

From sewer to saviour — targeting the lymphatic system to promote drug exposure and activity

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Abstract | The lymphatic system serves an integral role in fluid homeostasis, lipid metabolism and immune control. In cancer, the lymph nodes that drain solid tumours are a primary site of metastasis, and recent studies have suggested intrinsic links between lymphatic function, lipid deposition, obesity and atherosclerosis. Advances in the current understanding of the role of the lymphatics in pathological change and immunity have driven the recognition that lymph-targeted delivery has the potential to transform disease treatment and vaccination. In addition, the design of lymphatic delivery systems has progressed from simple systems that rely on passive lymphatic access to sophisticated structures that use nanotechnology to mimic endogenous macromolecules and lipid conjugates that ‘hitchhike’ onto lipid transport processes. Here, we briefly summarize the lymphatic system in health and disease and the varying mechanisms of lymphatic entry and transport, as well as discussing examples of lymphatic delivery that have enhanced therapeutic utility. We also outline future challenges to effective lymph-directed therapy.

The lymphatic system comprises a network of vessels and nodes that circulate immune cells and provide a site for antigen presentation and immune activation¹. The lymphatic system also transports dietary lipids, in the form of lipoproteins, from the intestine to the general circulation² and clears fluid, macromolecules (including proteins), particulates (including infectious materials such as bacteria) and small molecules packaged into endogenous carriers (such as plasma lipoproteins, vesicles or exosomes) from the peripheral tissues^{2–4} into the systemic circulation.

Entry into the lymphatics is via the initial lymphatic capillaries in the interstitium. From lymphatic capillaries, lymph flows through progressively larger pre-collecting and collecting lymphatic vessels, lymph nodes and post-nodal (efferent) lymphatic vessels, each segmented frequently by semilunar valves to facilitate unidirectional flow. The collecting lymphatic vessels are also surrounded by smooth muscle that pumps lymph via contractions initiated by pacemaker cells. The majority of lymph is returned to the venous system at the junction of the left jugular and subclavian veins through the thoracic lymph duct (FIG. 1).

Historically, lymph was thought to form in the capillary beds from arterial exudate through a passive process dictated by Starling forces^{5,6}. Accordingly, measurements of hydrostatic and osmotic pressures in blood, interstitial

fluid and lymph combined with known lymph and blood flow rates led to the assumption that ~90% of the capillary filtrate in the interstitium was directly reabsorbed into post-capillary venules, whereas the remainder drained into lymphatic vessels. More recent evidence, however, suggests that a far greater proportion of the capillary filtrate may be drained via the lymph⁶. The importance of the lymphatics in capillary filtrate reabsorption is underscored by the development of tissue oedema caused by either an intrinsic (genetic) fault in the structure or function of lymphatics (primary lymphoedema) or trauma or surgery interrupting the lymphatic structure (secondary lymphoedema), such as a lymph node biopsy in cancer⁷.

Immunologically, the lymphatic vessels provide channels for antigens, antigen presenting cells (APCs) and lymphocytes to traffic from tissues to draining lymph nodes, where antigen presentation by APCs to resident lymphocytes regulates immune responses^{1,8}. Recent studies have further demonstrated that lymphatic endothelial cells (LECs) may actively modulate the immune response by controlling lymph flow and the delivery of antigens and immune cells to lymph nodes via the coordinated expression of nitric oxide, chemokines and adhesion molecules⁸. In this way, the lymphatics have key roles in promoting

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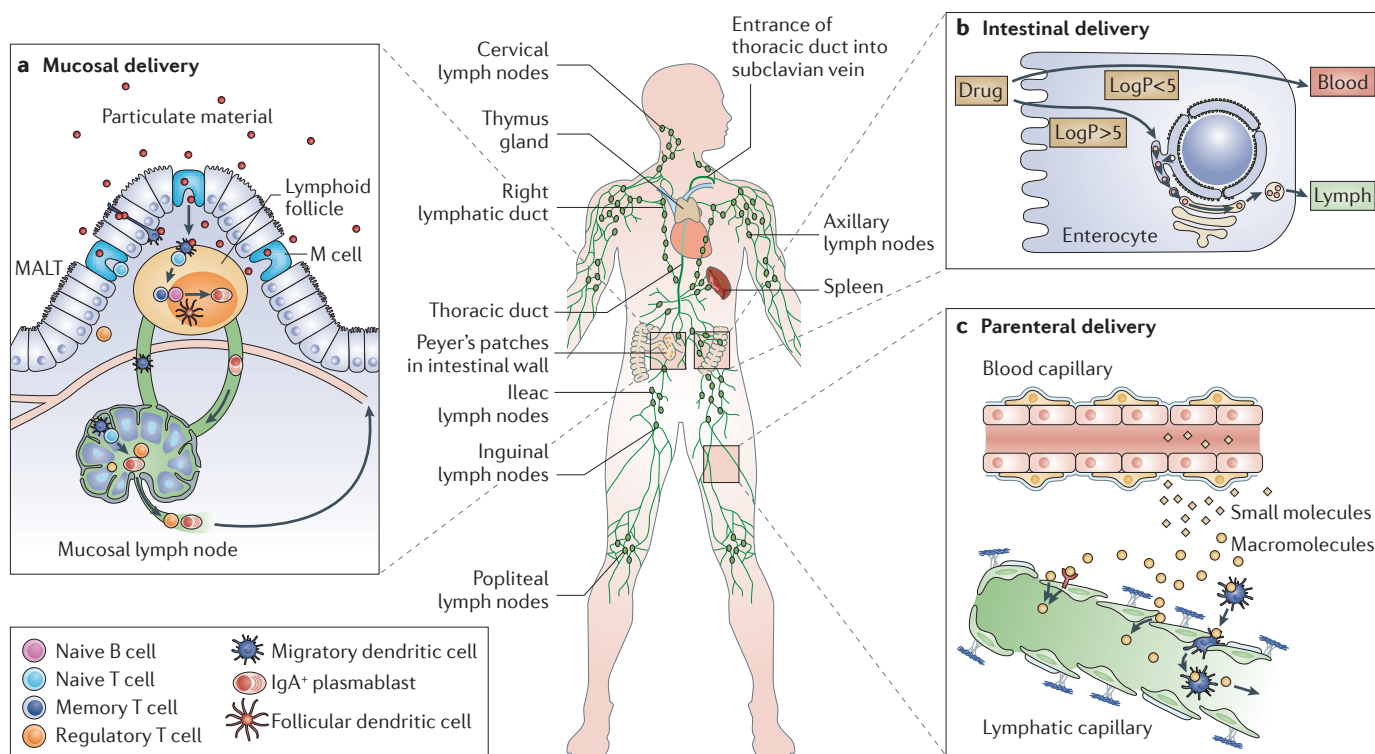


Figure 1 | Access routes to the lymphatics after oral and parenteral delivery. The lymphatic system consists of a network of lymphatic vessels, tissues and nodes. Fluid, immune cells, macromolecules and molecules packaged into carriers such as lipoproteins, vesicles or exosomes enter the initial lymphatic capillaries to form lymph fluid. From here, lymph flows through a network of progressively larger collecting (afferent) lymphatic vessels, lymph nodes and post-nodal (efferent) lymphatic vessels to converge at either the left (or right) thoracic lymph duct. Lymph empties from the major lymph ducts directly into the venous system. Therapeutics can be targeted to the lymphatic system via mucosal, intestinal or parenteral routes. **a** | Mucosal delivery of particulate materials leads to their absorption across the epithelium into mucosa-associated lymphoid tissue (MALT) (FIG. 5). **b** | Intestinal or oral delivery of lipophilic drugs (typically logP values >5) leads to their incorporation into the process of intestinal lipoprotein assembly and transport into the intestinal lymphatics (FIG. 4). **c** | Parenteral or interstitial delivery of macromolecular materials leads to their entry into lymphatic capillaries as these materials are too large to access the blood capillaries draining the injection site (FIG. 3). IgA, immunoglobulin A.

Lipoproteins

Biochemical complexes of lipids and soluble apolipoproteins that transport lipids in lymph fluid and blood to tissues throughout the body. The largest and least dense lipoproteins, chylomicrons, are assembled in the small intestine. Very low-density lipoproteins (VLDLs) and the smallest and most dense lipoproteins, high-density lipoproteins (HDLs), are assembled in both the liver and the intestine. Low-density lipoproteins (LDLs) are formed following removal of lipids from VLDL by tissues.

Initial lymphatic capillaries

Small blind-ended lymphatic vessels in the tissue periphery that have a discontinuous basement membrane, lack smooth muscle and are characterized by button-like interendothelial junctions and short anchoring filaments that are tethered to elastin fibres in the surrounding tissue. Initial lymphatics are adapted for the uptake of fluid and cells.

immune activation or the development of immune tolerance. Additionally, the mesenteric lymph nodes (MLNs) have a central role in the induction of local and systemic tolerance to self and food proteins, and in local tolerance to commensal bacteria and their by-products, as well as providing a firewall against systemic entry and immune responses to the commensal bacteria. Consequently, food allergies⁹ and inflammation and infection resulting from mucosal and systemic entry of commensal bacteria and their by-products are prevented^{10,11}.

In the intestine, dietary lipids are packaged into lipoproteins by enterocytes and preferentially drain into intestinal lymphatic capillaries, rather than blood capillaries, thereby avoiding the liver upon their return to the systemic circulation^{2,12}. Recent studies also provide evidence of broader roles for the lymphatics in lipid metabolism. For example, two separate studies suggest that the clearance of excess cholesterol from tissue macrophages is mediated via cholesterol transfer to high-density lipoprotein (HDL) followed by the transport of HDL to the systemic circulation via the lymphatic vessels, ultimately for excretion by the liver^{13,14}. These data implicate the lymphatics in HDL reverse cholesterol transport and

atherosclerosis. Indeed, functional lymphatics appear to be required to facilitate the clearance of atherosclerotic plaques¹³. Lymphatic vessels and nodes are also typically embedded in adipose tissue^{15,16}, and increased fat deposition around the lymphatics is seen in transgenic mouse strains with hyperpermeable lymphatics^{17,18} as well as in patients with lymphoedema¹⁵. High-fat diets promote changes to lymphatic permeability, contractility and transport properties^{18–21}, alter lymph node structure^{22,23}, and expand the surrounding adipose tissue^{18,19}. Together, these results suggest close links between lymphatic function, immunity, metabolism and diet.

A growing appreciation of the diversity of physiological functions that lymphatics modulate has led to the realization that they influence a wider range of diseases than once thought^{7,24–26}. These include lymphoedema⁷, cancer and metastases²⁷, immune and inflammatory conditions^{28,29} (for example, inflammatory bowel disease^{30,31}, psoriasis³², rheumatoid arthritis³³ and asthma³⁴) and metabolic disease (for example, obesity^{15,17,18}, hypertension³⁵ and atherosclerosis¹³). Liver disease and ascites³⁶, cardiovascular disease³⁷, infection (for example, HIV^{38,39}, hepatitis⁴⁰, filariasis⁴¹ and Ebola virus⁴²), acute and critical illness⁴³,

solid organ transplant rejection^{7,44}, and tolerance to self and food proteins^{9,45} are also influenced by lymphatic function. In many of these diseases, there are changes to lymphangiogenesis, lymphatic vessel density, dilation, contraction and/or lymph flow (FIG. 2), although the functional importance of these changes are not yet known. In cancer, metastatic dissemination from the primary tumour most often occurs via the transfer of tumour cells to the lymph nodes through tumour-associated lymphatic vessels²⁷. Similarly, in infection, the lymphatics appear to be a major site of viral replication and/or dissemination, notably in HIV^{38,39}, Ebola virus⁴² and hepatitis⁴⁰.

Recognition that the lymphatics have key roles in disease has driven an increased interest in targeted delivery to the lymphatics to enhance therapeutic outcomes^{29,46–49}. Mirroring the advances in our understanding of lymphatic function, the design of lymphatic delivery systems has also progressed to include sophisticated systems that mimic or integrate into endogenous lymphatic transport processes. This Review describes the latest findings regarding the mechanisms by which drugs, prodrugs, vaccines and delivery systems access the lymphatic vessels and lymph nodes after oral, parenteral and mucosal delivery. Particular focus is given to novel systems that utilize recently identified routes of lymph entry. Finally, we highlight examples of lymphatic delivery approaches that have demonstrated enhanced drug exposure or utility in immunotherapy, vaccination, viral therapy and cancer metastasis.

Collecting lymphatic vessels

These lymphatic vessels are characterized by a continuous smooth muscle cell layer and the presence of semilunar valves that facilitate the unidirectional transport of lymph and associated components. Afferent collecting lymphatics carry lymph into lymph nodes whereas efferent collecting lymphatics carry lymph from lymph nodes.

Thoracic lymph duct

The largest lymphatic vessel, sometimes called the left thoracic lymph duct, that collects most of the lymph in the body apart from the lymph draining the right thorax, arm, head and neck. The latter drain instead into the right lymphatic duct. Lymph empties from the thoracic lymph duct into the systemic circulation at the junction of the left subclavian and left internal jugular veins.

Antigen presenting cells

(APCs). A heterogeneous group of immune cells that initiate the cellular immune response by processing and presenting antigens for recognition by lymphocytes such as T cells. Classical APCs include dendritic cells, macrophages, Langerhans cells and B cells.

Tolerance

A state of immune unresponsiveness to an antigen that results from the suppression of immune responses to antigens that have been administered or encountered previously.

Lymphangiogenesis

The formation of new lymphatic vessels from pre-existing lymphatic vessels.

Routes of drug entry into lymphatic vessels

For most small molecules, drainage from the interstitial space occurs primarily via the blood capillaries because blood flow rates in these vessels are ~100–500-fold higher than lymph flow. Indeed, for most molecules and drugs delivered orally or parenterally, transport from the site of administration is via blood capillaries. By contrast, macromolecular constructs such as proteins and large peptides are able to promote entry into the lymphatics because their size precludes ready access to the blood but does not restrict lymphatic access (BOX 1). Consequently, lymphatic-targeting strategies have centred on macromolecular constructs (either unmodified or modified) that are transported from interstitial tissues via the lymphatics rather than blood capillaries.

