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Targeting lymph nodes for enhanced cancer vaccination: From nanotechnology to tissue engineering

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ABSTRACT

Lymph nodes (LNs) occupy a critical position in initiating and augmenting immune responses, both spatially and functionally. In cancer immunotherapy, tumor-specific vaccines are blooming as a powerful tool to suppress the growth of existing tumors, as well as provide preventative efficacy against tumorigenesis. Delivering these vaccines more efficiently to LNs, where antigen-presenting cells (APCs) and T cells abundantly reside, is under extensive exploration. Formulating vaccines into nanomedicines, optimizing their physiochemical properties, and surface modification to specifically bind molecules expressed on LNs or APCs, are common routes and have brought encouraging outcomes. Alternatively, porous scaffolds can be engineered to attract APCs and provide an environment for them to mature, proliferate and migrate to LNs. A relatively new research direction is inducing the formation of LN-like organoids, which have shown positive relevance to tumor prognosis. Cutting-edge advances in these directions and discussions from a future perspective are given here, from which the up-to-date pattern of cancer vaccination will be drawn to hopefully provide basic guidance to future studies.

1. Introduction

The hundreds of lymph nodes (LNs) in the body are one of the primary places for acquired immunity to occur. The multiple types of immune cells in LNs, together with the peripheral lymphocytes that can migrate to LNs, form a massive and dynamic system that monitors the entrance of immunogenic foreign substances into the body [1,2]. Foreign substances will be carried by interstitial flow or by migratory dendritic cells (DCs) to LNs, where they will be further processed to evoke cognate immune responses [3,4]. In antitumor immune responses, LNs are essential tissues sensing and regulating the progression of cancer. The functioning of immunotherapy, regardless of modality (immune checkpoint blockade, chimeric antigen receptor T-cell transfer, cancer vaccination or other strategies), relies on the efficient priming of lymphocytes in LNs [1,5]. Specifically, cancer vaccines are expected to eliminate tumor cells by providing antigens to the immune system to bare cancer cells' true identity, while LNs are the primary place where APCs and T cells reside. Delivering antigens into LNs to enable *de novo* recognition of tumor cells and boost specific immune responses is a promising treatment strategy [6,7].

The most straightforward route for LN-targeted delivery is intranodal administration. Compared with intravenous, subcutaneous, intradermal or intratumoral administration, the utilization rate of antigens and adjuvants can be improved via intranodal injection when operated rightly [8]. Antigens will directly encounter antigen-presenting cells and T cells, reaching the ultimate and optimal place for exerting their immunogenic capability. A clinical trial testing intranodal administration of melanoma antigen plasmids and peptides into melanoma patients yielded an overall immune response rate of 50 % [9]. Another more recent trial tested the percutaneous administration of poly-neoepitope mRNA into inguinal LNs of melanoma patients, and achieved neoepitope-specific T cell responses in all patients and vaccine-related objective responses in 40 % metastasis-bearing patients [10]. Despite these encouraging results, intranodal administration may raise some

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masty concerns such as the damage to LNs caused by injection, and the technical difficulty of accurate injection (ultrasound guidance is generally necessary) [11]. Therefore, researchers are putting more efforts on leading drugs to LNs through the lymphatic vessels.

Particles or molecules in blood hold the possibility to enter LNs via high endothelial venules (HEVs), but this pathway may lead to a relatively low LN-targeting efficiency since substances in blood will have many ways to go or be captured [12,13]. On the other hand, a LN is "protected" by a fibrous capsule that is basically not permeable, and therefore reaching into LNs generally need to enter the lymphatics which have higher permeability [14], and then arrive LNs via the afferent lymphatic vessels. Particles entering lymphatics via a cell-free passive diffusion can be classified as the "passive transport", while particles reaching into LNs by using cells (*e.g.*, migratory DCs) as carriers is the "active transport" [12]. The most commonly applied administration routes for vaccines include intradermal, subcutaneous and intramuscular injections [15], and the vaccine particles will need to penetrate interstitial matrix to achieve the passive drainage into LNs, thereby raising the need to carefully tailor the physiochemical properties of vaccine particles. For the active transport which involves the capture by migratory DCs, particle-cell interaction is also governed by the morphological and surface features of particles. Nanotechnologies devoting to the modulation of size [16], shape [17] and surface chemistry [18] are constantly contributing to nanovaccine development, since all these features have been demonstrated to exert great influence on *in vivo* distribution, efficacy and toxicity of not only vaccines, but also nanomedicines in a broad sense.

Targeting LNs is the key to release the therapeutic potential of immunotherapeutics, while in the context of cancer, LNs may be silenced after bathed with tumor-associated antigens [19]. DCs may be suppressed by the abundance of tumor-derived factors in LNs [20], and exhibit an immature and M2 macrophage-like phenotype with poor T-cell stimulatory ability [21]. These limitations have motivated great research efforts on developing LN-like niches or organoids to enhance tumor-specific immune responses. Biomaterial scaffolds encapsulating suitable cytokines have been shown to be capable of recruiting DCs, which then carry antigens to the draining LNs when antigens are co-loaded [12,22]. LN-like organoids are widely found in tumor tissues and are demonstrated to be positively relevant to cancer prognosis, and inducing the formation of LN-like organoids is becoming a new research focus [23]. Generally, neither the scaffold-based niches nor organoids can match LNs in structure and function, but they are offering a promising option to enhance LN-mediated immune stimulation and expansion. Therefore, advances in fields spanning from nanotechnology to tissue engineering will all contribute to the improvement of LN-targeting.

2. Lymphatic biology

2.1. LNs and tertiary lymphoid structures

LNs are a type of secondary lymphoid organs (which also include spleen, tonsils and Peyer's patches) that distribute all over the body. They are bean-shaped lymphoid tissue encapsulated by a capsule of connective tissue, which grow in connection with the network of lymphatic vessels [12,24]. Each LN can be broadly divided into two parts, the capsule (the outer connective tissue), and the cortex and paracortex regions inside the capsule [12]. As shown in Fig. 1, in the cortex region there are specialized compartments for B cells, often called the B-cell follicles, while in the paracortex region T cells and DC cells abundantly live [12,25]. Traversing the cortex and paracortex regions, high endothelial venules (HEVs) carry abundant B and T cells, which may enter the LNs when passing through. For each LN, lymph enters through afferent lymphatic vessels and leave via efferent vessels; as a whole, lymph moves in a unidirectional manner via the synchronized movement of lymphatic vessel compartments [26]. Small substances (typically smaller than 100 nm) including antigens in the interstitial space may enter the lymphatic vessels by passing through the fenestrated lymphatics, and then reach the draining LNs. When draining the peripheral excess fluid, LNs provide continuous monitoring of the presence of antigens and inflammatory factors in the fluid. LNs respond to the cues via the activation of immune cells and further recruitment through HEVs [27], which is commonly accompanied by LN enlargement.

Some inflammatory or pathological conditions may lead to lymphogenesis, producing organoids termed tertiary lymphoid structures (TLSs) which has similar but simplified structures compared with LNs. TLSs are ectopic neogenesis that form in non-lymphoid tissues, typically at sites of chronic inflammation including tumor [28]. They are organized aggregates of immune cells that contain partitioned B and T cell zones, APCs, fibroblastic reticular cells (FRCs) and HEVs, resembling the structure of LNs [29]. Unlike those generated via implanting artificial

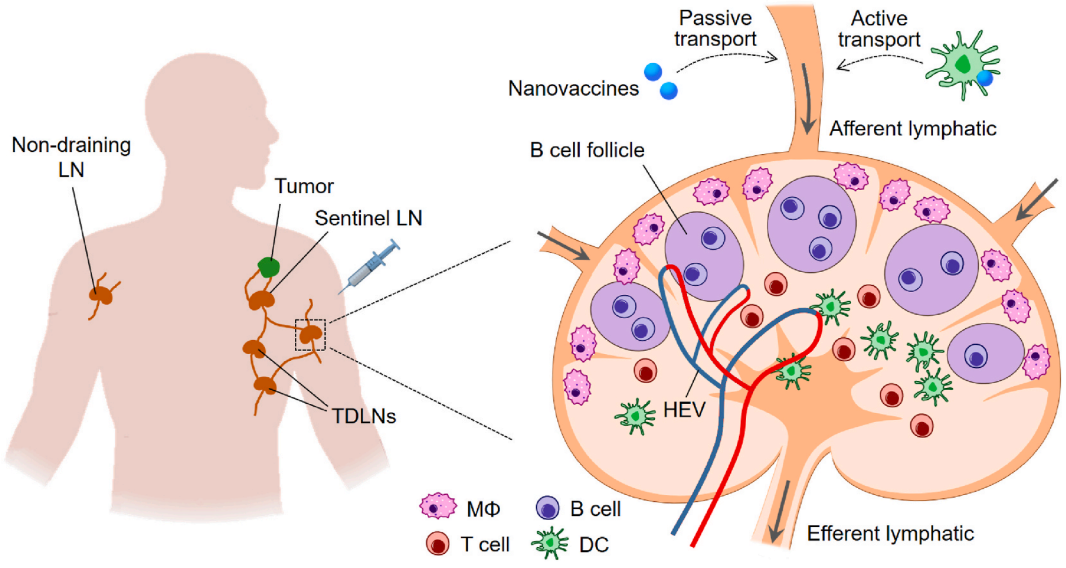


Fig. 1. Lymph node biology. Schematic illustration of the definitions of sentinel LN, TDLNs and non-draining LNs, the basic structure and cell composition of LNs, as well as the passive and active transport pathways of nanovaccines to LNs.

biomaterials, TLSs are made from autologous compositions and are functionally and phenotypically more comparable to LNs. Due to the variation in cellular composition and organization of TLSs in different tumor types, as well as the invasiveness of immunohistochemical staining, identification of TLS may need to rely on the signature of an array of genes, such as the 12-gene signature used to predict the presence of TLSs in melanoma and colorectal cancer [30]. TLS induction is emerging as a promising type of immunotherapy, due to the mounting evidence showing its positive relevance to cancer regression [31].

2.2. TDLNs and association with cancer immunotherapy

LNs drain the interstitial substances from nearby areas of the body. After establishing their presence, tumors are also drained by the nearby LNs which are consequently defined as tumor-draining LNs (TDLNs). The first TDLN draining the tumor is called sentinel LN (Fig. 1). Existence of cancer cells in TDLNs is an important prognosticator for patients, and surgeons often need to resect these LNs to ensure a good prognosis [32]. Tumor-associated antigens, released by secretion of exosomes, cell exfoliation from tumor tissue, or tumor cell necrosis, can reach TDLNs through lymphatics. DCs in tumor tissue which are exposed to tumor-associated antigens can also travel to TDLNs [33,34]. Meanwhile, regulatory cytokines and leukocytes will also be drained from tumor microenvironment, creating conditions in TDLNs for metastasis [35]. Therefore, TDLNs are often simultaneously bathed with antigens and immunosuppressive molecules/cells, creating an immunosuppressive microenvironment despite the abundance of immune cells. Targeting TDLNs has become an effective route to realize immuno-reeducation and metastasis control [36].

