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Nanoparticles for nucleic acid delivery: Applications in cancer immunotherapy

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ABSTRACT

Immunotherapy has recently emerged as a powerful tool for cancer treatment. Early clinical successes from cancer immunotherapy have led to a growing list of FDA approvals, and many new therapies are in clinical and preclinical development. Nucleic acid therapeutics, including DNA, mRNA, and genome editing systems, hold significant potential as a form of immunotherapy due to its robust use in cancer vaccination, adoptive T-cell therapy, and gene regulation. However, these therapeutics must overcome numerous delivery obstacles to be successful, including rapid *in vivo* degradation, poor uptake into target cells, required nuclear entry, and potential *in vivo* toxicity in healthy cells and tissues. Nanoparticle delivery systems have been engineered to overcome several of these barriers as a means to safely and effectively deliver nucleic acid therapeutics to immune cells. In this Review, we discuss the applications of nucleic acid therapeutics in cancer immunotherapy, and we detail how nanoparticle platforms have been designed to deliver mRNA, DNA, and genome editing systems to enhance the potency and safety of these therapeutics.

1. Introduction

Despite advances in understanding the underlying mechanisms of cancer progression, chemotherapy, radiation, and surgery remain the current standards-of-care for many cancers [1]. The use of these strategies has become more focused and personalized based on the type and stage of disease, which has led to a decline in cancer-related mortality over the past three decades [2]. However, these therapies are often highly invasive, have substantial adverse side effects, and therapeutic results are variable [3–5]. Thus, there is a dire need to develop non-invasive, minimally toxic, and highly specific alternatives. Towards this goal, cancer immunotherapy has emerged as a powerful alternative to conventional therapies, and substantial research efforts are ongoing to improve upon their efficacy and safety.

The overarching goal of cancer immunotherapy is to introduce the necessary molecular tools to harness the immune system to halt disease progression. Thus, immunotherapy can be personalized for specific types and stages of cancer, with higher safety profiles and longer therapeutic windows compared to traditional cancer therapeutics [6]. The field encompasses several classes of immunotherapy, including gene therapy, cellular vaccines, checkpoint inhibitors, agonistic antibodies, and cytokines [7]. Of these, checkpoint inhibitors and cytokines are the most widely studied to date, and multiple therapies are currently used in the clinic [7]. More recently, nucleic acid therapeutics including DNA, mRNA, and CRISPR/Cas9 gene editing systems have emerged as an important branch of cancer immunotherapy. The vast potential of nucleic acids for treating cancer can be demonstrated by the use of CRISPR/Cas9 to inactivate PD-1/PD-L1 interactions between cancer cells and T-cells [8]. In one example of this, CRISPR/Cas9 genome editing was used to generate PD-1 deficient anti-CD19 chimeric antigen receptor (CAR) T-cells, resulting in enhanced killing of PD-L1 + tumor xenografts [8]. Similarly, gene therapy approaches are highly prevalent in adoptive T-cell immunotherapy to induce T-cells to express CARs. In 2017, Novartis gained the first FDA approval for a cell-based gene therapy, Kymriah, which utilizes CAR T-cells to treat leukemia [9,10]. The early success of Kymriah and the ability for CRISPR/Cas9 to enhance T-cell-mediated killing form the basis for the development of other types of gene therapy to treat cancer, with reduced adverse effects and higher success rates than traditional approaches [9,10].

Although the examples described above demonstrate the therapeutic potential of nucleic acid therapeutics, their translation into the clinic is hindered by several delivery challenges for both *ex vivo* and *in vivo* applications. Nucleic acids are highly unstable, and they degrade quickly in the presence of nucleases before reaching the desired tissues

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[11]. Further, nucleic acids are unable to enter cells alone, requiring the use of transfection reagents or physical techniques (such as electroporation) that are highly toxic to cells *ex vivo* and are not feasible for *in vivo* use [12,13]. Several nucleic acid therapeutics, such as gene editing components and DNA, are faced with another delivery barrier of crossing the nuclear membrane to be transcribed in the nucleus [14]. Thus, there is great interest in developing novel delivery platforms that can encapsulate and protect nucleic acids, as well as mediate their delivery into the desired tissues and cells, in order to exploit their powerful therapeutic potential.

Nanoparticles (NPs), which are typically defined as particles that are 1–1000 nm in diameter, are being developed to overcome the delivery barriers faced by nucleic acids (Fig. 1). NPs can be comprised of a range of materials such as lipids, polymers, or metals, all of which offer unique delivery advantages that have been thoroughly reviewed elsewhere [11,15,16]. Importantly, NP features such as material composition, size, and surface chemistry can be carefully engineered for nucleic acid delivery. NPs can encapsulate or bind to nucleic acid therapeutics via electrostatic interactions or chemical conjugation to overcome the therapeutic challenges faced by unbound nucleic acids [6,7]. Additionally NPs can reduce therapeutic toxicity, by promoting site-specific accumulation and reducing off-target effects. Further, NPs offer protection over the therapeutic cargo, to avoid nuclease degradation and to extend circulation half-life. In addition to protecting nucleic acids, NPs can be engineered to respond to environmental cues, such as the acidic environment within solid tumors or within the endosomes of cells, to degrade and release therapeutic cargo on-demand [17]. By enabling control over nucleic acid delivery, NPs can minimize toxicity in healthy tissues while maximizing delivery to cancer cells, which may be highly beneficial for solid tumor immunotherapy [18]. Lastly, NPs can be modified with targeting ligands and other molecules, to promote both cellular and nuclear uptake to targeted tissues that overexpress the targeted protein [19].

Here, we review the design of NP platforms for nucleic acid delivery - including mRNA, DNA, and genome editing therapies – and their applications in cancer immunotherapy. Several NP platforms have demonstrated preclinical success in delivering nucleic acids to target cells, and significant efforts are now underway to translate these technologies into the clinic. Of note, lipid NPs (LNPs) complexed with mRNA are currently being evaluated in clinical trials of melanoma (NCT02410733) [20]. Further, Alnylam Pharmaceuticals received the first FDA approval of an RNA therapeutic for their lipid-siRNA NP, Onpattro, in 2018 [7,21]. Below, we overview applications of NPs for delivering DNA, mRNA, and genome editing systems for cancer immunotherapy, and we discuss future directions of gene therapy towards the goal of clinical translation.