However, lymph-targeted delivery of low molecular mass therapeutics is also achievable. Strategies include associating the low molecular mass therapeutic with synthetic macromolecular carriers (for example, nanoparticles, polymers and liposomes) or via *in situ* association with endogenous macromolecular constructs (for example, lipoproteins and proteins) or cells (leukocytes) that possess inherent lymphotropic properties. These methods have been used following parenteral, oral and, in some cases, mucosal delivery (for example, pulmonary, nasal or genital) (FIG. 1).

Parenteral delivery. The lymphatic uptake of exogenous therapeutic macromolecules follows a similar pathway to interstitial fluid, endogenous macromolecules and cells (FIG. 3). After interstitial administration (for example,

subcutaneous, intramuscular or intradermal injection) small molecules or moderately sized macromolecules that are <10 nm⁴⁸ in size (or ~16–20 kDa for proteins⁵⁰) are absorbed primarily via the blood capillaries draining the injection site rather than lymphatic capillaries. Particles >100 nm in diameter are also poorly transported into lymph owing to reduced diffusion and convection through the interstitium (the water channels that provide conduits for transfer within the interstitium are typically 100 nm in diameter^{48,51}). Between these extremes, therapeutic proteins and macromolecules 20–30 kDa^{50,52–57} in size and particles 10–100 nm in size^{48,58,59} are able to move through the interstitium and enter lymphatic vessels. Entry from the interstitium to the initial lymphatics occurs via interendothelial cell junctions and may also involve active transcytosis^{2,12,14,60–62}. The potential mechanisms that underlie the size dependency of lymphatic transport are outlined in more detail in BOX 1.

The interstitium is primarily composed of entangled collagen fibres and glycosaminoglycans that are cross-linked in a gel-like matrix⁴. The principle glycosaminoglycan is hyaluronic acid, which carries a net negative charge. Migration through the interstitium is therefore usually lower for materials carrying a net positive charge, whereas a neutral or negative charge commonly promotes interstitial transfer^{63,64}, although there are exceptions^{48,65}. Movement of hydrophilic macromolecules through the interstitial water channels is thought to occur more effectively than for hydrophobic macromolecules⁶⁶.

The transport of macromolecules into the lymph is promoted by fluid flow from the interstitium to the lymphatics (BOX 1). Factors that alter interstitial fluid pressure and flow therefore alter lymphatic transport. As such, administration at different injection sites, where interstitial pressures and fluid flows vary, leads to different degrees or rates of lymphatic transport. The foot, for example, has a high interstitial pressure, and subcutaneous injection into this region results in higher lymphatic transport than injection into the flank or abdomen of rats⁵⁸ and sheep⁵⁴. Intradermal injection may also promote enhanced lymphatic uptake compared with intramuscular or subcutaneous injection owing to higher interstitial pressure and higher lymph flow rates in the skin relative to other interstitial sites^{67–69}. Factors that increase interstitial oncotic pressure, such as the co-administration of albumin⁷⁰, dextrans⁷¹ or proteins that increase vascular extravasation (for example, bradykinin and histamine)⁷² may also promote lymphatic uptake and transport.

Albumin drains from the interstitium and is returned to the systemic circulation via the lymphatics. Therapeutic macromolecules that bind to albumin are therefore expected to preferentially drain from the interstitium into the lymphatics. Liu *et al.*⁷³ recently took advantage of this approach to develop vaccines that target lymph nodes after subcutaneous administration (see below). Derivatization of macromolecular therapeutics or carriers with targeting agents that bind targets expressed on LECs or tumours has also been used to promote interaction with and uptake across the lymphatic endothelium. For example, hyaluronic acid⁷⁴ and LyP-1 (REFS 62,75–78) have been used to target lymphatic vessel endothelial

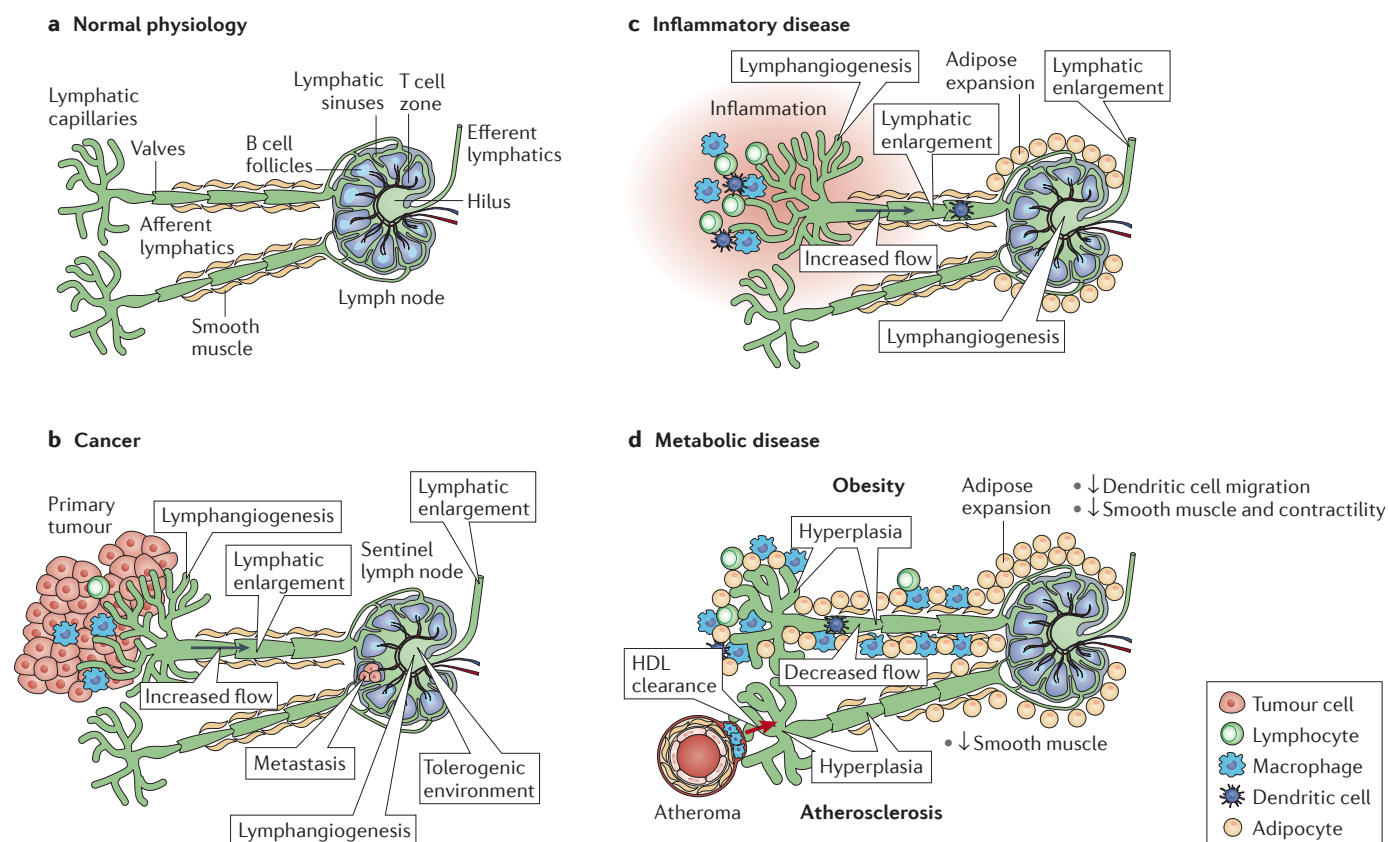


Figure 2 | Lymphatic function in health and disease. **a** | Under normal physiological conditions, entry into the lymphatics is via the initial lymphatic capillaries in the interstitium. From lymphatic capillaries, lymph flows through progressively larger pre-collecting and collecting (afferent) lymphatic vessels, through the lymph nodes via lymphatic sinuses and then to post-nodal (efferent) lymphatic vessels. The collecting and post-nodal lymphatic vessels are segmented frequently by semilunar valves and are surrounded by smooth muscle cells that facilitate unidirectional lymph flow. In disease, there are substantial changes to the lymphatic system compared with normal physiological conditions. **b** | In cancer, metastatic dissemination from the primary tumour often occurs via lymph vessels to the sentinel (first draining) lymph node. Tumour cells and associated macrophages induce lymphangiogenesis at the tumour site and in the draining lymph nodes via the release of pro-inflammatory and lymphangiogenic factors^{27,29,269}. Lymphangiogenesis, lymph vessel dilation and increased interstitial pressure modulate lymph flow from tumours and therefore alter immunity^{27,29,269}. Tumours may also release factors that promote immune tolerance^{27,29,269}. **c** | In inflammatory disease, immune cells (for example, macrophages and lymphocytes) release pro-inflammatory and lymphangiogenic factors that promote lymphatic hyperplasia^{29,33,34}. These changes stimulate alterations in the flow of fluid, inflammatory mediators and dendritic cells from inflamed tissue to lymph nodes and therefore modulate immunity and inflammation. In chronic inflammation, there is also expansion of the adipose tissue surrounding the lymph node^{29,33,34}. **d** | In metabolic disease, lymphatic function is markedly altered by high-fat diets and hypercholesterolaemia^{14,15,19,270}. High-fat diets and/or obesity alter lymph node structure^{22,23}, promote lymphatic vessel hyperplasia and dilatation, reduce lymphatic smooth muscle coverage and contractility, and reduce lymph transport of fluid and dendritic cells^{18–21,271}. The lymphatics are surrounded by adipose, and impairments in lymphatic function typically increase lipid deposition in adipose, promoting obesity^{15,17,18}. Mice with hypercholesterolaemia exhibit lymphatic vessel hyperplasia in the skin and loss of smooth muscle coverage^{14,270}. Recent data also suggest that lymphatic vessels facilitate high-density lipoprotein (HDL)-mediated cholesterol clearance from atheromas¹³. In this way, the lymph and lymphatics are broadly implicated in the development and progression of metabolic disease.

hyaluronic acid receptor 1 (LYVE1) on LECs and p32 on tumours, respectively. LECs also express a range of receptors (for example, integrins⁷⁹) and secrete chemoattractants (for example, chemokines⁸) that promote the adhesion and trafficking of immune and tumour cells through lymphatic vessels to lymph nodes. Delivery systems that bind or respond to these agents may provide additional routes to lymphatic-specific delivery.

Alternatively, targeted lymphatic uptake after parental delivery may be achieved by exploiting the mechanisms by which antigens are presented to lymphoid tissues. Antigens are phagocytosed by APCs such as dendritic cells in the extracellular matrix. APCs subsequently mature, enter the lymph and migrate to lymph nodes where they present antigen to effector cells (lymphocytes). Materials that are taken up by APCs in the

Box 1 | Mechanisms of entry into the lymphatics from the interstitium

Most descriptions of lymphatic entry focus on the enhanced permeability of lymphatic capillaries to macromolecules in comparison with vascular capillaries. Thus, small molecules and macromolecules <10 nm are relatively freely permeable across both blood and lymphatic capillary endothelium. However, because the flow of blood through vascular capillaries is ~100–500-times faster than the flow of lymph through lymph capillaries, small molecules and macromolecules are predominantly transported away from the interstitium via the blood capillaries. With increasing molecular size, transport across the vascular endothelium is reduced, whereas entry into the more permeable lymphatic capillaries is retained or becomes the preferred route of uptake.

Alternatively, lymphatic uptake may be regulated by interstitial transport rather than, or in addition to, endothelial permeability²⁵². Molecules are transferred across the interstitium via diffusion and convection. Diffusion is slow for larger materials and so convection becomes the major driver of interstitial transfer. As convective flow is typically directed from the blood to the lymph, it is expected that after interstitial injection, larger materials are delivered more effectively to the lymph than to the blood. By contrast, the diffusion of smaller molecules is more rapid and allows more effective transfer to blood capillaries. The transfer of larger (>100 nm in diameter) materials through the interstitium is ultimately limited by the dimensions of the water channels that provide the conduits for interstitial transfer. These channels are typically ~100 nm in diameter. Particle sizes between 10–100 nm thus appear to be preferred for lymphatic transfer^{46,48}.

The mechanism by which fluid and macromolecules are transferred across the lymphatic endothelium is contentious^{4,12,252}. Historically, entry from the interstitium into the lymphatics was thought to occur via the intercellular junctions between lymphatic endothelial cells (LECs). However, more recent evidence suggests that macromolecular constructs and even fluid²⁵³ may enter lymphatic capillaries via transcytosis facilitated by receptors and/or vesicular transport processes. Consistent with this suggestion, hyaluronic acid may be cleared by the lymphatics via interactions with lymphatic vessel endothelial hyaluronic acid receptor 1 (LYVE1)²⁵⁴. Ultrastructural studies have also suggested that chylomicrons are taken up across LECs via paracellular and active (transcellular) transport pathways^{12,14}. Dixon *et al.*^{60,255} further demonstrated that lipoproteins and albumin-bound fatty acids cross LEC monolayers via paracellular and transcellular pathways. Lim *et al.*¹⁴ have presented *in vitro* and *in vivo* evidence that the scavenger receptor class B member 1 (SRB1) regulates the uptake and transcytosis of interstitial high-density lipoprotein (HDL) across LECs into the lymph. Martel *et al.*¹³ also recently concluded that HDLs are cleared from the interstitium via the lymphatics, implicating the lymphatics as an integral part of the reverse cholesterol transport pathway (although the same research group also proposed that HDL enters the lymphatics largely via paracellular pathways²). Albumin is transported across vascular endothelium by the transporter gp60 and caveolae^{256,257}; however, this has not yet been demonstrated in LECs. Although the importance of receptors in facilitating the entry of macromolecules into lymphatics remains unclear, it seems likely that receptor-mediated uptake mechanisms provide a potential conduit for enhanced lymphatic delivery.

extracellular matrix can therefore traffic to draining lymph nodes in association with the APC. Larger antigens are more likely to be phagocytosed and carried to the lymph node by APCs such as dendritic cells, whereas smaller antigens (<70 kDa) or particles (<50 nm) are more likely to drain directly into the lymphatics^{59,80}. In addition, materials carrying a positive charge are generally more readily taken up into APCs than materials carrying a neutral charge⁸¹. However, the efficiency of uptake into the draining lymphatics is typically lower for larger and positively charged materials owing to enhanced retention within the interstitium^{59,82}, despite their affinity for APCs. Improved targeting to dendritic cells and draining lymph nodes has also been achieved via the derivatization of delivery systems with carbohydrates (such as mannose) that are recognized and internalized by mannose receptors^{82,83} and with mAbs to lymphocyte antigen 75 (also

known as DEC-205), a transmembrane protein found on dendritic cells⁸⁴. Finally, uptake into dendritic cells and draining lymph nodes is enhanced after intradermal vaccination compared with other parenteral routes owing to the higher number of dendritic cells in the skin.

