Minireview

Overcoming physiological barriers by nanoparticles for intravenous drug delivery to the lymph nodes

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Impact statement

The lymph nodes are clinical targets for drug delivery, due to their role in coordinating the adaptive immune response, as well as their involvement in a wide range of pathologies such as cancer, autoimmunity, and viral infection. This article describes the unique biological challenges to drug delivery to the lymph nodes upon intravenous administration and how nanoparticles can be designed to overcome such barriers. In sum, the information presented herein will inform nanoparticle development to improve drug delivery based on our understanding of lymph node physiology and lymph node-nanoparticle interactions.

Abstract

The lymph nodes are major sites of cancer metastasis and immune activity, and thus represent important clinical targets. Although not as well-studied compared to subcutaneous administration, intravenous drug delivery is advantageous for lymph node delivery as it is commonly practiced in the clinic and has the potential to deliver therapeutics systemically to all lymph nodes. However, rapid clearance by the mononuclear phagocyte system, tight junctions of the blood vascular endothelium, and the collagenous matrix of the interstitium can limit the efficiency of lymph node drug delivery, which has prompted research into the design of nanoparticle-based drug delivery systems. In this mini review, we describe the physiological and biological barriers to lymph node targeting, how they inform nanoparticle design, and discuss the future outlook of lymph node targeting.

Keywords: Nanoparticles, drug delivery, barrier, lymph node, targeting, hitchhiking

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Introduction

The lymphatic system is a network of organs, including the thymus, bone marrow, spleen, tonsils, and lymph nodes, interconnected by lymphatic vessels through which lymph flows. The primary functions of the lymphatic system are to maintain fluid homeostasis and regulate the adaptive immune response. Approximately 10% of blood plasma leaks out of the blood vessel capillaries and enters the interstitial space, with approximately 5–8 L of plasma pushed into the interstitium each day.[1,2] As shown in Figure 1, this fluid enters the lymphatic system through highly permeable lymphatic capillaries, which feed the fluid, now called lymph, into the larger lymphatic vessels. Since there is no central pump in the lymphatic system, fluid flow in lymphatic vessels is generated by the contraction and relaxation of the surrounding tissues. One-way valves in lymphatic vessels ensure that lymph flows unidirectionally from the lymphatic capillaries to the subclavian veins, where lymph mixes with venous blood and re-enters the cardiovascular system.[3] Hence, one major function of the lymphatics is to maintain fluid balance in the circulatory system.[4,5]

Another important function of the lymphatic system is immune surveillance.[6,7] As lymph flows from the lymphatic capillaries toward the subclavian veins, it passes through

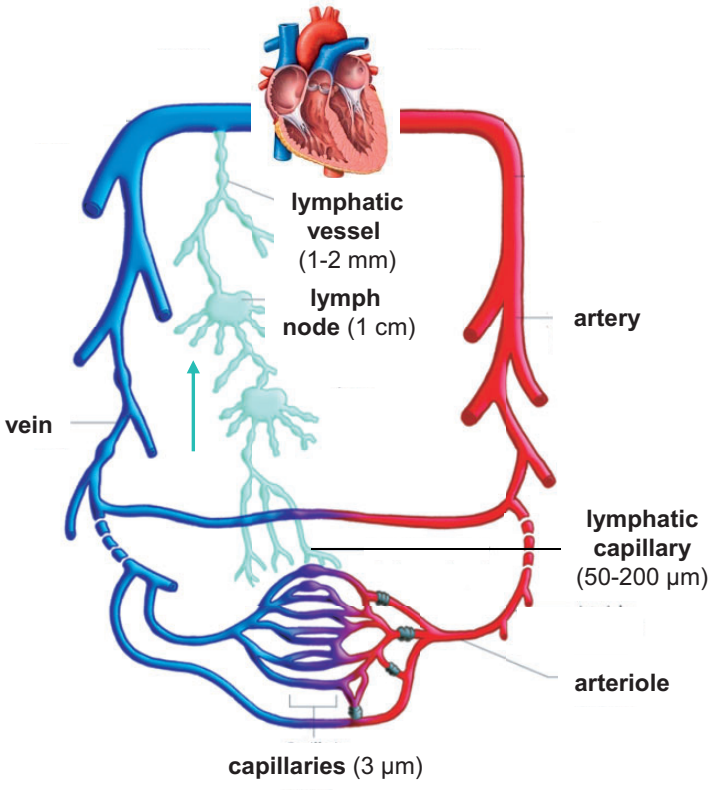

Figure 1. Lymphatic vessels drain interstitial fluid secreted by blood vessels and tissues. Lymph flows unidirectionally toward the subclavian veins, where it mixes with venous blood and re-enters circulation. (A color version of this figure is available in the online journal.)

several lymphoid organs called lymph nodes, which are densely populated with immune cells, such as macrophages, dendritic cells, B cells, and T cells.[8] As shown in Figure 2, lymph enters the lymph node through the afferent lymphatic vessels and flows through the node via the subcapsular sinus into the medullary sinus and out through the efferent lymphatic vessels.[9] The lymph node is home to several populations of resident immune cells, such as subcapsular sinus (SCS) macrophages, and dendritic cells (DCs) that line the subcapsular sinus or the conduits formed by the fibrous matrix inside the node. Their primary function is to sample incoming lymph for antigens to present to naïve B cells and T cells to initiate the adaptive immune response.[10–12] Circulating B cells and T cells use an array of cell surface proteins such as CCR7 (C-C chemokine receptor 7), LFA-1 (lymphocyte function-associated antigen 1), and L-selectin to migrate into the lymph nodes through specialized blood vessels called high endothelial venules (HEVs) that innervate the lymph node, where they undergo clonal expansion in their respective zones following antigen exposure.[13] Because there is a rich population of immune cells within the lymph nodes, especially the effector cells of adaptive immunity (B cells and T cells), these lymphoid tissues have been fervently studied in the development of immunotherapeutic strategies.[14–20]

