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In vivo fate and intracellular trafficking of vaccine delivery systems ☆

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ABSTRACT

With the pandemic of severe acute respiratory syndrome coronavirus 2, vaccine delivery systems emerged as a core technology for global public health. Given that antigen processing takes place inside the cell, the intracellular delivery and trafficking of a vaccine antigen will contribute to vaccine efficiency. Investigations focusing on the in vivo behavior and intracellular transport of vaccines have improved our understanding of the mechanisms relevant to vaccine delivery systems and facilitated the design of novel potent vaccine platforms. In this review, we cover the intracellular trafficking and in vivo fate of vaccines administered via various routes and delivery systems. To improve immune responses, researchers have used various strategies to modulate vaccine platforms and intracellular trafficking. In addition to progress in vaccine trafficking studies, the challenges and future perspectives for designing next-generation vaccines are discussed.

Contents

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1. Introduction

The unprecedented outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-mediated coronavirus diseases 2019 (COVID-19) has brought greater interest and reliance on vaccines than ever before. Unlike therapeutic agents, vaccines have the advantage in being able to prevent the rapid spread of epidemics and contribute to social net benefits by reducing the social burden on health services (e.g., hospitalization and ambulatory care) [1]. Given the effectiveness of vaccines from the social point of view, the World Health Organization organized the COVID-19 Vaccines Global Access to promote global collaboration for COVID-19 vaccine development. Moreover, various social vaccine programs to prevent the spread of COVID-19 are being implemented in countries around the world, including the COVID-19 Vaccination Eligibility Guidance issued by the United States Centers for Disease Control and Prevention [2].

A vaccine effectively suppresses viral infection via its ability to train immunological memory [3]. When an administered vaccine comprises part of the infection-causing virus, the body's immune system establishes a defense system that includes humoral and cellular immunity [4]. Once trained, the immune system will work to effectively and vigorously neutralize an invasion of the actual virus. For this purpose, various types of vaccines, such as those based on live attenuated virus, recombinant protein subunits, DNA, and RNA, have been operationalized against numerous infectious diseases, including COVID-19 [5].

To properly prepare the immune systemdeal with a pathogen invasion, a vaccine must be distributed to the right place(s) in the right form. Most vaccine molecules are susceptible to degradation and are not themselves efficiently recognized by the immune system due to limited accessibility and/or inefficient cell permeation [6]. Although our immune system is widespread throughout the body, it is particularly important to move the vaccine molecules to immune-specialized organs in which immune cells are centralized, such as lymph nodes and the spleen, [7,8]. In addition, although a network of various cells contributes to the immune response, certain cells, such as antigen-presenting cell (APC), play central roles [9]. Therefore, when seeking to develop a vaccine that can respond quickly and efficiently to a rapidly spreading pandemic, we must understand the in vivo behavior and intracellular trafficking of various vaccines.

One key technology is the vaccine delivery system, which can directly change the fate of a vaccine in the body to give it increased efficacy. A tailored vaccine nano-formulation may enable the vaccine to reach its target with high accuracy and stability. Representative examples of how vaccine delivery systems contribute to determining the in vivo behavior of a vaccine [10] include the ability to protect the vaccine from enzymatic degradation [11], the use of surface engineering (e.g., PEGylation) to improve pharmacokinetic properties [12], the use of active targeting to transport the vaccine to a specific organ or cell type [13], and the engineering of sophisticated vaccine release in a sustained or controlled manner. For example, researchers incorporated ligands or membrane fusogenic agents to enable the strategic targeting of an appropriate intracellular transport pathway and facilitate endosomal escape, enabling the vaccine to be distributed to the cytosol [14]. By choosing among the various delivery systems, researchers can hope to develop an optimal vaccine formulation that is suitable for the characteristics and purpose of a given vaccine.

This review will focus on the in vivo behavior of vaccine delivery systems and the subsequent intracellular transport of the vaccine. Current vaccine delivery systems and the formulation strategies used to optimize them for different vaccine types and administration routes will be introduced. Finally, challenges and opportunities for the design and development of future vaccines will be discussed. In this review, we focused on the non-viral delivery systems.

2. Vaccine delivery systems

2.1. Nanomaterials for vaccine delivery systems

Although lipid nanoparticles represent the first nano-scale delivery system approved for delivering COVID-19 mRNA, various types of delivery systems are currently under clinical and preclinical development for this purpose and broader clinical use. Vaccine delivery systems resemble conventional drug delivery systems in that they aim to transport their cargo to the target in a safe and efficient manner. As with conventional drug delivery systems, the nanomaterials used for vaccine delivery are mainly categorized as lipid, polymer, inorganic, peptide/protein, and cell membrane-based systems. The characteristics of each material and their potential as a vaccine carrier are briefly described in this section (Table 1).

2.1.1. Lipid-based vaccine delivery systems

Lipid nanoparticles, which are the most representative lipid-based vaccine delivery system, are already widely used in the clinic [15]. A key component of recent lipid nanoparticles developed for mRNA vaccine delivery is an ionizable lipid that can undergo a pH-dependent charge conversion [16]. An ionizable lipid that has a neutral charge under physiological conditions can take on a positive charge under an acidic condition, such as that found in endosomes. A positively charged ionizable lipid in an endolysosome will tend to interact with the negatively charged endosomal membrane, thereby facilitating the endosomal escape of a loaded vaccine. Since the addition of a permanent cationic charge can induce cytotoxicity, the temporary charge conversion of a lipid nanoparticle offers advantages in terms of safety [17]. Among the various ionizable lipids, ALC-0315 was clinically used for the Pfizer COVID-19 mRNA vaccines, while SM102 lipid was used for the Moderna COVID-19 mRNA vaccine [16].

A typical lipid nanoparticle includes other constituent lipids [15]. Structural helper lipids, such as 1,2-distearoyl-sn-glycero-3-phosphocholine or 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, may form the lipid layer and support the overall structure of the lipid nanoparticle. PEGylated (i.e., polyethylene glycol (PEG)-conjugated lipids) can confer hydrophilic properties on the nanoparticle surface and improve stability by steric hindrance. Cholesterols may be included in the lipid membrane to directly affect its permeability and fluidity. Dioleoyl phosphatidylethanolamine lipid may also be included as a helper lipid to enhance endosomal escape.

Table 1
Nanomaterials for vaccine delivery systems.

Material	Delivery system	Vaccine type	Disease / Antigen	Injection route	Ref
Lipid	Lipid Nanoparticle	mRNA	COVID-19	Intramuscular	[16,18,114]
	Lipid Nanoparticle	Protein	Tuberculosis	Intranasal	[62]
	Dry powder	Protein	Human papillomavirus	Intrapulmonary	[101]
	Lipid Nanoparticle	Protein	Cancer	Subcutaneous	[64]
	Lipid Nanoparticle	mRNA	Human papillomavirus	Subcutaneous	[106]
Polymer	Chitosan silica nanoparticle	Protein	Bovine serum albumin	Oral	[20]
	Chitosan alginate gel	mRNA	Ovalbumin	Subcutaneous	[23]
	PEG alginate gel	Peptide	Acute myeloid leukaemia	Subcutaneous	[24]
	PLGA lipid nanoparticle	Protein	Ovalbumin	Oral	[26]
	PEI-PLGA nanoparticle	Protein	Ovalbumin	Subcutaneous	[28]
	PEG-PEI nanoparticle	Peptide	Cancer	Intratumoral	[30]
	PEI nanoparticle	pDNA	Cancer	Intratumoral	[30,31]
Inorganic material	Gold nanoparticle	Protein	Cancer	Subcutaneous	[184]
	Gold nanoparticle	Protein	Influenza	Intranasal	[36,185]
	Gold nanoparticle	Protein	Influenza	Intraperitoneal	[37]
	Iron oxide nanoparticle	Protein	Cancer	Subcutaneous	[38]
	Iron oxide nanoparticle	Protein	Malaria	Intraperitoneal, Subcutaneous, Intramuscular	[39]
Self-assemble protein	Dry powder	Peptide	Tuberculosis	Subcutaneous	[43]
	Peptide fibril	Peptide	Ovalbumin	Subcutaneous	[44]
	Nanosphere	Peptide	Influenza	Intramuscular	[45]
	Nanobarrel	Protein	Influenza	Subcutaneous	[46]
	Nanosphere	Protein	COVID-19	Intraperitoneal	[47]
Cellular subunit	Exosome	mRNA	COVID-19	Intramuscular	[51]
	Cell membrane coated nanoparticle	Protein	Cancer	Subcutaneous	[66]
	Cell membrane coated nanoparticle	Protein	Cancer	Subcutaneous	[186]
	Virus-like particle	mRNA	Human papillomavirus	–	[54]

Various ingredients can be combined to obtain optimal delivery efficacy, and the diversity and flexibility of the formulation procedure is a key feature of lipid-based delivery systems. These systems are generally considered to be biocompatible and biodegradable, and exhibit low toxicity [18]. The facile production and potential for mass production of lipid nanoparticles are other key advantages that have contributed greatly to their rapid commercialization [18,19]. However, lipid-based delivery systems remain challenged by their low stability and the need for strict cold-chain management during vaccine distribution.

2.1.2. Polymer-based vaccine delivery systems

Based on the source of their component material, polymers can be classified as natural or synthetic polymers. Typical natural polymers have been intensely investigated as vaccine carriers due to their distinctively high biocompatibility, biodegradability, and mucoadhesiveness. For instance, chitosan, which is derived from a natural polysaccharide, can be formulated as nanoparticles that were found to enhance the bioavailability of an oral vaccine by improving mucoadhesive properties [20]. The positive charge of chitosan also offers an advantage for nucleic acid-based vaccine delivery [21]. Chitosan can interact with nucleic acid antigens through the protonation of its primary amines. Transfection efficiency of chitosan can be optimized by fine tuning of degree of acetylation and degree of polymerization [21]. A recent study exploited the cationic feature of chitosan for nucleic acid antigen delivery. Intranasally administered chitosan-based SARS-CoV-2 DNA vaccine was found to induce humoral and cellular immunity in mouse model [22].

Alginate, which is another natural polymer often used for vaccine delivery, has a hydrogel-forming ability that can be exploited to enable a sustained vaccine release and thereby induce a durable immune response [23,24].

Synthetic polymers, mainly versions of poly(lactic-co-glycolic acid) (PLGA), have also been widely studied for vaccine delivery. PLGA is a copolymer composed of lactide and glycolide connected by ester bonds; its degradation rate can be controlled by adjusting the molecular weight and the ratio of the two components [25]. PLGA-based biodegradable polymers were approved as a biomaterial by the Food and Drug Administration (USA) in 1989 and have been investigated for use in classical vaccine delivery systems ever since [26,27]. Vaccine molecules can be encapsulated in a nano- or micron-sized particle during PLGA synthesis, or PLGA can serve as a core scaffold for surface engineering and vaccine adsorption [28]. Proven safety from long-term use, well-controlled nanoparticle synthesis, and the availability of various surface modification strategies makes PLGA a popular candidate for a vaccine carrier.

Synthetic polymers, particularly polyethyleneimine, can also be used for nucleic acid-based vaccine delivery systems. Polyethyleneimine takes on various structures, including linear and branched; it can have secondary or tertiary amine groups, and has a positive charge that enables it to efficiently bind negatively charged nucleic acids. Polyethyleneimine nanocomplexes show excellent gene transfection efficiency and have exhibited great promise as versatile gene delivery vehicles for delivering nucleic acids, including plasmids, siRNA, and mRNA [29,30]. However, their clinical application has been somewhat challenged by cytotoxicity and low function. Recently, various attempts have been made to overcome these issues. For example, chemical modification with PEG [29] or farnesylthiosalicylic acid [31] to form self-assembled nanostructures and functionalization with a lipid or sugar coating [32,33] have been used to produce various polyethyleneimine derivatives for use in delivering advanced cancer vaccines.

2.1.3. Inorganic vaccine delivery systems

Metallic nanoparticles have unique properties that are not found in other materials, such as a finely tunable particle size and shape, high surface area, facile surface modification, and characteristic optical properties; thus, they are of particular interest in drug delivery [34]. These properties have also been applied to the delivery of vaccines. As a representative example, gold nanoparticles, which are characterized by high biocompatibility and low immunogenicity, have been widely used for vaccine delivery. Gold nanoparticles can be loaded with antigens using chemical bonds

such as disulfide bonds or multi-layer on the surface [35]. They can be engineered to have various particle sizes, ranging from precisely 5 nm to around 1000 nm, and can exhibit various shapes, including nanospheres and nanorods. The size and shape of a particle are critical factors that greatly affect the distribution, lymph node drainage, and cellular uptake pattern of a vaccine carrier, thereby significantly affecting vaccine efficacy [36,37].

Iron oxide nanoparticles are also widely used for vaccine delivery. The superparamagnetic properties of iron oxide can be exploited for targeted guidance to and retention of a vaccine in a lymph node under a magnetic field [38]. In addition, several studies have reported that the iron oxide itself has adjuvant properties that can activate immune cells [39]. Given these unique features, metallic nanoparticles are valuable for advanced vaccine development.

2.1.4. Self-assembled protein-based vaccine delivery systems

Since peptides or proteins can be self-assembled into supramolecular nanostructures, attempts have been made to deliver vaccines via protein nanoparticles [40,41]. Complex molecular interactions based on electrostatic interaction, hydrophobic interaction, aromatic interaction, or hydrogen bonding can induce the self-assembly of peptides or proteins into a thermodynamically more stable structure [41]. Whereas a single soluble protein antigen has limited immunogenicity, a highly organized supramolecular particle can yield an improved immune response by regularly and repeatedly exposing an antigen in a form that better mimics the original pathogen [42]. In addition, the self-assembled nanostructure contributes to enhancing the physical and chemical stability of a protein-based vaccine to improve its utility [43]. For instance, nanofibers were capable of inducing strong immune response based on the multivalency. In the study, nanofibers were composed of repeats of Q11 peptide conjugated with short epitope of Mycobacterium tuberculosis antigen. The nanofiber was found to maintain immunogenicity until six months when stored at 45 °C due to the thermal stability of short synthetic peptides [43].

Various types of supramolecular structures have been proposed. The reported structures of self-assembled protein nanoparticles for vaccine delivery include nanofibers composed of Q11 peptide [44], nanospheres based on ferritin [45], and nanobarrels based on BP26 from Brucella outer membrane proteins [46]. These protein vaccine delivery systems demonstrated vaccine efficacy against influenza virus. Recently, a ferritin-based self-assembled nanosphere was investigated as a COVID-19 vaccine and showed promise by eliciting robust humoral and cellular immune responses in an animal model [47].

