Quantitative Imaging of Lymphatic Function with Liposomal Indocyanine Green

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Abstract

Lymphatic vessels play a major role in cancer progression and in postsurgical lymphedema, and several new therapeutic approaches targeting lymphatics are currently being developed. Thus, there is a critical need for quantitative imaging methods to measure lymphatic flow. Indocyanine green (ICG) has been used for optical imaging of the lymphatic system, but it is unstable in solution and may rapidly enter venous capillaries after local injection. We developed a novel liposomal formulation of ICG (LP-ICG), resulting in vastly improved stability in solution and an increased fluorescence signal with a shift toward longer wavelength absorption and emission. When injected intradermally to mice, LP-ICG was specifically taken up by lymphatic vessels and allowed improved visualization of deep lymph nodes. In a genetic mouse model of lymphatic dysfunction, injection of LP-ICG showed no enhancement of draining lymph nodes and slower clearance from the injection site. In mice bearing B16 luciferase-expressing melanomas expressing vascular endothelial growth factor-C (VEGF-C), sequential near-IR imaging of intradermally injected LP-ICG enabled quantification of lymphatic flow. Increased flow through draining lymph nodes was observed in mice bearing VEGF-C–expressing tumors without metastases, whereas a decreased flow pattern was seen in mice with a higher lymph node tumor burden. This new method will likely facilitate quantitative studies of lymphatic function in preclinical investigations and may also have potential for imaging of lymphedema or improved sentinel lymph detection in cancer.

Introduction

The lymphatic vascular system has recently garnered an increasing amount of attention, as its diverse roles in tumor metastasis, chronic inflammation, and lymphedema have been better understood (1, 2). In particular, there is increasing evidence that tumor-associated lymphatic vessel growth promotes lymph node metastasis (3–5) and that tumor-induced lymphangiogenesis within sentinel lymph nodes enhances further metastatic spread (6–8). Because novel therapeutic approaches for the treatment of tumor lymphangiogenesis, as well as for the therapy of postsurgical lymphedema in cancer patients, are currently being developed, there is a critical need for quantitative imaging methods to measure lymphatic function and to quantify lymphatic flow.

Lymphatic imaging has traditionally been performed clinically with direct lymphangiography methods using computed tomography or lymphoscintigraphy with 99mTc bound to either sulfur colloid or albumin (9). These methods have several shortcomings, particularly when applied to animal models. These include the need to directly inject contrast agent into lymphatic vessels, the use of radioactive tracers, and poor anatomic spatial resolution (9). More recently, magnetic resonance imaging (MRI) methods that can detect gadolinium- or iron-based contrast agents in lymphatic vessels and within lymph nodes have been developed (10–14). However, the costly and technically demanding nature of MRI, especially when applied to preclinical research, has led to investigation of alternative modalities to image the lymphatic system.

Fluorescence imaging methods focusing on contrast agents that have spectral properties in the near-IR (NIR) region have recently shown great promise for lymphatic imaging (15, 16). NIR light, in the wavelength range between 700 and 900 nm, allows deeper penetration of photons into living tissue due to reduced absorption and scattering and minimal tissue autofluorescence (17). The NIR dye indocyanine green (ICG) has been approved by the Food and Drug Administration (FDA) as an agent to evaluate hepatic function, cardiac output, and for ophthalmic angiography (16, 18). ICG binds quickly to serum albumin after intravenous injection and is rapidly cleared through the liver and bile duct (19–21). Recent studies have shown the potential of this dye to image lymphatics in animal models and in humans (22). However,
several aspects of the dye make it nonideal for quantitative lymphatic imaging. First, ICG is unstable in solution. It aggregates and self-quenches (23) and therefore must be injected within hours as it otherwise rapidly loses its fluorescence. In vivo, plasma proteins, such as albumin, bind preferentially to the monomeric ICG molecules and reduce the aggregation (24, 25). Thus, prevention of aggregation of ICG is dependent on the availability of plasma proteins at the injection site, placing a limitation on the concentration that can be administered. Second, the quantum yield of ICG is inherently low, although this is improved to some extent with protein binding (26). Third, because of its low molecular weight, ICG rapidly leaks out of collecting lymphatics into the extravascular space or is being taken up by venous capillaries at the site of injection or by high endothelial venules within the lymph nodes. For these reasons, ICG has been complexed to human serum albumin (HSA) before injection, improving the optical characteristics and preventing aggregation (26, 27). In attempts to increase ICG stability in biological fluids without altering its biocompatibility, the dye has been encapsulated in PLGA nanoparticles (28) or conjugated to polyether polyol dendrons (29).

