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

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1. Abstract

The lymphatic system serves integrative roles in fluid homeostasis, lipid metabolism and immune defense. In cancer, the lymph nodes that drain solid tumours are a primary site of metastasis and recent studies suggest intrinsic links between lymphatic function, lipid deposition, obesity and atherosclerosis. Advances in 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 concert, the design of lymphatic delivery systems has also progressed, from simple systems that rely on passive lymphatic access, to sophisticated structures that employ nanotechnology to mimic endogenous macromolecules, and lipid conjugates that 'hitchhike' onto lipid transport processes. Here we briefly summarise the lymphatics in health and disease, address the varying mechanisms of lymphatic entry and transport, discuss examples where lymphatic delivery has enhanced therapeutic utility and finally outline future challenges to effective lymph-directed therapy.

2. Introduction

The lymphatic system comprises a network of vessels and nodes that i) circulate immune cells and provide a site for antigen presentation and immune activation[1], ii) transport dietary lipids, in the form of intestinal lipoproteins, from the intestine to the general circulation[2] and iii) clear 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 (afferent) 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, while 10% drained into lymphatic vessels. More recent evidence, however, suggests that a far greater proportion of the capillary filtrate may be drained via the lymph[6]. The important role of the lymphatics in capillary filtrate reabsorption is underscored by the development of tissue edema in cases where there is either an intrinsic (genetic) fault in the structure or function of lymphatics (a condition referred to as primary lymphedema) or where trauma or surgery interrupt lymphatic structure such as lymph node biopsy in cancer (secondary lymphedema)[7].

In immunity, the lymphatic vessels provide channels for antigens, antigen presenting cells (APCs), and lymphocytes to traffic from tissues to draining lymph nodes, where antigen presentation to resident lymphocytes regulates immune responses[1,8]. As part of this process, lymphatic endothelial cells (LEC) actively modulate the immune response by controlling lymph flow and the delivery of antigens and immune cells to lymph nodes via coordinated expression of NO, chemokines and adhesion molecules[8]. In this way, the lymphatics prime immune responses or promote the development of immune tolerance. The mesenteric lymph nodes (MLNs), in particular, play a central role in the induction of local and systemic tolerance

to self and food proteins, and local tolerance to commensal bacteria and their byproducts whilst also providing a firewall against systemic entry and immune responses to the commensal bacteria. This prevents food allergies[9] and inflammation and infection resulting from mucosal and systemic entry of commensal bacteria and their byproducts[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 return to the systemic circulation[2,12]. Recent studies also provide evidence for broader roles for the lymphatics in lipid metabolism. For instance, two separate studies suggest that the clearance of excess cholesterol from tissue macrophages is mediated via cholesterol transfer to HDL followed by transport of HDL to the systemic circulation via the lymphatic vessels, ultimately for excretion via the liver[13,14]. This implicates the lymphatics in HDL reverse cholesterol transport and atherosclerosis. Indeed, functional lymphatics appear to be required to facilitate the clearance of atherosclerotic plaques[13]. 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 lymphedema[15]. 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] suggesting links between lymphatic function, immunity, metabolism and diet.

Appreciation that the lymphatics control a range of physiological functions has led to the realization that they influence a far more diverse range of diseases than once thought[7,24-26]. These include lymphedema[7], cancer and metastases[27], immune and inflammatory conditions[28,29] (e.g. inflammatory bowel disease[30,31], psoriasis[32], rheumatoid arthritis[33] and asthma[34]), metabolic disease (obesity[15,17,18], hypertension[35], atherosclerosis[13]), liver disease and ascites[36], cardiovascular disease[37], infection (e.g. HIV[38,39], hepatitis[40], filiarasis[41], ebola virus[42]), acute and critical illness[43], solid organ transplant rejection[7,44] and tolerance to self and food proteins[9,45]. In many of these diseases there are changes to lymphatic vessel density, dilation, contraction and/or lymph flow and lymphangiogenesis (**Fig 1**), although the functional importance of these changes may not be known. In cancer, metastatic dissemination from the primary tumour most often occurs via transfer of tumour cells to the lymph nodes through tumour associated lymphatic vessels[27]. Similarly in infection, the lymphatics appear to be a major site of viral replication and/or dissemination, notably in HIV[38,39], ebola virus[42] and hepatitis[40].

Recognition that the lymphatics play key roles in disease has driven increased interest in targeted delivery to the lymphatics to enhance therapeutic outcomes[29,46-49]. Mirroring advances in 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 advances in our understanding of 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 new literature and to novel systems which employ recently identified routes of lymph entry. Finally, examples where lymphatic delivery has been shown to enhance drug exposure or utility in immunotherapy, vaccination, viral therapy and cancer metastasis are highlighted.

3. Routes of drug entry into lymphatic vessels

For most small molecules, drainage from the interstitial space occurs primarily via the blood capillaries, since blood flow rates are ~100-500 fold higher than lymph flow. After oral or parenteral administration, therefore, most molecules, including drugs, are transported from the site of administration or absorption via blood capillaries. Macromolecular constructs, on the

other hand, are able to promote specific entry into the lymphatics since their size precludes ready access to the blood, but does not restrict lymphatic access, see **Box 1**.Consequently, lymphatic targeting strategies have centred on macromolecular constructs that are transported from interstitial tissues via lymphatic rather than blood capillaries. Lymph targeted delivery has been achieved via the administration of i) unmodified or modified macromolecular therapeutics (proteins, peptides), ii) lower molecular weight therapeutics in association with macromolecular carriers (nanoparticles, polymers, liposomes etc) or iii) lower molecular weight therapeutics that associate, in situ, with macromolecular constructs (lipoproteins, proteins) or cells (leukocytes) with inherent lymphotropic properties. These methods have been employed following parenteral and oral administration and in some cases after mucosal delivery (e.g. pulmonary, nasal, genital) (**Fig 1**).

Parenteral delivery

The lymphatic uptake of exogenous therapeutic macromolecules follows a similar pathway to interstitial fluid, endogenous macromolecules and cells (**Fig 2**). After interstitial administration (eg subcutaneous, intramuscular or intradermal injection) small molecules or moderately sized macromolecules that are <10 nm[48] in size (or ~16-20 kDa for proteins[50]) 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 due 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] and 10-100 nm particles[48,58,59] are able to move through the interstitium and are ideally sized to selectively 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 main components of the interstitium are entangled collagen fibers and glycosaminoglycans that are cross-linked in a gel-like matrix[4]. 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 neutral or negative charge commonly promotes interstitial transfer[63,64,74,76] (although there are exceptions)[48,65]. Movement of hydrophilic macromolecules through the interstitial water channels is thought to occur more effectively than for hydrophobic macromolecules[66].

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 high interstitial pressure and subcutaneous injection into this region results in higher lymphatic transport than injection into the flank or abdomen of rats[58] and sheep[54]. Intradermal injection may also promote enhanced lymphatic uptake when compared to intramuscular or subcutaneous injection due 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 albumin[70] or dextrans[71], and proteins that increase vascular extravasation (e.g. 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 interstitum into the lymphatics. Liu recently took advantage of this approach to develop vaccines that target lymph nodes after subcutaneous administration[73]

(see section 5). Derivatisation with molecular targeting agents (e.g. hyaluronic acid[74] and Lyp-1[75-79] that bind targets expressed on LEC, (such as Lyve-1 and p32 respectively), particularly in tumours, has also been employed to promote interaction with, and uptake across, the lymphatic endothelium. LEC also express a range of receptors (e.g. integrins[80]) and secrete chemo-attractants (e.g. chemokines[8]) 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 lymph specific delivery.

Alternatively, targeted lymphatic uptake after parenteral delivery may be achieved by exploiting the mechanisms by which antigens are presented to lymphoid tissues. Antigens are phagocytised by antigen presenting cells (APCs), such as dendritic cells (DCs), 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 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 DCs whereas smaller antigens (<70kDa) or particles (<50 nm) are more likely to drain directly into the lymphatics[59,81]. In addition, materials carrying a positive charge are, in general, more readily taken up into APCs than materials carrying a neutral charge [82]. However, the efficiency of uptake into the draining lymphatics is typically lower for larger and positively charged materials due to enhanced retention within the interstitium[59,83], in spite of their affinity for APCs. Improved targeting to DCs and draining lymph nodes has also been achieved via derivatisation of delivery systems with carbohydrates (such as mannose) that are recognised and internalised by mannose receptors[84,85] and with monoclonal antibodies to DEC-205, a transmembrane protein found on DCs[86]. Finally, uptake into DCs and draining lymph nodes is enhanced after intradermal vaccination, when compared to other parenteral routes due to the increased number of DCs in the skin (section 5).

Lymphatic transport and/or lymph node distribution has also been reported following intravenous delivery of PEGylated proteins, liposomes and nanoparticles[65,87-89]. 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(s) of extravasation are not well defined, although transfer across more permeable fenestrated or sinusoidal endothelium might be expected to be enhanced. The brain lacks classical lymph vessels, but a series of recent studies have demonstrated that paravascular transport pathways (the 'glymphatic system') facilitate the transport and clearance of small, drug-like molecules and proteins from the brain interstitium, serving a lymphatic-like function (**Box 2**)[90-94].