2.1.5. Cellular membrane-based vaccine delivery systems

Biological delivery systems derived from cellular membranes hold potential for efficient vaccine delivery, as they reflect the original function of the source membrane. Cellular membranes have natural surface proteins and carbohydrates that can be recognized as antigens [48]. We do not yet know the exact molecular mechanism through which cell-membrane-derived particles deliver intrinsic biomolecules to their target(s), but such particles exhibit a distinct distribution and high delivery efficiency compared to synthetic nanomaterials [49]. Based on this natural phenomenon, researchers have made various attempts to modify the original membrane-producing cells to produce multifunctional membrane-based particles.

Exosomes, which are a type of released extracellular nanovesicle, have recently attracted attention for their ability to deliver endogenous functional proteins and RNA to induce meaningful effects in recipient cells [49]. For vaccine delivery, exosome-producing cells can be genetically engineered to increase their ability to load therapeutic cargo. Host cells can be transfected with a vaccine-expressing plasmid or transduced by viruses for stable expression of an mRNA vaccine [50]. Physical entrapment via electroporation or fusion with an mRNA-loaded liposome can be utilized to increase the loading of a vaccine into an exosome [50]. A recent study demonstrated that an exosome-based mRNA vaccine induced long term humoral and cellular immune responses against COVID-19 [51].

Virus-like particles are engineered membrane particles generated by the virus-assembly mechanism [52]. Structural viral proteins are self-assembled to form virus-like particles that bud out of the host cell within an external cellular membrane coating. The virus-like particle mimics the structure of the original pathogenic virus but lacks an infectious genome, and thus can safely induce potent immune responses [53]. Virus-like particles have long been developed as vaccine delivery platforms, such as clinically used human papillomavirus vaccine, Gardasil. Recently, a novel retrovirus-based virus-like particle was developed for mRNA delivery [54]. The utilized method of selective endogenous encapsidation for cellular delivery can selectively load target mRNA in a tailored manner with the help of the retroviral-like protein, PEG. It demonstrated efficient mRNA delivery, and thus may have potential for delivering an mRNA vaccine.

The basic principle of vaccine delivery is no different from that of conventional drug delivery. For instance, the recently approved COVID-19 mRNA vaccines from Moderna and Pfizer were conceptually drawn from ionizable lipid-based nanoparticles and microfluidic technologies that were originally developed for delivering siRNA to the liver [55,56]. Similarly, the adenoviral vectors that have been approved for COVID-19 vaccines have long histories in gene therapy [57]. The characteristics of the vaccine delivery systems therefore reflect those of conventional drug delivery systems, and understanding basic features of the materials and gaining new insights into their delivery may facilitate the development of future innovative vaccines. Specific vaccine nanoformulations that are currently being studied will be introduced in more detail in a later chapter.

2.2. Vaccine delivery systems in clinical trial

Currently, nanovaccines are clinically approved or under clinical investigations (Table 2). Lipid nanoparticle formulations of COVID-19 mRNA vaccine were authorized by U.S. Food and Drug Administration, and clinically used worldwide. Protein antigen-based nanoparticle of Novavax was also approved for COVID-19 prevention. Clinical trials are in the way for various antigen modalities such as mRNA, peptide or protein-based antigens. Delivery systems include lipid nanoparticle, virus like particle, or gold nanoparticle. Target diseases are mainly viral infectious diseases such as influenza, ebola, rabies, zika, respiratory syncytial virus (RSV) infections. With recent progresses in tumor vaccines, nanovaccines for immunotherapy of specific tumors may be in clinical trials, in the near future.

2.3. Factors that influence in vivo fate of vaccine delivery system

For a vaccine to efficiently induce immunogenicity, its behavior in the body must be properly controlled and it must be efficiently recognized by the immune system. Certain immune-oriented organs and tissues can be targeted by vaccines to induce desirable effects. As each target has its own characteristic physiology, the first consideration in vaccine design should be the selection of an appropriate administration route.

Until now, various routes have been studied for administration of vaccine delivery systems. Oral routes would be beneficial to prevent infection in the gastrointestinal tract from enteric virus and bacteria such as rotavirus and Escherichi coli [58]. Nasal and

Table 2
Clinical applications of vaccine delivery systems.

Disease	Vaccine type	Phase	Name	Delivery system	Organization	NCT number
COVID-19 (SARS CoV-2)	mRNA	Approved	mRNA-1273	Lipid nanoparticle	Moderna	NCT 04470427
	mRNA	Approved	BNT162b2	Lipid nanoparticle	BioNTech Pfizer	NCT 04368728
	Protein	Approved	NVX-CoV2373	Self-assmebled protein nanoparticle	Novavax	NCT 05249816
	mRNA	III	CVnCoV	Lipid nanoparticle	CureVac	NCT 04860258
	Self-amplifying mRNA	I/II	ARCT-021	Lipid nanoparticle	Arcturus Therapeutics	NCT 04480957
	Virus like particle	I	Coronavirus-Like Particle	Virus like particle	Medicago	NCT 04450004
	Peptide	I	PepGNP-SARSCoV2	Gold nanoparticle	Emergex Vaccines Holding	NCT 05113862
Influenza	Protein	III	NanoFlu	Self-assmebled protein nanoparticle	Novavax	NCT 04120194
	mRNA	I	mRNA-1851	Lipid nanoparticle	Moderna	NCT 03345043
	mRNA	I	mRNA-1440	Lipid nanoparticle	Moderna	NCT 03076385
Ebola	Protein	I	EBOV GP	Self-assmebled protein nanoparticle	Novavax	NCT 02370589
	Protein	I	EBV gp350-Ferritin	Ferritin nanoparticle	National Institute of Allergy and Infectious Diseases	NCT 04645147
Rabies	mRNA	I	CV7202	Lipid nanoparticle	CureVac	NCT 03713086
	Self-amplifying mRNA	I	GSK3903133A	Cationic nanoemulsion	GSK	NCT 04062669
Melanoma	mRNA	I	RNA-NP	Liposome	University of Florida	NCT 05264974
Ovarian cancer	mRNA	I	W_ova1 Vaccine	Liposome	BioNTech	NCT 04163094
Zika	mRNA	I	RNA-1325	Lipid nanoparticle	Moderna	NCT 03014089
RSV	mRNA	I	mRNA-1345	Lipid nanoparticle	Moderna	NCT 04528719
Chikungunya	mRNA	I	mRNA-1944	Lipid nanoparticle	Moderna	NCT 03829384
Cytomegalovirus	mRNA	I	mRNA-1647	Lipid nanoparticle	Moderna	NCT 03382405
Human Metapneumo-virus + Parainfluenza virus 3	mRNA	I	mRNA-1653	Lipid nanoparticle	Moderna	NCT03392389

pulmonary routes aim to enhance the induction of secretary IgA antibody in nasal passages and lung mucosal sites [59]. Indeed, nasal and pulmonary routes were shown to be effective to provide immune responses against influenza [60,61] and tuberculosis [62,63], respectively. Subcutaneous route can allow the migration of vaccine antigens to lymph nodes, the primary organs of adaptive immune response [64]. Subcutaneous route has been actively studied for therapeutic cancer vaccine for promoting adaptive immune responses against tumor antigens [65,66].

The administration route (e.g., intradermal, subcutaneous, and intramuscular) is the first gate in determining the in vivo fate of a vaccine. Depending on the administration route, vaccines will face various barriers to accomplishing their goal in each characteristic physiological environment. Overcoming these barriers are key to the success of a vaccine. For this purpose, various pharmaceutical strategies are being utilized to change the fate of vaccines for each route of administration (Fig. 1, Table 3).

In vivo fates of vaccine delivery systems can be affected by several factors such as size, shape, charge, and surface moieties. The surface charge of a nanoparticle is an important factor in the design of a lymph-node-targeting vaccine delivery system. Collagen and glycosaminoglycan, which constitute the interstitium, are mostly negatively charged; thus, positively charged nanoparticles are likely to bind and become trapped in the extracellular matrix and fail to migrate to lymph nodes. Compared to a positively charged nanoparticle, neutral or negatively charged nanoparticles are typically more efficient in terms of lymphatic delivery [67].

The size of the vaccine delivery system can modulate the in vivo fate. A size range of 10 to 100 nm has been reported to be the most suitable for the lymph node delivery of a vaccine delivery system. Particles with sizes less than 10 nm was reported to escape from the loose lymphatic vessels and transit the bloodstream to accumulate in the spleen [67]. In contrast, particles larger than 100 nm may not efficiently diffuse to the lymphatic vessels. The size is critical especially in pulmonary vaccine delivery systems due to size-dependent deposition in the respiratory tract.

The geometrical feature of vaccine delivery systems is also known to play a role in cellular internalization [68]. Spherical shape of nanoparticles has been known to undergo phagocytosis faster than the nanoparticle that has higher curvature. Moreover, filamentous shape nanoparticles showed longer retention in the blood compared to spherical nanoparticles [68].

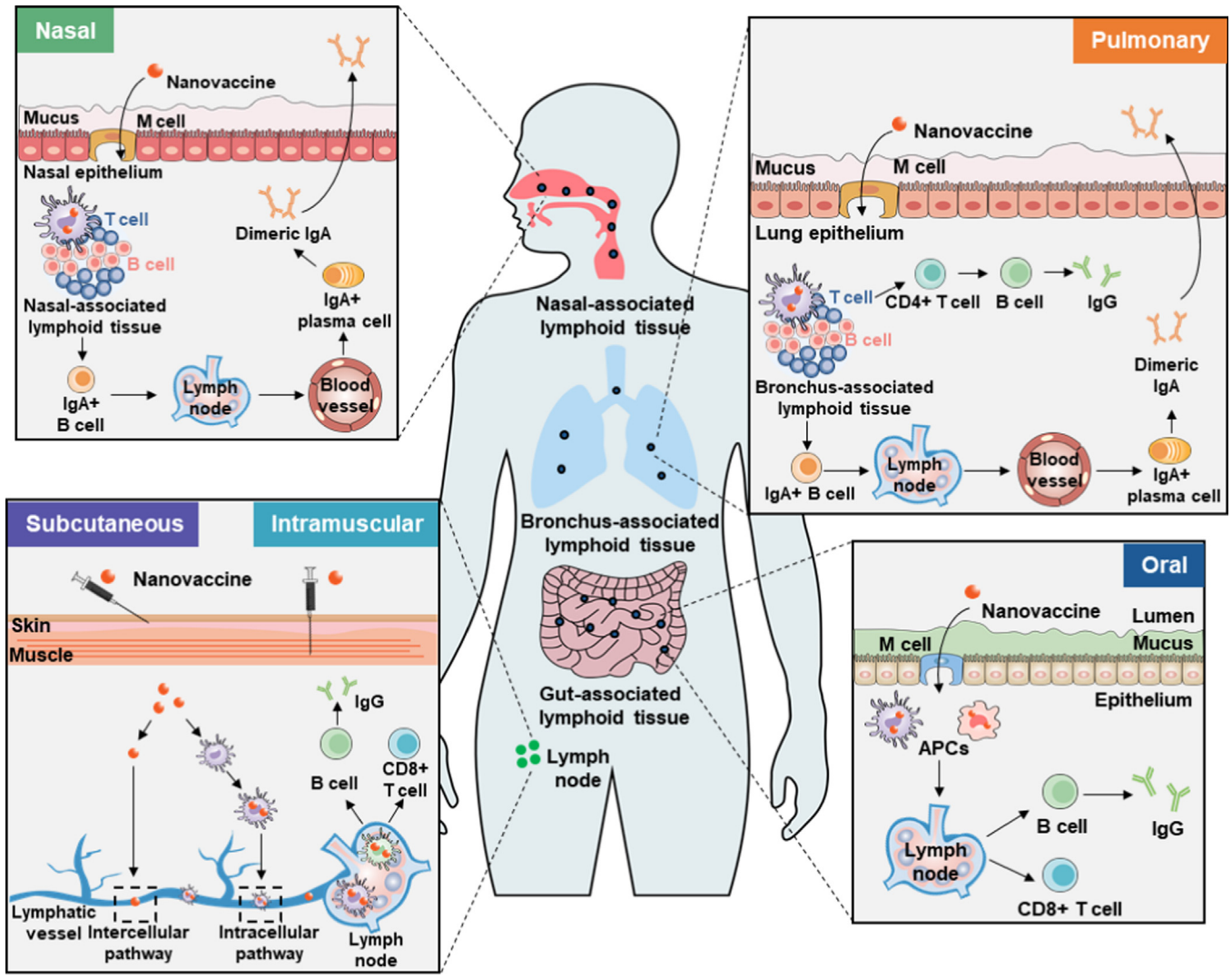

Fig. 1. In vivo fates of nanovaccines administered by various routes. Nanovaccines have been administered by various routes including nasal, pulmonary, subcutaneous, intramuscular, and oral routes. Depending on the routes of administrations, in vivo fates of nanovaccines to reach lymph nodes can vary.

Surface modification can modulate the stability and biodistribution of vaccine delivery systems. Hydrophilic polyethylene glycol coating can confer stability during storage by preventing aggregation of hydrophobic nanovaccines. In addition, pegylation can prolong the blood circulation time of nanoparticles [69]. In another approach, the surfaces have been modified to increase distribution of nanovaccines to target organs. Conjugation of antibody or ligand specific to target receptor can direct the vaccine delivery system to suitable target cells [70]. Further studies on lymph node targeting ligands or antibodies would be useful for designing nanovaccines for enhanced lymphatic delivery.

The distribution of vaccine delivery systems can be modulated by making use of endogenous lymph trafficking mechanisms. The concept of "albumin hitchhiking" may be a useful strategy for targeting lymph nodes [71]. Albumin is a macromolecule that is present in subcutaneous tissue and is specifically transported to lymph nodes rather than the bloodstream. As albumin can also serve as a fatty acid transporter, attempts have been made to conjugate albumin-binding lipids to vaccines. Indeed, this natural lymph node transporter can successfully guide a lipid-conjugated cargo to lymph nodes, and recent studies utilized albumin hitchhiking for COVID-19 vaccine development [72,73].

The spleen is another immune-oriented organ that holds potential for inducing an efficient systemic immune response. Spleen distribution of nanovaccines is attributed to the large blood flow and distinct anatomical vasculature structure of spleen [74]. The size of slit between endothelial cells in the spleen is known to be around 200 nm. Such physiological feature supports that nanoparticle size can play a crucial role in spleen accumulation [75]. In spleen, nanovaccines in systemic circulation can leak out of the blood vessel and be captured by phagocytic cells. The processing of antigens carried by the delivery systems can boost immune responses. Therefore, several studies sought to modulate the distribution of a vaccine to the spleen based on these features.