The lymph nodes also represent an important clinical target for chemotherapeutic drug delivery. In addition to being a primary site for lymphoid cancers such as lymphoma, the lymph nodes are the most common site of metastases for solid cancers, as an estimated 80% of cancer metastasis occurs through the lymphatics.[21–24] Lymphatic vessels are prone to cancer cell invasion for several reasons. Firstly, lymphatic vessels are highly permeable especially in the initial lymphatics, which lack a complete basement membrane. Additionally, endothelial cells in the initial lymphatics are joined via discontinuous button-like junctions of vascular endothelial cadherins spaced 3 μm apart, which is in contrast to endothelial cells in downstream vessels that are joined via continuous zippers.[25–30] Secondly, tumor cells secrete lymphangiogenic factors like VEGF-C and VEGF-D that stimulate the formation and dilation of lymphatic vessels near the tumor.[31] Thirdly, the lower rate of fluid flow in lymphatic vessels compared to blood vessels (0.4 dyne/cm^2 vs. >30 dyne/cm^2) results in less fluid shear stresses experienced by circulating cancer cells in lymphatic vessels, increasing their likelihood of survival.[32–37]

Early preclinical studies for lymph node drug delivery have focused on subcutaneous drug administration, as this route circumvents clearance by the mononuclear phagocyte system that patrols the blood vasculature.[38–40] Comparatively, intravenous drug delivery to the lymph nodes has been less explored. However, intravenous drug delivery to the lymph nodes holds great clinical potential, because it can deliver drugs systemically to all lymph nodes, while subcutaneous injection concentrates drugs to lymph nodes downstream of the injection site and provides a local effect.[41,42] Despite the clinical significance of the

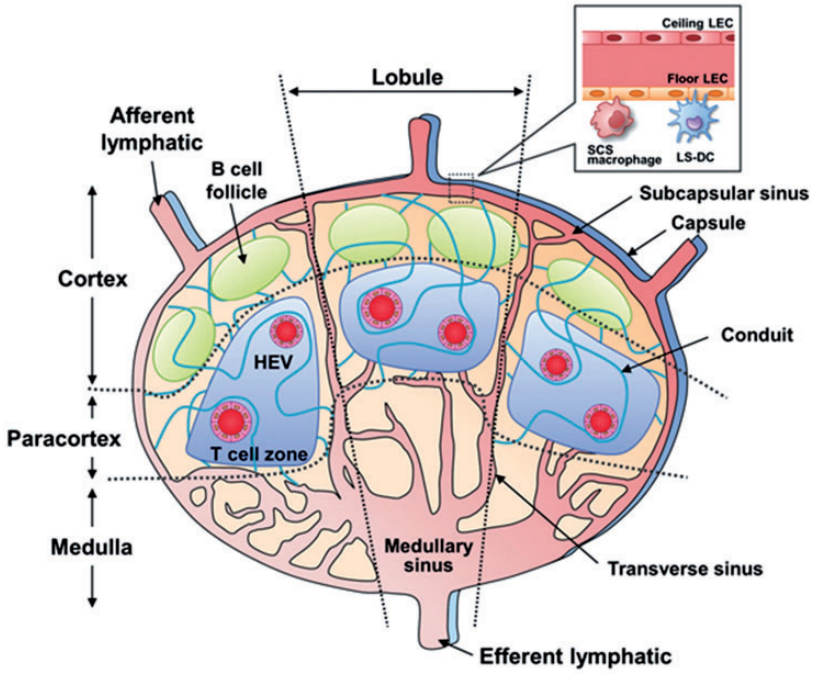

Figure 2. Structure of the lymph node. Lymph enters via the afferent lymphatic vessels and exits via the efferent lymphatic vessels. Antigens from the lymph are sampled by node-resident immune cells such as lymphatic sinus associated dendritic cells (LS-DCs) and subcapsular sinus (SCS) macrophages and presented to B cells and T cells to generate an immune response. Antigen-naïve B cells and T cells enter the lymph node through specialized blood vessels called high endothelial venules (HEVs) and undergo expansion following antigen exposure in the B cell follicles and T cell zones, respectively. Reprinted with permission from Nakamura and Harashima.[9] (A color version of this figure is available in the online journal.)

lymph nodes as a therapeutic target, intravenous drug delivery to the lymph nodes has challenges.[39,43,44] This mini review will describe the physiological and biological barriers to lymphatic drug delivery, discuss the parameters for nanocarrier design to overcome such barriers, and highlight drug delivery systems that target the lymph nodes by "hitchhiking" with endogenous biomolecules and immune cells that regularly traffic through the lymph nodes.

Physiological barriers to lymph node drug delivery

Once encapsulated into a nanoparticle, the *in vivo* fate of therapeutic drugs is determined by the carrier's interaction with plasma as well as the cellular and biological environment. Size and surface charge are two key physical parameters that influence a nanocarrier's interaction with its environment as well as its biodistribution following intravenous injection.[42,45–59] Notably, rational design of nanoparticle size and charge can be leveraged to bypass certain physiological barriers and instruct nanoparticle accumulation into specific tissues like the lymph nodes.[60–62] As shown in Figure 3, nanocarriers designed for lymph node delivery must (1) avoid clearance from the bloodstream by the mononuclear phagocyte system (MPS), (2) extravasate out of fenestrated blood vessels, and (3) traverse the extracellular matrix of the interstitium.[63] Hence, a deep understanding of each of these physiological barriers and the size and charge constraints they place on nanoparticle design are critical to develop successful nanocarrier systems to the lymph nodes.