Oral delivery

Following oral administration, drugs or drug delivery systems must first pass through the intestinal epithelium in order to access the underlying interstitial space that is drained by the blood and lymph capillaries. Since selective lymphatic access from the interstitium requires a macromolecular construct (see above), the stability of macromolecules within the GI tract and low permeability across the GI mucosa provide significant 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, that can readily diffuse into both blood or lymph capillaries, are therefore absorbed and transported from the intestine via the blood circulation rather than the lymphatic system due to higher mass transport. Nonetheless, significant lymphatic transport can occur after oral administration when macromolecular access to the GI interstitium is possible and where access to blood capillaries

is restricted. This has been described for i) lipophilic small molecule drugs or prodrugs that are absorbed and then associate with intestinal lipoproteins during passage across enterocytes, and ii) macromolecular constructs such as antigens, tolerogens, peptides, proteins and nano-sized delivery systems that are stable in the GI tract and are permeable, at least to some extent, across the GI epithelium. These are discussed briefly below and in **Fig 3 and 4,** 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 main route of transport to the systemic circulation following oral delivery[47]. For these drugs, lymphatic access occurs via association with lipid absorption and lipoprotein assembly pathways during diffusion across intestinal absorptive cells (enterocytes)[47,49] (**Fig 3**). 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 significant when the drug is administered with a source of lipid (from food or a formulation) since 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 ($>C_{14}$) lipids are assembled into intestinal lymph lipoproteins whereas the reverse is true for medium chain lipids ($<C_{12}$) where the majority diffuse across enterocytes to enter the blood circulation directly[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 initially suggested that the physicochemical properties required to promote drug association with intestinal lipoproteins (and therefore to promote lymphatic transport), were log P>5 and solubility in long chain triglyceride (TG) >50 mg/g[103]. 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 TG solubilities[104] and substantial lymphatic transport for drugs with relatively low TG solubilities[105,106]. In the latter cases, drug affinity for the interfacial region of lipoproteins rather than the TG rich core, or affinity for an unidentified active transport process, have been suggested as alternate 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, using i*n vitro* drug affinity for 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 thus 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 candidates[112] and commonly raises questions regarding lipophilic efficiency (LiPE) and toxicity[113]. An alternate approach is to temporarily boost lipophilicity, via the synthesis of a lipophilic prodrug, where 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. In contrast, lipophilic prodrugs that integrate into lipid processing pathways such as TG or phospholipid (PL) resynthesis, are typically more effective[96,114,115] (see **Fig 5**). In recent studies

for example (illustrated in **Fig 5**), we have shown that TG mimetic prodrugs of the immunosuppressant mycophenolic acid are far more effective in promoting lymphatic transport than simple alkyl esters or amides[114]. Interestingly this study[114], and others[95,115], reveal significant 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 appear to promote lymphatic transport most effectively[96,114,115], although this is not always the case[116].

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 brain[96]. Phospholipid prodrugs for lymphatic transport are less common[117,118], but enhanced lymphatic transport of the phospholipid prodrug dipalmitoylphosphatidylfluorouridine (DPPF) and more recently DP-VPA, a phospholipid prodrug of valproic acid, has been shown[117,118].

Lymphatic uptake of orally administered macromolecular constructs

Drug delivery systems that facilitate the oral absorption of macromolecules (including therapeutic proteins and nano/micro sized 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 whilst an increasing number of reports suggest the possibility of the absorption or sampling of macromolecules or particulates, whether the quantity absorbed is sufficient to promote biological activity across a range of applications is less clear (see **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 effect[99]. Indeed, several oral vaccines have been developed and are commercially available[97,121] (see **section 5**). But, whether the intestinal absorption of macromolecular therapeutics will ever occur in sufficient quantities to provide reliable and consistent therapeutic endpoints for applications other than vaccination and conditions other than those localized in the GIT, or where highly potent therapeutics can tolerate very low bioavailability, remains in question. Several encouraging reports have emerged (for example[122,123]), but none have yet translated into a clinically and commercially successful product.

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]. **Fig 4** summarises 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. In contrast, soluble and lower molecular weight antigenic material such as polypeptides may more readily cross the normal villous epithelium from where they enter mesenteric lymph vessels directly, or following interaction with DCs in the lamina propria[9,97].

Translocation across the FAE into 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 (see **Fig 4**). 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]. Whether they represent a major mechanism of antigen and particulate uptake *in vivo* however, has been questioned[9].

For nano/microparticulate delivery systems, the route and extent of intestinal uptake is likely to be dependent on the characteristics of the particles[99] such as physical and chemical stability, size, surface charge, shape and elasticity. The presence (or absence) of targeting ligands such as lectins[128], invasins[129], RGD[130] and others[120,131] may also enhance uptake. Uptake may occur either via the M-cells in 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 by the realisation that 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 >10μm in diameter appear to be inefficiently taken up by M cells or epithelial cells whereas particles in the nanometer to low micrometer size range may be taken up more effectively[133,134].

Absolute quantification of the extent of absorption of nano/micro-particulates 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 nano/micro-particles that is absorbed intact has been reported to range from essentially zero[135-137] to quite large quantities (5-40% of the dose)[123,129,132,134,138-140]. In some of the first reported studies, between 6-34 % of the dose of 50 nm–3 μm polystyrene microspheres or nanoparticles or 50-500 nm polymeric or squalenated nanoparticles was found to be absorbed after oral administration[129,134,138,139]. More recently, Zhang et al reported relative bioavailabilities of 4.9-7.1 % for solid lipid nanoparticles loaded with insulin when compared to subcutaneous injection of insulin in saline[140] and Ralay-Ranaivo described oral bioavailabilities of 9% for fondaparinux loaded nanoparticles administered in gastroresistant capsules[139]. Prigden et al also report absorption efficiencies of 13.7% per hour for insulin administered in Fc conjugated ~60 nm polymeric nanoparticles[123] and docetaxel administration in nanocapsules embedded in microparticles has been suggested to result in oral plasma AUCs that are 1.77 fold higher than that after IV administration of the same dose of the commercial solution (Taxotere®)[122]. Finally, Reineke et al recently reported the absorption of 30-45 % of the dose of polystyrene microsphere particles of varying diameters (500 nm - 5μm) after injection into isolated loops of jejunum or ileum[132].

In contrast, perhaps as many studies suggest essentially zero absorption of nano or micro sized particles. For example, absorption of <0.0055 and 0.01% of the dose of 27 nm and 170-250 nm latex particles[137] and <0.01% of the dose of 2.65 μm particles (with 0.0006% of particles detected in Peyer's patches)[135]. In one of the few studies to quantify the uptake of nano/micro particles into the mesenteric lymphatics directly, Jenkins et al[136] found that less than 0.2% of the dose of a range of particles 0.15 to 10.0 μm 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 is available oral absorption is also very low[141,142]. Perhaps unsurprisingly the widely disparate oral bioavailabilities reported for orally administered nano/microparticles has led to controversy and questions regarding the consistency of studies and the differing methodologies used (see **Box 3**).

Mucosal and other routes of delivery

There has been a recent surge in interest in targeted delivery to (non-GI) mucosal lymphoid tissues and lymph nodes, such as the nasal, pulmonary and genital mucosa. Much of this has

focussed on the delivery of vaccines to mucosal surfaces (and is described further in section 5)[46,143,144]. Different mucosal surfaces share commonalities in the structure of the associated lymphatic systems. In most, 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 epithelium[144]. The normal epithelium is interrupted, at varying frequencies depending on the mucosal site and animal species, by mucosa associated lymphoid tissue (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 have been shown to transit the nasal mucosa via M cells, interact with nasal-associated lymphoid tissues and resident APCs and elicit protective immune responses[145]. In a follow on 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 vaccination[146]. To this point, 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 dictates that the absorption of small molecule drugs from the deep lung into the systemic circulation is rapid and almost immediate in many cases[148]. The lung is also drained via deep and superficial lymphatic vessels[149]. The superficial lymphatic vessels lie beneath the pleural lining of the lung while the deep lymphatic vessels and pulmonary lymph nodes reside mainly 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 (e.g. rats, rabbits) although it is not normally present in healthy lungs of adult humans and mice but can be induced by antigens, infection or inflammation[150].

The deep lymphatic vessels and lymph nodes associated with the conducting airways appear to be 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 mucocilliary escalator or captured by macrophages and cleared via the lymphatics[151,152]. In contrast, the lymphatics in the lung parenchyma are relatively sparse, although small (10-20 μm) interlobular lymph vessels have been observed within inter-alveolar walls[153]. The primary function of these lymphatic vessels has been suggested to be the collection of interstitial fluid and extravasated proteins that surround the interalveolar septa[153]. 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 role in the uptake of nanomaterials from the lungs[150]. Several studies have demonstrated uptake of inhaled nanoparticles, including liposomes[154], 20-70 kDa dextrans[155] , non-cationic organic and inorganic nanoparticles of ≤ 34 nm[156] and antigen carrying lipid nanocapsules[157] into lung lymph nodes. Several other studies have inferred lung lymphatic involvement in the systemic availability of inhaled solid lipid nanoparticles[158], liposomes[159] and 50-900 nm polystyrene nanoparticles[160]. However, the mechanisms of lymphatic uptake of materials from lung 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 vary considerably to humans. Direct evidence of

the role of the lung lymphatics in the clearance of particulate materials in larger animals and human patients is therefore lacking.

