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EXPERT REVIEW

Nanotechnology for Enhanced Cytoplasmic and Organelle Delivery of Bioactive Molecules to Immune Cells

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Abstract

Immune cells stand as a critical component of the immune system to maintain the internal environment homeostasis. The dysfunction of immune cells can result in various life-threatening diseases, including refractory infection, diabetes, cardiovascular disease, and cancer. Therefore, strategies to standardize or even enhance the function of immune cells are critical. Recently, nanotechnology has been highly researched and extensively applied for enhancing the cytoplasmic delivery of bioactive molecules to immune cells, providing efficient approaches to correct *in vivo* and *in vitro* dysfunction of immune cells. This review focuses on the technologies and challenges involved in improving endo-lysosomal escape, cytoplasmic release and organelle targeted delivery of different bioactive molecules in immune cells. Furthermore, it will elaborate on the broader vision of applying nanotechnology for treating immune cell-related diseases and constructing immune therapies and cytopharmaceuticals as potential treatments for diseases.

KEY WORDS bioactive molecules · cytoplasmic and organelle delivery · immune cells · nanotechnology

INTRODUCTION

The body's immunity incarnates the essence of the "security officers" by recognizing and eliminating invading pathogens and maintaining the homeostasis of the internal environment through coordinating with other systems. The adaptive and innate immunity enclosures are collectively responsible for the body's immunity. Furthermore, the innate immunity enclosure is mainly constituted with phagocytic cells (dendritic cells and macrophage) and granulocytes (mast cells, eosinophils, basophils, and neutrophils) acting as the front line of defense of the body. When a pathogen invades the body, it generates molecular patterns and signals that can be interpreted by the immune cells, which then respond by activating and recruiting themselves to the damaged, inflamed, and infected tissues (1). The dysfunction of such immune cells may lead to immune-mediated inflammatory diseases that lack cure, including rheumatism, asthma, cutaneous inflammatory conditions, autoimmune neurological disorders, type 1 and type 2 diabetes, Crohn's disease, ulcerative colitis, inflammatory bowel disease, and connective tissue disorder (2). In addition to the ability to progress over time, these diseases are frequently aligned with several concomitant medical conditions that affect the cognitive system, cardiovascular function, metabolic environment, and bone structure (3). Therefore, the treatment for restoring the function of innate immune cells in immune-mediated inflammatory diseases through targeted cell therapy is also a subject that needs extensive exploration (4).

Besides, the immune system's role is not only responsible for fighting infection and inflammation but also it performs immune surveillance. Tumors are usually regarded as genetic diseases due to genetic mutations that cause cells to gain infinite proliferation. Hence, the mutation burden of tumor cells plays an essential role on the immune recognition and sensitivity to immune therapy (5). As an element of the innate immune enclosure, dendritic cells are responsible for promoting subsequent antitumor immune reactions by phagocytosis and presenting tumor antigens due to their vital role as antigen-presenting cells (APCs). The adaptive immune enclosure performs a crucial role in the

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antitumor immune response through T cells. APCs generate co-stimulatory signals and tumor antigens resulting in T cells transformation to effector T cells within lymphoid organs; these tumor-specific effector T cells can relocate and infiltrate cancerous tissues to induce tumor cell apoptosis. On the other hand, the tumors will not comply with immune system manipulation. The tumor defense against the immune system relies on "fast growth" and actively adapts various strategies to delay, change, or even prevent antitumor immunity. It can achieve efficient immune suppression by implementing features that help evade immune system detection or by stimulating immune-related negative regulatory pathways (6). The current enhanced immunotherapies are generally divided into two strategies: 1) highly effective immune enhancement, such as enhancing direct apoptosis of tumor cells using adaptive immune cell therapies (engineered T cells such as chimeric antigen receptor T cells, CAR-T). 2) enhancing the immune system activation by modulating endogenous immune mechanisms. One of the representatives of these therapies is the dendritic cells-based vaccine, which can enhance antigen uptake, processing and presentation to T cells for facilitating activation and growth of naive T cells (7, 8). Therefore, standardizing and improving immune cells' effectiveness is the key to curing many diseases.

However, as mentioned above, to achieve immune cell modulation, many bioactive molecules such as small molecules (chemicals) and some macromolecules (nucleic acids, proteins) must be delivered efficiently to the immune cells. Especially for macromolecules such as nucleic acids and proteins, instability and low penetration through cell membranes directly hinder their application in immune cells (9). In the past twenty years, nanotechnology's growth has enhanced bioactive molecule delivery. With the help of nanocarriers, macromolecules such as proteins can better penetrate cells and escape from lysosomes, while nucleic acids and easily degradable macromolecules, can remain stable until reaching their intracellular targets. Various nanocarriers have been implemented as means for transporting bioactive molecules to immune cells (10). Nevertheless, the intracellular barrier of immune cells is still a major obstacle that affects bioactive molecules' effectiveness. Nanocarriers can transport cargo into immune cells by endocytosis or by enhancing the permeability of cell membranes (11). Subsequent to internalization, almost all major internalization methods deliver cargos to the endocytic vesicles (12), which will successively fuse with the early and late endosomes, resulting in their compartmentalization in the lysosomal vesicles. As the fusion occurs, there is a gradual decrease of pH together with the accumulation of digestive enzymes (nucleases, lipases, and proteases) within vesicles, which may lead to degradation of nanocarriers and their payload, thereby affecting their efficacy (13). Extensive research has shown that nanocarriers with endo-lysosomal escape functions could enhance cytoplasmic delivery (14). Figure 1 summarizes these concrete methods. Meanwhile, after entering the cytoplasm, the payloads in nanocarriers need to be released before exerting their biological action. It has been reported that the cytoplasmic release of nanocarriers can be triggered by endogenous stimuli (pH, enzyme, and

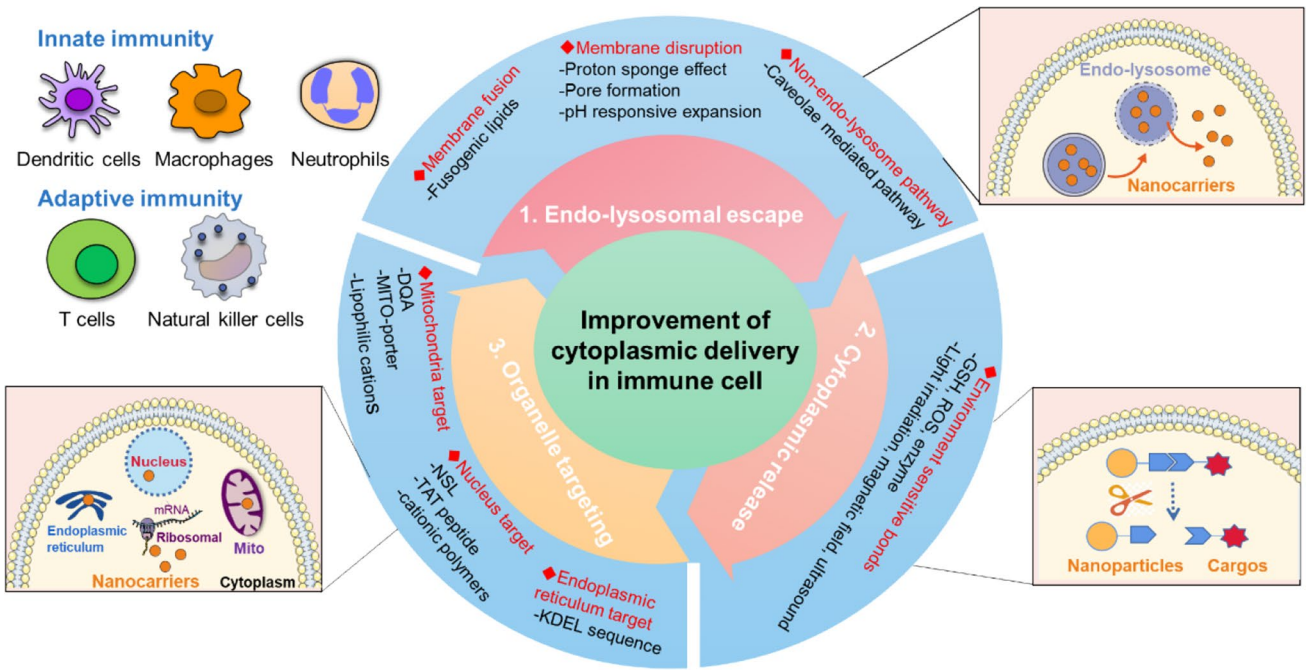

Fig. 1 Schematic diagram of improving cytoplasmic delivery of bioactive molecules to immune cells by nanotechnology.