Mononuclear phagocyte system

The first challenge faced by nanocarriers following intravenous administration is to resist clearance from the bloodstream mediated by macrophages and monocytes of the mononuclear phagocyte system (MPS), as well as by neutrophils, which have been shown to have phagocytic capacity and affect nanoparticle clearance *in vivo*.[54,64,65] Macrophages can be found in all major organs, such as the liver, kidneys, spleen, lungs, brain, and lymph nodes, while monocytes and neutrophils patrol the bloodstream.[66] The recognition of nanocarriers as foreign materials in the bloodstream is largely influenced by the adsorption of serum proteins called opsonins onto the particle surface, which are recognized by phagocytes through surface receptors.[54] This relationship between serum protein adsorption and subsequent phagocytosis and clearance has driven the investigation of antifouling polymer coatings like polyethylene glycol (PEG), as well as zwitterionic polymers like poly(carboxybetaine) (PCB), which have gained wider use due to potential PEG immunogenicity.[67–71] In addition, the physical properties of nanoparticles, such as size and surface charge have been reported as important determinants of *in vivo* stability and clearance.

Nanoparticle size has been considered a major factor in phagocyte-mediated clearance that can be tuned to enhance nanoparticle circulation. Generally, nanoparticles that are <100 nm are associated with less protein adsorption and longer circulation half-lives.[45,46] For example, Fang *et al.* incubated 80 nm, 171 nm, and 243 nm PEG-poly(cyanoacrylate-co-*n*-hexadecyl cyanoacrylate) (PEG-PHDCA) nanoparticles in 50% mouse serum found that protein

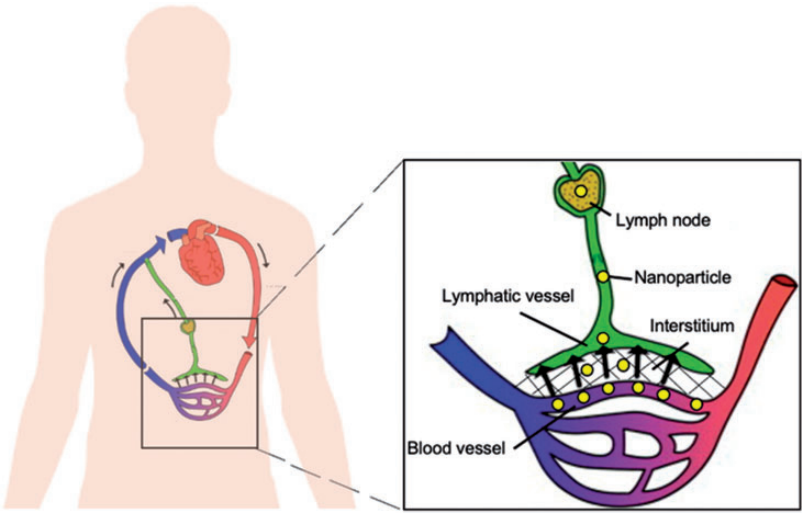

Figure 3. To reach the lymph nodes, nanoparticles must avoid clearance from the blood stream by the mononuclear phagocyte system, extravasate out of the blood vessel endothelium, and diffuse past the extracellular matrix in the interstitium. Adapted with permission from Hong *et al*. [63] (A color version of this figure is available in the online journal.)

adsorption increased with nanoparticle size (6%, 23%, and 34%, respectively), which was correlated to macrophage uptake, as the 80 nm particles were phagocytosed at a 40% lower rate than 243 nm particles when incubated with RAW 264.7 murine macrophages *in vitro*.[45] The same dependence of phagocytosis on nanoparticle size was also reported in a study by Bisso *et al.*, who tested the uptake of 20 nm, 50 nm, 100 nm, and 200 nm polystyrene beads in human neutrophils *in vitro*.[72] To study the effects of nanoparticle size on *in vivo* half-life, Choi *et al.* injected PEG-coated gold nanoparticles of different sizes, ranging from 5 nm to 98 nm intravenously into BALB/c mice via the tail-vein while controlling particle concentration and surface charge and found that nanoparticle half-life decreased with size.[46] This trend was also found in studies by Perrault *et al.* who also found that increasing PEG weight prolonged the half-lives of PEGylated gold particles of similar core sizes.[73]

The surface properties of nanoparticles like charge are also important design criteria to consider for avoiding clearance from circulation by phagocytes. Because most serum proteins are negatively charged, nanoparticles with strong positive surface charge (+30 mV) are generally phagocytosed and eliminated quicker than uncharged or negatively charged particles.[47–49,74,75] For example, Feng *et al.* incubated 10 nm iron oxide nanoparticles coated with polyethylenimine (PEI, +29 mV) or PEG (−1 mV) with RAW264.7 macrophages *in vitro* and observed that the positively charged particles were phagocytosed at 3-fold the rate of the neutral particle.[74] When injected intravenously into mice, PEI-covered iron oxide particles (225 nm) were cleared from the bloodstream within 10 min, and had 78-fold lower AUC than negatively charged particles.[75] However, other studies report that negatively charged nanoparticles can also be prone to phagocytosis and clearance, due to greater interaction with the positively charged scavenger receptors of phagocytic cells.[50–54,59] For example, Metz *et al.* reported 21 nm negatively charged iron oxide nanoparticles were internalized by phagocytic monocytes at a higher rate than similarly sized uncharged iron oxide particles.[50] Interestingly, this study also reported that 62 nm negatively charged iron oxide particles were phagocytosed at 3-fold the rate of 150 nm uncharged iron oxide particles, despite being much smaller, which suggests that particle surface charge may be more influential on clearance than nanoparticle size. These physical properties govern the ability of nanoparticles to resist phagocyte-mediated clearance from the bloodstream, which is a critical consideration for lymph node targeting strategies, as longer-circulating particles have more opportunities to extravasate from the blood endothelium and accumulate in lymphatic tissues.