Swartz et al. showed the importance of targeting TDLNs, but no the non-TDLNs, in stimulating antitumor immune responses [19,37]. Adjuvant-conjugated polymeric NPs of about 30 nm in diameter efficiently accumulated within TDLNs only when NPs were injected in the site ipsilateral to the tumor. Increased frequency of antigen-specific $CD8^+$ T cells and slowed tumor growth were observed in a B16F10 melanoma and E.G7-ovalbumin (E.G7-OVA) tumor models. In comparison, administration in the contralateral site led to NP accumulation in non-TDLNs and did not suppress tumor growth [19]. The reason for this difference might be ascribed that tumor-derived antigens had already infiltrated in TDLNs and delivery of adjuvants could evoke effective immunity against tumor, while in non-TDLNs the antigens were relatively rare [33]. Activated T cells in TDLNs can also arrive tumors more efficiently than those in non-TDLNs. Intratumorally administrated agents hold the capability to be drained to TDLNs, but more direct delivery may be needed. Park et al. reported that peritumoral injection of a micellar formulation of toll-like receptor 7/8 agonist led to accumulation in both tumors and TDLNs, and induced significantly stronger innate and adaptive immune responses than intratumoral delivery alone [36]. Tumor-specific T cells generated in TDLNs could efficiently infiltrate into tumor tissues.

Like the researches above, employing the drainage to target TDLNs requires the nanovaccines to be administrated in the tumor-draining area (or into the tumor) rather than distant sites [37]. Administration to an intradermal site ipsilateral to tumor could induce efficient accumulation of nanovaccines in TDLNs, while administration to a contralateral site could not [19]. Meanwhile, targeting TDLNs through the drainage requires suitable physiochemical properties of the nanovaccines, as will be discussed below. For example, ferritin nanocages of about 20 nm in diameter were used to carry programmed cell death protein 1 (PD-1) and target TDLNs [38]. Obvious accumulation in TDLNs after intratumoral administration was observed. Both the employed tumor cells (CT26.CL25 cells) and the DCs in LNs expressed high-level PD-L1 [38], indicating a necessity of specific recognition between nanovaccines and targets.

Sentinel LN as the first TDLN is also an important target for immunotherapy, and it has another clinical significance which is for the identification of metastasis. A negative sentinel LN biopsy indicates that cancer cells has not yet spread and therefore good prognosis can be expected [39]. For the identification of sentinel LNs, many imaging agents are available, such as the blue dye (*e.g.*, methylene blue), radioactive colloid (*e.g.*, Technetium-99 m) and indocyanine green. Combined utilization of blue dye and radioactive colloid is regarded as a golden standard for sentinel LN identification in breast cancer [40]. Nanomaterial-based laboratory advancements are constantly reported, including the colored NPs [41], fluorescence imaging agents [41], and even MRI-CT-PET-fluorescence multimodal agents [42].

3. Optimizing physiochemical properties of nanovaccines

Adjuvants are routinely co-delivered with antigens to promote innate immune responses (*e.g.*, promote the maturation of APCs and production of co-stimulatory signals), which in turn benefit the antigen-specific immunity [43]. When the delivery vehicles can serve as adjuvants, we will obtain "self-adjuvanted" nanovaccines [44]. While such nanocarriers can provide additional biological clues to strengthen the immune responses, the following are the critical physiochemical properties of nanocarriers that are directly relevant with the ability to travel across lymphatics or be internalized by migratory DCs.

3.1. Effect of particle size

Size is one of the most critical parameters influencing LN transport efficiency and pathway. Smaller particles are generally more inclined than large particles to travel under interstitial flow and permeate through vasculatures [12], thereby having greater passive transport efficiency. On the other hand, particle size cannot be too small. It is easy to imagine that if a particle was down-scaled to the size of small molecules, it will be highly permeable, diffusible and not easy to be actively internalized by cells and will enter blood circulation, accumulating in other organs [45]. It has been reported that drug formulations that stayed longer at the administration site resulted in enhanced lymphatic uptake [46]. Generally, NPs with hydrodynamic diameter smaller than 5 nm are permeable through blood endothelial cell junctions, leading to hampered LN-targeting [25]. Meanwhile, active transport efficiency for ultra-small particles will be relatively low if the particle size is too small. Migratory DCs tend to engulf particles with suitable size, not too small and not too large [47]. Therefore, the overall LN transport efficiency is not monotonically relevant with particle size.

The necessity of formulating vaccines into particulate nanoformulations to improve LN-targeting efficiency has been confirmed by a number of studies (Table 1). For example, nanovaccines prepared via the self-assembly of OVA and CpG showed a LN-targeting capacity of about 10 times as that of free OVA [48]. The small size (~80 nm when hydrated) and negative surface charge enabled quick drainage to LNs 2–4 h post subcutaneous injection. On the other hand, increasing particle size to around 100–200 nm may attenuate LN-targeting (Table 1). For example, when revealing the size-dependent accumulation of pluronic-stabilized polypropylene sulfide NPs, it was found that 25-nm NPs entered the dermal lymphatic capillary network much more efficiently than 100-nm NPs did [49]. Up to 50 % of 25-nm NPs targeted the LN-residing DCs, 10 times as efficient as the 100-nm NPs [49]. In another study, biodegradable poly(lactic-*co*-glygolic)-*b*-poly(ethylene-glycol) NPs showed similar preference for LN trafficking and paracortex penetration [50]. After subcutaneous injection, the 20-nm NPs were easily drained by proximal and distal LNs and retained there more effectively than the 40- and 100-nm NPs. Interestingly, the 100-nm NPs showed slightly higher uptake efficiency by DCs, suggesting that the greater LN-targeting of 20-nm NPs should be ascribed to the easier free drainage [50].

Increasing particle size to microscale will further attenuate the pathway of free drainage to LNs and cell-involved active transport will be necessary. This was demonstrated by Bachmann et al. using DC-

Table 1
Representative studies reporting the effect of particle size on LN targeting efficiency.

NP formulation	Size	Animals and Ad. route	LN's preference for size	Ref.
Pluronic-stabilized polypropylene sulfide NPs	25 and 100 nm	BALB/c, C57BL/6, C3$^{-/-}$, OT-II and CD45.1 mice; *i.d.*	25-nm NPs reached LNs via interstitial flow 10 times more efficiently than 100-nm ones	[49]
Polystyrene NPs	20–2000 nm	C57BL/6 mice; *i.c.*	20–200 nm NPs could reach LNs via free drainage, while 500–2000 nm ones needed DCs to arrive	[16]
PLGA-PEG NPs	20, 40 and 100 nm	SKH1 Elite mice and CD-1 IGS mice; *s.c.*	20-nm NPs reached and retained in LNs more easily than 40- and 100-nm ones, and also were more penetrable in paracortex region	[50]
Melittin-lipid NPs	10–20 nm	C57BL/6 mice; *s.c.*	NPs achieved higher LN-targeting than free melittin, yielding 3.6-fold increase in CD8$^+$ T cell response	[51]
OVA-CpG assembled NPs	80 nm after hydration	C57BL/6 mice; *s.c.*	Accumulation in LNs of OVA was improved by 10-fold after prepared into NPs	[48]
Micelle NPs composed phospholipid-PEG-antigen peptide	20–30 nm	C57BL/6 mice; s.c.	NPs had improved accumulation in LNs and increased antigen-specific T cells to 10 fold of free vaccines	[52]
OVA-conjugated gold NPs	5, 15, 50 and 100 nm	C57BL/6 mice; *i.d.*	50–100 nm NPs had 175-fold more antigen delivery at FDC dendrites than 5–15 nm ones	[53]
Assembled PAMAM NPs	100 nm (breakable into 5 nm)	C57BL/6 and Balb/c mice; *i.v.*	Size reduction from 100 to 5 nm improved intratumoral perfusion and enhanced migration to LNs	[54]
Nanodiscs composed of phospholipids and ApoA1-like peptide	10 nm in lateral size	C57BL/6 mice; *s.c.*	Nanodiscs had increased antigen trafficking to draining LNs over free antigens	[55]
Thiolated PPS NPs	27-nm NPs that could release small molecules	C57BL/6 mice; *i.d.*	NPs accumulated in draining LNs and remained at the periphery but the released molecules were in the cortex/paracortex region, realizing partitioning of NPs and cargos	[56]
OVA-conjugated gold NPs	10, 22 and 33 nm after hydration	C57BL/6 mice; *s.c.*	22- and 33-nm NPs showed higher accumulation in draining LNs than 10-nm ones; 22-nm NPs induced the most polyfunctional CD8$^+$ T cells	[57]
MOF-gated mesoporous silica NPs	100 nm	EG7-OVA tumor-bearing C57BL/6 mice; *s.c*	OVA carried by the NPs reached the draining LNs more efficiently than free OVA	[58]

ApoA1: apolipoprotein-A1; PPS: poly(propylene sulfide); *i.d.*: intradermal; *i.c.*: intracutaneous; *s.c.*: subcutaneous.

depleted control experiments [16]. It was found that NPs traffic to draining LNs by targeting distinct DC populations according to their size. After injected into the footpads of mice, the 500–2000 nm particles were mostly associated with DCs from the injection site, suggesting the vital role of active transport; 20–200 nm ones were found in local DCs but also in LN-resident DCs and macrophages, suggesting that the involvement of free drainage (passive transport). Using CD11c-diphtheria toxin receptor transgenic mice whose DCs could be conditionally depleted, it was found that large (500–2000) particles could not reach LNs upon DC depletion, confirming the necessity of active transport pathway for microparticles [16].

Particularly, exploring the overall intra-lymph accumulation of antigens is convenient but may be vague. LNs have advanced physiological structure and multiple types of immune cells inside, and looking into the cell-specific uptake of antigens will be more helpful. A number of studies have showed the feasibility of site- or cell-type-specific delivery. Chan et al. showed that OVA-conjugated gold NPs have size-dependent retention and presentation in LN follicles (Fig. 2a) [53]. 5–15 nm NPs quickly appeared in the follicles after intradermal injection but were cleared via extracellular vesicles within 48 h; 50–100 nm NPs showed delayed follicle accumulation but were retained for up to 5 weeks. Smaller NPs preferentially entered into follicular DCs, stayed in endolysosomes but then be cleared, while larger NPs were mostly aligned on cell surface and dendrites. As a result, larger particles located outside and exhibited easier antigen presentation to stimulate B cells [53]. Therefore, it may be not enough to simply improve NP accumulation in LNs, and their fate and subcellular location are also critical aspects determining the efficiency of antigen presentation. More recently, a programmable multistage delivery pattern has been developed to realize delivery of small-molecule cargos to specific lymphocyte subpopulations within LNs (Fig. 2b–e) [56]. NPs were designed to have the capability to release small molecules with a controlled release half-life. NPs (~27 nm in diameter) quickly accumulated within the draining LNs after intradermal administration, and upon intra-lymphatic release, the small-molecule dyes were efficiently delivered to the cortical cells (including T and B cells and DCs) in LNs, while the NPs remained at the peripheral barrier cells, thereby realizing partitioning of NPs and cargos. The different size of NPs and small molecules was believed to account for their difference in accumulation site [56].