2. Nanoparticles for DNA delivery

DNA vaccine-based cancer immunotherapy, in which cells are transfected with plasmid or chemically synthesized DNA to elicit immune responses against the encoded antigen, is a powerful tool to engage the immune system to attack cancer cells [22]. Early studies in mice demonstrated the ability of DNA plasmids to drive immune responses against transgene products related to influenza, human immunodeficiency virus-1, and cancer, which established DNA as a promising immunization platform [23]. However, initial clinical applications of DNA vaccines revealed only low levels of immunity, indicating that naked DNA was not feasible as an independent vaccination strategy largely due to the delivery barriers discussed above (Fig. 1) [23]. For example, the negatively charged DNA typically cannot cross the anionic cell membrane without an exogenous transfection reagent or delivery vehicle [24], and once within cells, DNA needs to surpass the nuclear membrane and enter the nucleus [24]. Lastly, it is critical that DNA is transfected into the desired cells with minimal off-target expression [25–28]. Several physical techniques to improve DNA delivery including gene guns, electroporation, and sonoporation are commonly used *ex vivo* and in small animals [29,30], but they are either not feasible for *in vivo* use, or they are limited to local delivery [24]. Utilizing NPs as DNA delivery vehicles can overcome the aforementioned limitations, and several unique applications are described below [24].

Several types of LNPs, including liposomes, ionizable lipids, and polymer-lipid NPs, have been developed to deliver DNA to target cells. Liposomes were among the first DNA delivery systems and are the furthest in clinical development, as they are currently used clinically to treat cancer [31]. Liposomes are composed of materials with polar head groups and non-polar tails, and they spontaneously self-assemble into vesicles at low concentrations [32,33]. Cationic lipids, such as DOTMA, DOTAP and zwitterionic DOPE, are commonly used to form cationic liposomes by exploiting electrostatic interactions between lipids and negatively-charged nucleic acids (Fig. 2a). When used to encapsulate DNA or other drugs, these cationic liposomes induce stronger therapeutic effects than free drug, which has led to several cationic liposomal drug formulations advancing into clinical trials [34–38]. However, the use of cationic liposomes is limited due to toxicity at the site of administration [39–41], undesired immune responses [42], and clot formation [43], all of which can limit the allowable administered dose [41,44–48].

As an alternative to traditional cationic liposomes, ionizable lipids that are neutral at physiologic pH (~7.4) but ionize under acidic conditions, such as those found within endosomes, have been developed for nucleic acid delivery [49–51]. The ability of these lipids to buffer endosomal compartments by taking on positive charges can promote endosomal escape and enable processing of the nucleic acids in the cytosol [51,52]. Ionizable LNPs typically have 3 components in addition to the ionizable lipid itself; a fusogenic helper phospholipid (DSPC, DOPE, DOTC, DOTMA, POPC) [53,54], cholesterol to increase stability and membrane fusion [55,56], and a lipid-anchored poly (ethylene glycol) (PEG) to extend their circulatory half-life and decrease non-specific protein adsorption (Fig. 2a and b) [54]. Ionizable LNPs have been used for DNA cancer immunotherapy by encapsulating CpG (a TLR-9 agonist) oligodeoxynucleotides (ODNs). In this application, CpG-NPs were subcutaneously co-administered with tumor associated antigens in murine models of thymoma and melanoma [57]. NPs exhibited preferential accumulation and uptake by immune cells in lymph nodes and augmented antigen-specific immune cell and cytokine/chemokine responses, ultimately leading to greater tumor rejection in a murine EG7-OVA tumor model [57]. Although ionizable LNPs have been shown to effectively load nucleic acids of relatively small size (e.g. short synthetic DNA, siRNA, and microRNA), encapsulating large cargo (e.g. pDNA) is challenging [58–60]. Thus, new classes of polymer-based NPs, such as polyplexes [61–63], chitosan-based NPs [64,65], and poly(beta-amino esters (PBAEs) [66–68], have been developed to effectively condense pDNA into NPs and enhance transgene expression as described below.

Cationic polymeric NPs can be engineered to possess specific physicochemical properties, such as hydrophobicity and charge, due to the diverse range of available polymers and chemical modifications [27]. This chemical diversity allows researchers to utilize polymeric NPs for delivery to a wide array of cell types [69]. Poly(L-lysine) (PLL) is a homopolymer of the amino acid lysine that has been shown to effectively condense DNA (Fig. 2c) [70]. Studies indicate that PLL generally has low transfection success, likely due to its low rate of endosomal escape [24]. However, one study used PLL-coated polystyrene NPs to deliver pDNA encoding OVA antigen as a model for a DNA-based prophylactic cancer vaccine against EG7 tumor cells [70]. Two vaccinations with these NPs inhibited tumor growth following a EG7 tumor cell challenge in mice [70]. Notably, immature dendritic cells (DCs) had higher levels of NP uptake compared to mature DCs [70], which is likely due to the reduced endocytic and phagocytic rates in mature DCs that lowers their capacity to internalize and process antigens [70].

