



Nanoformulations targeting immune cells for cancer therapy: mRNA therapeutics

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ABSTRACT

The approved worldwide use of two messenger RNA (mRNA) vaccines (BNT162b2 and mRNA-1273) in late 2020 has proven the remarkable success of mRNA therapeutics together with lipid nanoformulation technology in protecting people against coronaviruses during COVID-19 pandemic. This unprecedented and exciting dual strategy with nanoformulations and mRNA therapeutics in play is believed to be a promising paradigm in targeted cancer immunotherapy in future. Recent advances in nanoformulation technologies play a prominent role in adapting mRNA platform in cancer treatment. In this review, we introduce the biologic principles and advancements of mRNA technology, and chemistry fundamentals of intriguing mRNA delivery nanoformulations. We discuss the latest promising nano-mRNA therapeutics for enhanced cancer immunotherapy by modulation of targeted specific subtypes of immune cells, such as dendritic cells (DCs) at peripheral lymphoid organs for initiating mRNA cancer vaccine-mediated antigen specific immunotherapy, and DCs, natural killer (NK) cells, cytotoxic T cells, or multiple immunosuppressive immune cells at tumor microenvironment (TME) for reversing immune evasion. We highlight the clinical progress of advanced nano-mRNA therapeutics in targeted cancer therapy and provide our perspectives on future directions of this transformative integrated technology toward clinical implementation.

1. Introduction

The authorized use and increasing global vaccination rate of two effective and safe mRNA-based vaccines (BNT162b2 [1] and mRNA-1273 [2]) against COVID-19 have successfully reduced the transmission of coronaviruses and deadly illness, making extraordinary contributions to helping people get through the difficult pandemic. The success of these lipid nanoformulation-mRNA-based COVID-19 vaccines have provided practical insights into the treatment of cancer patients by introducing tumor antigen-encoding nano-mRNA technology [3].

Malone and colleagues first reported the successful protein expression of mRNA delivered by a synthetic cationic lipid nanoparticle (LNP) in vitro in 1989 [4]. Soon after, Wolff and co-workers demonstrated the success of in vivo transfection of reporter luciferase protein by direct

injection of naked mRNA into mice in 1990 [5]. The study of administration of mRNA expressing vasopressin into rats first demonstrated the biological response of mRNA platforms in 1992, though the biological function did not last long [6]. In 1993, liposome-mRNA expressing influenza virus protein showed the induction of antigen specific cytotoxic T lymphocytes (CTLs) against virus infected cells in mice [7], indicating immunogenic features of mRNAs. In 1995, the first naked cancer mRNA vaccine encoding carcinoembryonic antigen presented antigen specific antibody immune response, protecting mice from tumor challenge [8]. Despite these early promising results, the development of mRNA therapeutics was not well invested at that time, largely owing to inadequate knowledge in mRNA biology and poor control on mRNA stability, inherent high immunogenicity, and in vivo delivery strategies.

Upon pathogen invasion, the innate immune system can recognise

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the foreign by sensing pathogen associated molecules (such as RNA), thereby orchestrating adaptive immune response [9]. The path from RNA sensing to the licensed mRNA products for human use took decades. Toll-like receptors (TLRs, such as TLR3 [10], TLR7 [11], TLR8 [12]) of antigen presenting cells (APCs) were found sensing RNA molecules in the early 21st century, but it was unknown how APCs can distinguish pathogen RNA from self-RNA released from apoptotic self-cells until 2005. The historic collaboration between two pivotal researchers, Karikó and Weissman from different disciplines, led to a landmark in RNA biology research [13]. It was discovered that RNA modifications, such as base alternations and pseudouridine incorporation, could ablate RNA mediated activation of TLRs 3, 7 and 8, subsequently reducing immunomodulatory function [13]. This significant finding uncovered the mechanism by which the innate immune cells can sense non-self RNA molecules, and fuelled the studies on RNA modifications and associated receptor-based immune recognition [14]. However, the precise mechanistical understanding of “how” and “to what degree” the receptors can tolerate the modified RNA molecules remains incompletely clear. Despite these knowledge gaps, this finding paved the way for current authorized use of synthetic COVID-19 mRNA vaccines in human, where pseudouridine incorporation enables acceptable immunostimulatory function (reducing adverse effects) and increased translation capability of target proteins (enhancing antigen production). Given the advances on mRNA optimization and purification, synthetic mRNA has been explored as a versatile technology to produce peptides and proteins as therapeutics in the host.

To enhance the efficacy of synthetic mRNA therapeutics, various nanoparticles have been engineered to protect mRNA molecules from degradation by ubiquitous RNases in vivo, deliver them to specific organs, and achieve endosomal escape after entering cells [15]. The prevalent mRNA delivery system at advanced clinical development is ionizable LNPs [16]. Accumulated knowledge in nano-mRNA technology is expected to advance the development of a wide range of therapeutics, such as preventative viral vaccines [17], therapeutic cancer vaccines [18], and replacement of protein-based immunotherapeutics [18].

To fight against infectious diseases, the prophylactic mRNA vaccines require robust antigen specific antibody immune response (humoral immunity) produced by B cells, which can neutralize pathogens with the help of CD4⁺ T cells [19]. In this context, CD8⁺ CTLs at a low level might be involved in the early control on viral infections prior to the production of sufficient antibodies. To combat existing cancer, it is vital for therapeutic cancer vaccines to evoke T-cell based immune response (cellular immunity), particularly potent and multifunctional CTLs, thereby eliminating tumor cells. The help signals from CD4⁺ T-helper 1 (Th1) cells is essential to promote the proliferation, functionality, and longevity of CTLs [20]. Guided by advanced nanoformulations, mRNA cancer vaccines enable simultaneous activation of CD8⁺ and CD4⁺ T cell immunity by targeting regulation of APCs in lymphoid organs, inducing potent and durable cytotoxic activity of CTLs against cancer [21–25]. Recently, Sahin and colleagues tuned the surface charge of mRNA-lipoplexes that encoded tumor neoantigens to be slight negative or near-neutral, which surprisingly allowed exclusive target to splenic plasmacytoid DCs (pDCs) and macrophages post intravenous administration, and elicited exceptionally potent and broad effector T cell immunity against tumor in both preclinical and clinical settings [21]. Instead of activation of TLR receptors like in the case of lipoplexes, the screened heterocyclic amine of LNPs reported recently enabled the activation of intracellular stimulator of interferon genes (STING) pathway, thus enhancing the immunogenicity, in vivo translation of tumor antigen and anti-tumor efficacy of nano-mRNA cancer vaccines [22].

Beyond the progress in targeted therapeutic vaccination, recent emerging nanoformulation technologies have advanced the development of mRNA therapeutics encoding immunostimulatory cytokines [25–27] (ClinicalTrials.gov number: NCT04455620, NCT03946800),

therapeutic antibodies [28–30] (NCT05262530, NCT04683939), or transcription factors [31], creating new modalities in targeted cancer immunotherapy. Success has been reported in nano-mRNA programming tumor associated macrophages (TAMs) [31], cytotoxic (adoptive) T cells [32–34] and NK cells [30], or indirect modulation of multiple immune cells [35]. The rapid advancement in engineered nanoformulations and mRNA technology enables nano-mRNA therapeutics functioning better in vivo, with increased safety, stability, translation, non-TLR associated immunogenicity, modulation of tumor infiltrating immune cells, and anti-tumor immunity.

The technology of nano-mRNA has been revolutionizing the development of new drugs for prevention of infectious diseases and cancer treatment. There is a large pipeline of advanced and innovative mRNA therapeutics under clinical studies, expecting to address many unmet clinical needs in future. Recent reviews on mRNA therapeutics offer timely summary data, focusing on mRNA vaccines for infectious diseases and/or cancers [17,18], LNPs [16] or diverse nano-platforms [15] for delivery of mRNA therapeutics. In this review, we discuss the basic principles and new developments of mRNA technology (Fig. 1-I), and fundamental chemistry and advanced engineering technology of fascinating nanoformulations (Fig. 1-II). We highlight the latest preclinical (Fig. 1-III) and clinical (Fig. 1-IV) paradigms of nano-mRNA therapeutics in regulation of specific subtypes of immune cells of interest for enhanced cancer immunotherapy, such as therapeutic cancer vaccines and protein-based cancer immunotherapy (e.g., nano-mRNA encoding cytokines or specific antibodies). We conclude this review by providing our perspectives on future directions of the integrated nano-mRNA technology from basic science to clinical practices in cancer immunotherapy. This review offers an introduction of nano-mRNA technology and up-to-date reference point in targeted cancer immunotherapy for researchers in this interdisciplinary field of nanotechnology, immunology, and RNA biology.

2. Basic principles of mRNA design and manufacture

mRNA is a single stranded of nucleotides, which was first discovered in 1961 by Brenner and colleagues [36]. It was found that endogenous mRNA was in an unstable intermediate step between the translation of genetic information of DNA and production of DNA encoded proteins by cytoplasmic ribosomes. Since then, numerous scientific breakthroughs in mRNA biology have brought the use of mRNA from basic science to clinical reality (top light blue rectangles, Fig. 2). For instance, Lockard and Lingrel in 1969 provided the first evidence of in vitro production of mouse proteins encoded by purified mRNA in a cell-free system (rabbit-reticulocyte lysate), demonstrating the success of translation of exogenous mRNA in different systems [37]. In 1971, intracellular production of mRNA encoded proteins was achieved in cells [38]. The success of first in vitro transcription (IVT) of functional mRNA using SP6 RNA polymerase in 1984 [39] was an important milestone in the development of mRNA-based therapy. This pioneer work enables commercial scale production of synthetic mRNAs.

In the synthesis of IVT mRNA, the construction of plasmid DNA (pDNA) is the first step, which contains the corresponding sequence of targeted proteins. Then, linearized pDNA is used as a template for DNA-dependent RNA polymerases (such as SP6, T3 or T7 RNA polymerases derived from bacteriophages) to synthesize mRNA in a cell-free approach [40,41]. Finally, pDNA template is degraded in the presence of DNases for mRNA purification. The synthesized mRNA contains five structural regions as shown in Fig. 3. From 5' to 3' end, mRNA includes a 5' cap, a 5' UTR (untranslated region), an ORF (open reading frame) encoding protein of target, a 3' UTR and a 3' poly (A) tail that consists of repeated sequences of adenine nucleotides.

The discovery of mRNA 5' cap modification by Muthukrishnan and colleagues in 1975 [42] was essential for the control on mRNA recognition, immunogenicity and translation. The evidence discovered in 1963 revealed that non-self mRNA from pathogens triggered the

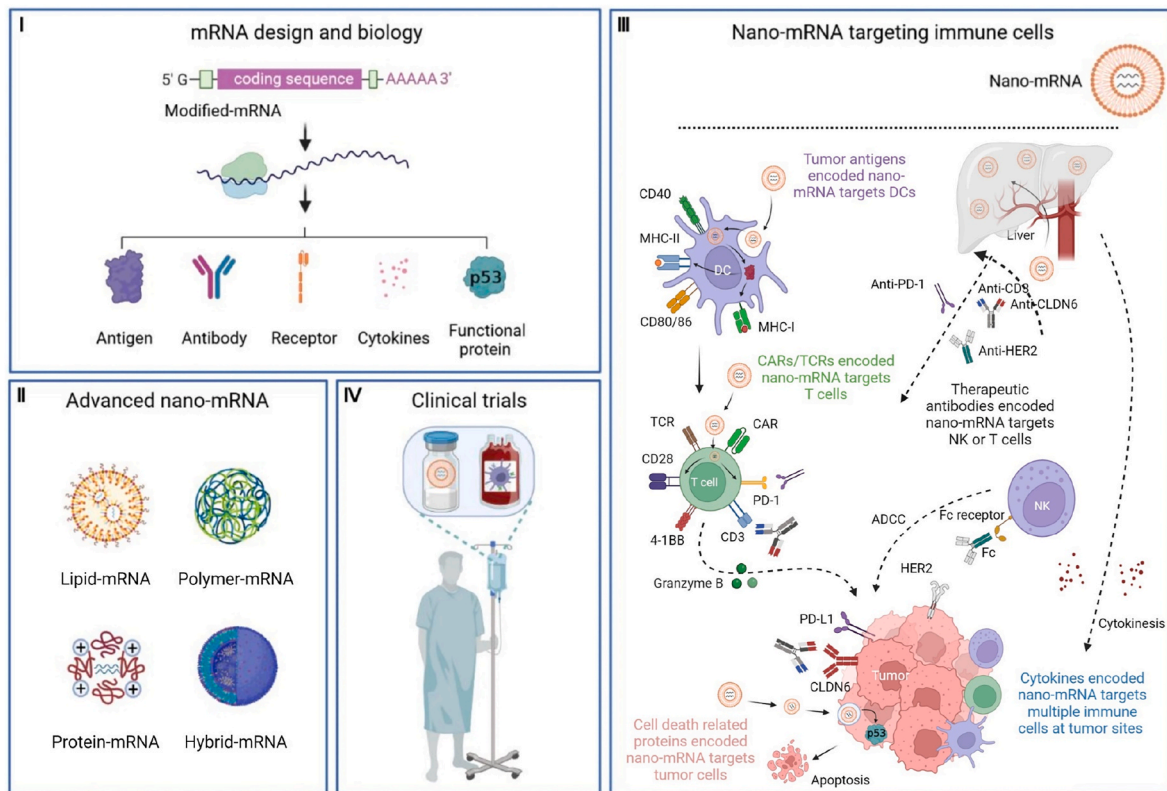


Fig. 1. The fundamental science and emerging concepts in mRNA biology and mRNA delivery nanoformulations underline new modalities of targeted cancer immunotherapy under clinical investigation. (I) The advancements in mRNA biology and modification strategies accelerate the progress of mRNA therapeutics encoding immunological molecules, such as tumor antigens and therapeutic antibodies. (II) Scientific advances in nanotechnology led to the development of cutting-edge platforms for mRNA delivery, including the representative lipid, polymer, protein, or lipid/polymer hybrid nanoparticles. (III) Nanoformulations can encapsulate and protect mRNA molecules from degradation and promote in vivo expression of encoded therapeutic immuno-molecules for targeted modulation of immune cells against cancerous cells in preclinical models. (IV) The encouraging outcomes from preclinical studies together with funding efforts have moved several fascinating nano-mRNA technologies from lab bench to clinical trials. Created with permission by BioRender.

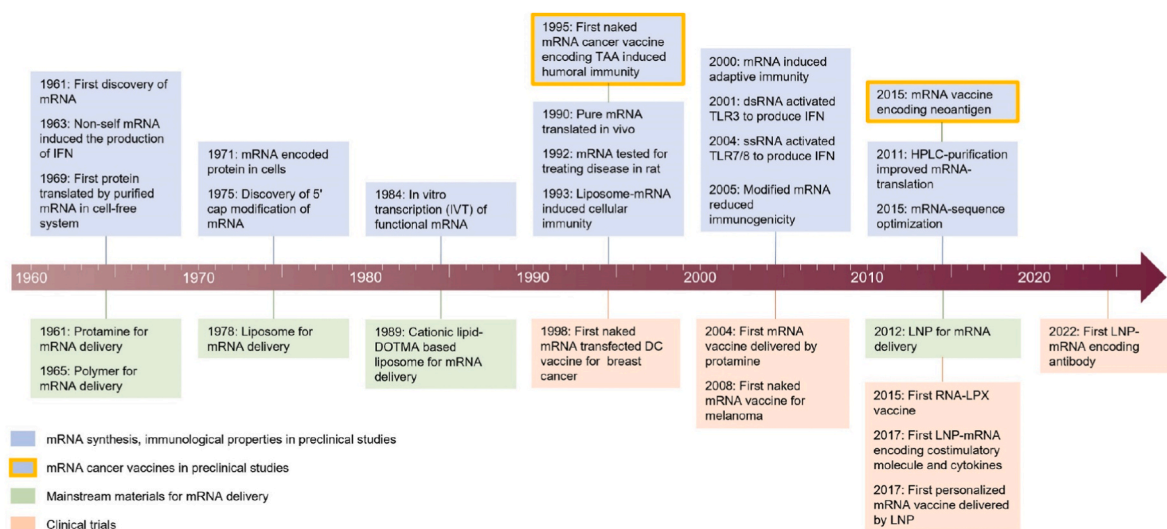


Fig. 2. A timeline of key milestones in the development of mRNA technology and nano-mRNA formulations in preclinical and clinical studies, of which targeted cancer immunotherapy related are highlighted with orange outlines.

production of interferon (IFN), suggesting that host cells can recognise foreigners by sensing non-self nucleic acids [43]. Mechanistically, unmodified exogenous RNA acting as PAMP (pathogen-associated molecular pattern), can be recognised by some key PRRs (pattern recognition receptors) in cells, such as TLR 7/8 [12], TLR 3 [10] and retinoic

acid-inducible gene (RIG)-like receptors (RIG-I and MDA-5(melanoma differentiation-associated protein 5)) [44]. The activation of these innate immune sensors leads to the secretion of type I IFN, degradation of mRNA and inhibition of mRNA translation. In contrast, 7-methylguanosine (m7G) modification of 5' terminal can protect mRNA from

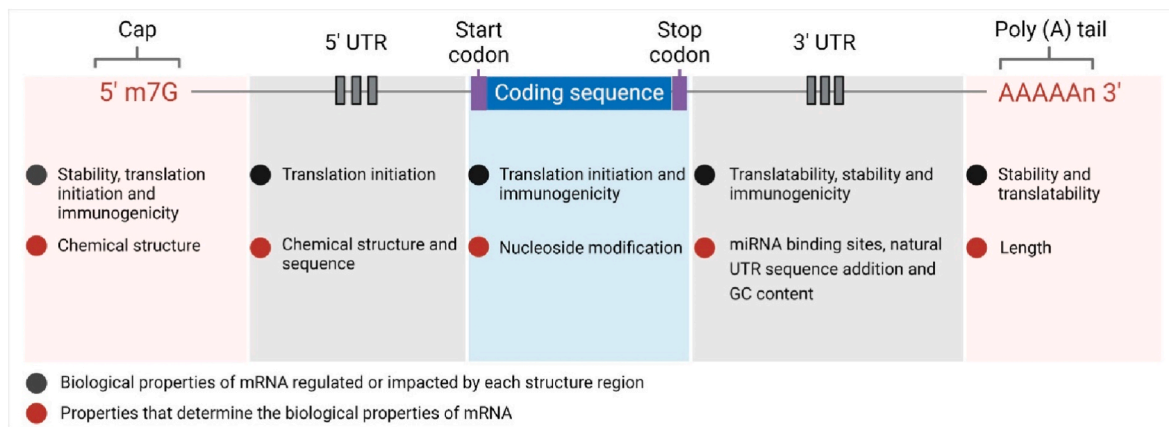


Fig. 3. A scheme of IVT mRNA construct and key functions of each structural region. Created with permission by BioRender.

degradation by 5'–3' exonucleases, thereby increasing the stability of mRNA. Beyond that, 5' cap plays a crucial role in recruiting the translation initiation factor, such as eukaryotic initiation factor 4F (eIF4F) [45], and assisting ribosomes to bind with mRNA sequence, thus improving mRNA translation efficiency. Thus, correct and complete cap structure is critically important in reducing immunogenicity and promoting translation of synthesized mRNA. Capped mRNA can be produced by incorporating a cap analogue in the DNA template during the process of IVT, or by enzymatic reaction after transcription [46,47].