3.2. Particle shape

Particle shape has great impact on nano-bio interactions [17]. It influences LN-targeting in multiple ways, including cellular uptake efficiency, permeability across vasculatures and migration capability in interstitial flow. For example, discoidal or sheeted particles can cross slits that are narrower than particle diameter and therefore can be more permeable to enter lymph vessels [59]; spiky particles have been reported to be internalized more easily than spherical particles [60,61], holding promise to target the migratory DCs at the injection site. Therefore, as illustrated in Fig. 3, active and passive transport pathways may have different preferences for particle shape, just like the case in particle size. Furthermore, it was reported that particles of different shapes induced different types of immune responses [62,63]. Tailoring

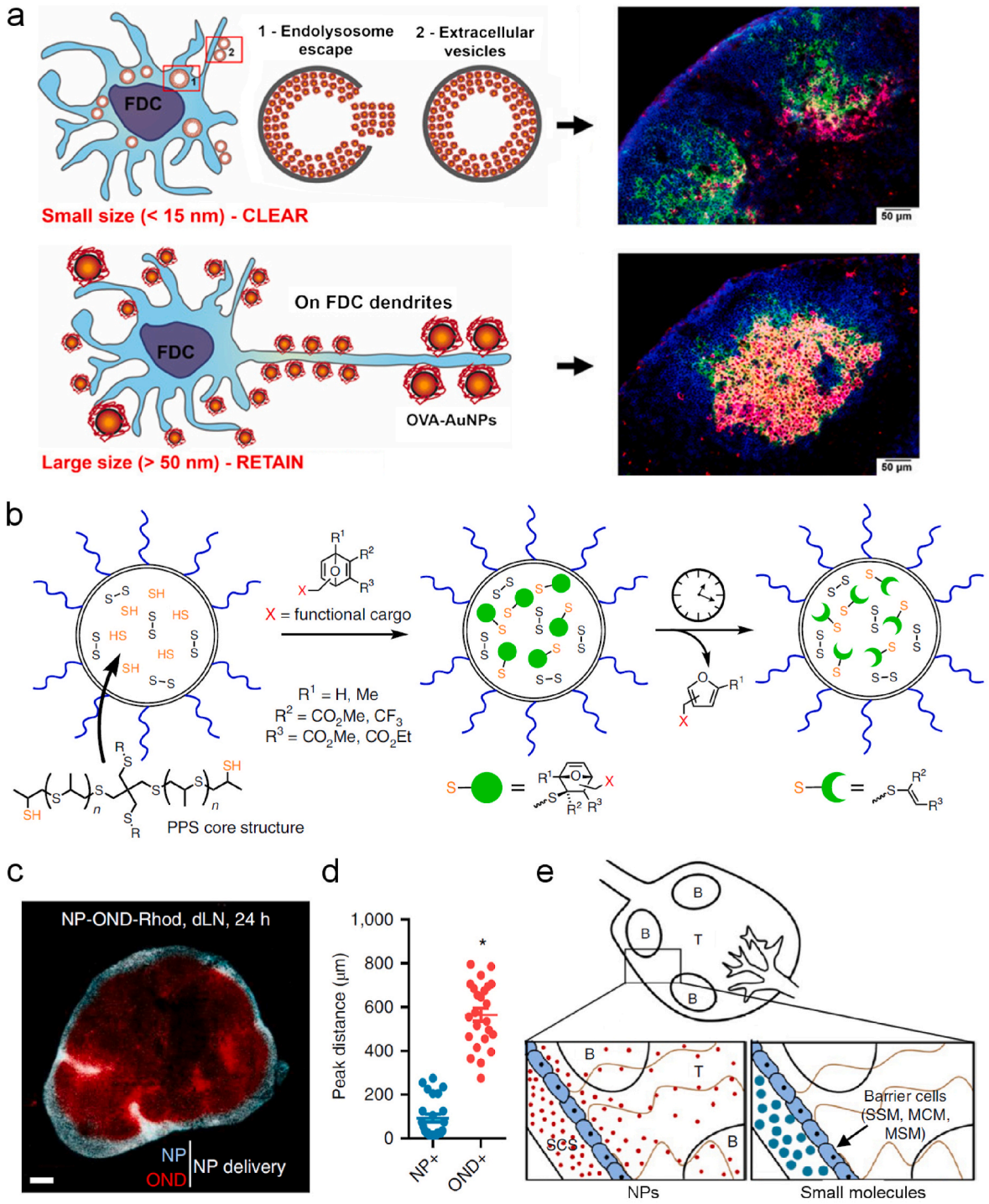


Fig. 2. Cell- and site-specific delivery enabled by size. (a) After intradermal injection, smaller OVA-gold NPs appeared in the follicles of draining LNs but were then cleared by FDCs within 2 days; larger NPs were retained within follicles for weeks. Reproduced with permission [53]. Copyright 2019, American Chemical Society. (b) Diagram of the programmable multistage release of furan-tagged cargos. (c) Fluorescence image of excised the draining LN after intradermal injection of NPs, showing NP accumulation at the periphery and cargo accumulation throughout the LN. (d) Peak distance from NPs and cargos to the LN boundary, calculated from images such as in c. (e) Illustration of the distribution of NPs and small-molecule cargos partitioned by size. Reproduced with permission [56]. Copyright 2020, Springer Nature.

NP shape is therefore powerful in vaccination.

Comparison of the rate and difficulty for cells to internalize NPs with different shapes has been ongoing since shape control becomes feasible. NPs with relative sharp edges were often reported to be internalized more easily, including triangular gold NPs [64], gold nanorods [65], spiky silica NPs [60], etc. There are also studies reporting contrary conclusions, computationally [66–68] and experimentally [69]. Several studies reported that the internalization of NPs depended on the orientation relative to cell membrane. Cellular uptake would be slower if the long axis of anisotropic NPs was perpendicular to cell membrane at the contact site, due to the need of laying down to obtained maximum NPs-membrane contact area and facilitate particle wrapping by cell membrane [66,70]. Recognition and uptake of NPs by DCs are also shape-dependent (Table 2). Mammalian cells were reported to

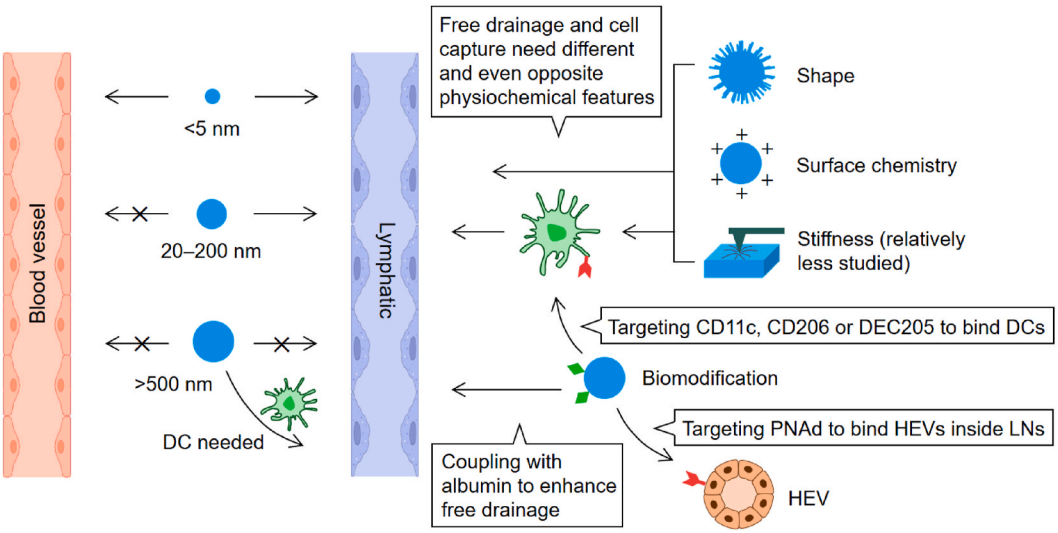


Fig. 3. Basic design rationales of physiochemical and surface features. NPs of different sizes in interstitial space have different destinies, especially for the microscale particles which can reach LNs only via active transport. The effect of particle shape, surface chemistry or stiffness is hard to generalize, and different transport pathways have different and sometimes opposite requirements for each of them. Surface biomodification can be employed to target DCs, enhance free drainage or target HEVs inside LNs.

preferentially uptake disc-shaped hydrogel NPs over the nanorods of similar volume, surface charge and material composition [71]. While the uptake kinetics and efficiency was cell type-specific, mouse BMDCs, Hela cells and HEK293 cells and HUVECs all preferred to internalize nanodiscs, and the former three cells even preferred the nanodiscs with high aspect ratio. Uptake pathways were also shape-specific [71]. In another study, nanofibers composed of self-assembled peptides showed enhanced uptake by DCs compared with the spherical counterparts, and induced Th1 phenotype immune response to a greater extent [72]. All these studies showcased the potential of shape optimization for improved cellular uptake, regardless of the controversy about optimal shape.

Impact of particle shape on the capability of interstitial migration towards LNs has been relatively less studied. Indeed, different shapes endow particles with different flow characteristics, as has been extensively studied on circulating lifetime and biodistribution [75,76]. Several works reported that nonspherical NPs exhibit improved lateral drift and are more likely to reside on vessel walls to contact with endothelial cells [77,78]. Researches by Ferrari et al. showed that nonspherical (*e.g.*, discoidal and ellipsoidal) NPs would travel in a trajectory nonparallel to vessel wall due to tumbling and rolling dynamics, which was different from spherical particles who traveled basically parallel to the vessel wall [79]. As a result, nonspherical NPs were more likely to contact and bind endothelial cells for extravasation. The flow velocity towards LNs in the subcutaneous interstitial space, as well as the viscous resistance upon movement, are obvious different from that in blood. Meanwhile, it is necessary to decouple the active transport pathway by depleting the migratory DCs to exclusively study the interstitial flowing capability of differently shaped NPs. To the best of our knowledge, this has yet been explored.