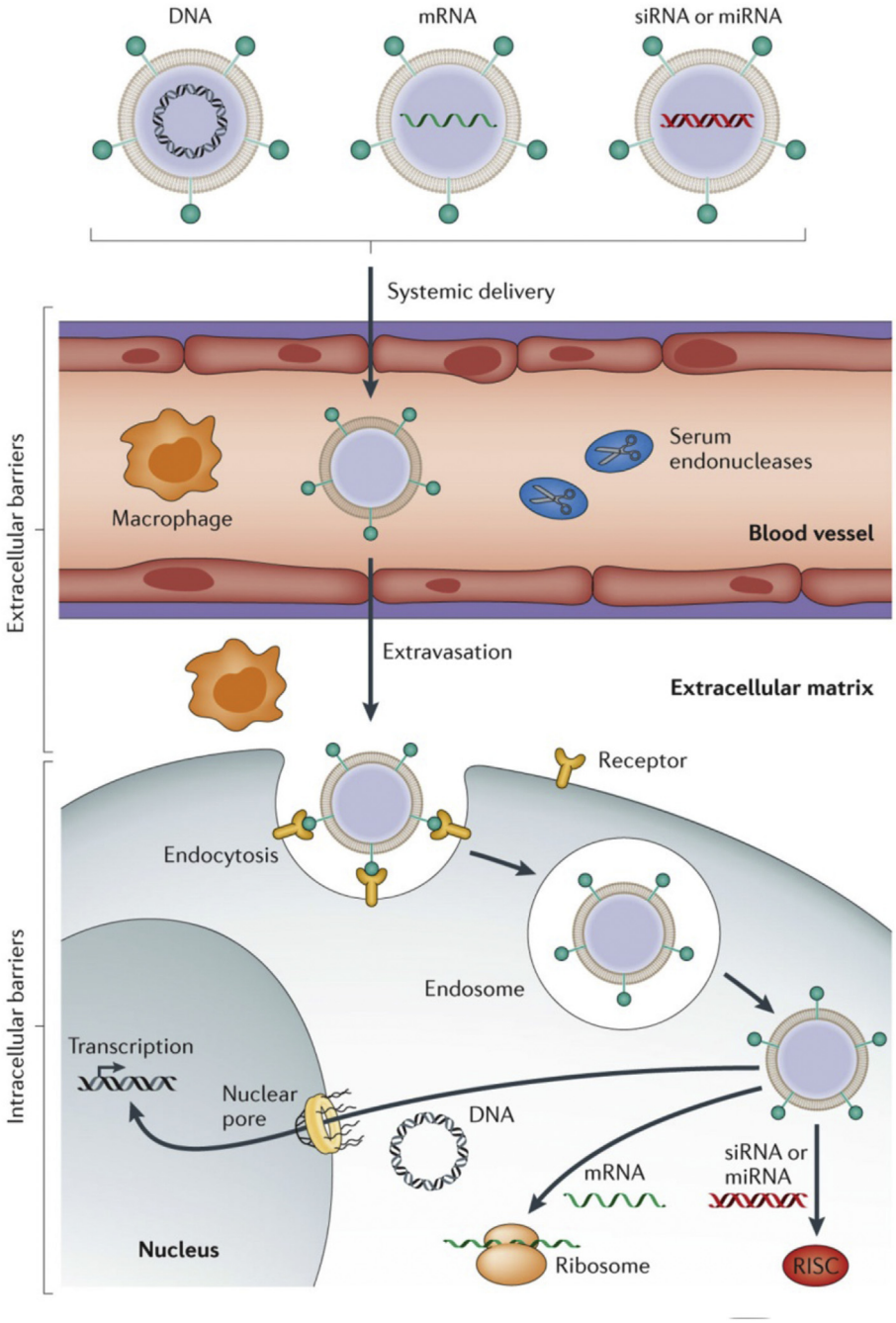

Fig. 1. The role of NPs in overcoming extracellular and intracellular barriers for nucleic acid delivery. In the circulation, NPs need to protect nucleic acids from serum endo- and exo-nucleases, evade immune detection, and avoid non-specific protein interactions within the blood. Further, NPs must avoid renal clearance (achieved through size modulation), while also promoting extravasation from the blood and into target tissues, upon which they promote cellular uptake and localization into the cytosol or nucleus. Adapted with permission from Ref. [24].

Similar to PLL, polyethylenimine (PEI) is another cationic polymer that is often used as a "gold-standard" for transfection efficacy (Fig. 2c) [24,71]. PEI exerts a high charge density at low pH, which enhances endosomal escape and makes it a potent transfection reagent, but it also confers high cytotoxicity [72–76]. Longer chain and higher charge density PEIs tend to have damaging interactions with cellular membranes that lead to potent cytotoxicity, and several strategies have emerged to address this including branched architectures, biodegradability, and PEG-grafting [74,75,77]. In one instance, modified branched PEI was synthesized to improve upon the cytocompatibility and transfection efficiency of unmodified PEI [74]. Of note, succinylated PEI induced better siRNA-mediated knockdown and 10-fold lower polymer toxicity compared to unmodified PEI [74]. This demonstrates the importance of balancing transfection efficiency and biocompatibility when designing PEI-based delivery vehicles. The high transfection ability of PEI was exploited for cancer immunotherapy by condensing IL-12-encoding pDNA [78]. This therapy was administered as an aerosol to mice bearing SAOS-LM7 tumors in a murine model of osteosarcoma lung metastasis [78]. Mice that received aerosolized PEI-IL-12 gene therapy exhibited IL-12 expression only in the lungs and had significantly fewer lung metastases than untreated controls [78]. The ability of PEI to condense DNA is also applicable to newer polymer-based NP delivery platforms, such as PBAEs, described below.