glutathione) (15, 16), and exogenous stimuli (light irradiation, magnetic field, and ultrasound) (17, 18) (Fig. 1). In addition, some cargoes with organelle-specific pharmacologic action require organelle-targeting nanocarriers for their delivery. With regard to those cargos, even if they reach the cytoplasm, they cannot achieve their subcellular targets because of the many obstacles to targeting organelles. For the targets of genetic drugs such as plasmids, the nucleus is surrounded by a double-layered nuclear membrane, leaving a nuclear pore complex (NPC) with a central channel of only 9 nm for nucleocytoplasmic transport. It turns out that only 0.1% of the free plasmids in the cytoplasm can reach the nucleus to exert their pharmacological effects, which significantly reduces the efficacy (19). In order to solve the problem of low transport capacity in the nucleus, nanotechnology can not only protect the easily degradable plasmid but also achieve nuclear targeting by adding some specific nuclear localization signaling peptides to the nanocarrier to simulate the way some DNA viruses enter the nuclear envelope. Similarly, mitochondria, which are called cellular energy factories, have also been extensively studied as a subcellular target commonly used in immune cells. Mitochondrion is a target for many cytotoxic drugs and a clearance target for lipid accumulation in some cardiovascular diseases. However, the negatively charged mitochondrial bilayer prevents most bioactive molecules from entering the mitochondria. Nanotechnology can use charge attraction or imitate high-density lipoprotein to help bioactive molecules accumulate in the mitochondria to play a better pharmacological effect. Targeting enzymes in endosomes/lysosomes of immune cells could help reduce inflammation or enhance killing. Regarding endosomal/lysosomal targeting, it is difficult to achieve endosomal/lysosomal accumulation of bioactive molecules by endocytosis while avoiding degradation by the endosomal/lysosomal environment. Several innovative designs are used to resolve this paradox (Fig. 1).

In this review, we will summarize different nanotechnology strategies in promoting the evolution of immune cell-based therapies based on three basic aspects: 1) endolysosomal escape; 2) cytoplasmic release, and 3) organelle targeting for improving the delivery of bioactive molecules into immune cells (neutrophils, natural killer cells, dendritic cells, T cells, and macrophages) (Fig. 1).

Cellular internalization

As the first barrier to entry, the cell membrane, immune cells also have different methods to overcome this barrier and ingest the nanoparticles, which may adjust and mediate various functions of immune cells. For example, different uptake methods in T cells can regulate T cell receptor signaling, antigen discovery and activating cell growth (20). The content of this part has been extensively explored as normal biology (21–23), and the different endocytosis methods and mechanisms of different immune cells have been summarized in Table I. Immune cells can internalize nanocarriers through different pathways depending on the shape, dimension, and surface chemistry (24). Internalization of nanoparticles with a diameter smaller than 200 nm occurs by clathrin-mediated endocytosis, caveolae-dependent endocytosis, and clathrin- and caveolae-independent endocytosis. Nanoparticles with a diameter larger than 200 nm necessitate the use of phagocytosis and macropinocytosis pathways (23). For phagocytic cells like macrophages, neutrophils, and dendritic cells, endocytosis is the usual way to uptake nanoparticles, while for relatively non-endocytotic cells such as T cells, strategies of disturbing membrane or enhancing the permeability of the cell membrane are widely used for

Table I The uptake mechanisms for various immune cells

Type of endocytosis	Mechanism of internalization	Cells
Phagocytosis	Phagocytic receptors and complement receptors led to the rearrangements in the actin cytoskeleton that induce internalization	Macrophages (25) Neutrophils (26) T cells (27)
Macropinocytosis	Several growth factors trigger plasma membrane ruffling, followed by sealing of aperture to form macropinosomes	Macrophages (28) Neutrophils (29) T cells (30) Dendritic cells (31)
Clathrin-mediated endocytosis	The ligand binds to the cell surface receptor, which results in the assembly of clathrin triskelions driving the formation of clathrin-coated pits (CCPs) which engulfs the particle	Dendritic cells (32) Macrophages (33) Neutrophils (34) T cells (35)
Caveolae-dependent endocytosis	Caveosomes (via caveolin protein) arise on the membrane to pack the granule	Macrophages (36) Neutrophils (37)
Clathrin- and caveolae-independent endocytosis	Various Mechanism	Dendritic cells (32) T cells (38) Nature kill cells (39)

the effective internalization of nanoparticles. Post-cellular uptake of nanoparticles, the intracellular barrier is a major challenge for the bioactive molecules.

CYTOPLASMIC DELIVERY IN DENDRITIC CELLS

As the primary APCs, dendritic cells (DCs) have a vital role in antigen presentation to generate innate or adaptive immune responses (40). The efficacy of induced immune response depends on antigens loading capability or the maturity of DCs. Poor cellular uptake of antigens by DCs leads to insufficient immune activation. Meanwhile, immature DCs express low CCR7 and MHC class I/II molecules, hindering further antigen presentation and immune responses. Thus, enhanced antigen delivery to DCs is the main challenge for generating successful DCs-based immunotherapies. At present, nanotechnology offers an excellent antigen delivery platform for therapies based on DCs (41). Various nanocarriers can directly deliver antigens into DC cells. Furthermore, nanocarriers' cytostatic gene delivery and knockout contribute to co-stimulatory factors or attenuating inhibitory signals in DCs. Another strategy for improving dendritic cell regulation of T cells' immune responses is to use adjuvants with nano-drug characteristics to overcome the tumor-micro environment immunosuppressing barrier.

Nanotechnology for Improved Endo-Lysosomal Escape in Dendritic Cells

Previous reports revealed that DCs have neutral pH in early endosomes and lower endo-lysosomal enzyme activity than the degradative macrophages, allowing antigen to escape into the cytoplasm (42). With the maturation of endosomes, proteins derived from the endoplasmic reticulum are reduced, resulting in a falling of pH levels and an increase in proteolysis, leading to faults in cross-presentation. However, they gain characteristics required by MHC class II molecules for peptide hauling (43). Variations in pH levels occur intermittently, approaching 4.5 during late endosomes in DCs. Hence, it is critical to apply pH-sensitive delivery systems and endosomal evasion behaviors to enhance the bioavailability of bioactive molecules. Ma *et al.* designed a pH-responsive poly (D, L-lactic-co-glycolic acid) (PLGA) NPs with NH_4HCO_3 and OVA encapsulation by integrating W/O/W emulsion diffusion extraction method with premix membrane emulsification method. By adjusting the internal water phase to oil phase ratio, PLGA NPs had larger internal space and thinner shells, which promoted NH_4HCO_3 and OVA encapsulation and pH-responsive antigen intracellular release behavior. Upon endocytosis by DCs, PLGA NPs were transported to endosomes and fused with lysosomes where the hydrogen ions could infiltrate the PLGA shell and react with NH_4HCO_3 to produce NH_3 and CO_2. Expansion of these gases destroyed the thinner shells and the lysosomal membrane, leading to effective antigen release and lysosomal escape. The enhanced cross-presentation of pH-responsive PLGA NPs stimulated the increase in levels of co-stimulatory molecules in DCs. Also, they mediated effective lymphocyte activation. Hence it is demonstrated as a potential approach for vaccine delivery and adjuvant systems (44).

Morphological changes of nanoparticles triggered by an acidic late endosomal environment can also induce endosome escape. Liang *et al.* designed a novel proton-driven nano transformer-based vaccine (NTV) composed of a polymer-peptide conjugate-based nanotransformer and loaded antigenic peptide ($OVA_{241-270}$). At pH 7.4, the nanotransformer is formed by auto-assembling amphiphilic polymer peptide synthesized by conjugation of p($DMAEMA_{22}$-$OGEMA_4$)-b-p$(MAVE)_{30}$ with the hydroxylated pyrene-coupled d-peptide (PDP) through a pH response cleavage of acetal bond. When instantaneous cleavage of PDP peptide is initiated within the acidic environment (pH 5.6) of the endosomes, NTV experiences rapid and extensive morphological transformation, from nanospheres (about 100 nm in diameter) into nanosheets (about 5–8 μm in length or width). This change disturbs the mechanical stability of the endosome and promotes cytoplasmic delivery of antigenic peptides. Furthermore, the nanosheets within the cytoplasm initiate the NLRP3 inflammasome pathway, thus sustaining antigen processing and growth of DCs. This role enhances the cross-presentation of antigens to $CD8^+$ T cells, resulting in sufficient inhibition of tumor development and providing a potent and secure DC-based immunotherapy, which can also serve as a potential therapy for various infectious diseases when packed with pathogenic antigens (45). To enhance DCs-based vaccine potency, Ma.Y *et al.* conjugated galactosyl dextran with all-trans-retinal (an analog of vitamin A) through a pH-sensitive hydrazone bond to construct galactosyl dextran-retinal (GDR) nanogels for cancer vaccine delivery, in which the addition of galactosyl allowed the targeting of DCs. When dissolved in water, pH-responsive nanogels were formed via GDR self-assembly, which facilitated the encapsulation efficiency of OVA antigen for the formation of GDR/OVA nanovaccine. Once in acidic endo/lysosomes, GDR is triggered to disassemble, causing rupture of the lysosome and facilitating the escape of antigens from lysosomes. The release of all-trans-retinal promoted DCs maturation through activation of retinoic acid receptor signaling and promoted both antigen uptake and cytosolic antigen release in DCs. The pH-sensitive GDR nanogels can promote MHC-I antigen presentation, triggering potent *in vivo* immune responses against cancer. Moreover, the lysosome rupture can initiate the formation of reactive oxygen