4. Routes of drug and drug delivery system entry and retention in lymph nodes

Lymph nodes provide a site for immune surveillance, the generation of immune responses and tumour cell metastasis[48,51]. The uptake of therapeutics into lymph nodes is therefore important for vaccination and 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 6**. High endothelial venules (HEVs) provide the primary point of entry for naïve T and B lymphocytes, plasmacytoid DCs and natural killer cells from the systemic circulation[1]. Naïve T and B lymphocytes extravasate across HEV via a multi-step cascade that is initiated by the recognition of lymph node addressins by the lymphocyte homing receptor L-selectin. This results in lymphocyte tethering, rolling and ultimately transit across the HEV wall[1]. 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 ligands[1].

Lymph, containing tissue fluid, antigens, proteins, lipoproteins and immune cells etc enters the lymph nodes via afferent lymphatic vessels[1,51] (**Fig 6**). The majority of the lymphocytes that enter the afferent lymph are memory T cells[161]. In contrast naïve T cells enter the lymph nodes primarily via HEVs[1]. The majority of T cells that exit lymph nodes via efferent lymphatics are also naïve T cells[161]. Chemokines modulate the entry, migration and retention of cells within the lymph node[1]. DCs 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 recognised by CCR7 and CCR8 receptors on the DC surface)[1,162].

Therapeutics may similarly enter the lymph node via afferent lymph vessels or HEVs. For example, nanoparticulate (20-40 nm) superparamagnetic iron oxide particles have been shown to accumulate within lymph nodes after intravenous injection due to a combination of transfer across HEV and extravasation into the interstitial space followed by transfer to lymph nodes via afferent lymphatic vessels[163]. For direct entry via HEVs, a ligand that binds to surface receptors on the HEV might be expected to enhance uptake, although to the best of our knowledge no such system has yet been described. Alternatively, entry via HEV may occur following uptake of therapeutics into immune cells followed by transfer of the immune cell into the node via the HEV. For example, the immunosuppressant fingolimod accumulates within lymph nodes in this way via association with lymphocytes in the systemic circulation and subsequent uptake up into the node, presumably via HEV[164].

Entry of therapeutics into the lymph nodes via the afferent lymphatic vessels has been studied in more detail than access via HEV[27,51] (**Fig 6**). 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 intersitium and subsequent entry of the APC into the lymph. In general, larger (500-2000 nm) and positively charged materials are preferentially taken up by APCs, and in particular DCs, at the injection site, whereas smaller and neutrally charged materials (20-200 nm) are trafficked directly to the lymphatics[27,59,165,82]. After entry into the lymph node, 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 zones[46]. 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 sinuses or conduits[46,59,165,168,169]. Sequestration within the lymph node via interaction with node

resident macrophages or DCs may also be promoted by the use of targeting ligands to APC-specific receptors such as mannose[83,84], antigens such as DEC-205[86] 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 SC injection site. For example, macromolecules of increasing size are more effectively retained in lymph nodes but drain poorly from interstitial injection sites[82,83,172]. Thus, mitomycin-C conjugated to higher molecular weight dextrans drained inefficiently from the injection site when compared to unconjugated mitomycin-C or mitomycin-C conjugated to low molecular weight dextrans, but was retained more effectively in lymph nodes[173]. 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 due 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 such as PEG on the surface of a nanoparticle typically enhances drainage from an SC injection site, but provides a steric barrier to opsonisation 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 interaction with resident APCs[82,174]. For example, cationic materials have recently been found to be more readily taken up by DCs and to be retained in the lymph node medulla and paracortex when compared to neutral materials[82]. 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 retention[66].

Importantly, recent studies have highlighted that even where enhanced uptake and retention of materials within lymph nodes is achieved, this does not always translate into enhanced therapeutic utility[81,175]. 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 toxicity[81].

5. Pharmacokinetic and pharmacodynamic advantages of lymphatic delivery

The benefits of lymphatic delivery are usually manifest in either an increase in exposure or an enhancement in therapeutic utility (ie efficacy/toxicity) or both. Several studies have shown that lymphatic delivery can enhance drug exposure, particularly following oral delivery, and others provide evidence that lymphatic transport can promote more 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 toward 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 are reviewed elsewhere[29,176].

Oral bioavailability

Intestinal lymphatic transport circumvents hepatic first pass metabolism. Unlike 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 5**). Increases in intestinal lymphatic transport therefore substantially enhance oral bioavailability where bioavailability is limited by first pass metabolism. An example of this approach is the commercial product testosterone undecanoate (Andriol®, Merck). The oral bioavailability of testosterone is essentially zero after oral administration due to complete first pass metabolism[177]. In contrast, testosterone undecanoate, an alkyl ester prodrug of testosterone, enables oral testosterone replacement therapy as a proportion of the dose of the prodrug is transported via the intestinal lymphatics avoiding first pass-metabolism[177]. Undecanoate esters have also been found to enhance the oral bioavailability of methylnortestosterone[178] and dimethandrolone[179]. Similar studies have shown that promoting the intestinal lymphatic transport of lipophilic drugs (rather than prodrugs) such as halofantrine[180] and CRA13[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 enterocyte[182].

Recently, Attili-Qadri et al suggested that lymphatic transport may be recruited to enhance the oral bioavailability of docetaxel[122]. When administered in standard formulations, docetaxel has poor oral bioavailability due to extensive Cyp3A4 mediated first-pass metabolism and P-glycoprotein mediated efflux. In contrast, after oral administration of docetaxel incorporated into nanocapsules that were then embedded in microparticles, exposure was significantly enhanced. The authors hypothesised 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 (ie. they became 'lipoproteinated').

Cancer Chemotherapy

Many cancers metastasise via the lymphatics, with cancer cells initially lodging and proliferating in the sentinel (ie first draining) lymph node[48]. Lymph node metastases are often removed surgically (lymphadenectomy) or obliterated using radiation therapy in an attempt to prolong survival. However, this is invasive and leads to morbidity associated with the disruption of lymphatic flow, including pain, swelling and oedema[48]. Lymph-targeted chemotherapy has the potential to enhance delivery to lymph resident cancers and to reduce systemic exposure that correlates with dose-limiting side effects.

After oral administration, a relatively limited number of studies have described benefits in the treatment of cancer via lymph targeting of drugs[183] or prodrugs[118,184], including the docetaxel studies described above[122]. A far greater 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 construct at or near the site of the primary tumour. Improved lymph node targeting has been demonstrated for PEGylated proteins[185], liposomes[228,274], dendrimers[88,186], polymeric micelles[187-189], nanoparticles[190,191] and hyaluronan conjugates[192]. 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 toxicity[66].

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 has been shown to enhance delivery to the lymph nodes draining a subcutaneous injection site

and to promote both anti-inflammatory and immunosuppressive effects[198]. In a similar approach, but in this case harnessing intestinal lymphatic transport processes, a highly lipophilic immunomodulator was shown to more effectively target lymphocytes and to increase the expression of the anti-inflammatory cytokines IL-4 and IL-10 in lymphocytes after ex vivo mitogen stimulation, when co-administered with lipid to stimulate lymph transport[199]. As described above, the efficacy of fingolimod, has also been linked to its accumulation in lymph nodes[164]. Lymphatic vessels themselves have recently emerged as therapeutic targets in inflammation, suggesting the potential for lymph targeted delivery to enhance anti-inflammatory therapy[29,200]. Interestingly, many current treatments for inflammatory disease such as anakinra, tocilizumab and infliximab affect lymphangiogenesis[201-203]. 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 play 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 vaccination) 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 disease[51]. The potential for lymphatic delivery to enhance tolerance is summarized in a 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/peptides, or DNA/RNA, administered in combination with adjuvants that facilitate the recruitment of immune cells and activate the appropriate immune responses[51,204]. 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 well reviewed elsewhere[51,204]. 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 binding and internalization of the antigen by DCs (or other APCs) at the injection site followed by trafficking to the lymph node where the DC presents antigen to T-lymphocytes. Alternatively, antigens can enter the lymphatic capillaries directly and drain to lymph nodes where antigen is internalized and presented to lymphocytes by resident DCs resulting in lymphocyte activation[205].

The importance of the lymphatics in the vaccine response has stimulated studies to enhance vaccine efficacy by promoting lymph node targeting. The methods employed usually involve targeting antigen and adjuvants to lymph nodes in a controlled manner. Most simply, direct injection of vaccines into lymph nodes (ie intranodal injection) has been shown to enhance vaccine potency and to be safe and efficacious in pre-clinical models and clinical trials[51,206-209]. However, intranodal injection requires a trained healthcare professional and the benefits of lymph node targeting following intranodal injection may be short-lived due to rapid flushing of the lymph node by lymph fluid. To address 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 has been shown to enhance immune responses to ovalbumin relative to intramuscular injection or intranodal injection of control formulations[208].