Blood vessel endothelium

In addition to limiting phagocytic clearance, nanoparticles must extravasate out of the blood vasculature to reach the lymph nodes. As depicted in Figure 4, blood vessels are comprised of flat, quiescent endothelial cells joined laterally via tight junction proteins and attached to a basement membrane comprising collagen, fibronectin, laminin, and glycosaminoglycans.[76,77] The blood vasculature functions primarily as a barrier restricting the movement of fluid, proteins, blood cells, and nanoparticles between the intravascular and interstitial compartments.[77] Before extravasation can occur, however, nanoparticles must first marginate towards the blood vessel walls, for which mathematical modeling and microfluidic studies have revealed that nanoparticles with higher aspect ratios like ellipsoid or rod-shaped particles can have superior margination to the vessel wall compared to spherical nanoparticles.[78–80] Upon reaching the vessel wall, nanoparticles must extravasate out of the blood vessel through fenestrations in the endothelium or through transcellular transport.

Fenestrations in the blood vessel endothelium can span 60 nm, which can be up to 800 nm in tumor vasculature, and these fenestrations are believed to be a major route of

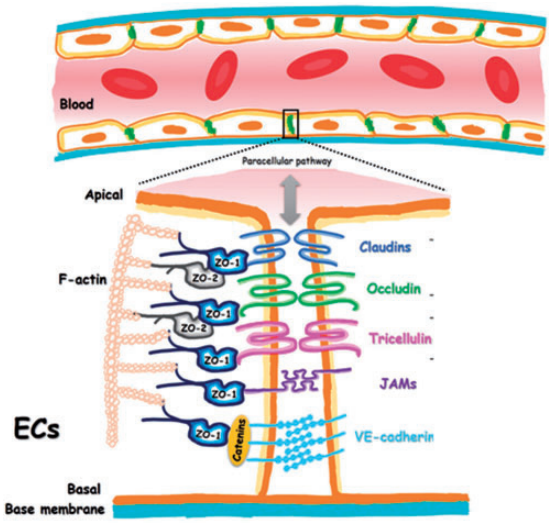

Figure 4. Junction proteins maintain tight cell–cell junctions between blood vessel endothelial cells. Adapted with permission from Cong and Kong.[76] (A color version of this figure is available in the online journal.)

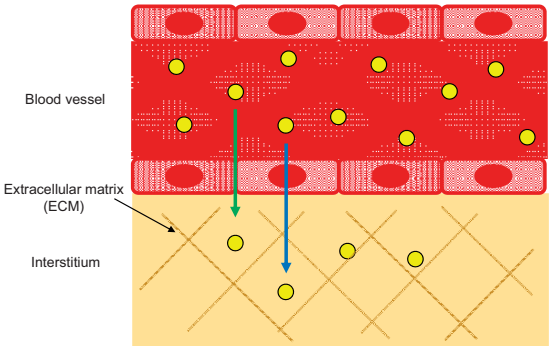

Figure 5. Nanoparticles can extravasate out of the blood vessel endothelium into the ECM-containing insterstitium through cell–cell junctions (green arrow) or transcytosis (blue arrow). (A color version of this figure is available in the online journal.)

extravasation for nanoparticles smaller than 200 nm out of the blood vasculature.[42,55–58] As such, nanoparticle extravasation out of the blood vessel endothelium into the interstitium has been reported to increase as nanoparticle size is decreased. For example, Vu *et al.* used human umbilical vein endothelial cells (HUVECs) as an *in vitro* model of blood vasculature and reported an over 10-fold increase in the extravasation of 40 nm polystyrene nanoparticles out of the blood endothelium compared to that of 70 nm and 130 nm particles.[81] Kong *et al.* corroborated these results *in vivo* by using a mouse model of ovarian cancer to evaluate the extravasation of liposomes sized from 100 nm to 400 nm out of tumor blood vessels under hyperthermic conditions and reported that particle extravasation decreased with nanoparticle size.[82] However, none of the liposomes were observed to extravasate under physiological conditions, which highlights growing concerns with the potency of the enhanced permeability and retention (EPR) effect that many researchers have assumed lead to increased nanoparticle delivery to tumor tissue.[83–85] Indeed, a study by Smith *et al.* reported that 25 nm quantum dots were able to extravasate out of the vasculature in only one of three murine tumor models.[86] These studies indicate that further research is necessary to characterize the complex interactions between nanoparticles and blood vasculature *in vivo*.

In addition to extravasation through gaps between endothelial cells, or paracellular transport, recent studies have suggested nanoparticles can take advantage of transcytosis through endothelial cells as an additional means of transport out of the blood vasculature (Figure 5).[87,88] Positively charged nanoparticles have been reported to initiate transcytosis through increased interaction and adsorption to the negatively charged cell membranes of endothelial cells.[89–91] Zhang *et al.* studied the transcytosis of neutral and positively charged polystyrene nanoparticles (22, 48, and 100 nm) through an *in vitro* model of blood vessel endothelium. While no size effects were reported to affect transcytosis in this study, positively charged particles entered the endothelium at 100-fold the rate of neutral particles, and a similar result was reported by Gil *et al.* comparing negatively charged (−12 mV) and positively charged (+14 mV) cyclodextrin nanoparticles.[90,91]

Extracellular matrix of the interstitium

Upon extravasation out of the blood vasculature, nanoparticles can encounter another barrier in the form of the extracellular matrix (ECM) in the interstitium before reaching the lymph nodes. The ECM contains hundreds of different proteins including collagen, glycosaminoglycans, laminins, and fibronectin that are primarily produced by fibroblasts.[92] Collagen fibrils crosslink to form a mesh-like structure with pore sizes ranging from 20 nm up to 130 nm[93,94] Given the variable nature of ECM pore sizes, smaller nanoparticles are believed to have increased diffusivity past the ECM.[95–97] Wong *et al.* compared the diffusivity of 10 nm quantum dots with 100 nm quantum dot-coated silica nanoparticles in a cell-free collagen matrix.[95] The 10 nm particles had a diffusivity of 2.3×10^{-7} cm^2/s, while the 100 nm particles were unable to diffuse in the matrix. While this study only compared nanoparticles of two sizes, Goodman *et al.* evaluated the ability of 20, 40, 100, and 200 nm polystyrene nanoparticles to penetrate the ECM of a tumor spheroid culture and found that diffusion past the ECM was inversely correlated with nanoparticle size, as 20 nm particles were most efficient in penetrating the ECM (57% of all particles), while 100 and 200 nm particles had <5% penetration.[96]

