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***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 is a prognostic indicator for cancer progression. There is a great demand for sensitive and non-invasive methods to detect metastasis to the lymph nodes. While conventional *in vivo* imaging approaches have focused on the detection of cancer cells, lymphangiogenesis within tumor draining lymph nodes might be the earliest sign of metastasis. In mouse models of lymph node lymphangiogenesis, we found that systemically injected antibodies to lymphatic epitopes accumulated in the lymphatic vasculature in tissues and lymph nodes. 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 lymph nodes with expanded lymphatic networks *in vivo* by positron emission tomography (PET). Anti-LYVE-1 immuno-PET enabled visualization of lymphatic vessel expansion in lymph nodes 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.

Keywords

lymphangiogenesis; lymph node; metastasis; inflammation; cancer; positron-emission tomography; non-invasive imaging; LYVE-1; immuno-PET

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 the regional lymph nodes (LNs) 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, non-invasive, and preferentially

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simpler methods to detect metastasis to the 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 on-set 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 the 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 non-invasively *in vivo* using positron emission tomography (PET) with radiolabeled antibodies to lymphatic specific epitopes (immuno-PET). PET is a non-invasive, 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/VEGF transgenic mice) (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 non-invasive 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, Switzerland, tested for microbial contaminations before the experiment) were transfected by Lipofectamine (Invitrogen, Carlsbad, CA) with full-length human-VEGF-C subcloned into the pcDNA3.1 vector (Invitrogen). 2×10^5 B16-F1-VEGF-C cells were injected into the left footpads of female C57BL/6 N mice (Charles River Laboratories, Wilmington, MA) as described (13). For bioluminescence imaging experiments, firefly expressing B16-F10-luc2 cells (Caliper Life Sciences, Hopkinton, MA, purchased before the experiment) were transfected with VEGF-C and injected into female C57BL/6J-*Tyr^{c-J}* (albino) mice (The Jackson Laboratory, Bar Harbour, ME) as described

above. All animal experiments were approved by the cantonal veterinarian office Zurich (protocols 123/2005, 149/2008 and 128/2008).

Ex vivo fluorescence experiments

Eighty-five micrograms of rat anti-mouse LYVE-1 antibody (clone 223322, R&D Systems, Minneapolis, MN, USA, <0.1 EU endotoxin/ μ g) or isotype-matched rat control IgG (AbD Serotec, Duesseldorf, Germany, <0.01 EU endotoxin/ μ g) 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, Zoeterwoude, Netherlands). Seven-micrometer sections were fixed with 4% paraformaldehyde in PBS, incubated with an Alexa Fluor (AF) 594 conjugated donkey anti-rat IgG antibody (Invitrogen) and co-stained with a rabbit anti-mouse LYVE-1 antibody (Angiobio, Del Mar, CA, USA) detected by an AF488 donkey anti-rabbit IgG antibody (Invitrogen), or co-stained with a biotinylated rat Meca32 antibody (BD Pharmingen, Franklin Lakes, NJ) detected by AF488 streptavidin (Invitrogen). Sections were counterstained with Hoechst 33342 (Invitrogen) and analyzed with an AxioScop2 mot plus microscope (Zeiss, Oberkochen, Germany). 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, Oxford, UK) were radiolabeled with Na¹²⁵I (Perkin Elmer, Waltham, MA, USA; radiochemical purity 99.0 %, radionuclide purity 99.95 % with <0.04 % contamination by ¹²⁶I) or with Na¹²⁴I (IBA Molecular, Louvain-La-Neuve, Belgium, radiochemical purity 95 %, radionuclide purity > 99 % with < 0.5 % contamination by ¹²³I and < 0.1 % contamination by ¹²⁵I) by adapting the standard chloramine-T method (20). Briefly, 70 – 380 μ Ci Na¹²⁵I and 5 μ l of 5 mg/ml chloramine T (Sigma-Aldrich) were added per 100 μ g antibody in PBS (1 mg/ml) or 2–2.7 mCi Na¹²⁴I, and 9 μ l of 5 mg/ml chloramine T were added to 180 μ g antibody in PBS (1 mg/ml). After 2 minutes, the radiolabeled antibodies were separated from free ¹²⁵I using PD10 columns (GE-Healthcare, Chalfont St. Giles, UK). The radioactivity of the samples was determined using a γ -counter (Cobra Autogamma, Packard Instrument Comp., Meriden, CT). 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 four days before an experiment and oral administration of sodium perchlorate one hour before antibody injection.