For example, layered double hydroxide nanoparticles of around 200 nm size were specifically distributed to the spleen when administered by intravenous injection, and were found to successfully induce an immune response as an anticancer vaccine [76]. This study showed the highest spleen distribution of nanoparticles with an average size of 215 nm. Nanoparticles with sizes greater than 400 nm could not pass through the red pulp fenestration in spleen, resulting relatively lower spleen distribution.

As another example, lipid nanoparticles composed of cell-penetrating peptides and ionizable lipids were found to specifically distribute to the spleen and act as an effective anticancer vaccine

Table 3
The routes and target organs of vaccine delivery systems.

Injection route	Delivery system	Vaccine type	Disease / Antigen	Target organ	Ref
Oral	Inorganic Nanoparticle	Protein	Ovalbumin	Intestine	[82]
	Polymeric Nanoparticle	Protein	Helicobacter pylori	Intestine	[83]
	Polymeric Nanoparticle	Protein	Staphylococcus aureus	Intestine	[84]
Nasal	Polymeric nanogel	Protein	Streptococcus pneumoniae	Nasal mucosa	[97]
/ Pulmonary	Polymeric nanoparticle	Protein	Swine influenza virus	Lung	[61]
	Lipid nanoparticle	Protein	Ovalbumin	Nasal mucosa	[98]
	Gold nanoparticle	DNA	COVID-19	Nasal mucosa	[22]
	Lipid nanoparticle	Protein	HPV	Lung	[101]
Subcutaneous					
	Lipoplex hydrogel	mRNA	Ovalbumin	Lymph node	[23]
	PEI nanoparticle	mRNA	Cancer	Lymph node	[33]
	Lipid nanoparticle	Protein	Cancer	Lymph node	[64]
	Lipid nanoparticle	mRNA	Cancer	Spleen	[106]
	Polymeric nanoparticle	Protein	Viral infection	Lymph node	[107]
	Proteinaceous nanoparticle	Protein	Bacterial infection	Lymph node	[108]
	Polymeric nanoparticle	Protein	Cancer	Lymph node	[65]
	Cell membrane coated nanoparticle	Protein	Cancer	Lymph node	[66,186]
	Gold nanoparticle	Protein	Cancer	Lymph node	[184]
Intramuscular	Lipid-polymeric nanoparticle	Protein	Ovalbumin	Lymph node	[111]
	PEI nanoparticle	Protein	Cancer	Lymph node	[112]
	Albumin emulsion	Protein	Cancer	Lymph node	[113]
	Lipid nanoparticle	mRNA	COVID-19	Lymph node	[114]
	Lipid nanoparticle	mRNA	Chlamydia	Lymph node	[187]
	Lipid nanoparticle	mRNA	Zika	Lymph node	[188]
	Lipid nanoparticle	mRNA	Influenza	Lymph node	[189]

delivery system [77]. In this study, nanoparticle with sizes higher than 150 nm was found to enhance spleen distribution.

Recent work showed that the behavior of nanoparticles in the body can differ significantly depending on the protein(s) attached to the nanoparticle surface [78]. The types of serum proteins that adhere to the surface vary by the surface charge of the lipid nanoparticle. Since each organ has a different receptor expression pattern, the distinct protein corona, the adsorption of nonspecific blood proteins surrounding the nanoparticle can direct distribution to organs that express the matching receptors. Using this concept, the authors generated neutral, positively, and negatively charged nanoparticles to target the liver, lung, and spleen, respectively. Therefore, researchers should consider the importance of surface charge for trafficking to these specific organs when seeking to modulate vaccine distribution, such as in the design of a spleen-targeting vaccine.

Other important factors in determining the fate of a vaccine in the body include how it is metabolized and eliminated. The biodegradability of a vaccine delivery system will deeply affect its metabolism and pharmacokinetic properties. When developing an ionizable lipid for lipid nanoparticle-mediated RNA delivery, for example, developers can introduce an ester bond within the hydrophobic alkyl lipid chain to promote its hydrolysis. This enhanced biodegradability was shown to remarkably accelerate the degradation and excretion of lipid nanoparticles [79-81].

The introduction of ester bonds was used for an intramuscularly administered ionizable lipid-based nanoparticle [81]. Significant amounts of the lipid were detected in the liver and spleen, that indicating systemic distribution occurred after local injection. Formulations whose lipids contained several ester bonds showed significantly lower levels of lipids (close to the baseline) at the injection site and in the liver and spleen.

The accelerated metabolism of an ester-containing lipid leads to its efficient excretion. Pharmacokinetic research on intravenously administered lipid nanoparticles showed that 30% of an ester bond-introduced ionizable lipid was excreted in the urine within 12 hr and 40% of the lipid was excreted in feces over 12 to 48 hr. Given this rapid excretion, the biodegradable lipid was completely eliminated from the body within 72 hr in mice [79]. The abilities of ester bond-introduced lipids to undergo enhanced metabolism and excretion were shown to reduce the bodily exposure to potentially toxic lipids [81]. In rat and monkey models, conventional ionizable lipid nanoparticles caused inflammatory responses (e.g., edema) at the site of intramuscular injection, whereas ester bond-introduced lipid-based lipid nanoparticles (LNP) did not show such toxicity.

In sum, modulating the administration, distribution, metabolism, and excretion of a vaccine will greatly impact its in vivo fate. Considering these factors in the design of a vaccine delivery system can maximize the potential of the vaccine.

3. Impact of the delivery routes on the in vivo fates of vaccines

3.1. In vivo fate of an oral vaccine

Although oral delivery is a highly convenient vaccine administration route for patients and a useful noninvasive way to increase their compliance over the injection route, it is not easy for an orally delivered vaccine to reach its target intact. Harsh conditions in the gastrointestinal tract, such as the presence of digestive enzymes and an acidic pH, can degrade vaccines and/or delivery system components. In oral vaccine design, efforts have been made to deliver vaccines to Peyer's patch; there, lymphoid tissues and immune cells are specifically localized to form gut-associated lymphoid tissue in the small intestine (Fig. 2A). Progress has been made in enhancing the delivery of oral vaccines to Peyer's patch, offering a representative example of how the use of a delivery system can change the in vivo fate of a vaccine.

One approach for modulating the behavior of an oral vaccine has been to liberate the antigen in an intestine-specific and sustained-release manner. For this purpose, enteric coating has been utilized to prevent the decomposition of protein antigens in gastric fluid. For example, calcium phosphate nanoparticles loaded with protein antigens were sequentially coated with positively charged chitosan and negatively charged alginate via electrostatic interaction [82]. C chitosan surface coating of the nanoparticle was observed to increase the uptake by APC, and mice treated with dual biopolymer-coated oral vaccine nanoparticles showed signif-

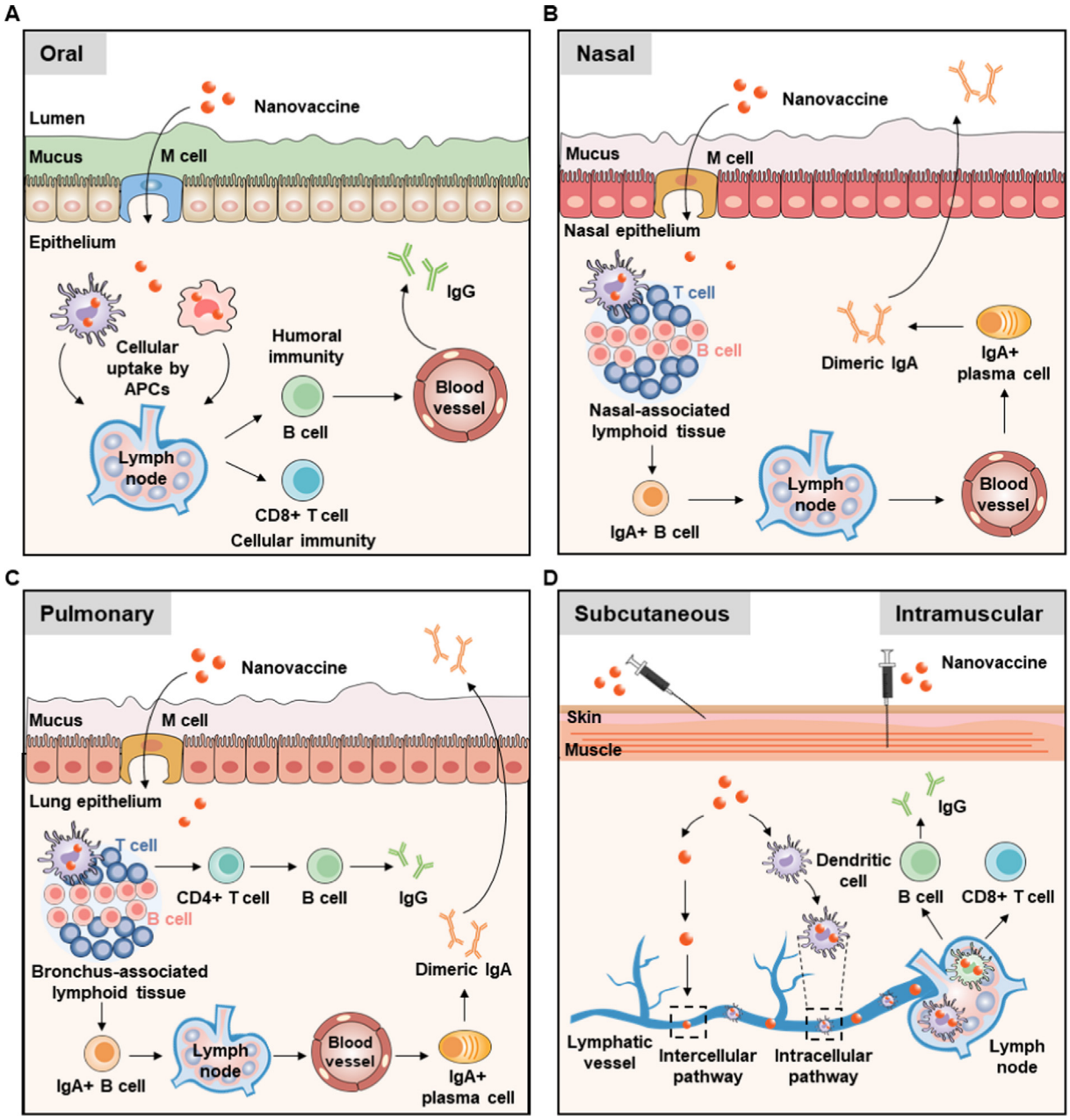

Fig. 2. In vivo fates of nanovaccines administered by various routes. (A) After oral administration, nanovaccines are taken up by M cells in the gastrointestinal tract, transcytosed, and processed by APC in the draining lymph nodes. Presentation of vaccine antigens on APC can induce humoral and cellular immunity. (B) Nasally administered nanovaccines are taken up by M cells and pass through the nasal epithelium. Dendritic cells capture the nanovaccines and migrate to the nasal-associated lymphoid tissues, resulting in the generation of antigen-specific IgA^+ B cells. The IgA^+ B cells migrate to the lymph nodes and enter the blood stream. IgA^+ B cells differentiate to IgA^+ plasma cells and generate dimeric IgA. (C) After pulmonary administration, nanovaccines are captured by dendritic cells and migrate to the bronchus-associated lymphoid tissues. Next, CD4+ T cells and B cells are activated, triggering the IgG secretion-mediated humoral immune response. In parallel, IgA^+ B cells migrate from the lymph node to systemic circulation, generating IgA^+ plasma cells and secreting dimeric IgA to the effector sites. (D) Subcutaneously and intramuscularly administered nanovaccines pass through the epidermis and subcutaneous tissues to reach lymphatic vessels via intercellular or intracellular pathways. In the lymphatic vessels, nanovaccines or nanovaccine-loaded dendritic cells traffic to the lymph node, where resident dendritic cells can be triggered to activate B cells and T cells in an antigen-specific manner.

icantly higher IgG and IgA levels than the free-protein vaccine-treated group.

Another challenge that can modulate the behavior of an oral vaccine in the gastrointestinal tract is its need to penetrate the mucosa and undergo transepithelial transport through tight junctions. To overcome the challenge, an oral vaccine against *Helicobacter pylori* was coated with PEG [83]. The nanoparticle core was generated by charge-charge interaction between the anionic protein antigen and the cationic cell-penetrating peptide, poly-L-arginine. The nanoparticle core was further coated with PEG-allyl glycidyl ether, which is a negatively charged copolymer. This PEG surface modification stabilized the nanoparticle in the mucus and enhanced its mucus-layer penetration compared to that seen for a plain complex of protein antigen and poly-L-arginine. Gradual dissociation of the PEG coating was expected to expose the cell-penetrating poly-L-arginine and enhance the transepithelial transport of the vaccine nanoparticle.

As an alternative way to control the distribution of an oral vaccine in the gastrointestinal tract, researchers have investigated the use of physical forces, such as propulsion. For example, the self-propelling force of a micromotor was employed to modulate the motility of a vaccine nanoparticle in the intestine [84]. The micromotor was fabricated by adding an asymmetric TiO_2 rigid coating onto a magnesium nanoparticle, leaving a small hole. As the chemical reaction between magnesium and water generated hydrogen gas, the particle moved within the gastrointestinal tract.

For use as an oral vaccine, the motor particles were coated with staphylococcal toxin A-bound membrane and further coated with chitosan for mucoadhesiveness and Eudragit L100-55 for protection against the harsh gastric environment. The moving particles could reach a speed exceeding 200 μm/s and showed significantly better retention in the intestinal wall and villi, compared to static particles. The enhanced retention and distribution of the moving particles in the intestine eventually resulted in higher antigen-specific IgA antibody titers, demonstrating that modulating the gastrointestinal transit time can modulate the efficacy of an oral vaccine.

As discussed above, the gastrointestinal tract is a good target for vaccination, as it can efficiently induce both local immune responses and systemic immunity. However, it is also a gate through which numerous pathogens and foreign antigens enter the body from the outside. The immune responses in the gastrointestinal tract are thus under tight regulation, and it is important to properly control the immune balance when seeking to induce an effective vaccine response.

Recent work showed that gut immunity can be greatly influenced by the commensal bacteria in the gut. Interestingly, the microbiome and its metabolites were identified as critical factors that directly affect the activity of surrounding immune cells [85]. In addition, the effectiveness of oral vaccines was reported to be lowered by regulatory T cells induced under an abnormal intestinal environment, such as those associated with chronic malnutrition or environmental enteric dysfunction [86]. Therefore, future efforts to develop oral vaccines should consider the role of the microbiome and inflammatory pathological conditions when seeking to maximize the immune efficacy.

