiogenesis by Immuno–Positron Emission Tomography

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