Lymphatic transport and/or lymph node distribution has also been reported following intravenous delivery of polyethylene glycol(PEG)ylated proteins, liposomes and nanoparticles^{65,85–87}. In this case, macromolecules must first extravasate from blood capillaries into the interstitium, from where lymphatic access is expected to occur in the same way that it would after direct interstitial administration. The site (or sites) of extravasation are not well defined, although transfer across more permeable fenestrated or sinusoidal endothelium might be expected to be enhanced. Until very recently, the brain has been considered an organ that is devoid of a classical lymphatic system. However, a series of recent discoveries has demonstrated the existence of a glymphatic system and a lymphatic system in the brain that facilitate the transport and clearance of fluid, drug-like molecules, macromolecules, proteins and immune cells from the brain^{88–94} (BOX 2).

Oral delivery. Following oral administration, drugs or drug delivery systems must first pass through the intestinal epithelium to access the underlying interstitial space that is drained by the blood and lymph capillaries. As selective lymphatic access from the interstitium requires a macromolecular construct (see above), the stability of macromolecules within the gastrointestinal tract and their low permeability across the gastrointestinal mucosa are substantial physical and biological barriers to lymphatic entry after oral delivery. The flow rate of blood through the intestinal blood capillaries and portal vein is also substantially higher (~500-fold) than the flow rate of lymph through the intestinal lymphatic system. The majority of small molecules, which can readily diffuse into both blood and lymph capillaries, are therefore absorbed and transported from the intestine via the blood circulation rather than the lymphatic system owing to higher mass transport. Nonetheless, substantial lymphatic transport can occur after oral administration when macromolecular access to the gastrointestinal interstitium is possible and where access to blood capillaries is restricted. This has been described for lipophilic small-molecule drugs or prodrugs that are absorbed and then associate with intestinal lipoproteins during passage across enterocytes, and with macromolecular constructs such as antigens, tolerogens, peptides, proteins and nanosized delivery systems that are stable in the gastrointestinal tract and are permeable, at least to some extent, across the gastrointestinal epithelium. These are discussed briefly below and in FIGS 4, 5, and reviewed in detail elsewhere^{47,49,95–99}.

Lymphatic uptake of orally administered lipophilic drugs and prodrugs. For some highly lipophilic drugs, intestinal lymphatic transport may be highly efficient and the predominant route of transport to the systemic circulation following oral delivery⁴⁷. For these drugs, lymphatic access occurs via association with lipid absorption and lipoprotein assembly pathways during diffusion across

Glymphatic system

A recently identified paravascular pathway that enables the exchange of cerebrospinal fluid with interstitial fluid in the brain and provides a function similar to the lymphatic system elsewhere in the body. In this way, the glymphatics facilitate the clearance of solutes and waste products from the brain.

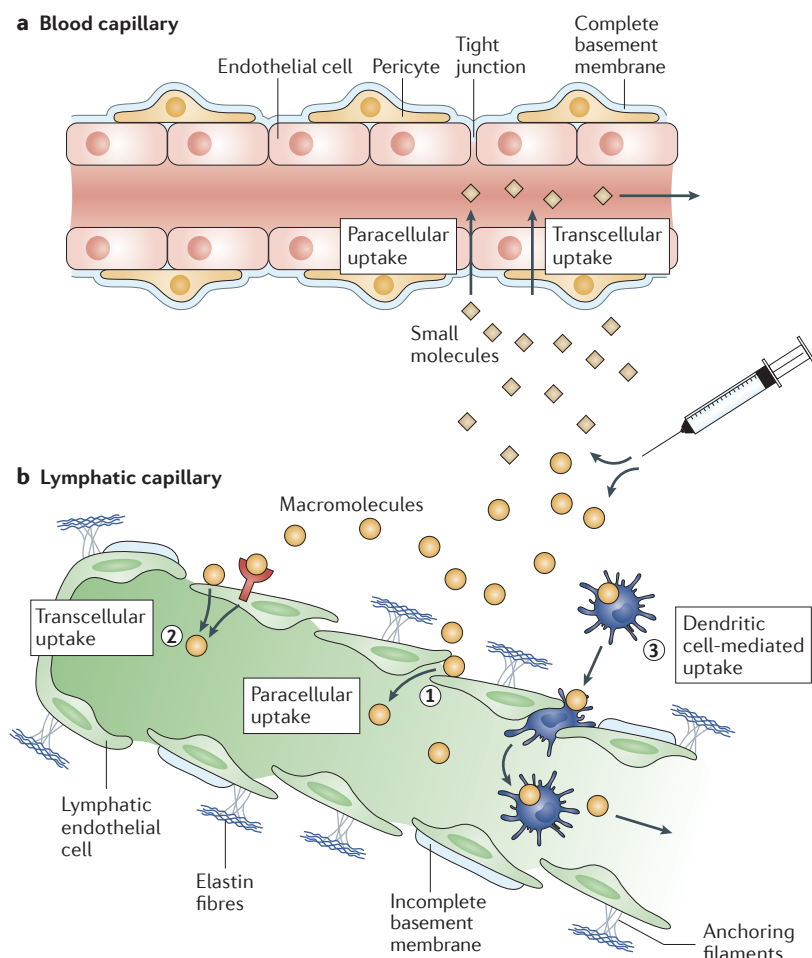


Figure 3 | Mechanisms of access to the lymphatics from the interstitial space.

a | Vascular capillaries are characterized by tight junctions between endothelial cells and the presence of an underlying basement membrane. By contrast, the initial lymphatics have a discontinuous basement membrane, lack smooth muscle and exhibit wide, button-like interendothelial junctions and short anchoring filaments that are tethered to elastin fibres in the surrounding tissue. Small-molecule drugs or moderately sized materials that are <10 nm in diameter (or ~16–20 kDa for proteins)⁴⁸ are preferentially absorbed from the interstitial space into the blood capillaries rather than lymphatic capillaries. This discrimination partly reflects the higher (~100–500-fold) flow in vascular versus lymphatic capillaries, but the mechanism is probably multidimensional (BOX 1). Uptake into the blood capillaries may occur via either paracellular or transcellular transport across the endothelium. **b** | Increasing molecular size leads to increasing uptake via the lymphatics. Transfer of materials across the lymphatic endothelium may occur via passive diffusion through interendothelial junctions (1) or transcytosis (2), either passively or following binding to receptors such as scavenger receptor class B member 1 (SRB1), lymphatic vessel endothelial hyaluronic acid receptor 1 (LYVE1) or p32 on the surface of lymphatic endothelial cells (LECs)^{14,62,74}. Materials may also access lymphatics indirectly via uptake into dendritic cells and subsequent migration of dendritic cells into the lymph (3). Uptake into dendritic cells (and thus uptake into the lymphatics) may be promoted via the attachment of targeting ligands to receptors on the dendritic cell surface^{81,82}. Lymphatic access and clearance from the interstitium is restricted for larger (>100 nm) particulates or hydrophobic molecules because diffusion and convection away from the injection site via interstitial water channels is reduced⁴⁸. Macromolecules with a partially anionic charge are repelled from negatively charged glycosaminoglycans in the extracellular matrix, facilitating improved transport through the interstitium and access to lymphatic capillaries²⁷². By contrast, the interstitial transfer of cationic materials is often restricted (although uptake into dendritic cells may be enhanced). Increases in osmotic pressure may also facilitate interstitial flow and promote lymphatic transport of macromolecules.

intestinal absorptive cells (enterocytes)^{47,49} (FIG. 4). Upon exocytosis from enterocytes, drug–lipoprotein complexes are transported across the basement membrane and trafficked from the intestinal lamina propria via the lymphatics. In general, intestinal lymphatic transport of lipophilic drugs is only substantial when the drug is administered with a source of lipid (from food or a formulation) because this is required to promote lipoprotein formation^{47,49,100}. The type and dose of lipid with which the drug is administered therefore becomes important in directing lymphatic transport. After absorption, the majority of long-chain (>C14) lipids are assembled into intestinal lymph lipoproteins, whereas the reverse is true for medium-chain lipids (<C12), for which the majority diffuse across enterocytes to directly enter the blood circulation^{101,102}. Drug administration with long-chain lipids therefore promotes lymphatic transport more effectively than administration with short- or medium-chain lipids^{47,101,102}.

Charman *et al.*¹⁰³ initially suggested that the physicochemical properties required to promote drug association with intestinal lipoproteins (and therefore to promote lymphatic transport) were a logP value of >5 and solubility of >50 mg per g in long-chain triglyceride. These approximations have been remarkably successful in predicting the potential for intestinal lymphatic transport, although some exceptions are evident, including examples of low lymphatic transport for compounds with high triglyceride solubilities¹⁰⁴ and substantial lymphatic transport for drugs with relatively low triglyceride solubilities^{105,106}. In the latter cases, drug affinity for the interfacial region of lipoproteins rather than the triglyceride-rich core, or affinity for an unidentified active transport process, have been suggested as alternative drivers of lymphatic transport^{105,107,108}. It is also apparent that drugs may influence their own disposition into the lymph by altering the production of lymph lipoproteins^{105,109}, further complicating predictive strategies. Nonetheless, the potential for drugs to associate with intestinal lymph lipoproteins *in vivo* and therefore to access the intestinal lymph has been estimated, with some success, via *in vitro* drug affinity assays using isolated or reassembled chylomicrons^{105,108,110} or via analysis of a range of molecular descriptors using *in silico* approaches^{107,111}.

Increases in lipoprotein affinity are therefore expected to enhance intestinal lymphatic transport. Most simplistically, this can be achieved via the introduction of structural modifications to enhance lipophilicity and thereby to generate highly lipophilic drug analogues. However, this is inconsistent with typical ‘rule of 5’-like progression gates for drug candidates¹¹² and commonly raises questions regarding lipophilic efficiency and toxicity¹¹³. An alternative approach is to temporarily boost lipophilicity via the synthesis of a lipophilic prodrug, whereby the parent drug is conjugated to a lipid or lipophilic moiety via a cleavable linker^{95,96}. The simplest of these prodrugs comprise alkyl esters that promote passive partition into lipoproteins in the enterocyte to facilitate lymphatic transport. However, these are relatively inefficient. By contrast, lipophilic prodrugs that integrate into lipid processing pathways, such as triglyceride or phospholipid resynthesis, are typically

Box 2 | **Lymphatic function in the brain**

Historically, the brain was considered to be an organ devoid of classical lymphatic vessels. This led to questions regarding the mechanism of clearance of fluid, waste products and immune cells from the brain. Recently, Illiff *et al.*^{88,89,91,258} identified a specific paravascular transport pathway, the 'glymphatic system', that serves a lymphatic-like function in the brain. In a series of seminal papers, the disposition of fluorescent tracer molecules introduced into the cerebrospinal fluid (CSF) was investigated after administration into either the ventricle or subarachnoid space^{88,89,91,258}. Tracers infused into the ventricle remained near the infusion site, whereas those infused into the subarachnoid space at the cisterna magna entered the brain via para-arterial pathways. Large tracers were unable to enter the paravascular space (for example, fluorescein isothiocyanate (FITC)-dextran, 2,000 kDa). By contrast, moderately sized hydrophilic tracers (for example, Texas red-dextran 3, 3 kDa) and small lipophilic tracers (for example, Texas red hydrazide, 621 Da) were confined to the paravascular space, did not enter brain parenchyma and instead moved from the para-arterial pathways to paravenous channels²⁵⁹. Smaller hydrophilic tracers (Alexa Fluor 594 hydrazine (759 Da) and mannitol) and the peptide amyloid- β were able to move from the paravascular pathways into the brain parenchyma^{88,89,91,258}. The depth of brain penetration thus appeared to be proportional to molecular size. From here, the tracers were cleared from the brain together with interstitial fluid (ISF) via a paravenous pathway. Clearance from the brain parenchyma was dramatically reduced in mice lacking astrocytic aquaporin 4 (AQP4), suggesting that clearance was dependent on convective flow of ISF facilitated by astrocytic water channels. As the pathway is dependent on glial water flux and provides a lymphatic-like function (interstitial solute clearance), the authors coined the term 'glymphatic system'.

Interestingly, both sleep and anaesthesia increased convective exchange of CSF with ISF and the clearance of amyloid- β via the glymphatic pathway owing to an increase in interstitial space⁹¹. Sleep may therefore function to clear the brain of neurotoxic waste products that accumulate while awake, providing a restorative function. Very recently, two separate labs subsequently identified the existence of conventional lymphatic vessels in the brain meninges^{93,94}. The meningeal lymphatic vessels exit the base of the skull alongside arteries, veins and nerves and connect to the deep cervical lymph nodes (dcLNs). Macromolecular dyes (a 20 kDa polyethylene glycol (PEG)-conjugated infrared dye and Alexa Fluor 488-conjugated OVA (45 kDa)) injected into the brain parenchyma were found to exit the brain along paravenous routes (the glymphatic pathway), enter the CSF space and from there were cleared via the meningeal lymphatics⁹³. The meningeal lymphatics thus appear to function downstream of the glymphatic system to transport ISF and CSF and associated macromolecules from the subarachnoid space to the dcLNs⁹³. The meningeal lymphatics also appear to transport immune cells⁹⁴ and thus display similar functionality to the lymphatics elsewhere in the body. The discovery of the glymphatic and lymphatic systems of the brain has substantial implications for immune control within the brain, the mechanism of amyloid- β accumulation in neurodegenerative disease and the identification of possible new treatment targets for neurological disorders. In the current context, the implications are in drug delivery to the brain, particularly clearance from the brain; that is, inadvertent drug accumulation might occur with sleep deprivation, ageing and disease as these pathways become less efficient.

more effective^{96,114,115}. In recent studies, for example (illustrated in FIG. 6), we have shown that triglyceride mimetic prodrugs of the immunosuppressant mycophenolic acid are far more effective in promoting lymphatic transport than simple alkyl esters or amides¹¹⁴. Interestingly this study¹¹⁴, and others^{95,115}, revealed substantial structural sensitivities in the absorption and lymphatic transport of glyceride prodrugs, in particular the point of conjugation and the nature of the conjugation chemistry. In general, conjugation at the *sn*-2 position and via an ester bond appears to promote lymphatic transport most effectively^{96,114,115}, although this is not always the case¹¹⁶.