species (ROS), which are essential for enhancing proteasome activity, a crucial enzyme for MHC I cytosolic antigen presentation. These discoveries provide powerful support for applying pH-sensitive nanocarriers to improve the efficacy of anti-cancer vaccines through antigen cross-presentation (46). Additionally, inhibition of immune-suppressing genes (SOCSI or PD/PD-L1) is considered to be a potent strategy to enhance DC function. Harashima *et al.* reported delivering siRNA of SOCS1 to DCs using octa-arginine (R8)-modified lipid envelope-type nanoparticles (R8-MEND). Intracellular trafficking revealed that endosome evasion is a substantial rate-limiting process for siRNA delivery. DOPE and PA with optimized ratio were applied to construct R8-MEND, promoting endosomal escape due to the high fusogenic activity in an acidic environment. The effective siRNA cytoplasm delivery induced the knockdown of immunosuppressive gene SOCS1 and consequently enhanced DCs-based vaccine potency *in vivo* (47).

Nanotechnology for Improved Cytoplasmic Release in Dendritic Cells

The effective lysosomal escape followed by the intracellular release of the cargos is equally essential. Taking advantage of the intracellular reductive environment derived from the abundant intracellular glutathione (GSH) in DCs, when exposed to extracellular conditions, the disulfide bonds possess moderate stability. However, the cleavage of the disulfide bonds can occur by exchange reaction with GSH, making it widely used to trigger intracellular drug release. Hence, Stayton *et al.* designed a pH and GSH responsive polymer micelle with a pH-sensitive hydrophobic core that can drive endosomal membrane destabilization and a neutral hydrophilic corona component with a hanging pyridyl disulfide moieties that can reversibly conjugate with thiolated OVA. Under the physiological environment, the diblock polymer exhibited self-assembling properties by forming micelles 25–30 nm in size. Investigations performed in murine dendritic cell lines (DC 2.4) showed that this polymer micelle could enhance the ability to retain intracellular antigens and antigen accumulation in the cytoplasm owing to endosomal escape and intracellular release related to the cleavage of disulfide bonds, finally resulting in improving antigen-specific $CD8^+$ T cell responses and antigen cross-presentation (48).

Coincidentally, Jon *et al.* prepared an antigen and adjuvant-loaded small lipid nanoparticles (OVAPEP-SLNP@CpG) as nanovaccine for inducing an antitumor immune response. OVAPEP-SLNP@CpG used DOPE as the endosomal escape lipid to improve the transfer of antigens from endosomes to the cytosol. The monoarginine-cholesterol (MA-Chol) acted as a cationic material for complexing the adjuvant of CpG oligodeoxynucleotide into lipid nanoparticles. In addition, the antigen of OVAPEP is connected to lipid nanoparticles through disulfide bonds with PEGylated phospholipid. After entering into (DC2.4) murine DCs, OVAPEP-SLNP@CpG could escape from the endosome and release its contents to the cytoplasm. The high cytosolic concentration of GSH can result in selective disulfide bond cleavage through a thiol-disulfide exchange reaction, finally promoting the release of OVAPEP in the cytoplasm. The nanovaccine prepared exhibited a high potency to induce tumor-specific $CD8^+$ T cell response and high efficacy against tumor in prophylactic and therapeutic E.G7 tumor models (49).

CYTOPLASMIC DELIVERY IN MACROPHAGES

Macrophages are among the essential cells of the immune system. They are responsible for the phagocytosis of cellular fragments and pathogens, and the activation of other immune cells in reaction to pathogens, thus playing various roles in maintaining physiological homeostasis (50). Moreover, macrophages perform complex roles in the tumor microenvironment. (51). As a result, they have attracted researchers to investigate their application in immuno-oncology and the pathogen-targeted treatment of chronic infections caused by intracellular pathogens. Due to the high plasticity in macrophages, either M1 or M2 activation can occur under different stimuli (52). M1 macrophages can cause incredible tumoricidal activity through tumor necrosis factor-alpha and nitric oxide. On the contrary, tumor-associated macrophages (TAMs) and other M2 macrophages are potential targets during tumor therapy because of their reciprocal interaction with cancer cells (53). In this part, we will discuss the current approaches that are used to attain effective delivery of bioactive molecules to macrophages, also we will analyze current obstacles facing this process.

Nanotechnology for Improved Endo-Lysosomal Escape in Macrophages

Phagocytic cells such as macrophages can internalize nanocarriers through phagocytosis. Nanocarriers are then transported into the endosomes' low pH (pH 5–6) environment upon entering macrophages. Afterward, they are transferred into the lysosomes, where the pH (pH 4–5) is lower than the endosomes due to the digestive enzymes such as cathepsins and glucosidases (54, 55). Furthermore, they can avoid degradation and accumulate effectively in the target organelles only after their timely release from the lysosomes. In order to achieve lysosomal escape, it is necessary to take advantage of the enzymes in the lysosomes, the high reductive potential, and the low pH in the organelles. For instance, Giorgio *et al.* utilized a mannosylated tri-block polymer

to deliver siRNA to TAMs for reprogramming TAMs to adopt an immunogenic *in vitro* and *in vivo* anti-tumor phenotype. Mannosylated triblock polymers were fabricated by RAFT polymerization of butyl methacrylate (BMA), 2-propylacrylic acid (PAA), and 2-(dimethylamino)ethyl methacrylate (DMAEMA), creating a terpolymer that can respond to pH changes and also possesses elastic properties for endosome escape. This mannosylated tri-block polymer could efficiently condense siRNA into nanoparticles and get transported into the endo-lysosome via mannose receptor-mediated endocytosis. The pH-sensitive portion provided by the combination of DMAEMA and PAA would lead to the endosomal escape through the proton sponge effect, facilitating the cytoplasmic transport of siRNA (56). Besides, in order to deliver antibiotics for treating bacterial infections, Chen *et al.* applied a PEGylated and mannosylated graphene oxide (GO-PEG-MAN) to load rifampicin (Rif@GO-PEG-MAN) for delivering into macrophages. The results showed that Rif@GO-PEG-MAN exhibited increased uptake by macrophages via mannose receptor-mediated endocytosis and was readily transported into lysosomes. The protonation of the amine group causes cleavage of the hydrogen bond between the multi-functionalized graphene oxide carriers and rifampicin; this will lead to an increase in the average diameter in Rif@GO-PEG-MAN to 1100 nm in acidic lysosomal condition, promoting the lysosomal escape and rifampicin release. Rif@GO-PEG-MAN improved the delivery of rifampicin into cells and increased the *in vivo* and *ex vivo* efficacy in the cessation of intracellular BCG and Mtb bacilli contaminated macrophages (57). Another pH-sensitive approach for implementing effective intracellular release is to use an acid-intolerant linker between drugs and vehicles. Couvreur *et al.* synthesized an amphiphilic nanoparticle based on benzylpenicillin (PNG) attached to squalene through a pH-sensitive acyloxymethyl ester bond that has extreme chemical and enzymatic hydrolysis sensitivity. This nanoparticle spontaneously self-assembled in aqueous media and entered into the macrophage cell line J774 through clathrin-dependent endocytosis, further accumulating in acidic late endosomes and lysosomes. Sequentially, the rupture of pH-sensitive bonds and PNG protonation would help PNG cross intracellular membranes and release into the cytoplasm, resulting in a significant antibacterial effect against intracellular S. aureus (58). Since macrophages can serve as hosts for many pathogens without damaging them, it is crucial to investigate their distribution pattern and ability to evade the host immune system to facilitate their application for drug delivery. Listeria monocytogenes demonstrate a revolutionary approach for maintaining its survival within microphages by releasing cholesterol-dependent pore-forming toxin listeriolysin-O (LLO). Under the effect of LLO, phagosome and lysosome fusion are interrupted by the imbalance of calcium and pH gradient across the phagosome membrane (59, 60). Also, bacterial escape from the phagosome to the cytoplasm can be promoted through a combined effect of LLO and phospholipases ((61, 62). Inspired by the endosome-perforating mechanism of Listeria monocytogenes, Lee *et al.* designed pH-sensitive liposomes that co-encapsulated with antigenic protein and LLO. Compared to DCs, LLO-mediated endo-lysosomal escape was more pronounced in macrophages due to its cholesterol content in the endo-lysosomal membrane and the rate of acidification, which were beneficial to the activity of LLO (63).