A number of studies have shown that vaccine efficacy is enhanced by increasing lymphatic uptake of vaccine antigens and adjuvants [81,168,210,211]. For example, administration into the skin has been shown to improve the vaccination response relative to administration via other parenteral routes. This reflects an increased number of DCs and a richer supply of lymphatic vessels in the skin and therefore enhanced antigen uptake and immune responses in draining lymph nodes[68,69]. Reddy et al have also reported enhanced humoral and cellular immunity to the model antigen ovalbumin after conjugation to surface modified biodegradable poly(propylene sulfide) nanoparticles. Conjugation to the nanoparticles increased delivery to DCs in lymph nodes following interstitial administration, and activated the complement cascade[59,168]. Targeting of ovalbumin or a malaria antigen to lymph node APCs via subcutaneous injection in interbilayer-crosslinked multilamellar vesicles (ICMVs) has been similarly shown to enhance humoral and cellular immune responses[212-171]. Targeting Trp2 peptide antigen to lymph node DCs via subcutaneous injection in cationic micelles also enhanced cellular immune responses and reduced tumour growth in a B6-F10 murine melanoma model when compared to non-lymph targeted Trp2[82]. In a different approach, St John et al[213] developed polymeric nanoparticles that mimicked mast cell granules and that targeted the lymph node draining an injection site, slowly releasing cytokines. This approach enhanced vaccination with an influenza virus antigen and increased survival from a lethal challenge of the virus. Most recently DNA or peptide vaccines and an adjuvant (CpG) have been targeted to lymph nodes using a 'albumin hitchhiking' approach leading to enhanced vaccination (see **Fig 5**)[73]. For therapeutic cancer vaccines, recent studies suggest that targeted delivery specifically to the lymph node(s) draining a primary tumour site may provide additional benefit when compared to targeting other lymph nodes[214,215]. 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 when compared to distal lymph nodes[215].

Mucosal vaccination

Mucosal vaccination provides 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 thus 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 nano/microparticles, liposomes, virus-like particles, bacterial ghosts, and immunostimulating complexes have been widely employed to enhance vaccine efficacy[116,147,148]. The benefits of particulate vaccines likely stem, at least in part, from enhanced translocation via M cells and access to mucosa associated lymphoid tissue (MALT) when compared to soluble antigens (see **Fig 4**). Immune responses are subsequently stimulated by antigen uptake by DCs in MALT, DC activation and antigen presentation to T and/or B lymphocytes in the MALT (or draining mucosal lymph node), ultimately leading to the generation of memory and effector lymphocytes (**Fig 4**).

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 cells[216]. 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. Zhu et al recently reported enhanced immune protection against vaccinia virus in the rectum and vagina via the administration of the vaccine and adjuvant in 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 patches[217].

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 polymer nanoparticles conjugated to ovalbumin and adjuvant, enhanced uptake into DCs 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 burden[218]. Pulmonary administration of the adjuvant CpG, and lymph node targeting of nanoparticle based carriers conjugated to ovalbumin or the influenza virus, has also been shown to lead to more robust immunization and protection when compared to (non-lymph targeted) soluble ovalbumin and CpG[219]. Finally, lipid nanocapsules containing a protein or peptide antigen have been shown to increase uptake into APCs and to promote transport to the draining lymph nodes after pulmonary administration when compared to pulmonary administration of soluble antigen or subcutaneous administration of the nanocapsule based system. When administered in combination with toll-like receptor (TLR) agonists, these antigen loaded nanocapsules improved the efficacy of both a therapeutic tumour vaccine and a prophylactic viral vaccine when compared to soluble antigen and adjuvant.[220]

Tolerance

Tolerance encompasses a range of mechanisms that are initiated to 'switch off' local and/or systemic immune responses to antigen[9,221-223]. Therapeutically, tolerance induction is being explored as means of overcoming poor inherent tolerance to food, animal and plant antigens[9] and to treat autoimmune disease[221,223] and allograft rejection[224], where 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 cells[222].

The lymphatics, and in particular, the lamina propria and MLN are crucial sites for the promotion of oral tolerance[9,221] (see **Fig 4**). It has been suggested that the preferred route of antigen entry for tolerance induction is via the villous epithelium and transport to the MLN via the intestinal lymphatics (either directly or indirectly following uptake into DCs) [12,252-254]. In contrast, M-cell-mediated antigen uptake into GALT may play 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 (see **Fig 4**) might therefore be expected to be more effective in promoting tolerogenic responses when compared to particulate antigens[225]. These suggestions are supported by studies that demonstrate impaired oral tolerance after removal of MLN[226,227], but normal tolerance induction in the absence of Peyer's patches[227-229]. Contrary findings are apparent, however[332,333], and targeted delivery of protein antigen directly to Peyer's patches via M cells has been shown to facilitate tolerance induction[230]. Similarly, oral antigen delivery in liposomes[231], nanoparticles[232,233] and microspheres[234] appear to enhance tolerance, although the mechanisms involved are not well understood.

An increasing number of studies have demonstrated that tolerance can be enhanced via targeted delivery to the lymphatic system following parenteral (rather than oral) delivery[81]. Systemic injection of PLGA nanoparticles loaded with mycophenolic acid enhanced distribution to spleen and lymph node resident macrophages and DCs and promoted tissue graft survival by limiting the ability of the APCs to prime and expand graft reactive T cells[235]. Similarly, co-delivery of the immunomodulator rapamycin with either protein or peptide antigens in nanoparticles enhanced distribution to lymph nodes after SC delivery, and induced potent and durable antigen-specific immune tolerance[236]. 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 evident[236]. In 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 molecular inhibitor of inflammatory $NF_{\kappa}B$ in liposomes has also been shown to enhance uptake into lymph node resident DCs, to reduce $NF_{\kappa}B$, to decrease the proliferation of self-reactive T cells and to diminish the severity of arthritis[237]. Tolerance induction in an animal model of multiple sclerosis, by administration of microparticles designed to mimic tolerogenic apoptotic cells, has also been shown to be dependent on microparticle delivery to marginal zone macrophages in the spleen, a primary lymphoid organ[238].

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 viral reservoirs was recently found to be correlated with the concentration of antiretroviral drugs in the lymph nodes of patients[38]. Other studies have also suggested that insufficient antiretroviral concentrations in lymphoid tissues may contribute to viral persistence[239-241]. Lymph targeted drug delivery is therefore increasingly being explored as a means of enhanced antiretroviral activity[242-244]. Thus, glycerolipidic prodrugs have been synthesised to promote drug delivery to HIV reservoirs in the gut lymphatics[245,246] and a range of nanomedicine platforms (e.g. drug polymer conjugates, dendrimers, micelles, liposomes, solid lipid nanoparticles, nanosuspensions and polymeric nanoparticles) have been employed to facilitate delivery of antiretrovirals to HIV reservoirs in lymphoid tissue[242-244]. These systems have been dosed via parenteral and/or oral routes, and attempts to enhance targeting to lymphoid reservoirs have been made via the use of targeting ligands for immune cells such as folic acid and mannose[242-244].

Unfortunately, whilst increases in lymphatic transport have been described, studies that describe 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 numbers[239]. In a second, 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-1p24+ cells in a mouse model of HIV[247].

5. Clinical application of lymphatic drug delivery

Whilst 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 in order to achieve pharmacokinetic or therapeutic benefits. Testosterone undecanoate provides one obvious example[248]. 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 in order to promote an immune response[51,177]. However, it seems likely that most were not designed with this property in mind. Similarly, a number of orally administered highly lipophilic drugs, parenterally administered biologics (modified or unmodified proteins, antibodies etc) and macromolecular and nanoparticulate delivery systems that are currently on the market or in clinical trial have properties that suggest the likelihood of lymphatic transport – but this has rarely been explored (or exploited).

To date the majority of macromolecular biologicals and delivery systems have been developed for the treatment of cancer or inflammatory diseases[29]. As such, uptake of these systems via the lymphatic system may play an important role 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 where administration routes are being switched from intravenous to subcutaneous administration in order to promote patient acceptability. Indeed, some of these materials (e.g. liposomal doxorubicin, pegylated interferons etc) have been shown in pre-clinical studies to readily enter the lymph and under some circumstances to enhance the treatment of cancer metastases by targeting lymph nodes[87,88,185]. The potential importance of lymphatic delivery to clinical outcomes is further supported by a recent clinical study that describes enhanced suppression of HIV replication in lymphoid tissues in patients with increased lymph node concentrations of antiretrovirals[38].

The lack of 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 on occasion in larger animal species (e.g. pigs, dogs, sheep)[249]. How well 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 increasing advances in minimally invasive techniques to catheterize the thoracic lymph duct in human patients[251] 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.

6. Concluding remarks

Historically, 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 understanding of the central role of the lymphatics in regulating diseases such as cancer, transplant rejection, infection, inflammation and metabolic disease has re-invigorated interest in the lymphatic system (and the cells contained within it) as a drug target. Growing evidence to support the benefit of targeting therapeutic and protective vaccines to antigen presenting cells in the lymph and lymph nodes provides further impetus to research in this area.