3.2. In vivo fate of nasal and pulmonary vaccines

The nasal and pulmonary administration routes have gained great interest as alternative routes for vaccine delivery because they offer several advantages over the intramuscular vaccination approach (Fig. 2B, 2C). In addition to being noninvasive, both nasal and pulmonary vaccines are exposed to mucosal sites, and thus can mimic the process of a natural respiratory infection. Mucosal immune systems inspect for foreign antigens and protect the body as a first line of defense. Because most infections occur on mucosal surfaces, the nasal or pulmonary delivery of a vaccine provides it with rational access to the immune systems while avoiding interference from systemic immunity [87].

The surface of the nasal cavity and the inner surface of the tracheal bronchus are covered in a layer of mucus; this adhesive gel contains several types of mucin that can trap pathogens [88]. The mucosal immune system, which protects the body against many pathogens that attempt to enter through the mucosal membranes, derives its functions from the mucosa-associated lymphoid tissues of the nose and lungs [89]. The so-called nasopharynx-associated lymphoid tissues and bronchus-associated lymphoid tissues [90] are targeted by nasal vaccines and pulmonary vaccines, respectively.

These vaccines pass through the mucosal layer and the vaccine antigens reach the outer epithelial layer of the nasopharynx- or bronchus-associated lymphoid tissue, which consists of memory cells [91] that are capable of transporting antigens across the epithelium [92]. APC such as macrophages and dendritic cells also exist in the nasopharynx- and bronchus-associated lymphoid tissues. Particulate pathogens or immunogenic substances are absorbed by memory cells and passed through the epithelium, while soluble antigens can be directly recognized by APC [93]. After being taken up by memory cells, particulate antigens are recognized by APC and presented to T cells. The antigen also drains to lymph nodes via APC migration, thereby triggering a systemic immune response [94].

Although nasal delivery is emerging as a convenient vaccine administration route for animals and human, additional studies are needed to enable the formulations to overcome some drawbacks. For example, a solution-form nasal vaccine, while easy to apply in the clinic, is not suitable for a massive vaccination setting due to the loss of vaccine antigens via leakage from the administration site or swallowing into the oral cavity [95]. Mucociliary clearance and enzymatic degradation at mucosal surfaces also serve as barriers to the success of nasal and pulmonary vaccines [96].

To reduce the losses experienced by vaccine antigens administered in liquid form and increase the retention at mucosal surfaces, other dosage forms, such as mucoadhesive gels, have been studied [97]. To reduce degradation at mucosal surfaces, researchers have studied the encapsulation of vaccine antigens in biodegradable poly (lactic-co-glycolic acid) nanoparticles [61] and liposomes [98].

The need to penetrate through mucosal tight junctions remains a challenge in mucosal vaccine development. However, chitosan coating of gold nanocarriers was shown to enhance the intercellular penetration of a DNA vector expressing the S protein of SARS-CoV-2 [22]. Moreover, intranasal delivery of a SARS-CoV-2 S protein-encoding DNA vaccine using gold-chitosan nanocarriers provided a higher antigen expression and pulmonary immune response than a DNA vaccine formulated using noncoated gold nanocarriers.

In pulmonary vaccines, the particle sizes can affect the lung deposition. Particles were found to deposit in different anatomical locations of the respiratory tract in size dependent manner. It has been reported that desirable sizes of pulmonary vaccines range from 1 to 5 μm [99]. Particles larger than 5 μm can be deposited in the upper respiratory tract or exhaled in the air. Particles less 5 μm can be easily phagocytosed by lung macrophages, inducing proliferation of antigen specific CD4+ T cells in draining lymph nodes [100]. For pulmonary vaccines, dry powder inhalation has been studied. Given the size-dependence of lung deposition, the particle sizes of dry power vaccines should be carefully controlled. Use of excipients which can prevent aggregation during storage needs to be considered in developing pulmonary dry powder vaccines.

Glucopyranosyl lipid A, an amphiphilic endotoxic derivative, was investigated as a coating agent to reduce the particle aggregation of a dry powder pulmonary vaccine [101]. Notably, glucopyranosyl lipid A was used in this study as both a coating agent and an adjuvant. Following intratracheal administration in mice, the glucopyranosyl lipid A-coated dry powder vaccine provided greater human papillomavirus 16 minor capsid protein L2 antigen deposition in the lung compared to other organs. Moreover, the glucopyranosyl lipid A-coated formulation could induce systemic immune responses against human papillomavirus, showing greater responses than those obtained with a noncoated version.

Although progress has been made in resolving the formulations for nasal and pulmonary vaccine deposition, the utilized devices can also affect the deposition of vaccines [102]. Device competency and patient compliance remain challenges for pulmonary delivery [103]. To overcome the limitations of nasal and pulmonary delivery, researchers should seek to develop in vitro or tissue models that can exquisitely reflect the nasal or pulmonary environment.

3.3. In vivo fate of subcutaneous vaccines

Subcutaneous injection is used to administer vaccines into the layer of skin below the epidermis and dermis [15]. In the clinic, live vaccines (e.g., varicella vaccine) have been administered via the subcutaneous route [104]. In subcutaneous vaccine delivery, the particle size plays a critical role in the subsequent distribution to lymph nodes. It has been reported that nanoparticles of 10–100 nm in diameter are more likely to diffuse into the lymphatic vessels, migrate to lymph nodes, and be taken up by lymph node-resident APC [105]. As the lymph node distribution is an important in vivo fate for a subcutaneous vaccine, researchers have sought to increase the lymph node distribution following subcutaneous injection (Fig. 2D).

Synthetic lipoprotein-based nanodisc formulations have been used for the subcutaneous co-delivery of neoantigens and immunostimulants [64]. The nanodiscs consisted of phospholipids, apolipoprotein A1-mimetic peptides, antigen peptides, and a cholesterol-modified 5′-C-phosphate-G-3′ (CpG) motif. After subcutaneous injection to mice, a fluorescent dye-labeled peptide antigen in the nanodisc showed increased fluorescence signals at draining lymph nodes compared with free-form antigen. Subcutaneous delivery of a neoantigen in nanodiscs yielded a 7-fold higher frequency of antigen-specific cytotoxic T cells in the peripheral blood of mice, compared to that seen in the free antigen-immunized group.

Over 1,000 ionizable lipid-based nanoparticles were screened for their potential in a subcutaneous mRNA vaccine delivery system [106]. The studied lipids shared an unsaturated lipid tail and dihydroimidazole-linked and cyclic amine head groups. Among the various lipid formulations, lipid nanoparticles A2 and A12 yielded very high antigen protein expression levels at the injection sites and draining lymph nodes, but A2 triggered greater immune responses than A12. This suggests that immune responses can be affected by factors other than the in vivo fate of the delivered vaccine.

Subcutaneously delivered chitosan and heparin-based nanoparticles have been investigated for the in vivo lymphatic trafficking of foot-and-mouth disease capsid protein antigens and immunostimulants [107]. The nanoparticles ranged from 90 to 130 nm in size. To visualize the in vivo fates of nanovaccines, the authors labeled the capsid protein antigens with Alexa Fluor 750. Molecular imaging revealed that the nanoparticles first reached the proximal lymph nodes by 30 min post-dose and were distributed to the distal lymph nodes by 9 hr post-dose. The signals at both proximal and distal lymph nodes were observed for up to 2 days post-dose. In contrast, free capsid protein antigen was observed only at the proximal lymph nodes at 9 hr post-dose.

Fully protein-based, self-assembling nanovaccines have been designed for the efficient lymphatic delivery of antigens via subcutaneous administration for anti-cancer therapy [108]. The all-protein nanoparticles, which were produced in *Escherichia coli* strains, consisted of pentamer domains from the bacterial AB_2 toxin (contributing to the self-assembly ability) and antigen peptides. The in vivo fate of this formulation was assessed with Cy7 fluorescent dye labeling and molecular imaging. Following subcutaneous injection to Balb/c mice, fluorescence corresponding to the free antigen was not detected in the lymph nodes; in contrast, that of the nanoparticles peaked in the lymph nodes at 12 hr post-dose and was retained for upto 48 hr.

A PEGylated reduced graphene oxide nanosheet (RGO-PEG) was studied for lymph node delivery after subcutaneous administration for cancer immunotherapy [109]. The nanosheets, which had an average thickness of 25 nm, were loaded with neoantigens and CpG as an immunostimulant. To investigate the in vivo fate following subcutaneous injection, the authors labeled the neoantigens with the radioisotope, ^{64}Cu. In the free ^{64}Cu-neoantigen-treated group, positron emission tomography (PET) imaging showed strong signals in the kidneys and bladder, indicating that there was rapid renal dissemination. In contrast, the nanosheet-treated group showed obvious ^{64}Cu signals in the axillary and inguinal lymph nodes beginning at 3 hr post-injection; the signals peaked at 48 hr and were sustained up to 72 hr post-injection. The PEGylation and flexible structure of the nanosheets were cited as contributing to the main distribution of this formulation to the lymph nodes after subcutaneous administration. This study is noteworthy in that the exact % of the injected dose was quantitatively observed not only at the lymph nodes, but also for blood, kidney, bladder, and other organs.

Lymph node trafficking was also studied for a subcutaneously administered nanovaccine delivered via aluminum hydroxide AlO(OH)-based nanoparticles [65]. The AlO(OH)-based nanoparticles were loaded with ovalbumin antigen and CpG oligonucleotides and coated with PEG-derivative polymers, which were utilized in part to keep the nanoparticles under 90 nm in size. Following subcutaneous administration, the PEGylated AlO(OH)-based nanovaccine was found to traffic to draining lymph nodes more effectively than free ovalbumin over 24 hr post-dose. Notably, this study examined the in vivo fate of the vaccine only as it pertained to draining lymph nodes. Before seeking to translate this system to the clinic, researchers should carefully assess the in vivo fate of PEGylated aluminum oxyhydroxide in other organs and examine the potential for toxicity.

In addition to its size, the rigidity of a nanoparticle may also affect the in vivo trafficking of subcutaneously administered vaccines. Coating of core nanoparticles with bacterial outer membrane vesicles and tumor cell membranes was studied for enhancing the distribution to lymph nodes and APC [66]. In this study, tumor-endogenous antigens in tumor cell membranes acted as vaccine antigens to induce tumor-specific immune responses. The core nanoparticles were formed of poly(lactic-co-glycolic acid), silicon dioxide (SiO_2), or colloidal gold. The authors found that the nature of the core nanoparticle was important for lymph node trafficking: All particles were coated with the same membranes, but the rigid poly(lactic-co-glycolic acid) nanoparticles showed greater trafficking to the distal inguinal lymph nodes, compared to SiO_2- and gold-based core nanoparticles.

3.4. In vivo fate of intramuscular vaccines

Intramuscular administration, which is one of the most widely used routes in the clinic, involves the use of a needle that passes through the epidermis and subcutaneous tissues to deliver the vaccine deeply into muscle [15]. Muscle tissues have a plentiful blood supply, which supports the rapid absorption of vaccines into the bloodstream. Compared with other administration routes, intramuscular injection offers a rapid onset of action, avoids first-pass metabolism, and provides a substantial space that can retain a large volume of drugs. Moreover, owing to the high degree of vascularization in muscle tissue, vaccines may distribute quickly to lymph nodes through lymphatic vessels [110]. For vaccines containing aluminum salt as an adjuvant (e.g., those for hepatitis A, hepatitis B, and diphtheria), intramuscular administration is the first choice since subcutaneous injection may cause severe adverse effects at the injection site. Recently, COVID-19 vaccines have been given by intramuscular administration into the deltoid muscle.

Since mannose receptor is expressed abundantly on the surface of APC, mannose modification was studied as a means to modulate the in vivo fate of intramuscularly administered nanovaccines [111]. The developed nanoparticles consisted of cationic lipid 1,2-dioleoyl-3-trimethylammoniumpropane for electrostatic interaction with ovalbumin antigens. After intramuscular injection to

C57BL/6 mice, mannose-decorated hybrid nanoparticles showed greater retention at the intramuscular injection site, with detectable signals present until 96 hr post-dose. The trafficking to inguinal lymph nodes was the highest for mannose-modified nanoparticles compared to ovalbumin in free form and in plain nanoparticles. The results from this study indicated that mannose modification could enhance the in vivo lymph node trafficking of intramuscularly administered nanoparticles. However, the authors did not address trafficking to antigen-presenting dendritic cells.

The in vivo fate of lipid components used for an mRNA nanovaccine was studied after intramuscular administration [81]. To trace the in vivo fate, the authors analyzed the lipid component, D-Lin-MC3-DMA, in biological samples. At 1 day post-dose, D-Lin-MC3-DMA had decreased to 50% of the initial dose at the injected muscle site, and was observed in the liver and spleen. It should be noted that these authors traced the in vivo fate by measuring the main lipid component of the nanoparticle. In the future, a parallel study of mRNA distribution may provide more information.

Genipin-crosslinked polyethyleneimine nanoparticles were studied for intramuscular ovalbumin antigen delivery [112]. Molecular imaging of rhodamine B-labeled nanoparticles showed that the fluorescence in the draining lymph nodes peaked at 3 hr after intramuscular administration and was retained until 12 hr post-dose. No significant signal was observed in the lymph nodes of the free antigen-treated group. The authors speculated that this may reflect the rapid elimination of low-molecular-weight antigens from the injection site but did not evaluate the dynamic of antigen exposure on the dendritic cells in draining lymph nodes or the duration of immune activation, which would be key components of this research. Further mechanistic and dynamic studies will be needed to support the utility of this strategy.

A deformable nanoemulsion was studied for its ability to modulate the in vivo trafficking of an antigen after intramuscular administration [113]. The oil-in-water nanoemulsion consisted of albumin, squalene, and lipopeptide ovalbumin. Due to its deformability, the nanoemulsion (330 nm in diameter) was expected to pass though cellular and endothelial gaps to undergo enhanced lymph node trafficking via an intercellular pathway. Indeed, the deformable nanoemulsion exhibited higher trafficking to APC at the injection sites compared with solid albumin particles. At 1 day after intramuscular administration, the deformable nanoemulsion exhibited improved lymph node accumulation and $\sim$ 2-fold and more than 10-fold higher antigen-infiltrated areas compared with the solid albumin nanoparticle and free antigen groups, respectively.