Along with 5' cap, 3' Poly (A) tail plays an active role as in increasing the stability and translation efficiency of mRNA. The average length of Poly (A) tail is 100–250 nucleotides. Studies have shown that the length of poly (A) influences the translation efficiency of mRNA, but the optimal length is still controversial [48–50]. The poly (A) tail can be synthesized by adding poly (T) sequences to a DNA template during the process of IVT, or by using poly (A) polymerases post transcription [51]. The former method can more precisely control the length of poly (A) tail. In the enzymatic polyadenylation, poly (A) polymerases are added to RNA 3'-OH terminal to ensure the formation of a sufficient length of poly (A) tail, however; the length can be influenced by the concentration of 3' end OH, reaction time, enzyme amount and ATP (adenosine triphosphate) concentration. The final product obtained from this approach is often a mixture of mRNAs with heterogeneous tail lengths, which leads to inconsistent quality control in different batches and hardly meets the regulatory requirements [52]. In contrast, the co-transcription of added poly-T nucleotides together with poly (A) enables production of mRNA molecules with a homologous tail length [50].

UTRs regulate the translation and half-life of mRNA. 5' UTR, together with 5' cap, affects ribosome recruitment and the initiation process of mRNA translation. For example, highly stable secondary and tertiary structures of 5' UTR prevent the recruitment of ribosome, but internal ribosome entry sites (IRES) that are in the regions of mRNA can bypass the 5' cap structure and recruit ribosomes directly [53–55]. Therefore, optimizing 5' UTR, such as reducing advanced structures and adding IRES sequences, is vital for enhanced mRNA translation. 3' UTR containing regulatory elements governs mRNA translatability, stability, and immunogenicity. For example, 3' UTR contains binding sites with its partner microRNA (miRNA, a translation repressor), which can regulate the interaction of miRNA and subsequent translation silence mediated by miRNA [56]. Thus, removing miRNA-binding sites from 3' UTR is an approach to promote encoded protein expression. Besides, 3' UTR is a key determinant of intracellular kinetics of mRNAs [57]. Long 3' UTR tends to decrease half-life of mRNAs though short 3' UTR facilitates mRNA translation. To optimise the length of UTRs, natural UTR sequences screened from endogenous long-lived mRNAs, such as globin mRNA [58], are broadly used in synthetic IVT mRNAs. Furthermore, optimizing the proportion of different bases in the 3' UTR sequences,

such as increasing GC (guanine, cytosine) contents and reducing U (uracil) contents [59], can improve the stability and decrease the immunogenicity of synthetic mRNA.

ORF is the most important part of mRNA, which directs the synthesis of proteins of interest. The amino acid composition and spatial structure of each functional protein are specific, so the overall room for adjustment is limited. Codon optimization is one efficacious strategy for increasing translatability and minimizing immunogenicity of mRNAs [60]. Except methionine and tryptophan, each amino acid can be encoded by more than one codon. Therefore, replacing the low frequency codon with the high frequency codon speeds up the translation [61]. However, codon replacement strategy needs to consider the effect of translation speed on the formation of correct spatial structure of proteins. Some rare codons may decrease translational speed but benefit optimization of protein folding [62]. Nucleoside modification appears the most attractive and widely used strategy. Studies confirmed that modified nucleosides profoundly avoid the recognition of mRNAs by PRRs [13,63], and reduce the negative regulation of type I-IFN response on adaptive immunity. Currently, modified nucleosides used in synthetic mRNAs include pseuduridine (ψ) [64,65], N1-methylpseuduridine [66,67], 5-methylcytidine (m5C), 5-methyluridine (m5U), N⁶-methyladenosine, and 2-thiouridine.

In addition to the optimization and post-transcriptional modification of mRNA, purification process used for the synthetic mRNA substantially impacts mRNA intrinsic immunogenicity. Upon IVT process, a wide variety of potentially immunogenic contaminants would induce inflammatory response associated side effects, such as residual templates, nucleotides, and double-stranded RNA (dsRNA). Most of these contaminants can evoke type I-IFN response by activating PRRs, thus inhibiting mRNA translation. The most common method used to purify IVT mRNA is high performance liquid chromatography (HPLC). In 2011, Kariko and colleagues proved that HPLC purification remarkably increased the translational efficacy of unmodified or m5C/ ψ -nucleoside modified mRNA, and almost completely eliminated the immunogenicity [68]. Moreover, another study in 2015 demonstrated that the sequence optimized mRNA purified by HPLC can also obviously avoid innate immune activation in large animals [69]. Nevertheless, the disadvantages of HPLC hamper its wide applications in large-scale production of mRNAs: 1) a large amount of mRNA consumed (with a low recovery rate of about 50%); 2) high economic costs; 3) hazardous waste generated during treatment; 4) long processing time (1–2 days). Currently, some new purification methods with a high recovery and low cost have been developed and applied in mRNA production, such as oligo(dT)-cellulose chromatography that can remove at least 90% dsRNA contaminants and achieve more than 65% recovery rate in less than 2 h [70], and ribonuclease III (RNase III) specific digestion with process time of approximately 30 min [71].

IVT is a rapid, versatile, and controllable method for manufacturing mRNAs, which addresses the major challenges associated with the perception of using mRNA molecules as therapeutic medicines. The first evidence of *in vivo* translation of naked mRNAs was reported by Wolff and colleagues in 1990 (Fig. 2) [5], laying the foundation of the concept that mRNAs can be utilised as therapeutics. Soon after, Jirikowski and colleagues demonstrated that intrahypothalamic injection of mRNA encoding angiotensin can temporarily reverse diabetes insipidus in rats in 1992 [6], which was the first preclinical studies of mRNA used for treating disease. The concept of mRNA vaccines can be dated back to 1993, when induction of cellular immunity was observed in mice immunised with liposome-mRNA encoding influenza virus proteins [7]. The first naked mRNA cancer vaccine designed in 1995 proved the successful induction of humoral immune response against encoded tumor associated antigens (TAAs) in mice [8]. In 2000, mRNA vaccine constructed in a liposome system was able to induce comparable balanced antigen specific adaptive immunity, but showed improved stability compared to the unprotected naked mRNA [72]. Continued research efforts on mechanisms by which host immune system senses endogenous or modified RNAs shared a determination to move mRNA platform from the lab to medical inventions. For example, dsRNA was found recognised by TLR3 in 2001 [10], while ssRNA was found sensed by TLR7/8 in 2004 [12]. The first evidence in 2005 that modified mRNAs were able to completely shut down the TLR-mediated massive inflammation [13]. These key hallmarks have inspired numerous studies on mRNA modifications to balance the immunologic effects and translation efficacy of mRNAs, enabling acceptable safety profile and potency. Naked mRNAs are easily degraded by ubiquitous RNases *in vivo*. As a result, a great variety of delivery platforms (Please refer to next section) have been established to encapsulate and protect mRNAs from degradation.

Compared with DNA-based therapy, mRNA therapeutics have several unique advantages: 1) The translational process of mRNA occurs immediately in the cytoplasm, without entering the cell nucleus. 2) mRNA will not integrate into the host genomic DNA, with reduced risk of mutagenesis and carcinogenesis [73]. 3) Conventional IVT mRNA has no self-replicating ability and degrade rapidly after translation, so mRNA regulation of host cells is transient, exhibiting a favourable safety profile.

Over the past decades, mRNA technology along with advanced delivery nanoplatforms served as the backbone for the success of mRNA therapeutics in human use. In regard of cancer treatment, mRNA therapeutics are expanding well beyond cancer vaccines, including the replacement of therapeutic antibodies, proteins, cytokines, receptors for immunotherapy. This paradigm is a potential modality with great therapeutic prospects for clinical translation in cancer therapy.

3. Delivery strategies for mRNA therapeutics

Inside the body, mRNA is easily engulfed by innate immune cells and degraded by nucleases. Given the negative charge and a relatively large molecular size of 10^4 – 10^6 Da (dalton), mRNA hardly cross the anionic cell membrane [17]. Diverse delivery strategies have been developed for intracellular delivery of mRNA molecules, including physical electroporation methods and nanotechnology-based solutions. The nanoformulation platforms usually contain cationic compositions that can capture mRNA inside the nano-space (Fig. 4), thereby protecting mRNA from *in vivo* degradation. The flexibility in the modulation of chemophysical features of nanoformulations enables targeting delivery to specific organs without causing unwanted toxicities. In terms of intracellular delivery, mRNA encapsulated nanoformulations enter the cells via endocytosis pathway and are trapped in the acidic environment, where the nanoformulations usually facilitate the cytosolic release of mRNA by disrupting the endo/lysosome membrane (Fig. 4). The delivered mRNA can then exert its function to guide ribosome to produce the encoded immunogenetic proteins, which will be secreted out to extracellular space, tethered to the cell membrane, processed by proteasome and presented on cell surface, or function inside the transfected cells. During the past decades, numerous advanced nanoformulations have proven their capability in protecting, delivering and potentiating mRNA therapeutics in animal and human studies (bottom light green and orange rectangles, Fig. 2). In this part, we will introduce the basic physicochemical properties of nanoparticle delivery platforms, advanced development in the formulation of nano-mRNA therapeutics, and the structure-function relationships in targeted cancer immunotherapy.

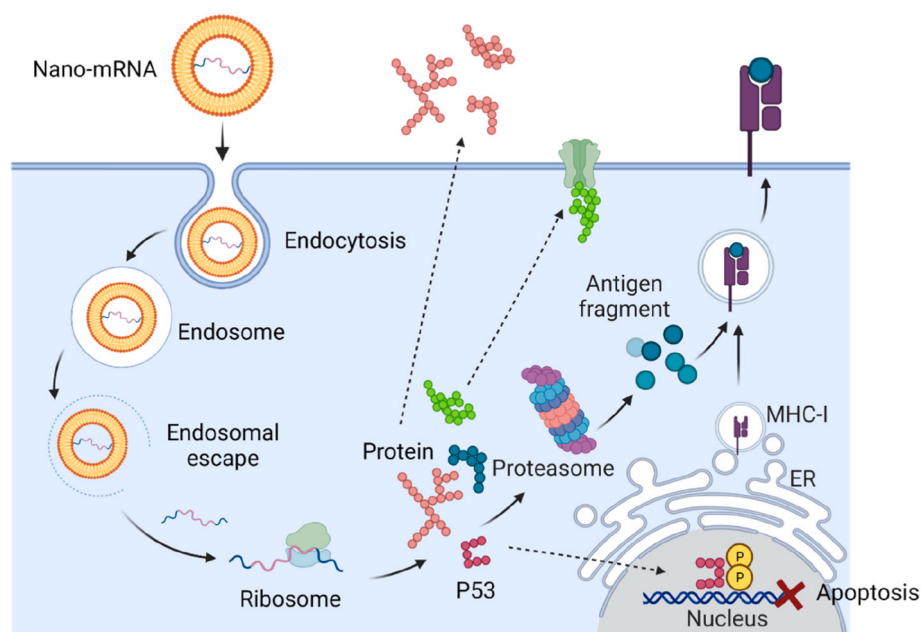


Fig. 4. A scheme showing the intracellular delivery and translation process of mRNA encapsulated in the nanoformulations, and the function modes of produced proteins. Created with permission by BioRender.

3.1. Physical delivery for mRNA therapeutics

The commonly used physical methods for mRNA delivery includes electroporation [74,75], gene guns [76], photoporation [77] and direct local injection [78]. Electroporation, also termed electrotransfection, is defined as the use of an external electric field between two electrodes to form small pores in the cell membrane, so that negatively charged mRNA can pass through the membrane barrier. Electroporation approach is often applied locally and relatively superficial area to transfect targeted cells rapidly and efficiently. Currently, electroporation is an efficient and rapid method utilised to generate genetically engineered DCs or T cells for cancer treatment in animal models and humans [79–81]. However, *in vitro* electroporation will inevitably damage the function of some target cells and even cause cell death. While at the same time, *in vivo* electroporation is also affected by the electrode contact area, so it can only be used in superficial area. Gene gun shots mRNA loaded on gold nanoparticles at a high speed into target cells by using a gas shock wave [82]. However, potential cell death caused by the high-pressure shock wave and high costs limit the practical application of gene gun in mRNA delivery. Photoporation is a relatively new technique, in which transient pores are created in the cell membrane using a femtosecond laser to increase cell membrane permeability of delivered molecules [83]. A recent study demonstrated that vapor nanobubble assisted photoporation increased mRNA transfection of T cells *in vitro* under a reduced laser intensity [77]. But until now, it has been mainly used at the single-cell or subcellular levels.

Direct local injection of naked mRNA molecules has shown some efficacy in cancer immunotherapy, while the protein expression level is relatively low. Enhanced protein expression was observed post intranodal injection of mRNA dissolved in buffers containing calcium ions [84]. Modification of mRNA construct is an alternative approach to increase the potency of direct injected mRNA *in vivo*. For example, Christian and colleagues constructed N1-methylpseudouridine modified and cellulose-purified mRNA that encoded multiple cytokines of GM-CSF (granulocyte-macrophage colony-stimulating factor), IL-12 (interleukin-12), IL-15, and IFN- α . Intratumoral injection of the modified mRNA enhanced the translation of mRNA and induced potent anti-tumor immunity in preclinical tumor models [78]. However, the promising results of naked mRNA therapeutics in preclinical animal models cannot be reflected in clinical studies, which could be contributed by species-specific difference in immune system. For instance, intradermal injection of naked mRNA encoding TAAs in patients with melanoma under a clinical study, did not show clinical effectiveness [85], though mice intradermally immunised with naked mRNA demonstrated successful protein expression and development of immune response against encoded proteins. Altogether, direct administration of naked mRNA locally could be a promising therapeutic utility in certain circumstances, such as certain administration routes, mRNA modification and research settings.

3.2. Nanoformulations for delivery of mRNA therapeutics

Physical methods are simple but have some intrinsic disadvantages, such as potential side effects in cell damage, limited access to the sites or cells of interest, inconsistent efficiency in small and large animals. Therefore, chemical nanomaterial-based delivery strategies have been developed as alternative strategies for delivery of mRNA therapeutics, greatly assisting the translation of promising mRNA therapeutics into clinical use. As immunotherapy landscape evolved, delivery nanotechnologies have rapidly adapted to meet specific delivery need for mRNA immuno-therapeutics [86]. The advantages of nanoplatfoms in mRNA delivery are profound compared to other viral delivery methods, such as low immunogenicity, excellent biocompatibility, high payload, and easy scale-up fabrication. The widely used nanoplatfoms for mRNA delivery mainly include lipid/liposome [16], protein/peptide [87], lipid/polymer hybrid [88], and inorganic nanoparticles [89]. Each type of

nanoparticles possesses unique chemical compositions and physical properties of solubility, size, surface charge and softness. Upon *in vivo* administration, these physicochemical features are determinants for their biodistribution, immunogenicity, and transfection efficacy of mRNA. Improved fundamental understanding on the mechanisms on mRNA-nanoformulation mediated immunological response will provide guidelines on further optimization toward the development of next generation of nanoparticle delivery systems.

3.2.1. Peptide or protein-based nanoparticles

Peptides and proteins are biological polymers comprising of amino acids as building blocks. Proteins but not peptides appear in a complex spatial structure. Protamine is a natural cationic polypeptide, which was the first mRNA delivery nanoparticles proposed by Amos in 1961 (Fig. 2) for enhanced transfection in cells [90]. The tight nanocomplexes of protamine-mRNA increased the stability of delivered mRNA molecules against degradation by serum RNases [72]. The first clinical trial of mRNA cancer vaccine delivered by protamine was initiated by Benjamin and co-workers in 2004 [91], revealing that protamine was a safe mRNA delivery system and was able to induce T cell immunity. The encouraging preclinical data supported the development of protamine-based mRNA cancer vaccine products under early clinical investigation (see the details of clinical trials in Section 5).