Colloidal particles can interact with ICG and induce changes in spectral properties (27, 30). For instance, it was found that large phospholipid vesicles reduced the self-quenching of the dye and slightly increased its quantum yield (30). More recently, ICG was stabilized by its association to surfactant micelles (31). Despite interesting in vitro data, the in vivo efficacy of these systems has not been assessed thus far. In the case of micelles, their dilution following injection may rapidly induce their dissociation (32) and premature release of ICG. Owing to their well-established safety profile, stability on dilution, small size, and versatility in terms of composition and surface properties, we hypothesized that liposomes might constitute a promising vehicle to image the lymphatic system.

In this study, we designed a liposomal formulation of ICG (LP-ICG) that possesses ideal attributes for lymphatic function imaging. We found that LP-ICG displays improved optical properties and prolonged fluorescence stability in solution over weeks. After intradermal injection, several draining lymph nodes were clearly visualized, allowing the release kinetics was monitored over 3 hours at 37°C. In vivo, plasma proteins, such as albumin, bind preferentially to the monomeric ICG molecules and reduce the aggregation (24, 25). Thus, prevention of aggregation of ICG is dependent on the availability of plasma proteins at the injection site, placing a limitation on the concentration that can be administered. Second, the quantum yield of ICG is inherently low, although this is improved to some extent with protein binding (26). Third, because of its low molecular weight, ICG rapidly leaks out of collecting lymphatics into the extravascular space or is being taken up by venous capillaries at the site of injection or by high endothelial venules within the lymph nodes. For these reasons, ICG has been complexed to human serum albumin (HSA) before injection, improving the optical characteristics and preventing aggregation (26, 27). In attempts to increase ICG stability in biological fluids without altering its biocompatibility, the dye has been encapsulated in PLGA nanoparticles (28) or conjugated to polyether polyol dendrons (29).

Materials and Methods

Chemicals

ICG (IR-25, laser grade pure) was purchased from Acros Organics. 1,2-Oleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG-DSPE) were a gift from Lipoid GmbH. D-(+)-Glucose monohydrate and HSA (97–99% purity) were purchased from Sigma-Aldrich. Chloroform (analytic grade, Fluka) and DMSO (Uvasol, spectroscopic grade, Merck) were used as received.

Liposome preparation

Liposomes were prepared by the film hydration/extrusion method (33). DOPC and PEG-DSPE (95:5 mol %, 3.9 mmol/L total lipid concentration) were codissolved in chloroform. The organic solvent was eliminated under nitrogen, and the lipid film was placed under vacuum overnight. The dried film was then hydrated with an isotonic glucose solution (5%, w/v) containing ICG (15 μmol/L, lipid/dye molar ratio of 260). The ICG-containing dispersion was freeze thawed 6 times and extruded 10 times through double-stacked 50-nm pore size polycarbonate membranes using a Lipex thermobarrel extruder (Northern Lipids) to yield small unilamellar vesicles. Free dye was removed by size exclusion chromatography on a PD MidiTrap G-25 column (GE Healthcare) using glucose buffer as eluent.

Formulation stability

Absorbance and fluorescence measurements were performed over 42 days on an Infinite M200 microplate reader (Tecan Ltd.) using black/clear (flat bottom, untreated) OptiLux 96-well plates (BD Biosciences). The scanned visible range was 600 to 900 nm. The set parameters for the fluorescence scans were as follows: λex = 720 nm and λem = 750 to 850 nm (manual gain optimization). During the time of observation, ICG-containing formulations were stored in the dark at room temperature. Stability of free and encapsulated ICG was monitored in a serum-containing medium [fetal bovine serum (FBS) mixed at 50% (v/v) with isotonic glucose] over 12 hours. Fluorescence spectra were recorded on a Cary Eclipse Fluorescence spectrophotometer (Varian, Inc.), equipped with a Peltier circulating water bath to strictly control the temperature. Serum-induced bilayer destabilization was monitored using a fluorescence dequenching assay (details provided as Supplementary Materials and Methods). DOPC/PEG-DSPE liposomes were incubated in medium containing a high or low concentration of serum (50% or 10% FBS, respectively), and the release kinetics was monitored over 3 hours at 37°C.