The in vivo trafficking of a core–shell-structured lipopolyplex was studied after intramuscular administration [114]. An mRNA encoding luciferase was condensed with a cationic compound and encapsulated in a lipid shell composed of an ionizable lipid, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, and PEGylated lipid. Following intramuscular administration, the bioluminescence of expressed luciferase was observed for more than 10 days post-dose. Quantitative polymerase chain reaction was used to measure the amounts of mRNA at each organ: Muscle showed the highest proportion of lipopolyplex-delivered mRNA (83%), followed by blood, spleen, fat, lung, and liver.

As described above, intramuscular and subcutaneous nanovaccines can show in vivo trafficking to lymph nodes (Fig. 2). Although intramuscular administration is a preferred route for the currently licensed vaccines due to its ease of administration and low risk for side effects at the injection site, the subcutaneous route is also being actively studied for new vaccine development. Compared with muscle, skin is highly immunocompetent; thus, it has long been considered a desirable route for improving immune responses. Many human studies have found that there is no significant difference in the ability of a vaccine administered via intramuscular injection versus subcutaneous injection in generating immune responses to hepatitis A, hepatitis B, influenza [115], and varicella [116]. Further work is needed, however, to fully compare the in vivo fates of nanovaccines between small rodents and human.

Although extensive studies focused on in vivo fates of nanovaccines, the relationship between the in vivo clearance and immunogenicity of nanovaccines should be studied. In one study comparing the in vivo fate of nanoparticles formulated with different ionizable lipids, the nanoparticle that showed the fastest in vivo clearance in muscle, spleen, and liver triggered higher humoral antigen-specific antibody titers [81]. This indicates that the rate of in vivo clearance does not always reflect the immunogenicity of a vaccine delivery system.

3.5. In vivo fate of intratumoral vaccines

Intratumoral route has been emerging for administration of tumor vaccines. In 2015, intratumorally administered talimogene laherparepvec was approved for oncolytic virotherapy by the FDA for melanoma treatment [117]. After intratumoral injection, nanovaccines with tumor antigens can be taken up by APC in tumor microenvironments. In tumor-draining lymph nodes, the processing of tumor antigens by APC can present tumor antigens to T cells. The recognition of tumor antigens by T cells can prime T cells, and activate cytotoxic T cells when the same tumor antigens were rechallenged. After intratumor injection of nanovaccines carrying CpG adjuvant, the activation of T cells in tumor-draining lymph nodes was observed, with regression of rechallenged tumors [118] (Fig. 3).

In intratumoral route, the solidity of tumors may affect the intratumoral distribution. It has been reported that the porosity of tumor tissues can affect the diffusion of nanoparticles in tumor tissues [119]. The size of nanovaccines can affect the intratumoral distribution. Modeling study of intratumoral particle distribution suggested that the sizes between 10 and 30 nm would be suitable for retention in tumor microenvironments [119].

To increase the retention in tumor tissues, a recent study introduced tumor-specific ligands. In the study, a collagen-binding protein, lumican, was used to anchor intratumorally administered cytokine, interleukin-12 [120]. Although the study used cytokine to increase the retention in tumor tissues, similar approach can be exploited to prolong the retention of intratumorally administered nanovaccines in tumor tissues.

In general, compared to the systemic administration, intratumor route was shown to reduce the distribution to the liver and lung, and increase retention at the local tumor tissues, followed by activation of immune cells in the lymph nodes and spleen [121]. In the future, the vivo fates of the intratumoral vaccines can be further controlled with the use of tumor tissue specific ligand modification and development of novel formulations.

4. Intracellular trafficking of vaccine delivery systems

Understanding the behavior of a nanocarrier in the intracellular environment is a crucial piece of grasping the cellular mechanism of a vaccine. This section will elaborate on the intracellular behavior of a nonviral nanocarrier as a vaccine delivery system by looking at fundamental mechanisms of vaccination inside the cell, intracellular trafficking pathways, intracellular barriers for vaccine delivery, and strategies for overcoming these challenges.

4.1. Processing of vaccine antigens at the cellular level

Innate immunity, which is the first line of defense against pathogenic antigens, involves not only neutrophils, eosinophils,

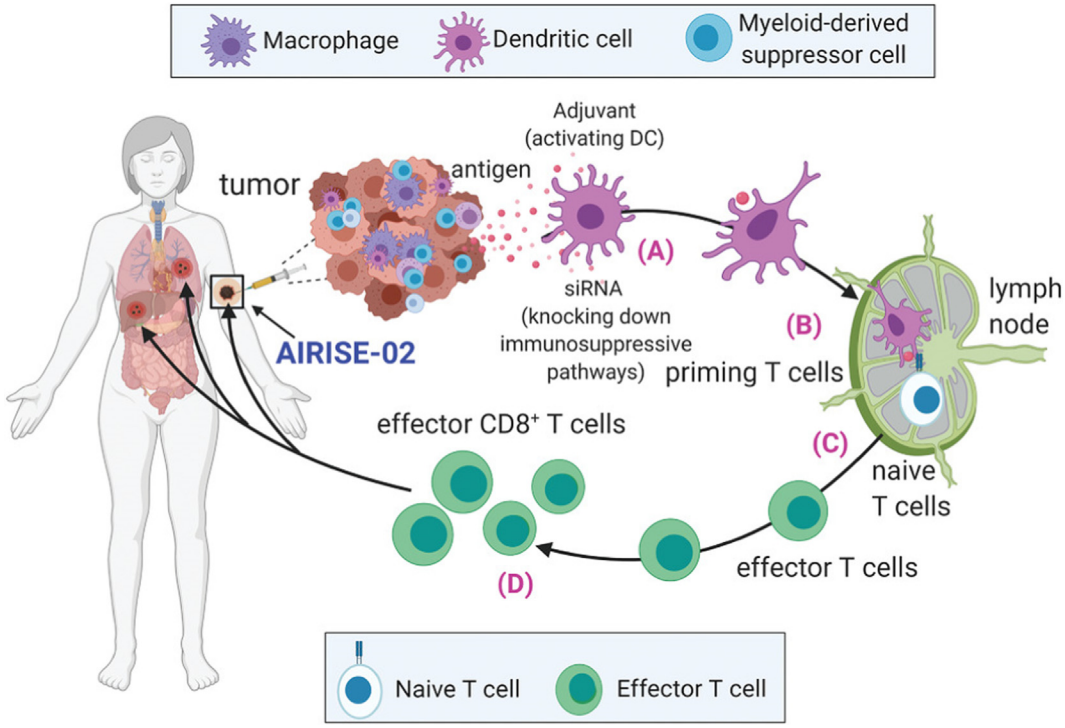

Fig. 3. In vivo fate of intratumorally administered nanovaccines. After intratumoral injection, nanovaccines containing CpG adjuvants activate local dendritic cells (A). The nanovaccine-loaded cells then traffic to tumor draining lymph node (B). Next, antigen-presenting cells prime antigen-specific T cells (C). Effector CD8+ T cells play a role in prevention of tumor recurrence (D). Reproduced with permission from [118].

macrophages, and dendritic cells, but also skin, mucous layers, and various antimicrobial peptides. This primary defense system reacts rapidly and nonspecifically against pathogens. However, the innate immune system occasionally fails to protect the body; in such cases, it may be supplemented by the adaptive and/or acquired immune systems. The adaptive immune response is relatively slow, but it is strong and long-lasting. The most important features of adaptive immunity include the specificity of its antigen response and its active recall under repeated exposure. For a vaccine to achieve a sufficient immune response against its target pathogen, the vaccine must evoke a strong adaptive immune response.

Antigen presentation, which is a vital step in activating key components of the adaptive immune system (especially T cells), is mediated by professional APC such as dendritic cells or macrophages. In order to prime T cells, an APC must take up the antigen, process it, and complex the antigen with MHC molecules to make it recognizable by T cells. The antigen-presentation procedure differs by the MHC class, leading to distinct immune responses.

Extracellular antigens, such as bacteria or toxins, are complexed with MHC class II molecules and presented to effector CD4+ T cells; this activates B cells to secrete immunoglobulin or signal other T cells. Briefly, the antigen is engulfed through phagocytosis. Unlike the situation in a granulocyte, the major object of phagocytosis by a professional APC (e.g. dendritic cell), is to prime the adaptive immune system through antigen presentation. Following phagocytosis, the antigen is digested and degraded to peptide fragments by the acidic pH and proteases in the phagosome and phagolysosome. The generated antigen peptide fragments are bound to MHC class II molecules that originate from the endoplasmic reticulum. Finally, the generated complex is displayed on the cell surface to be recognized by the T cell receptors of CD4+ T cells. This process occurs specifically in professional APC.

In contrast, cytosolic antigens, such as those derived from the viral infection, are complexed with MHC class I molecules and presented to effector CD8+ T cells, which induce apoptosis of the invading cells. In this procedure, the proteasome, which comprises the 20S catalytic core and two 19S regulatory caps, degrades the antigen into peptides within the cytoplasm. These peptide fragments are delivered to the ER and bound to MHC class I molecules, and the generated complex is presented on the cell surface to interact with the T cell receptors of CD8+ T cells. In general, all cell types possess MHC class I molecules, meaning that this process is not limited to professional APC.

The same processes may be undertaken by antigens that are introduced artificially by vaccination. Protein-based vaccine antigens are processed like exogenous bacterial antigens and presented by MHC molecules for immune cell activation. Consequently, when seeking to deliver a vaccine antigen to its optimal target cells, the developers must consider the intracellular uptake of the antigen and how it is processed at the cellular level.

4.2. Modulation of nanovaccine uptake

For vaccination to yield a sufficient effect and prevent disease, antigens and (where relevant) adjuvants must be internalized to the cytoplasm. However, the plasma membrane is a highly complex cellular structure and the cellular entry of biomolecules is sophisticated. In nature, endocytosis is a natural means to internalize a biomolecule to the cytoplasmic space. Therefore, it is reasonable to expect that this mechanism could be applied to a vaccine delivery system. In vaccine delivery, modulation of phagocytosis and pinocytosis is an important first step in enhancing cellular uptake.

Intracellular vaccine delivery via phagocytosis is one of the most promising strategies for cellular uptake modulation. Phagocytosis mainly occurs in professional APC, which are suitable target cells for vaccine delivery. Moreover, certain phagocytosis-initiating receptors, such as dectin-1, can stimulate innate immune responses. Consequently, phagocytosis-initiating receptors have been widely researched for their potential use in vaccine delivery.

C-type lectin receptors, scavenger receptors, complement receptors, and Fc receptors are well-known phagocytosis-triggering molecules. Accordingly, the ligands of these receptors have been explored in the context of vaccine delivery.

C-type lectin receptors are expressed on macrophages, granulocytes, and dendritic cells. Indeed, all leukocytes, including those of myeloid and lymphoid lineages, express C-type lectins. However, each leukocyte type expresses a distinct class of C-type lectins. Transmembrane C-type lectin receptors contribute to a broad range of physiological functions, ranging from cellular modulation and development to immunological responses [122].

Mannose receptor (CD206) is a C-type lectin that is mainly expressed on macrophages and dendritic cells. Mannose receptors trigger phagocytosis, making them a reasonable target for vaccine delivery. For mannose receptor-mediated APC uptake, a mannose-conjugated infectious vaccine antigen was studied [123]. *Plasmodium falciparum* circumsporozoite protein, which was used as the antigen, was modified with mannose and TLR-7-activating imidazoquinolines via click chemistry. Conjugation with mannose was observed to enhance the endocytosis of the antigen by dendritic cells, increase the antigen-specific IgG level, and decrease the malaria parasite burden in hepatocytes.

Mannose-mediated cellular uptake modulation was also studied for the delivery of a cancer vaccine [111]. Mannose-conjugated liposomes were formulated to include imiquimod and monophosphoryl lipid A, which are agonists of TLR7/8 and −4, respectively. As a model antigen, ovalbumin was loaded to the liposomes. Mannose modification was found to increase the cellular entry of the vaccine liposomes to bone marrow-derived dendritic cells, increase humoral and cellular immunity responses, and improve the survival of thyoma-bearing tumor model animals in vivo [111].

Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (CD209) was also studied for its ability to enhance the dendritic cell uptake of nanovaccines [124,125]. CD209 is a C-type lectin receptor with high affinity for intercellular adhesion molecule 3, which is expressed by macrophages and dendritic cells. Researchers exploited the high-mannose glycoprotein-binding feature of CD209 and developed apoptotic extracellular vesicles expressing high-mannose glycan as a cancer vaccine [124]. In another study, CD209 aptamer modification was examined in the context of tumor-derived cell membrane vesicles [125]. A cholesterol-conjugated form of CD209 aptamer was used to modify a membrane vesicle with surface expression of CD209. The CD209-modified vesicles exhibited increased internalization to dendritic cells and enhanced lymph node accumulation.

Due to their diverse ligand-binding and endocytic activities, scavenger receptors have been targeted with the goal of enhancing the uptake of nanovaccines by APC [126]. Protein corona-coated nanovaccines were found to easily bind to scavenger receptors, such as MARCO, and undergo engulfment by APC [127,128]. Class A scavenger receptors bind negatively charged ligands, and carboxylic acid group-modified negatively charged nanoparticles were shown to be taken up by dendritic cells via class A scavenger receptor-mediated endocytosis. Meanwhile, scavenger receptor class B1-mediated endocytosis was modulated using a high-density lipoprotein-mimicking peptide. The peptide-modified nanovaccine was found to be taken up by dendritic cells and suppress tumor growth in vivo [129].

Complement system has been studied asWith the goal of engineering a vaccine delivery system to deliver its antigen to the cell via CR3, researchers developed dextran-coated iron oxide nanoparticles [130]. The dextran coating was proposed to form a protein corona in serum and activate complement system C3, leading to the CR3-mediated opsonization of nanoparticles by dendritic cells. The dextran coating was also proposed to increase the interaction with CR1 and CR2 on B cells and follicular lymphocytes, respectively leading to activation of humoral immune responses [130].

As described above, clathrin-mediated endocytosis is a representative pathway of receptor-mediated endocytosis. Taking advantage of this, crystalline aluminum oxyhydroxide (AlO(OH))-polymer nanoparticles were internalized to dendritic cells via clathrin-mediated endocytosis. The entry route of the nanoparticle was supported by the ability of the clathrin-mediated endocytosis inhibitor, chlorpromazine, to suppress nanoparticle entry [65]. Another study reported clathrin-mediated endocytosis of a microscale cancer vaccine [131]. Cancer cells were coated with epigallocatechin-3-gallate and an Al(III) cation coordination layer. The resulting microparticle cancer vaccine was ∼ 7–10 μm in size, which is similar to the size of bone marrow-derived dendritic cells. Even though the particle was too big to be suitable for clathrin-mediated endocytosis, chlorpromazine was found to block the endocytosis of the microparticles.