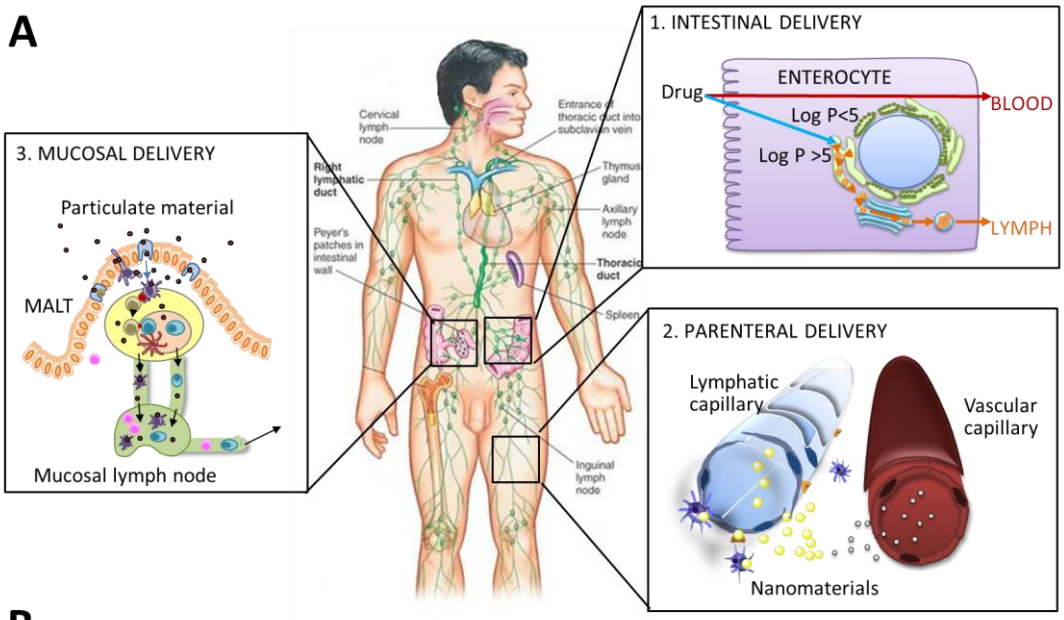
Looking forward, drug delivery efforts will continue to be driven by increases in understanding of lymphatic biology and in particular the mechanisms of uptake and entry into the lymph and the role of the lymphatics in disease. Progress in material and pharmaceutical sciences and, in particular, the construction of macromolecular conjugates and constructs with specific

lymphatic affinity will further advance efforts to promote lymphatic targeting. Areas of focus will likely 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, whilst it is apparent that the lymphatics and lymphoid tissues play a central role in a range of diseases, it is equally apparent that this is highly interactive and that the same disease states also impact on 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.

Figures

Figure 1: Targeting lymphatic vessel function in health and disease

Panel A. 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 **1.** Oral/intestinal delivery of lipophilic drugs (typically log P >5) that incorporate into the process of intestinal lipoprotein assembly and transport into the intestinal lymphatics (see Fig 3), **2.** Parenteral/interstitial delivery of macromolecular materials that are too large to access blood capillaries draining the injection site and instead access lymphatic capillaries (see Fig 2), **3.** Mucosal delivery of particulate materials that are absorbed across the epithelium into mucosa associated lymphoid tissue (see Fig 4). **Panel B.** In disease there are substantial changes to the lymphatic system when compared to *normal physiological conditions*. In *cancer*, metastatic dissemination from the primary tumour often occurs via lymph vessels to the sentinel 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[27,29,252]. Lymphangiogenesis, lymph vessel dilation and increased interstitial pressure modulate lymph flow from tumours and thus alter immunity[27,29,252]. Tumours may also release factors that promote immune tolerance[27,29,252]. In *inflammatory disease*, immune cells (macrophages, lymphocytes etc) 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 (DCs) from inflamed tissue to lymph nodes and in doing so modulate immunity and inflammation. In *metabolic disease*, lymphatic function is markedly altered by high fat diets and hypercholesterolemia[14,15,19,253]. 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 DCs[18-21,254]. The lymphatics are surrounded by adipose and impairments in lymphatic function typically increase lipid deposition in adipose, promoting obesity[15,17,18]. Mice with hypercholesterolemia exhibit lymphatic vessel hyperplasia in the skin and loss of smooth muscle coverage[14,253] Recent data also suggest that lymphatic vessels facilitate HDL mediated cholesterol clearance from atheromas[13]. In this way the lymph and lymphatics are broadly implicated in the development and progression of metabolic disease.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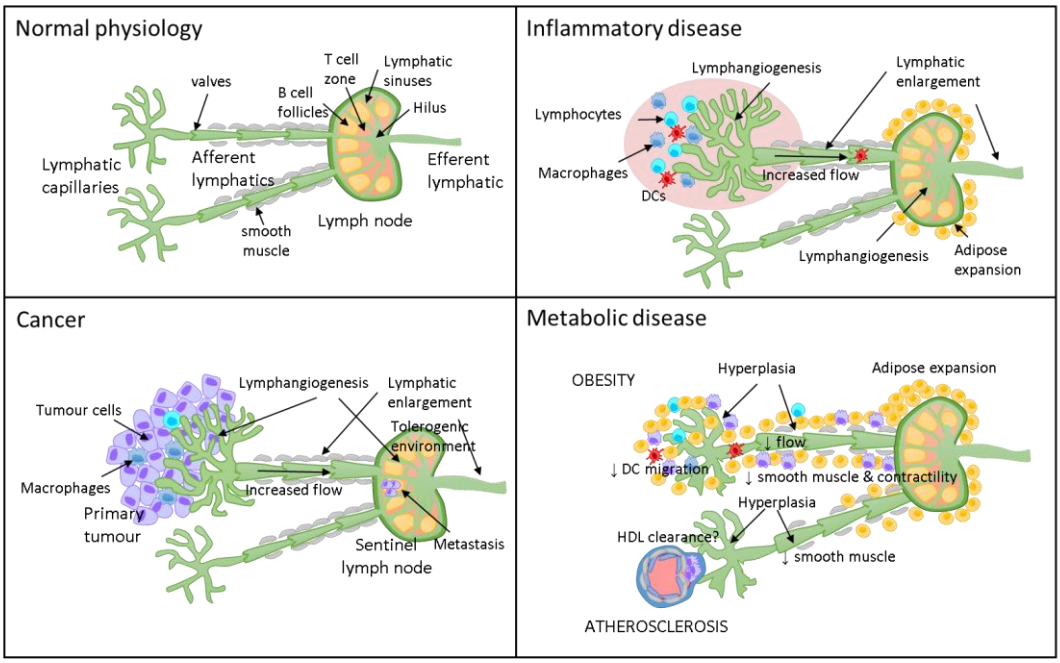
2. PARENTERAL DELIVERY
Lymphatic capillary
Vascular capillary
Nanomaterials
Cervical lymph node
Entrance of thoracic duct into subclavian vein
Right lymphatic duct
Thymus gland
Axillary lymph node
Peyer's patches in intestinal wall
Thoracic duct
Spleen
Inguinal lymph node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Lymphatic capillaries
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smooth muscle
Lymph node
Inflammatory disease
Lymphangiogenesis
Lymphatic enlargement
Lymphocytes
Macrophages
DCs
Increased flow
Lymphangiogenesis
Adipose expansion
Cancer
Lymphangiogenesis
Lymphatic enlargement
Tumour cells
Tolerogenic environment
Macrophages
Increased flow
Primary tumour
Sentinel lymph node
Metastasis
Metabolic disease
OBESITY
Hyperplasia
Adipose expansion
↓ flow
↓ DC migration
↓ smooth muscle & contractility
Hyperplasia
HDL clearance?
↓ smooth muscle
ATHEROSCLEROSIS

Figure 2: Mechanisms of access to the lymphatics from the interstitial space

Vascular capillaries are characterized by tight junctions between endothelial cells (1) and the presence of an underlying basement membrane. In contrast, the initial lymphatics have a discontinuous basement membrane, lack smooth muscle, and exhibit wide, button-like inter-endothelial junctions and short anchoring filaments that are tethered to elastin fibers 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 (1). This reflects, in part, higher (~ 100-500 fold) flow in vascular vs lymphatic capillaries, but the mechanism is likely multidimensional (**Box 1**). Increasing molecular size leads to increasing uptake via the lymphatics (2). Transfer of materials across the lymphatic endothelium may occur via passive diffusion through inter-endothelial junctions (2) or active transcytosis (3) following binding to receptors such as SR-BI, Lyve-1 or p32 on the LEC surface[14,62,74]. Materials may also access lymphatics indirectly via uptake into dendritic cells (DCs) and subsequent migration of the DC into the lymph (3). Uptake into DCs (and thus uptake into the lymphatics) may be promoted via the attachment of targeting ligands to receptors on the DC surface[82,83]. Lymphatic access and clearance from the interstitium is restricted for larger (>100 nm) particulates or hydrophobic molecules since diffusion and convection away from the injection site via interstitial water channels is reduced[48]. Macromolecules with partial anionic charge are repelled from negatively charged glycosaminoglycans in the extracellular matrix, facilitating improved transport through the interstitium and access to lymphatic capillaries[255]. In contrast, the interstitial transfer of cationic materials is often restricted (although uptake into DCs may be enhanced). Increases in osmotic pressure may also facilitate interstitial flow and promote lymphatic transport of macromolecules.