It was reported by Sawa et al. that gold NPs of different shapes not only had different uptake efficiencies by DCs but also elicited different immune responses [65]. Spherical gold NPs induced higher antigen-specific antibody production than the nanorods and cubic NPs, while this was not caused by the difference in cellular uptake since the nanorods showed higher uptake by BMDCs. Meanwhile, the spherical and cubic NPs elicited the secretion of inflammatory cytokines (TNF-α, IL-6, IL-12 and GM-CSF), while the nanorods activated the production of inflammasome-dependent cytokines (IL-1β and IL-18) [65]. Mitragotri et al. reported size- and shape-dependent immune response induced by antigen-carrying polystyrene particles [62]. After subcutaneously injected into mice, smaller spherical particles (193 nm in diameter) induced Th1-biased immune response, while large rod-shaped particles (1530 nm in length) elicited Th2-biased response against the delivered antigen. Variation in the rods' maximum length, but not width, was proposed responsible for the difference in immune type [62]. Therefore, shape modulation by itself is a powerful strategy for dictating the immune response strength and type.

3.3. *Effect of surface chemistry*

Surface charge is probably the most influential surface property. A negative surface charge will enable prolonged circulation in blood, easier diffusion in nanoporous tissue matrix and inhibited clearance by macrophages [80]. Most nanovaccines are designed as negatively charged or non-ionic for LN-targeting [12]. Specifically, PEGylation is considered as a useful modification to inhibit non-specific binding of biomolecules and to prolong the circulation half-life in blood [81]. Positively charged NPs will instantly absorb a protein corona after local or systemic administration, thereby increasing the overall hydrodynamic size and blocking the original surface ligands [82]. NPs with positive surface charge can also cause inflammation to the local tissue and hepatotoxicity when administrated intravenously [83]. For example, it was reported that positively charged lipid NPs induced obviously increased release of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase in mice liver compared with neutral and negatively charged NPs, indicating a higher hepatotoxicity [84]. They also induced a significant pro-inflammatory response by elevating the expression of Th1 cytokines 10–75-fold higher compared with control NPs [84]. Therefore, positively charged NPs are rarely used for drug delivery despite some evidences about their capability in inducing innate immune responses [84,85]. Nevertheless, NH_2-modified silica NPs, partially covered by CpG DNA, with a final surface charge of 15 mV, were employed for vaccine delivery [86]. The positively charged NPs exhibited high colloidal stability, and after subcutaneously injected, complexation between NPs and albumin from interstitial space occurred, which reduced nonspecific interaction with local tissue and promoted drainage towards LNs (note that endogenous albumin has intrinsic capability to trace LNs [87]). This provides a unique route to improve passive LN transport efficiency and suppress the recognition by macrophages due to the decoration of autologous proteins on NP surface [86]. Meanwhile, positively charged NPs generally have higher affinity

Table 2
Representative studies reporting the effect of particle shape on internalization by DCs.

NP formulation	Shape and size	Surface chemistry	DC's preference for shape	Ref.
polyethylene glycol diacrylate hydrogel NPs	Discs (d × h): 80 × 70 nm; 220 × 100 nm; 325 × 100 nm Rods (l × w × h): 400 × 100 × 100 nm; 800 × 100 × 100 nm	ZP: ~-57 mV for all particles	BMDCs internalized nanodiscs with high aspect ratios more easily than nanorods, and larger NPs were internalized more than smaller ones	[71]
Self-assembled peptide amphiphile molecules coated with CpG-ODN	Fibers: 15–20 nm in diameter, >200 nm in length Spheres: 15–20 nm	+6 mV for nanofibers; +21 mV for nanospheres	Nanofibers achieved higher uptake into both DCs and plasmacytoid DCs than nanospheres	[72]
Gold NPs coated with WNVE protein	Spheres: 20 and 40 nm Rods (l × w × h): 40 × 10 × 10 nm Cubes: 40 × 40 × 40 nm	ZP: ~-24, −11 and −9.9 mV for spheres, cubes and rods	BMDCs internalized rods more easily than spheres and cubes, but 40-nm spheres induced the most WNVE antibody	[65]
Gold NPs coated with CpG-ODN and conjugated with MUC1 antigen	Spheres: 20 and 45 nm Rods (l × w × h): 20 × 6 × 6 nm; 46 × 14 × 14 nm Stars: 70 nm (inter-spike)	ZP: 17.7–11.4 mV for nanorods	Nanospheres and nanostars showed weaker uptake by DCs but induced higher level of T-helper-1 immune responses	[73]
Hydroxyapatite rods adsorbed with OVA	Rods of ~100, 200, 500, 1000 and 10000 nm in length, and ~3.5, 4, 7.1, 12.7 and 25.4 in aspect ratio, respectively	not indicated	Shorter rods with lower aspect ratio showed higher uptake by DCs and better LN-targeting	[74]

d × h: diameter × height; l × w × h: length × width × height; ZP: zeta potential; WNVE: West Nile virus envelope, MUC1: human mucin 1.

to cell membrane [88], and the resulting improved uptake by DCs can be leveraged for enhanced active transport.

Surface charge is usually measured as the overall net charge value in a solvent (typically water), while irrespective of surface charge, each chemical moiety on particle surface will have an impact on biodistribution. The principle of structure-defines-function also governs the design of LN-targeting NPs. Truong et al. explored the impact of chemical structure of lipid NPs on LN-targeting efficiency [89]. When stabilized by an amphiphilic molecule consisting of a branched PEG and a lipid tail, the lipid NPs realized highly specific delivery of antigens to LNs; in comparison, if the stabilizing molecule had a longer unsaturated lipid tail, the delivery efficiency was significantly reduced. Given that most lipid tails were "hidden" in the interior, the difference in LN-targeting efficiency showcased the importance of chemical structure. Meanwhile, if the PEG was linear, the targeting efficiency was also much lower, which should be instructive since most lipid NPs have been using linear PEG for surface modification [89]. Ko et al. studied four protein NPs including *E. coli* DNA binding protein (DPS) and human ferritin heavy chain (hFTN), and found that hFTN exhibited the best LN-targeting [90]. hFTN migration through lymphatic vessels was detected instantly after injection into the foot-pad of mice, and strong accumulation in LNs was observed within 10 s. The DPS with similar size, morphology and zeta potential showed much lower LN-targeting [90]. Xu et al. realized cell-specific delivery to LNs after screening a library of lipids with different tail lengths, linkers and head structures (Fig. 4) [91]. The influence of the variation in pK_a and particle size caused by the alteration of lipid structure was negligible. LN-targeting capacity was obviously influenced by the subtle variation in lipid structure (Fig. 4 a–c), and was better than the commercial COVID-19 mRNA vaccine (ALC-0315 from Pfizer/BioNTech) after further optimization (Fig. 4 d–e). DCs and macrophages but not T cells, B cells nor NK cells in draining LNs were effectively transfected after *s.c.* administration (Fig. 4 f–g), thereby enabling cell-specific delivery [91].

3.4. Particle stiffness

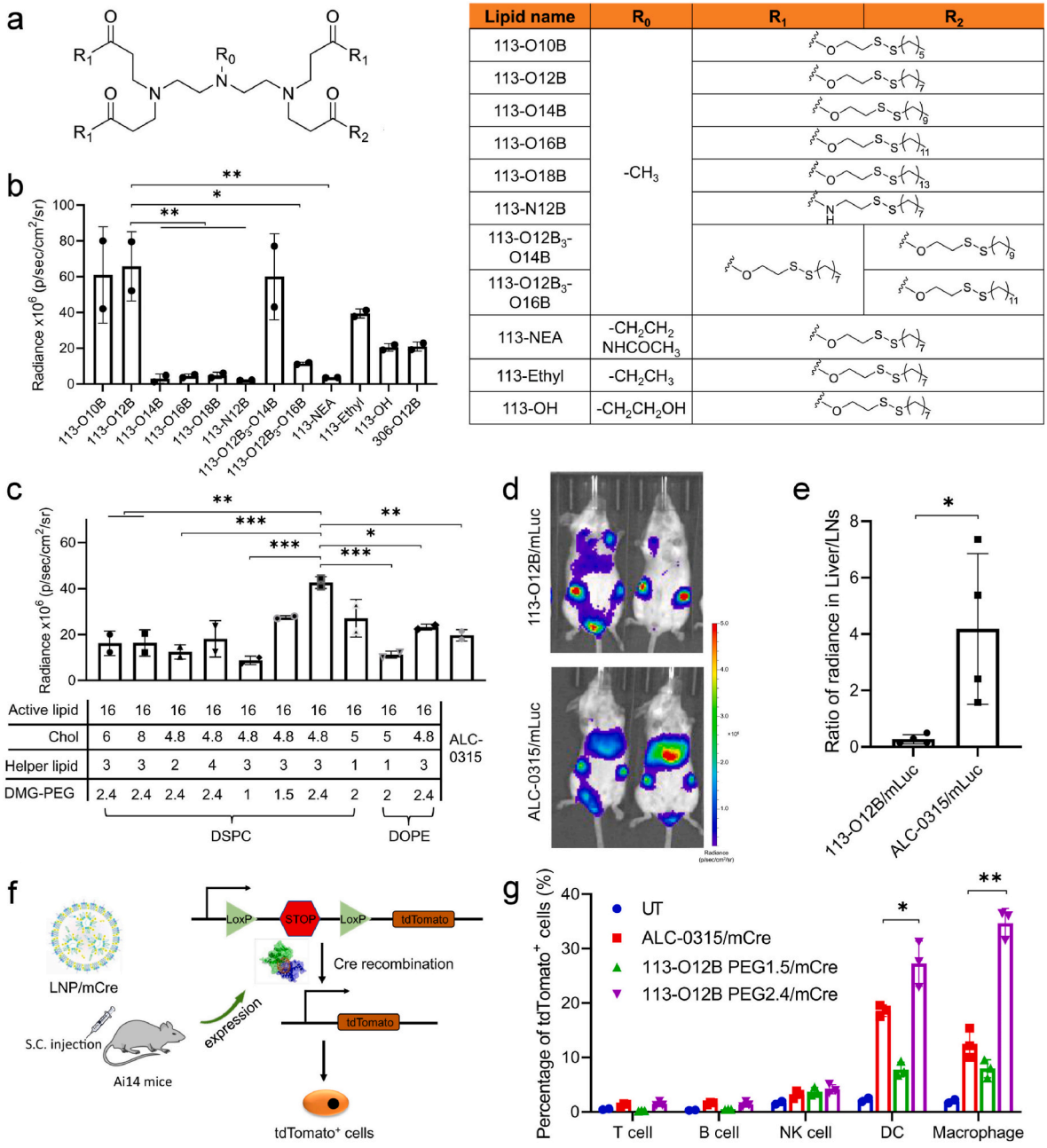
Particle stiffness affects bio-nano interactions. Various strategies are currently available for modulating NP stiffness, primarily via tuning the cross-linking density of polymeric particles [92], tailoring particle shape (*e.g.*, into biconcave shape) [59], changing thickness of shelled/layered/fibrous particles [93,94], *etc*. Soft NPs can travel across narrower slits than stiff NPs of the same hydrodynamic diameter can do [59]. The maximum distance between endothelial cells on lymphatic vessels was reported to be around 100 nm [95,96], while for soft NPs they can be bigger than that for intra- or extravasation. Therefore, soft NPs may hold greater promise for entering lymphatic vessels. Soft NPs are often reported to exhibit inhibited cellular uptake and longer circulation time in blood than hard NPs [97], partly due to the unique protein corona composition modulated by NP stiffness [97,98]. This feature, together with the better extravasation capability, suggests that nanovaccines with low stiffness may be administrated intravenously to realize entrance into the lymphatic system.