Nanotechnology for Improved Cytoplasmic Release in Macrophages

When bacteria infect a cell, the resulting microenvironment can induce drug release in the cytoplasm. For example, Wang *et al.* developed a lipase-sensitive polymeric triple-layered nanogel (TLN) comprised of a polyphosphoester core and PEG shell inserted by lipase-sensitive poly (ε-caprolactone) (PCL) layer, which forms a packed hydrophobic molecular fence. The PCL layer in the TLN would degrade upon encountering the intracellular lipase-secreting bacteria in Raw264.7 macrophages. This degradation results in vancomycin release from the nanogel, further enhancing the antimicrobial activity on intracellular S. aureus infection compared to the effect of free vancomycin (64). Exogenous stimuli have also attracted attention in improving the intracellular release of bioactive molecules. Hubbell *et al.* developed light-responsive polymersomes to interrupt endosomes and enhance the release efficiency of cargo into the cytoplasm. The polymersomes form by an oxidation-sensitive block copolymer poly(ethylene–glycol)-block-poly(propylene sulfide) (PEG_{17}-b-PPS_{30}) loaded with ethyl eosin as photosensitizer within the PPS-rich membrane's inner leaflet. Upon optical excitation, the polarity of ethyl eosin would change from hydrophobic to hydrophilic, disrupting the polarity balance of the block copolymer components, resulting in the rupture of polymersomes and the release of payload. The polymersomes can quickly escape from the endosome whenever RAW 264.7 macrophages endocytose them under the optofluidic interaction. This process is due to the polymersomes' ability to transform into micellar surfactants, thus causing pore formation in the endosomal membrane. Subsequently, a rapid (milliseconds) release and distribution of the polymersomes payload occur all over the cytoplasm. This optofluidic approach can be a potential strategy for using polymersomes rupture as a means of precise intracellular delivery, which can be used in research involving single and population cells for controlling cellular activities and therapeutics (65).

Nanotechnology for Improved Organelle Targeted Delivery in Macrophages

Previous studies revealed that in all phases of atherosclerosis development, macrophages and monocytes have an essential role and are involved in local inflammatory responses, plaque growth, and cholesterol deposition (66). When macrophages within arteries extensively internalize oxidized low-density lipoproteins, they initiate foam cell growth, resulting in plaque. With the development of plaques, macrophages finally become apoptotic due to lipid accumulation and metabolic stress, releasing their lipid content and other inflammatory debris, contributing to the formation of necrotic cores and further thrombus development (67). It is apparent that cholesterol transport to mitochondria through steroidogenic acute regulatory (StAR) protein is the rate-limiting step of cholesterol degradation (68). Correspondingly, mitochondria in macrophages are a promising target and an excellent detection site for anti-atherosclerosis strategies. However, the implementation of proper delivery to mitochondria faces several obstacles. The first challenge is macrophage-iconic endocytosis that confines nanocarriers within endo-lysosomes and hinders the diffusion of nanocarriers. At the same time, after attaining endosomal escape, the nanocarriers migrate to mitochondria. The negatively charged bilayer membrane of mitochondria becomes another obstacle. Although the outer membrane resembles cell membranes, its inner membrane contains a high concentration of cardiolipin, which possesses two negative charges per molecule. Consequently, a hydrophobic, positively charged ligand is required to overcome these obstacles (69, 70).To target delivery into mitochondria, Dhar *et al.* developed targeted high-density lipoprotein (HDL)-mimicking hybrid nanoparticle, which carried a contrast agent for prior identification of susceptible plaques using HDL's reverse cholesterol transport function to reduce the incidence of thromboembolism. The HDL-mimicking hybrid nanoparticle consisted of a PLGA and cholesteryl oleate (CO) core encapsulated with quantum dot (QD) for optical imaging. Meanwhile, the HDL-mimicking hybrid nanoparticles are wrapped in a 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE)-PEG-COOH lipid layer embedded with cholesterol and apoA-I mimetic 4F peptide sequence (FAEKFKEAVKDYFAKFWD). Also, it contained a stearyl-triphenyl phosphonium (TPP) cation to achieve mitochondrial targeting on the surface. It is explicit that the lipophilic TPP cation can remarkably accumulate in mitochondria within cells; hence it has been applied for guiding a range of molecules to the mitochondria for probing or treatment (71).The above design showed significant cell association and mitochondrial targeting in healthy RAW 264.7 cells and remarkably decreased the serum levels of triglyceride and total cholesterol in male rats.

CYTOPLASMIC DELIVERY IN NEUTROPHILS

As the predominant circulating leukocytes in humans, neutrophils handle an essential role in the innate immune response to infection or tissue injury (72). Neutrophils experience constitutive or spontaneous apoptosis after regulating inflammation to maintain the host's immune system (73). Acute and chronic inflammation can be triggered by excessive activation, uncontrolled infiltration, and delayed/impaired apoptosis of neutrophils, leading to the progression of many diseases (74, 75). Therefore, neutrophils, as the first step in the inflammatory response, can be regarded as a target for improving the efficacy of therapeutics in inflammatory disorders such as rheumatoid arthritis, sepsis, stroke, and even cancer (76, 77). In recent years, neutrophils on their own have been extensively researched as drug carriers. However, the fate of nanocarriers and their payload in neutrophils still lacks research. Therefore, enhancing the cytoplasmic delivery into neutrophils can be beneficial to cure neutrophils related diseases and further promote the development of neutrophil-based cytopharmaceutics.

Nanotechnology for Improved Phagosomes/Phagolysosome Escape in Neutrophils

Previous studies in neutrophils demonstrated that the phagosomal pH was elevated between 7 and 9 for a minimum of 30 min after phagocytosis of bacterium or fungus (78, 79). The NADPH oxidase (NOX2) activity dramatically contributes to the alkalinization in neutrophil phagosomes and facilitates the generation of superoxide, which leads to an oxidized environment within phagosomes. Subsequently, the phagosome pH would decline slowly after two hours. It had been reported that the decline in phagosome pH coincided with the tendency of lysosome fusion with the phagosome, finally forming the phagolysosome (80). However, up to now, whether the phagocytosis of nanocarriers into the neutrophils and formation of the vesicular phagosomes contributes to the elevated pH levels is still unknown. Moreover, few studies were focused on using nanotechnology to enhance phagosomes/phagolysosome escape. Therefore, since hardly degraded small molecule drugs constitute the majority of the cytoplasmic delivered drugs, exploring the release and diffusion of small molecule drugs in neutrophils becomes critical.

Nanotechnology for Improved Cytoplasmic Release in Neutrophils

To improve cytoplasmic release in neutrophils, Deng's group designed a sialic acid-modified liposomal doxorubicin

(DOX-SAL) for efficient targeting of inflammatory neutrophils in the peripheral circulation followed by intracellular delivery of the drug substance. Within the first 15 min of endocytosis, the liposomes were reported in lysosomes and induced DOX release. The transport of DOX into the nucleus resulted in neutrophil apoptosis and blockage of neutrophil migration, further suppressing the excess inflammation corresponding to the dysfunction of neutrophils (81). To inhibit the excessive neutrophil activation, Wang *et al.* synthesized pH-sensitive doxorubicin (DOX) prodrug by connecting DOX with bovine serum albumin (BSA) through a hydrazone linkage. The prodrug could construct into nanoparticles (DOX-hyd-BSA NPs) by desolvation. Inflammatory neutrophils could selectively uptake DOX-hyd-BSA NPs owing to the recognition by Fcγ receptors overexpressed on the surface of inflammatory neutrophils. They used HL-60 cells (a human promyelocytic leukemia cell line) to mimic inflammatory neutrophils. Upon entry into the acidic environment of neutrophils, DOX-hyd-BSA NPs release DOX due to the breakup of the pH-sensitive bond between BSA and DOX. The diffusion of DOX into cells effectively induced neutrophil apoptosis and bypassed mouse neurological trauma during reperfusion procedure to ischemic stroke. This study provided a novel venue for the effective delivery of therapeutics to inflammatory neutrophils and can be applied to other anti-inflammatory agents for NEs related inflammatory treatment (82). To investigate the release of antibiotics in neutrophils to treat intracellular bacterial infections, Jayakumar *et al.* designed rifampicin (RIF) loaded amorphous chitin nanoparticles (RIF-ACNPs) by the ionic cross-linking reaction. The environment-friendly nature, biocompatibility, and safety of chitin and amorphous chitin significantly contributed to their extensive usage in drug delivery. 60% of RIF was released from nanoparticles within 24 h in polymorphonuclear leukocytes. However, the sustained release of the drug was maintained for up to 72 h. The diffusion, followed by swelling, and breakdown of ACNPs inside the cell explain the observed release pattern of RIF from the nanoparticles (83).