Biodistribution studies

K14/VEGF mice were injected intravenously with either 7 μ g (16 μ Ci, i.e. 2.29 μ Ci/ μ g antibody; n=5), 35 μ g (80 μ Ci, i.e. 2.29 μ Ci/ μ g antibody; n=5), or 90 μ g (55 μ Ci, i.e. 0.61 μ Ci/ μ g antibody; n=3) of ¹²⁵I-anti-LYVE-1 antibody, or with equal amounts of ¹²⁵I-rat control IgG at 6 or 8 days after challenging one ear with oxazolone. Mice were sacrificed 24 h 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 timecourse experiments, K14/VEGF mice were given intravenous injections of 37 μ g (52 μ Ci, i.e. 1.4 μ Ci/ μ g antibody) of ¹²⁵I-anti-LYVE-1 antibody 13 days after oxazolone challenge. Four animals each were analyzed at 2 and at 3 days after injection. To assess the metabolic stability of ¹²⁵I-anti-LYVE-1, serum was collected (3 mice) and applied to PD MiniTrap G-25 gel filtration units (GE-Healthcare). Fractions of 300 μ l were collected and radioactivity was measured.

Microradiography

^{125}I -anti-LYVE-1 antibody (35 μg , 150 μCi , i.e. 4.3 $\mu\text{Ci}/\mu\text{g}$ antibody) or ^{125}I -rat control IgG (35 μg , 120 μCi , i.e. 3.4 $\mu\text{Ci}/\mu\text{g}$ antibody) were injected intravenously into K14/VEGF mice at 10 days after the challenge ($n = 2$ per group). In an additional experiment, mice ($n = 3$) were pre-injected with 625 μg unlabeled antibody one day before injection of ^{125}I -anti LYVE-1 antibody (3 mice with pre-injection, 2 without pre-injection). 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., Rochester, NY), and developed according to the manufacturer's instructions after 2 weeks exposure time.

Positron emission tomography

^{124}I -anti-LYVE-1 (38 μg , 0.37–0.42 mCi, i.e. 9.7–11 $\mu\text{Ci}/\mu\text{g}$ antibody) or ^{124}I -labelled rat control IgG (38 μg , 0.34–0.36 mCi, i.e. 8.9–9.4 $\mu\text{Ci}/\mu\text{g}$ antibody) were injected intravenously into K14/VEGF mice after oxazolone treatment of the ear skin (3 mice per treatment). PET scans were performed approximately 24 h (2 mice per group) or 48 h (1 mouse per group) after intravenous 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, Abbott Park, IL) in an air/oxygen mixture as described previously (22). Auricular LNs were dissected and the mice were re-scanned *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 min acquisition time per position) and were reconstructed in a single time frame, with pixel sizes of 0.3875 mm and 0.775 mm in the transverse and axial directions, respectively. Series of coronal image slices and maximum intensity projections (MIPs) (23) as well as MIP movies, were generated using the software PMOD (PMOD Technologies Ltd., Adliswil, Switzerland). Coronal PET sections were displayed with a fixed grey 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, ^{124}I -anti-LYVE-1 (30 μg , 0.26–0.38 mCi, i.e. 8.7–12.7 $\mu\text{Ci}/\mu\text{g}$ antibody) or ^{124}I -rat control IgG (30 μg , 0.32–0.33 mCi, i.e. 10.7–11 $\mu\text{Ci}/\mu\text{g}$ antibody) were injected intravenously into tumor bearing mice 19 or 20 days after tumor cell injection. PET and biodistribution analyses of selected organs were performed approximately 18 h after antibody injection (two mice per group) as described above. For ^{18}F -FDG-PET, 0.3 – 0.6 mCi ^{18}F -FDG was injected intravenously 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 s. 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 following systemic injection of an antibody against a lymphatic-specific epitope, the antibody accumulates in the lymphatic vasculature in an established mouse model of chronic skin inflammation (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 ear draining (auricular) LNs (18). K14/VEGF mice were given intravenous injections of a rat antibody to the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) (25,26), or control IgG. In tissue sections of auricular LNs obtained 24 h after injection, the anti-LYVE-1 antibody was detected by a fluorescently labeled anti-rat 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 co-staining with a rabbit antibody to LYVE-1 (Fig. 1C and K). The anti-LYVE-1 antibody did not co-localize 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}I in the biodistribution experiments and therefore assessed the quality of ^{125}I -anti-LYVE-1 antibody. The immunoreactivity of ^{125}I -anti-LYVE-1 antibody to LYVE-1 was 92%. To compare the binding capacities of ^{125}I -anti-LYVE-1 and unlabeled antibody, we performed a competitive radioimmunoassay. Unlabeled anti-LYVE-1 antibody dose-dependently inhibited binding of ^{125}I -anti-LYVE-1 antibody to immobilized LYVE-1 (Supplementary Figure 1). Half-maximal binding of ^{125}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}I -anti-LYVE-1 antibody into K14/VEGF mice with unilateral LN lymphangiogenesis. Microradiography of tissue sections obtained 24 h after injection revealed localization of the ^{125}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}I -control IgG (Fig. 2O-U). The localization pattern of the ^{125}I -anti-LYVE-1 antibody determined by microautoradiography overlapped with staining for LYVE-1 with a rabbit antibody in serial sections (Fig. 2V-Y). Pre-injection of a 17.8-fold excess of unlabeled anti-LYVE-1 antibody inhibited binding of ^{125}I -anti-LYVE-1 antibody to lymphatic vessels (Fig. 2. H-N) and also reduced the uptake of radiolabeled antibody into the lymph nodes (Fig. 3C), confirming that the binding of ^{125}I -anti-LYVE-1 antibody was specific.