On the other hand, migratory DCs have different internalizing capability for NPs of different stiffness. Cells can sense material stiffness and response accordingly. Multiple studies found that mammalian cells grown on stiffer substrate were more adherent to the substrate [99,100], and could uptake NPs more efficiently on a per cell basis [99] or less efficiently in a size-dependent manner [100]. Uptake results of polymeric nanorods by breast cancer cells showed that stiffer nanorods exhibited easier cellular uptake, although the influence of nanorod composition was not perfectly decoupled [101]. In comparison, a recent study explored a library of spherical PEGylated polymeric NPs and showed that two human cancer cell lines would uptake 100-nm softer NPs faster and threefold more than hard NPs of the same size, while no significant difference was observed for 50-nm NPs [102]. Therefore, the exact effect of stiffness is controversial and may be conditional. Effect of stiffness on DC uptake efficiency is studied to a much less extent. Cell line also matters, since it was reported that different cells can be directed to varied degree of stress state by material stiffness, which finally influences the NP uptake efficiency [103].

4. Bio-modifications for LN-targeting

4.1. Employing the endogenous intra-lymphatic drainage

Some endogenous biomolecules such as albumin and immunoglobulins can be used as natural carriers for LN-targeting (Fig. 3). Albumin was reported to be blocked from disseminating into blood but instead migrated efficiently into the lymphatics due to its large molecular weight (66 kDa) [104], leading to the exploration of albumin-binding strategies for LN-targeting. Dye-based sentinel LN visualization is actually such a 'albumin hitchhiking' route relying on the binding of dyes to endogenous albumin. Amphiphiles consisting of an antigen or adjuvant linked to an albumin-binding lipophilic tail have been demonstrated to have increased accumulation in LNs and reduced systemic dissemination compared with free antigens and adjuvants, yielding 30-fold enhancement in T cell priming [105]. In another example, maleimide-functionalized Evans blue, which could bind human serum



Lipid name	R_0	R_1	R_2
113-O10B	$-CH_3$		
113-O12B			
113-O14B			
113-O16B			
113-O18B			
113-N12B			
113-O12B$_3$-O14B			
113-O12B$_3$-O16B			
113-NEA	$-CH_2CH_2NHCOCH_3$		
113-Ethyl	$-CH_2CH_3$		
113-OH	$-CH_2CH_2OH$		

Fig. 4. Cell-specific delivery enabled by chemical structure. (a) Chemical structures of the studied lipids. (b) Bioluminescence intensity within inguinal LNs obtained with lipid NPs made from different lipid structures. (c) Bioluminescence intensity acquired using 113-O12B lipid NPs but with different formulations. (d) Bioluminescence results of mice treated with luciferase-mRNA-transfected 113-O12B lipid NPs or the commercial ALC-0315. (e) Liver-to-LN ratio of bioluminescence intensity in the indicated groups. (f) Diagram of the 'lightning' of tdTomato$^+$ cells for evaluating NP entrance into different cells. (g) Percentage of tdTomato$^+$ cells in different types of cells after the indicated treatments. Reproduced with permission [91]. Copyright 2022, National Academy of Science of the USA.

albumin, was used to modify vaccines, and the obtained nanocomplexes realized almost 100-fold more efficient LN accumulation of CpG-ODN compared with the unfunctionalized emulsion of the same antigen [106].

4.2. Cell membrane-coated nanovaccines

Cell membranes have been widely used to fabricate liposome-like delivery systems [107]. Membranes from different cell types are extracted to encapsulate cargos, and some of the biological functions of the original cells may be retained due to the good preservation of cell membrane structures. Tumor cell membranes are also employed in the fabrication of cancer vaccines, as both the source of tumor-specific epitopes and delivery vehicles [108]. Also following this strategy, membranes of mature DCs have been extracted in a recent study to encapsulate mRNA and achieve improved accumulation in lymphoid organs [109]. BMDCs were separated from mice and stimulated with resiquimod to induce maturation, followed by membrane extraction. Membrane proteins including CD80, CD86 and MHC-I were well reserved. Compared with commercial lipid NPs without DC membrane, the nanovaccines showed improved accumulation in spleen and LNs after intravenous injection, possibly due to the LN-homing capability given by the DC membranes [109]. Generally, cell membrane-derived nanoplatforms represent a promising system in not only vaccination, but also drug delivery and bio-sensing, despite some limitations such as the difficulty in maintaining a specific differentiation state of immune

cells after extensive expansion *ex vivo* [110,111].

4.3. Targeting the molecules over-expressed on DCs or LNs

Ligands that specifically bind the cells that can migrate to, or already reside within LNs, have been widely designed and employed for LN-targeting. This can be considered as an "active targeting" route [1], as distinguished from the "passive targeting" pathway which relies on passive cellular uptake. Active targeting has been widely explored in nanomedicine design, by employing surface ligands (*e.g.*, cRGDyk peptide) to target the receptors overexpressed on cells (*e.g.*, αvβ3 receptor in MDA-MB 435 cells) [112]. Targets that can be employed for DC-targeting include CD11c [113], MR/CD206 receptor on DCs that can bind mannose [114], and DEC-205 receptor which is used by DCs for antigen processing [115] (Fig. 3). Nanovaccines will be delivered to LNs by virtue of the homing capability of DCs towards LNs. Targeting different receptors is able to interfere specific steps in immunogenicity and finally induce varied immunity at varied intensity. For instance, mannose receptor has been reported to trigger meta-inflammation apart from its well-known role in facilitating the internalization of antigens [116], and therefore NP modification with mannose may also lead to inflammation modulation.

Given the highly specific recognition and motivated by the development of antibody drugs [117], antigens were widely modified with antibodies against receptors on DCs. A clinical trial (NCT03358719) is testing the safety and efficacy of anti-DEC-205-NY-ESO-1 fusion protein (NY-ESO-1 is a cancer-testis antigen expressed in multiple cancer types) in combination with poly-ICLC, decitabine and nivolumab. Preclinically, a nanovaccine modified with anti-CD40 antibody was transdermally administrated via a microneedle, and promoted accumulation in TDLNs was observed [118]. Probably due to the high cost and technical complexity of antibody conjugation, modification with other synthetic ligands or short peptides is explored increasingly. Relevant preclinical studies are in larger number. A recent example is a porcine circovirus type 2 Cap protein fused with a DC-binding peptide, which has been demonstrated to induce stronger Cap-specific antibody production compared with the Cap protein without DC-binding peptide [119]. Similarly, using an antigen peptide that can target the scavenger receptor class B1 (SR-B1), which were modestly expressed on the intralymphatic DCs, the LN-targeting of ultra-small nanovaccines was enhanced [120].

In comparison with antibodies and peptides, small-molecule-based synthetic ligands are more stable, characterizable and can avoid protein-induced immune side effects. Hubbell et al. reported an antigen-polymer platform with DC-targeting capability, where the polymer was composed of mannose and adjuvant monomers [121]. Presence of mannose led to improved antigen delivery to multiple DC subsets and finally increased antigen localization to the draining LNs after intradermal injection. It was also shown that OVA conjugated to the polymer enhanced the humoral and cellular immunity compared with the OVA conjugated to polymers lacking either mannose or the adjuvant [121]. Many mannose-conjugated nanoformulations are currently available, for delivering therapeutic agents [122–124], detecting sentinel LNs [125–127], *etc*.

Another active targeting strategy is targeting the molecules in LNs. This is different from the DC-targeting method since it does not rely on the migration of DCs but leads vaccines directly to LNs. An example was reported by Abdi et al. who realized efficient targeting of TDLNs by targeting the peripheral node addressin (PNAd), a family of glycoproteins on high endothelial venules (HEVs) [128]. By developing a monoclonal antibody against the PNAd and conjugating paclitaxel to it, the obtained antibody-drug conjugate showed an accumulation in TDLNs higher than all other major organs after intravenously injected into tumor-bearing mice. HEV-containing primary and metastatic tumors were also targeted by the antibody-drug conjugate, leading to reduced tumor size and metastatic number [128].

5. DC-based delivery to LNs

An important class of immunotherapy is DC vaccines, which infuse antigen-stimulated autologous DCs back into patients to activate anti-tumor immune responses [129]. The use of autologous cells inhibits immune side effects and increases the survival rate of DCs after infusion, and *ex vivo* stimulation with patient-derived antigens realizes personalized therapy [130]. *Ex vivo* stimulation is vital for DCs to acquire the migratory capacity and the ability to induce antigen-specific T cells [131,132]. By virtue of the intrinsic migrating capability, the infused DCs are able to self-deliver into LNs and prime immunity. One DC vaccine therapy, the Sipuleusel-T, has been approved by FDA in 2010 to treat castrate-resistant prostate adenocarcinoma, in which autologous DCs loaded with engineered fusion protein (which combines prostate antigen with GM-CSF) are intravenously administrated [133]. Greater efforts have since then been put in this field.

Despite the extensive exploration and the FDA approval of Sipuleusel-T, DC vaccines are often less effective compared with the efficacy they theoretically have. *Ex vivo* manufacturing procedures may change the phenotype and function of the original DCs, leading to difficulty in maintaining intact immunogenic capacity [129]. Several other limitations exist, including the technical complexity of the whole procedures, the difficulty in screening suitable biomarkers, the low viability of engineered DCs after administration [134], as well as the relatively low cell percentage that can finally arrive LNs [135]. For example, de Varies et al. found via scintigraphic imaging and immunohistochemistry that maximally 4 % of intradermally injected DCs reached the draining LNs, and large number of DCs stayed at the injection site and lost viability irrespective of administration route (intradermal or intranodal) [135]. Interestingly, intradermal administration of DC vaccines was found to have superior antitumor T cell induction compared with intranodal vaccination in HLA-A2.1$^+$ melanoma patients. DC migration into adjacent LNs after intranodal administration was significant but highly variable, that about 30 % patients had a complete absence of migration [136]. Strategies have been developed to address these hindrances, including the use of a combination of cytokines and proteins to improve DC maturation during *ex vivo* culture (the maturation cocktails) [137], and notably, the modulation of migrating capability to enhance LN-targeting.