Mice

C57BL/6J-TyrRS/ Albino mice (The Jackson Laboratory) were maintained in pathogen-free conditions until imaging. NMRI mice with a mutation in VEGFR3 (Chy mice) were a kind gift of Dr. Kari Alitalo (University of Helsinki; ref. 35). Mice were fed an alfalfa-free diet (Experimental diet 2222, Provimi Kliba) to reduce tissue autofluorescence. All animal experiments were approved by the cantonal veterinarian office Zurich (Kantonales Veterinäramt Zürich; protocol 128/2008).

B16 murine melanoma model

B16.F10-luc2 murine melanoma cells were purchased from Caliper Life Sciences. Cells were maintained in culture with...
DMEM (Invitrogen) containing 10% FBS. Cells were transfected by electroporation with full-length human vascular endothelial growth factor-C (VEGF-C) subcloned into the pcDNA3.1 (Invitrogen) vector. Stable clones (B16.F10-VEGF-C cells) were selected, and human VEGF-C expression was confirmed by reverse transcription-PCR and ELISA in comparison with B16.F10.pDNA empty vector–transfected cells. We detected 0.8 ng/ml of human VEGF-C in the cell culture supernatant of the stably VEGF-C–transfected clone that was used for further studies, as assessed by quantitative ELISA (Bender MedSystems) at 24 hours after seeding 1 × 10^5 cells. These levels are similar to those reported for the highly metastatic PC3 human prostate tumor cell line (36, 37). B16.F10-VEGF-C or B16.F10.pDNA cells (2 × 10^5) in 10 μL PBS were injected into the right footpads of 9- to 11-week-old female C57BL/6-Tyr^−/− mice (38). The tumors were allowed to grow for 21 days, at which time they reached a diameter of ∼6 mm.

**In vivo NIR fluorescent imaging**

Mice were anesthetized with 2% isoflurane, and the fur was removed from the leg and abdomen using a shaver and topical hair removal cream. The mice were then positioned inside an IVIS Spectrum (Xenogen, Caliper Life Sciences) on their ventral side, and precontrast injection was performed with the above settings: exposure time = 6 seconds, f/stop = 2, medium binning, field of view = 6.6 × 6.6 cm^2. Five microliters of either ICG dye or the ICG liposomes (15 μmol/L in 5% glucose each) were intradermally injected at the top of the foot. Immediately after injection, serial images were acquired every 30 seconds for 10 minutes and every 60 seconds thereafter. Total acquisition time varied from 25 to 70 minutes depending on the model. Imaging of the site of injection was performed with the above settings but with exposure time of 0.1 second.

For image analysis, Living Image software (Caliper Life Sciences) was used. Regions of interest (ROI) were placed over the popliteal lymph node, medial iliac lymph node (when visible), and liver. Average signal intensity values were recorded for each ROI and plotted versus time in GraphPad Prism. For assessments of flow through the popliteal lymph node, the data were analyzed via normalizing the values based on a percentage of total enhancement (maximum average ROI signal intensity minus baseline average ROI signal intensity). Beginning at the time point of maximum enhancement in the popliteal lymph node, the data were fit to an exponential decay model:

\[
\text{Normalized signal enhancement} = \text{Span}^* e^{-K_{ex}t} + \text{Plateau}
\]

with maximum signal enhancement = 1 at time = 0 and a plateau = 0, this reduces to:

\[
\text{Normalized signal enhancement} = e^{-K_{ex}t}
\]

The rate of signal decay in the lymph node was expressed by either \(K_{IN}\) or half-life (∼0.67/\(K_{IN}\)).