Phospholipid mimetic prodrugs have been described for a range of purposes, including improved oral bioavailability and reduced toxicity, controlled drug release, and enhanced delivery to the brain⁹⁶. Phospholipid prodrugs for lymphatic transport are less common^{117,118}, but enhanced lymphatic transport of the phospholipid prodrug dipalmitoylphosphatidylfluorouridine and more recently DP-VPA, a phospholipid prodrug of valproic acid, has been demonstrated^{117,118}.

Lymphatic uptake of orally administered macromolecular constructs. The oral absorption of macromolecules (including therapeutic proteins and nanosized or microsized delivery systems) have been a focus of the

pharmaceutical sciences for decades^{99,119,120}. The subsequent extent of lymphatic transport of these materials is rarely studied; however, their size suggests that a large proportion of an absorbed dose might be expected to drain into intestinal lymphatic capillaries. The intestine, however, is a substantial barrier to macromolecule absorption^{99,119,120} and although an increasing number of reports suggest the possibility of the absorption or sampling of macromolecules or particulates by cells of the intestinal tract, whether the quantity absorbed is sufficient to promote biological activity across a range of applications is less clear (BOX 3). For certain applications, such as vaccination and tolerance induction, the absorption of a small proportion of the dose may be sufficient to achieve a clinically relevant effect⁹⁹. Indeed, several oral vaccines have been developed and are commercially available^{97,121} (see below), but whether the intestinal absorption of macromolecular therapeutics will ever occur in sufficient quantities to provide reliable and consistent therapeutic end points for applications other than vaccination and conditions other than those localized in the gastrointestinal tract, or where highly potent therapeutics can tolerate very low bioavailability, remains in question. Several encouraging reports have emerged (for example, REFS 122,123), but none has been translated into a clinically and commercially successful product.

LogP

The logarithm of the ratio of the concentrations of un-ionized solute (drug) in two immiscible liquid phases (usually octanol and water) at equilibrium. LogP provides one measure of drug lipophilicity, with high logP values indicating higher lipophilicity.

Lipophilicity

The affinity of a molecule for a lipophilic environment (lipid or lipid-like). Lipophilic literally means 'fat loving'.

Rule of 5

Limits to a series of molecular properties of drugs (logP, molecular weight, hydrogen bond donors and acceptors), suggested by Lipinski to increase the likelihood of good oral absorption.

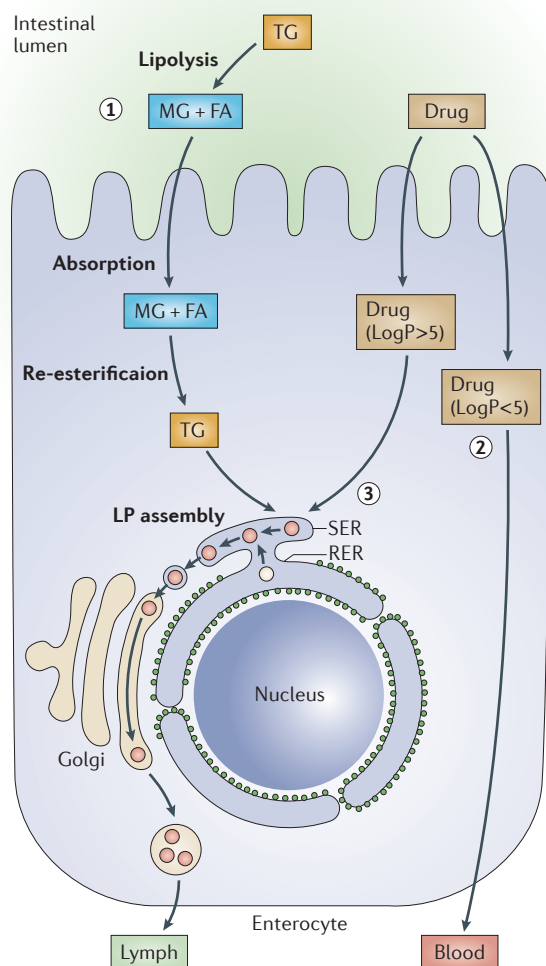


Figure 4 | Lipid and lipophilic drug access to the intestinal lymphatics after oral administration. Dietary lipids (including triglycerides (TGs)¹²) and lipophilic drugs^{47,49} access the mesenteric lymph vessels following absorption across enterocytes. (1) TGs are digested within the gastrointestinal lumen at the *sn*-1 and *sn*-3 position to release fatty acids (FAs) and 2-monoglyceride (MG). FAs and MG are absorbed from the gastrointestinal lumen into enterocytes where they are re-synthesized to TG in the smooth endoplasmic reticulum (SER). The TG droplets formed in the SER combine with 'primordial lipoproteins' consisting of phospholipids and apolipoproteins that are assembled in the rough endoplasmic reticulum (RER), ultimately resulting in the assembly of nascent lipoproteins (LPs)^{47,49}. Intestinal LPs are trafficked to the Golgi apparatus, exocytosed from the enterocyte and transported away from the intestine via the mesenteric lymphatics¹². (2) Most drugs are absorbed across the enterocyte into the vascular capillaries that drain the small intestine and are transported to the systemic circulation via the portal vein (as the rate of fluid flow in the portal vein is ~500-times higher than that of the mesenteric lymph). (3) By contrast, highly lipophilic drugs (typically, but not exclusively^{105,106}, those with logP values >5 and solubility >50 mg per g in long-chain TG lipid¹⁰³) partition into developing LPs in the enterocyte, providing a mechanism of preferential access to the intestinal lymph. Drug delivery to the intestinal lymph avoids first-pass metabolism in the liver as lymph drains directly into the systemic circulation via the thoracic lymph duct.

Mesenteric lymphatic vessels

The lymphatic vessels that collect lymph from the intestine. This includes the initial lymphatic capillaries ('lacteals'), pre-nodal (afferent) collecting lymphatic vessels and the post-nodal (efferent) mesenteric lymph duct. The mesenteric lymph duct collects almost all lymph from the small intestine.

For macromolecular constructs that do cross the intestinal epithelium, the primary route of absorption is likely to be via the intestinal lymphatic system. The intestinal lymphatic system comprises the gut-associated lymphoid tissue (GALT), including the Peyer's patches and isolated lymphoid follicles, and the mesenteric lymphatic vessels and MLNs^{9,97}. FIGURE 5 summarizes the modes of access to the GALT and mesenteric lymphatics. The characteristics of the macromolecular construct are likely to influence the site of uptake^{9,97}, although this is incompletely understood. In general, particulate antigens, including bacteria and viruses, and particulate delivery systems, cross the follicle-associated epithelium (FAE) to enter the GALT. By contrast, soluble and low molecular mass antigenic materials, such as polypeptides, may more readily cross the normal villous epithelium from where they enter mesenteric lymph vessels directly or, following interaction with dendritic cells, in the lamina propria^{9,97}.

Translocation across the FAE into the GALT typically occurs via M cells. M cells constitute only 10% of the cells in the FAE but have a higher transcytotic activity for macromolecular constructs than normal epithelial cells (enterocytes)¹²⁴. Lymphatic access can also occur across the normal villous epithelium via several mechanisms (FIG. 5). In addition to uptake across the FAE or villous epithelium, a population of myeloid cells^{9,125} in the lamina propria are able to extend their cellular processes between adjacent villous epithelial cells and sample the contents of the intestinal lumen directly, including antigens and microbiota^{126,127}. However, whether they represent a major mechanism of antigen and particulate uptake *in vivo* has been questioned⁹.

For nanoparticulate and microparticulate delivery systems, the route and extent of intestinal uptake is likely to be dependent on the characteristics of the particles⁹⁹, including physical and chemical stability, size, surface charge, shape and elasticity. The presence (or absence) of targeting ligands, such as lectins¹²⁸, invasins¹²⁹, RGD peptide¹³⁰, and others^{120,131}, may also enhance uptake. Uptake may occur either via the M cells in the GALT or via the normal villous epithelium^{119,132}. Much of the early research in this area focused on absorption via M cells in the GALT, although this route of entry may be limited because the GALT is located largely in the lower intestine and comprises less than 10% of the surface of the intestine (and, as described above, only ~10% of the epithelial cells in GALT are M cells). In general, particles >10 µm in diameter appear to be inefficiently taken up by M cells or epithelial cells, whereas particles in the nanometre to low micrometre size range may be taken up more effectively^{133,134}.

Absolute quantification of the extent of absorption of nanoparticulates and microparticulates has been investigated in few studies, and even fewer have examined lymphatic transport directly (although this is often inferred). The proportion of the dose of nanoparticles or microparticles that is absorbed intact has been reported to range from essentially zero^{135–137} to relatively large quantities (5–40% of the dose)^{123,129,132,134,138–140}. In some of the first reported studies, between 6 and 34% of

the dose of 50 nm to 3 μ m polystyrene microspheres or nanoparticles or 50–500 nm polymeric or squalenated nanoparticles were absorbed after oral administration^{129,134,138,139}. More recently, relative bioavailabilities of 4.9–7.1% were reported for solid lipid nanoparticles loaded with insulin compared with subcutaneous injection of insulin in saline¹⁴⁰, and oral bioavailabilities of 9% for fondaparinux-loaded nanoparticles administered in gastroresistant capsules¹³⁹. Absorption efficiencies of 13.7% per hour were also described for insulin administered in ~60 nm polymeric nanoparticles conjugated with crystallizable antibody fragments (Fc) that readily bind to the neonatal Fc receptor (FcRn) in the intestinal epithelium¹²³, and docetaxel administration in nanocapsules embedded in microparticles has been suggested to result in oral plasma area under the curves (AUCs) that are 1.77-times higher than that after intravenous administration of the same dose of the commercial solution (Taxotere)¹²². Finally, a recent study demonstrated that 30–45% of the dose of polystyrene microparticles of varying diameters (500 nm to 5 μ m) was absorbed after injection into isolated loops of jejunum or ileum¹³².

By contrast, perhaps as many studies suggest essentially zero absorption of nanosized or micro-sized particles; for example, absorption of <0.0055 and 0.01% of the dose of 27 nm and 170–250 nm latex particles¹³⁷ and <0.01% of the dose of 2.65 μ m-sized particles (with 0.0006% of particles detected in Peyer's patches)¹³⁵. In one of the few studies to directly quantify the uptake of nanoparticles and microparticles into the mesenteric lymphatics¹³⁶, <0.2% of the dose of a range of particles 0.15–10.0 μ m in diameter was absorbed and transported in lymph. The absorption of smaller dendrimer-based polymeric materials (2.5–15 nm) has been less well-studied *in vivo*, but where data are available, oral absorption is also very low^{141,142}. Perhaps unsurprisingly, the widely disparate oral bioavailabilities reported for orally administered nanoparticles and microparticles has led to controversy and questions regarding the consistency of studies and the differing methodologies used (BOX 3).

Mucosal and other routes of delivery. There has been a recent surge in interest in targeted delivery to non-gastrointestinal mucosal lymphoid tissues and lymph nodes, such as the nasal, pulmonary and genital mucosa. Much of this interest has focused on the delivery of vaccines to mucosal surfaces (described further below)^{46,143,144}. Different mucosal surfaces share commonalities in the structure of the associated lymphatic systems. In most mucosal surfaces, the epithelium consists of a single layer of columnar epithelial cells, although some surfaces, such as the oral mucosa, upper respiratory tract and lower genital tract comprise multilayered squamous epithelium¹⁴⁴. The normal epithelium is interrupted, at varying frequencies depending on the mucosal site and animal species, by MALT such as Peyer's patches or isolated lymphoid follicles. The MALT is covered by M cells that, as described above for the gastrointestinal tract, more readily transport antigens and particulate matter than normal epithelial cells. Antigens, macromolecular

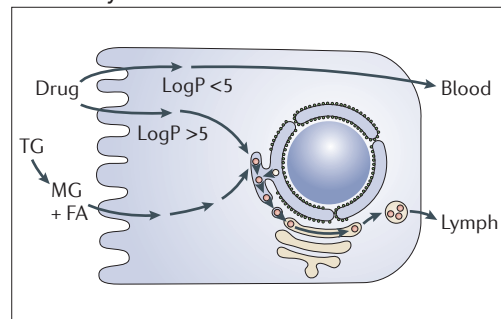
drugs and delivery systems that cross the epithelium are transported to the draining mucosal lymph nodes via lymphatic capillaries and collecting vessels.

For example, following nasal administration, 50 nm polypropylene sulfide nanoparticles transit the nasal mucosa via M cells, interact with nasal-associated lymphoid tissues and resident APCs and elicit protective immune responses¹⁴⁵. In a follow-up study, the model antigen ovalbumin was conjugated to similar nanoparticles, but in this case, of different sizes (30–200 nm). The 200 nm particles provided the most effective immune response¹⁴⁶. However, it is unknown whether this reflects enhanced uptake into the MALT, improved capture by APCs or enhanced immunogenicity of the particles.