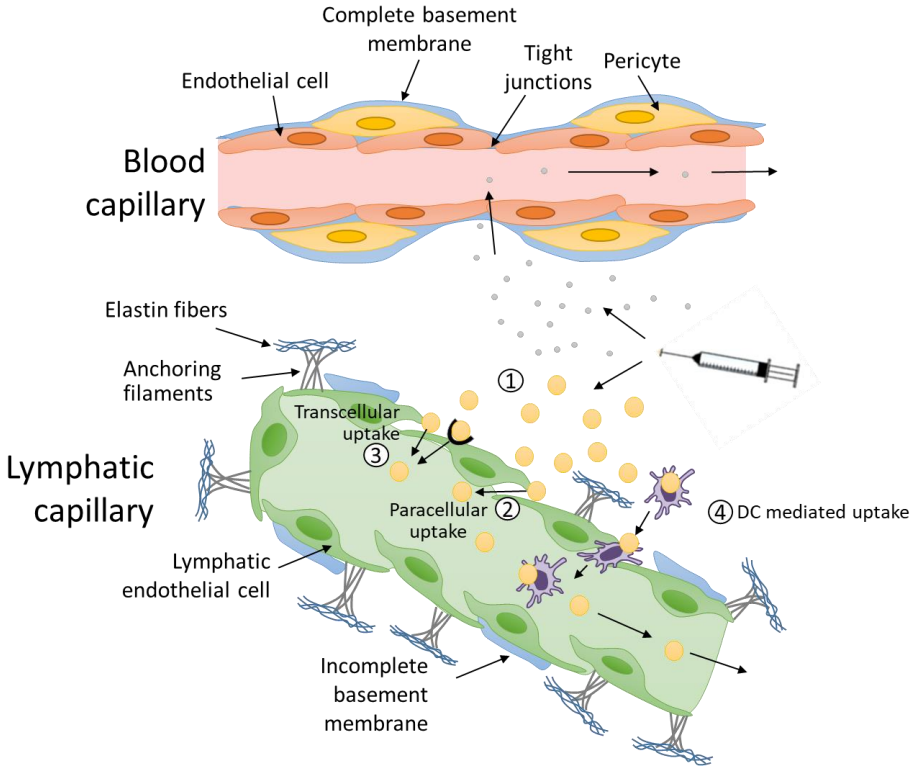


Figure 3: Lipid and lipophilic drug access to the intestinal lymphatics after oral administration

Dietary lipids including triglycerides (TG)[12], and lipophilic drugs[47,49] access the mesenteric lymph vessels following absorption across enterocytes. (**1**) TG are digested within the GI 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 GI lumen into enterocytes where they are re-synthesised to TG in the endoplasmic reticulum and assembled into 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 lymphatics[12]. (**2**) Most drugs are absorbed across the enterocyte into the vascular capillaries that drain the small intestine and transported to the systemic circulation via the portal vein (since the rate of fluid flow in the portal vein is ~500 times higher than that of the mesenteric lymph). (**3**) In contrast, highly lipophilic drugs (typically, but not exclusively[105,106], those with log P> 5 and solubility greater than 50 mg/g in long chain triglyceride lipid[103]) partition into developing LP 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 since lymph drains directly into the systemic circulation via the thoracic lymph duct.

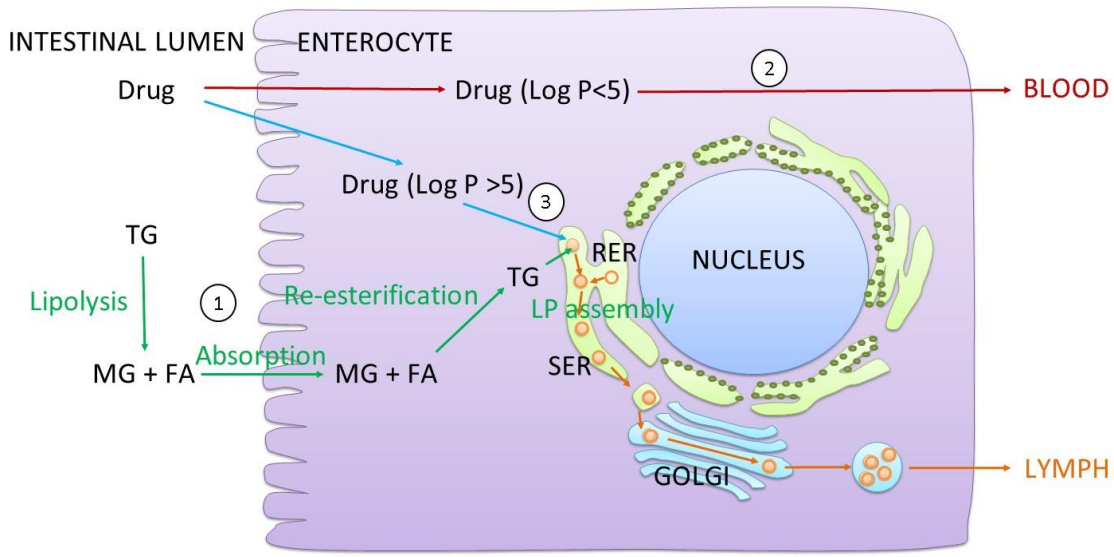


Figure 4: Mechanisms of macromolecular construct access to the intestinal lymphatics after oral administration

The intestinal lymphatic system comprises i) the gut associated lymphoid tissue (GALT) including the Peyer's patches, ii) the lacteals and mesenteric lymphatic vessels that drain both the intestinal microvilli and Peyer's patches, and iii) the mesenteric lymph node (MLN) into which the mesenteric lymphatic vessels flow[9].

A. 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 DCs prior to entry into the mesenteric lymphatics and the MLN[9,221]. Mechanisms proposed for the transport of soluble antigens across the villous epithelium include: (1) paracellular diffusion between adjacent epithelial cells[9,225], (2) incorporation into and exocytosis within MHCII expressing exosomes from enterocytes[256], (3) transcytosis across enterocytes following fluid phase uptake [257], (4) incorporation into enterocyte lipoproteins[122,258] 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)[259]. Within the lamina propria and MLN, the immune response to soluble antigen is biased towards induction of tolerance (reviewed elsewhere[9,221]). Antigenic material is commonly phagocytized by DCs and transported to the MLN where DCs present the antigen to naïve T cells resulting in the generation of inducible regulatory T cells (iTregs) that preferentially home back to the gut after exiting the MLN and entering the blood circulation[9,221].

B. In contrast to soluble antigens, particulate antigens (e.g. bacteria, viruses) and delivery systems, are preferentially absorbed across the follicle associated epithelium (FAE) and enter Peyer's patches[99,260]. Translocation across FAE typically occurs via the M cells, but may also occur via sampling by myeloid cells[126,127]. Below the FAE a subepithelial dome comprising T and B cell follicular regions and DCs is drained by efferent lymphatics that transport particulates to the MLN. In vaccination, the induction of a secretory IgA response typically requires uptake and processing by DCs in the subepithelial dome [97,121]. DCs loaded with antigen subsequently induce the conversion of naïve T cells to effector T cells[97,121]. The effector T cells, B cells and follicular DCs (FDCs) together induce the formation of a germinal centre in the B cell zone in the Peyer's patch leading to B cell expansion, differentiation and affinity maturation to IgA+ plasmablasts[121,143]. In an alternate scenario, primed B cells migrate to germinal centres in the MLN where they also undergo affinity maturation with the assistance of DCs and T cells[121]. IgA+ plasmablasts subsequently migrate to the blood bone marrow, GALT or the intestinal lamina propria[97,143].

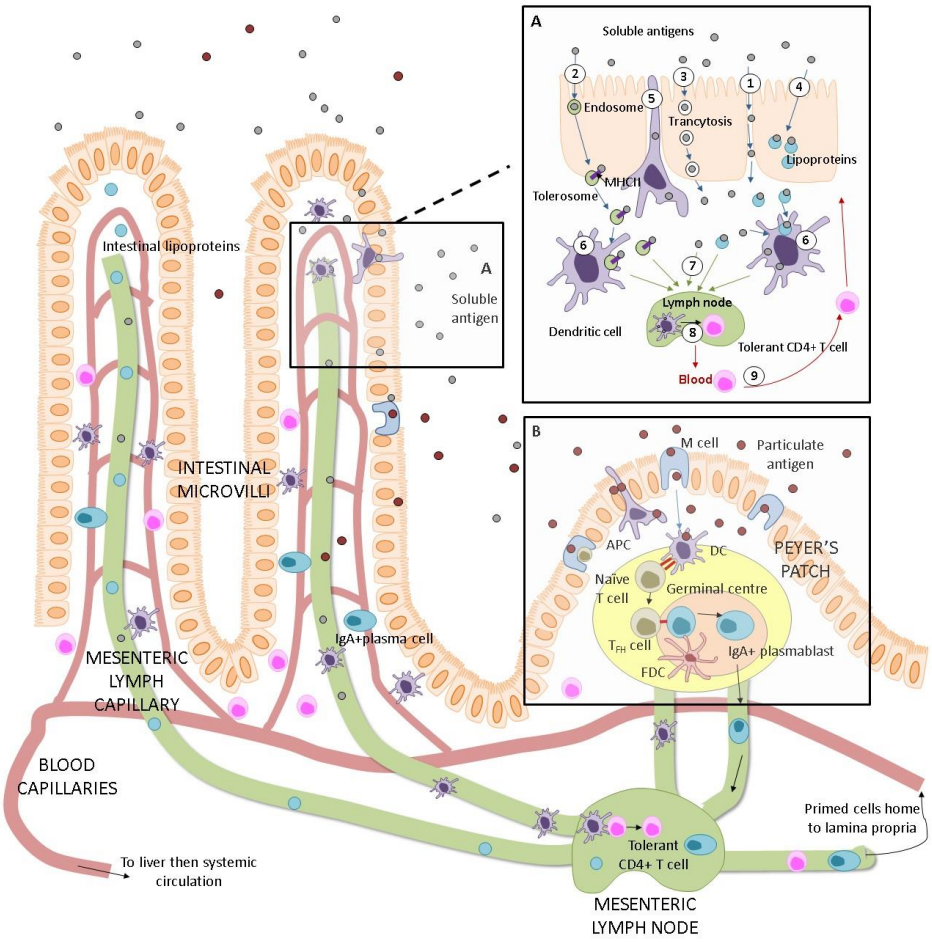

A
Soluble antigens
Endosome
Trancytosis
Lipoproteins
MHCII
Tolerosome
Lymph node
Dendritic cell
Tolerant CD4+ T cell
Blood
Intestinal lipoproteins
A
Soluble antigen
INTESTINAL MICROVILLI
B
M cell
Particulate antigen
APC
DC
PEYER'S PATCH
Naive T cell
Germinal centre
T_{FH} cell
FDC
IgA+ plasmablast
IgA+plasma cell
MESENTERIC LYMPH CAPILLARY
BLOOD CAPILLARIES
To liver then systemic circulation
Primed cells home to lamina propria
Tolerant CD4+ T cell
MESENTERIC LYMPH NODE