In caveolin-mediated endocytosis (see above), 50–100 nm-wide pits are formed in a plasma membrane containing caveolins with sphingolipids or cholesterol-rich regions [132]. Caveolin-mediated endocytosis is an attractive endocytic pathway for vaccine delivery due to its unique ability to avoid the lysosome during intracellular trafficking. It is also a major viral invasion pathway [133,134].

Inhibitors of specific endocytic pathways have been used to assess the main entry pathways of nanovaccines. It should be noted that a nanovaccine may enter cells by more than one pathway and may, in fact, use several pathways for entry. One study found that the clathrin-dependent, phagocytosis, and micropinocytosis pathways were all involved in the entry of a developed microscale particle. The particle was not always engulfed whole; rather, it was sometimes fragmented, and the fragments were taken up by the cells via the different pathways [131]. The entry of whole versus partially broken particles may explain the diverse entry pathways utilized by this vaccine delivery system.

With continued progress in the fields of immunology and cell biology, the list of receptors that can be used to promote entry into APC will keep increasing. Although the inclusion of ligands that bind to the receptors on immune cells was found to increase the cellular entry of nanovaccines, these receptors may be expressed on non-immune cells that could also become delivery targets. Most studies to date have focused on immune cell uptake and have not assessed possible uptake by other cell types. Efforts to identify receptors that are exclusive to APC may facilitate the design of more specific vaccine delivery carriers.

4.3. Modulating the intracellular trafficking of vaccine antigens

4.3.1. Cellular entry pathways

A cell uses various tactics to maintain cytoplasmic homeostasis under exogenous stress. The plasma membrane surrounding a cell is a key barrier that sets up the distinction between the inside and outside of the cell. An amphiphilic phospholipid bilayer represents the fundamental structure of the plasma membrane. Additional structure and functions are provided by other lipids, such as cholesterol and/or sphingolipids, along with biomolecules of varying complexity. The entry of external molecules or nanoparticles is tightly regulated by sophisticated mechanisms. Delivering the antigen of interest to the cytoplasm of an appropriate target cell is the first step in successful vaccination. Thus, we must work to understand the cellular entry mechanisms of delivery systems.

Unlike small molecules, most nanovaccines cannot enter the cells by plasma membrane penetration; instead, they use a special process called endocytosis [134]. Based on the property of the carrier structures and components, endocytosis pathways involving various plasma membrane proteins, such as clathrin or dynamin

can be involved. For a nanoparticle, the action of the endocytic pathway begins with an interaction between the nanoparticle and the membrane, which leads to the generation of a membrane-bound vesicle. This early endosome, which is the first component of intracellular trafficking, will deliver the vesicle cargo to the lysosome, thereby forming the late endosome (or endolysosome); from there, the cargo will go to the ER, *trans*-Golgi network (TGN), or endosomal recycling network depending on the specific organelle markers.

Phagocytosis, which is a major pathogen-clearance procedure in the mammalian immune system, is mainly performed by professional phagocytes, including macrophages, granulocytes, dendritic cells, neutrophils, and B cells. Phagocytosis is also how the cell deals with apoptotic cell debris and nanoparticles. The phagocytosis of a nanoparticle is initiated by physical interaction with a membrane receptor. Actin rearrangement causes pseudopodia to develop and engulf the nanoparticle. The fusion of the engulfing membrane generates the phagosome, which develops to the phagolysosome and fuses with a lysosome. The acidic environment of the lysosome contains antimicrobial enzymes including acid hydrolase and oxidase, which can produce radicals when degrading a vaccine antigen.

In the context of vaccine delivery, phagocytosis is closely linked with antigen presentation because macrophages and dendritic cells act as both phagocytes and professional APC. A number of receptors are capable of initiating phagocytosis, including C-type lectin receptors, scavenger receptors, complement receptors, and Fc receptors. These receptors have been widely studied as entry route targets for dendritic cells, macrophages, and/or lymphoid organ distribution.

A cell may internalize nanoparticles via macropinocytosis [135-137]. Macropinocytosis, which is a cellular uptake pathway for the nonspecific engulfment of extracellular fluid and solutes through actin signaling, occurs via large endocytic vesicles called macropinosomes. Unlike the other endocytic pathways, the initiation of this process does not require any interaction between a ligand and the cellular membrane. Instead, polymerization of actin and membrane ruffling provokes micropinocytosis. Macropinocytosis plays important physiological roles in antigen presentation: Immature dendritic cells consistently take up extracellular fluid in search of antigens present in the extracellular environment. In this regard, vaccine delivery via micropinocytosis has been considered to be a promising strategy.

Nanovaccines can be taken up by the cells via clathrin-dependent endocytosis. Clathrin-dependent endocytosis is triggered when the cytoplasmic tail of a receptor recruits adapter protein 2, which then recruits the clathrin triskelia from the cytosol to induce clathrin coat assembly. Following clathrin-coated vesicle formation, dynamin mediates scission to generate the endosome [138]. In addition to biomolecule ligands, nanoparticles can enter the intracellular space via clathrin-dependent endocytosis, meaning that this should be considered among the potential endocytic pathways for a nanovaccine [139].

Caveolin-dependent endocytosis is another potential pathway for nanovaccine internalization [140,141]. Caveolins are a family of integral membrane proteins found in the membranes of caveolae, which are a distinct type of lipid raft characterized by flask-shaped plasma membrane invaginations of 50–100 nm in diameter [132,133]. This structure includes cholesterol and sphingolipids, providing strong hydrophobicity and membrane rigidity [142,143]. Generally, caveolin-coated vesicles move to the ER or TGN. Notably, this pathway does not pass through an acidic or enzymatic environment that could damage a nano-delivery system. Thus, caveolin-dependent endocytosis is emerging as a cellular entry route for the vaccine delivery of lysosome-sensitive nucleic acid-based platforms [144].

In controlling the cellular entry pathway of nanovaccine, the size is a crucial consideration factor. The size of LNP used for mRNA delivery is known to affect the pathway by which it enters the cell. In an in vitro experiment with 300–400 nm lipid nanoparticles, the presence of the micropinocytosis inhibitor, 5-(N-ethyl-N-isopropyl)-amiloride', was found to decrease the expression of the loaded mRNA, whereas the clathrin inhibitor, Pitstop2, had no such effect [145]. The size dependence might be due to the size of the clathrin-coated endosomal vesicles, which is reported to be around 85–100 nm [138].

4.3.2. Endosomal trafficking

An endosome is a small intracellular vesicular structure that is especially important for innate immunity due to the surface expression of pattern-recognition receptors (PRR), TLR, and MHC molecules, which are major vaccine targets. Following invagination of a nanoparticle, the cargo will be delivered to the early endosome, which is the first component of the endocytic pathway. Due to the mildly acidic condition (pH 6.0–6.8) inside the early endosome, engulfed materials dissociate from the receptor, which is transported back to the cell surface.

Endosome will then fuse with a lysosome to form an endolysosome, which has an acidic environment that is rich with degrading enzymes [146]. Endosomal maturation and endolysome formation are essential steps in this pathway for antigen-presentation, but they also act as barriers for vaccine delivery systems, especially for DNA- or RNA-based vaccine platforms. In endolysosomal environments, the nucleic acid cargoes of nanovaccines can be degraded, not reaching the cytoplasm and nucleus for vaccine antigen presentation. Therefore, strategies to allow endosomal escape have been extensively studied [147]. A recent study reported nanovaccines with acidic cleavable linkers for improving endosomal escape and immune response [148]. In the study, cyclodextrin-based gel system containing doxorubicin, CpG derivatives, and photoresponsive indocyanine green was designed. Photothermally released CpG derivatives were taken up by immature dendritic cells. Lysosomal release and endosomal escape of CpG was found to promote the maturation of dendritic cells and immune responses.

Intracellular trafficking of nanovaccine-loaded endosome is not limited to lysosomal fusion (Fig. 4). The endosome to TGN traveling pathway can start from early endosome/recycling endosome or the late endosome. Several proteins are known to meditate cargo recognition at endosomes, and thereby mediate TGN delivery. Retromer is a one of TGN-sorting protein that mediates endosome-TGN transport. [149].

Another intracellular trafficking pathway of nanovaccine-loaded endosome is exocytosis, which moves cytoplasmic molecules from the Golgi apparatus to the plasma membrane for secretion. It is an essential pathway due to its secretion function, but it can also decrease vaccine delivery efficiency. Therefore, blocking the exocytosis pathway is also a reasonable strategy for enhancing vaccine delivery [150].

4.4. Modulation of endosomal escape

In efforts to modulate the intracellular trafficking of nanovaccines, strategies to facilitate endosomal escape have been extensively studied. Endosome escape is the process that the antigen cargoes of nanovaccines move from endosomes to the cytoplasm. After a nanovaccine is internalized by an endocytic pathway, it is entrapped in an endosome, through which it will come into contact with the lysosome and undergo degradation or recycling. Among the different types of vaccine antigens, nucleic acid vaccine antigens in particular need to escape the endosome to reach the cytosol (for RNA vaccines) or eventually the nucleus (for DNA vaccines),

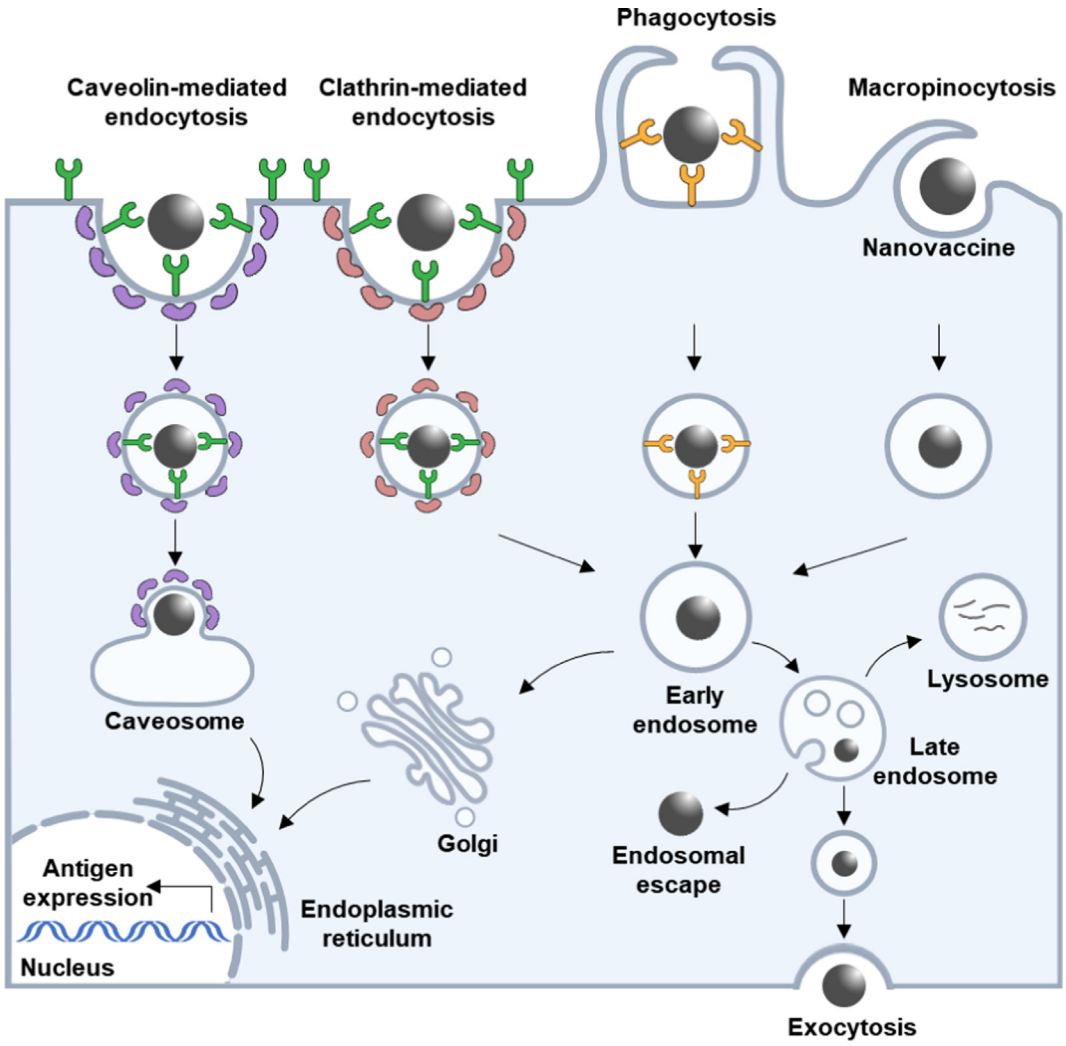

Fig. 4. Intracellular trafficking pathways of nanovaccines. Nanovaccines enter cells through various routes, such as micropinocytosis, phagocytosis, clathrin-mediated endocytosis, and caveolin-mediated endocytosis. Endosomes formed by caveolin-mediated endocytosis do not fuse with lysosomes. For nucleic acid antigen-carrying nanovaccines, endosomal escape is critical for effective antigen expression in the cytosol.

and this must occur before the nucleic acid antigens are degraded in the lysosome [151]. Since the endosome escape is a non-natural event, vaccine developers must force it.

The mechanism underlying endosomal escape is not fully understood, but it is thought to involve the osmotic swelling effect, membrane fusion, and/or membrane destabilization. The most widely suggested mechanism is the osmotic swelling effect, which is also known as the "proton sponge effect" [152]. In an acidic pH environment, the amine groups of a cationic delivery system interact with protons to form a cationic charge in an endolysosome. This leads to the recruitment of chloride ions into the endo/lysosome, which is followed by the swelling (due to the movement of water under osmotic pressure) and eventual rupture of the endo/lysosome. Ionizable lipids with pH responsive charge-conversion properties may have advantages over nonionizable cationic materials for endosomal escape. The clinical successes of the SARS-CoV-2 mRNA vaccines, BNT162b2 (Pfizer–BioNTech) and mRNA-1273 (Moderna), have been attributed in part to the use of ionizable lipids, which can enhance endosomal escape [153].

Modification with functional groups that react with the endosomal membrane has been exploited to induce membrane destabilization and rupture (Fig. 5). Other strategies include the use of cell-penetrating peptides that reportedly destabilize membranes, and the formulation of nanoparticles with a fusogenic lipid to enable membrane fusion. For a negatively charged nanoparticle, combination with an endosome-disrupting agent was shown to enhance the endosomal escape of the cargo [154].