Nanotechnology for Improved Organelle Targeted Delivery in Neutrophils

Neutrophils can further promote the damage and destruction of inflammatory tissues by mediating the discharge of highly active substances such as reactive oxygen species and lytic enzymes (hydrolases, proteases, peroxidases) (84). Among them, neutrophil elastase, stored within azurophilic granules, is a principal proteolytic enzyme responsible for destroying elastin in the tissue. A promising tactic for inhibiting neutrophil elastase activity is to directly transport or release anti-neutrophil elastase substances like α1-antitrypsin into the neutrophil azurophilic granules. Therefore, Lessig *et al.* developed a layer-by-layer (LbL)-coated system as an α1-antitrypsin transporter vehicle for targeting polymorphonuclear leukocytes. The LbL coating was performed utilizing oppositely charged bio-polyelectrolyte of protamine sulfate (PRM) and dextran sulfate (DXS). This allows the formation of capsule materials on the surface of $CaCO_3$ microparticles, providing enhanced transport capacities. Various layers of PRM/DXS-coated $CaCO_3$ microparticles contained the α1-antitrypsin, which formed a negatively charged coating in different positions. Once polymorphonuclear leukocytes phagocytosed α1-antitrypsintransporter vehicle, α1-antitrypsin would reach its targeted cell compartment via the fusion between phagosomes and azurophilic granules. This enables the step-by-step release of α1-antitrypsin during multilayer decomposition and directly inhibits neutrophil elastase (85).

CYTOPLASMIC DELIVERY IN T CELLS

As a crucial element of adaptive immunity, dysfunctional T cells are aligned with various disorders, such as viral infections, blood cancers, and inflammation; thus, effective targeting of T cells can benefit disease prevention and treatment. Recently, the antitumor effect of engineered T cells has been emphasized. Notably, CAR T cell therapy has demonstrated clinically successful outcomes. In numerous researches, nanocarriers can serve as vehicles to convey medicinal agents to T cells for pathological treatment and T cell-mediated immune enhancement. However, the relatively nonphagocytic nature of T cells, compared to the phagocytic immune cell such as macrophages and neutrophils, sets obstacles to the efficient delivery of nanocarriers to T cells (86). Current research utilizes viruses and electroporation to transport nucleic acids to T cells. Nevertheless, viral delivery has shown limitations linked to cargo size, insertional mutagenesis, and high-titer production. On the other hand, electroporation is restricted by its tendency to affect the viability and normal functioning of T cells. A rational design of nanocarriers for improving intracellular delivery tends to be a better choice. For example, Braeckmans *et al.* designed light-sensitive iron oxide nanoparticles placed in biocompatible electrospun nanofibres, which promoted effective internalization by enhancing membrane permeability with photothermal effects. Positively charged nanofibres could adhere to the surface of T cells. After light irradiation, the photosensitive nanoparticles were excited to produce a photothermal effect on the cell membrane, elevating cell membrane permeability and promoting the diffusion of biomacromolecule into T cells. This strategy showed better performance than traditional electroporation by efficient knockdown of PD-1 resulting from siRNA transport to

primary human T cells, without significant effect on T cells' function, morphology, phenotype, and activation state (87).

Nanotechnology for Improved Endo-Lysosomal Escape in T Cells

Evidence shows that cationic polyplexes can interact with cell surface proteoglycans, allowing them to enter cells (88). Two kinds of cationic polymers, namely polyethyleneimine (PEI) and poly (2-dimethylaminoethyl methacrylate) (pDMAEMA), are shown to promote lysosome escape, and their ability to promote gene delivery in T cells is under extensive research. Being critical mediators in asthma development, activated T cells (ATCs), mainly T helper 2 cells (Th2) in the lungs, can promote asthma progression by producing pro-inflammatory cytokines, which induce a series of airway inflammatory responses (89). The silencing of asthma-related genes by siRNA in Th2 cells (e.g., IL-5 (90) and GATA-3 (91)) has been well investigated. Effective transfection of IL-5 related siRNA to T cells faces several challenges such as instability and insufficient uptake owing to siRNA's hydrophilic and polyanionic character. In order to overcome these limitations, Merkel *et al.* used a disulfide linkage to construct transferrin-polyethyleneimine (Tf-PEI) to design a siRNA delivery system to the lungs. In this system, Tf could trigger fast internalization in ATCs due to the interaction with transferrin receptors (TfR) on the surface of T cells. Meanwhile, the low molecular weight PEI (5 kDa) could completely condense and protect IL-5 related siRNA from facilitating cellular internalization and endosomal escape via the proton sponge mechanism with minimal toxicity (92). After entry into the endosome compartment, the disulfide bonds were reduced and ruptured to release PEI/siRNA polyplexes, facilitating the rapid recycling of TfR and efficiently delivering siRNA to the cytoplasm after escaping from lysosomes in ATCs or Jurkat cells (93), finally mediating significant gene knockdown (94).

The intracellular delivery into T cells requires maintaining their original functional properties and high survival rate. These requirements limit the application of traditional cationic polymers for intracellular delivery into the T cells because of their inevitable toxicity. Although low molecular weight PEI (5 kDa) was used to minimize toxic effects in previous studies, there was still a need for a more efficient and less toxic gene delivery material. Therefore, Pun *et al.* discovered that modification of linear-branched (comb) structure of pDMAEMA polymer to cyclic-branched (sunflower) could significantly reduce the toxicity and increase gene delivery efficacy in T cells. Transfection efficacy and cell toxicity were checked in the Jurkat human T cell line by screening different synthetic polymers. A subset of linear-branched and cyclic-branched pHEMA-g-pDMAEMA polymers demonstrated minimal cell toxicity (> 90% viability) and high transfection efficiencies (up to 50%) in the Jurkat human T cell line (95). CAR-T cell therapies are currently a new frontier and focus in immunology, and they can further arm T cells against tumors by delivering the plasmids, mRNA, or CARs. Unlike plasmids, mRNAs are easy to synthesize *in vitro*, and they can be translated into proteins in the cell cytoplasm without nuclear localization or genomic integration. Adequate transportation of mRNAs across the cellular lipid membrane can be challenging due to their negative charge and high molecular weight. Hence, Waymouth *et al.* reported an amphiphilic charge-altering releasable transporter containing dynamic polycationic α-amino esters for mRNA complexation by simple mixing. After transport to endosomes, oligo(α-amino ester) cations form bio-degradable (uncharged) amides resulting from ester-to-amide rearrangement, thus providing a mechanism for endosomal rupture and mRNA release. This finally results in endosomal escape and cytosolic mRNA delivery to Jurkat cells (96).

Nanotechnology for Improved Cytoplasmic Release in T Cells

Due to the tremendous applications of engineered T cells, nanotechnology can help achieve fast and safe engineering methods for T cells by employing effective endocytosis and mRNA release into the cytoplasm. Stephan *et al.* designed targeted mRNA nanocarriers by mixing with Jurkat-E6 T cells in order to reprogram them via transient expression. These bioengineered polymeric NPs are composed of four active elements: 1) surface-anchored targeting ligands including anti-CD3 and anti-CD8 antibodies that selectively bind to T cells and initiate rapid internalization via receptor-mediated endocytosis. 2) a negatively charged polyglutamic acid (PGA) layer to prevent off-target delivery. 3) a biodegradable poly(β-amino ester) (PBAE) with a half-life (appropriate for gene therapy) of 1 and 7 h in an aqueous environment. Upon encapsulation into the endosome, PBAE condenses and protects the mRNA from enzymatic degradation; however, expression of the encoded protein will occur immediately after its transfer into the cytoplasm. 4) Gene editing or transient protein expression can alter the phenotype of the T cells due to the mRNA released from the delivery system. This simple yet effective approach leads to the "hit-and-run programming" of therapeutic T cells, facilitating the development of T cells therapies (97).