Along with positively charged collagen fibrils, the ECM also contains thin fibers of negatively charged glycosaminoglycans, namely hyaluronic acid, that effectively make the ECM a patchwork of positive and negative charges as shown in Figure 6.[98,99] Le Goas *et al.* studied nanoparticle surface charge effects on the diffusion of 12–20 nm polymethacrylate-coated gold nanoparticles in Matrigel and found that negatively charged nanoparticles (<−20 mV) exhibited greater diffusivity, while positively charged particles (+10 mV) did not diffuse, a result also confirmed by Kim *et al.*[100,101] However, others have reported that neutral particles have increased diffusion through the

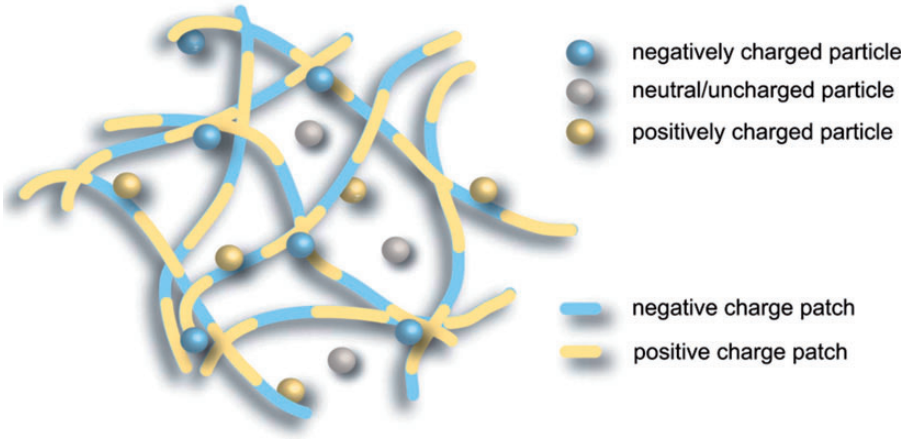

Figure 6. The ECM in the interstitium forms a mesh with positively and negatively charged protein fibers. Reprinted with permission from Lieleg *et al*.[98] (A color version of this figure is available in the online journal.)

ECM compared to both positively and negatively charged nanoparticles.[99,102] The observation of charged particles having hindered diffusion in ECM has been attributed to their interaction and binding with positively charged (collagen) and negatively charged (glycosaminoglycans) matrix proteins in the ECM.[98,99] Interestingly, modeling studies performed by Stylianopoulos *et al.* using 1–10 nm quantum dots concluded that charge effects diminish with decreasing nanoparticle size, suggesting that smaller nanoparticles, regardless of surface charge, may have superior diffusivity based on their decreased interaction with the proteins of the ECM.[98] Once past the ECM and the other physiological barriers described in this section, nanoparticles can enter the lymphatic system via highly permeable lymphatic vessels and be carried by lymph into the lymph node to unload a therapeutic payload.

In vivo application of nanoparticles for lymph node targeting

The nanoparticle size and charge constraints imposed by the mononuclear phagocyte system, blood vessel endothelium, and extracellular matrix of the interstitium suggest that small, uncharged nanoparticles may target the lymph nodes more efficiently following intravenous injection. Nanoparticle delivery to the lymph nodes has been used in numerous clinical and pre-clinical applications, such as the imaging and treatment of lymph node cancer metastases, as well as for vaccine delivery.[41,103–106] Although the majority of nanoparticle formulations are sequestered in the liver and spleen following intravenous administration, leading to lymph node concentrations of <0.1% of the injected dose (ID), engineering the physical characteristics of nanoparticles such as size and charge have been reported to boost lymph node accumulation.[107–109] For example, dextran-coated ultrasmall superparamagnetic iron oxide (USPIO) nanoparticles with hydrodynamic diameters of approximately 40 nm have been explored clinically for the identification of lymph node metastases in prostate cancer using MRI, with preclinical studies reporting up to 12% ID in the lymph nodes after intravenous administration in rats.[42,110,111] The USPIO particles function as contrast agents by entering the lymph nodes and retention by node-resident macrophages.[42] The efficacy of USPIO particles for lymph node metastases detection was evaluated in a clinical study involving 80 patients and 334 lymph nodes; USPIO-aided MRI following intravenous injection improved the sensitivity of lymph node metastases detection from 35.4% to 90.5% compared to MRI alone.[110]

In addition to cancer imaging, nanoparticles targeted to the lymph nodes have also been investigated for cancer therapy. Cabral *et al.* investigated the ability of uncharged polymeric micelles administered intravenously to accumulate in the metastatic lymph nodes of B16F10 tumor-bearing mice and deliver a platinum-based drug.[41] While mice treated intravenously with 8 mg/kg of free oxaliplatin had similar tumor growth to an untreated control, mice treated with 3 mg/kg of the drug loaded into 30 nm polymeric micelles had significantly smaller tumors and lymph node metastases after nine days (three total injections given on days 0, 2, and 4). Moreover, the lymph node targeting of the micelles was reported to be size-dependent, as 30 nm micelles were reported to have approximately 4 times better accumulation in lymph node metastases than 70 nm micelles. Another study by this group investigated the ability of 55 nm micelles to deliver the chemotherapy drug epirubicin to the metastatic lymph nodes of a luciferase-expressing murine breast cancer model.[103] To limit off-target toxicity associated with drug release in healthy lymph nodes, the epirubicin was loaded into the micelles via pH-sensitive hydrazine linkers, to facilitate increased drug release in the acidic environment of the primary tumor and lymph node metastases. Eight hours after intravenous injection, the epirubicin concentration in the metastatic lymph nodes of micelle-treated mice was 10-fold higher than in the nodes of mice treated with free epirubicin, and the increased drug concentration resulted in significant reduction in the size of lymph node metastases (Figure 7). Moreover, the drug concentration in metastatic lymph nodes was almost 3-fold higher than the drug concentration in healthy nodes 48 h after injection.