The expression of C–C chemokine receptor type 7 (CCR7) on DCs is essential for their directional migration to the draining LNs. DCs swarm into the T cell zones in LNs via the interaction of CCR7 and its ligands, chemokine C–C motif ligand 19 (CCL19) and CCL21 [138]. Upregulating the expression of CCR7 has been exploited as an effective route to improve LN-targeting. A micellar nanovaccine containing CCR7-encoding plasmid DNA has been reported to exhibit promoted DC migration to LNs and induce strong antitumor immunity [139]. The migration of regulatory macrophages to the T cell zones of LNs after intranodal injection was also improved via the retroviral over-expression of CCR7 [140]. Meanwhile, the specific engagement of CCR7 and CCL19/21 suggests that it is possible to guide the migration of DCs by modulating ligand expression. This was supported by a study where intrapulmonary treatment with CCL21 gene-modified DCs led to increased intratumoral infiltration of CD11c$^+$DEC205$^+$ DCs in murine bronchoalveolar cell carcinoma [141]. This success has led to a clinical study testing CCL21 gene-modified DCs intratumorally administrated into patients with non-small cell lung carcinoma, in which systemic antitumor immunity and enhanced CD8$^+$ T cell infiltration were observed [142]. Apart from these methods, it was shown that migratory capability of DCs could be improved during radiation treatment. Combining radiation with exogenous adjuvant increased the number of migrating DCs in poorly radioimmunogenic tumors [143].

DCs can also serve as carriers to deliver the NPs that are designed for LN-targeting but turn out to be less efficient. *Ex vivo* co-incubation with NPs leads to improved cellular uptake efficiency by DCs compared with direct administration of NPs which may be internalized by local cells;

the loaded NPs can modulate the immunogenicity of DCs or provide concomitant imaging signals [144]. For example, antigen-loaded upconversion NPs were used to label and stimulate DCs, realizing DC maturation before administration and *in vivo* DC tracking after administration [145]. OVA-conjugated Bi_2S_3 NPs were used to stimulate DCs and monitor their *in vivo* spatiotemporal fate via computed tomography (CT) imaging (Fig. 5a–c) [144]. Bi_2S_3 NPs with X-ray attenuation capability served as the CT contrast agent. The NP-loading DCs rapidly and durably accumulated in draining LNs and triggered greater T-cell responses than OVA-stimulated DCs. Iron oxide NP-loaded DCs was shown useful for early prediction of antitumor responses via magnetic resonance imaging, in which the volume of draining LNs 48 h after intradermal administration was identified as a criterion of the responsiveness to the therapy (Fig. 5d) [146]. MRI signal intensity in LNs was found negatively correlated with survival time in murine tumor models.

6. Creating LN-like structures

6.1. Biomaterial-based niches

Implanting biomaterial scaffolds with chemokine gradients can attract immune cells including DCs to create artificial immune niches. A rationale for this strategy is that the autologous LNs are often bathed with tumor-associated antigens and become immunosuppressive [19,

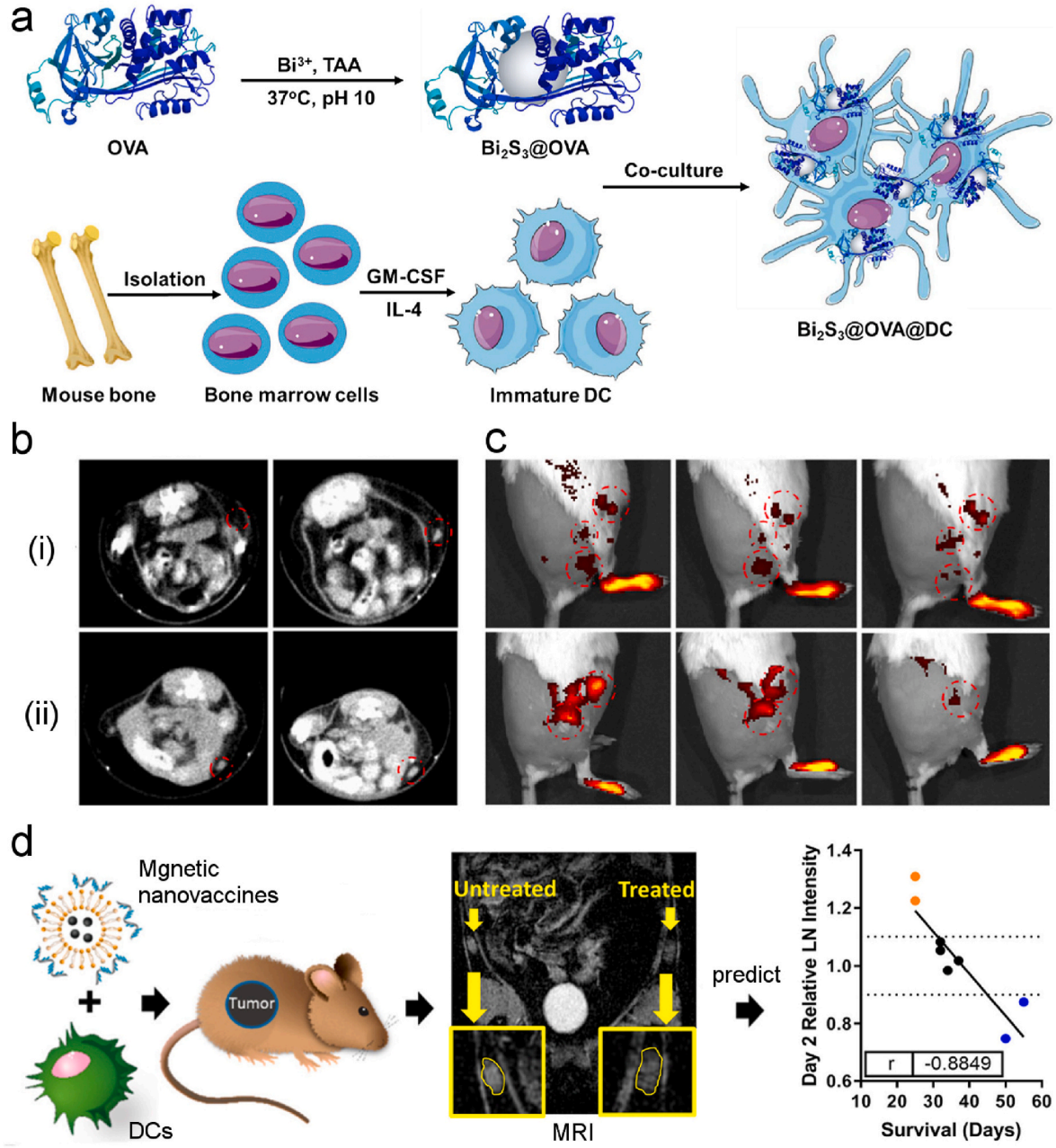


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Fig. 5. LN-targeting via co-incubation with DCs. (a) Illustration of the preparation of Bi_2S_3@OVA NP-loaded DC vaccines. Monitored accumulation of (i) Bi_2S_3@OVA NPs and DC-carried Bi_2S_3@OVA NPs in draining LNs via (b) CT and (c) fluorescence imaging after *s.c.* injection. Reproduced with permission [144]. Copyright 2022, Wiley. (d) Illustration of the preparation of magnetic nanovaccines for predicting treatment responses based on the MRI signal intensity in LNs. Reproduced with permission [146]. Copyright 2019, American Chemical Society.

147], and during cancer treatment the LNs may be resected to ensure a good prognosis [148]. Creating artificial niches with a desired combination of antigens, cytokines, chemokines and other immunostimulators can guide and enhance the immune activation. Meanwhile, the viability and proliferation of administrated cells can be improved under the protection of scaffolds [149]. Choosing suitable cytokines has been demonstrated highly important. For example, it was shown that GM-CSF loaded in macroporous poly(lactide-*co*-glycolide) matrix was more chemotactic compared with the Fms-related tyrosine kinase 3 ligand (Flt3L) and C–C motif ligand 20 (CCL20) in the same matrix [150]. GM-CSF significantly improved the expression of MHC(II) and CD86 on conventional DCs, while Flt3L led to greater number of plasmacytoid DCs, suggesting the possibility to modulate specific DC subsets using the chosen cytokine.

From the perspective of material science, implantable or injectable biomaterials with high biocompatibility are desired, and considerable porosity is needed for the recruited cells to reside and expand [151]. Uploading with cytokines and chemokines facilitates the recruitment of DCs, and the presence of antigens and adjuvants stimulates the DCs to mature, expand and migrate (Fig. 6a). Surface chemistry of the scaffold has a great impact on tissue compatibility, as modification with PEG helped to reduce inflammation [152], and surface coating with collagen could enhance the residence stromal cells [153]. High porosity will allow the entrance of multiple types of cells to facilitate their communication and growth, as demonstrated in the porous hydrogel scaffolds where the 'new tissue' was even vascularized [153]. A sponge-like collagenous scaffold embedding stromal cells was shown to grow into a functional organoid after transplanted into the renal subcapsular space in mice [154]. The use of collagenous scaffold rather than the segmented polyurethane was essential in attracting B cells. Similar to real LNs, the formed organoid contained compartmentalized B-cell and T-cell clusters, HEV-like vessels, germinal centers and follicular DC networks [154]. Strong secondary immune responses (*i.e.*, antibody production) were induced and maintained for weeks after immunization (*i.e.*, antigen challenge), in both normal and lymphocyte-deficient SCID mice [155]. Scaffolds with different textures are currently available, including porous polymeric sponges [156,157], nitinol porous films which were employed for delivering living cells (Fig. 6a) [149], *etc*. Biodegradable matrices are commonly preferred since surgical removal will be avoided.

Injectable scaffolds are increasingly explored to avoid the invasive surgical implantation. For the pre-formed scaffolds, polymeric sponges are usually used due to the high deformability. An example was the injectable cryogel, made from alginate that were conjugated with RGD peptides, as carriers of whole-cell cancer vaccines (Fig. 6b) [159]. The large, continuously interconnected macropores significantly lowered the overall rigidity and also facilitated the residence of cells. Modification with RGD peptides enhanced the adhesion of tumor cells through binding with integrin, and uploading irradiated tumor cells led to significant recruitment, infiltration, maturation and trafficking of DCs [159]. Another category is the *in situ* formed scaffolds, which usually involves the occurrence of chemical reaction or phase transition from liquid precursors to indiffusible scaffolds in the site of administration [160]. Particularly, subcutaneously injecting the suspension of mesoporous silica rods with high aspect ratio led to the spontaneously assembly of the rods during the outward diffusion of solvent, finally generating a 3D porous depot upon the interlaced stacking of rods (Fig. 6b) [158]. Immunostimulators including GM-CSF and CpG-ODN were loaded into the pores of silica rods and released *in vivo* in a sustained manner, resulting in the recruitment of substantial number of DCs to the scaffolds and subsequent homing to LNs. Potent adaptive immune responses were induced when antigens were co-loaded [158]. Generally, *in situ* formed scaffolds hold the merit of noninvasiveness during administration, but the exact structure formed *in vivo* may be variable and dependent on the injection site and rate, thereby raising concerns about the repeatability of treatment efficacy.