Cationic cell-penetrating peptide (CPP) is a class of small molecule polypeptide, generally composed of 8–30 amino acids that are capable of mediating cell membrane penetration and endosomal membrane disruption [92]. Thus, CPP-based nanoparticles were explored as antigen mRNA delivery systems, enabling induction of antigen specific CTLs [93]. Different from protamine that delivers mRNA into cells through classical endocytic pathway, CPP assists intracellular delivery of mRNA molecules via receptor independent direct transmembrane, which is more efficient and less limited by the type of cells to be delivered [94]. In addition, CPP, as a short peptide, shows advantages in large-scale production compared with natural extracted protamine with a spatial structure [95]. CPP started late in the field of mRNA delivery and the development of CPP-mRNA in cancer immunotherapy has significantly lagged behind protamine systems. However, the application prospects of CPP in mRNA delivery for cancer immunotherapy are very broad.

Virus-like particles (VLPs) are formed by the self-assembly of one or several viral structural proteins in the absence of viral DNA or RNA genomes. Natural VLPs (20–300 nm) often act as subunit antigen sources in prophylactic vaccine products [96], such as human papillomavirus (HPV) vaccine Gardasil. The potential of VLPs as delivery platforms was recently investigated for exogenous mRNA encoding reporter proteins [97]. VLPs encapsulate mRNA by binding internal amino acid residues of VLPs with specific motif sequence in mRNA molecules [98]. In some cases, the encapsulation of mRNA in VLPs is driven by the electrostatic interaction between the positively charged arginine sequence at the N-terminal of the capsid protein and the negatively charged RNA skeleton [99]. Recently, VLP-mRNA paradigm was used for cancer immunotherapy in preclinical animal models. For instance, recombinant bacteriophage MS2 VLPs was reported to deliver mRNA encoding prostate cancer associated antigen PAP (prostatic acid phosphatase) and GM-CSF to DCs, inducing balanced Th1/Th2 responses against murine prostate cancer [100]. Recently, Zhang and colleagues reported that PEG10 (paternally expressed 10), a mammalian retrovirus like protein, enabled successful encapsulation of exogenous mRNA by flanking delivered mRNA genes with 5' and 3' UTRs of PEG10 [87]. VLPs resemble the capsid structure of viruses that can be recognised by PRRs of innate immune cells, possessing immunogenic effects [101]. Beyond that, the ease of large-scale production and purification endows VLPs as potential mRNA delivery platforms in targeted cancer immunotherapy, though this paradigm is still under early investigations.

3.2.2. Lipid nanoparticles

Lipids are a class of amphiphilic molecules comprising a hydrophilic

head, a hydrophobic tail, and a linker conjugated two domains. Different types of lipids, such as natural phospholipid, ionizable lipids and cationic lipids, have been extensively investigated for mRNA delivery in preclinical animal models and humans. Liposomes are spherical nanovesicles with single or double layers formed by phospholipids and cholesterol (Fig. 5). In 1978 (Fig. 2), scientists used membrane-structured liposomes to transport mRNA into mouse [102] and human [103] cells, inducing intracellular expression of encoded proteins. Now, solid LNPs that were first explored in delivering self-amplifying mRNA vaccines in 2012 [104], are favourably used in clinic. Solid LNPs are made of four-lipid cocktail (Fig. 5): 1) a helper phospholipid that resembles the cell membrane lipids forming a bilayer structure; 2) a cholesterol that modulates the integrity, rigidity and stability of the bilayer structure, 3) a polyethylene glycol (PEG)-lipid that increases the colloidal stability and reduces opsonization, and 4) an ionizable lipid that is protonated with a positive charge at a low pH and cling to mRNA molecules in the core of LNPs. Of note, the ionizable lipids lose the positive charge under bloodstream alkaline conditions, decreasing systemic toxicity. In addition, the protonation of ionizable lipids in acidic endo/lysosomes promote endosomal escape of delivered mRNA by destabilizing the membrane. The incorporation of ionizable lipids is the key for the success of LNP-mRNA vaccines, such as ionizable lipid SM102 in mRNA-1273 and ALC-0315 in BNT162b2 (Fig. 5). In addition to delivery

function, zwitterionic ionizable lipids containing a heterocyclic amine group in the head domain were found capable of activating STING pathway and potentiating anti-tumor immunity of LNP-mRNA vaccines [22].

By contrast, cationic lipids have a permanent positively charged head [105]. The cationic lipids of DOTMA (1,2-di-O-octadecenyl-3-trimethylammonium propane (chloride salt)) and DOTAP (1,2-dioleoyl-3-trimethylammonium-propane, chloride) (As demonstrated in Fig. 5) were the first lipids explored for mRNA delivery in 1989 [4] (Fig. 2). In 2015, cationic lipids demonstrated successful delivery of mRNA expressing neo-antigens that can be recognised by CD4⁺ T cells, with complete rejection of established tumors in mice [106]. Cationic lipids together with cholesterol and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (Fig. 5) are commonly used in the LNP-nanoformulations for mRNA delivery, such as the therapeutic mRNA cancer vaccine product of lipoplexes that are actively investigated under clinical studies [21,23]. DOPE is the most widely used auxiliary lipid, which can stabilize the bilayer membrane structure of LNP and reduce the cytotoxicity of positive components of LNPs. In addition, the inverted hexagonal H(II) phase of DOPE leads to the instability of endosomes, which plays a crucial role in assisting endosome escape of LNPs [107]. By modulating the surface properties of lipoplexes to slightly negative charge, lipoplexes can direct mRNA

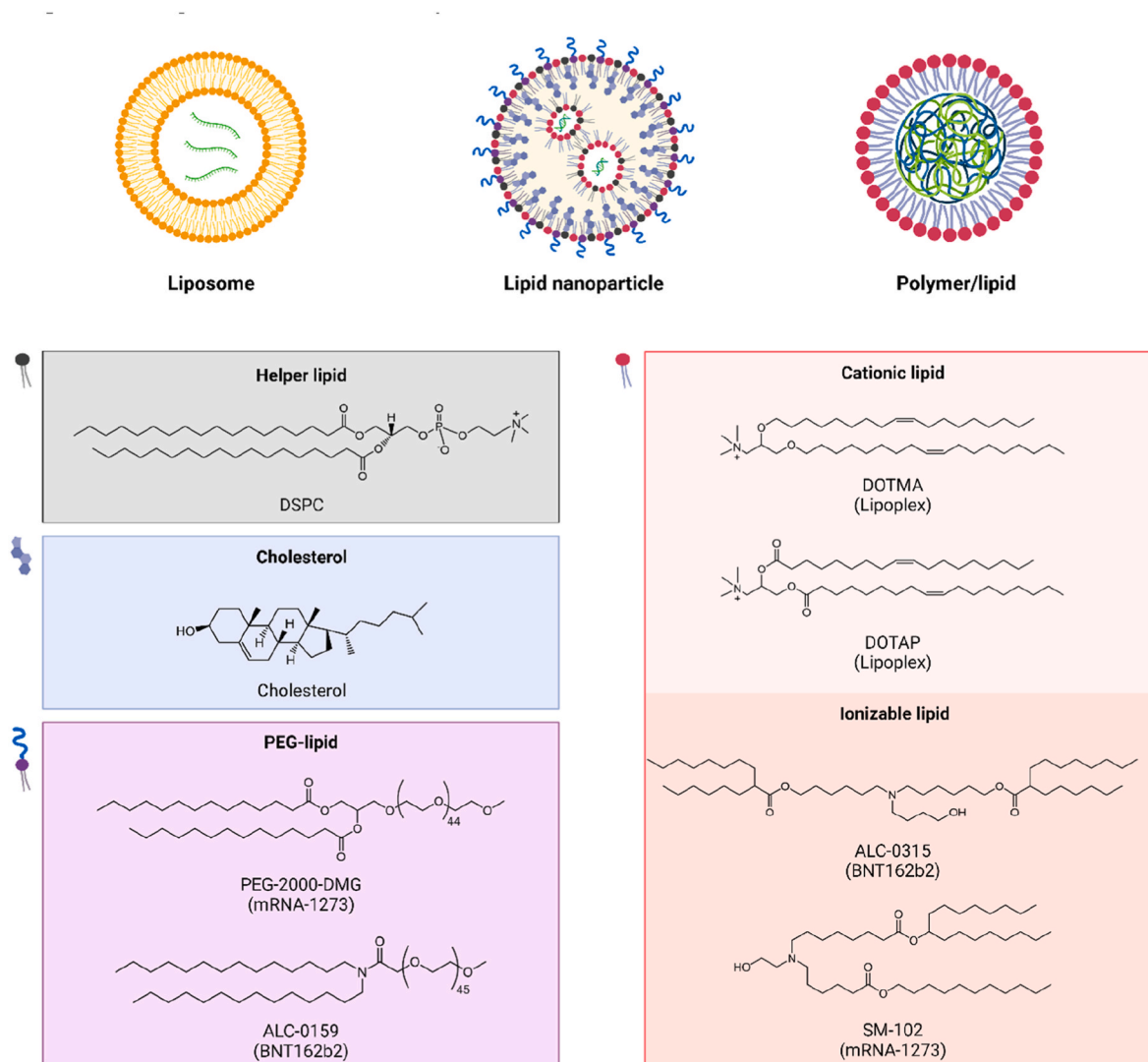


Fig. 5. Schematic illustration of representative cationic or ionizable lipid-based nanoformulations complexed with mRNA and chemical structures of the components in LNPs. Created with permission by BioRender.

specifically to spleens for potent vaccine-induced immunity (see details in Sections 4 and 5). Diverse cationic lipids have been developed to adapt into the need of specific nano-mRNA therapeutics, which are clarified in detail in an excellent review focusing on the LNPs for mRNA delivery [16]. The delivery efficacy and inherent adjuvancity of cationic-based LNPs can be widely adjusted by alternating the chemical compound structures of each component as well as physical properties of nanoformulations.

3.2.3. Polymer nanoparticles

A polymer is a class of macromolecules composing numerous repeating natural or synthetic subunits, which can be formed in a variety of nano-constructs [108]. Cationic diethylaminoethyl-dextran was the first type of polymer, being found capable of enhancing poliovirus RNA infection in 1965 [109]. This chemical now is a product in the market for gene transfection. Compared to LNPs, polymer-based nanoparticles are less clinically advanced, but offer similar functions for mRNA delivery. For instance, the cationic polymers condense mRNA molecules into the nanoformulations, promoting cellular up by cells and cytosolic delivery by proton sponge mediated osmotic swell and endosome membrane rupture [110].

Polyethylenimine (PEI) is the most often used polymers for gene delivery, while its applications are limited by high charge density mediated toxicity [111]. To mitigate the toxicity, biocompatible or degradable components are incorporated into PEI-based formulations, such as PEG [112] and redox sensitive disulfide linkers [113]. Alternatively, biodegradable polymers that contain with disulfides have been explored for mRNA delivery, such as poly (β -amino ester)s (PBAE) that combined with PEG-lipid specifically targeting lungs [88,114,115]. In addition, pH-responsive poly(aspartamide)s linked with ionizable aminoethylene facilitated cytosolic delivery of mRNA, of which the hydrophobic properties and length of aminoethylene impacted delivery efficacy [116]. However, most of these polymers alone were not often studied in nano-mRNA-based cancer immunotherapy.

3.2.4. Hybrid nanoparticles and other candidates

Hybrid nanoparticles integrate the physicochemical features of dual or multiple materials, evolving as a promising platform for mRNA delivery, such as liposome-protamine nanoparticles [117] and lipid/polymer nanoparticles (Fig. 5) [28,118–121]. One lipid/polymer product, called TransIT-mRNA Transfection kit that is manufactured by Mirus Bio, remarkably enhanced the efficacy of mRNA encoding bispecific antibodies against established large tumors [28]. This strategy is currently under clinical evaluation (see Section 5). Recently, cationic lipids termed G0-C14 formed complexes with mRNA, which was then coated with PLGA (poly(lactic-co-glycolic acid)/PEG. The hybrid nanoformulation adhered to local mucosal sites, increasing mRNA-mediated inhibition of bladder cancer [118].

Organic/inorganic hybrid nanoparticles were also examined for mRNA delivery in cancer cells, such as natural polymer chitosan/selenium nanoparticles that were decorated with tumor targeting folic acid molecules [122], and dendrimer polymer/gold/folic acid nanoparticles [123]. However, in vitro validation of nano-mRNA efficacy is limited. In contrast, inorganic calcium phosphate nanoparticles that were coated with LNPs demonstrated as a powerful delivery system in vivo for targeted cancer immunotherapy [124].

Other candidates that are at the early stage in mRNA delivery potentially offer new opportunities in cancer immunotherapy, mainly including mesoporous silica nanoparticles with a high porosity [89], Zn²⁺ ions [125], outer membrane vesicles (OMVs) derived from bacteria [24]. The advantages and disadvantages of representative types of mRNA delivery nanoparticles are summarized in Table 1.

Table 1

Summary of the advantages of disadvantages of representative types of nanoparticles for mRNA delivery.

Type of nanomaterials	Advantages	Disadvantages
Peptides	Low toxicity Easy membrane penetration Low immunogenicity	Short half-life Lack of targeting
Proteins	Low toxicity Good stability Natural sources	Undesired immunogenicity Lack of targeting
Lipid	High biocompatibility Adjustable compositions	Potential immunogenicity Low encapsulation efficiency
Polymer	Easy surface modification and functionalization Easy mass production Adjustable compositions	Potential immunogenicity and toxicity Low degradation
Hybrid	Easy surface modification and functionalization Customizability of functions	Complex design and composition Potential immunogenicity and toxicity

4. Recent advanced nanoformulation-mediated mRNA immunotherapies in preclinical studies

4.1. Nano-mRNA targeting DCs

Cancer vaccines are an appealing immunotherapeutic approach to stimulate the immune system to produce antigen specific immune response against cancerous cells [126,127]. Such vaccines typically consist of exogenous well-defined tumor specific antigens (TSAs) or TAAs and immunogenetic adjuvants that can efficiently activate DCs and boost immune response against tumor antigens [126]. APCs comprise a heterogenous group of immune cells that can process and present endogenous and exogenous antigens via the major histocompatibility complex (MHC) molecules, which is essential for initiating the interaction between innate and adaptive immunity. DCs are the main subtype of APCs with a key function of priming T cell immunity. Therefore, the advancements in cancer vaccines focus on targeted delivery of antigens and adjuvants to DCs [21,22,24]. T cells rely on DCs to capture and transport the antigens to the draining lymph nodes (dLNs) from the immunization sites. Generally, the captured exogenous protein or peptide antigens are processed in endo/lysosomes and then presented on MHC class II molecules, which can be recognised by TCRs (T-cell receptors) of CD4⁺ Th cells [128]. By contrast, CTLs are restricted to MHC class I presented antigens [129]. The mRNA cancer vaccine platform promotes MHC class I antigen presenting pathway by endogenously translating encoded tumor antigens, thereby effectively priming CTLs [130], which are the key mediators for tumor eradication. Beyond that, mRNA itself can be sensed by TLRs of DCs, showing inherent immunogenicity to boost immune response of mRNA vaccines against antigens [131]. Apart from these characteristics, mRNA strategy is versatile and can be used to encode multiple peptides or proteins, not only providing tumor antigens but also offering additional molecule adjuvants to peripheral or lymphatic DCs [132]. The mRNA platform also shows potential in in situ vaccination, by reprogramming DCs to restore their functions in antigen presentation and T cell priming [133]. The success of this deliberate mRNA strategy heavily relies on the powerful delivery systems, promoting the translation of mRNA in vivo [16]. Transformative advances in nanoformulations have demonstrated their unique capability in harnessing the potential of mRNA cancer vaccines in preclinical and clinical studies [15].

4.1.1. Ex vivo nano-mRNA DC vaccines

DC vaccines are generated ex vivo prior to infusion back patients, where the isolated DCs are appropriately activated by adjuvants and loaded with tumor antigens [134]. DCs are often stimulated with whole tumor lysate derived from debulking surgery to prime the immune system of patients [135], while this strategy might potentially induce a tolerogenic transformation caused by immunoregulatory cytokines. DC vaccines loaded with nano-mRNA encoding TAAs or TSAs offer a complementary approach [136]. In term of tumor antigens, ovalbumin (OVA) derived from egg white is commonly used as model antigens in preclinical studies of cancer immunotherapy, which can sensitize immune reactions and elicit antigen specific antibodies (humoral) and T cell (cellular) immune response. To enhance the potency of nano-mRNA DC vaccines, Mitchell and colleagues screened cationic liposomes for optimized DC activation and antigen translation ex vivo, and then engineered hybrid nanoformulations by incorporation of commercially available iron oxide nanoparticles (IONPs) and OVA-mRNA in the optimized liposomes (denoted as IO-RNA-NPs, Fig. 6a) [137]. Under magnetic field, IO-RNA-NPs enhanced DC activation and T cell priming ex vivo (Fig. 6b). Mice received IO-RNA-NPs-loaded DC vaccine and adoptive OT-I T cells with transgenic TCRs that specifically recognise MHC I restricted OVA peptide, significantly inhibited the growth of B16F10-OVA melanoma. The incorporated IONPs enabled tracking DC migration from the injection site to dLNs under magnetic resonance imaging (MRI) (Fig. 6a), which acted as predictors in anti-tumor performance of DC vaccines (Fig. 6c and d). The potential of vitamin

E-scaffolds/lipoplex/cationic peptide-delivered OVA-mRNA was explored in the development of ex vivo DC vaccine against E.G7-OVA lymphoma in mice [138]. Instead of pulsing DCs with mRNA encoding tumor antigens, Moghaddam and co-workers engineered DCs stimulated with chitosan nanoparticles-mRNA encoding costimulatory molecules of CD40 and ICOSL (inducible costimulator ligand) and isolated tumor cell mRNA, which enabled T cell proliferation and significant inhibition of 4T1 mouse breast tumor post intratumor injection [139].