**In vivo bioluminescent imaging**

The IVIS Spectrum was also used for in vivo bioluminescent imaging of B16-F10-luc2 tumors and draining lymph node metastases. Mice were injected intraperitoneally with 150 mg/kg body weight β-luciferin substrate (Caliper Life Sciences). Pilot studies revealed that the peak bioluminescent intensity of tumors was reached at ∼25 minutes after β-luciferin injection; therefore, this time point was chosen for imaging. Images of the footpad tumor were taken under the following settings: exposure time = 5 seconds, f/stop = 1, medium binning, field of view = 6.6 × 6.6 cm^2. Afterwards, the tumor was covered with black tape and one image (exposure time = 3 minutes, f/stop = 1, medium binning, field of view = 3.9 × 3.9 cm^2) of the region comprising the popliteal lymph node was collected. On the final day of imaging, mice were sacrificed via cervical dislocation and images of the dissected lymph nodes were taken with exposure time of 1 minute. Living Image software was used to quantify the bioluminescent signal, reported as units of tissue radiance (photons/s/cm^2/sr).

**Statistics**

Mean and SD are reported for all data. Comparisons between two groups at the same time points were made with two-tailed \(t\) tests. Comparisons between multiple groups or multiple time points were made with two-way ANOVA analyses. Spearman rank correlations were made to test for association between two variables.

**Results**

**Spectral properties and stability of LP-ICG**

Several liposomal formulations differing in composition, size, and loading procedures were first screened (data not shown). Figure 1 shows the absorption (Fig. 1A) and fluorescence (Fig. 1B) spectra of ICG dissolved in isotonic glucose buffer compared with the spectra of optimized LP-ICG (DOPC/PEG-DSPE, 95:5 mol/mol, 60 nm; Fig. 1C). The remarkable red shift (22 nm) in the absorption spectrum of ICG liposomes confirmed the affinity of ICG for lipid bilayers (24, 25). The emission spectrum of the ICG-loaded lipid dispersion exhibited a 4-fold increase in fluorescence intensity, as well as an expected red shift in \(\lambda_{max,em}\). The seemingly minor shifts toward longer wavelengths resulted in a dramatic decrease of in vivo background signal during detection (Supplementary Fig. S1), leading to an improved signal-to-noise ratio in vivo.

The optical properties of ICG and LP-ICG were monitored as a function of time. As illustrated in Fig. 1D, the free ICG completely lost its fluorescence signal within 7 days. In contrast, LP-ICG was highly stable with unaltered spectral properties for ∼14 days. The effect of HSA on the spectral properties of ICG and LP-ICG was then compared. At an HSA/ICG molar ratio of 1, HSA had no influence on the absorption and emission spectra of LP-ICG (Supplementary Fig. S2). As reported previously, the complexation of HSA with free ICG induced a red shift of the maxima, which was similar to the one observed on the encapsulation of...
ICG in liposomes, but the fluorescence intensity remained 2-fold lower. To assess the stability of the liposomal bilayer, the liposomes were incubated in medium containing either a high or a low concentration of serum (50% or 10% FBS, respectively), and leakage was monitored using a fluorescence dequenching assay (Supplementary Fig. S3). The high-serum concentration was used to mimic the blood, whereas the low-serum concentration was used as a surrogate of the lymph. At the low-serum concentration, we found that <10% of the ICG content was released within 3 hours, indicating that the liposomes maintained their integrity. In contrast, there was a rapid destabilization at high-serum concentrations, suggesting that the liposomes are not stable in the systemic circulation.

**In vivo imaging of C57BL/6 albino mice**

LP-ICG was compared with ICG after intradermal injection into the paws of normal C57BL/6 albino mice. Surprisingly, no major differences in the dynamics of flow of the contrast agents through the popliteal lymph node were seen in the initial hour after injection (Fig. 2) despite the larger hydrodynamic diameter (∼60 nm) of LP-ICG. Both imaging agents showed extremely rapid enhancement of the popliteal lymph nodes, indicating that the increased interstitial pressure created by the injection volume in the intradermal space was sufficient to open gaps between the lymphatic endothelial cells and quickly fill the lymphatic space with contrast agent. There was no significant difference in the signal decay at the popliteal lymph node between groups, indicating that the free ICG did not flow faster through draining lymph nodes than the LP-ICG.