Escape from the late endosome/lysosome stage was found to be crucial for the successful delivery of an mRNA vaccine [155]. Conventionally, the early endosome stage is considered to be the main trafficking stage at which lipid nanoparticles should seek to escape the endosome. A number of proteins are characteristic of endosomes. Among them is Rab7A, which contributes to endosomal maturation and late endosome/lysosome trafficking. Patel et al. showed that the expression of a protein encoded by lipid nanoparticle-delivered mRNA was significantly decreased by knock-out of Rab7A.

Various materials have been studied for their ability to promote the endosomal escape of vaccine cargoes, including pH-sensitive cationic ionizable lipids, cell-penetrating peptides, cationic polymers, exosomes, pH-sensitive polymers, and light-responsive materials [156]. Lipid nanoparticles have demonstrated their ability as clinical delivery systems for mRNA vaccine antigens. Despite this clinical success, however, these systems deliver mRNA to the cytoplasm at an efficiency of only $\sim$ 2% [157]. Recently, with the pandemic of COVID-19, increasing numbers of studies have

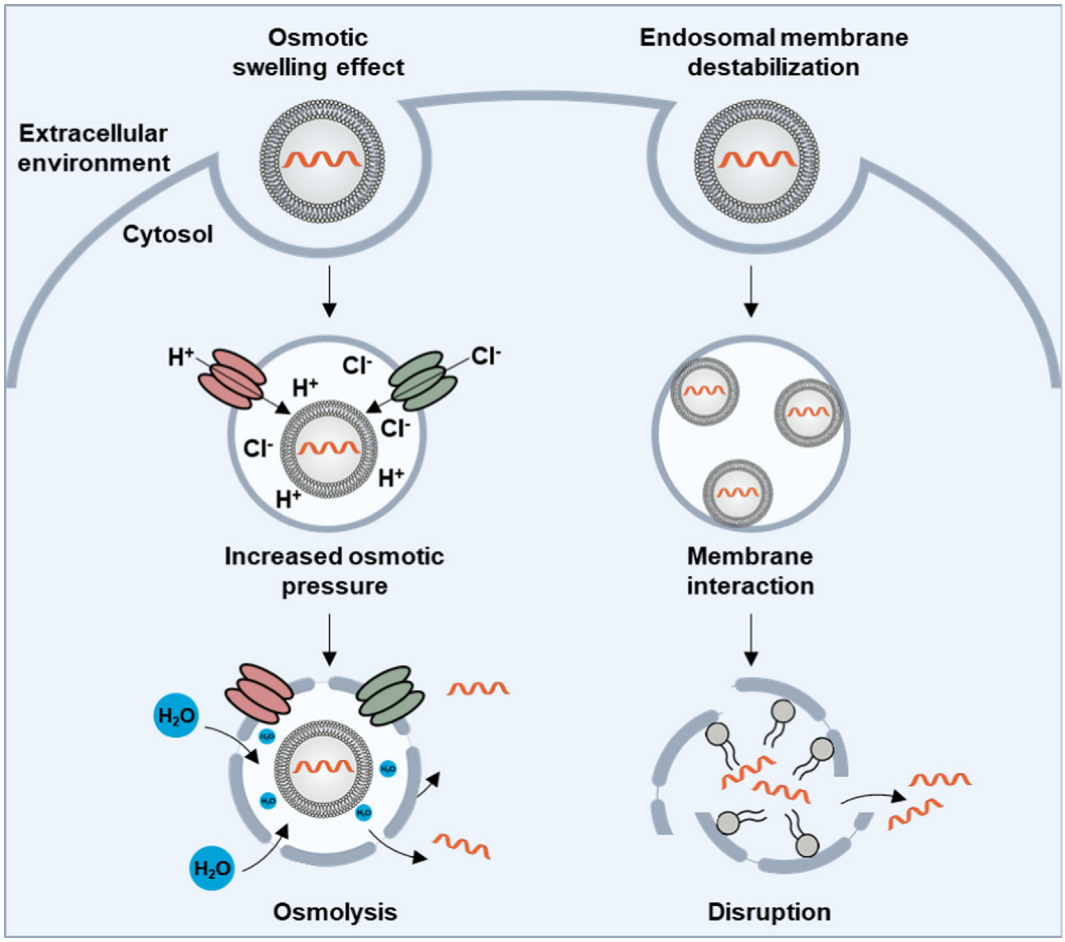

Fig. 5. Modulation of endosomal escape of nucleic acid antigens. mRNA or DNA delivered in nanovaccines need to exit the endosome and migrate to the cytosol or nucleus, respectively. Osmolysis and membrane disruption mechanisms have been used to modulate the endosomal escape of nucleic acid antigens delivered by various carriers. Osmolysis, which is induced by increased osmotic pressure inside endosomes, is also called the "osmotic swelling effect". Some polymers or lipid-based delivery systems have been proposed to interact with the endosomal membrane to disturb the endosome and thereby release nucleic acid vaccine cargoes to the cytosol.

focused on the endosomal escape of nucleic acid vaccine cargoes [158,159].

Many candidate ionizable lipids have investigated for their ability to enhance the endosomal escape of lipid nanoparticle-delivered cargoes [160,161]. Screening and structural modification are common strategies for optimizing ionizable lipids. For example, a zwitterion ionizable lipid with one ionizable amine and phosphate groups in three hydrophobic tails, can transition to a hexagonal shape and thereby enhance the escape of cargo from endosomes via membrane destabilization [162]. The tertiary amine structure of the ionizable lipid becomes protonated under the acidic pH of the endosome, leading it to fuse to the endosomal membrane. The multiple hydrophobic tails form the hexagonal structure, resulting in membrane disruption and improving downstream mRNA expression and gene editing efficiency.

Other strategies investigated for their ability to increase the endosomal escape of lipid nanoparticles have included efforts to change the molar ratio of ionizable lipids and mRNA nucleotides (Fig. 6) [139]. The authors found that an mRNA-to-ionizable lipid ratio of 1:1 could yield the most effective transport of mRNA from endosomes to the cytoplasm via the fusion of the lipid nanoparticles to endosomal membrane. Chaudhary and researchers reported that substitution of cholesterol with β-sitosterol could increase the transportation of a nanovaccine-carried mRNA to the cytosol [6]. Moreover, researchers studied the modification of phospholipids with a fusogenic moiety [163]. The use of phosphoethanolamine head group-containing phospholipids in lipid nanoparticles was shown to improve endosomal escape compared to that of lipid nanoparticles lacking the head group. In PEGylated lipids, the molecular size of polyethyleneglycol or the length of the alkyl chain were found to affect endosomal escape [164].

The lytic peptide, melittin, has been exploited to facilitate the endosomal escape of a polymeric nanovaccine-loaded nucleic acid cargo. Sylvestre et al. developed virus-inspired polymer for endosomal release (VIPER) for the pH-responsive and enhanced endosomal escape-enabled release of melittin [165]. The polymer component of VIPER, 2-diisopropylaminoethyl methacrylate, can transition from the hydrophobic to hydrophilic state at pH 6.3. Given this, VIPER can form micelles that entrap melittin within the polymer under a physiological environment but, upon exposure to the acid environment of the endosome, release melittin to disrupt the endosomal membrane. The authors found that the transfection efficiency was approximately 2 times greater when they used VIPER compared to a control polyethyleneimine polymer in a HeLa GFP transfection test in vitro.

In addition to facilitating the delivery of nucleic acid vaccines, endosomal escape can also enhance the therapeutic effect of subunit vaccines. The endosomal escape of a subunit vaccine was shown to decrease its protein degradation in the lysosome to yield more efficient epitope production in the cytosol [158]. Polymers conjugated with fluoroalkyls or fluoroaromatics enhanced the endosomal escape of cargoes, which may be attributed to the fluo-

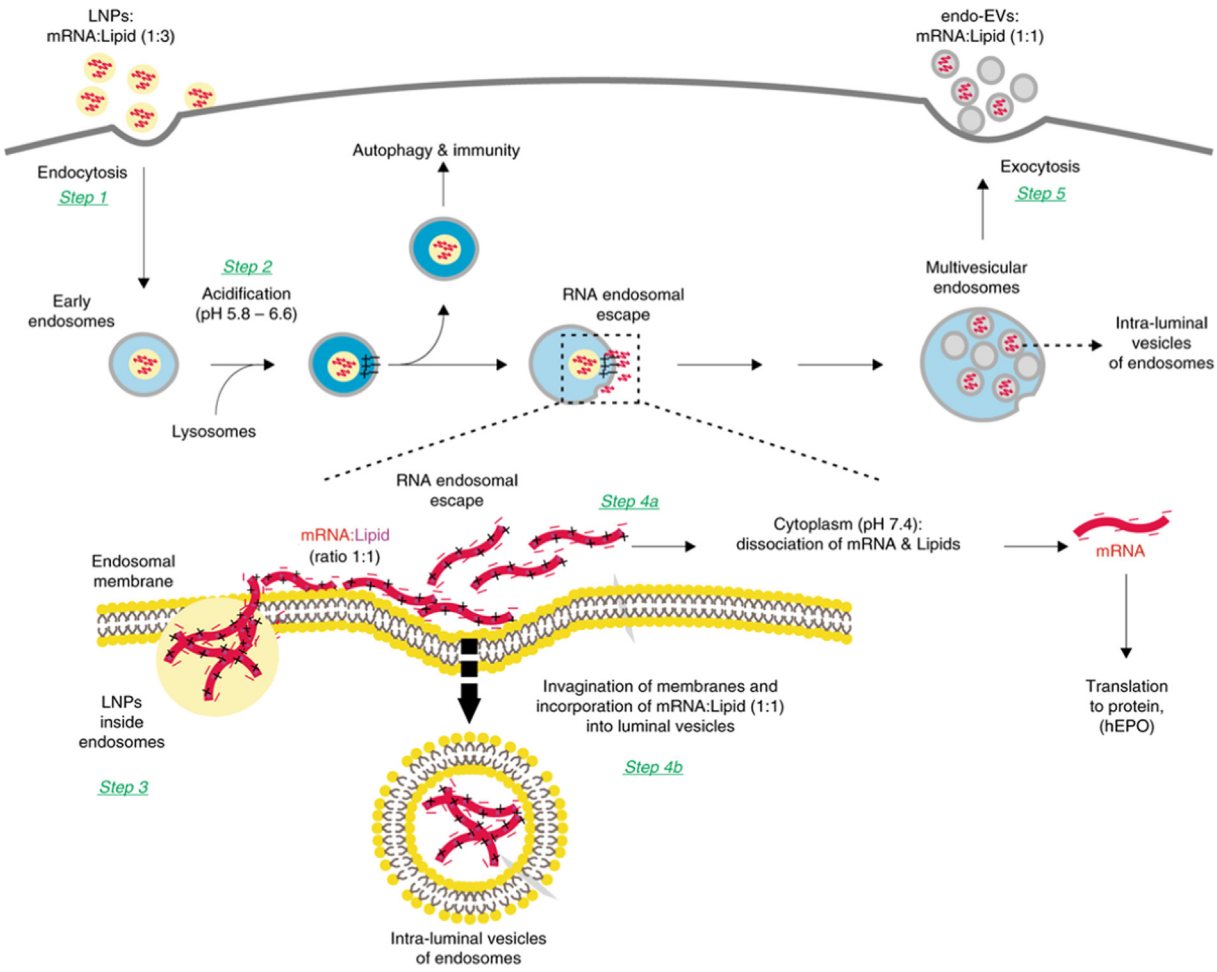

Fig. 6. Intracellular fate of mRNA-loaded lipid nanovaccines. After cellular entry of mRNA-loaded LNP nanovaccines, early endosomes fused with lysosomes. In the resulting acidic endolysosomes, the surface charges of LNP converted to positive charge due to ionizable lipids. The interaction between LNP with the endosomal membrane promoted the escape of mRNA from endosome to the cytosol. Reproduced with permission from [139].

rine atoms on the fluoropolymer. Enhanced delivery efficiency into HeLa Cells was observed with fluoropolymers carrying BSA-FITC, β-Gal, and CP11 proteins.

Protein vaccine antigens that escape from the endosome can be cleaved into optimal epitopes by the cytosol proteasome-mediated cleavage of amide bonds [159]. Senapati et al. designed a novel amphiphilic pentablock copolymer (PBC) to enhance the endosomal escape of an antigen [166]. The authors synthesized the pentablock copolymer (PDEAEM–PEO-PPO–PEO-PDEAEM) and investigated its intracellular trafficking. PBC is a pH-responsive polycationic polymer that can induce the proton sponge effect in the endosome, yielding improved cytosolic delivery in APC and enhanced humoral immunity.

Furthermore, hexa-histidine metal assembly (HmA) of a subunit vaccine was reported as a means to deliver the GP100 antigen peptide with rapid endosomal escape. The colocalization of HmA nanoparticles and endosomes in DC2.4 cells was strongly decreased compared to that in the antigen-only treatment group. The endosomal escape of the antigen was expected to protect the antigen from degradation and enhance its cross-presentation to the MHC I complex [166,167].

4.5. Other strategies for modulating intracellular trafficking

Other strategies for modulating the intracellular trafficking of nanoparticles have been exploited to enhance vaccine efficiency. Light-stimulated cytosolic delivery is one such approach (Fig. 7). Cyclodextrin-based near infrared-to-ultraviolet upconversion nanoparticles were designed to release siRNA upon irradiation with near infrared light [168]. Azobenzene-tagged siRNA formed an inclusion complex with cyclodextrin via host–guest interaction. Upon near infrared light irradiation, the nanoparticle converted the light to ultraviolet and azobenzene underwent trans-to-cis photoisomerization, releasing the siRNA to the cytosol. This strategy was notable in that it used an external light stimulus to control the endosomal escape of cargoes.

The ER and Golgi apparatus have also been reported as potential intracellular targets for vaccines (Fig. 8). ER targeting was reported to increase trafficking of nanovaccines to the nucleus via the ER-nucleus route [170], but relatively few studies have examined this strategy. Pardaxin peptide-modified cationic liposomes were shown to enhance the accumulation of nanoparticles in the ER. Additionally, the gene editing efficiency was increased by the improved nuclear trafficking of these nanoparticles via the ER-nucleus route [170,171]. For trafficking to the TGN, an amphipol-class amphipathic polymer derived from SARS-CoV-2 envelope protein was exploited [172]. The study showed that the amphipol-class polymer-based delivery of an antigen could increase its accumulation in the TGN.