PBAEs are simple to synthesize and they provide an additional

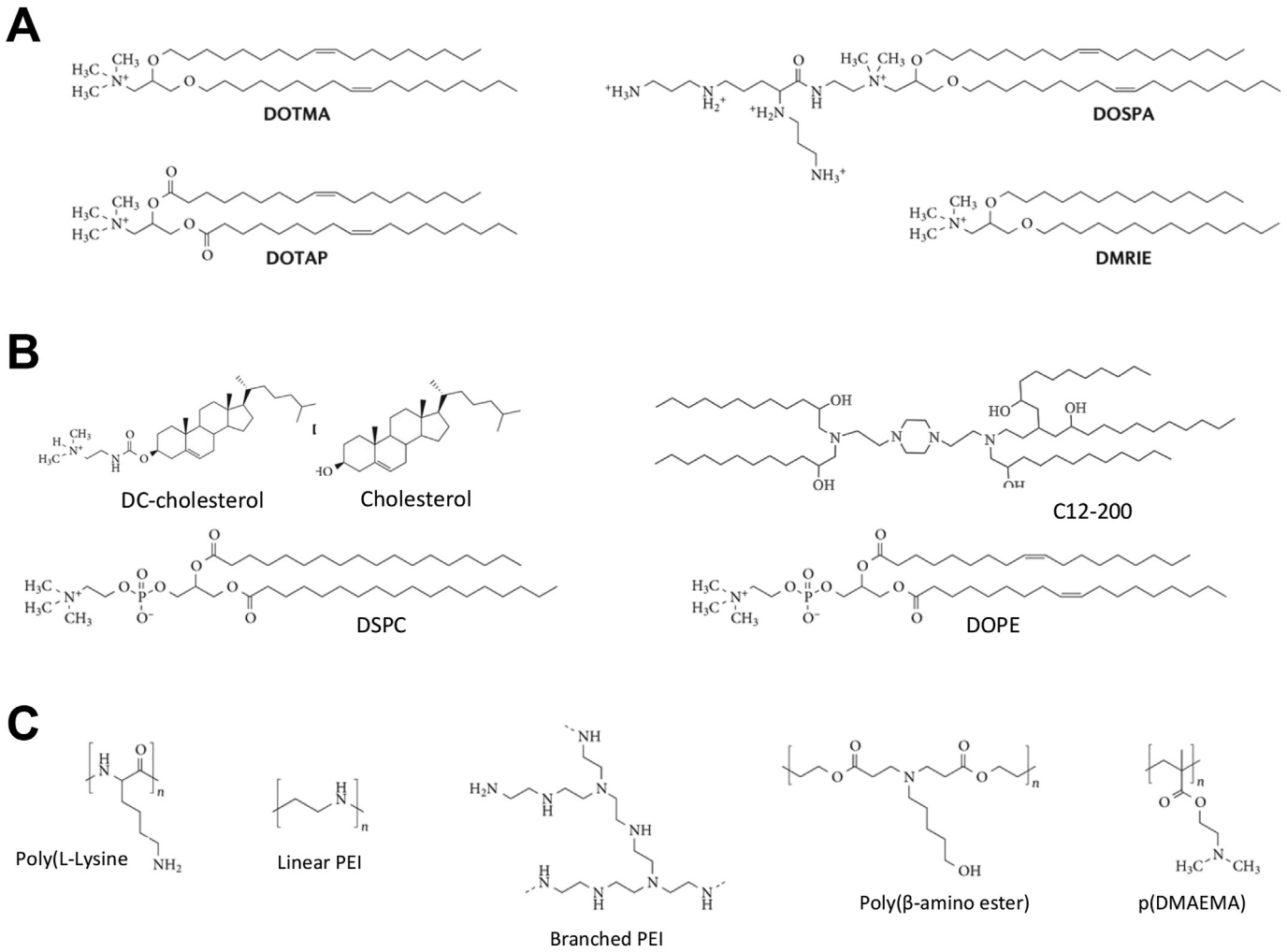

Fig. 2. Chemical structures of lipids and polymers used to engineer NPs for nucleic acid delivery. **A.** Common lipids used for liposomal formulations including DOTMA, DOSPA, DOTAP, DMRIE and DC-cholesterol, which are used to condense and encapsulate nucleic acids. Structurally, cationic lipids are defined as having a cationic head group, linker region, and hydrophobic tails. **B.** Ionizable lipid LNP formulations are comprised of four components: ionizable lipids, such as C12-200, phospholipids (DOPE, DSPC), cholesterol, and lipid-anchored PEG. **C.** Cationic polymers and biopolymers used as vectors for nucleic acid delivery. PEI and PLL were two of the initial vectors used for DNA delivery but are faced with safety (PEI) and efficacy (PLL) concerns. PBAEs and pDMAEMA are newer polymer vectors developed for nucleic acid delivery with improved safety and efficacy. Adapted with permission from Ref. [24].

benefit of having tunable biodegradation (Fig. 2c) [79]. A major advantage of their simple, parallelizable synthesis is the ability to generate diverse libraries of PBAE structures that can be screened for DNA delivery to identify key structures for potent gene delivery [80]. In the context of immunotherapy, PBAE NPs functionalized with an anti-CD3e T-cell-targeting antibody fragment were used to deliver leukemia specific CD194-1BBz CAR pDNA to T-cells *in situ* in a murine leukemia model (Fig. 3a) [81]. These NPs also contained microtubule-associated sequence (MTAS) and nuclear localization signal (NLS) peptides to mediate nuclear translocation of the therapeutic pDNA cargo [81]. NP-programmed CAR T-cells generated tumor regression similar to that of traditionally prepared CAR T-cells, with only a small portion of NPs transducing phagocytic cells, likely due to successful antibody targeting to T-cells [81]. PBAEs have also been used to deliver cyclic dinucleotides (a STING agonist) or CpG nucleic acid adjuvants (Fig. 3b) [82,83]. Notably, results from these studies indicated that the PBAE:DNA ratio is a critical factor for NP stability and *in vivo* functionality. Specifically, PBAE-NPs with higher PBAE:DNA ratios yielded better protection of the CpG cargo. However, lower ratios exhibited better CpG uptake and activation of tumor-specific T-cells, resulting in improved survival in a mouse melanoma model [82,83].

With the aid of NP delivery systems, DNA-based therapeutics have shown great promise in the field of cancer immunotherapy [84]. Although using pDNA as an antigen source has shown encouraging outcomes in many preclinical studies, the same success has not been found in human clinical trials, and interest in using DNA as antigen sources has decreased [84,85]. However, there is substantial ongoing work to develop DNA NPs in immunotherapy for CAR T-cells or as adjuvants [81,82,86]. More recently, mRNA has emerged as a potent tool for gene immunotherapy for cancer, and several unique applications are described below.

3. Nanoparticles for mRNA delivery

Early interest in mRNA stemmed from its use as an alternative to conventional and DNA-based vaccines [87]. mRNA therapeutics are a promising alternative to DNA owing to their lower mutational risk, fewer intracellular delivery barriers, and transient expression [24,88,89]. Further, mRNA only needs to cross the cell membrane and reach the cytosol – in contrast to DNA which requires nuclear entry - to induce protein translation [88–90]. Finally, protein expression induced by mRNA is transient and does not require integration into the genome, thereby avoiding the risk of insertional mutagenesis that can occur from DNA [24,88,89]. When used as a vaccine, mRNAs encoding for antigens