The utilization of cytoplasmic release mediated by degradation is evident in Irvine's research. The amphiphilic organic ligand-protected gold nanoparticles (amph-NPs) were synthesized with hydrophobic pockets in ligand shells for loading small molecule drugs. Additionally, the amp-NPs had a mixed monolayer of alkanethiols terminated by hydrophobic methyl and water solubilizing sulfonate groups

for embedding within lipid bilayers and exhibiting membrane-penetrating activity. Conjugation of amph-NPs with targeting antibodies or camelid-derived nanobodies could temporarily suppress the cell penetrability of amph-NPs and promote targeted uptake in specific lymphocyte subpopulations. After degrading the targeting moieties in the endosome, amph-NPs can recover their cell-penetrating activity and be released into the cytoplasm of primary CD8 + T cells. By using a rational nanoparticle design, the amph-NPs showed a 40-fold increase in penetration to $CD8^+$ T cells compared to ordinary nanoparticles and effectively delivered TGF-β inhibitors to relieve TGF-β inhibition of T cell proliferation and function (98).

Nanotechnology for Improved Organelle Targeted Delivery in T Cells

As the terminus of transportation, organelle targeted delivery in T cells is also essential to enhance the cargo's therapeutic effect. Plasmids should localize the nucleus to encode the transgene of interest. The efficiency of nuclear localization is still an immense challenge for delivering CAR plasmids to T cells. In previous studies, poly (β-amino ester) was defined as a suitable vector for DNA delivery (99, 100). Due to its safety and efficacy, a peptide containing microtubule-associated sequences (MTAS) and nuclear localization signals (NLS) facilitated fast-track nuclear import of their genetic cargo via the microtubule transport machinery (101). For *in situ* programming of circulating T cells with leukemia-targeting CAR plasmids for enhanced tumor-recognizing capabilities, Stephan *et al.* chose poly (β-amino ester) modified with MTAS and NLS to condense plasmid DNA into nanosized complexes. T cells targeting anti-CD3e f(ab′)2 fragments were modified on the surface of nanosized complexes by electrostatic adsorption to achieve effective endocytosis by T cells. The final nanoparticles could effectively recognize the circulating T cells and improve the nuclear import of transgenes via MTAS and NLS. The *in-situ* transduction showed 19.7% ± 4.1% of CAR transduction efficiency in circulating T cells after post injections of T cells targeted nanoparticles co-delivering transgenes of 194-1BBz CAR and iPB7 transposase in B-cell acute lymphoblastic leukemia mouse model. On day 24, CAR expression was kept with 7.1% ± 1.7% in circulating T cells. The antitumor effects revealed that the in-situ CAR T cells programming exhibits similar efficacy to ex vivo lentiviral transduced 194-1BBz CAR T cells. These results further demonstrate the therapeutic potential of 'on demand' DNA-carrying nanoparticles (102). Although adoptive T cell immunotherapy like CAR-T cells was endorsed for tackling B cell lymphoma, its application in solid tumors still faces various challenges, owing to the immunosuppression of adoptive T cells in the solid neoplasm. The suppressed T cells exhibit inadequate tumor targeting ability and activity. One of their apparent features is a sharp reduction of potent toxic agents in the tumor microenvironment. Here, Zhang *et al.* designed a lysosome-targeting nanoparticle (LYS-NP) to reprogram lysosomes of $CD8^+$ T cells, which enhanced the antitumor effect of T cells (Fig. 2). The nanoparticle contained an acid-degradable metal–organic framework (ZIF-8) as the core for encapsulating perforin and granzyme B and was covered by Ca^{2+} via mineralization, which gave ZIF-8 good biocompatibility and acid degradability, thus enhancing the functions of granzyme B and perforin. Ultimately, a CD63

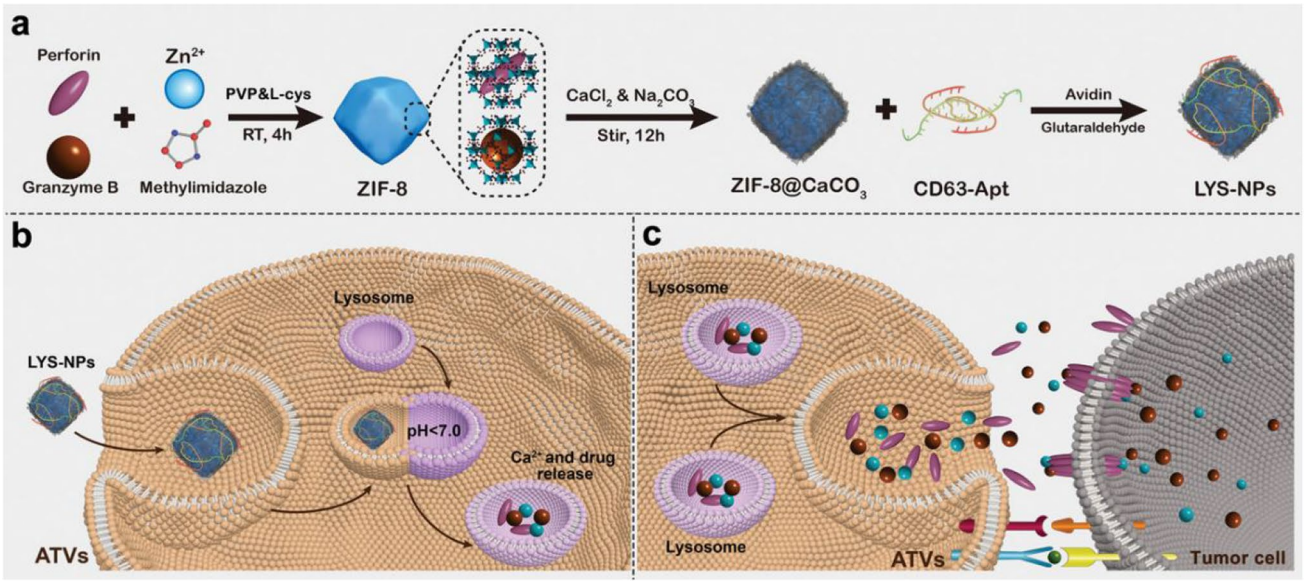

Fig. 2 Schematic diagram of designing and preparation of LYS-NPs and their function in lysosome-targeted drug delivery to T cells for promoting T cells-medicated antitumor effects (103).

aptamer (CD63-Apt) was adjusted on the mineralized ZIF-8 to equip LYS-NP with T-cell targeting ability. Upon entry into the lysosomes, LYS-NP degraded and released perforin, granzyme B, and Ca^{2+}. After activation by MHC, the lysosome discharged its contents to immunological synapses. It is evident that the autonomously controlled release of Ca^{2+}, granzyme B, and perforin can enhance the antitumor effects in solid tumors. In summary, it is clear that LYS-NP accomplishes the lysosome-targeted drug delivery to T cells and promotes T cells-medicated tumor cell apoptosis by transforming its lysosomes into the armory (103).

CYTOPLASMIC DELIVERY IN NATURAL KILLER CELLS

Natural killer cells (NK cells) belong to cytotoxic lymphocytes of the body's innate immunity, and like $CD8^+$ T cells, they play a crucial role in tumor therapy (104). Although as adaptive cell therapy NK cell therapy showed comparable potency to CAR-T cell therapy, it possesses several benefits compared to T cell therapy. Numerous receptors on the cell surface control NK cells' killing activity by affecting equilibrium between stimulatory and inhibitory signals (105). Thus, NK cells can fight tumor cells while avoiding tumor antigens or clonal expansion. This process allows the scanning for modified protein expression on cells and promotes accurate distinction of healthy cells from tumor cells without inducing graft *versus* host disease (GVHD) (106). Hence, NK cell therapy can become an attractive potential technique for cancer immunotherapy. Autologous/allogeneic NK cells and human NK cell lines found massive applications in NK cell therapy. Nowadays, NK-92 cells have become a potential platform for NK cells therapy, and they provide continuous activation because they lack nearly all inhibitory receptors (107, 108). Gene expression modification in NK-92 cells by nanocarriers is a viable approach for enhancing the capability of clinical NK cell therapy.