4.1.2. Nano-mRNA therapeutic cancer vaccines activating TLRs

The clinical efficacy of ex vivo generated DC vaccines are often comprised by the poor control on their migration in vivo to the therapeutic sites. In contrast, the advancement in nanoformulations allowed targeting delivery of nano-mRNA cancer vaccines to endogenous DCs in lymphoid organs. Sahin who is known for BioNTech company and COVID-19 vaccine (BNT162b2) and his team discovered that the surface charge of lipoplex-mRNA determined its biodistribution post intravenous administration [21]. Tuning lipoplex-mRNA to be slight negative or neutral led to exclusive target to splenic DCs as well as macrophages, thereby substantially promoting translation of encoded tumor antigens in DCs and inducing profound expansion of antigen specific T cells. Lipoplex-mRNA demonstrated universal and potent anti-tumor capability in multiple aggressive murine tumor models, including B16-OVA and B16F10-Luc (luciferase) melanoma, CT26 and CT26-Luc colon carcinoma, and TC-1-Luc cervical cancer [21]. Mechanically, lipoplex-mRNA activated splenic DCs via TLR-7 signalling pathway,

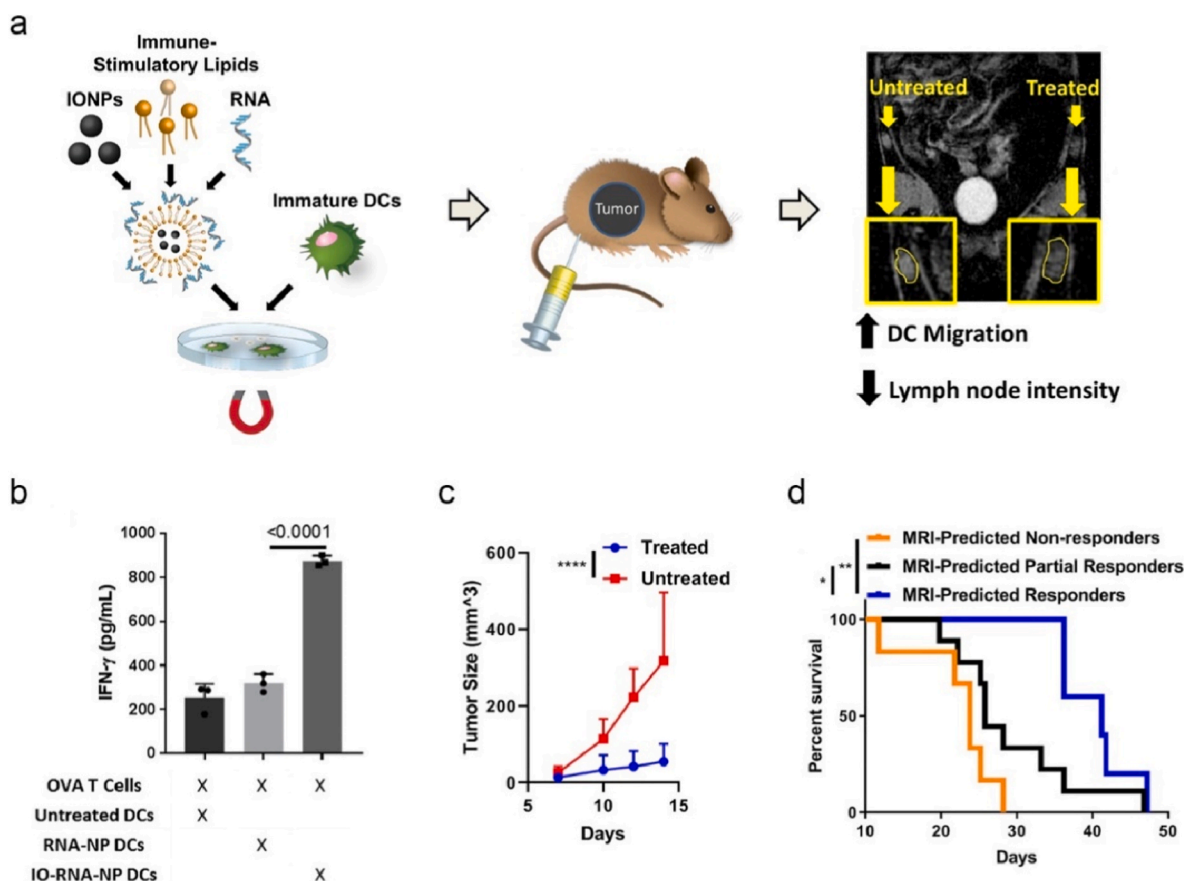


Fig. 6. Cationic liposome/IONPs promote OVA-mRNA translation, ex vivo DC maturation, tracking DC vaccines in vivo with MRI, thereby effectively predicting anti-tumor effects. (a) Schematic illustration of the synthesis of IO-RNA-NPs, activating DCs ex vivo under magnetic field, and monitoring trained DC migration from the injection site to dLNs with an MRI-instrument. (b) Ex vivo DCs pre-treated with IO-RNA-NPs or RNA-NPs were co-cultured with OVA experienced T-cells for 48 h. IFN-gamma levels were measured by enzyme-linked immunosorbent assay (ELISA). (c) Average B16F10-OVA tumor growth curves of mice received IO-RNA-NPs loaded-DC vaccine and adoptive OT-I T cells, and untreated mice. (d) Survival curves of mice grouped based on the response prediction by MRI data Reprinted with permission from Ref. [137]. Copyright 2019, American Chemical Society.

leading to type 1 IFN α secretion and cooperation of innate and adaptive immune response against tumor. The preliminary data of this intriguing nano-mRNA platform in 3 patients with advanced malignant melanoma displayed supportive results of systemic IFN α secretion and amplification of T cells against encoded tumor antigens. The full report on the results of lipoplex-mRNA clinical trial (NCT02410733) will be introduced in Section 5.

Instead of priming endogenous T cells, Sahin team demonstrated a great success of lipoplex-mRNA vaccine in promoting the proliferation of adoptively transferred chimeric antigen receptor (CAR)-T cells to treat solid tumors [34]. CAR-T cell therapy has shown clinical success in curing patients with B-cell malignancies [140,141], while remains challenging in the treatment of patients with solid tumors [142]. To increase cancer specific targets and expansion of CAR-T cells, Sahin team engineered a lipoplex-mRNA vaccine encoding TAAs of claudin (CLDN) 6 (denoted as CLDN6-LPX), which showed effective expression of CLDN6 in ex vivo human DCs under an optimal dose of 100 μ g/ml. Following intravenous administration, CLDN6-LPX enabled targeted

transfection of splenic CD8+DCs, CD8-DCs, and F4/80+ macrophages, providing proliferation signals to engrafted CLDN6-CAR-T cells against established solid OV-90 human ovarian cancer in immunodeficient NSG (NOD scid gamma) mice. Following engraftment, the adoptive CLDN6-CAR-T cells alone even at a high dose tended to decline rapidly in vivo, while repeated vaccination with CLDN6-LPX maintained CLDN6-CAR-T cells at therapeutic levels, even at an initial low dose. The kinetic expansion results explained the superior capability of vaccine-stimulated CLDN6-CAR-T cells in the treatment of solid tumors. This fascinating nano-mRNA platform in combined with CLDN6-CAR-T cell therapy currently is under clinical studies, which will be discussed in next section.

LNPs not only enable protection and targeting delivery of mRNA to endogenous DCs, but also offer self-adjuvancy via activation of TLRs that are not associated with mRNA sensing [25]. Xia and colleagues screened a library of cationic LNPs via the interaction of poly (-amidoamine) (PAMAM) and epoxide consisting of different R structures [25]. The optimal C1 LNP containing C12 tail enabled effective

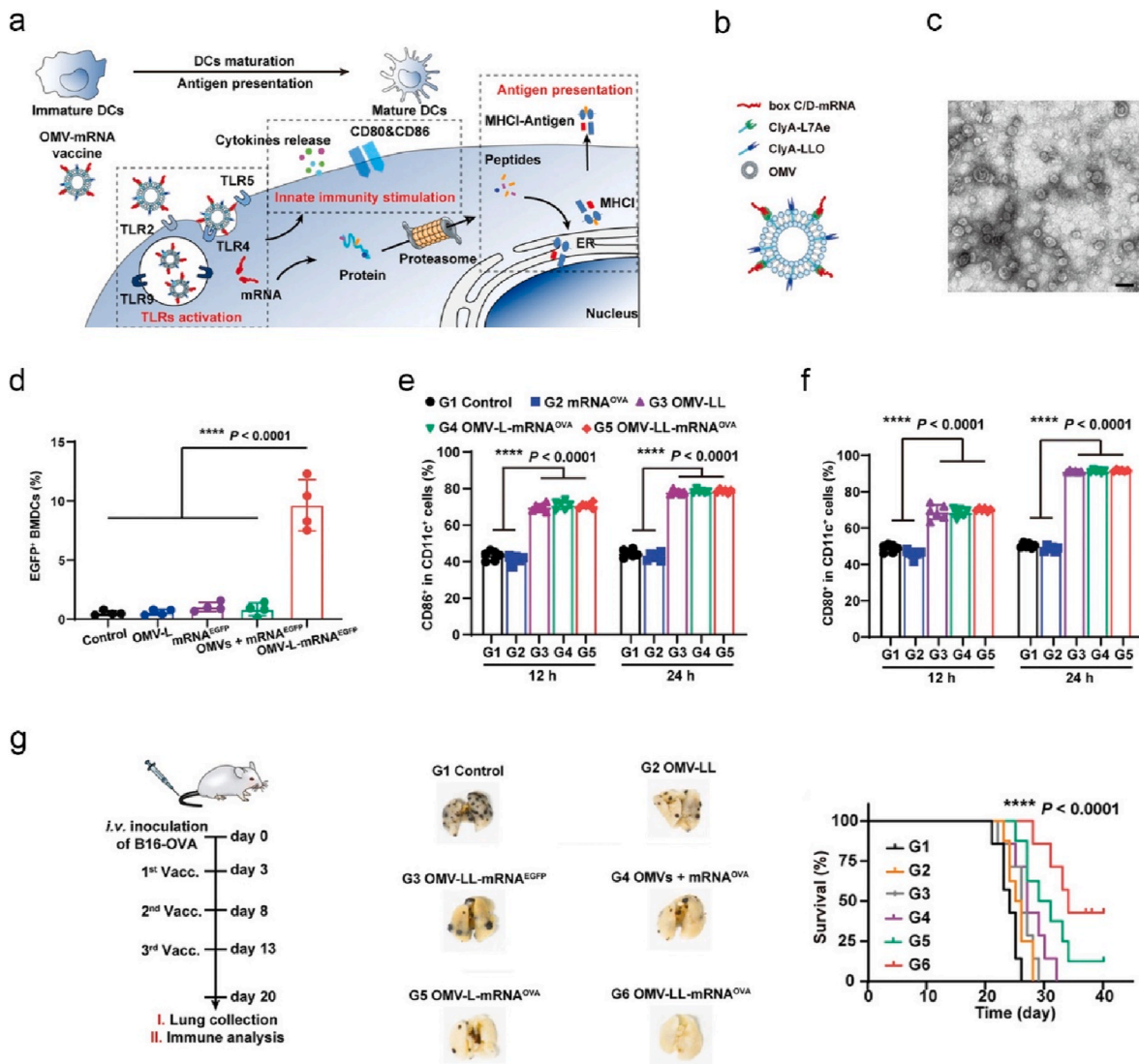


Fig. 7. An OMV-mRNA vaccine activates DCs via multiple TLR receptor pathways, and promotes cross-presentation of expressed antigens, thereby exerting profound anti-tumor effect. (a) A scheme for DC activation mechanism mediated by OMV-mRNA vaccine. (b) Schematic diagram of the composition of OMV-mRNA. (c) A TEM (transmission electron microscope) image of L7Ae-modified OMVs. Scale bar: 50 nm (d) The percentages of enhanced green fluorescent protein (EGFP)+ BMDCs (bone marrow-derived dendritic cells) transfected with different mRNA nanoformulations as indicated. (e–f) The maturation markers of CD86 (e) and CD80 (f) expressed on CD11c+ DCs were analysed by flow cytometry after incubated with various nanoformulations. (g) The experimental timeline of mice inoculated intravenously with B16-OVA cells and vaccinated with different mRNA nanoformulations. Shown are the representative digital photos of resected lungs with tumor nodules and survival curves of mice [24].

targeting of mRNA to dLNs, promoted encoded antigen expression and presentation via stimulating TLR4 pathway and secreting pro-inflammatory cytokines (such as IL-12 and IL-1 β). Mice immunised with C1 LNP-mRNA encoding OVA exhibited potent anti-tumor immunity in B16-OVA tumor model.

To complement LNPs, OMVs have been exploited as delivery vehicles in the development of potent cancer vaccines [143]. OMVs possess heterogeneity in size and composition, and immunomodulatory features endowed by abundant PAMPs [143,144]. Very recently, Nie and colleagues first explored the potential of OMV-platform derived from *E. coli* in targeted delivery of mRNA cancer vaccines to endogenous DCs and stimulating DCs via multiple TLRs (Fig. 7a) [24]. The authors genetically engineered OMVs with surface expressed L7Ae (RNA binding protein) and listeriolysin O (lysosomal escape protein) (denoted as OMV-LL, ~30

nm in diameter) (Fig. 7b and c). OMV-LL enabled antigen expression (Fig. 7d) and cross presentation, and DC maturation (Fig. 7e and f). Mice vaccinated with OMV-LL-mRNA encoding OVA or ADPGK (ADP-dependent glucokinase) exhibited remarkable regression of B16-OVA metastatic melanoma (Fig. 7g) or MC38 colon cancer, respectively.

Direct incorporation of TLR agonists provides an alternative approach to promote immunogenicity of nano-mRNA cancer vaccines [119,145–147]. Wang lab engineered a transformable hydrogel that was formed by small graphene oxide nanoparticles and low molecular weight PEI (denoted as GLP-RO Gel, Fig. 8a), to deliver a nano-mRNA vaccine consisting of R848 (TLR7/8 agonist), and OVA encoding mRNA (denoted as GLP-RO, Fig. 8b) [145]. The hydrogel enabled sustained release of GLP-RO nanoparticles, effective OVA expression in DCs in vitro (Fig. 8c), and targeted delivery to skin dLN-DCs post

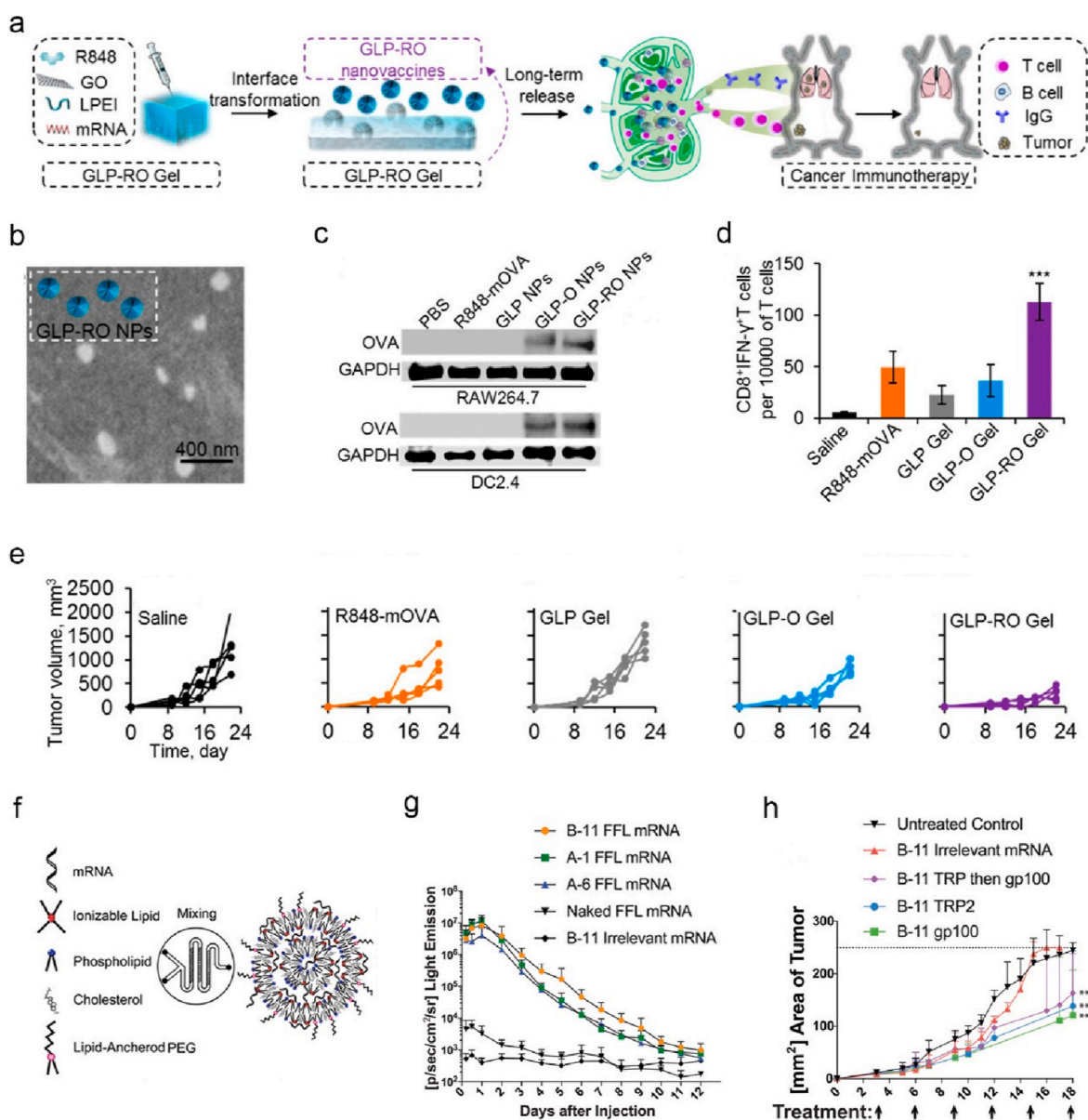


Fig. 8. Nanoformulations incorporated with TLR7/8 or TLR4 agonists target dLN-DCs, promoting translation of mRNA and anti-tumor T cell immunity. (a) Illustrated is the preparation of GLP-RO nano-mRNA vaccines in the transformable hydrogel and associated mechanism for enhanced cancer immunotherapy. (b) A SEM (scanning electron microscope) image of released GLP-RO nanoparticles. (c) Western blot analysis of OVA expression levels of RAW264.7 and DC2.4 cells treated with different formulations as indicated. (d, e) Splenic CD8⁺IFN- γ ⁺ T cells (d) and individual B16-OVA tumor growth curves (e) of mice received different treatments as indicated. (f) A schematic illustration of the construct of optimized LNP-mRNA. (g) Kinetic expression profiles of FFL at the injection sites of mice received different nano-mRNA formulations as indicated. (h) Average B16F10 tumor areas of mice treated with different nano-mRNA formulations as indicated. Reprinted with permission from Refs. [145,146]. Copyright 2017 and 2021, American Chemical Society.

subcutaneous injection. Mice immunised with GLP-RO Gel induced strong systemic functional CD8⁺ T cells against OVA (Fig. 8d), thus significantly regressed B16-OVA tumor (Fig. 8e). Blankschtein and co-workers developed PEG-LNPs to deliver mRNA encoding melanoma TAAs (TPR2 (terminal protein region 2) and gp100 (glycoprotein 100)) and lipopolysaccharide (LPS, TLR4 agonist) (Fig. 8f) [146]. The optimized B11 LNPs among three promising candidates (B-11, A-1, and A-6) that were screened from a lipid library with various components, induced the strongest antigen specific CD8⁺ T cells, and effectively increased translation of mRNA encoding firefly luciferase (FFL) at the injection sites in mice (Fig. 8g). Along with lipid component optimization, mRNA modification was also optimized. Unmodified mRNA potentially activates TLRs 3, 7 and 8 [148], which could enhance vaccine immunogenicity but also hinder the translation and promote degradation of mRNA [149]. To address these issues, the authors designed modified mRNAs replaced with ψ and 5-mC. The optimized B11-modified mRNA displayed potent therapeutic effects against B16F10 tumor in mice (Fig. 8h). Beyond these representative platforms, the potential of other types of LNPs were also explored by co-delivering of mRNA encoding tumor antigens and TLR agonists to endogenous DCs [119,147].