At later time points, two major differences were found between ICG and LP-ICG. First, a second draining lymph node, the medial iliac, was visualized repeatedly after LP-ICG injection but never seen with ICG. This lymph node is much deeper (∼1 cm) compared with the popliteal lymph node (39). Second, LP-ICG showed a more specific uptake by the lymphatic system than ICG alone. By placing a region of interest over the liver, we were able to determine when the contrast agent had reached the bloodstream, as both imaging agents distribute to this organ after intravenous injection (data not shown). As shown in Fig. 2B, after intradermal injection of LP-ICG, enhancement peaks were seen in both draining lymph nodes before the liver showed enhancement at 60 to 70 minutes. In contrast, ICG (Fig. 2A) showed much earlier enhancement at the liver (∼10 minutes), suggesting partial uptake of the free dye by venous capillaries at the injection site and/or by high endothelial venules in the lymph nodes.

We also evaluated the clearance of LP-ICG from the injection site and from several tissues in mice after intradermal administration in comparison with ICG (Supplementary Fig. S4). No significant differences in tissue fluorescence were detected between LP-ICG-injected animals versus ICG-injected animals at 24 or 48 hours after injection. At 48 hours after injection, there was no significantly increased signal

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**Figure 1.** Spectral properties of LP-ICG. Typical normalized absorption (A) and fluorescence (B) spectra of LP-ICG (C) with respect to free ICG. Solid line, ICG in 5% glucose; dashed line, LP-ICG. The spectra are normalized for the maximum absorbance ($\lambda_{max} = 780$ nm) and the maximum fluorescence emission ($\lambda_{em} = 810$ nm) of free ICG. The stability of the spectral properties of LP-ICG over time is compared with that of ICG in buffer. The fluorescence maximum emission over time (D) is normalized for the initial maximum emission (ICG $\lambda_{em} = 810$ nm; LP-ICG $\lambda_{em} = 831$ nm). ●, ICG; ○, LP-ICG.
intensity in any of the evaluated tissues above normal values, indicating that LP-ICG is efficiently cleared with no evidence of retention by the reticuloendothelial system.

**Imaging in Chy mutant mice after intradermal injection**

LP-ICG was then tested in a model of lymphatic dysfunction. *Chy* mice, which have a mutation in VEGFR3, lack dermal lymphatics and develop lymphedema in the hind paws (35). Three male *Chy* mice and three wild-type (WT) littermates were injected with 5 μL of LP-ICG, and sequential imaging was performed for 1 hour. Images were also taken shortly after injection, at 4 hours, and at 24 hours to track clearance of the contrast agents from the injection site.

Representative sequential images after injection in *Chy* mice (Fig. 3A, left) showed no enhancement of the popliteal or iliac lymph nodes for up to 1 hour after imaging. There was additionally no signal increase in the liver, indicating that the liposomes remained confined to the interstitial space. Conversely, all WT NMRI littermates showed rapid enhancement and flow through popliteal lymph nodes after injection (Fig. 3A, right), with similar dynamics to those observed in normal albino C57BL/6 mice.

Evaluation of the clearance of LP-ICG in *Chy* mice at later time points indicated that despite the lack of dermal lymphatics, liposomes were cleared from the site of injection (Fig. 3B, right). However, this process occurred at a reduced rate compared with WT littermates. Whereas the signal intensity at the injection site was comparable in both groups at 10 minutes, there were significant differences at both 4 and 24 hours after injection (Fig. 3C). The clearance in *Chy* mice seemed to occur via interstitial diffusion, as a diffuse fluorescent signal shifted proximally up the hind limb at later time points (Fig. 3B, left).

**Lymphatic imaging in B16 tumor-bearing animals**

We next investigated whether LP-ICG could be used to quantify tumor-associated lymph flow. We hypothesized that...
lymphatic flow through tumor draining lymph nodes would be increased in comparison with normal nodes and that tumors overexpressing VEGF-C would have a further acceleration in flow. To test this, B16-F10-luc2 mouse melanoma cells expressing luciferase were stably transfected with a human VEGF-C overexpression vector. After injection into the footpad of C57BL/6 albino mice, the dynamics of tumor growth were evaluated using bioluminescent (data not shown) and caliper measurements. No significant differences in tumor growth were found between tumors overexpressing VEGF-C or control vector (Fig. 4A).