Nanotechnology for Improved Endo-Lysosomal Escape in Natural Killer Cells

For improving the cytoplasm delivery of siRNA in NK-92 cells, Harashima *et al.* synthesized YSK12-C4, a fusogenic cationic lipid that facilitates the endosomal escape, to construct a pH-sensitive cationic lipid-based multifunctional envelope-type nanodevice (YSK12-MEND) loaded with antihuman GAPDH siRNA. YSK12-MEND showed an increased ability of endosomal escape and cytoplasmic delivery due to the pH-sensitive endo-lysosome membrane fusion of YSK12-C4. Additionally, replacing YSK12-C4 with a polycation such as protamine generated a core derived from siRNA's electrostatic interactions and further modulated the toxicity of YSK12-MEND in NK-92 cells. The final nanodevice YSK12-MEND/core (CR5) possessed good biocompatibility and gene silencing activity in NK-92 cells; thus, it can act as a potential nanodevice for enhanced clinical NK cell therapy (109). Extensive research on structure–activity relationships of pH-sensitive cationic lipids showed that the cationic lipid's polar head greatly influences the apparent pKa of the final product, playing a critical role in endosomal escape. The hydrophobic tail will significantly affect the phase transition temperature of the lamella phase to the inverted hexagonal HII phase in the process of endosomal escape mediated by membrane fusion (110). Based on the above consideration, Harashima *et al.* further identified a new pH-sensitive cationic lipid (CL1H6) to prepare siRNA-loaded LNP for efficient delivery of siRNAs to NK-92 cells with low cytotoxicity (Fig. 3). They found that the oleate tail in CL1H6 contributed to an increased GADPH gene

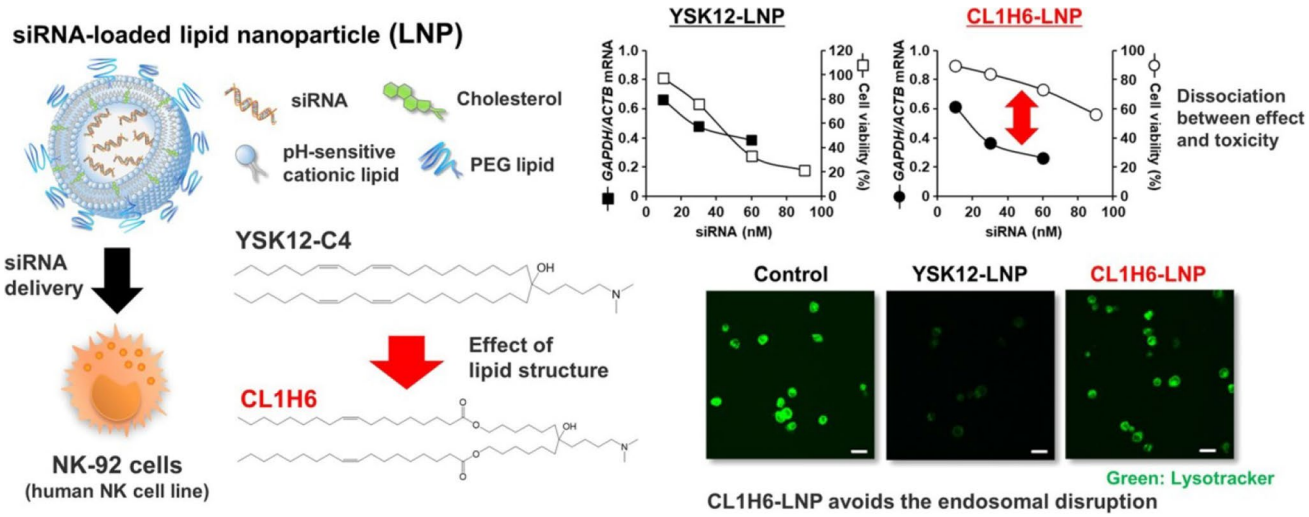

Fig. 3 Schematic diagram of the design and development of LNP and their efficient low-toxic transfection effect for NK cells (112).

silencing and cell viability in NK-92 cells when compared with YSK12-LNP. The main mechanism of reduced cytotoxicity and enhanced gene silencing in CL1H6-LNP would be ascribed to increase the transition temperature from the lamellar phase to the hexagonal HII phase by (111) reducing the number of double bonds, resulting in mild membrane fusion without damaging the cell membrane in NK-92 cells. This finding is of great significance for the rational development of pH-sensitive cationic lipids with appropriate endosomal escape efficiency for cytoplasmic delivery of siRNA to NK-92 cells (112).

Nanotechnology for Improved Cytoplasmic Release in Natural Killer Cells

The adequate decorating of nanocarriers at the final destination is essential to the nucleic acid cargo to show a pharmacological effect. In contrast, decapsulation in the endo-lysosome impairs the efficiency of transfection owing to nucleic acid instability (113). Therefore, a stability/instability switch is essential prior to and after the endosomal escape. In order to investigate this issue, Harashima *et al.* designed LNP containing several cationic lipids and observed the relationship between the cationic lipids structure and intracellular release patterns. The cationic lipids developed in this study included CL4H6, a lipid comprising biodegradable ester bonds as part of the hydrophobic tail, while YSK13-C3 did not. After escaping from the lysosome and entering the cytoplasm, the ester linkage in CL4H6 by cytoplasmic lactase cleavage resulted in a hydrophilic alkanol amine production and the release of oleic acid. The hydrophilicity of alkanol amine would lead to its rapid elimination, making it unable to link with nucleic acid even if it has cationic properties. Produced oleic acids with a negative charge neutralize the positive charge of the remaining pH-sensitive cationic lipids, thus further promoting the cytosolic release of siRNAs. These findings proved that the immediate release of siRNA in the cytoplasm of NK-92cells could be induced by introducing enzyme-cleavable and chemically stable ester bonds in the lipids' hydrophobic tail (114).

THE FACTORS CONSIDERED IN RATIONAL DESIGN OF INTRACELLULAR NANOCARRIERS FOR IMMUNE CELLS

With the continuous development of immunomodulatory drugs and immune cell-based cytopharmaceuticals, the issue about the rational design of intracellular nanocarriers in conforming to the properties of immune cells has drawn attention. The following steps should be taken into account (Fig. 4).

Considerations of the bioactive molecules

The bioactive molecules for intracellular delivery into immune cells can be mainly divided into small molecules and macromolecules. Generally, compared with small

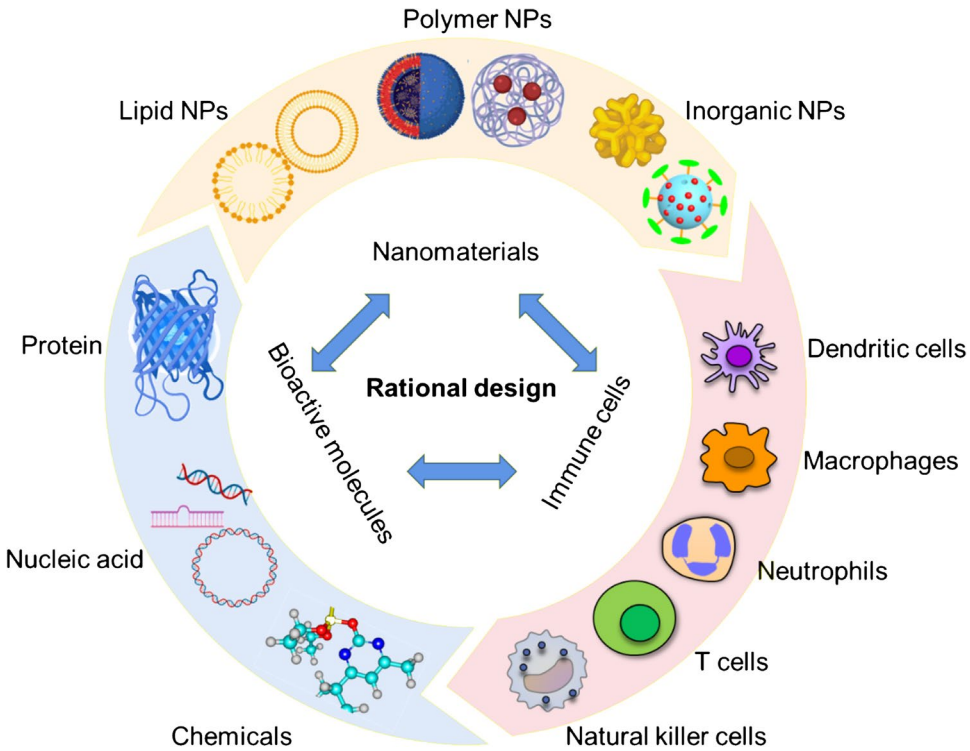

Fig. 4 The factors considered in rational design of intracellular nanocarriers for immune cells.

molecules, macromolecules would be confronted with more physiological and pathological barriers before interacting with their targets due to the lower membrane permeability, the easier degradation in the intracellular environment and the lower diffusion capacity. Hence, rational design of intracellular nanocarriers for bioactive molecules should not only enhance the cellular uptake by immune cells, but also protect bioactive molecules from degradation before reaching their target site. In addition, the entire release of bioactive molecules from nanocarriers would play an essential role on enhancing the therapeutic effect and attenuating toxicity. Hence, the process of cytoplasmic release should be taken into account and also varies accordingly. Furthermore, due to the different target site of the cargos, special design for targeting delivery to organelles would be other important factors that need to consider and some organelle delivery strategies were summarized in Table II.