Because the majority of solid cancers metastasize via the lymphatics, lymph node-targeted nanoparticles have also

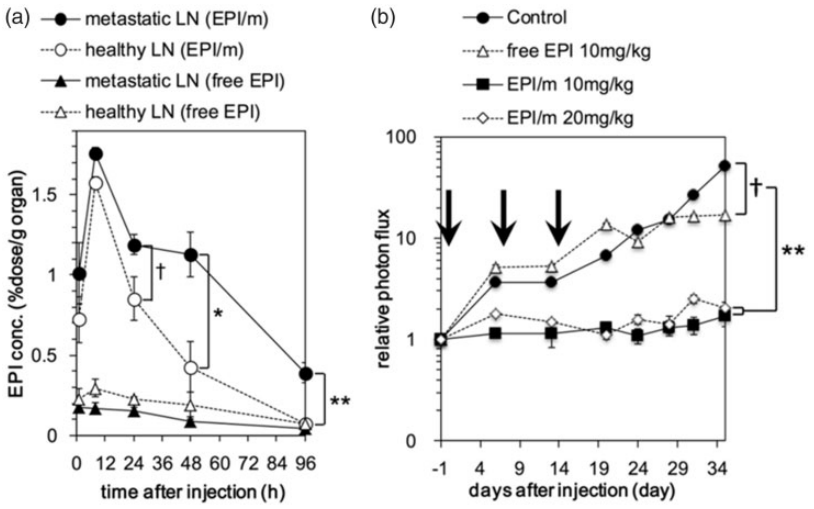

Figure 7. (a) Epirubicin-loaded micelles deliver drugs to metastatic lymph nodes. (b) Epirubicin micelle treatment inhibits the growth of lymph node metastases in a luciferase expressing murine breast cancer model. $^{*}P < 0.05$, $^{**}P < 0.01$. Adapted with permission from Chida *et al*.[102]

been investigated for their ability to inhibit the formation of lymph node metastases.[112] Liu *et al*. characterized the distribution of nanoparticle clusters 100 nm in size assembled from 5 nm dendrimers loaded with a cisplatin prodrug and tested the nanoparticle's ability to inhibit lymph node metastases. Particles injected intravenously were found to colocalize with FITC-labeled lymphatic vessels, confirming their ability to enter the lymphatics following systemic injection. Moreover, nanoparticle treatment inhibited the formation of lymph node metastases in a metastatic 4T1 breast cancer model and improved the median survival of mice from 45 days to 56 days relative to a PBS control, validating the efficacy of lymph node-targeted nanoparticles for cancer therapy.

In addition, nanoparticles have been used to deliver vaccines to the lymph nodes for immunization against cancer. Specifically, Baharom *et al*. explored intravenous delivery of 20–50 nm micelles loaded with MC38 tumor peptide neoantigens for anti-tumor vaccination of mice.[105] Mice challenged with MC38 colon cancer cells 28 days after vaccination were observed to have significantly reduced tumor growth compared to unvaccinated mice. Interestingly, intravenous vaccination of mice bearing established MC38 tumors was observed to control tumor growth, while subcutaneous vaccination was ineffective. These studies reported the success of small, uncharged nanoparticles for lymph node targeting, which coincides with the ideal nanoparticle physical properties described above. The rising number of studies regarding lymph node drug delivery for cancer therapy reflects the growing recognition of the lymphatic system's role in mediating cancer metastasis and recurrence.

Hitchhiking strategies for lymph node drug delivery

Albumin-mediated drug delivery to the lymph nodes

An alternative approach to drug delivery to the lymph nodes is to design a delivery system that can bind to endogenous molecules or immune cells that are regularly trafficked to the lymph nodes. Albumin is the most abundant protein in blood, interstitial fluid, and lymph.[34] Albumin is produced in the liver and released into the bloodstream as a globular protein with 4×15 nm dimensions, with an average half-life of 19 days.[113,114] The main functions of albumin are to maintain the osmotic pressure of blood, and to transport other biomolecules, such as fatty acids, hormones, ions, and steroids.[115,116] About 5% of the albumin in circulation is pushed out of the blood vasculature every hour into the interstitial space, and nearly 100% of this albumin is absorbed into the lymphatics before re-entering circulation.[34]

Given albumin's excellent stability and half-life, as well as its frequent trafficking to the lymphatics, subcutaneous delivery systems leveraging albumin-hitchhiking for the delivery of vaccines and imaging molecules, such as the albumin-binding dye Evans Blue, to draining lymph nodes have been explored.[34,117,118] Albumin has also been investigated as a nanocarrier for anticancer therapies, due to its ability to passively accumulate in primary tumors via leaky tumor vasculature, as well as its active transport into tumor cells via the Cav-1 protein.[119,120]

Additionally, albumin hitchhiking has been adopted for intravenous delivery systems targeted to the lymph nodes in preclinical and clinical studies.[121–125] For example, Yu *et al.* synthesized combination nanoparticles by loading nanospheres comprised of crosslinked albumin with the chemotherapy drug gemcitabine and photodynamic therapy agent pheophorbide-a and evaluated therapeutic efficacy in the lymph node metastases of a murine BxPC-3 pancreatic cancer model.[125] Twenty-four hours after intravenous administration, the combination nanoparticle was observed to have increased accumulation in metastatic lymph nodes compared to free pheophorbide-a. Furthermore, *in vivo* efficacy studies demonstrated reduced volume and weight of metastatic lymph nodes of mice treated with nanoparticle.