4.1.3. Nano-mRNA therapeutic cancer vaccines activating STING pathway

Type I IFNs consisting of IFN α and IFN- β , can potentiate the induction of CD8⁺ T cell immunity of mRNA vaccines [150]. Thus, it is desired in the development of nano-mRNA cancer vaccines that mRNA platforms or nanoformulations are capable to induce strong type I IFNs for enhanced function and durability of CTLs against cancer. TLRs signal activates the transcription factor myeloid differentiation primary response 88 (MyD88), inducing production of inflammatory cytokine IFN α [151]. Different from RNA sensing TLRs, STING is activated by cytosolic dsDNA (double stranded DNA) in response to viral infection [152]. STING signalling plays a central role in production of type I IFNs in innate immune system [152]. STING now is an appealing target for therapeutic cancer vaccines and cancer immunotherapy [153]. Anderson and co-workers engineered LNPs containing heterocyclic amine groups (A18), which enabled targeted activation of dLN-DCs via STING pathway, mRNA translation, subsequent secretion of type I IFNs and initiation of T cell priming. Mice vaccinated with A18 LNP-mRNA cancer vaccines showed profound therapeutic effect in multiple murine tumor models. In addition to LNP-mediated STING activation, Huang and co-workers designed a mRNA encoding active mutations in STING (V155M) [154] that caused hyperactivity in STING activation, which was encapsulated into LNPs together with HPV-E6/E7 antigen mRNA [155]. This nanoformulation enabled potent secretion of type I IFN cytokines via STING pathway, which in turn increased the expression and cross presentation of HPV-E6/E7 and subsequent proliferation of CD8⁺ T cells against E7. Vaccination with LNP-antigen mRNA-STING^{V155M} significantly regressed HPV + TC-1 tumor and prolonged the survival of mice.

4.1.4. Nano-mRNA therapeutic cancer vaccines activating the receptors of polysaccharides

PRR activation is critical to establish a pro-inflammatory milieu and initiate the cross-talk between non-specific innate and specific adaptive immune response, thus the strategies targeting PRRs have been harnessed in the development of therapeutic cancer vaccines [126]. Among PRRs, TLRs and STING have been extensively explored, while the immunology of CLR (C-type lectin receptors) and their immunostimulatory potentials in vaccine development, particularly in nano-mRNA cancer vaccines, have been much less investigated. In response of fungal infection, CLR will be activated by sensing cell wall polysaccharides, leading to potent cytokine production and subsequent development of humoral and cellular immune response. Polysaccharides β -glucans and mannans can activate CLR Dectin-1 and Dectin-2, respectively, while these CLR are not the sole receptors for the

defined polysaccharides, depending on their source and physical properties (such as soluble or particulate) [156,157]. Thus, the studies on polysaccharides-based formulations in nanoscale for mRNA delivery would provide new understandings on their receptor signalling and associated vaccine immunological activity, which potentially offers guidelines in engineering advanced nano-mRNA systems. For this reason, Moon and colleagues engineered sugar nanocapsules (Fig. 9a and b) by coating silica nanoparticles (around 200 nm, Fig. 9b) with mannan (Mann) or dextran (Dex) and core removal, to deliver antigen mRNA with the aid of PEI [158]. Mann-capsule and Dex-capsule were found activating DCs via Dectin-2/TLR4 and CD206/CD209 signalling pathways, respectively. Following subcutaneous injection, Mann-capsule allowed more efficient drainage to dLNs (Fig. 9c) compared to Dex-capsule, potentially owing to the abundant mannan receptors on DCs. Mice vaccinated with Mann-capsule-mRNA encoding OVA antigen displayed substantially enhanced regression of B16-OVA than those with Dex-capsule-mRNA (Fig. 9d), which can be explained by the higher frequency of systemic and tumor infiltrating OVA-specific CD8⁺ T cells (Fig. 9e). In the formulation of nano-mRNA cancer vaccine, mannan showed superior capability over Dextran in targeting DCs and priming T cells. In another study, Pichon and colleagues decorated lipopolyplex with trimannans to deliver pseudouridine nucleoside modified mRNA (denoted as LPR, Fig. 10a), to reduce unmodified mRNA associated inflammatory side effects while maintain the immunogenicity of modified mRNA. LPR enabled targeting splenic DCs (Fig. 10b) post intravenous administration [132]. Mice immunization with LPR delivered a mixture of mRNAs (encoding E7, and stimulatory molecules of CD40L, CD70, and TLR4) elicited strong antigen E7 specific CD8⁺ T cell immune response (Fig. 10c), and profound efficacy in the treatment of TC-1 tumor (Fig. 10d). Cytotoxic T cell response was dependent on type I IFN induced by LPR, irrespective of encoded stimulatory molecules. It would be interesting to explore the specific receptor signalling pathway activated by the unique trimannans-nanostructure.

To promote cytosolic delivery of mRNA vaccines in targeted DCs, inorganic calcium phosphate was incorporated into LNPs, followed by decoration with mannoses [124]. The designed hybrid nano-mRNA encoding antigen mucin 1 (MUC1) significantly inhibited the growth of breast cancer in combination with anti-CTLA-4 (anti-cytotoxic T lymphocyte-associated antigen 4) monoclonal antibody (mAb). The strategies targeting polysaccharide receptors expressed on APCs are a versatile approach in nano-mRNA vaccine paradigm.

4.1.5. Nano-mRNA therapeutic cancer vaccines enhanced by other therapeutic modalities

The discoveries of inhibitory markers on immune cells and associated pathway mechanisms that modulate the immune system against cancer led to the great success of non-antigen specific cancer immunotherapy, such as immune checkpoint inhibitors (ICIs). ICIs that block the inhibitory receptor of PD-1 (programmed cell death protein 1) or CTLA-4 expressed on T cells have demonstrated potent clinical efficacy in the treatment of patients with advanced cancer. However, only a small fraction of cancer patients can benefit from this treatment modality. Low prevalence of tumor infiltrating T cells expressing such receptors could be one of the key factors for non-responders. Thus, vaccination with therapeutic cancer vaccines could potentially increase response rate of cancer patients to ICI treatment by expansion of antigen specific T cells. To this end, Dewitte and colleagues evaluated the potency of engineered nano-mRNA vaccine combined with anti-PD-1 mAbs in cancer treatment [159]. To increase the potency, the authors co-encapsulated α -GC (glucosidase) that activated iNKT (invariant NKT) cells and nucleoside-modified antigen mRNA in LNPs (denoted as mRNA Galsomes, Fig. 11a). The modified mRNA promoted its *in vivo* translation (Fig. 11b) and the nanoformulation of mRNA Galsomes enabled targeting splenic DCs post intravenous administration (Fig. 11c). Mice vaccinated with OVA mRNA Galsomes elicited strong systemic OVA specific CD8⁺ T cells, non-antigen specific iNKT and NKT cells, while

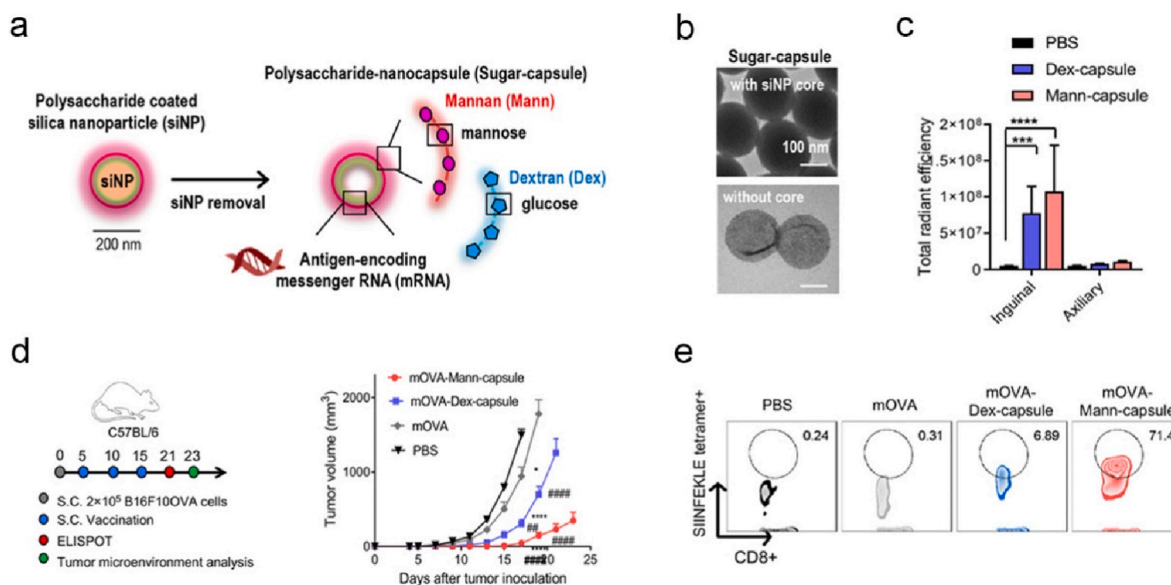


Fig. 9. mRNA-sugar-capsule decorated with mannan or dextran promotes DC maturation via the activation of multiple pattern recognition receptors, cross-presentation of translated antigens, thus elicits effective anti-tumor T cell response. (a) Illustrated is the preparation process of mRNA-sugar-capsule. (b) Representative TEM images of sugar-capsules. (c) Cy5.5 signal intensity of Cy5.5 (cyanine5.5) tagged Man-capsule or Dex-capsule in inguinal and axillary LNs harvested from C57BL/6 mice post injection. (d) The experimental timeline of mouse B16F10-OVA model and the average tumor growth curves of mice received with different formulations. (e) Flow cytometry data showing the frequency of tumor-infiltrating OVA specific tetramer + CD8⁺ T cells. Reprinted with permission from Ref. [158]. Copyright 2020, American Chemical Society.

these effector cells displayed upregulated expression of PD-1 after infiltrating into B16-OVA tumor sites (Fig. 11d). As expected, vaccination with OVA mRNA Galsomes significantly promoted the response of mice with B16-OVA tumor to anti-PD-1 mAbs therapy (Fig. 11e). It remains a challenge to identify TAAs for vaccine development. For this reason, Mitchell lab developed a personalised nano-mRNA vaccine, where whole tumor derived mRNA molecules were loaded into LNPs (Fig. 12a) [160]. Following intravenous injection, the nanoformulation systemically targeted the peripheral (such as lung, liver, and lymphoid organs, Fig. 12c) and tumor PD-L1 (programmed cell death ligand 1) + myeloid cells, most of which were PD-L1+ APCs (Fig. 12a). DC transfection by mRNA-LNP was visualised (Fig. 12b) with a high expression of PD-L1. Vaccination with this mRNA-LNP potentiated the therapeutic effect of anti-PD-1 mAbs against B16-OVA (Fig. 12d), owing to the significantly increased frequency of PD-1+CD8⁺ T cells infiltrated in tumor sites (Fig. 12e). In contrast, co-administration of anti-IFN α receptor 1 (IFNAR1) mAbs that block type I IFN receptor signalling led to abrogation of anti-tumor efficacy (Fig. 12d and e), suggesting that the therapeutic effect of mRNA-LNP was dependent on type I IFN.

Inorganic mesoporous silica nanoparticles were also explored their potential in delivery of mRNA cancer vaccines in combined with other therapeutic inhibitors [89]. The designed inorganic platform enabled the slow-release of loaded PKR (protein kinase R) inhibitor C16 that blockades the inhibitory factor of translation initiation, thereby improving antigen mRNA-translation in DCs and eliciting strong anti-tumor immunity.

Local radiotherapy promoted the capability of tumor associated DCs in sensing endogenous antigens from dying cancer cells, thereby potentiating the therapeutic effect of systematically administrated nano-mRNA cancer vaccines [161–163]. The combined strategy of nano-mRNA cancer vaccines with therapeutic immuno-molecules (e.g., ICIs [164]) or other treatment modalities represents a promising approach for potent and durable anti-tumor immunity, with some fascinating paradigms under clinical investigation [23].

4.2. Nano-mRNA targeting cytotoxic T cells and NK cells

Tumor antigen specific T cells and NK cells are two central cytotoxic effector cells in eliminating cancer. T cell-based therapy requires prior antigen exposure to target tumor cells by recognition of epitopes presented by MHC class I molecules on tumor cells. By contrast, NK cells that are a subtype of innate immune cells can recognise and target tumor cells with absent expression of MHC class I molecules or TAAs/TSAs. Instead of single receptor mediated recognition like CTLs, NK cells target the altered antigen MICA/B (MHC class I chain related proteins A and B) expressed by tumor cells via an array of stimulatory (such as NKG2D and NKp46) and inhibitory (such as NKG2A and KIRs) receptors. In fact, the inhibitory receptors were activated by MHC class I molecules, thereby avoiding undesired destruction on healthy cells.

Adoptive transfer of genetically engineered cytotoxic T cells have emerged as potentially curative options in patients with certain cancers, such as B-cell malignancies, making extraordinary contributions to new cancer treatment modalities in clinic [165]. In these personalised cancer immunotherapies, the isolated patient T cells are engineered with expression of CARs or TCRs ex vivo prior infusion back to patients, enabling the redirection of T cells against tumor cells by recognizing tumor specific antigens. Beyond T cells, CAR-based therapeutics have recently explored the potential in CAR-NK cell-based cancer therapy [166]. To reduce the complexity and high cost associated with ex vivo engineering strategy, the advanced nano-mRNA technology that allows specific target to T cells, has been investigated in engineering circulating T cells with CARs or TCRs in both preclinical and clinical studies.