Systemically injected anti-VEGFR-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 vascular endothelial growth factor receptor-3 (VEGFR-3). Detection of the injected anti-VEGFR-3 antibody in tissue sections by fluorescently labeled secondary antibodies (Supplementary Figure 2) and microradiographies of tissues sections of mice injected with ^{125}I -anti-VEGFR-3 (Supplementary Figure 3) indicated the specific accumulation of anti-VEGFR-3 antibody in lymphatic vessels. Despite this 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 ^{125}I -anti-LYVE-1 antibody and ^{125}I -control rat IgG. Seven, thirty-five, or ninety micrograms of antibody were injected intravenously 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 – 1.9 fold more anti-LYVE-1 antibody than the control LN (Fig. 3A). Calculation of the percentage of the injected dose [ID]/g of tissue for different mouse tissues revealed that increasing the antibody dose from 7 μg to 35 μg led to a 4.6-fold increased uptake in the inflamed LNs (Supplementary Fig. 4A – C). Elevation of the dose to 90 μg did not further increase the antibody concentration in the LNs compared to other organs (Supplementary Fig. 4A – C). The uptake of control IgG in the 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. 5). Therefore, and since 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 h, 24 h, and 52 h after antibody injection by gel filtration revealed increasing relative amounts of free compared to antibody-bound ^{125}I over time (Supplementary Figure 6). 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 ^{124}I . The immunoreactivity of ^{124}I -anti-LYVE-1 antibody to LYVE-1 was 94%. We performed competitive radioimmunoassays and found that the affinity of LYVE-1 antibody to LYVE-1 was unchanged after radiolabeling (Supplementary Figure 7).

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 lymph nodes at this time point in the biodistribution studies with ^{125}I -anti-LYVE-1 antibody (Fig 3B). In mice injected with ^{124}I -anti-LYVE-1 antibody, strong radioactive signals were produced at the sites of the auricular, brachial and axillary LNs (Fig. 4A, and Supplementary Video 1). Importantly, the inflamed auricular LNs with on-going LN lymphangiogenesis produced stronger radioactive signals than the contra-lateral LNs (Fig. 4A, Supplementary Video 1) what was also imaged at day 2 after antibody injection (Supplementary Video 5). ^{124}I -control IgG had a different distribution pattern; with localization primarily in the blood (Fig. 4B and Supplementary Video 2 and 6).

There was an increased accumulation of the ^{124}I -anti-LYVE-1 antibody, compared with ^{124}I -control IgG, in the LNs. The ^{124}I -anti-LYVE-1 antibody accumulation was clearly visible in planes of auricular, axillary, and brachial LNs (Fig. 4C, Supplementary Fig. 8) in normalized coronal PET sections. Meanwhile, no lymph nodes could be discerned from the

corresponding sections of mice injected with ^{124}I -control IgG (Fig. 4D, Supplementary Fig. 9).