Nab-paclitaxel (Abraxane) is a nanoformulation of the anti-cancer drug paclitaxel bound to albumin that is FDA

approved for the treatment of metastatic breast cancer, non-small cell lung cancer, and pancreatic adenocarcinoma.[126,127] Given its lymphatic targeting properties, it has also recently been examined in lymph node-resident cancers such as non-Hodgkin lymphoma.[121–124] In two separate case reports of patients presenting diffuse large B-cell lymphoma, each achieving only partial responses after multiple lines of chemotherapy, the patients were prescribed combination therapies including nab-paclitaxel. The first patient received a weekly dose of nab-paclitaxel over six months after initial treatment with the chemotherapy drug azacitine, and PET-CT scans revealed complete disease remission after the third month of treatment.[122] Follow-up scans performed five years after the end of treatment showed no evidence of disease recurrence. The second patient was prescribed a combination of nab-paclitaxel and liposomal doxorubicin as a fourth-line chemotherapy regimen and achieved complete disease remission after four cycles of treatment, with PET-CT scans showing no disease recurrence after seven years.[121] Based on the success of these case reports, a phase II clinical trial involving 13 patients with relapsed or refractory diffuse large B cell lymphoma was performed using a combination therapy of rituximab, nab-paclitaxel, and liposomal doxorubicin.[123] Of the 13 patients in the study, 5 achieved complete response, 6 achieved partial response, and 2 patients had stable or progressive disease. Although clinical studies reported therapeutic success in patients unresponsive to other chemotherapies, they were limited by small sample sizes. Thus, the use of nab-paclitaxel as a monotherapy for diffuse large B cell lymphoma warrants further study.

Immune cell-mediated drug delivery to the lymph nodes

Another approach for delivering therapeutics to the lymph nodes is to hitch nanoparticles with immune cells that regularly traffic to the lymph nodes, such as T cells monocytes, and dendritic cells (DCs) which can enter the lymph node via the lymphatics, as well as through the blood vessels in the lymph node. Naïve T cells constantly travel between the blood circulation and lymphatics in search of antigen. Upon activation, T cells express chemokine receptors CCR5, CCR7, and CCR8 that facilitate their migration to the lymph nodes through the lymphatic vasculature and lymph node blood vasculature.[128–130] Huang *et al.* exploited the lymph node homing ability of T cells by covalently linking cultured luciferase$^+$ T cells to lipid nanocapsules (NCs) loaded with the anticancer drug SN-38.[131] After intravenous injection into a mouse model of lymphoma, T cells conjugated with the NCs accumulated in the lymph nodes within 20 h, reaching peak levels at 60 h post-injection, and were retained until the end of the study (80 h). Importantly, these kinetic properties were similar to unmodified T cells, confirming that conjugation to the NCs did not affect T cell trafficking. When drug concentrations in the tumor-bearing lymph nodes were evaluated, T cell-mediated NC delivery resulted in increased drug concentration, over 60-fold greater than the concentration achieved by the injection of unbound NCs. The increased drug concentration associated with T cell-NC treatment translated to an approximately 10-fold reduction in tumor burden compared to unbound NC treatment.

Additionally, T cell hitchhiking can also be achieved *in situ*. For example, Schmid *et al.* evaluated the ability of intravenously administered T cell-targeted PEG-PLGA nanoparticles to accumulate in the tumor-draining lymph nodes of a mouse colorectal cancer model and deliver the immunomodulatory drug SD-208.[132] SD-208 functions by inhibiting the TGF-BR1 kinase in cancer cells, which promotes immunosuppression.[133] T cell targeting was achieved by reacting maleimide-terminated nanoparticles with free thiol groups of PD1 or CD8 antibody fragments, as both PD1 and CD8 have been reported to be expressed in active T cells.[132,134–136] Twenty-four hours following intravenous injection, T cell-targeted nanoparticles were observed to accumulate in the tumor-draining lymph nodes, with approximately 25% of T cells in the node bound to nanoparticles, while nanoparticles functionalized with non-targeting isotype control antibody fragments were not observed in the lymph nodes (Figure 8(a)). The increased lymph node accumulation of the targeted nanoparticles led to delayed tumor growth in a subcutaneous MC38 colorectal cancer model. While treatment with non-targeted nanoparticles had no effect on tumor growth, mice treated with T cell-targeted nanoparticles delayed tumor growth and prolonged mouse survival by approximately 12 days (Figure 8(b)).

Like T cells, dendritic cells (DCs) are immune cells that migrate to the lymph nodes and are critical for mediating adaptive immunity. As the primary antigen-presenting cell of the immune system, DCs are dispersed throughout the body and are typically found in higher concentrations in tissues exposed to the external environment, such as the skin, intestinal lining, and nasal passages.[137,138] Although DCs have been reported to migrate to the lymph nodes to some degree during steady-state conditions, the majority of DC migration is reported in the context of post-antigen exposure, after which DCs begin to express surface receptors like CCR7 that are used to follow chemokines such as CCL19 and CCL21 to the lymphatics, where DCs initiate adaptive immune responses via antigen presentation to naïve T cells and B cells.[139–141] Due to the role of DCs in adaptive immunity, a plethora of studies and reviews have been conducted with regards to targeting nanoparticles to DCs for immunotherapy and vaccination.[142–145]

Although many of the studies have used the subcutaneous or intradermal route for DC-targeting, intravenous injection of nanoparticles actively targeted to DCs has also shown success in immunotherapeutic and vaccination strategies. Sancho *et al.* utilized DC, NK lectin group receptor-1 (DNGR-1) for targeted antigen delivery to DCs for the generation of CD8 T cell responses against B16F10 melanoma tumors.[146] Specifically, anti-DNGR-1 antibodies conjugated to the immunogenic peptide SIINFEKL were injected intravenously into mice bearing B16F10-OVA tumors and were found to colocalize with DCs in the lymph nodes. This resulted in CD8 T cell responses that significantly mitigated tumor metastases in the lungs, confirming the potency of DC-targeting for immune stimulation. In addition, Stead *et al.* investigated DC-targeting in the context of generating immune tolerance to allogenic