Tumors expressing high levels of VEGF-C have been shown to increase lymph node metastases (3–5, 40). Thus, we next performed in vivo bioluminescent imaging of the popliteal lymph node region. No metastases were detected in either group at days 10 and 15. At day 21, an evaluation of in vivo bioluminescence intensity indicated a significant increase in popliteal lymph node signal in the VEGF-C group (Fig. 4B). We found that seven of nine mice bearing VEGF-C–overexpressing tumors had popliteal lymph node metastases (Fig. 4C), whereas only two of eight mice bearing pcDNA control tumors (Fig. 4D) had metastases. These metastasis rates are similar to those previously reported for the B16-F10 model (36). The luciferase signals of dissected lymph nodes detected ex vivo were comparable with the in vivo–detected signals (Fig. 4C and D, insets).

The lymphatic flow dynamics after peritumoral, intradermal injection of LP-ICG were then evaluated in VEGF-C–overexpressing tumor-bearing animals and compared with pcDNA tumor-bearing and normal mice. To quantify the dynamics of flow, the data of signal intensity in the popliteal lymph node versus time were fitted to a model of exponential decay (Supplementary Fig. S5). Data analysis yielded the elimination ($k_{LN}$) constant and half-life of signal decay. It was found that pcDNA tumor-bearing mice (Fig. 5A, left) had increased lymphatic clearance through popliteal lymph
nodes in comparison with non–tumor-bearing animals. Surprisingly, the VEGF-C–overexpressing tumor-bearing mice had a much greater variability in clearance rates, with one group of mice showing rapid flow (Fig. 5A, middle) and another showing dramatically reduced flow (Fig. 5A, right). A direct comparison of the in vivo bioluminescence values of popliteal lymph node metastases to the $k_{LN}$ values in VEGF-C tumor-bearing mice resulted in a negative correlation (Fig. 5B, left; $P < 0.05$). By setting a threshold of average in vivo bioluminescence of the popliteal lymph nodes to $1 \times 10^7$ photons/s/cm$^2$/sr, we separated the VEGF-C tumor-bearing mice into two groups: a low (or "early") metastatic burden group and a high metastatic burden group. With this threshold, it was found that low metastatic VEGF-C tumor-bearing mice had accelerated lymphatic flow that was significantly higher than that in both non–tumor-bearing mice and pcDNA tumor-bearing mice (Fig. 5, middle and right). In contrast, VEGF-C–overexpressing tumor-bearing mice with a higher metastatic burden in tumor draining lymph nodes had a much slower lymphatic flow, which was not significantly different from non–tumor-bearing mice. Immuno-fluorescence stains for the melanoma cell marker gp100 showed that large tumor cell masses could be found in the subcapsular sinus region of lymph nodes of mice with high metastatic burden (Supplementary Fig. S6). In comparison, lymph nodes from mice with a low metastatic burden revealed only few gp100-positive cells.

**Discussion**

In this study, a novel NIR contrast system containing ICG was developed to image lymphatic flow and clearance in several mouse models. The LP-ICG formulation has several advantages over ICG, including (a) increased fluorescence signal with a shift toward the longer wavelength absorption and emission, (b) vastly improved stability in solution and biological fluids, (c) specific uptake into the lymphatic system, (d) increased clearance from the injection site with no retention in lymph nodes or other organs, and (e) improved visualization of deeper lymph nodes in vivo. These characteristics of LP-ICG have enabled development of quantifications of lymphatic function in mice.

ICG is considered one of the most attractive exogenous contrast agents for in vivo NIR fluorescent (NIRF) imaging,
thanks to its spectral properties, minimal toxicity, low cost, and FDA-approved status as a medical diagnostic compound (16). The design of an efficient ICG delivery system should preserve its optical properties and improve them whenever possible. The chemical and physical features of the molecule and the physiology of the target tissue have to be taken into consideration in the development process. For the current application, the formulation was optimized to include (a) a hydrophobic domain capable of preventing dye oligomerization, prolonging lifetime and reducing self-quenching; (b) a flexible hydrophilic polymeric coating (PEG) to prevent phagocytosis after injection (41); and (c) a suitable size for specific uptake into lymphatic vessels. The optimized formulation (DOPC/PEG-DSPE, 95:5 mol/mol, 60 nm) exhibited an increased absorbance, a bathochromic shift in the absorption spectrum, and a total fluorescence yield \( \sim 4 \)-fold higher than ICG. The spectral properties of LP-ICG were preserved in serum-containing medium over the time range of the in vivo experiments. The low liposomal content release in low-serum medium indicates bilayer stability in the lymphatic fluids during the time course of the imaging studies.