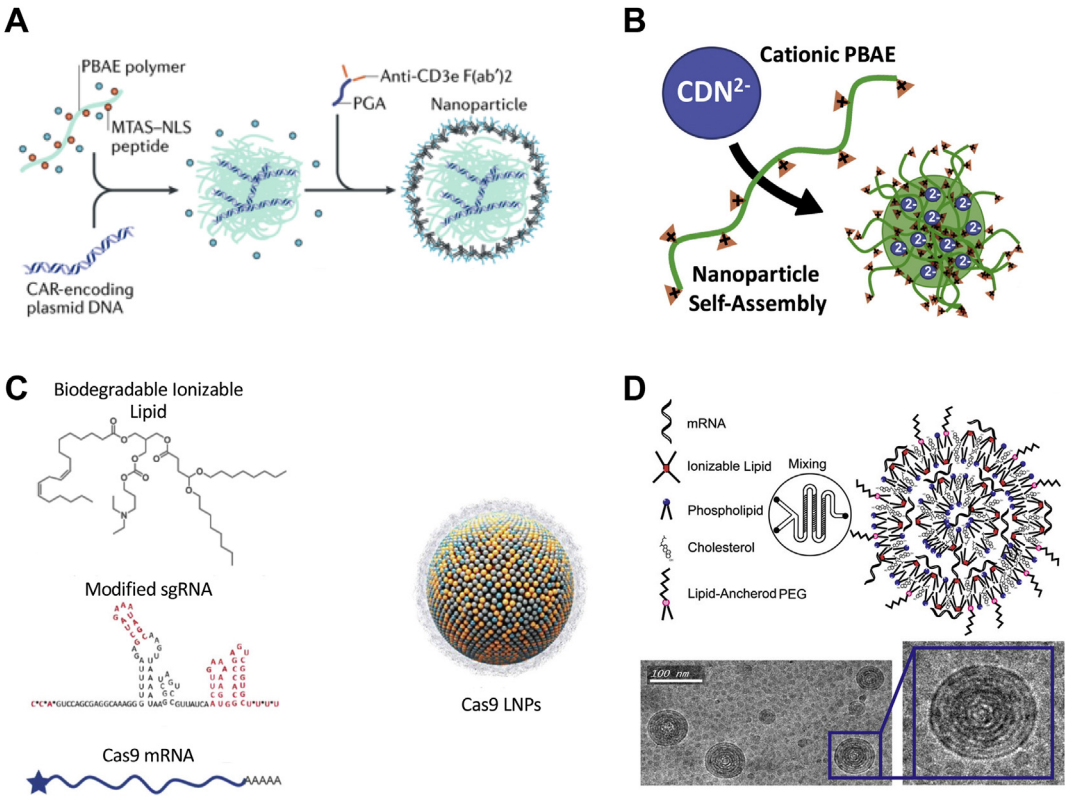

Fig. 3. NPs for nucleic acid delivery and their applications in cancer immunotherapy. **A.** A PBAE polymer functionalized with an MTAS-NLS peptide was used to condense CAR-encoding plasmid DNA. In this application, an anti-CD3e-poly(glutamic acid) (PGA) conjugate was adsorbed to the surface of the PBAE core to enable T-cell targeting and *in situ* generation of CAR T-cells. Adapted with permission from Ref. [81]. **B.** PBAE polymer was used to deliver a Stimulator of Interferon Receptor Genes (STING) antagonizing cyclic dinucleotide (CDN) in combination with a PD-1 blocking antibody and demonstrated potent inhibition of tumor growth. Adapted with permission from Ref. [83]. **C.** A biodegradable ionizable lipid was used to co-deliver a modified sgRNA and Cas9 mRNA that achieved potent gene editing in the liver for 12 weeks. sgRNA was modified with phosphothiorate bonds at both ends of the strand (indicated by *) and 2′-O-methylation of nucleotides (shown in red). Adapted with permission from Ref. [126]. **D.** Multilamellar ionizable LNPs generated potent CD8 T-cell activation upon antigen delivery, and were used to deliver tumor antigens gp100 and TRP2 that led to tumor shrinkage and elongated survival in a B16F10 melanoma mouse model. Adapted with permission from Ref. [49]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

are delivered to antigen presenting cells, either through *ex vivo* transfection or under systemic administration. Antigen presenting cells then translate mRNA into its encoded cancer-associated antigen that is presented to T-cells for activation and induction of cytotoxic T lymphocyte responses [87]. However, the large size (10^3-10^5 nucleotides), negative charge, and hydrophilicity of mRNA, combined with its susceptibility to nucleases, hinder the ability of naked mRNA to reach and enter target cells upon systemic administration [14,24,88–90]. NPs can overcome these barriers and facilitate its intracellular delivery, and several NP platforms for mRNA delivery are described below.

Similar to DNA delivery, ionizable LNPs have also been used for mRNA delivery. In one example of this, LNPs comprised of an ionizable lipid, a helper phospholipid, cholesterol, lipid-anchored PEG , and mRNA were designed to induce expression of luciferase and erythropoietin following systemic injection in BALB/c mice [91]. The study utilized Design of Experiment (DoE) methodology to optimize a top-performing LNP for siRNA delivery to now deliver mRNA to the liver of mice [91]. In the context of immunotherapy, multilamellar ionizable LNPs were used to deliver tyrosine-related protein 2 (TRP2) and glycoprotein 100 (gp100) tumor self-antigen mRNAs to antigen presenting cells to induce a cytotoxic CD8 T-cell response (Fig. 3d). Subcutaneous administration of these LNPs led to reductions in tumor volume, extended survival in a B16F10 tumor model, and yielded potent $CD8^+$ activation [49]. Interestingly, these LNPs were able to transfect neutrophils, macrophages, and dendritic cells, demonstrating that they may be useful to deliver mRNA to a range of immune cells.

Similar to DNA delivery, the chemical diversity of polymers and polymer-lipid systems allows for identification and incorporation of structures that can improve biocompatibility and bioavailability of the encapsulated materials [27]. Polymers used for mRNA delivery are positively charged and can condense mRNA into nanometer sized electrostatic complexes [88]. PBAEs are a major class of pH-responsive and bioreducible polymers known for their biocompatibility, but they have had limited *in vivo* success due to their poor serum stability [92,93]. Recent PBAE work has explored the incorporation of PEG-lipids and new PBAE architectures to improve uptake, stability, and biodistribution [94–96]. In one approach, PBAEs were modified to include internal alkyl tails to enable their formulation with PEG-lipids and improve their stability under physiologic conditions [96]. mRNA-loaded PBAE terpolymer NPs formulated with PEG-lipids demonstrated greater serum stability *in vitro* compared to those formulated without PEG-lipids, and they achieved selective luciferase expression in the lungs of mice following intravenous injection [96]. PBAEs have also been used for mucosal immunization, where lipid-enveloped NPs with pH-responsive PBAE cores delivered mRNA to immune cells [97]. Mucosal immunization has drawn interest for two reasons: 1) many pathogens invade through mucosal surfaces and 2) mucosal immunizations can elicit both systemic and mucosal immunity [98,99]. These NPs successfully delivered GFP mRNA to difficult-to-transfect dendritic cells *in vitro* as well as luciferase mRNA to mucosal tissue compartments following intranasal administration *in vivo* [97].

Vaccination is a central application of mRNA for cancer immunotherapy. Electroporation is typically used to introduce mRNA into T-cells *ex vivo*, which can induce cellular toxicity [13]. Further, this *ex*

vivo cell engineering process is time, labor, and cost intensive, which creates significant challenges towards broader clinical translation [100]. Thus, NPs are ideal to deliver mRNA to T-cells without the need for electroporation. An early mRNA cancer vaccine approach utilized PEGylated histidine-rich polylysines mixed with L-histidine-(N,N-di-n-hexadecylamine)ethylamide (HDHE) and cholesterol liposomes, referred to as histidylated lipopolyplexes, to deliver mRNA encoding human melanoma antigen MART1 to T-cells. Immunization with MART1 histidylated lipoplexes induced priming of B16-specific $CD4^+$ and $CD8^+$ T-cells, leading to a ~10-fold reduction in tumor volume and a 75% reduction in detectable lung metastases compared to control mice in a B16/F10 melanoma model [101]. More recently, PBAE NPs were coated with CD3 or CD8 antibodies and used to target T-cells in order to induce receptor-mediated endocytosis. Antibody-coated NPs improved *ex vivo* T-cell transfection 10-fold compared to non-targeted PBAE NPs [12]. Building upon this success, this robust platform has been used to deliver two different mRNAs. In one study, these NPs delivered megaTAL nuclease mRNA to knockout endogenous T-cell receptors that may cause graft-versus-host disease [12]. Separately, NPs loaded with an mRNA encoding the $Foxo1_{3A}$ transcription factor were used to guide $CD62L^+$ T-cells away from terminal differentiation and senescence, and towards a central memory phenotype [12]. Taken together, these results indicate that this robust platform can be adapted to generate several distinct immune responses.