Figure 5: 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 where diacyl lipids are linked to adjuvants, promotes binding to albumin in the interstitum. Albumin preferentially enters the lymph and 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 cancer[73]. 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 (1) "biochemically integrate" into the pathway of triglyceride (TG) hydrolysis-absorption-resynthesis resulting in incorporation into intestinal lipoprotein (LP) assembly pathways and access to the lymph. TG-mimetic prodrugs are digested to form monoglyceride (MG)-derivatives, absorbed into enterocytes and re-synthesised back to TG-derivatives. The TG-derivatives are assembled into LPs in the endoplasmic reticulum and transported from the intestine via the mesenteric lymphatics[95,96]. Alkyl ester prodrugs (2) are highly lipophilic and partition into intestinal LPs during transport across the enterocyte. Association with the colloidal LPs again promotes preferential access to the lymph[96,114]. Alkyl ester prodrugs are typically less efficient than TG-mimetics since 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, since intestinal lymphatic transport bypasses portal vein transport to the liver[177]. Entry into the systemic circulation within intestinal lymph lipoproteins may also alter drug clearance and disposition[261].

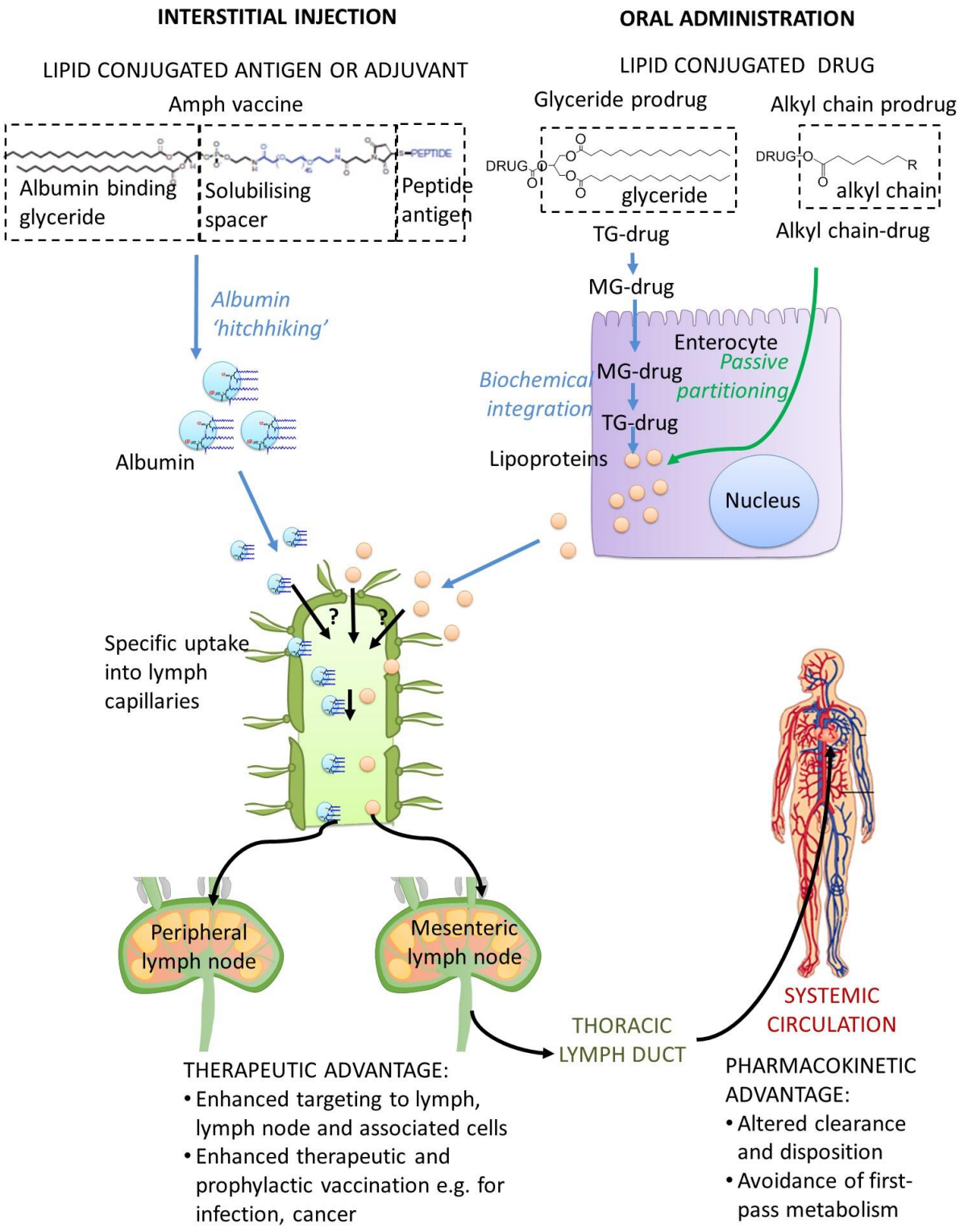
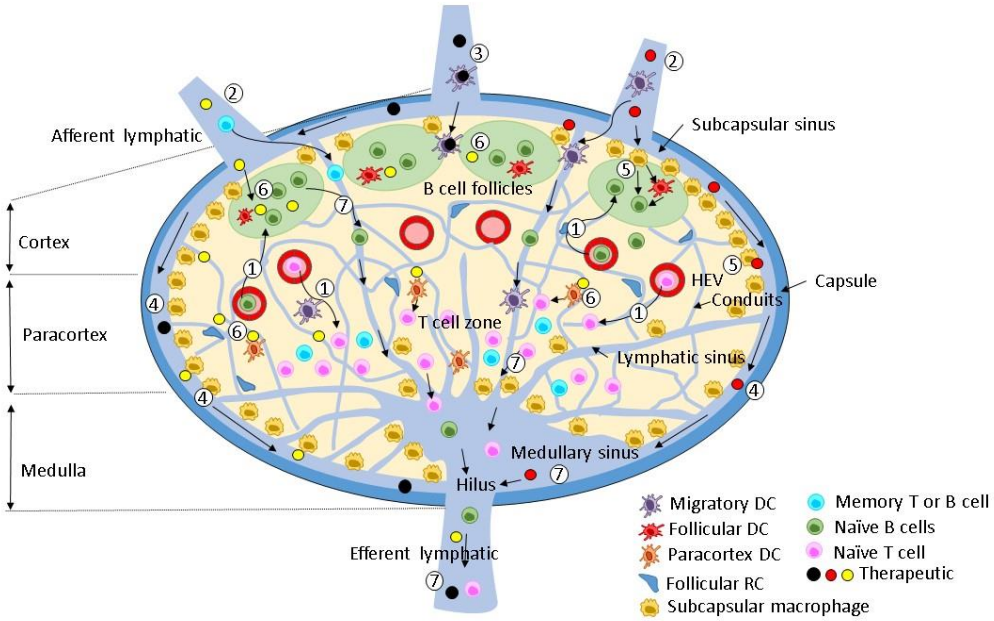

INTERSTITIAL INJECTION
ORAL ADMINISTRATION
LIPID CONJUGATED ANTIGEN OR ADJUVANT
Amph vaccine
Albumin binding glyceride
Solubilising spacer
PEPTIDE
Peptide antigen
LIPID CONJUGATED DRUG
Glyceride prodrug
Alkyl chain prodrug
DRUG
glyceride
alkyl chain
TG-drug
Alkyl chain-drug
MG-drug
Albumin 'hitchhiking'
Enterocyte
Biochemical integration
MG-drug
Passive partitioning
TG-drug
Lipoproteins
Nucleus
Albumin
Specific uptake into lymph capillaries
Peripheral lymph node
Mesenteric lymph node
THORACIC LYMPH DUCT
SYSTEMIC CIRCULATION
THERAPEUTIC ADVANTAGE:
• Enhanced targeting to lymph, lymph node and associated cells
• Enhanced therapeutic and prophylactic vaccination e.g. for infection, cancer
PHARMACOKINETIC ADVANTAGE:
• Altered clearance and disposition
• Avoidance of first-pass metabolism

Figure 6: 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, respectively[1]. Blood is supplied by specialized high endothelial venules (HEV) that terminate within the paracortex[1]. (1) Naïve T and B lymphocytes enter the node via extravasation across HEV[1]. Therapeutics that associate with lymphocytes in the systemic circulation can enter the lymph node across HEV together with lymphocytes[164]. (2) Afferent lymph, contains particulates, macromolecules, DCs 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 DCs at the injection site enter nodes in association with DCs[46,59,165]. (4) Lymph (and its contents, including therapeutics) either flows around the node via the subcapsular sinus to the efferent lymphatic[46] 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[262,263] and can transfer material to DCs or B cells[46,59,165-167,169]. Cellular uptake may be promoted by targeting ligands or highly charged molecules[65,82] and avoided by PEGylation which provides a steric barrier to opsonisation and phagocytosis[65]. 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 lymph[1]. 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/molecules can enter the B or T cell zones through lymphatic sinuses and conduits and may be taken up by B cells and DCs that interact with T cells[51,59,165,169]. (7) Particles/molecules that pass through the node, and naïve lymphocytes that do not meet their specific antigen in the node, leave via the efferent lymph[1].