Selection of nanomaterials

Although nanotechnology has made significant contributions to the field of cytoplasmic drug delivery in immune cells, the potential biological safety of nanomaterials and their impact on the function of immune cells should be taken into account (115). For some metal oxide nanoparticles, previous research has shown that rare-earth oxides (REOs) NPs could induce bone marrow-derived macrophages and macrophage cell lines to undergo pyroptosis and increase IL-1β production, while transition metal oxides (TMOs) NPs could trigger cell apoptosis due to the activation of caspases 3 and 7 in primary Kupffer cells (116). Besides, carbon black (CB) nanoparticles were reported to induce lactate dehydrogenase discharge from RAW264.7 cells owing to the reduced plasma membrane integrity, eventually inducing cell pyroptosis (117). Therefore, delivering nanomaterials into immune cells can be a double-edged sword and lesson has also raised concerns about the safety of nanomaterials. Besides, many biodegradable materials have gained sustainable attention for the purpose of enhancing the safety profile and further application to construct nanoparticles (118). Polymer- and lipid-based nanoparticles have become the most comprehensively used carriers for delivering bioactive molecules due to their high biocompatibility and biosafety (119, 120). However, both of the above biodegradable nanoparticles have their drawbacks. The release of polymer nanoparticles seems to be related to their degradation kinetics and is easily affected by the environment (121), which makes it difficult to achieve the desired drug release profile (122). Meanwhile, their low and selective drug loading also limits their application (123). For lipid-based nanoparticles such as liposome, the high clearance rate and low stability also need to further resolve. In order to achieve higher drug loading, some inorganic materials such as mesoporous silicon nanoparticles and gold nanoparticles have also used to deliver bioactive molecules to immune cells (98), but the long-term cumulative toxicity of these materials should be strictly monitored and considered. Therefore, to choose suitable nanomaterials, researchers should not only pay attention to its delivery efficiency, but also critically consider the nanomaterials' safety aspect. Furthermore, establishing toxicological evaluation of different nanomaterials in various immune cells will assist in assessing their safety, thereby promoting its further application and clinical transformation.

Considerations of immune cells

Due to the different characteristics of various immune cells, there is a crucial need to diversify the focus of cytoplasmic delivery. For instance, due to phagocytosis and digestion in macrophages or neutrophils, avoiding the degradation of substances in the endo-lysosomes by effective endo-lysosomal escape can be essential to achieve effective intracellular delivery. Different pH-responsive materials can be added to promote the rupture of endo-lysosomal membranes. Furthermore, taking advantage of the experience from bacterial phagocytic escape by adding bacteria-specific membrane-penetrating peptides and perforins to the nanocarrier will enhance lysosomal escape as mentioned above. For the intracellular release of the nanocarriers, the powerful phagocytosis and digestion function of phagocytes can be utilized by adding biodegradable materials to promote the release of the cargos or to further promote a stronger release through exogenous stimulation. For the

Table II The different organelle delivery strategies

Organelles	Delivery strategies	Key points of delivery
Nucleus	1. Ultra-small NP can penetrate the nuclear pore complex 2. Some specific nuclear localization signals peptide can guide NPs to the nucleus	Enter the nucleus
Mitochondria	1. Nanocarrier with high cationic group can be used to target mitochondria 2. Nanoparticles comprising the mitochondriotropic dequalinium chloride (DQAsome) guide cargo to the mitochondria	Accumulate in mitochondria
Lysosome	1. Various enzyme inside lysosome offers target for stimuli-responsive delivery system 2. Lysosome-targeting chimeras	Retained in lysosomes

Table III A summary of nanotechnology for enhanced cytoplasmic and organelle delivery of bioactive molecules to immune cells for treating diseases

Nano-drug delivery systems	Immune cells	Delivery strategies	Diseases	References
Novel proton-driven nano transformer-based vaccine	Dendritic cell	Lysosomal escape	Tumor and infectious diseases	(45)
pH and GSH responsive polymer micelle	Dendritic cell	Lysosomal escape and cytoplasm release	Tumor	(48)
PEGylated and mannosylated graphene oxide	Macrophage	Lysosomal escape	Bacterial infections	(57)
Lipase-sensitive polymeric triple-layered nanogel	Macrophage	Cytoplasm release	Bacterial infections	(64)
HDL-mimicking hybrid nanoparticles	Macrophage	Mitochondria targeting	Cardiovascular thromboembolism	(71)
DOX-hyd-BSA NPs	Neutrophil	Cytoplasm release	Ischemic stroke	(82)
PRM/DXS-coated $CaCO_3$ microparticles	Neutrophil	Phagosome targeting	Inflammatory injury	(85)
Amphiphilic charge-altering releasable polymer	T cell	Lysosomal escape	Tumor	(96)
Amphiphilic organic ligand-protected gold nanoparticles	T cell	Cytoplasm release	Tumor	(98)
Mineralized Metal–Organic Framework	T cell	Lysosome targeting	Tumor	(103)

primary APCs such as DCs, most cargos need to accumulate effectively in the cytoplasm. Thus, designing nanoparticles that can both evade endo-lysosomes and release their cargo in the cytoplasm is critical. For non-endocytic cells like T cells, promoting effective internalization, endo-lysosomal escape, cytoplasmic release, and organelle targeting is essential for the maximum efficacy. Professional phagocytes uptake nanoparticles that undergo nonspecific macropinocytosis and phagocytosis, while among non-phagocytic cells, these pathways are only found in activated T cells and are generally considered inefficient. Thus, many factors such as the kinetics of receptor endocytosis, particle size, and cell activation state should be taken into account (124). Recently, to promote the internalization of nanoparticles into T cells, more research on transport techniques such as viral vectors, electroporation, and other methods to enhance the permeability of membranes and promote internalization is needed. After internalization, nanocarriers also face additional barriers to achieving endosomal escape due to slower endosomal acidification in T cells (125). Hence, unlike macrophages and neutrophil, nanomaterials that utilize pH changes in lysosomes to achieve lysosomal escape need to be carefully selected in the condition of T cell. At the same time, the toxicity and biodegradability of functional nanomaterials also need critical considerations in the intracellular delivery of T cells.

CONCLUSION AND OUTLOOK

Nanotechnology can overcome the limitations of conventional cytoplasmic delivery approaches through cell-specific targeting and organelle-specific transportation (126), taking advantage of a more controlled and precisely targeted drug delivery for improving the therapeutic efficacy of bioactive molecules in various diseases caused by the dysfunction of the immune cells. A summary of nanotechnology for enhanced cytoplasmic and organelle delivery of bioactive molecules to immune cells for treating diseases was showed in Table III.

In recent years, immune cells themselves have become an effective platform for tackling the challenges of classic nanotechnology; they can overcome multiple physiological/pathological barriers faced by traditional nanocarriers (127). For instance, our group constructed neutrophils-based cytopharmaceuticals via loading liposomal paclitaxel into neutrophils for enhancing post-operational glioma chemotherapy. Due to the inflammation-mediated chemotaxis, neutrophils-based cytopharmaceuticals significantly enhanced the targeting efficiency of paclitaxel to the lesions, which was 1162- and 86-fold higher than that of Taxol and liposomal paclitaxel (128). Similar results were found on neutrophil-based cytopharmaceuticals that improved targeting efficiency to the tumor after photothermal therapy and radiotherapy (129, 130). In addition to neutrophils, T cells and macrophages were also applied to design the cytopharmaceuticals by loading or anchoring nanomedicines to immune cells to amplify targeted therapeutic efficiency (131–133).

Taking the future clinical translation into consideration, the extensive funding has been invested for developing nanomedicines and a large number of published articles focus on nanomedicines, however, the success of the approved products and clinical translation are infrequent. There are still many obstacles for intracellular delivery systems to fulfill commercialization including low-efficiency of delivery due to systemic clearance of nanoparticles, ability to design stable formulations with adequate pharmaceutical shelf-life and the availability of safe biomaterials that enable exploitation of biomimetic principles. Building the bridge to fill the gap between basic pharmaceutical research and clinical translation of

products and technology is of great importance for clinical application. First, the development of biodegradable and biocompatible materials can improve the formulation's safety and feasibility for clinical application. Meanwhile, advanced computing technology and algorithms can help establish a rapid screening system to screen out suitable, safe, and efficient nanomedicines. In addition, it is essential to design integrated automation equipment that will simplify preparation steps and establish a quality evaluation system for nanomedicine, consequently, promoting further production and marketing.

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Declarations

Conflict of interest statement The authors declare no conflict of interest and no competing financial interests to disclose.

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