Mitochondrial trafficking was also studied as a means to modulate the intracellular trafficking of nanoparticles. Mitochondria play important roles in immune signaling and the activation of

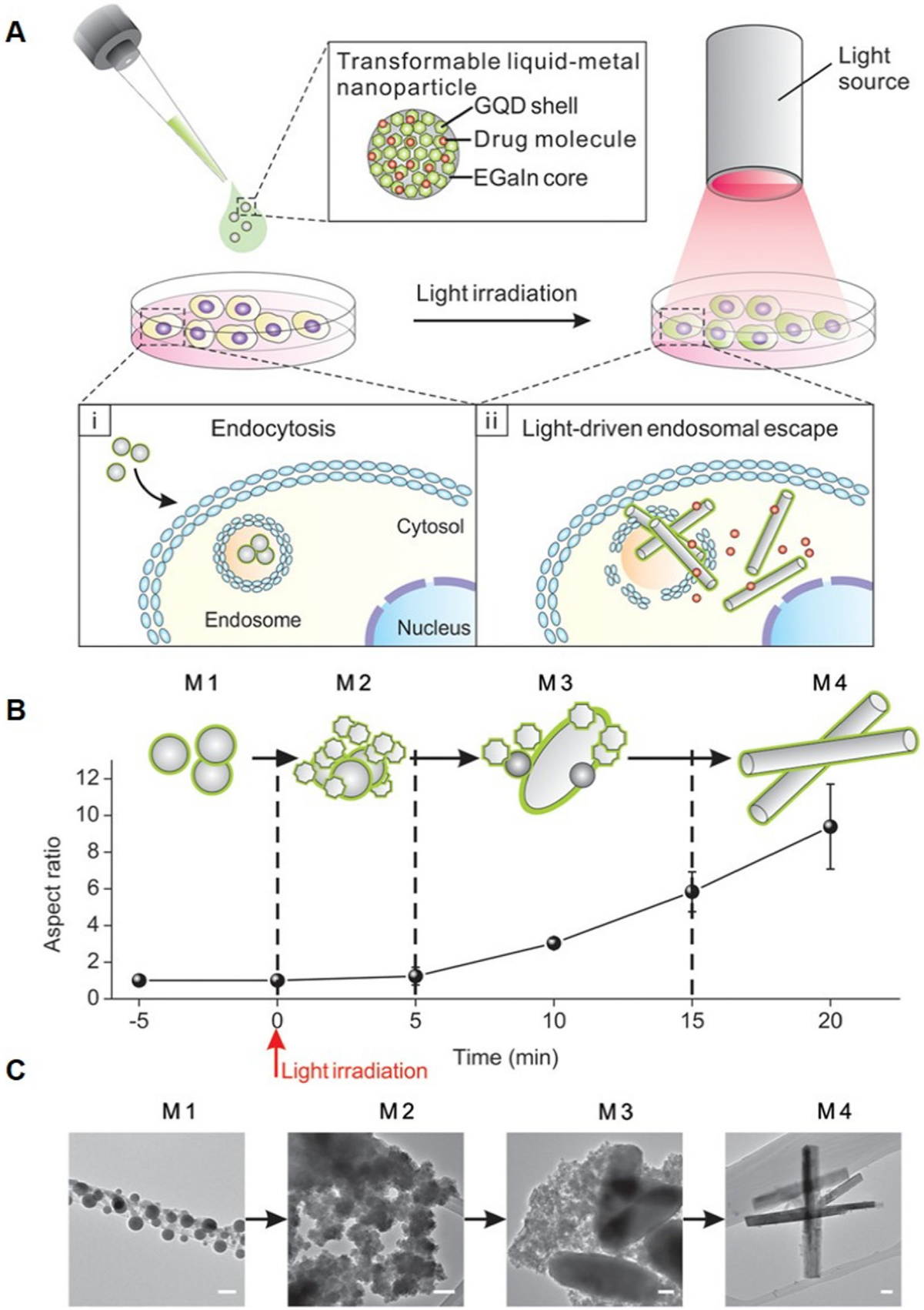

Fig. 7. Examples of a nanovaccine designed for light-responsive endosomal escape. (A) Graphene quantum dots (GQD)-coated transformable nanoparticles were exploited for light-triggered endosomal escape. (i) Endocytosis of nanoparticles. (ii) Light-responsive intracellular morphological transformation induced physical disruption of endosomal membrane. Aspect ratio (B) and representative transmission electron microscopy image (C) under different light irradiation time. Reproduced with permission from [169].

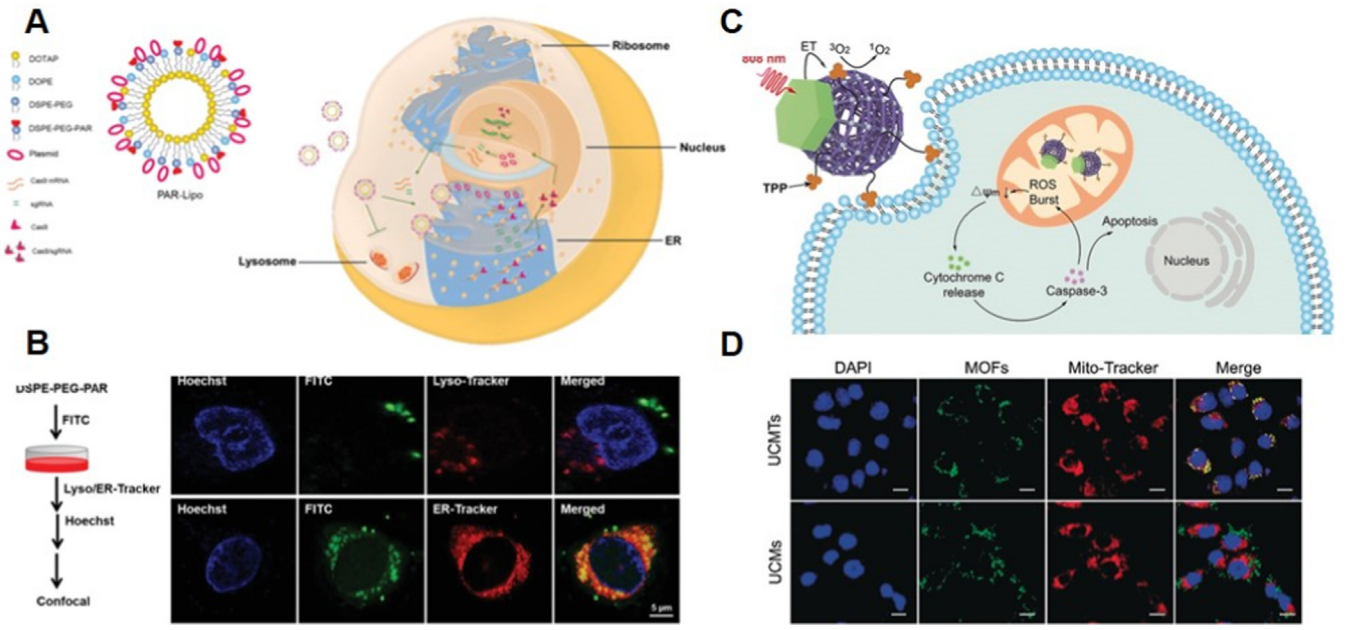

Fig. 8. Examples of nanovaccines targeting intracellular organelles. (A) Nanovaccines were proposed to bypass lysosomes capture and increase trafficking to ER. Nuclear trafficking was enhanced via ER-nucleus route, resulting in improved gene editing efficiency. (B) Co-localization images of fluorescent dye-labeled ER-targeting nanoparticles. Reproduced with permission from [170]. (C) Intracellular trafficking of triphenylphosphonium-modified metal organic framework (MOF) nanoparticles, showing accumulation in mitochondria. (D) Co-localization images of MOF nanoparticles and mitochondria. Reproduced with permission from [173].

cytotoxic T cells [174-176]. Several studies monitored the trafficking of nanoparticles to mitochondria using triphenylphosphonium as a mitochondria-targeting ligand [177]. Here, mitochondrial trafficking must be preceded by endosomal escape. Going forward, therefore, researchers should consider both endosomal escape and subsequent mitochondrial targeting when designing a mitochondria-trafficking nanovaccine.

In addition to the intracellular trafficking of cargoes, the interaction of cargo materials with cellular receptors needs to be studied further. Molecular interaction between cargoes and cellular receptor can affect the triggering of innate or adaptive immunity. If mRNA vaccine antigens bind to pattern-recognition receptors (e.g., TLR-3 and −7/8) on immune cells, innate immunity will be induced and proinflammatory cytokines will be produced [178]. Inhibition of the binding of mRNA antigens to pattern-recognition receptors, in contrast, may trigger adaptive immunity [179].

5. Current challenges and perspectives

The existing studies on the in vivo fate and intracellular trafficking of vaccine platforms have provided a background to support the development of potent vaccine formulations, but their clinical translation still faces a number of challenges. Modulations of vaccine formulations have been investigated as a means to increase the induction of immune responses and overcome the hurdles facing the current vaccine delivery systems. Various administration routes and intracellular delivery strategies have been reported as promising and effective in both in vitro and in vivo experiments.

The fate of vaccines in vivo has mainly been studied in small rodents. Although recent studies have reported substantial achievements for vaccine delivery systems and their in vivo fate monitoring in mice, the potential for species-level differences must be considered. Because the relevant anatomical structures, such as the digestive and respiratory tracts, differ from model animals to human, preclinical results related to vaccine delivery will not accurately reflect the clinical results for oral, nasal, and pulmonary delivery systems. The thickness and strength of the skin also differ by species and age, making it difficult to generalize the accumulating results pertaining to the in vivo distribution of vaccines after intramuscular and intradermal administration in model animals. To address these issues, several preclinical studies have been performed in nonhuman primates [180,181]. Since some recent vaccines for COVID-19 were inevitably used before the clinical trials were completed, preclinical studies on the in vivo fate of these vaccine should be done in nonhuman primates.

Most of the published studies have examined the efficacy of vaccine formulations in 2-dimensional cell culture in vitro. This cannot reflect the in vivo extracellular environment of various immune tissues and does not enable the researcher to investigate the interactions of the vaccine with immune cells. Therefore, it would be useful to develop 3-dimensional culture systems that could more closely mimic the extracellular matrix of various immune tissues. Indeed, a 3-dimesional system modulated by extracellular matrix was used to examine the host-pathogen interactions of *Mycobacterium tuberculosis* [182]. Going forward, the inter- and intracellular trafficking of vaccines could be investigated using a 3-dimensional cell culture system that more closely reflects the extracellular environment of immune tissues.

The need to establish in vitro experiments with appropriate cells is another concern when studies use primary dendritic cells to evaluate the immune response triggered by various vaccine formulations. Since various APC are involved in the immune response at each organ, the origin and culture conditions of primary dendritic cells should be carefully controlled. For example, bone marrow-derived dendritic cells were used for in vitro studies after being ex vivo-induced from bone marrow cells; thus, it is relevant that the features of bone marrow-derived dendritic cells can vary depending on the composition of the culture medium. In addition, different laboratories have used cells of different phenotypes and reactive patterns. Going forward, researchers should strive to standardize the cells used to study vaccine fates in vitro.

It has been reported that dendritic cells can show heterogeneous expression of receptors [183], which may affect the

receptor-targeted trafficking of nanovaccines to dendritic cells. Strategies to overcome the physiological heterogeneity of receptor expression levels on APC need to be studied further. In addition, the in vivo formation of protein coronas on the surfaces of nanoparticles may cloak the targeting moiety of nanovaccines. Further design efforts should be made to prevent the formation of a nonspecific corona coating on the surfaces of ligand-modified nanovaccines.

In the published in vivo studies of vaccine fate, fluorescence markers or radioisotopes were used to specifically label the antigen or delivery system, but not both. The colocalization of antigens and delivery systems should be addressed. The distribution of DNA or mRNA vaccines has been monitored by assessing the expression patterns of the encoded proteins. To compare these findings to the distribution of the delivered materials, researchers should assess the distribution of the nucleic acid cargo itself using a quantitative detection assay (e.g., quantitative polymerase chain reaction).

In vivo fates have been tested by monitoring either nanovaccine components or vaccine antigens. As these distributions may overlap and/or differ, dual labeling of the vaccine carrier and loaded antigens could provide new information on the retention of antigens in the carrier vaccines and/or their liberation from nanomaterials. Finally, relatively few studies have reported on the in vivo trafficking of nanovaccine carriers and antigen cargoes.

Most of the relevant studies followed the in vivo trafficking of nanovaccines in lymph nodes but did not extensively examine that in other organs. Among the studies that focused on lymph nodes, most examined draining lymph nodes near the injection sites. Further work is needed to assess distribution kinetics to the proximal and distal lymph nodes. The few studies that assessed in vivo trafficking at various organs beyond lymph nodes tended to report only qualitative imaging information. The proportion of the injected dose found at various organs has been radioisotopically quantified in a few cases. Although the distribution to other organs may not reflect the immune efficacy of the administered vaccine nanoparticle, these data would be meaningful in helping to predict the potential safety of a vaccine.

In vivo vaccine fates have mainly been studied at the tissue or organ levels. Given that the final target cells of vaccines are APC, more elaborate analyses of in vivo trafficking to APC is expected to provide new insights. More studies should undertake lymph node tissue staining for identification of APC and analysis of their colocalization with the administered antigen-carrying nanovaccines.

Cold-chain maintenance during distribution remains as challenges of lipid- based vaccines. Further studies are needed to develop stable lipid-based vaccine formulations. To increase the stability during storage, solid dosage forms of vaccine formulations may be one approach. Powder vaccines can be manufactured by spray drying or lyophilization. In spray drying process, thermal stability of vaccine antigens would be carefully checked. In lyophilization, the selection of cryoprotectants which can retain the integrity of lipid-based nanovaccine structures need to be studied further. Another approach would be the design of new lipid molecules which can resist to temperature-dependent physicochemical degradation pathways.

6. Conclusion

In light of the unprecedented pandemic situation we find ourselves in, huge efforts are being made worldwide to develop effective vaccines. The development of vaccine delivery systems is important, as is the generation of stable and efficient antigens. There are various antigen vectors, vaccine carrier materials, and vaccine administration routes, meaning that there is near limitless potential for further development of vaccine delivery systems. However, if we hope to accurately control the efficacy of various vaccine formulations at the tissue and cellular levels, we must continue accumulating knowledge on the in vivo fate and intracellular trafficking of vaccines. A more effective vaccine delivery system can be developed through strategies aimed at modifying intracellular penetration, enabling endosomal escape, enhancing translation, and/or utilizing a route of administration that is relevant to the point of action of an infectious disease. Although various hurdles face studies on the in vivo fate and intracellular trafficking of vaccines (e.g., limitations specific to a given administration route, species specificity, thermal stability, etc.) such work will form a cornerstone for the future development of effective vaccine delivery systems.

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.

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