Boxes

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

The majority of descriptions of lymphatic entry focus on the enhanced permeability of lymphatic capillaries to macromolecules when compared to vascular capillaries. Thus, small molecules and macromolecules < ~10nm are relatively freely permeable across both blood and lymphatic capillary endothelium, but are transported away from the interstitium via the blood capillaries as the flow of blood through vascular capillaries is ~ 100-500 times faster than the flow of lymph through lymph capillaries. With increasing size, transport across the vascular endothelium is reduced, whereas entry into the more permeable lymphatic capillaries is retained. Increasing molecular size therefore leads to preferential uptake via the lymphatic capillaries.

Alternatively, lymphatic uptake may be regulated by interstitial transport rather than, or in addition to, endothelial permeability[264]. This reflects the realization that 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. Since convective flow is typically directed from the blood to the lymph, after interstitial injection larger materials are expected to be delivered more effectively to the lymph than the blood. In 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) nanoparticulates through the interstitium is ultimately limited by the dimensions of the water channels that provide the conduits for interstitial transfer. These 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[4,12,264]. Historically, entry from the interstitium into the lymphatics was thought to occur via the intercellular junctions between lymphatic endothelial cells (LECs). But, more recent evidence suggests that macromolecular constructs and even fluid[265] 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 interaction with Lyve-1[266]. Ultrastructural studies have also suggested that chylomicrons are taken up across LEC via paracellular and transcellular transport pathways[267,268]. Dixon et al[60,269] 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 B class 1 (SR-B1) regulates the uptake and transcytosis of interstitial HDL across LEC into the lymph. Martel et al[13] also recently concluded that HDL are cleared from the interstitium via the lymphatics, implicating the lymphatics as an integral part of the reverse cholesterol transport pathway (although the same group proposed that HDL entered the lymphatics largely via paracellular pathways[2]). Albumin is transported across vascular endothelium by the transporter gp60 and caveolae[270,271], however, this has not yet been demonstrated on LEC's. Whilst 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.

Box 2: Glymphatics serve a lymphatic like function in the brain

Classical lymph vessels are notably absent from the brain parenchyma. This has led to questions regarding the mechanism of clearance of fluid and waste products from the interstitium surrounding the cells in the brain. Illiff et al recently recognized a specific paravascular transport pathway that serves a lymphatic-like function in the brain[90,91,93,272]. 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[90,91,93,272]. Tracers infused into the ventricle remained near the infusion site, but those infused into the subarachnoid space at the cisterna magna entered the brain via para-arterial pathways bound by smooth muscle cells and astrocytic end feet that expressed aquaporin-4 (AQP4) water channels. Large tracers were unable to enter the paravascular space (e.g. FITC-dextran 2000, 2000 kDa). In contrast moderated sized hydrophilic tracers (e.g. Texas red-dextran 3, 3 kDa) and small lipophilic tracers (e.g. Texas red hydrazide, 621 Da) were confined to the paravascular space, did not enter brain parenchyma and instead moved to para-venous channels[273]. Smaller hydrophilic tracers (Alexa Fluor 594 hydrazine (759 Da) and mannitol) and the peptide β-amyloid were able to move into the brain parenchyma[90,91,93,272]. 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) and solutes via a paravenous pathway. The draining ISF was subsequently cleared along wide draining veins. CSF flux through the interstitium, and the clearance of tracers/solutes injected into brain tissue was dramatically reduced in mice lacking AQP4, suggesting that trans-astrocytic water movement via these channels mediated the flux of CSF and ISF and associated solutes through the brain parenchyma. As this pathway was dependent on glial water flux and provides a lymphatic-like function (interstitial solute clearance), the authors coined the term ‘glymphatic’. Interestingly, both sleep and anaesthesia increased convective exchange of CSF with ISF, and the clearance of β-amyloid via the glymphatic pathway due to an increase in interstitial space[93]. Sleep may thus function to clear the brain of neurotoxic waste products that accumulate while awake, providing a restorative function. These findings have implications for the mechanism of β amyloid accumulation in neurodegenerative disease and the identification of possible new treatment targets. In the current context, the implications are in drug delivery to the brain and in particular in clearance from the brain, with possible drug accumulation in the brain with sleep deprivation and ageing as these pathways become less efficient.

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

Realisation that various endogenous macromolecules, antigens and bacterial products are transported from the intestine into the lymphatics has inspired the use of synthetic nano or microparticulate delivery systems as a means of enhancing oral bioavailability and/or intestinal lymphatic transport of drugs, therapeutic peptides/proteins and vaccine antigens[99,119,120,274-276]. However, the intestinal epithelium acts as a substantial barrier to the absorption of macromolecules[120] and under normal physiological conditions, particulate or macromolecular antigens are 'sampled' from the intestinal tract in relatively low quantities. Whether sufficient quantities of nanoparticles may be absorbed to deliver a typical therapeutic load is therefore less clear. Nonetheless, several recent studies suggest that nanoparticles can be absorbed across the intestinal tract and transported into the lymphatics, in some cases in large quantities[123,139,140]. Re-examination of the likelihood of particle absorption is therefore timely.

The proportion of the dose of nano/micro-particles that is absorbed intact has been reported to range from essentially zero[135-137] to quite large quantities (5-40% of the dose)[123,129,132,134,138-140]. (see **section 3**). 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 IV administration. This is further complicated by the labelling of nanoparticles with fluorophors or radiolabels to track disposition where the presence of free label, or liberation of free label in the GIT or enterocyte, make 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. A number of authors have taken advantage of an indirect model of lymphatic transport that compares plasma levels in the presence and absence of cycloheximide[122,183,277-280]. Cycloheximide inhibits protein synthesis and therefore inhibits intestinal lipoprotein secretion into lymph[281]. Dahan et al initially utilised this model to study the intestinal lymphatic transport of Vitamin D[281]. Lymphatic transport was assessed directly in lymph-cannulated animals and indirectly in animals administered cycloheximide via the reduction in plasma exposure on cycloheximide pre-treatment. In this case good correlation between the two methods was shown providing support for the approach. However, cycloheximide is a non-specific protein synthesis inhibitor that perturbs enterocyte ultrastructure[282]. Broader effects on nanoparticle processing are therefore likely, and studies where lymphatic transport is not verified directly are difficult to interpret. Visualisation of nanoparticles in lymph or lymph nodes via imaging techniques is also commonly used to support the suggestion that lymphatic transport of nanoparticles is significant following oral delivery. This technique provides evidence of nanoparticles in the lymph, but fails to provide a means of absolute quantification. This is further complicated by the use of highly lipophilic fluorophors 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.

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Glossary terms

Lipoproteins: a biochemical complex of lipids and soluble apolipoproteins that transports 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 (HDL), are assembled in both the liver and intestine. Low density lipoproteins (LDL) are formed following removal of lipids from VLDL by tissues.

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

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.

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.

High endothelial venules (HEVs): specialized post-capillary venules that are characterised 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.

Antigen presenting cells (APCs): are 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.

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

Initial lymphatics: 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 fibers in the surrounding tissue. Initial lymphatics are adapted for the uptake of fluid and cells

Collecting lymphatics: Larger lymphatics that are characterized by a continuous smooth muscle cell layer and the presence of semiluminar valves which facilitate the unidirectional transport of lymph and associated components. **Afferent** collecting lymphatics carry lymph into lymph nodes and **efferent** collecting lymphatics carry lymph from lymph nodes.

Mesenteric lymph duct: The major efferent collecting lymphatic vessel that exits the superior mesenteric lymph node and collects almost all lymph from the small intestine.

Thoracic lymph duct: the largest lymphatic vessel, sometimes called the left thoracic lymph duct, that collects most of the lymph in the body aside from lymph draining the 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.

Interstitium: the extracellular matrix or tissue space. The interstitium varies across tissue types but is typically composed of collagens, elastin, and glycosaminoglycans (mucopolysaccharides, such as hyaluronate and proteoglycans) that are mechanically entangled and cross-linked to form a gel-like matrix.

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