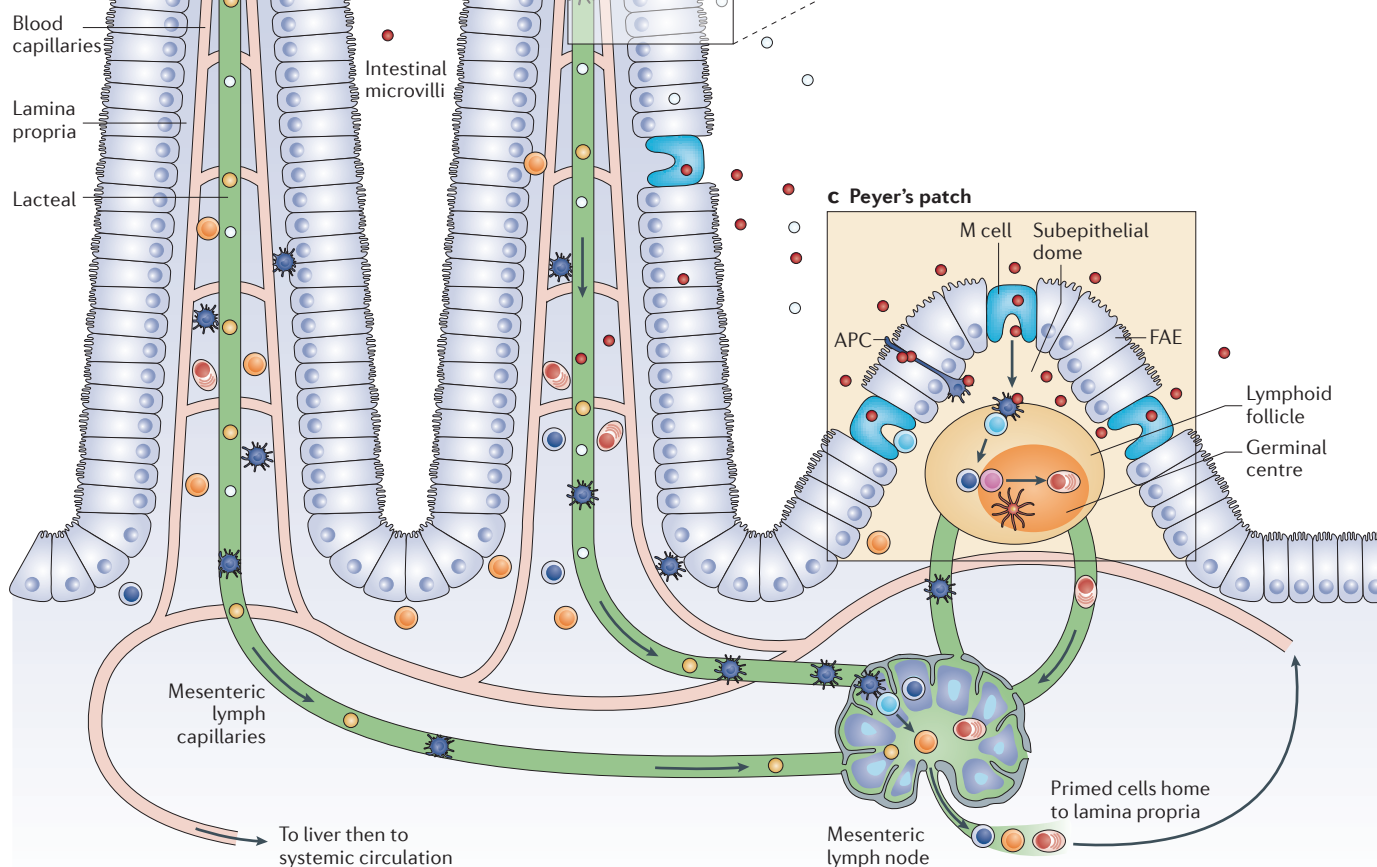
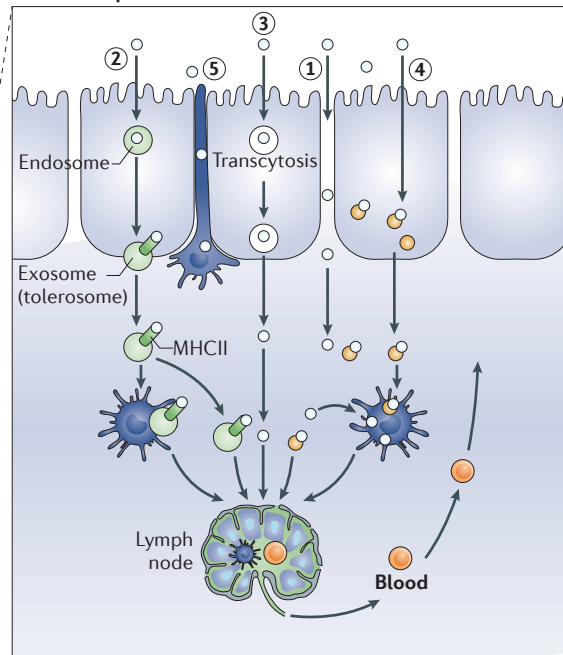
The lungs have a dense vascular supply, particularly in the respiratory region (alveoli, alveolar ducts and respiratory bronchioles)¹⁴⁷. The permeability of the lung epithelium and the density of vascular capillaries in the alveoli dictate that the absorption of small-molecule drugs from the deep lung into the systemic circulation is rapid and almost immediate in many cases¹⁴⁸. The lung is also drained via deep and superficial lymphatic vessels¹⁴⁹. The superficial lymphatic vessels lie beneath the pleural lining of the lung whereas the deep lymphatic vessels and pulmonary lymph nodes reside primarily along non-capillary vessels and the major conducting airways (trachea, bronchi and bronchioles). Lymphoid tissue is also present in the bronchus in healthy lungs of some species (for example, rats and rabbits) although it is not normally present in healthy lungs of adult humans and mice but can be induced by antigens, infection or inflammation¹⁵⁰.

The deep lymphatic vessels and lymph nodes associated with the conducting airways appear to be the most important for the elimination of inhaled foreign materials, which are normally trapped by the mucosa and cilia in the upper conducting airways and moved either to the throat and swallowed via the mucociliary escalator or captured by macrophages and cleared via the lymphatics^{151,152}. By contrast, the lymphatics in the lung parenchyma are relatively sparse, although small (10–20 μ m) interlobular lymph vessels have been observed within interalveolar walls¹⁵³. The primary function of these lymphatic vessels has been suggested to be the collection of interstitial fluid and extravasated proteins that surround the interalveolar septa¹⁵³. Inhaled nanomaterials may be removed from the respiratory region or conducting airways by the lymphatics, either following direct uptake across the pulmonary epithelium or following uptake by the large number of APCs present in the lung. Under basal conditions or after intentional induction, lymphoid tissue may also play a part in the uptake of nanomaterials from the lungs¹⁵⁰. Several studies have demonstrated uptake of inhaled nanoparticles, including liposomes¹⁵⁴, 20–70 kDa dextrans¹⁵⁵, non-cationic organic and inorganic nanoparticles of ≤ 34 nm¹⁵⁶ and antigen-carrying lipid nanocapsules¹⁵⁷ into lung lymph nodes. Several other studies have inferred lung lymphatic involvement in the systemic availability of inhaled solid lipid nanoparticles¹⁵⁸, liposomes¹⁵⁹ and 50–900 nm polystyrene nanoparticles¹⁶⁰. However, the mechanisms of

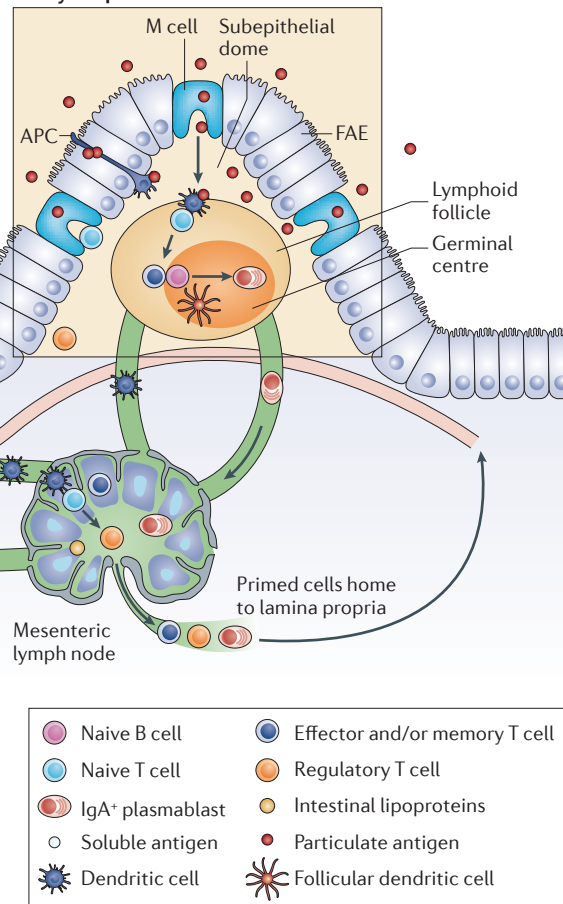
a Enterocyte



b Villous epithelium



c Peyer's patch



- | | |
|------------------------------|-------------------------------|
| Naive B cell | Effector and/or memory T cell |
| Naive T cell | Regulatory T cell |
| IgA ⁺ plasmablast | Intestinal lipoproteins |
| Soluble antigen | Particulate antigen |
| Dendritic cell | Follicular dendritic cell |

◀ **Figure 5 | Mechanisms of access to the intestinal lymphatics after oral administration.** The intestinal lymphatic system comprises the gut-associated lymphoid tissue (GALT) including the Peyer's patches, the lacteals and mesenteric lymphatic vessels that drain both the intestinal microvilli and Peyer's patches, and mesenteric lymph nodes (MLNs) into which the mesenteric lymphatic vessels flow⁹. **a** | Dietary lipids, including triglycerides (TGs)¹² and some highly lipophilic drugs^{47,49} (typically logP values >5), access the mesenteric lymph vessels following absorption across enterocytes, as described in FIG. 4. **b** | Soluble antigens, polypeptides and haptens cross the normal villous epithelium to the lamina propria from where they access the mesenteric lymphatics directly or via phagocytosis by dendritic cells prior to entry into the mesenteric lymphatics and MLNs^{9,219}. Transport of soluble antigens across the villous epithelium includes the following proposed mechanisms: (1) paracellular diffusion between adjacent epithelial cells^{9,224}; (2) uptake into endosomes and subsequent incorporation into, and exocytosis within, MHC class II (MHCII)-expressing exosomes²⁷³ (which have been referred to as tolerosomes); (3) transcytosis across enterocytes following fluid phase uptake²⁷⁴; (4) incorporation into enterocyte lipoproteins^{122,275}; and (5) sampling by lamina propria myeloid cells that extend their cellular processes between villous epithelial cells^{126,127}. Uptake via M cells and goblet cells has also been described (not shown)²⁷⁶. Within the lamina propria and MLNs, the immune response to soluble antigen is biased towards induction of tolerance (reviewed elsewhere^{9,219}). Antigenic material is commonly phagocytosed by dendritic cells and transported to MLNs where dendritic cells present the antigen to naive T cells, resulting in the generation of inducible regulatory T cells that preferentially home back to the gut after exiting the MLNs and entering the blood circulation^{9,219}. **c** | In contrast to soluble antigens, particulate antigens (for example, bacteria and viruses) and delivery systems are preferentially absorbed across the follicle associated epithelium (FAE) into Peyer's patches^{99,277}. Translocation across the FAE typically occurs via M cells, but may also occur via sampling by antigen presenting cells (APCs)^{126,127}. Below the FAE is a subepithelial dome and below this are lymphoid follicles that comprise T and B cell follicular regions and dendritic cells. The Peyer's patches are drained by efferent lymphatics that transport particulates and cells to MLNs. In vaccination, the induction of a secretory immunoglobulin A (IgA) response typically requires uptake and processing by dendritic cells in the subepithelial dome^{97,121}. Dendritic cells loaded with antigen subsequently induce the conversion of naive T cells to effector T cells^{97,121}. The effector T cells, B cells and follicular dendritic cells together induce the formation of a germinal centre in the B cell zone in the Peyer's patches, leading to B cell expansion, differentiation and affinity maturation to IgA⁺ plasmablasts^{121,143}. In an alternative scenario, primed B cells migrate to germinal centres in the MLNs where they also undergo affinity maturation with the assistance of dendritic cells and T cells¹²¹. IgA⁺ plasmablasts subsequently migrate to the blood, bone marrow, GALT or the intestinal lamina propria^{97,143}. FA, fatty acid; MG, 2-monoglyceride.

lymphatic uptake of materials from the lungs has rarely been assessed directly, and, with the exception of a relatively limited number of studies, has been conducted in rodents in which lung size and/or lymphatic anatomy are considerably different to humans. Direct evidence of the role of the lung lymphatics in the clearance of particulate materials in larger animals and humans is therefore lacking.