Recently, mRNA vaccines have begun testing in clinical trials. The clinical translation of mRNA vaccines is being led, in part, by Curevac (NCT03291002) with an RNA-based adjuvant that is being tested in patients with melanoma and squamous cell carcinoma, among others. With the introduction of these vaccines into the clinic, researchers are increasingly working towards introducing mRNA delivery systems as well. LNPs complexed with NY-ESO-1, MAGE-A3, tyrosinase, and TPTE mRNA are currently being evaluated in clinical trials. Early phase 1 dose escalation data has demonstrated that neutral or negatively charged LNP-mRNA complexes are well tolerated, and there were dose dependent IFN-α and antigen-specific T-cell responses in three melanoma patients [20]. Together with the preclinical studies described above, this demonstrates that NPs can aid in overcoming challenges associated with mRNA delivery, and enable potential use for multiple forms of cancer immunotherapy.

4. Nanoparticles for gene editing

CRISPR/Cas9 has emerged as a powerful tool in understanding and treating the genetic cause of various diseases (Fig. 4) [102,103]. In the context of cancer immunotherapy, CRISPR has been applied to disease modeling [104–106], target identification [107], and immune cell engineering [108–111]. CRISPR/Cas9 can be delivered as a nucleic acid-loaded protein (ribonucleoprotein, or RNP) or as nucleic acids [112,113]. Protein delivery presents several challenges, and strategies for overcoming these barriers have been reviewed elsewhere [114–116]. In contrast to nucleic acids, the chemical diversity and size of proteins often necessitates modifications to enable delivery with a vector [117,118]. For example, a successful approach is to "supercharge" proteins through the addition of densely charged moieties to enable electrostatic complexation with carriers [117,118], and this approach has been particularly effective in the localized delivery of Cas9 RNP NPs [119–121].

Delivery of Cas9 protein and mRNA offer transient protein expression, which is potentially beneficial because constitutive Cas9 expression can increase the risk for off-target editing and stimulation of pre-existing adaptive immune responses to the Cas9 protein [122–124]. However, the most successful attempts at formulating Cas9 RNP into NPs have been limited to localized delivery, whereas Cas9 mRNA has been successfully delivered via systemic administration [119–121,124–126]. Since CRISPR/Cas9 technology is still relatively new, there have been few attempts to deliver Cas9 components using NPs for the purpose of cancer immunotherapy. Here, we highlight NP delivery systems that have delivered CRISPR/Cas9 to treat non-cancer diseases, as well as viral delivery mechanisms for cancer immunotherapy. Moving forward, we anticipate that the critical insights gained from the gene editing studies discussed below will form the basis for NP-mediated gene editing for cancer immunotherapy.

One of the first successful Cas9 NP approaches utilized viral and non-viral NP delivery to achieve homology directed repair in hepatocytes in a mouse model of hereditary tyrosinemia type 1 [124]. A lipidoid-based LNP was used to encapsulate Cas9 mRNA and, due to size constraints, the sgRNA expression cassette and homology directed repair template were delivered using an adeno-associated virus (AAV) [124]. After optimizing the timing of LNP and AAV administration to maximize the overlap between peak Cas9 and sgRNA expression, this system achieved gene editing in 6% of hepatocytes at a 24.1% indel rate measured by deep sequencing of the target locus in total liver genomic DNA [124]. In a different proof-of-concept study, ionizable lipids and helper lipids were used to co-deliver Cas9 mRNA and modified sgRNA to reduce serum concentrations of transthyretin, and achieved 70% gene editing and > 97% knockdown in hepatocytes following a single intravenous injection in mice (Fig. 3c) [126]. In another example of NP-mediated Cas9 delivery, Miller et al. synthesized a library of zwitterionic amino lipids (ZALs) to co-deliver Cas9 mRNA and Lox sgRNA to mice expressing a Lox-Stop-Lox tdTomato cassette, and demonstrated stable gene editing two months after NP administration [125]. ZALs demonstrated potent protein expression with luciferase mRNA at doses < 600 pM *in vitro* and l mg/kg *in vivo* [125]. Together, these early examples of NP delivery platforms for genome editing preface their use for CRISPR/Cas9 nucleic acid delivery for cancer immunotherapy.

In the preceding text, we highlighted successful preclinical applications of NPs for the delivery of Cas9 components. Here, we describe the use of CRISPR/Cas9 for cancer immunotherapy mediated by viral and physical delivery methods. One important use of CRISPR/Cas9 in immunotherapy is towards more robust T-cell engineering. Allogeneic CAR T-cells are an attractive alternative to traditional autologous CAR T-cells because they can be distributed "off-the-shelf" to patients [127,128]. However, allogeneic transplant T-cell receptors (TCRs) can be reactive to host antigens in healthy tissues, leading to graft-versus-host disease (GVHD) [128,129]. Additionally, alloantigens present on transplanted cells, such as human leukocyte antigen-1 (HLA-1), can elicit unwanted host immune responses [128,129]. CRISPR/Cas9 could be employed to knock-out surface molecules to improve the compatibility of allogeneic CAR T-cells [128–131]. An early study utilized a combination of CRISPR mRNA and gRNA to target the T-cell Receptor alpha-constant (TRAC) locus and knock out TCRs. Subsequent transfection with an AAV encoding CAR cDNA was used to induce expression of CD19-specific CAR under transcriptional control of the TRAC promoter. These T-cells were more resistant to tonic signaling and had delayed differentiation and exhaustion, ultimately leading to greater tumor rejection when compared to retrovirally transduced CARs, both with and without TCR knockout [108]. CRISPR/Cas9 targeting enabled the identification of a specific transcriptional regulator that modulated CAR expression to maximize therapeutic benefit.

While single gene editing has several applications, the simplicity of CRISPR/Cas9 gives rise to the capability of multiplexed gene editing to simultaneously knock-out several genes. In one example, Cas9 mRNA and gRNAs were delivered to primary T-cells via electroporation and used to knock out PD-1, a suppressor of CD8 T-cell activity, in addition to TCR and HLA-1 [111,132]. A lentivirus was used to transduce CD19 or prostate-stem cell antigen (PSCA) CARs. Double knockout (TCR- and HLA-1-) CAR T-cells yielded reduced alloreactivity compared to single knockout (TCR-) CAR T-cells while maintaining potent antitumor activity, measured by enhanced survival in a Nalm6 mouse tumor model [111]. Triple knockout (TCR-, HLA-1-, and PD-1-) CAR T-cells demonstrated quicker and complete elimination of tumor cells compared to double-ablated CAR T-cells in an aggressive Nalm6-PDL1 leukemia