After intradermal injection, the increased brightness of LP-ICG and the reduction of background signal due to the bathochromic shifts, along with specificity of uptake into the lymphatic system, allowed clear visualization of not only the popliteal lymph nodes but also the medial iliac lymph nodes. These lymph nodes, downstream of the popliteal in the lymphatic drainage route from rear paws in mice (42), are located deep in the abdomen of the mouse (\( \sim 1 \) cm below the surface of the skin under the intestines), a region of high autofluorescence in the visible spectrum.

Unlike sentinel lymph node localization, in which it is considered to be advantageous to have the injected contrast agent retained by lymph node macrophages for sustained detection, we aimed to develop an agent that would passively flow through the lymphatic system. This feature is necessary to enable development of quantification methods of lymphatic flow with no interference from phagocytic cells. It is generally accepted, mostly from experiences with lymphoscintigraphy, that the ideal size for lymphatic uptake ranges from 5 to 100 nm (9). Smaller particles may be taken up by venous capillaries and enter the bloodstream directly. Larger particles cannot enter the endothelial cell junctions and may be retained for long periods at the site of injection (43). Instead of size, surface characteristics of liposomes play a more important role in lymph node retention (44, 45). By coating the surface of the 60-nm LP-ICG with PEG, retention in the lymph nodes was avoided. In addition, LP-ICG was not retained in any other organ of the mice, similar to the clearance dynamics of ICG. Both the free ICG and the encapsulated ICG showed no evidence of uptake by the reticuloendothelial system, and they were quickly cleared through the liver and bile, indicating that LP-ICG was destabilized once in the systemic circulation. This is an important difference in comparison with other nanoparticles tested as lymphatic tracers in animals. Quantum dots, even when coated with PEG,
are retained in many organs after injection (46). This efficient clearance, as well as the fact that all excipients used in this study are already used in humans, indicates a potential excellent safety profile for LP-ICG.

Our findings of increased lymphatic flow from murine melanoma tumors are consistent with recent studies using PEGylated quantum dots (38) or intravital microscopy methods (47). However, in our study, we collected sequential images, enabling clear visualization of the dynamics of signal at draining lymph nodes. By normalizing our measures of signal intensity of ICG to a peak enhancement value, we were able to develop a novel quantification method based on an exponential decay model over time that revealed significant increases in lymphatic flow from B16 tumors compared with non-tumor-bearing animals.

NIRF imaging with LP-ICG could be combined with bioluminescence imaging of luciferase-expressing tumor cells during a single imaging session to assess lymphatic flow, primary tumor bioluminescent signal, and lymph node metastases. The noninvasive nature of these modalities, as well as the fact that LP-ICG clears rapidly from the mouse, enables longitudinal imaging to be performed in the same animal. These methods will be powerful new tools to test novel therapeutic agents intended to improve or decrease lymphatic function in preclinical studies.

The combined bioluminescent/fluorescent imaging revealed an interesting phenomenon in mice with an increased lymph node tumor burden. The clearance rates of LP-ICG through the draining lymph nodes in these mice were dramatically reduced in comparison with tumor-bearing animals with low lymph node tumor cell burden. It has been well described that tumor cells after metastasizing through lymphatic systems seed in subcapsular sinus of the lymph node (47, 48), in agreement with our findings (Supplementary Fig. S6). It is conceivable that proliferation of tumor cells at this location could hinder flow of the liposomes through the lymph node. A similar phenomenon has been reported in several clinical studies during sentinel lymph node mapping, in which total occlusion by tumor cells prevents blue dye and/or radioisotopes from identifying sentinel nodes (49, 50). We are currently undertaking further studies to evaluate whether similar methods may be sensitive to predict which draining lymph nodes are positive for tumor cells. With the recent development of intraoperative NIR imaging systems (51), there may be a possibility to evaluate liposome-based contrast agent drainage patterns during surgery to improve the sensitivity of sentinel lymph node detection.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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