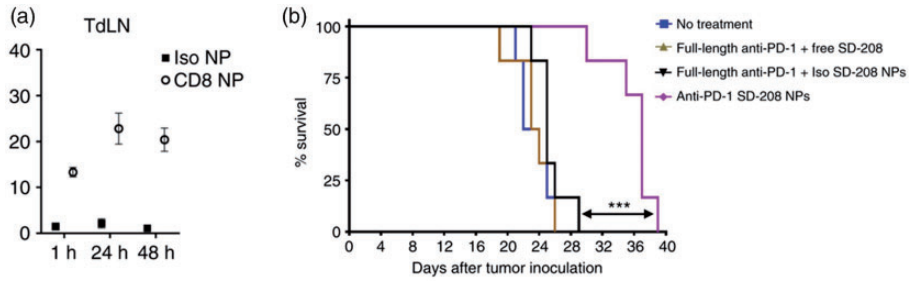

Figure 8. (a) T cell-targeted nanoparticles accumulate in the tumor-draining lymph nodes and (b) improve mouse survival (b). ***$P < 0.001$. Adapted with permission from Schmid *et al*.[131] (A color version of this figure is available in the online journal.)

transplants in mice by using targeted silicon nanoparticles (160 nm) to deliver the immunosuppressive drug, rapamycin, to induce expansion and proliferation of regulatory T cells.[147] DC-targeting was achieved by functionalizing the particles with antibodies targeted to the DC marker CD11c. Mice received a total of three intravenous injections on days 0, 14, and 28 of the study and were euthanized after 40 days. When harvested spleens were evaluated for the presence of regulatory T cells, DC-targeted nanoparticle therapy showed a five-fold increase in the number of regulatory T cells compared to mice treated with nanoparticles functionalized with isotype control antibodies. Collectively, these studies demonstrate the feasibility of leveraging DC-targeted nanoparticles for the delivery of antigens and small molecule drugs to the lymph node to induce an immunomodulatory effect.

In addition to T cells and DCs, monocytes are immune cells that rapidly accumulate in the lymph nodes through blood vessels during and immediately following inflammation, with functions spanning antigen transport and presentation and differentiation into DCs, although they have also been reported to be present in steady-state conditions.[12,148–153] The influx of monocytes during inflammatory states has been reported to be a result of the accumulation of lymph-borne chemokines such as monocyte chemoattractant protein-1 (MCP-1) in the lymph node, entering the lymph nodes via the afferent lymphatic vessels.[149–152] The increased trafficking of monocytes to the lymph nodes during inflammatory states such as cancer makes monocyte targeting a useful means of drug delivery to the lymph nodes. Our group has explored this by synthesizing micelles functionalized with peptides derived from the CCR2-binding motif of the MCP-1 chemokine.[154–158] When injected intravenously into B16F10 tumor-bearing mice, micelles containing the CCR2-binding motif were observed to accumulate in the lymph nodes at twice the rate of a non-targeted micelle 3 h post-injection, although further study is required to elucidated the mechanisms behind this.[154] The development of nanoparticle systems that can strongly associate with immune cells that migrate through the lymphatics offers a novel means of drug delivery to the lymph nodes.

Conclusions and future outlook

While early nanoparticle characterization and cancer efficacy studies focused on prolonging nanoparticle circulation in the blood vasculature and tumor accumulation, the prominent role of the lymphatic system, particularly the lymph nodes, in the progression of numerous pathologies, such as cancer, cardiovascular disease, autoimmunity, and viral infections, is now widely recognized.[6,159,160] As a result, improving drug delivery to the lymph nodes has become a heavily researched topic.[41,103–105,121–125,131,132,154] As reviewed here, intravenous drug delivery to the lymph nodes is challenged by numerous biological barriers, such as the MPS, blood vessel endothelium, and ECM, and overcoming these delivery barriers will be critical to their successful implementation in the clinic.[55–57,93,94,161–163]

An area for further research is to design nanocarriers that incorporate ligands that actively target lymph node-specific biomarkers to increase nanoparticle retention in the lymph nodes. For example, targeting nanoparticles to the lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) that is expressed by all lymphatic endothelial cells may promote nanoparticle accumulation deep into the cortex and paracortex of lymph nodes, similar to the use of peptides containing the integrin-binding RGD motif for tumor targeting and penetration.[164–166] Another biomarker that can be used for lymph node targeting is cluster of differentiation 169 (CD169), which is expressed by macrophages lining the subcapsular sinus of lymph nodes, as well as macrophages in the bone marrow.[167,168] Subcapsular sinus macrophages come into direct contact with lymph that flows into the node via the afferent lymphatic vessels and take up lymph-borne antigens that are presented to naïve B cells of the cortex.[167,169] Thus, nanocarriers actively targeted to subcapsular sinus macrophages have the potential to improve antigen delivery for immunization purposes. Although CD169 ligands have been used to target liposomes to bone marrow macrophages, their use in targeting the $CD169^+$ macrophages in the lymph nodes has yet to be explored.[170]

An alternative route for lymph node entry is through extravasation out of the blood vessels that innervate the lymph nodes, called high endothelial venules (HEVs). HEVs are specialized venules whose primary function is to transport lymphocytes, such as T cells, in and out of the lymph nodes by producing lymphocyte-attracting chemokines like CCL21.[171] Compared to the flat, endothelial cells of normal blood vessels, HEV endothelial cells are cuboidal in shape and are supported by a thicker basement membrane. While several nanoparticle studies have reported HEVs as a primary means of lymph node

accumulation, the mechanisms underlying HEV-mediated transport have not been well-characterized and require more rigorous study.[42,110] Although intravenous drug delivery to the lymph nodes is challenged by several physiological barriers, studies characterizing these barriers have paved the way to rational nanocarrier design that can improve therapeutic outcomes. However, the design rules reviewed here apply to intravenous administration, and likely differ from other administration routes; hence, the intended route of administration must be taken into consideration when designing nanomedicine strategies for *in vivo* use.

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