For a definitive proof that the ^{124}I -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 3). The isolated LNs emitted a radioactive signal, while the radioactive signals in the throat region that had been present in the *in vivo* scans were gone (Fig. 4E and F, Supplementary Fig. 10). In mice injected with control IgG, no lymph nodes could be detected (Supplementary Fig. 11, Supplementary Video 4). 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 ^{124}I -anti-LYVE-1 antibody or ^{124}I -control IgG into C57BL/6N mice bearing B16-F1-VEGF-C tumors in the footpads, that were approximately 90 mm³ in size. Strikingly, in ^{124}I -anti-LYVE-1 antibody injected mice, tumor-draining popliteal LNs were clearly visible by PET, in contrast to contra-lateral control LNs (Fig. 5A, Supplementary Video 7). In comparison, ^{124}I -control IgG was mainly localized in the blood (Fig. 5B, Supplementary Video 8). Normalized serial sections confirmed that ^{124}I -anti-LYVE-1 antibody but not ^{124}I -control IgG accumulated in the tumor draining LNs (Fig. 5C and D, Supplementary Fig. 12 and 13). 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, while 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 to control LNs (Fig. 5E and F). Tissue distributions of ^{124}I -anti-LYVE-1 antibody and ^{124}I -control IgG were quantified directly after PET (Supplementary Fig. 14 A), with a 4.1- to 5.5-fold enhanced ^{124}I -anti-LYVE-1 antibody accumulation in tumor draining compared to control LNs (Supplementary Fig. 14 B).

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 -fluorodeoxyglucose-PET (^{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-VEGF-C tumor cells into the footpads of 16 C57BL/6J-*Tyr^{c-J}* mice. Three mice showed a strong and one mouse 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 one 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 non-invasive *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 intravenous 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 lymph node 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 on-going 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 since immunoblot analyses of LN lysates also revealed an increased amount of LYVE-1 protein in inflammation-draining LNs, compared to 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, that is associated with significant side effects such as lymph edema (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 as well cause lymphatic vessel expansion in LNs (18,37) and these conditions could co-exist 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 humans cancer patients. Potential issues with high uptake in the lungs might be avoided by a pre-injection of unlabeled anti-LYVE-1 antibody to block the LYVE-1 molecules in the lung, since 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 pathological 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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) co-localized 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 panels B and C. (H) Merged panels F and G. (L) Merged panels J and K. (P) Merged panels N and O. The injected control IgG (R) did not co-localize with LYVE-1-positive lymphatic vessels (S). Panels 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) Pre-injection 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 .

Figure 3. Concentration- and time-dependent accumulation of ^{125}I -anti-LYVE-1 antibody in LNs

(A) Concentration dependent accumulation in the ear draining LNs of different doses of ^{125}I -anti-LYVE-1 antibody or ^{125}I -control IgG in mice. The results were expressed as counts per minute (cpm) $\pm$ 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 ^{125}I -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) Pre-injection of a 17.8-fold excess of unlabeled anti-LYVE-1 antibody inhibits binding of injected ^{125}I -anti-LYVE-1 antibody in the ear draining lymph nodes.

Figure 4. *In vivo* PET of inflammation- induced lymphangiogenesis in auricular LNs using ^{124}I -anti-LYVE-1 antibody

(A, B) MIPs of *in vivo* scanned mice injected with ^{124}I -anti-LYVE-1 antibody (A, n = 3) or ^{124}I -control IgG (B, n = 3). (A) The inflamed auricular LN (black arrow) accumulated more ^{124}I -anti-LYVE-1 antibody than the contra-lateral control auricular LN (grey arrow). Brachial and axillary LNs were also detected (arrow heads). (B) *In vivo* PET of a mouse injected with ^{124}I -control IgG; the black arrow indicates the heart. (C, D) Normalized coronal PET sections of mice injected with ^{124}I -anti-LYVE-1 antibody (C) or ^{124}I -control IgG (D). ^{124}I -anti-LYVE-1 antibody accumulation in the inflamed LN (black arrow), its control LN (grey arrow), brachial and axillary LNs (arrow heads). (D) (E, F) Normalized coronal PET sections of the ^{124}I -anti-LYVE-1 antibody injected mouse from panel C with dissected auricular LNs.

Figure 5. *In vivo* PET of tumor-induced lymphangiogenesis in popliteal LNs using ^{124}I -anti-LYVE-1 antibody
 (A, B) MIPs of *in vivo* scanned mice injected with ^{124}I -anti-LYVE-1 antibody (A, n = 2) or ^{124}I -control IgG (B, n = 2). (A) The tumor draining popliteal LN (black arrow) is clearly visible, in contrast to the contra-lateral control popliteal LN. (C, D) Normalized coronal PET sections of mice injected with ^{124}I -anti-LYVE-1 antibody (C) or ^{124}I -control IgG (D) reveal ^{124}I -anti-LYVE-1 antibody accumulation in the tumor draining popliteal LN (C; black arrow), whereas in corresponding sections of a ^{124}I -control IgG injected mouse, no signals in LNs were detected. (E, F) LYVE-1-positive lymphatic sinuses in sections of tumor draining (E) and contra-lateral control (F) popliteal LNs. Scale bars = 100 μm .

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.