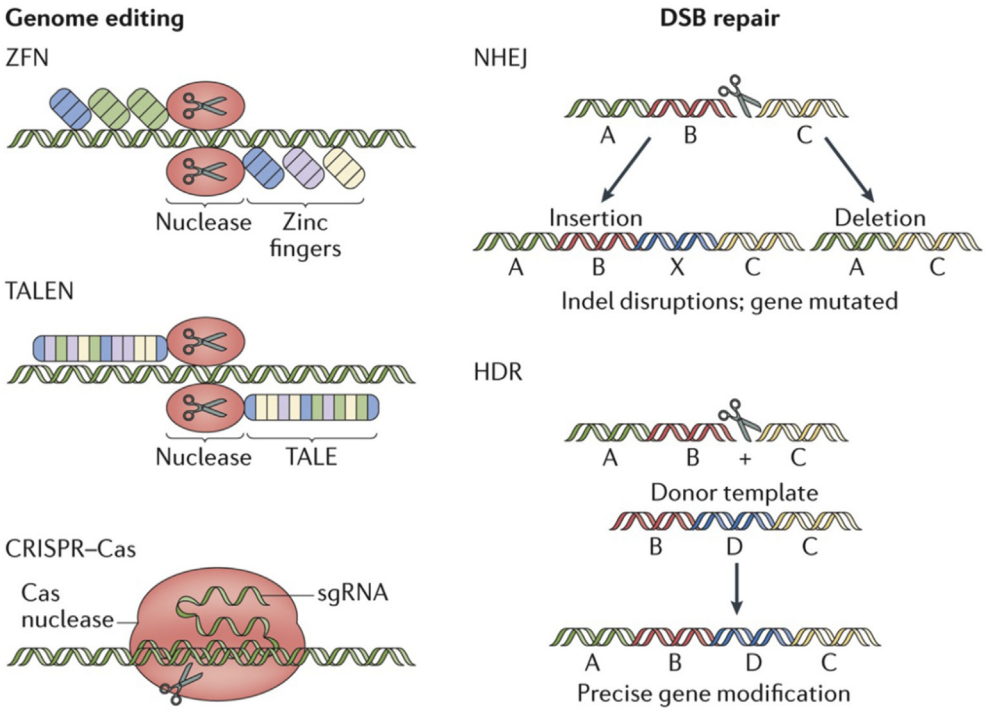

Fig. 4. Methods for genome editing systems. Zinc-finger nucleases (ZFN), transcription activator-like nuclease (TALEN), or CRISPR-Cas systems can be delivered with non-viral delivery platforms such as NPs. These gene editing systems can edit mammalian genomes by introducing double stranded breaks in a highly specific, sequence-dependent manner. Repair occurs through non-homologous end-joining or by homology-directed repair. Adapted with permission from Ref. [112].

model [111]. These results highlight the utility for CRISPR/Cas9 technologies for improving CAR T-cell therapy and their potential for clinical application.

While CRISPR/Cas9 is a potent new tool for treating and understanding disease, but effective intracellular delivery remains a challenge. The applications of CRISPR/Cas9 in cancer immunotherapy previously mentioned rely on combinations of physical and viral delivery methods to achieve gene editing [108,111]. Additionally, Cas9 transfection was performed *ex vivo* and in homogenous cell populations, further simplifying the challenge of delivery [108,111]. However, there has been successful NP mediated delivery of Cas9 components to immune cells. In one example of targeted CRISPR/Cas9 expression, CRISPR/Cas9 pDNA was delivered using cationic lipid-assisted polymeric NPs (CLANs) that were administered intravenously to type 2 diabetic mice. The CRISPR/Cas9 gene was designed to be under transcriptional control of the CD68 promoter unique to monocytes and macrophages in order to decrease off-target Cas9 expression [133]. These particles were used to knock out Ntn1, a guidance cue that blocks macrophage migration in adipose tissue and leads to insulin sensitivity and inflammation. Gene editing was achieved *in vivo* primarily in macrophages and monocytes, with little editing occurring in neutrophils or other off-target immune cells [133,134]. In another example of NP-mediated Cas9 delivery, Li et al. screened a library of PEG-PLGA, PLGA, BHEM-Chol, and DOTAP NPs for delivery of Cas9 plasmids to B cells *in vivo*. After identifying a lead candidate based on accumulation in the spleen and lymph node, the lead formulation was used to correct B cell dysfunction in a rheumatoid arthritis model [135]. Although NPs are still being developed to deliver CRISPR/Cas9 components to immune cells, these early examples of successful *in vivo* delivery preclude their use for gene editing in cancer.

As previously mentioned, the field of NPs to deliver CRISPR/Cas9 for cancer immunotherapy is nascent. The early research into the role of gene editing in cancer immunotherapy, especially the examples of T-cell engineering mentioned above, has yielded promising results as well as insight into the future directions in immunotherapy [108,111]. Concurrently, NPs are being developed to deliver Cas9 components, and have demonstrated efficacy in doing so in both immune and non-immune cells [124–126,133,135].

5. Future directions and conclusions

Currently, most forms of immunotherapy and their NP-based delivery systems have been primarily effective at targeting hematological cancers or melanoma [7]. Moving forward, there is great opportunity to study how NPs can be utilized to treat solid tumors by exploiting their unique physicochemical properties. This engineering challenge can be faced by carefully choosing materials that are known to efficiently transfect cells (such as cationic lipids and polymers) [11,50], have highly controllable sizes and surface chemistry [16,24,136], and those that can be functionalized with targeting ligands to promote uptake by tissues and cells of interest. In an elegant example of NP targeting for solid tumor immunotherapy, LNPs loaded with a DNA plasmid and cationic protamines were functionalized with targeting ligands to activate dendritic cells [137]. These targeted NPs were intravenously injected into mice with orthotopic colorectal tumors, and mice treated with NPs and chemotherapy experienced greater tumor inhibition compared to mice treated with chemotherapy alone [137]. The improvements gained by incorporating targeting ligands onto NPs can be attributed to enhanced target binding strength, biodistribution, and uptake that may decrease the doses required to produce therapeutic effects [138].

The improvements afforded by incorporating targeting ligands onto NPs are particularly attractive for transfecting various subtypes of lymphocytes directly *in vivo* for adoptive T-cell therapy. For example, PEG-PEI targeted to T-cells using CD3 antibodies induced transfection and functional regulation of T-cells *in vivo* in a rat allogeneic heart transplantation model [139]. In the context of cancer immunotherapy, antibody-targeted PLGA NPs have been engineered to bind PD-1 + T-cells in blood, lymphoid tissues, and tumors to deliver TGFβ inhibitors [136]. These complexes were shown to improve survival of melanoma-bearing mice compared to administration of free drug, an effect attributed to targeting this specific population of T-cells. This study demonstrated the concept that NPs can be used to target specific types of lymphocytes *in vivo,* which has the potential to expand the field of cancer immunotherapy in several ways. First, targeting T-cells *in vivo* can initiate specific anti-tumor immune responses for solid tumor immunotherapy, as described in the study above [136]. Second, this