Notably, a unique strategy of constructing LN-like organoids is using decellularized LNs (dLNs) as scaffolds (Fig. 7). In a pioneering study

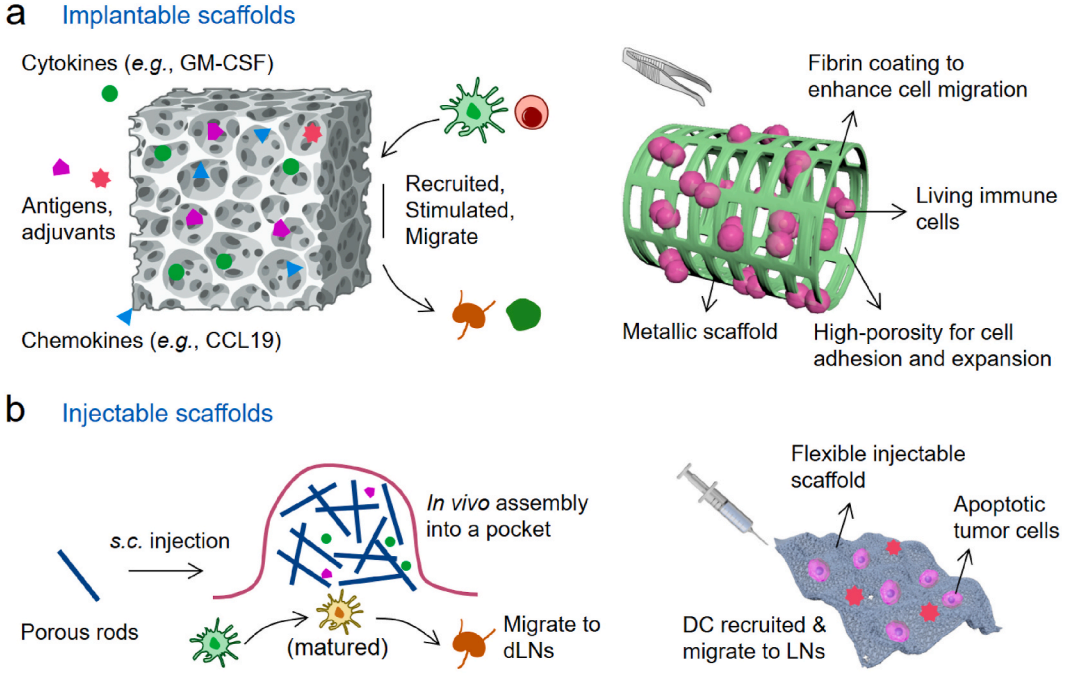

Fig. 6. Biomaterial scaffold-based immune niches. (a) Representative implantable scaffolds. Porous biomaterial scaffolds with the capability to sustainedly release cytokines, antigens and adjuvants, and to provide space for cells to reside, are widely developed for implantation [153,156,157]. Metallic scaffolds can also be implanted to deliver living immune cells after suitable surface modification [149]. (b) Representative injectable scaffolds. Microscale rod particles can self-assemble into a porous pocket, realizing DC recruitment, maturation, and migration when antigens and suitable cytokines are loaded [158]. Flexible sheeted scaffold absorbing apoptotic tumor cells can be injected to provide a full-spectrum tumor-associated antigens for vaccination [159]. The illustration of porous scaffold in left panel of (a) was acquired from BioRender.com with a publication license.

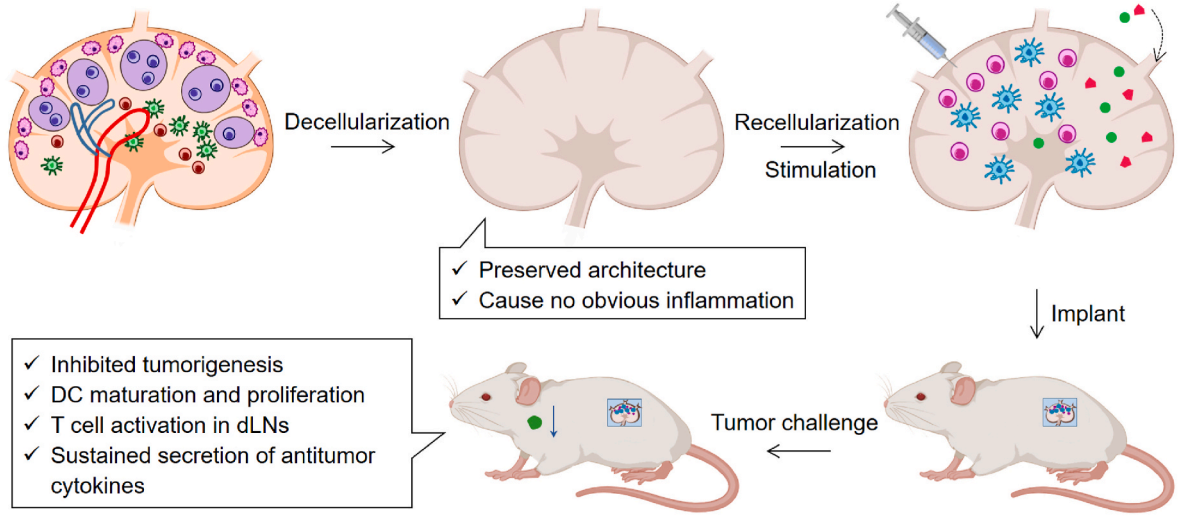

Fig. 7. Decellularized LNs as scaffolds. Illustration of the employment of decellularized LNs as scaffolds for delivery of immune cells after recellularization and stimulation, and ultimately for improved cancer vaccination.

[161], the architecture of murine LNs was maintained after decellularization by the detergent sodium dodecyl sulfate, and the scaffolds did not induce significant antigenic responses after implanted into syngeneic or allogeneic mice. When repopulated with splenocytes, the scaffolds realized *in vivo* cell delivery after submuscular implantation [161]. In a more recent study, LNs were decellularized using organic acids (*e.g.*, formic acid and citric acid) [162]. Nuclear acids and cell debris were efficiently removed but the extracellular matrix architecture was well preserved. BMDCs grew preferentially inside the dLNs, and when stimulated with OVA and CpG-ODN, significant DC maturation and secretion of cytokines were achieved. Complete rejection of E.G7-OVA tumor was realized by implanting the stimulated BMDC-dLNs into the original place of the removed inguinal LN [162]. Whether or not the dLNs still maintain some immunological functions such as supporting natural recellularization after implantation should be an interesting subject to study. It has been demonstrated that transplanted LNs could spontaneously reconnect with the host lymphatic system, restore the lymphatic flow and maintain the T- and B-cell populations [163]. Meanwhile, successful lymphangiogenesis using dLNs has been realized in lymphedema rat models, in which human adipose-derived stem cells (hADSCs) were used to recellularize the dLNs [164]. Upon transplanted into rats whose inguinal LNs were removed, the recellularized dLNs led to lymphangiogenesis with high expression of vascular endothelial growth factor A and lymphatic vessel endothelial hyaluronan receptor 1, indicating a sign of generating new lymph vessels [164]. Therefore, dLNs hold the promise to become histologically and functionally substitutes for resected or damaged LNs when suitably recellularized.

6.2. *Inducing LN-like organoids in vivo*

The presence of LN-like organoids, which are generally defined as TLSs, have been confirmed in many cancer types (Fig. 8a) [28,165]. In a "matured" TLS, there are FRCs in periphery, partitioned zones for the residence of B cells and T cells, as well as HEVs at vicinity (Fig. 8b). Evidences showing the correlation between TLS presence and treatment efficacy in cancer patients are accumulating. For example, TLS density in tumor has been observed to be positively correlated with the patient prognosis in multiple cancer types [28,166], and the induction of antitumor immune responses by immune checkpoint antibodies may require intratumoral TLSs [167]. A recent study indicated that the presence of mature TLSs in solid tumors could predict the efficacy of anti-PD-1 or anti-PD-L1 antibody independently of PD-L1 expression [168]. These results have motivated great efforts on inducing the intra- or peri-tumoral formation of TLSs, by using chemokines, cytokines or synthetic scaffolds. It was shown that a 12-chemokine gene expression signature could predict the degree of lymphoid infiltration and TLS formation [169], suggesting a need of screening suitable tumor models for this therapy. Meanwhile, whether or not upregulating the expression of the set of chemokine genes could facilitate TLS formation should be worthy to explore.

Since stromal cells in LNs play important roles in architectural support, lymphogenesis and lymphocyte recruitment [170], researchers have successfully induced TLS formation via subcutaneous injection of stromal cell lines [171]. The TLSs attracted infiltration of host immune cells, and their presence enhanced the efficacy of DCcell therapy in a MC38 tumor model. An improved cell-based strategy is using cells that overexpress lymphorganogenic chemokines. For example, the stromal TEL-2 cells, which were transfected with lymphotoxin-α and thereby secreted CCL19, CCL21 and CXCL13, were mixed with BMDCs [154]. The obtained formulation was useful in inducing the formation of lymphocyte-rich cell aggregates after incorporated into collagen sponges. To simplify the whole procedure, cell-free methods are under extensive exploration, such as replacement of stromal cells using a combination of lymphorganogenic chemokines and cytokines. A collagen matrix containing lymphotoxin-$\alpha_1\beta_2$ and additional chemokines was demonstrated useful in inducing TLSs [172]. Collagen sponge scaffolds containing slow-releasing lymphotoxin-$\alpha_1\beta_2$, CCL19, CCL21, CXCL12, CXCL13 and soluble RANK ligand were transplanted into the renal subcapsular space of mice, and 3 weeks later TLSs were produced with segregated B and T cell areas, DCs in T cell areas, follicular DCs and fibroblastic reticular cell networks. The induced TLSs were capable of initiating a strong antigen-specific secondary immune response [172].