Routes of entry and retention in lymph nodes

Lymph nodes provide a site for immune surveillance, the generation of immune responses and initial tumour cell metastasis^{48,51}. The uptake of therapeutics into lymph nodes is therefore important for vaccination and for the treatment of immune-related disease and cancer^{29,46,48}. The interior structure of the lymph node and the routes of access to the node via the blood and the lymph are summarized in FIG. 7. High endothelial venules (HEVs) provide the primary point of entry for naive T and B lymphocytes, plasmacytoid dendritic cells and natural killer cells from the systemic circulation¹. Naive T and

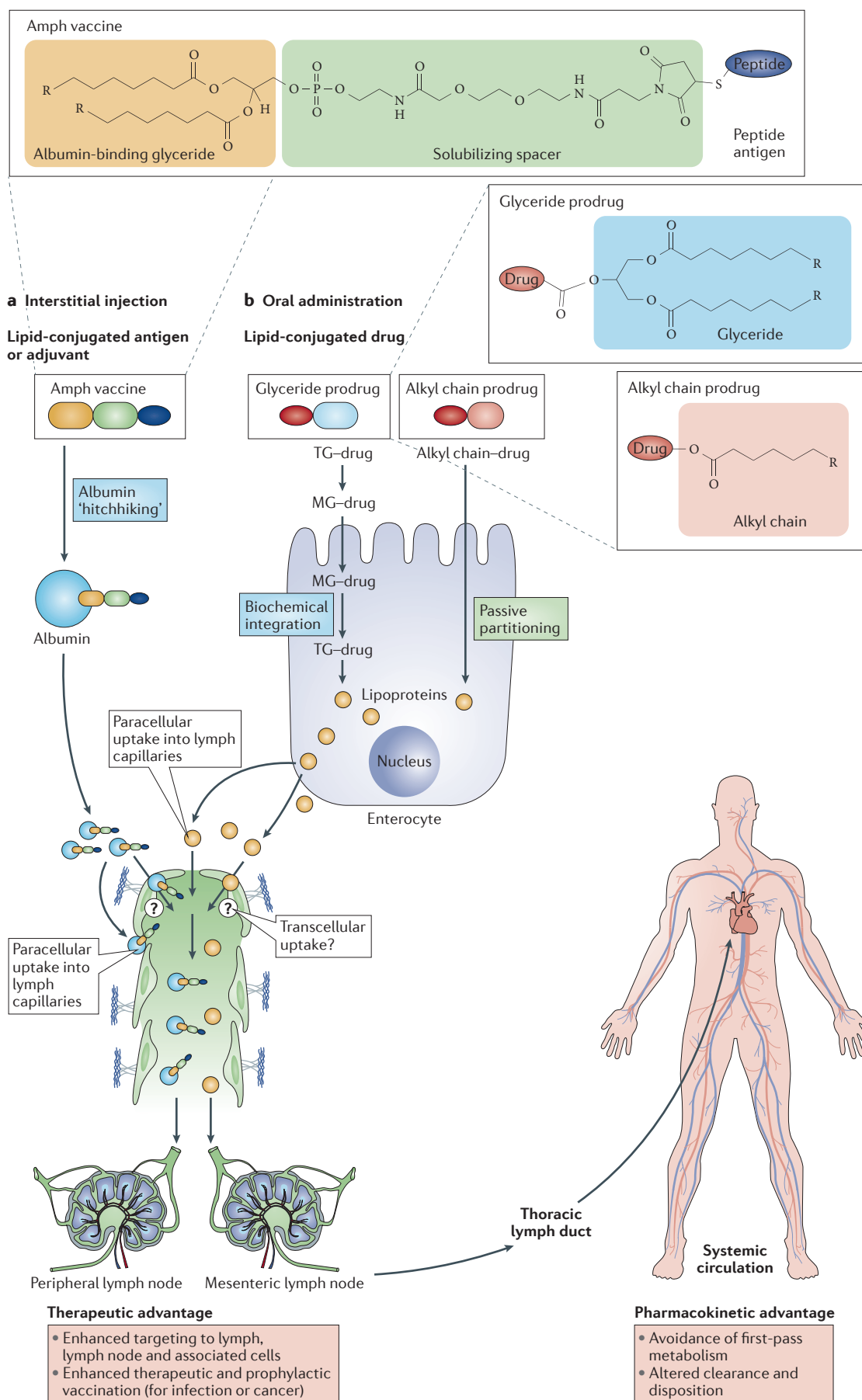
B lymphocytes extravasate across HEVs via a multi-step cascade that is initiated by the recognition of lymph node addressins by the lymphocyte-homing receptor L-selectin. This initial step results in lymphocyte tethering, rolling and ultimately transit across the HEV wall¹. T and B cells subsequently migrate through the lymph node and position themselves in the T and B cell regions of the node under the control of chemokine ligands¹.

Lymph, which contains tissue fluid, antigens, proteins, lipoproteins and immune cells, enters the lymph nodes via afferent lymphatic vessels^{1,51} (FIG. 7). The majority of the lymphocytes that enter the afferent lymph are memory T cells¹⁶¹. By contrast, naive T cells enter the lymph nodes primarily via HEVs¹. The majority of T cells that exit lymph nodes via efferent lymphatics are also naive T cells¹⁶¹. Chemokines modulate the entry, migration and retention of cells within the lymph node¹. Dendritic cells also migrate into the initial lymphatics, crawl along the lymphatics to the lymph node and migrate to the T cell-rich paracortex under the control of CCL21 and CCL1 (chemokines that are recognized by CCR7 and CCR8 receptors, respectively, on the dendritic cell surface)^{1,162}.

Therapeutics may similarly enter lymph nodes via afferent lymph vessels or HEVs. For example, nanoparticulate (20–40 nm) superparamagnetic iron oxide particles accumulate within lymph nodes after intravenous injection, which is due to a combination of transfer across HEV and extravasation into the interstitial space followed by transfer to lymph nodes via afferent lymphatic vessels¹⁶³. For direct entry via HEVs, a ligand that binds to surface receptors on HEVs might be expected to enhance uptake, although to the best of our knowledge no such system has yet been described. Alternatively, entry via HEVs may occur following uptake of therapeutics into immune cells followed by transfer of the immune cells into lymph nodes via HEVs. For example, the immunosuppressant fingolimod (Gilenya, Novartis) accumulates within lymph nodes via association with lymphocytes in the systemic circulation and subsequent uptake up into lymph nodes, presumably via HEVs¹⁶⁴.

Entry of therapeutics into lymph nodes via afferent lymphatic vessels has been studied in more detail than access via HEVs^{27,51} (FIG. 7). Access via afferent lymph occurs via direct uptake of an antigen, protein or particulate delivery system into the lymphatics from the interstitial tissue or following uptake into APCs in the interstitium and subsequent entry of the APC into the lymph. In general, larger (500–2,000 nm) and positively charged materials are preferentially taken up by APCs, particularly dendritic cells, at the injection site, whereas smaller and neutrally charged materials (20–200 nm) are trafficked directly to the lymphatics^{27,59,81,165}. After entry into lymph nodes, antigens or particles may pass around the outside of the lymph node via the subcapsular sinus and leave directly via the efferent lymph. Alternatively, materials may be retained via uptake into subcapsular macrophages or cells within the B and T cell zones¹⁶⁶. Larger particles are typically taken up by subcapsular macrophages^{166,167}, whereas smaller particles or small molecules enter the B or T cell zones through the lymphatic

High endothelial venules (HEVs). Specialized post-capillary venules that are characterized by plump, as opposed to thin, endothelial cells. HEVs are found in lymph nodes and other lymphoid tissues and support high levels of lymphocyte extravasation from the blood into these tissues.



◀ **Figure 6 | Lipid conjugates for enhanced lymphatic delivery.** **a** | Conjugation of peptide antigens to diacyl lipids via a water soluble polyethylene glycol (PEG) spacer ('amph vaccines'), and similar structures in which diacyl lipids are linked to adjuvants, promotes binding to albumin in the interstitium. Albumin preferentially enters the lymph (via paracellular and potentially transcellular access pathways) and subsequently the lymph nodes and provides a lymph-directed carrier for both antigen and adjuvant. Increased access to the lymph and lymph nodes promotes interaction with immune cells and has been shown to enhance therapeutic vaccination in cancer⁷³. Broader applicability to protective vaccination or drug delivery to lymph-resident targets may also be envisaged. **b** | Lipid prodrugs comprising drug conjugated to a glycerol backbone or fatty alkyl chain promote drug uptake into intestinal lymph after oral administration. Glyceride mimetic prodrugs 'biochemically integrate' into the pathway of triglyceride (TG) hydrolysis–absorption–resynthesis, resulting in incorporation into intestinal lipoprotein assembly pathways and access to the lymph via paracellular or possibly transcellular access routes. TG mimetic prodrugs are digested to form 2-monoglyceride (MG) derivatives, absorbed into enterocytes and re-synthesized back to TG derivatives. The TG derivatives are assembled into lipoproteins in the endoplasmic reticulum and transported from the intestine via the mesenteric lymphatics^{95,96}. Alkyl ester prodrugs are highly lipophilic and partition into intestinal lipoproteins during transport across the enterocyte. Association with the colloidal lipoproteins again promotes preferential access to the lymph^{96,114}. Alkyl ester prodrugs are typically less efficient than TG mimetics as they are more susceptible to instability in the intestine, brush border and enterocyte, leading to release of free drug and transport from the intestine to the systemic circulation via the portal vein and liver. Oral lymph-directed prodrugs provide potential pharmacodynamic advantage (via the delivery of high drug concentration to the intestinal lymph and mesenteric lymph nodes) and pharmacokinetic advantage (via the avoidance of first-pass hepatic metabolism), because intestinal lymphatic transport bypasses portal vein transport to the liver¹⁷⁷. Entry into the systemic circulation within intestinal lymph lipoproteins may also alter drug clearance and disposition²⁷⁸.

sinuses or conduits^{46,59,165,168,169}. Sequestration within lymph nodes via interaction with node-resident macrophages or dendritic cells may also be promoted by the use of targeting ligands to APC-specific receptors such as mannose^{82,83}, antigens such as DEC-205 (REF. 84) and peptide MHC and co-stimulatory receptor ligands^{170,171}.

In general, the properties that promote lymph node retention are the opposite of those required for efficient drainage from the subcutaneous injection site. For example, macromolecules of increasing size are more effectively retained in lymph nodes but drain poorly from interstitial injection sites^{81,82,172}. Thus, mitomycin-C conjugated to high molecular mass dextrans drained inefficiently from the injection site compared with unconjugated mitomycin-C or mitomycin-C conjugated to low molecular mass dextrans, but was retained more effectively in lymph nodes¹⁷³. Similar results have been observed for liposomes of varying diameter (40 nm to 400 nm); although in this case the proportion of the dose retained within the nodes was similar for all liposomes owing to a balance between decreased lymphatic uptake and increased lymph node retention as size increased^{58,167}.

Similarly, the assembly of a layer of a hydrophilic polymer, for example, PEG, on the surface of a nanoparticle typically enhances drainage from a subcutaneous injection site, but provides a steric barrier to opsonization and phagocytosis, and reduces uptake into lymph node-resident cells and lymph node retention^{65,166}. More highly charged macromolecules also show limited convection from interstitial injection sites but may be retained more effectively within lymph nodes via interactions with

resident APCs^{81,174}. For example, cationic materials are more readily taken up by dendritic cells and retained in the lymph node medulla and paracortex compared with neutral materials⁸¹. Conversely, anionic charge can promote drainage from the interstitium via electrostatic repulsion of negatively charged glycosaminoglycans at the injection site^{58,65,66} and may also promote lymph node retention⁶⁶.

Importantly, recent studies have highlighted that even when enhanced uptake and retention of materials within lymph nodes is achieved, this does not always translate into enhanced therapeutic efficacy^{80,175}. This may be because the nanomaterial does not release its cargo, does not enter the desired lymph node region, fails to interact with and activate the appropriate cells within the node or passes through the lymph node too rapidly to exert an effect. Focus is therefore now directed towards nanomaterials that not only promote lymph node sequestration, but also facilitate controlled release to the desired regions and cell types within the lymph node, modulate the lymph node microenvironment to generate appropriate immune responses and/or minimize systemic distribution and toxicity⁸⁰.

Therapeutic advantages of lymphatic delivery

The benefits of lymphatic delivery usually manifest as either an increase in exposure or an enhancement in therapeutic efficacy (that is, higher efficacy versus toxicity) or both. Several studies have shown that lymphatic delivery can enhance drug exposure, particularly following oral delivery, and other studies have provided evidence that lymphatic transport can promote effective vaccination, tolerance induction, immune therapy and the treatment of viral infections and cancer. Here, we provide a brief description of the most recent studies where lymphatic delivery has been shown to be beneficial. This is followed by a discussion of the clinical application of lymphatic delivery. Focus is directed towards benefits in drug delivery; however, lymphatic delivery strategies have also been used extensively to image lymphatic function or involvement in disease, outcomes that may also lead to enhanced therapeutic outcomes. These topics are reviewed elsewhere^{29,176}.

Oral bioavailability. Intestinal lymphatic transport circumvents hepatic first-pass metabolism. In contrast to absorption via the blood into the portal vein (and transport to the liver), the intestinal lymph flows from the intestine to the thoracic lymph before emptying directly into the systemic circulation via the major veins in the neck (FIG. 6). Increases in intestinal lymphatic transport therefore substantially enhance oral bioavailability when bioavailability is limited by first-pass metabolism. An example of this approach is the commercial product testosterone undecanoate (Andriol; Merck). Testosterone exhibits minimal bioavailability after oral administration owing to complete first-pass metabolism¹⁷⁷. By contrast, testosterone undecanoate, an alkyl ester prodrug of testosterone, enables oral testosterone replacement therapy because a proportion of the dose of the prodrug is transported via the intestinal lymphatics, thus avoiding first-pass metabolism¹⁷⁷.

Box 3 | Are nanoparticles absorbed and transported to the lymphatics after oral administration?

The realization that various endogenous macromolecules, antigens and bacterial products are transported from the intestine into the lymphatics has inspired the use of synthetic nanoparticulate and microparticulate delivery systems as a means of enhancing oral bioavailability and/or intestinal lymphatic transport of drugs, therapeutic peptides and proteins and vaccine antigens^{99,119,120,260–262}. However, the intestinal epithelium is a substantial barrier to the absorption of macromolecules¹²⁰, and under normal physiological conditions, particulate or macromolecular antigens are sampled from the intestinal tract in relatively low quantities. Whether sufficient quantities of nanoparticles are absorbed to deliver a typical therapeutic load is therefore less clear. Nonetheless, several recent studies have suggested that nanoparticles can be absorbed across the intestinal tract and transported into the lymphatics, in some cases in large quantities^{123,139,140}. A re-examination of the likelihood of particle absorption is therefore timely.

The proportion of the dose of nanoparticles or microparticles that is absorbed intact has been reported to range from essentially zero^{135–137} to relatively large quantities (5–40% of the dose)^{123,129,132,134,138–140} (see the main text for more details). Across these studies, the efficiency of uptake and the role of lymphatic transport have been assessed using a range of techniques, making cross-comparison difficult. In general, few studies report nanoparticle bioavailability using detailed pharmacokinetic analyses of exposure after oral and intravenous administration. Cross-comparison of results is further complicated by the labelling of nanoparticles with fluorophores or radiolabels to track disposition; the presence of free label, or liberation of free label in the gastrointestinal tract or enterocyte, making absolute quantification difficult. Many studies have also utilized gel permeation chromatography to detect polymer levels or cytometry to count particle numbers in blood or tissues.