Along with CARs or TCRs therapies, engineered therapeutic antibodies (Fig. 13) with one or multiple targets offer an alternative efficacious approach to boost the therapeutic efficacy of T or NK cells against cancer, by enhancing the target of these immune cells to cancer cells in preclinical and clinical studies [167,168]. However, serum short life together with manufacturing challenges are the key factors limiting their far-reaching potential of T/NK cell-engaging specific antibodies. For this reason, Sahin and his colleagues have explored the potential of their nano-mRNA technology encoding antibodies in producing endogenous long-lasting therapeutic antibodies for the treatment of

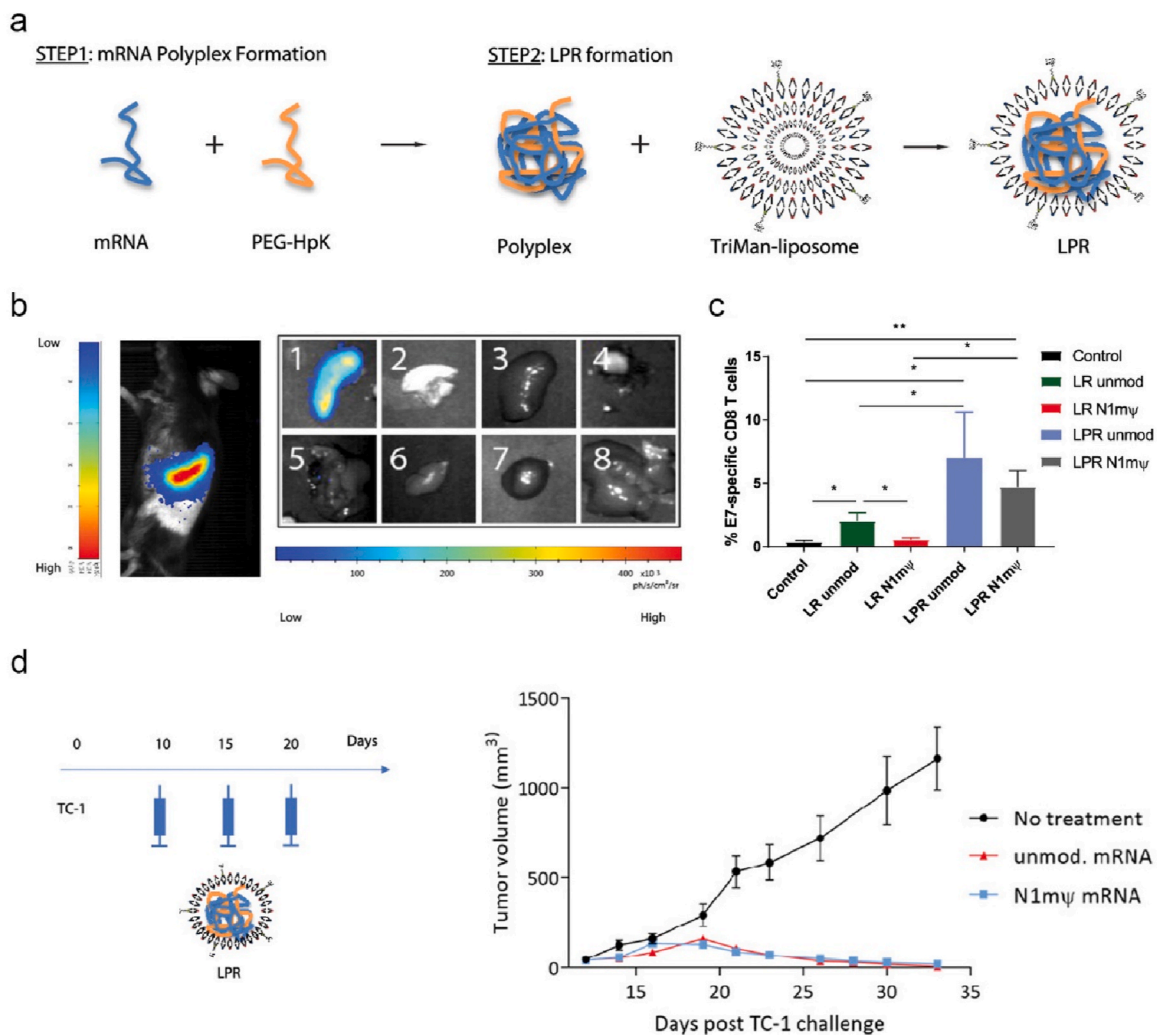


Fig. 10. Trimannose decorated lipopolyplex targets splenic DCs for enhanced delivery and translation of modified mRNA, hence increases anti-tumor immunity. (a) Schematic illustration of the preparation of LPR nanoparticles. (b) Bioluminescence imaging revealed that FFL-LPR-mRNA targeted the spleens of mice at 24 h after systemic administration. (c) E7-specific CD8⁺ T cells of mice immunised with different nanoformulations as indicated. (d) An experimental timeline of a therapeutic TC-1 tumor model and average TC-1 tumor growth curves of mice vaccinated with different nanoformulations as indicated. Reprinted with permission from Ref. [132]. Copyright 2018, American Chemical Society.

established large tumors [28]. The encouraging results from the clinical trials might pave the way to another breakthrough of nano-RNA technology in therapeutic mAbs-based cancer immunotherapy.

Far beyond the encoded therapeutics introduced above, the versatile and adaptable nano-mRNA could potentially replace a wide range of therapeutic immuno-molecules, such as IL-12 cytokines [121] or T cell inhibitory receptors [29], with improved local modulation of T/NK cell-mediated anti-tumor immunity.

4.2.1. Nano-mRNA engineering cancer-specific T cells

To enhance the potency of CAR-T cells, Stephan and colleagues developed cationic polymer PBAE nanoparticles modified with anti-CD3 and anti-CD28 mAbs for targeted translation of encoded transcription factor Foxo1 in human CAR-T cells *in vitro*, reprogramming the differentiation and phenotypic changes to memory CAR-T cells with competent functions [33]. The nano-mRNA engineered CAR-T significantly enhanced therapeutic activity against CD19⁺ human B-cell lymphoma established in NSG mice. To bypass T cell isolation and *ex vivo* culture of CAR-T cells, the authors recently utilised PBAE-mRNA technology for *in vivo* genetically engineering T cells with encoded CARs or TCRs (Fig. 14a) [32]. The decoration of anti-CD3 mAbs allowed active targeting to the spleen and lymph nodes (Fig. 14b), exclusively transfecting

CD45⁺CD3⁺ T cells, but not APCs (Fig. 14c). The infusion of PBAE-mRNA engineered adoptive human T cells with CARs, showed profound regression of leukemia and solid LNCap C42 prostate tumor (Fig. 14d and e) in NSG mice with comparable efficacies to *ex vivo* engineered CAR-T cell therapy. To advance the implementation of this intriguing paradigm in clinic, the authors proposed to perform comprehensive biosafety evaluation in large animal models.

4.2.2. Nano-mRNA engineering T cell stimulatory receptor

In contrast to ICI-based immunotherapies that target and obstruct tumor or T cell inhibitory receptor signalling pathway, agonistic mAbs against CD137 (4-1BB) or CD134 (OX40) promote the activation of T cell stimulatory receptors of 4-1BB or OX-40, thereby stimulating the proliferation and expansion of CD8⁺ T cells with profound anti-tumor activity in animal models [169]. Despite the success in preclinical studies, the low clinical efficacy (e.g., anti-4-1BB mAb agonist, Utomilumab) [170] and severe side effects (e.g., anti-4-1BB mAb, Urelumab) [171] have hampered their implementation in clinic. Several anti-OX40 mAb agonists entered clinical trials with a very low clinical activity [172]. To increase the therapeutic efficacy of anti-OX40 mAb agonists, Dong and co-workers engineered biomimetic phospholipid nanoformulations (denoted as PL1) for intratumor delivery of mRNA

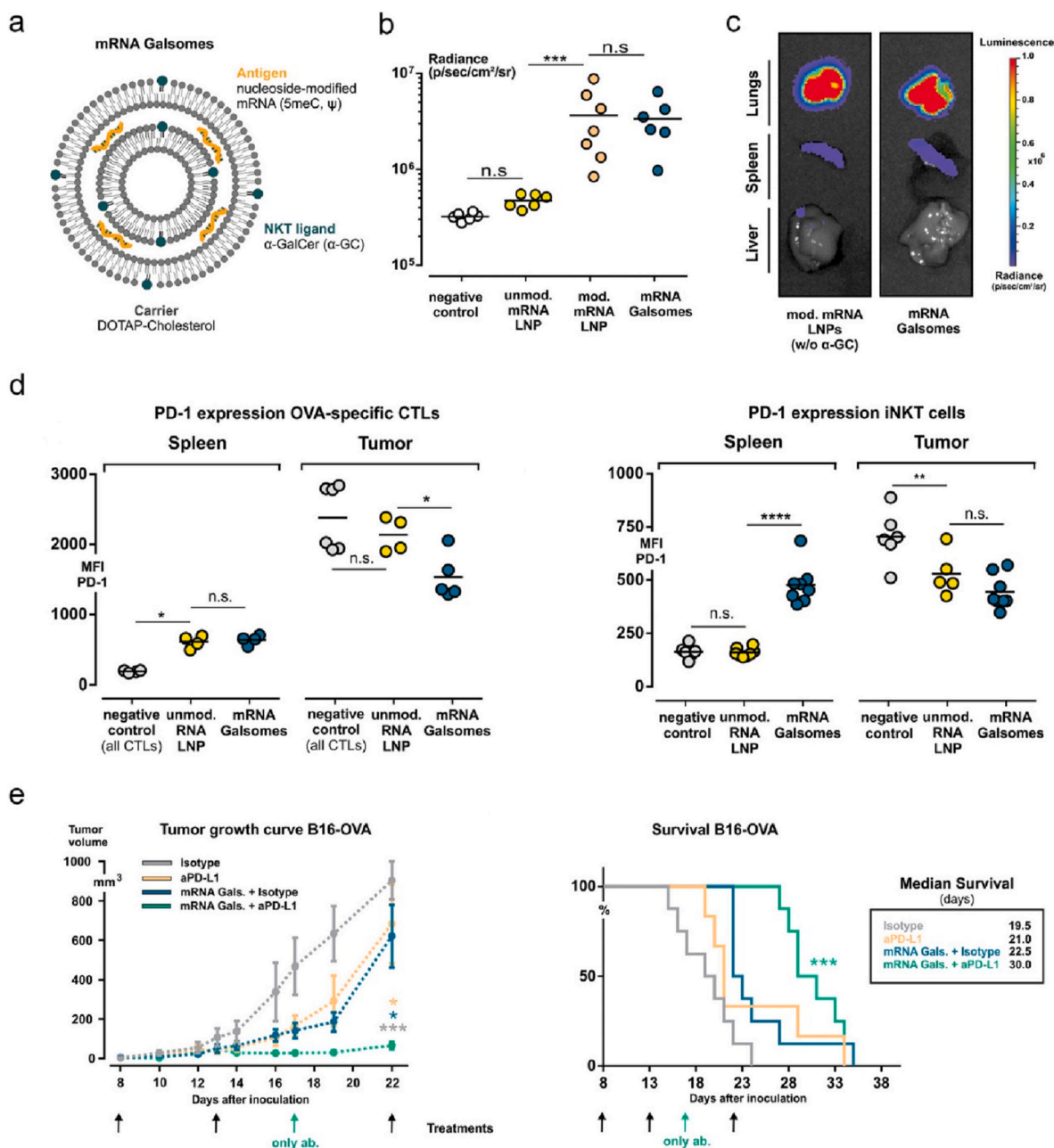


Fig. 11. Liposomes co-deliver nucleoside modified mRNA and immunopotentiator α -GC for enhancing antigen cross-presentation and iNKT activation. (a) Illustrated is the structure and composition of mRNA Galsomes. (b, c) The bioluminescence expression levels in the whole body (b) and major organs (c) in mice vaccinated with different fluc-mRNA nanoformulations. (d) Flow cytometry analysis of PD-1 expressed on OVA specific CTLs and iNKT cells harvested from spleen and tumor tissues of mice treated with different nanoformulations as indicated. (e) Average B16-OVA-tumor growth and survival curves of mice received different treatments. Reprinted with permission from Ref. [159]. Copyright 2019, American Chemical Society.

encoding OX40 (Fig. 15a) [98]. Intratumor injection of PL1-OX40 mRNA enabled upregulation of OX-40 expressed on tumor infiltrating T cells, which significantly promoted the therapeutic activity of anti-OX40 mAb against established B16F10 melanoma (Fig. 15b and c) or A20 tumor in mice, and enhanced the prevention immunity against the same tumor rechallenge (Fig. 15d). Mechanistically, the administration of PL1-OX40 mRNA substantially increased the infiltration of CD8⁺ and CD4⁺ T cells at tumor sites and decreased the ratio of Foxp3⁺ regulatory T (Treg) cells/CD4⁺ T cells (Fig. 15e). This nano-mRNA strategy was also applicable under systematic administration. Of note, the combination of PL1-OX40 mRNA and ICIs (anti-PD-L1 and anti-CTLA-4 mAbs) dramatically reduced tumor metastasis, indicating a potential treatment regimen in future clinical studies. Incorporating

mRNAs encoding cytokines (such as IL-23 and IL-36 γ) is an alternative strategy to promote the efficacy of nano-OX40 mRNA platform with long-term anti-tumor immunity [173].

4.2.3. Nano-mRNA expressing therapeutic antibodies

HER2 (human epidermal growth factor receptor type 2) overexpressed on breast cancer is associated with overall low survival rate. Trastuzumab is a clinically approved humanised antibody targeting HER2 for the treatment of invasive breast cancer [174]. Mechanistically, trastuzumab targets HER2 and abrogates the down-stream proliferative pathway of breast cancers. Additionally, trastuzumab induces NK-mediated ADCC (antibody dependent cell mediated cytotoxicity, Fig. 13), where Fc receptor expressed on NK cells interacts with Fc

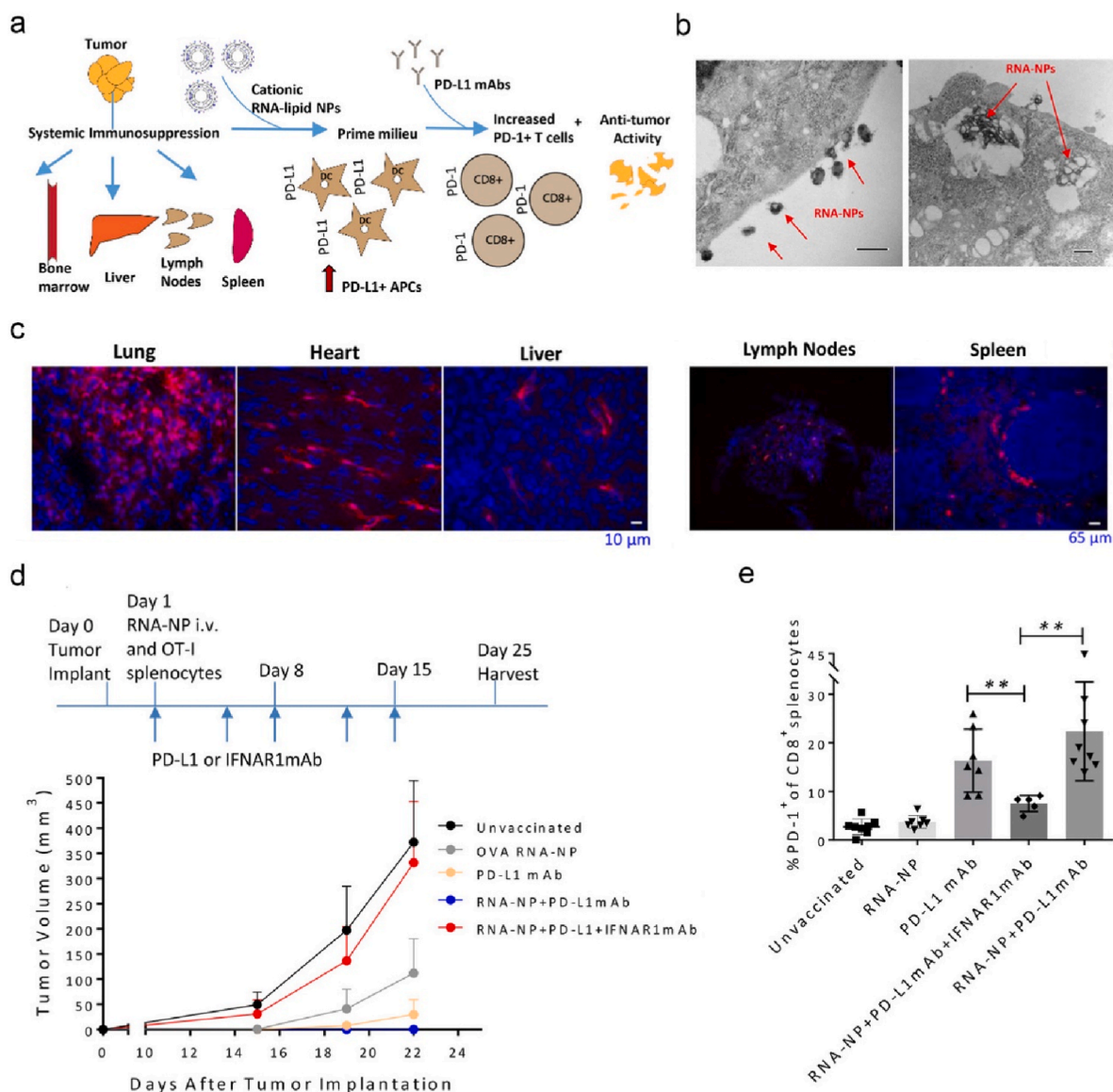


Fig. 12. Personalised LNP-mRNA induces systematic DC maturation, and reverses the immunosuppression in TME, therefore enhances ICI cancer immunotherapy efficacy. (a) Schematic representation of anti-tumor mechanism of cationic lipid-tumor derived mRNA vaccine combined with anti-PD-L1 mAbs. (b) Representative TEM images of LNP-mRNA internalized by DC2.4 cells (scale bars: 500 nm). (c) In vivo distribution of LNP-mRNA encoding Cre (cAMP response element) in different organs. (d) The experimental timeline and B16F10-OVA tumor growth curves of mice received different nanoformulations as indicated. (e) The percentages of PD-1+CD8+ splenocytes derived from B16F10-OVA bearing mice Reprinted with permission from Ref. [160]. Copyright 2018, American Chemical Society.

domain of trastuzumab, promoting NK activation and lysis of breast cancer cells. To improve the pharmacokinetic profile of trastuzumab with increased serum lifetime, Anderson and co-workers engineered LNP-mRNA targeting liver for in vivo production of trastuzumab, showing profound therapeutic effect against HER2-positive human breast cancer [30]. This nano-mRNA paradigm was applicable in expression of other types of therapeutic antibodies in vivo, such as anti-human CD20 mAbs (rituximab) against non-Hodgkin's lymphoma [175], anti-PD-1 mAbs for the treatment of intestinal cancer [29].

Instead of monospecific antibodies, Sahin team engineered a lipid/polymer nanoformulation encapsulated with nucleoside-modified mRNA for in vivo production of CD3 \times CLDN6 bispecific T-cell engager antibodies against established large tumors [28]. Post intravenous injection, the nano-mRNA enabled endogenous expression of encoded CD3 \times CLDN6 bispecific antibodies in liver, with potent pharmacological activity. NSG mice injected with three doses of lipid/polymer-CD3 \times CLDN6 mRNA completely rejected the established large OV-90 human ovarian carcinoma (200–300 mm^3). Abundant T

cells were found infiltrated into CLDN6+ tumor, proving bispecific antibodies mediated strong T cell engagement with tumor cells for potent anti-tumor activity. Exogenous CD3 \times CLDN6 bispecific antibodies was able to achieve comparable therapeutic efficacy but requiring as much as ten doses. The safety and potency of this nano-mRNA platform is currently evaluated under clinical trials (Please refer to next section for details).