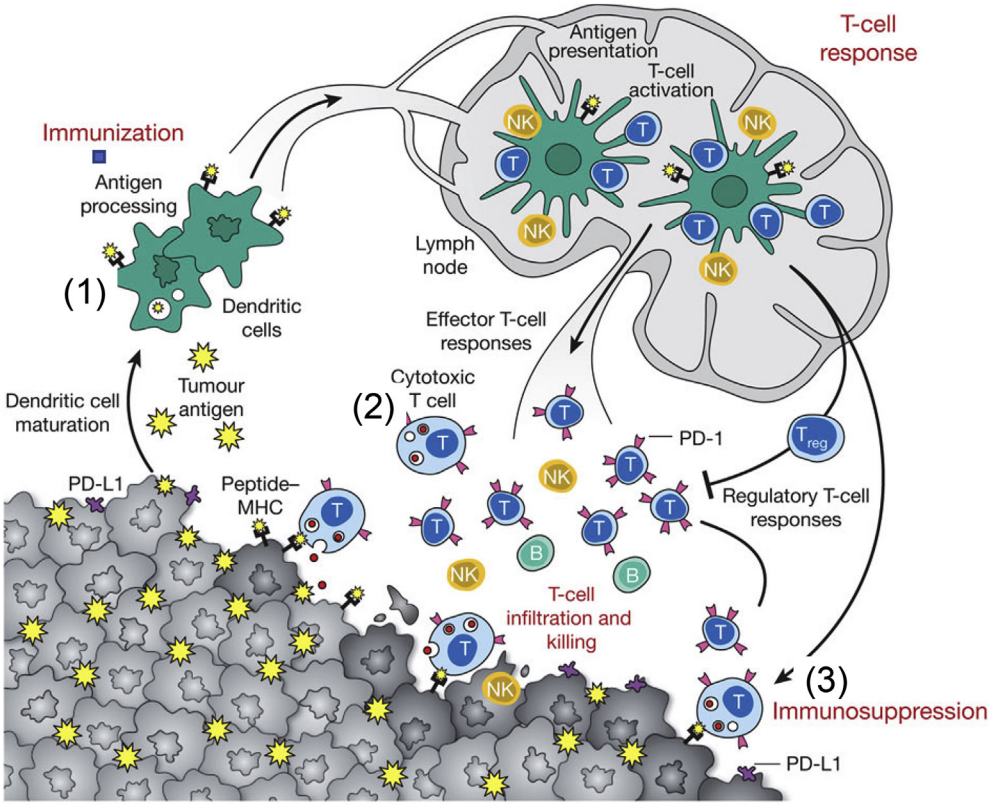

Fig. 5. Sites of therapeutic intervention for NPs to generate anti-tumor immune responses in solid tumors. Anti-tumor immune responses result from the presentation of tumor-associated antigens (TAAs), stimulating protective T-cell responses, and overcoming the immunosuppressive tumor microenvironment (TME). NPs can be used to activate these pathways to successfully deliver immunotherapeutics to solid tumors by: (1) enhancing delivery of nucleic acids encoding TAAs to improve delivery to antigen presenting cells for immune activation; (2) delivering nucleic acids to T-cells to promote their survival, proliferation, and anti-tumor phenotypes; and (3) alleviating the immunosuppressive signaling within the tumor microenvironment. Figure adapted with permission from Ref. [141].

technique can be used to generate CAR T-cells directly *in vivo* to overcome the many manufacturing limitations of *in vitro* CAR T-cell development, including high costs and production time [12]. Lastly, the ability to activate specific subpopulations of immune cells in circulation may be exploited to treat metastatic secondary tumors in addition to primary tumors, as these engineered cells can target any cancer cells throughout the body that express the target protein [101].

Another means of utilizing nanotechnology for immunotherapy in solid tumors is by alleviating immunosuppressive signaling within the microenvironment to improve native T-cell responses (Fig. 5) [140]. For example, delivering siRNA against Snail, a critical transcription factor that accelerates cancer metastasis by inducing immunosuppression, to tumor cells was shown to promote the infiltration of tumor-specific lymphocytes into melanoma tumors [140]. This increase in lymphocyte infiltration led to inhibited primary and metastatic tumor growth [140]. By utilizing NPs to enhance anti-tumor immune responses against solid tumors, researchers can exploit the inherent function of immune cells to attack cancer cells, which may be more effective for treating solid tumors compared to treating tumor cells themselves [141]. Moving forward, this technique to potentially make solid tumors more susceptible to native immune activity could be combined with *in vivo* targeting of immune cells for a multi-pronged approach to solid tumor immunotherapy [141].

In addition to developing delivery systems to enable solid tumor immunotherapy, it is also critical to engineer NPs to deliver gene editing tools [24]. Previously, we discussed the use of NPs to deliver CRISPR/Cas9 gene editing material to immune cells to treat diabetes and rheumatoid arthritis, as well as the viral delivery of gene editing technology for cancer immunotherapy [108,111,133,135]. There are several challenges to address as researchers develop nanotechnology to deliver gene editing components *in vivo* for cancer immunotherapy. A critical consideration is developing NPs that offer precise control over timing of nucleic acid delivery and release, as successful gene editing requires delivery of both guide RNA as well as the Cas9 protein or mRNA [124]. This challenge can be met by developing NPs using materials with highly tunable degradation profiles to release the nucleic acid cargo on-demand [18]. Additionally, NPs carrying gene editing components need to successfully edit a sufficient number of cells to mediate the desired therapeutic result, which can be challenge *in vivo* where immune cells are circulating throughout the body. This requirement can be met by attaching targeting ligands to NPs to promote their binding and uptake to target cells [19,136]. Ultimately, NPs that can deliver gene editing technology for cancer immunotherapy can exploit the delivery capabilities of the carriers as well as the highly specific editing afforded by CRISPR/Cas9 systems.

The NP delivery platforms discussed in this article represent novel and recent developments in nucleic acid delivery, with several applications in cancer immunotherapy. However, challenges remain before these systems can be used broadly in the clinic. For example, several of the examples discussed above utilize materials that have not yet been used in clinical trials or are not yet approved by the FDA. NPs comprised of FDA approved materials may have a simpler and expedited path towards clinical translation. Importantly, several NP-based gene therapeutics are in clinical trials or have been recently approved by the FDA. For example, in 2018 Alnylam Pharmaceuticals gained FDA approval for their lipid-siRNA NP Onpattro to treat polyneuropathy caused by transthyretin amyloidosis [142]. This introduction of NP-nucleic acid complexes into the clinic is a critical milestone for the entry of other NP-based immunotherapies into clinical trials and ultimately for FDA approval. As new NP delivery systems enter the clinic, physicians and scientists can begin to shift the current paradigm of cancer therapy towards potent and biocompatible nucleic acid delivery systems for immunotherapy.

Author contributions

A.J.M, R.S.R., R.Z., and M.J.M. conceived the ideas, performed research for the manuscript, discussed the manuscript content and wrote the manuscript. All authors reviewed and edited the manuscript before submission.

Competing interests

The authors have no conflicts of interest to declare.

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