The extent of lymphatic transport of nanoparticles has rarely been quantified directly. Many authors have taken advantage of an indirect model of lymphatic transport that compares plasma levels in the presence and absence of cycloheximide^{122,183,263–266}. Cycloheximide inhibits protein synthesis and therefore inhibits intestinal lipoprotein secretion into lymph²⁶⁷. Dahan *et al.*²⁶⁷ initially utilized this model to study the intestinal lymphatic transport of vitamin D. Lymphatic transport was assessed directly in lymph-cannulated animals and indirectly in animals administered cycloheximide via the reduction in plasma exposure on cycloheximide pretreatment. In this case, good correlation between the two methods was shown, thus providing support for this approach in measuring intestinal lymphatic transport. However, cycloheximide is a nonspecific protein synthesis inhibitor that perturbs enterocyte ultrastructure²⁶⁸. Broader effects on nanoparticle processing are therefore likely, and studies in which lymphatic transport is not verified directly are difficult to interpret. The visualization of nanoparticles in lymph or lymph nodes via imaging techniques is also commonly used to support the suggestion that lymphatic transport of nanoparticles is substantial following oral delivery. This technique provides evidence of nanoparticles in the lymph but fails to provide a means of absolute quantification. This observation is further complicated by the use of highly lipophilic fluorophores that associate with lipoproteins and might be expected to be transported into lymph via lipid transport pathways regardless of association with nanoparticles. More work is therefore required to validate indirect lymphatic transport models, such as the cycloheximide model (ideally via direct comparison with data in lymph-cannulated animals), especially for nanoparticle-based systems.

Undecanoate esters also enhance the oral bioavailability of methyltestosterone¹⁷⁸ and dimethandrolone¹⁷⁹. Similar studies have shown that promoting the intestinal lymphatic transport of lipophilic drugs (rather than prodrugs) such as halofantrine¹⁸⁰ and CRA13 (REF. 181) via co-administration with lipids also increases systemic drug exposure by reducing hepatic first-pass metabolism. In the case of halofantrine, there is also evidence to suggest a reduction in enterocyte-based metabolism via drug sequestration in lipoproteins in the enterocyte¹⁸².

Recently, it was suggested that lymphatic transport may be harnessed to enhance the oral bioavailability of docetaxel¹²². When administered in standard formulations, docetaxel has poor oral bioavailability that is due to extensive cytochrome P450 3A4 (CYP3A4)-mediated first-pass metabolism and P-glycoprotein (also known as MDR1/ABCB1)-mediated efflux. By contrast, after oral administration of docetaxel incorporated into nanocapsules that were then embedded in microparticles, exposure was significantly enhanced. The authors hypothesized that the orally administered docetaxel nanocapsules were transported into the intestinal lymphatics after receiving a surface coat of apoproteins and phospholipids during passage across the enterocytes (that is, they became 'lipoproteinized').

Cancer chemotherapy. Many cancers metastasize via the lymphatics, with cancer cells initially lodging and proliferating in the sentinel (that is, first draining) lymph node⁴⁸. Lymph node metastases are often removed surgically (lymphadenectomy) or obliterated using radiation therapy in an attempt to prolong survival. However, this process is invasive and leads to morbidity associated with the disruption of lymphatic flow, including pain, swelling and oedema⁴⁸. Lymph-targeted chemotherapy has the potential to enhance delivery to lymphatic-resident cancers and to reduce systemic exposure that correlates with dose-limiting side effects.

A relatively limited number of studies have described benefits in the treatment of cancer via lymph targeting of orally delivered drugs¹⁸³ or prodrugs^{118,184}, including the docetaxel studies described above¹²². A far larger body of literature, however, describes the therapeutic benefits of access to the lymphatics and sites of lymphatic cancer metastasis after parenteral administration. Enhanced drug exposure to metastasis-bearing lymph nodes has been shown after interstitial administration of a range of macromolecular constructs at or near the site of the primary tumour. Improved lymph node targeting has been demonstrated for PEGylated proteins¹⁸⁵, liposomes^{86,186}, dendrimers^{86,186}, polymeric micelles^{187–189},

nanoparticles^{190,191} and hyaluronan conjugates¹⁹². Lymph node targeting using these approaches has been shown to reduce the growth, or promote the regression, of metastatic and/or primary tumours^{73,185,193–197} and to reduce systemic toxicity⁶⁶.

Immunomodulation. Promoting the delivery of small-molecule immunosuppressant drugs to targets within the lymphatics has the potential to enhance immune modulation. For example, the incorporation of immunomodulatory drugs into 50 nm micelles formed from amphiphilic block copolymers enhanced the delivery of the immunomodulatory drugs to the lymph nodes draining the subcutaneous injection site and promoted both anti-inflammatory and immunosuppressive effects¹⁹⁸. In a similar approach, but in this case harnessing intestinal lymphatic transport processes, a highly lipophilic immunomodulator more effectively targeted lymphocytes and increased the expression of the anti-inflammatory cytokines interleukin-4 (IL-4) and IL-10 in lymphocytes after *ex vivo* mitogen stimulation when co-administered with lipid to stimulate lymph transport¹⁹⁹. As described above, the efficacy of fingolimod has also been linked to its accumulation in lymph nodes¹⁶⁴. Lymphatic vessels themselves have recently emerged as therapeutic targets in inflammation, suggesting the potential for lymph-targeted delivery to enhance anti-inflammatory therapy^{28,29}. Interestingly, many current treatments for inflammatory diseases, such as anakinra (Kineret; Sobi), tocilizumab (Actemra; Genentech) and infliximab (Remicade; Janssen) affect lymphangiogenesis^{200–202}. These therapeutic proteins are administered via subcutaneous and/or intravenous injection and their relatively large size suggests that at least a proportion of the dose will distribute via the lymphatics. Lymph vessel effects stemming from enhanced lymphatic exposure may therefore have a role in their anti-inflammatory activity.

Parenteral vaccination. Vaccines promote immune protection (or activation) or stimulate the development of immune tolerance. Thus, they prevent disease (prophylactic vaccines) by providing immune protection against future encounters with pathogens, or prevent allergy, organ transplant rejection or autoimmune disease by promoting immune tolerance to innocuous self or foreign antigens. Vaccines can also treat disease (therapeutic vaccines) by promoting immune activation and eradication of, for example, viruses or tumour cells and by enhancing immune tolerance to treat allergy or autoimmune disease⁵¹. The potential for lymphatic delivery to enhance tolerance is summarized in the following section. The potential for lymphatic delivery to enhance immune protection (prophylactic vaccination) or activation (therapeutic vaccination) is discussed below.

Vaccines typically comprise live-attenuated microorganisms, purified proteins or peptides, or DNA or RNA, administered in combination with adjuvants that facilitate the recruitment of immune cells and activate the appropriate immune responses^{51,203}. Vaccine-mediated immune protection or activation is a complex

process that is highly dependent on vaccine delivery to immune cells within the lymphatic system and the development of an immune response. The typical immune response to a vaccine antigen is reviewed in depth elsewhere^{51,203}. Most commonly, vaccine antigens are administered into interstitial tissues and are transported from the injection site to lymph nodes via lymphatic vessels. Upon reaching the lymph nodes, antigens generate an antigen-specific immune response via the activation of T lymphocytes, which in turn activate B lymphocytes to produce protective antibodies. This usually occurs via the binding and internalization of the antigen by dendritic cells (or other APCs) at the injection site followed by trafficking to lymph nodes where dendritic cells present antigens to T lymphocytes. Alternatively, antigens can enter the lymphatic capillaries directly and drain to lymph nodes where antigens are internalized and presented to lymphocytes by resident dendritic cells, resulting in lymphocyte activation²⁰⁴.

The importance of the lymphatics in the vaccine response has stimulated studies to enhance vaccine efficacy by promoting lymph node targeting. The methods used usually involve targeting antigen and adjuvants to lymph nodes in a controlled manner. Most simply, direct injection of vaccines into lymph nodes (that is, intranodal injection) has been shown to enhance vaccine potency and to be safe and efficacious in preclinical models and clinical trials^{51,205–208}. However, intranodal injection requires a trained health care professional and the benefits of lymph node targeting following intranodal injection may be short-lived owing to rapid flushing of the lymph node by lymph fluid. To investigate these concerns, a combination of a model vaccine (ovalbumin) and a biodegradable microparticle formulation to provide sustained release of an adjuvant (a Toll-like receptor 3 ligand) to the lymph node were tested and shown to enhance immune responses to ovalbumin relative to intramuscular injection or intranodal injection of control formulations²⁰⁷.

Many studies have shown that vaccine efficacy is enhanced by increasing the lymphatic uptake of vaccine antigens and adjuvants^{80,168,209,210}. For example, administration into the skin can improve vaccination responses relative to administration via other parenteral routes. This enhanced response reflects the higher number of dendritic cells and the richer supply of lymphatic vessels in the skin and therefore enhanced antigen uptake and immune responses in draining lymph nodes^{68,69}. Conjugation of the model antigen ovalbumin to surface-modified biodegradable polypropylene sulfide nanoparticles also increased delivery to dendritic cells in the lymph node following interstitial administration. This led to enhanced activation of the complement cascade, humoral and cellular immune responses^{59,168}. Similarly, targeting of ovalbumin or a malaria antigen to lymph node APCs via subcutaneous injection in interbilayer-crosslinked multilamellar vesicles enhanced humoral and cellular immune responses²¹¹. Targeting TRP2 peptide antigen to lymph node dendritic cells via subcutaneous injection in cationic micelles also enhanced cellular immune responses and reduced tumour growth in a

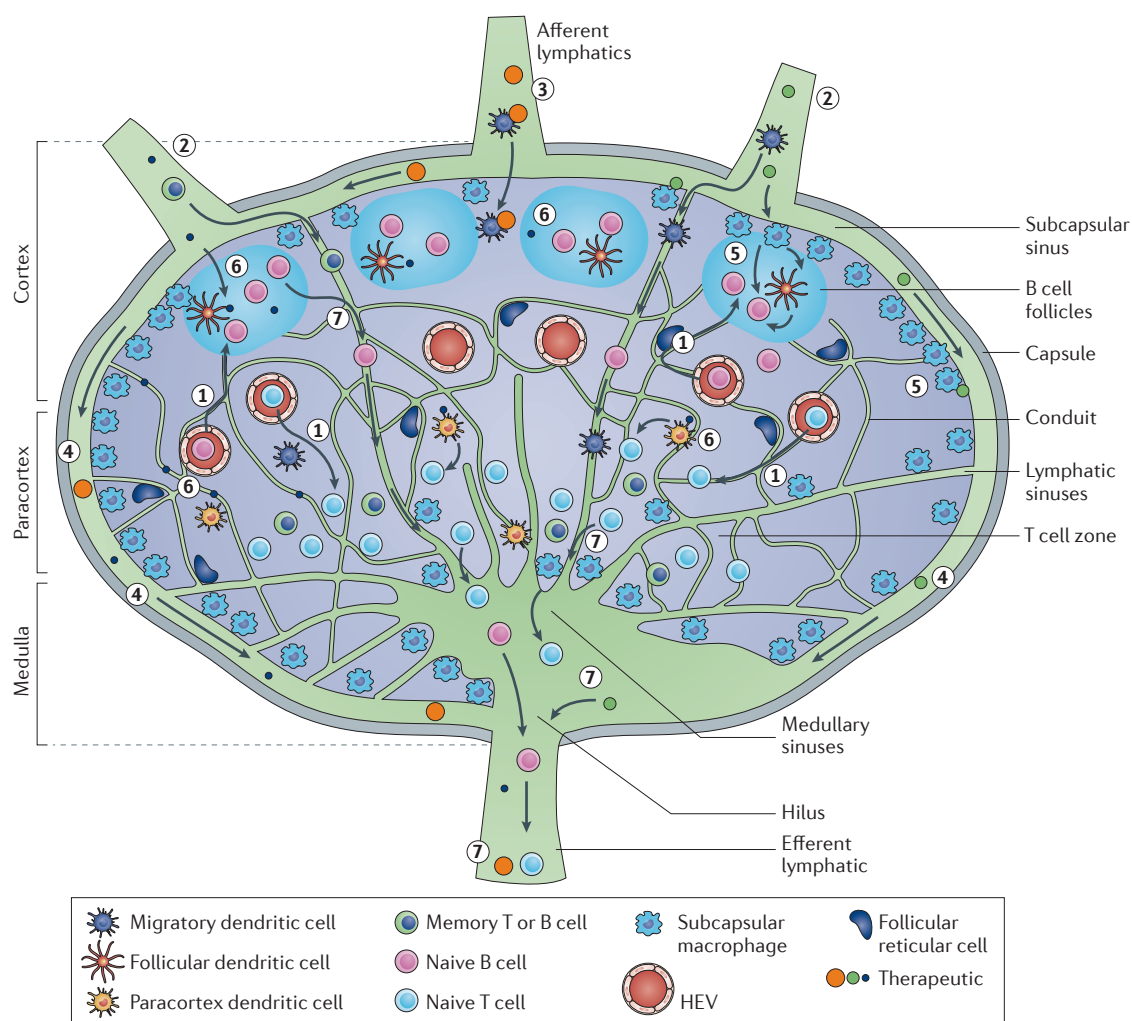


Figure 7 | Lymph node entry and trafficking mechanisms. Lymph nodes comprise the cortex, paracortex and medulla that are populated with B cell follicles, T cells and lymphoid tissue, respectively¹. Blood is supplied by specialized high endothelial venules (HEVs) that terminate within the paracortex¹. (1) Naive T and B lymphocytes enter the node via extravasation across HEVs¹. Therapeutics that associate with lymphocytes in the systemic circulation can enter the lymph node across HEVs together with lymphocytes¹⁶⁴. (2) Afferent lymph, containing particulates, macromolecules, dendritic cells and memory T cells that enter lymphatic capillaries in the periphery, enters the node through the outer capsule^{1,162}. (3) Larger therapeutics (>500 nm) that are internalized by dendritic cells at the injection site enter nodes in association with dendritic cells^{46,59,165}. (4) Lymph (and its contents, including therapeutics) either flows around the node via the subcapsular sinus to the efferent lymphatic⁴⁶ or flows to lymphatic sinuses that enter into the centre of the node^{1,51}. (5) Large and/or opsonized delivery systems are often taken up by the macrophages that line the subcapsular sinus^{279,280} and can transfer material to dendritic cells or B cells^{46,59,165–167,169}. Cellular uptake may be promoted by targeting ligands or highly charged molecules^{65,81} and avoided by polyethylene glycol (PEG)ylation, which provides a steric barrier to opsonization and phagocytosis⁶⁵. The lymphatic sinuses run from the subcapsular sinus, through the B and T cell zones and converge at the medullary sinus passing lymph to the hilus and ultimately efferent lymph¹. Within the B and T cell zones, narrow conduits (~3 or 5 nm wide, respectively) branch off from the lymphatic sinuses^{1,51}. (6) Smaller particles and/or molecules can enter the B or T cell zones through lymphatic sinuses and conduits and may be taken up by B cells and dendritic cells that interact with T cells^{51,59,165,169}. (7) Particles or molecules that pass through the node, and naive lymphocytes that do not meet their specific antigen in the node, leave via the efferent lymph¹.