4.3. Nano-mRNA targeting multiple immune cells in TME

Cytokines are stimulatory proteins that can target and modulate multiple subsets of immune cells by activating cytokine receptor downstream signalling. Clinically, administration of cytokines is one central approach to potentially increase the fraction of cancer patients responding to ICIs [176]. However, the systematic administration of stimulatory cytokines, such as IL-12, inevitably induces off-target associated toxicities [176,177]. Direct administration of cytokines into local sites is an alternative strategy with reduced toxicities to non-malignant

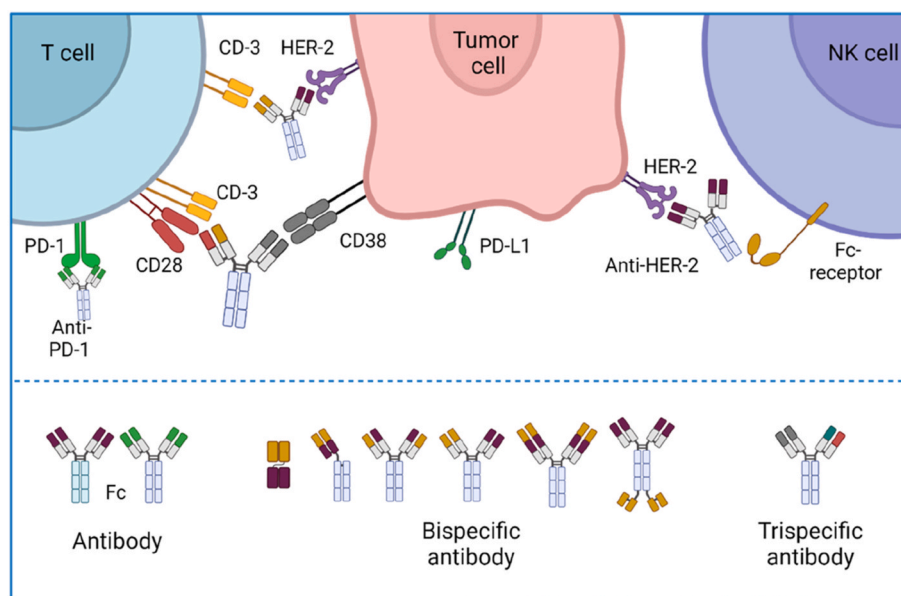


Fig. 13. The action modes and representative structures of therapeutic antibodies. Monospecific, bispecific and triple-specific antibodies that target antigens expressed on cancer and/or NK or T cells enable increased engagement of cytotoxic T/NK cells with cancer cells, thereby promoting cancer cell lysis (top). Engineering strategies on the chain structures of therapeutic antibodies allow a wide range of biological functions (bottom). Created with permission by BioRender.

regions. Luheshi and colleagues engineered non-toxic LNP-mRNA nanoformulation for intratumor injection in mice, inducing systemic immune response against primary and distant tumors [133]. The authors designed IL-12 mRNA incorporating the sequences of IL-12a and IL-12b together with a linker IL12p70, which was then formulated into LNPs. LNP-mRNA encoding IL-12 provoked strong anti-tumor immunity in a CD8⁺ T cell dependent manner. Locally administrated LNP-IL-12-mRNA in primary tumor sites induced systematic preventive immunity against distant tumor rechallenge in multiple tumor models (A20 murine B cell lymphoma, PD-L1 sensitive and resistant MC38 colon carcinoma). The secreted IL-12 showed advantages over membrane-tethered isoform of IL-12 in potentiating anti-PD-L1 mAbs. Anti-tumor immunity of LNP-IL-12-mRNA was correlated with promoted Th1 immunity in TME, though in-situ production of secreted IL-12 broadly activated DCs, NK and NKTs. To develop synergistic effect, LNPs locally delivered both IL-12 mRNA and IL-27 mRNA that targeted tumor cells and multiple immune cells (B cells, monocytes and macrophages), inducing robust tumor infiltration of cytotoxic T cells and NK cells against murine B16F10 melanoma [26].

In addition to local administration, systematic injections (such as intravenous and intraperitoneal injection) of nano-mRNA can target malignant tissues via ERP (enhanced permeability and retention) effect [35,178,179], or driven by the interactions between active targeting molecules and receptors expressed on tumor cells [180]. Following administration into bloodstream, it is crucial for the nanoformulation strategy to protect mRNA molecules from degradation during circulation and transportation period before entering tumor sites. For example, PEG are hydrophilic and neutral, which are often anchored on nanoformulations to increase the circulation time of nanoformulations, avoiding non-specific binding with proteins in serum components that can be recognised and cleared by innate immune cells (e.g., macrophages) [181]. Ionizable LNPs that are commonly used in clinical studies are neutral, which demonstrate enhanced circulation time and improved safety profiles compared with cationic LNPs [182]. Once ionizable LNPs enter acidic subcellular compartment of endosomes, they turn to be positively charged and promoted the release of mRNA molecules into the cytoplasm by fusing with endosomal membrane [183].

To modulate local immunosuppressive environment against cancer, nanoformulation strategies have been engineered for targeted delivery

of mRNA encoded cytokines of interest into tumor sites, such as IL-12 [184], IL-15 [120]. Beyond cytokines-mediated immune modulation of TME, targeted nano-mRNA platform can translate other stimulatory proteins, dramatically altering the functions and crosstalk of tumor infiltrated immune cells towards anti-tumor immunity, such as transcription factors driving polarization of M1-type macrophages [31], proinflammatory chemokine CCL5 (C-C motif ligand 5) recruiting multiple leukocytes [27], combined co-stimulatory molecules of OX-40L/CD80/CD86 activating APCs and T cells [185].

4.4. Indirect targeting immune cells by nano-mRNA mediated immunogenetic cancer cell death

Along with direct modulation of immune cells in TME, nano-mRNA encoding functional proteins can indirectly trigger immune cells infiltration in tumor through reprogramming tumor cells. Clinical studies revealed that the loss of suppressor gene, such as PTEN (gene of phosphate and tension homology deleted on chromosome ten), in human cancers was significantly associated with immunosuppressive environment and resistance to immunotherapy of anti-PD-1 mAbs [186]. To understand whether PTEN restoration could promote immunogenetic cell death-mediated immune modulation at TME, Shi and colleagues developed polymeric nanoparticles encapsulated with PTEN mRNA (denoted as mPTEN@NPs, with a size of around ~ 200 nm) to genetically reprogram cancer cells [179]. Following intravenous administration, mPTEN@NPs restored the expression of PTEN in PTEN-mutated B16F10 tumor, thereby inducing autophagy and associated immunogenic cell death. mPTEN@NPs enhanced the therapeutic effect against established B16F10 melanoma and prostate tumor. Mechanistically, cancer cell death induced the release of DAMPs (damage associated molecular patterns), which remarkably activated DCs and T cells, thereby promoting T cell infiltration in tumor sites with increased response to anti-PD-1 mAbs.

The loss of p53 tumor suppressor gene was also found associated with therapeutic resistance to cancer immunotherapy. Recently, the same lab engineered polymer/lipid hybrid nanoparticles decorated with peptide CTCE to encapsulate p53 mRNA (referred as CTCE-p53 NPs, Fig. 16a and b), which enabled active targeting to chemokine receptor CXCR4 expressing hepatocellular carcinoma (HCC) [35]. Intravenously

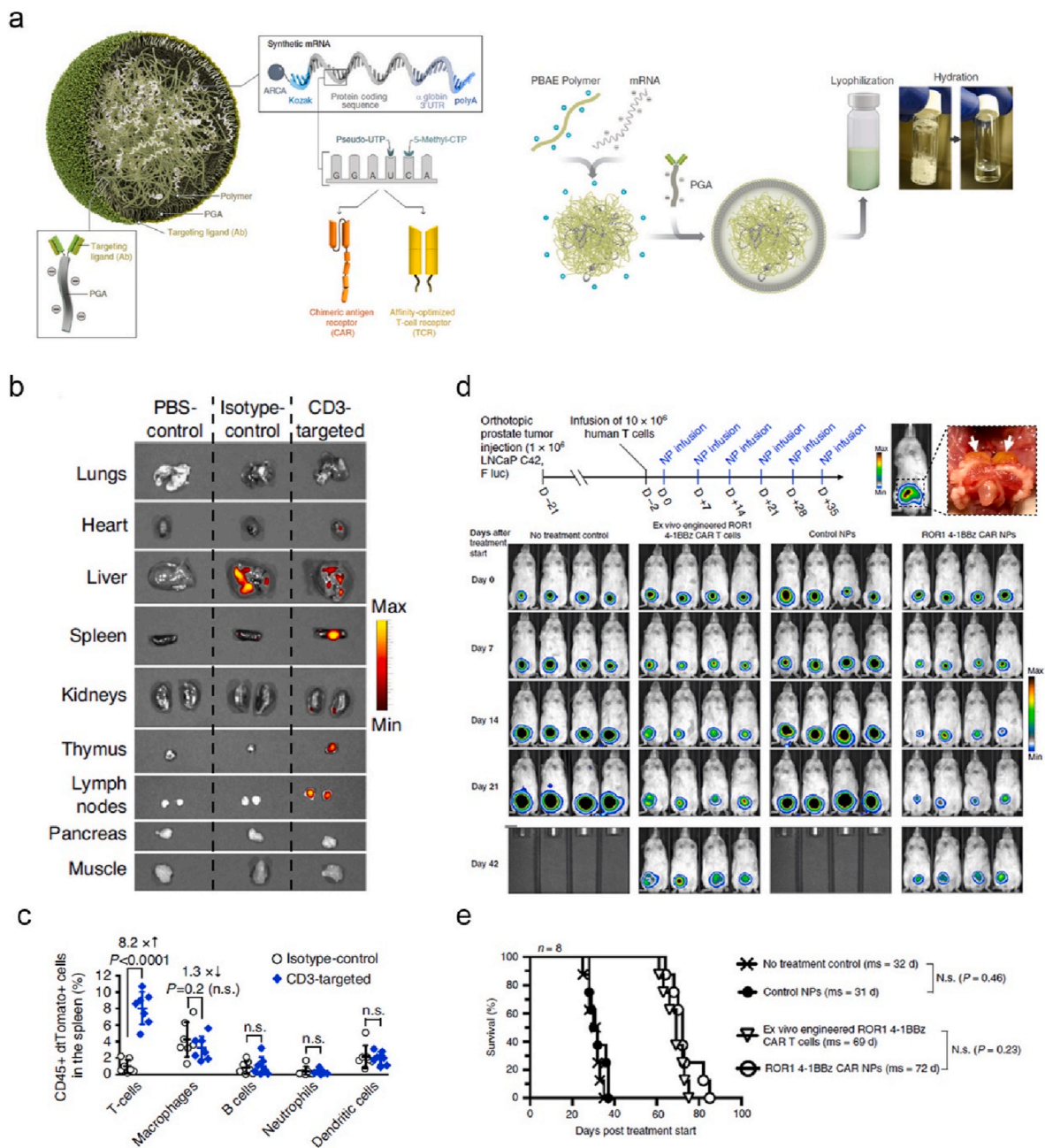


Fig. 14. PBAE nanoparticles functionalized with anti-CD3 enable targeted delivery of CAR or TCR mRNA for reprogramming circulating T cells in vivo, thereby effectively recognizing and eliminating tumor cells. (a) Illustrated is the preparation process of PBAE-mRNA NPs. (b) Fluorescence imaging revealed dtTomato expression in the organs of mice after intravenous injection of different dtTomato-mRNA-formulations as shown. (c) Flow cytometry analysed the percentages of different types of transfected CD45+dtTomato+ cells harvested from the spleen of mice. (d) The experimental timeline and representative bioluminescent images of mice with orthotopically transplanted luciferase-LNCap C42 prostate tumor and treated with different formulations as indicated. (e) Survival curves of mice received different treatments [32]. Reproduced under the terms of the Creative Commons CC BY license. Copyright 2020, The Author(s), published by Springer Nature.

administered CTCE-p53 NPs accumulated in orthotopic p53-null RIL-175 murine HCC and genetically restored the expression of p53 (Fig. 16c). CTCE-p53 NPs alone or combined with anti-PD1 mAbs exhibited a significant regression of established RIL-175 tumor (Fig. 16d). The restoration of p53 in HCC reversed the immunosuppressive environment, with increased secretion of inflammatory cytokines and infiltration of anti-tumor immune cells, such as M1 macrophages, effector T cells (Fig. 16e) and NK cells. Liposome-protamine hybrid nanoparticles were investigated as another promising delivery platform for in vivo targeting delivery of mRNA encoding survivin-T34A, inducing potent anti-tumor efficacy [187].

Immunosuppressive characteristics of specific type of tumor heavily

rely on the crosstalk among malignant cells and heterogeneous populations of immune cells. Genetically reprogramming tumor cells by nano-mRNA therapeutics provides a potential opportunity to remodel the distribution and function of immune cells infiltrated at TME for enhanced anti-tumor immunity.

Recent advanced nano-mRNA formulations that we introduced in preclinical studies for targeted cancer immunotherapy are summarized in Table 2. In the context of modulating tumor infiltrating immune cells or cancer cells, it is crucial to increase cancer selectivity of nano-mRNA, preventing potential chronic immune-related adverse events (irAEs) [188]. For example, nano-mRNA expressing anti-PD-1 or anti-CTLA-4 mAbs can concurrently inhibit non-cancer immune cells, augmenting

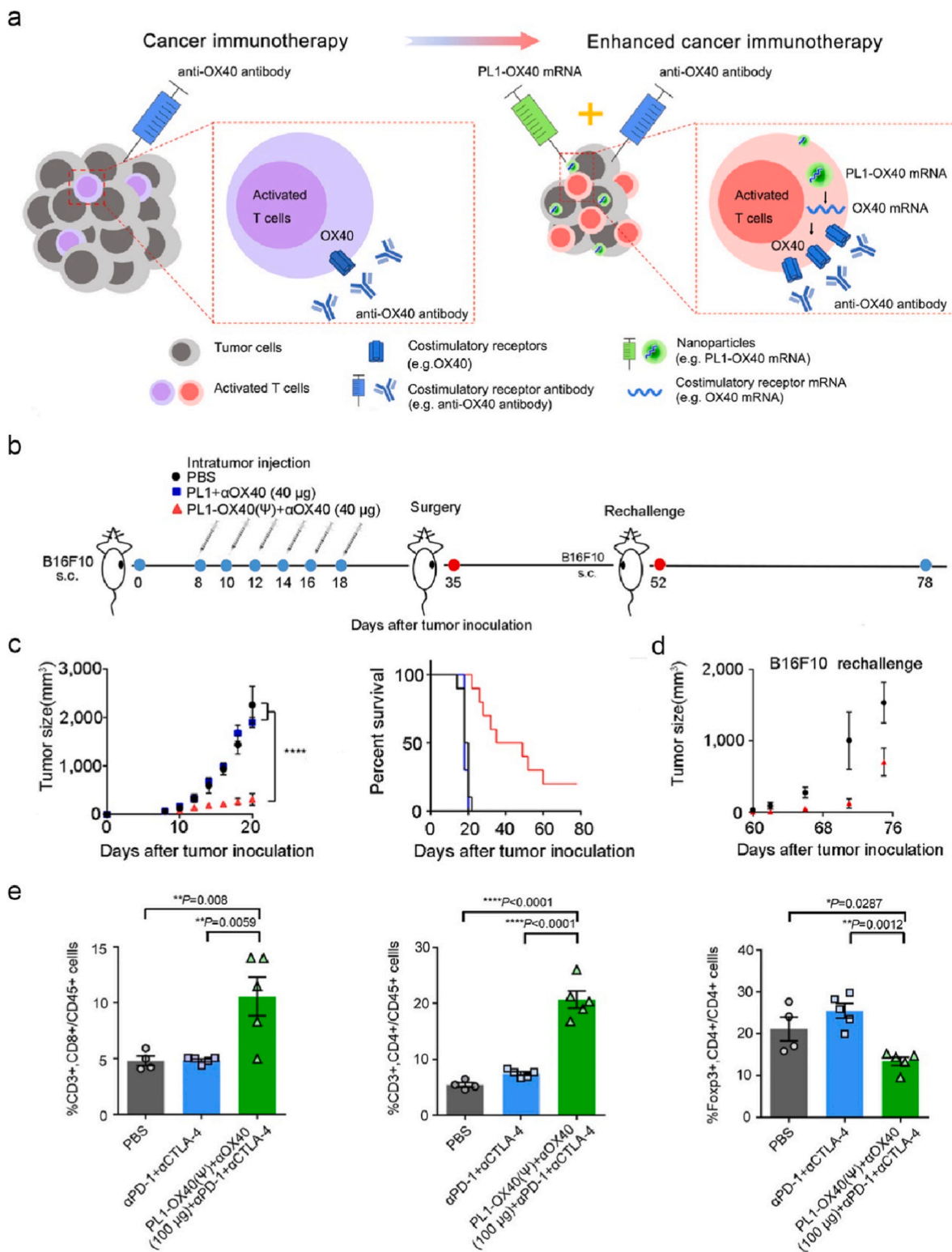


Fig. 15. Biomimetic phospholipid nanoparticles effectively deliver OX40 mRNA to tumor-infiltrating T cells with increased OX40 expression, thus potentiate the therapeutic effect of anti-OX40 mAbs against tumor. (a) Illustrated is the mechanism of PL1-OX40 mRNA nanoformulation for enhanced cancer immunotherapy of anti-OX40 mAbs. (b–d) Shown are the experimental timeline of B16F10 tumor bearing mice received different treatments as indicated (b), tumor growth profiles and survival curves of mice with primary tumor (c), and tumor sizes of rechallenged mice (d). (e) The percentages of CD8⁺, CD4⁺ T cells and Tregs infiltrated in A20-tumor derived from mice received different treatments as shown [98]. Reproduced under the terms of the Creative Commons CC BY license. Copyright 2021, The Author(s), published by Springer Nature.