Overall, the clinical significance of TLS-induction is still under exploration, and whether the abundance of TLS is the reason, or just an indication, of an effective antitumor immune response is still ambiguous. This is because treatment with lymphorganogenic chemokines may interfere other immunological pathways, and immunotherapy *per se* can induce TLS formation. It was recently reported that systemic delivery of immunostimulatory agonistic CD40 antibody induced the formation of TLSs in the brain in murine glioma models, and unexpectedly, this attenuated the response to immune checkpoint inhibitors [173]. Nevertheless, the accumulating evidence on the association between TLS presence and immune responsiveness is attracting continuous research efforts, especially considering that the proximity of TLSs and tumor tissue make them potential substitutes of TDLNs to overcome the possible immune suppression in TDLNs. Due to the attachment or

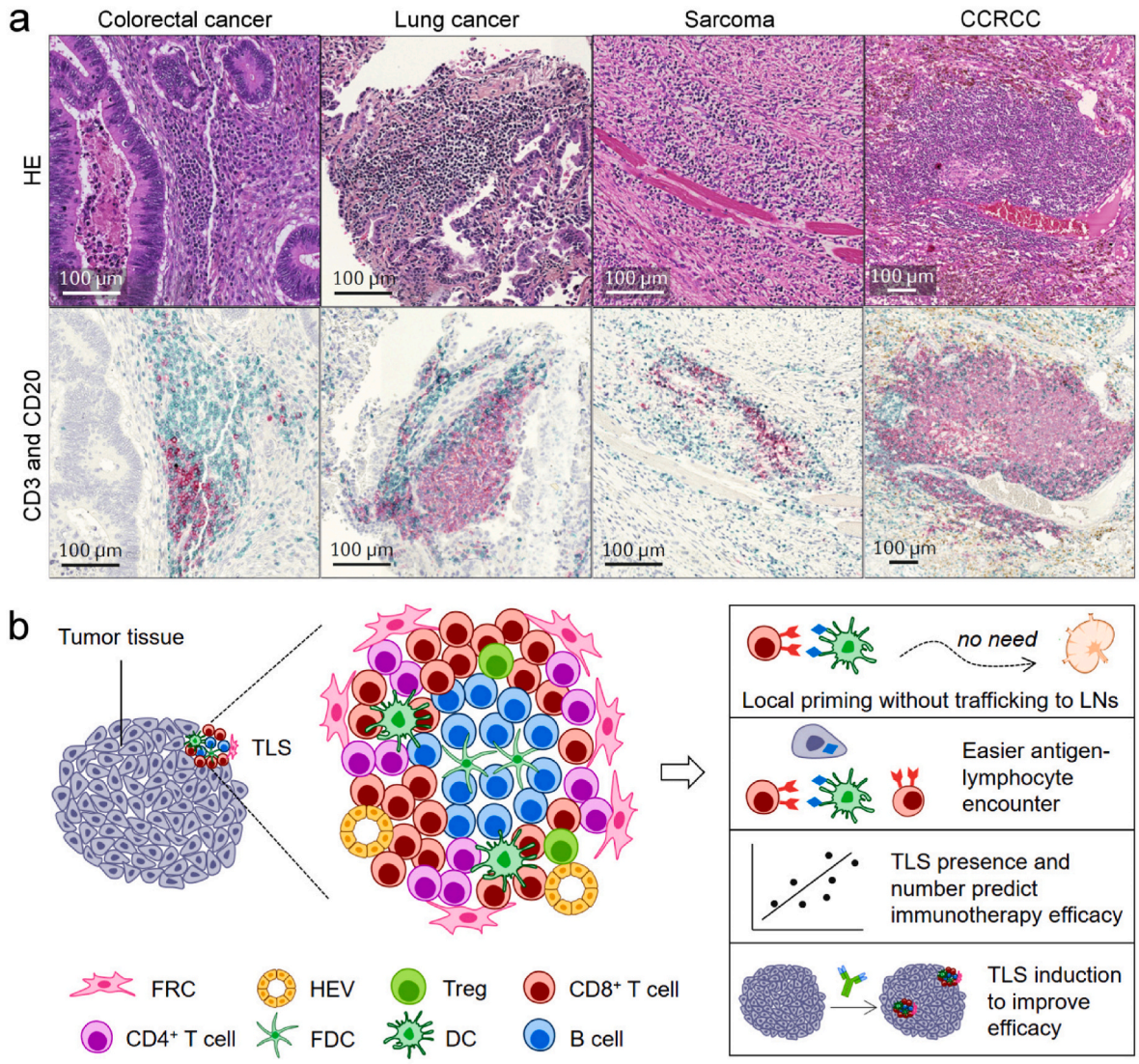

Fig. 8. TLS structure and therapeutic significance. (a) HE and $CD3^{+}CD20$ staining results of TLSs in colorectal cancer, lung cancer, sarcoma and clear-cell renal cell carcinoma (CCRCC). Reproduced with permission [28]. Copyright 2019, Springer Nature. (b) Illustration of the typical cell composition and the potential therapeutic benefits of TLSs.

proximity of TLSs to tumors, APCs will acquire abundant tumor-associated antigens and present them directly to the T cells within TLSs, thereby bypassing the need to migrate to draining LNs (Fig. 8b).

7. Future perspectives and conclusions

Most immunotherapies, especially cancer vaccination, exploit the central role of LNs in harnessing antigen-specific immune responses. As an optimization of common vaccinations which involves interdermal, subcutaneous or intramuscular injection of inactivated pathogens, the most straightforward strategy to enhance LN-targeting is to tailor the physiochemical properties of the injected matters. Particulate vaccines with suitable nanoscale diameter have been widely reported to reach LNs more easily than the sub-10-nm unmodified free vaccines. The reasons include inhibited entrance into blood due to the increased size, as well as higher possibility to be captured by migratory DCs due to longer residence time at administration site. The optimal size for DC internalization (active transport) was reported to be several times larger than that for passive intralymphatic drainage, suggesting the possibility to employ a specific transport pathway. Future studies may need to exclusively explore passive or active transport pathway to obtain a clearer view on size effect. Similarly, particle shape, surface chemistry and stiffness are all influential, but interplay between them makes it difficult to draw a generalized conclusion for all conditions. Compared with the abundance of preclinical studies, clinical success of nanovaccines is relatively limited. The reasons should include the failure to demonstrate notable clinical benefit, the lack of standardization for preparing nanovaccines (especially for those containing multiple components), the toxicity of artificial synthetic NPs, the safety concern regarding biogenic nanocarriers (*e.g.*, bacterial outer membrane vesicles and viral vectors) [174]. Representative examples of nanovaccines in clinical trials are listed in Table 3.

The concept of *ex vivo* stimulating DCs or T cells followed by infusion back into patients has achieved clinical success [175]. Antigen-pulsed mature DCs have the capability to migrate to LNs, which can be leveraged to realize LN-targeting. However, the objective response rates of DC vaccines in clinical trials are commonly below 15 % [1,176]. Low percentage of cell that can finally reach LNs, and the low cell viability after infusion, are among the main reasons. Given that CCR7 is essential for DCs to directionally migrate to LNs, cell engineering to improve the expression of CCR7 or its ligands is a useful approach to improve LN-targeting and has been explored in preclinical studies. For the low

Table 3
Representative clinical trials of carrier-based nanovaccines and scaffold-based vaccines for cancer treatment.

Carrier	Active components	Administration	Indication	Trial ID and Phase
Carrier-based nanovaccines				
Small spherical gold NPs	Spherical nucleic acid targeting Bcl2L12	*i.v.* injection	Gliosarcoma, recurrent glioblastoma	NCT03020017; Early Phase 1
Lipid NPs	mRNAs Encoding Human OX40L, IL-23, and IL-36γ	*i.t.* injection	RRSTM or lymphoma	NCT03739931; Phase 1
Lipid NPs	mRNA Encoding Human OX40L	*i.t.* injection	RRSTM or lymphoma	NCT03323398; Phase 1/2
Lipid NPs	Plasmid encoding TUSC2 tumor suppressor gene	*i.v.* infusion	Non-small cell lung cancer	NCT05062980; Phase 1/2
PLGA NPs	NY-ESO-1 antigen and iNKT cell agonist	*i.v.* infusion	Advanced solid tumor	NCT04751786; Phase 1
PEG-PEI-cholesterol lipopolymer	IL-12 DNA Plasmid Vector	*i.p.* administration	Ovarian/fallopian tube/primary peritoneal carcinoma	NCT01489371; Phase 1
Scaffold-based vaccines				
Porous PLGA scaffolds	Autologous melanoma cell lysate, GM-CSF and CpG	Implanting into an incision in skin	Melanoma	NCT01753089; Phase 1
Hydrogel made from poloxamer and satiaxane	RFA, mifamurtide and GM-CSF	Injection into the lesion of RFA	Unresectable colorectal liver metastases	NCT04062721; Phase 1
Biodiffusion chambers	Personalized whole tumor-derived cells and antisense ODN	Implantation	Glioblastoma	NCT04485949; Phase 2
Biodiffusion chambers	Insulin-like growth factor receptor-1 antisense ODN	Implantation	Malignant glioma neoplasms	NCT02507583; Phase 1
Biodiffusion chambers	Insulin-like growth factor receptor-1 antisense ODN	Implantation	Malignant glioma neoplasms	NCT01550523; Phase 1

RFA: radiofrequency ablation; RRSTM: relapsed/refractory solid tumor malignancies; *i.p.*: intraperitoneal.

cell viability, one solution may be giving constant stimulation at the injection site, which is also helpful in maintaining the mature state. On the other hand, strong stimulation may make DCs exhausted. More efforts need to be devoted to monitor not only the biodistribution, but also the viability and activation status of DC vaccines.

Biomaterial-based scaffolds are also a solution to the low cell viability. Uploading immunostimulators such as GM-CSF will enable constant stimulation of DCs, facilitating their survival and proliferation. Immunostimulators also help to recruit DCs. Therefore, uploading the antigens used for *ex vivo* DC stimulation into scaffolds will yield an *in vivo* niche for DCs to mature, expand and then migrate to LNs. Porosity of scaffolds allows the entrance and growth of stromal cells, which may lead to the formation of a vascularized "tissue" with multiple cell types. Placing the scaffolds in proximity to tumor, or instead, establishing a robust drainage between scaffolds and tumors, should be more helpful to develop a tumor-specific immunity; the latter has yet been realized. A number of scaffold-based cancer vaccinations have reached clinical trials (Table 3). Compared with biomaterial scaffolds, TLSs bearing compartmentalized B-cell and T-cell clusters are more like LNs in structure and function. They are emerging as a potential indicator for predicting the potency of immunotherapies such as PD-1/PD-L1 blockade. While the therapeutic benefit of inducing TLS formation has been preliminarily established, whether or not that was the result of the interference of other immune pathways needs more attention, and the robustness of this benefit should be confirmed in more cancer types. Meanwhile, the development process of TLSs in number and structure during tumor progression, as well as the underlying reason why TLSs emerge more frequently in some cancer types than others, are interesting to explore.

Generally, LNs have intrinsic capability to drain substances in the draining area, while modern immunotherapy pursues more targeted and specific immunostimulation. Advances in bioengineering and material science have provided many tools to achieve more effective vaccination than early immunologists do, and future efforts on looking into the molecule-level dynamics of the immune system, manipulating material-bio interactions, and creating advanced organoids will provide more.

CRediT authorship contribution statement

Jie Wang: Writing – original draft, Funding acquisition, Conceptualization. **Zongying Zhang:** Writing – original draft. **Rongxiang Liang:** Writing – original draft. **Wujun Chen:** Writing – original draft. **Qian Li:** Writing – original draft. **Jiazhen Xu:** Writing – review & editing. **Hongmei Zhao:** Writing – review & editing. **Dongming Xing:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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