B6-F10 murine melanoma model compared with non-lymph targeted TRP2 (REF. 81). In a different approach, polymeric nanoparticles were developed that mimicked mast cell granules and targeted the lymph node draining an injection site, slowly releasing cytokines²¹². This approach enhanced vaccination-induced responses to an influenza virus antigen and increased survival from a

lethal challenge of the virus. More recently, DNA or peptide vaccines and an adjuvant (CpG) have been targeted to lymph nodes using an albumin 'hitchhiking' approach, leading to enhanced immune responses⁷³ (FIG. 5). For therapeutic cancer vaccines, recent studies have suggested that targeted delivery specifically to lymph nodes draining a primary tumour site may provide additional

benefit compared with targeting other lymph nodes^{213,214}. For example, vaccination with a polymeric nanoparticle-based therapeutic vaccine induced stronger local and systemic cytotoxic CD8⁺ T cell responses following delivery to the tumour draining lymph node compared with distal lymph nodes²¹⁴.

Mucosal vaccination. Mucosal vaccination provides a biological advantage via the promotion of systemic immunity as well as immunity at local (and distal) mucosal sites^{121,143,144}. However, mucosal immunity, particularly in the intestine, is biased towards the development of tolerance. Mucosal vaccines therefore require adjuvants to promote immunogenicity and immunity rather than tolerance. Inadequate immunogenicity remains a major challenge to the effective development of mucosal vaccines^{121,143,144}.

In the design of mucosal vaccines, particulate antigens are usually more effective than soluble antigens^{116,147,148}, and nanoparticles or microparticles, liposomes, virus-like particles, bacterial ghosts and immunostimulating complexes have been widely used to enhance vaccine efficacy^{116,147,148}. The benefits of particulate vaccines probably stem, at least in part, from enhanced translocation via M cells and access to MALT compared to soluble antigens. Immune responses are subsequently stimulated by antigen uptake by dendritic cells in MALT, dendritic cell activation and antigen presentation to T and/or B lymphocytes in the MALT (or draining mucosal lymph nodes), ultimately leading to the generation of memory and effector lymphocytes (FIG. 5).

Delivery of antigens and adjuvants to the mucosal lymphatics is therefore critical to effective mucosal vaccination, a suggestion supported by many studies that describe enhanced immune responses after targeting particulate vaccines to the MALT via M cells²¹⁵. In oral vaccination, protection from degradation within the gastrointestinal lumen, as well as efficient uptake into the mucosal lymphatics, is important in ensuring an adequate immune response. A recent report showed enhanced immune protection against vaccinia virus in the rectum and vagina via the administration of the vaccine and adjuvant in poly(lactic-co-glycolic acid) (PLGA) nanoparticles encapsulated in pH-responsive microspheres. The microspheres selectively dissolved in the terminal ileum (protecting against degradation) and were subsequently taken up into Peyer's patches²¹⁶.

Enhanced mucosal vaccination has also been described via targeted delivery to the mucosal lymphatics after nasal or pulmonary administration. For example, intranasal administration of degradable polymeric nanoparticles conjugated to ovalbumin and adjuvant enhanced uptake into dendritic cells in the nasal-associated lymphoid tissue and promoted mucosal responses to ovalbumin in the lung and also at more distal mucosal sites such as the vagina and rectum^{145,146}. Similarly, pulmonary administration of lymph node-targeted nanoparticles conjugated to tuberculosis antigen enhanced T cell responses and reduced lung mycobacterial burden²¹⁷. Pulmonary administration of the adjuvant CpG, and lymph node targeting of nanoparticle-based carriers conjugated to ovalbumin or

the influenza virus, led to more robust immunization and protection compared with (non-lymph targeted) soluble ovalbumin and CpG²¹⁸. Finally, lipid nanocapsules containing a protein or peptide antigen showed increased uptake into APCs and promoted transport to the draining lymph nodes after pulmonary administration compared with pulmonary administration of soluble antigen or subcutaneous administration of the nanocapsule-based system. When administered in combination with Toll-like receptor agonists, these antigen-loaded nanocapsules improved the efficacy of both a therapeutic tumour vaccine and a prophylactic viral vaccine compared to soluble antigen and adjuvant¹⁵⁷.

Tolerance. Tolerance encompasses a range of mechanisms that are initiated to suppress local and/or systemic immune responses to antigen^{9,219–221}. Therapeutically, tolerance induction is being explored as a means of overcoming poor inherent tolerance to food, animal and plant antigens⁹, and to treat autoimmune disease^{219,221} and allograft rejection²²², in which tolerance to certain self-antigens is lost. Tolerance can be induced via mucosal (usually oral) or systemic administration of regulatory signals, soluble peptides, *in situ* production of peptide antigens via DNA vaccination, or the injection of peptide-coupled cells²²⁰.

The mesenteric lymphatics and, in particular, the lamina propria and MLNs are crucial sites for the promotion of oral tolerance^{9,219} (FIG. 5). It has been suggested that the preferred route of antigen entry for oral tolerance induction is via the villous epithelium and transport to the MLNs via the intestinal lymphatics (either directly or indirectly following uptake into dendritic cells)^{9,223}. By contrast, M cell-mediated antigen uptake into the GALT may have a subordinate role in oral tolerance induction, at least to protein antigen (although it may be more important in tolerance to commensal bacteria). Soluble antigens that are more readily taken up across the villous epithelium (FIG. 5) might therefore be expected to be more effective in promoting tolerogenic responses compared to particulate antigens²²⁴. These suggestions are supported by studies that demonstrate impaired oral tolerance after removal of MLNs^{225,226}, but normal tolerance induction in the absence of Peyer's patches^{226–228}. Contrary findings are apparent, however²²⁹, and targeted delivery of protein antigen directly to Peyer's patches via M cells facilitated tolerance induction²³⁰. Similarly, oral antigen delivery in liposomes²³¹, nanoparticles^{232,233} and microspheres²³⁴ appeared to enhance tolerance, although the mechanisms involved are not completely understood.

Several studies have also demonstrated that tolerance can be enhanced via targeted delivery to the lymphatic system following parenteral (rather than oral) delivery⁸⁰. Systemic injection of PLGA nanoparticles loaded with mycophenolic acid enhanced distribution to spleen and lymph node-resident macrophages and dendritic cells and promoted tissue graft survival by limiting the ability of the APCs to prime and expand graft-reactive T cells²³⁵. Similarly, co-delivery of the immunomodulator rapamycin with either protein or peptide antigens in

nanoparticles enhanced distribution to lymph nodes after subcutaneous delivery and induced potent and durable antigen-specific immune tolerance²³⁶. In the latter study, inhibition of antigen-specific hypersensitivity reactions, attenuation of relapsing experimental autoimmune encephalomyelitis (an animal model of multiple sclerosis) and reduced antibody responses against coagulation factor VIII in haemophilia A mice were also evident²³⁶. By contrast, administration of non-lymph targeted (free) rapamycin and separate administration of rapamycin and antigen did not promote immune tolerance. Co-formulation of a self antigen with a small-molecule inhibitor of inflammatory nuclear factor- κ B (NF- κ B) in liposomes has also been shown to enhance uptake into lymph node-resident dendritic cells, reduce NF- κ B (and thereby decrease the proliferation of self-reactive T cells) and to diminish the severity of arthritis²³⁷. Tolerance has also been induced in an animal model of multiple sclerosis, by administration of microparticles designed to mimic tolerogenic apoptotic cells, and was found to depend on microparticle delivery to marginal zone macrophages in the spleen, a primary lymphoid organ²³⁸.

Viral infection. Targeted delivery to the lymphatic system is expected to enhance therapy against viruses that reside, replicate within and/or disseminate via the lymphatics. In support of this contention, the effectiveness of antiretroviral suppression of HIV replication in lymphoid tissue was recently found to be correlated with the concentration of antiretroviral drugs in the lymph nodes of patients³⁸. Other studies have also suggested that insufficient antiretroviral concentrations in lymphoid tissues may contribute to viral persistence^{239–241}. Lymphatic-targeted drug delivery is therefore increasingly being explored as a means of enhancing antiretroviral activity^{242–244}. Thus, glycerolipidic prodrugs have been synthesized to promote drug delivery to HIV reservoirs in the gut lymphatics^{245,246}, and a range of nanomedicine platforms (for example, drug–polymer conjugates, dendrimers, micelles, liposomes, solid lipid nanoparticles, nanosuspensions and polymeric nanoparticles) have been used to facilitate delivery of antiretrovirals to HIV reservoirs in lymphoid tissue^{242–244}. These systems have been delivered via parenteral and/or oral routes, and attempts to enhance targeting to lymphoid reservoirs have been made via the use of targeting ligands, such as folic acid and mannose, for immune cells^{242–244}.

Unfortunately, although increases in lymphatic transport have been described, studies that show parallel increases in treatment benefit are less common. In one example, subcutaneous administration of indinavir in nanoparticles prolonged plasma residence times and enhanced lymph node concentrations of indinavir in HIV-2 infected macaques, leading to significantly reduced viral RNA load and increased CD4⁺ T cell numbers²³⁹. In a second example, a nanoformulated antiretroviral therapy targeted to the folic acid receptor increased drug levels in macrophage-rich regions of the spleen and lymph nodes and potently reduced viral loads, tissue viral RNA and numbers of HIV-1 p24⁺ cells in a mouse model of HIV²⁴⁷.

Clinical application of lymphatic drug delivery

Although substantial advances in lymphatic drug delivery have been made in recent years, a relatively small number of current or previously marketed pharmaceutical products have been designed to intentionally increase lymphatic delivery to achieve pharmacokinetic or therapeutic benefits. Testosterone undecanoate provides one obvious example²⁴⁸. A series of recent clinical trials to evaluate the utility of intranodal delivery of vaccines provide a further example of a direct attempt to enhance therapy through delivery to lymph nodes^{51,179–182}. In reality, many parenteral or orally administered vaccines are likely to be taken up into the lymphatic system to promote an immune response^{51,177}. However, it seems likely that most were not designed with this property in mind. Similarly, several orally administered highly lipophilic drugs, parenterally administered biologics (for example, modified or unmodified proteins and antibodies) and macromolecular and nanoparticulate delivery systems that are currently on the market or in clinical trials have properties that suggest the likelihood of lymphatic transport, but this has rarely been explored (or exploited).

To date, the majority of macromolecular biologics and delivery systems have been developed for the treatment of cancer or inflammatory diseases²⁹. As such, uptake of these systems via the lymphatic system may play an important part in their capacity to eradicate cancer metastases and alleviate inflammation, even though this has not been directly demonstrated in patients. This is likely to be especially true in the growing number of examples whereby administration routes are being switched from intravenous to subcutaneous administration to promote acceptance of the treatment by patients. Indeed, some of these materials (for example, liposomal doxorubicin and PEGylated interferons) have been shown in preclinical studies to readily enter the lymph and under some circumstances to enhance the treatment of cancer metastases by targeting lymph nodes^{85,86,185}. The potential importance of lymphatic delivery to clinical outcomes is further supported by a recent clinical study that described enhanced suppression of HIV replication in lymphoid tissues in patients with increased lymph node concentrations of antiretrovirals³⁸.

The lack of substantial clinical evidence of lymph targeting approaches reflects the fact that assessment of lymphatic drug exposure in humans is complex and has therefore rarely been attempted (with the exception of a handful of studies that have quantified uptake via the collection of lymph nodes or thoracic lymph^{193,248}). Instead, lymphatic delivery is most often studied in rodents and occasionally in larger animal species (for example, pigs, dogs and sheep)²⁴⁹. How accurately various animal, *in vitro* and *in silico* models predict lymphatic distribution in humans remains unknown. In the past, the quantitation of lymphatic transport in humans has required invasive surgery to cannulate the lymph duct or to collect lymph nodes. Recent advances in lymphatic imaging^{29,250} and in minimally invasive techniques to catheterize the thoracic lymph duct in humans²⁵¹ suggest that more detailed studies to collect lymph and/or quantify lymphatic delivery in humans are increasingly possible. The availability of these models, coupled with further work to validate *in vitro* and

in silico models, looks set to substantially enhance the ability to predict and quantitate lymphatic delivery in humans and, in doing so, to support translation of lymphatic drug delivery advances to the clinic.

Concluding remarks

Historically, the lymphatics have been seen largely as a 'sewerage system' for the clearance of fluid, proteins and debris from the interstitial space and as a transport mechanism for dietary fat. As such, drug absorption into the lymphatics or drug targeting to the lymphatics has been viewed as possible, even likely in some cases, but of little importance. However, recent increases in our understanding of the central role of the lymphatics in regulating diseases such as cancer, transplant rejection, infection, inflammation and metabolic disease has reinvigorated interest in the lymphatic system (and the cells contained within it) as a drug target. Accumulating evidence to support the benefit of targeting therapeutic and protective vaccines to APCs in the lymph and lymph nodes provides further impetus to research this area.

Looking forward, drug delivery efforts will continue to be driven by a more detailed understanding of lymphatic biology, particularly the mechanisms of uptake and entry into the lymph and the role of the lymphatics in disease. Progress in materials and pharmaceutical sciences — especially the construction of macromolecular conjugates and constructs with specific lymphatic affinity — will further advance efforts to promote lymphatic targeting. Areas of focus will probably revolve around the increasing realization that lymphatic access is not simply a function of size but instead harnesses a range of transport and metabolic processes. Finally, although it is apparent that the lymphatics and lymphoid tissues have a central role in a range of diseases, it is equally apparent that this is highly interactive and that the same disease states also affect lymphatic structure and function. Future efforts might usefully address the impact of disease-mediated changes in lymphatic function on lymphatic access of drugs, vaccines and drug delivery systems to better drive the development of robust lymphotropic delivery vehicles.

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Competing interests statement

The authors declare **competing interests**; see Web version for details.