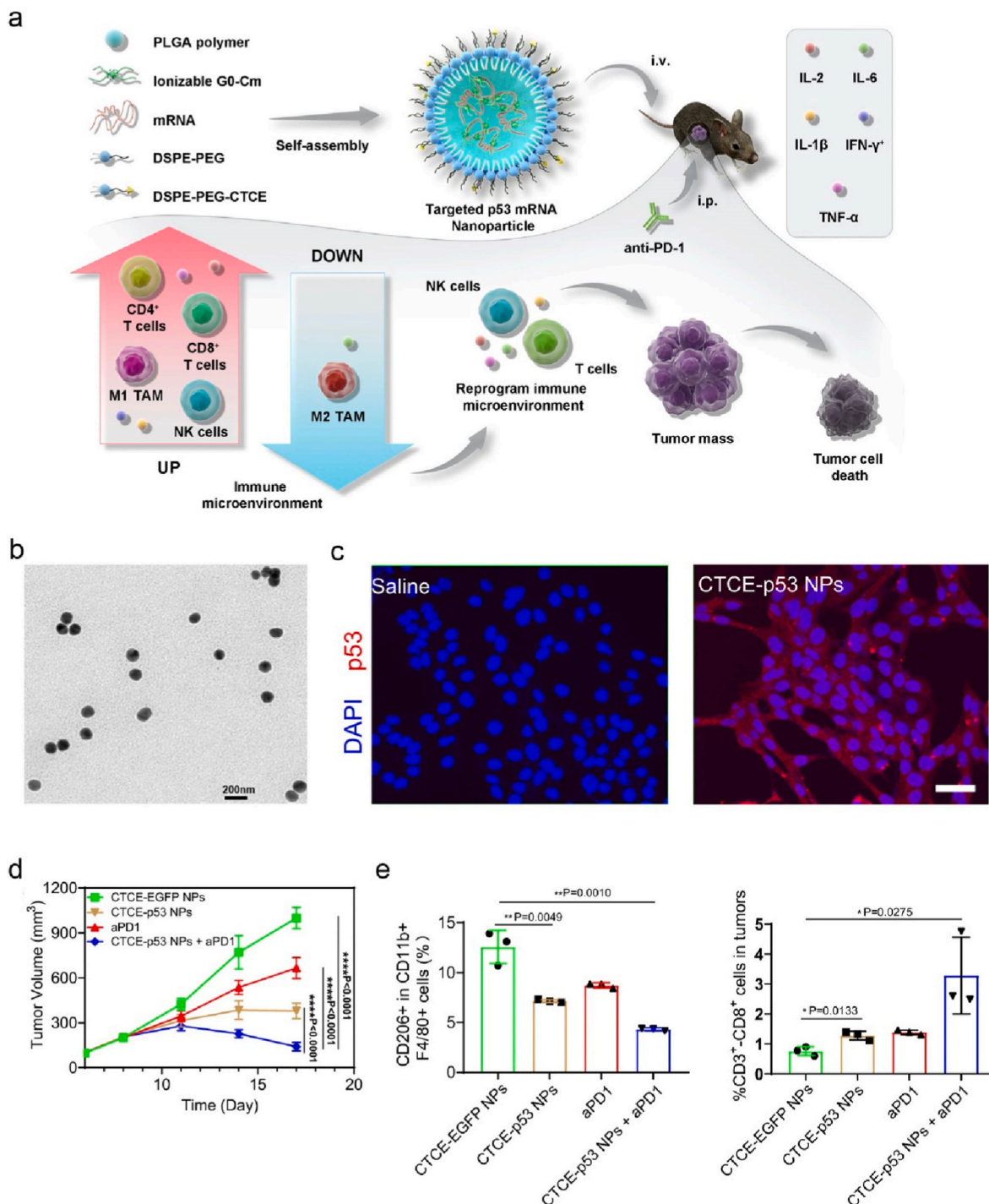


Fig. 16. CTCE decorated p53 mRNA-nanoparticles restore the expression of encoded p53 protein in HCCs, secreting multiple cytokines that modulate immune cells for efficacious cancer treatment. (a) Illustrated are the composition of synthetic CTCE-p53 NPs and the mechanism of CTCE-p53 NPs evoking strong anti-tumor response. (b) A representative TEM image of CTCE-p53 NPs. (c) Immunofluorescence images of p53 knockout RIL-175 cells showed the expression of encoded p53 (scale bar: 50 μ m). (d, e) p53 null RIL-175 tumor growth curves (d), and the percentages of tumor infiltrating CD3⁺CD8⁺ T cells and M2-like macrophages (e) of mice treated with different formulations as indicated [35]. Reproduced under the terms of the Creative Commons CC BY license. Copyright 2022, The Author(s), published by Springer Nature.

autoimmune toxicities and increasing the incidences of irAEs. By precisely engineering their physiochemical properties, nano-mRNA formulations can selectively target the organs of interest, promoting their clinical implementation in targeted cancer immunotherapy (please refer to the last section for our discussion on this point).

Despite immense potential of nano-mRNA strategies in cancer immunotherapy, their clinical translation has faced some obstacles with

nanotoxicity being one of the most often-encountered challenges. Once mRNA delivery systems engineered to nanoscale sizes, these nano-formulations actively interact with biological systems, potentially resulting in damage to human body at cellular or intercellular levels. The associated nanotoxicity is accentuated by the chemical components, morphology, surface charge and softness of nanoformulations [189]. For example, inorganic nanoparticles that can release metal ions (e.g., Cd²⁺.

Table 2
Summary of advanced nanoformulations for targeted delivery of mRNA to specific subsets of immune cells.

Targeted immune cells	Nanoformulations	mRNA encoded molecules	Size (nm)	Surface charge (mV)	Tumor models	Mechanisms	Therapeutic outcome	Administration routes	Ref
Nano-mRNA targeting DCs									
Ex vivo DCs	Cationic liposomes/iron oxide NPs	OVA	207.9 ± 67.2	44.1 ± 4.5	B16F10-OVA murine melanoma	Activating DCs and promoting antigen expression	Suppressing tumor growth	i.d.	[137]
Ex vivo DCs	Lipoplex/vitamin E scaffold/peptide	OVA	/	/	E.G7-OVA murine lymphoma	As above	Suppressing tumor growth	s.c.	[138]
Ex vivo DCs	Chitosan NPs	CD40+ICOSL	35	/	4T1 murine breast cancer	Activating DCs, promoting T proliferation and accelerating cytokine secretion	Suppressing tumor growth	i.t.	[139]
Splenic conventional DCs, pDCs and macrophage	Lipoplex	OVA; gp70; TRP-1; CT26-M90; HPV E6/E7	200–400	/	B16-OVA and B16F10-Luc murine melanoma, CT26 and CT26-Luc murine colon carcinoma and TC-1-Luc murine cervical cancer	Activating DC and priming CD4 ⁺ and CD8 ⁺ T cells via TLR7-IFN α signaling pathway	Regression of tumor/suppressing tumor growth	i.v.	[21]
As above	Lipoplex	CLDN6	/	/	xenograft OV-90 human ovarian cancer	Driving proliferation of adoptive CLDN6-CAR-T cells	Regression of tumor/suppressing tumor growth	i.v.	[34]
DCs	C1 LNP	OVA	150	16.37 ± 0.404	B16-OVA murine melanoma and MC38-OVA murine colon carcinoma	Activating DCs via TLR4 pathway	Suppressing tumor growth	s.c.	[25]
dLN-DCs, NK cells, macrophages and B cells	OMV/clyA-L7Ae/clyA-LLO	OVA; ADPGK	28.1	/	B16-OVA murine melanoma and MC38 murine colon carcinoma	Activating DC via multiple TLR pathways	Regression of tumor/suppressing tumor growth	s.c.	[24]
dLN-DCs	Hydrogel (PEI + GO)	OVA	220	−0.52	B16-OVA murine melanoma	Sustained release of delivered R848 and mRNA for enhanced DC activation via TLR7/8 pathway	Suppressing tumor growth	s.c.	[145]
DCs	PLGA/lipid NPs	OVA	400	20	B16-OVA murine melanoma	Delivery of gardiquimod for DC activation via TLR7 pathway	Suppressing tumor growth	i.v.	[119]
dLN-DCs, neutrophils, macrophages, and B cells	LNPs	Gp100; TRP2; OVA;	200	−14.1–2.0	B16F10 and B16-OVA murine melanoma	LNPs delivered modified mRNA and LPS for enhanced DC activation via TLR4 pathway	Suppressing tumor growth	s.c.	[146]
DCs	PEG/lipid/polymer NPs	OVA	137 ± 2.8	11.2 ± 2.1	E.G7-OVA murine lymphoma and RM1-OVA murine prostate cancer	Delivery of C16-R848 for enhanced DC activation via TLR7/8 pathway	Suppressing tumor growth	s.c.	[147]
dLN-macrophages, DCs, and monocytes	Heterocyclic LNPs	OVA;	100	/	B16F10-OVA murine melanoma and TC1 murine cervical cancer	Activating DCs via heterocyclic amine group-mediated STING pathway	Suppressing tumor growth	s.c.	[22]
DCs	LNPs	E7 E6/7; STING ^{V155M}	80–100	/	TC-1 murine cervical cancer	Activating DCs via hypersensitive STING ^{V155M} pathway	Suppressing tumor growth	i.m.	[155]
DCs	Manna/PEI nanocapsules	OVA	220	32 ± 0.5	B16F10-OVA murine melanoma	Activating DCs via dectin-2/TLR-4	Suppressing tumor growth	s.c.	[158]
Splenic DCs and macrophages	Trimannose/lipid/polymer hybrid NPs	OVA; E7; CD40L + CD70 ⁺ cATLR4	230	45	TC-1 murine cervical cancer and B16-OVA murine melanoma	Priming anti-tumor T cells via nanoparticle mediated-type I-IFN pathway	Regression of tumor/suppressing tumor growth	i.v.	[132]
dLN-DCs	Mannose/lipid-calcium/phosphate NPs	MUC1	58	38	4T1 murine breast cancer	Activating DCs via mannose receptor pathway	Suppressing tumor growth	s.c.	[124]
Splenic DCs	α -GC/liposomes	OVA	190	47	B16-OVA murine melanoma and E.G7-OVA murine lymphoma	Activating DCs, iNKT and NK cell	Suppressing tumor growth	i.v.	[159]
Systematic PD-L1+ APCs	LNPs	Whole tumor antigens; OVA	70–200	40–50	B16F10 and B16-OVA murine melanoma; glioma	Activating PD-L1+DC	Suppressing tumor growth	i.v.	[160]

(continued on next page)

Table 2 (continued)

Targeted immune cells	Nanoformulations	mRNA encoded molecules	Size (nm)	Surface charge (mV)	Tumor models	Mechanisms	Therapeutic outcome	Administration routes	Ref
DCs	Mesoporous silica NPs	OVA; GM-CSF;	115	/	E.G7-OVA murine lymphoma	Promoting mRNA translation by loaded PKR inhibitor C16	Regression of tumor/ suppressing tumor growth	s.c.	[89]
Lymphoid organ resident DCs	Lipoplex	PME1	340	/	CT26 murine colon carcinoma	Local radiotherapy induced CD8 ⁺ T cells combined with nano-mRNA induced CD4 ⁺ T immunity for enhanced therapeutic effect	Regression of tumor/ suppressing tumor growth	i.v.	[161]
DCs	Lipoplex	E7	200–250	–20–30	TC-1 murine cervical cancer	Activating DCs and inducing antigen-specific effector and memory CD8 ⁺ T cells	Regression of tumor/ suppressing tumor growth	i.v.	[164]
DCs	Lipoplex	E7	200–250	–20–30	TC-1 murine cervical cancer	Local radiotherapy promoted nano-mRNA vaccine efficacy	Regression of tumor/ Suppressing tumor growth	i.v.	[162]
DCs	Protamine	OVA	/	/	E.G7-OVA murine lymphoma	As above	Suppressing tumor growth	s.c.	[163]
Nano-mRNA targeting cytotoxic T cells and NK cells									
T cells	Anti-CD3/CD28 mAbs/PbAE/PGA(polyglycolic acido) polymer NPs	Foxo1	109.6 ± 26.6	1.1 ± 5.3	Raji murine lymphoma	Reprogramming CAR-T cells to a memory phenotype with competent functions	Suppressing tumor growth	i.v.	[33]
T cells	Anti-CD8 mAbs/PbAE/PGA polymer NPs	CARs; TCRs	106.9 ± 7.2	/	Eμ-ALL01 leukemia, Raji lymphoma, LNCaP C42 prostate cancer and HepG2 hepatitis B-induced hepatocellular carcinoma models	In vivo reprogramming of circulating T cells with CARs or TCRs	Suppressing tumor growth	i.v.	[32]
T cells	Phospholipid NPs	OX40; CD137	120–230	8–36	B16F10 murine melanoma, A20 B cell lymphoma	Promoting T cell activation and therapeutic response to anti-OX40mAbs	Regression of tumor/ Suppressing tumor growth	i.t./i.v.	[98]
T cells, macrophages, monocytes, granulocytes, DCs, tumor cells	LNPs	IL-23, IL-36γ, OX40L	/	/	H22 murine hepatocellular carcinoma, MC38 murine colon carcinoma, and B16F10-AP3 murine melanoma	Promoting the infiltration of multiple immune cells into tumor sites via cytokine- and OX-40L-mediated pathways	Regression of tumor/ Suppressing tumor growth	i.t./s.c./i.d.	[173]
NK cells	LNPs	Anti-HER2 mAbs	/	/	mouse xenograft MDA-MB-231-HER2 human breast cancer	Enhancing NK-mediated ADCC	Suppressing tumor growth	i.v.	[30]
T cells	LNPs	Rituximab antibody	/	/	mouse xenograft human Raji lymphoma	CDC and ADCC	Regression of tumor/ Suppressing tumor growth	i.v.	[175]
T cells	LNPs	Anti-PD-1 mAbs	/	/	MC38 murine colon carcinoma	Activating T cells by in vivo expression of therapeutic anti-PD-1 mAbs	Suppressing tumor growth	i.v.	[29]
T cells	Lipid/polymer NPs	CD3× CLDN6 bispecific antibodies	/	/	mouse xenograft OV-90, ES-2, ES-2/hCLDN6 human ovarian cancer	Bispecific T cell-engager mediated cytotoxic T cells to tumor cells	Regression of tumor	i.v.	[28]
Nano-mRNA targeting multiple immune cells in TME									
T cells, NK cells, macrophages	LNPs	IL-12a + IL12b	/	/	MC38-S and MC38-R murine colon carcinoma, B16F10-AP3 melanoma and A20 B cell lymphoma.	Driving Th1 immunity and activating DCs, T cells and NK cells	Regression of tumor/ Suppressing tumor growth	i.t.	[133]

(continued on next page)

Table 2 (continued)

Targeted immune cells	Nanoformulations	mRNA encoded molecules	Size (nm)	Surface charge (mV)	Tumor models	Mechanisms	Therapeutic outcome	Administration routes	Ref
T cells, DCs, NK cells	LNPs	IL-12, IL-27, GM-CSF	150–200	/	PDX model: ME 12057, ME 12058, HN 5111, HM5116 B16F10 murine melanoma	Inducing robust infiltration of NK and CD8 T cells in tumor	Suppressing tumor growth	i.t.	[26]
T cells, NK cells, macrophages	LNPs	IL-12	/	/	MYC-driven murine hepatocellular carcinoma	Promoting tumor infiltrating activated Th cells and production of IFN- γ	Suppressing tumor growth	i.v.	[184]
NK cells, T cells,	liposome-protamine complex	IL-15	221.33 \pm 2.52	48.03 \pm 1.429	C26 murine colon cancer	Enhancing proliferation of NK, B, and T cells in tumor	Suppressing tumor growth	i.p./i.t./i.v.	[120]
TAMs, monocytes, DCs, neutrophils	Di-mannose/PbAE/PGA polymer NPs	IRF5 and IKK β	99.8 \pm 24.5	3.40 \pm 2.15	ID8 murine ovarian cancer, B16F10 melanoma, and glioblastoma	Driving the polarization from M2-like macrophages to M1-like macrophages in tumor	Suppressing tumor growth	i.p./i.v.	[31]
T cells, DCs, NK cells	LNPs	CCL5	/	/	B16F10 murine melanoma	CCL5 chemokine mediated recruitment of multiple Leukocytes in tumor	Suppressing tumor growth	i.t.	[27]
Tumor cells, T cells, DCs, macrophages	Charge-altering releasable transporters (CART)	CD70, OX40L, CD80, CD86, IL-12, IFN- γ	/	/	A20 murine B cell lymphoma and CT26 murine colon carcinoma	Cytokine mediated robust local T-cell activation and systematic immune response	Regression of tumor/ Suppressing tumor growth	i.t.	[185]
Indirect targeting immune cells by nano-mRNA mediated immunogenetic cancer cell death									
Tumor cells	Lipid/mPEG-PLGA/G0-C14 hybrid NPs	PTEN	111.8 \pm 15.3	/	Pten-mutated B16F10 murine melanoma and Pten-null/Pten-Cap8 murine prostate cancer	Inducing autophagy of cancer cells and release of immunogenic DAMPs	Suppressing tumor growth	i.v.	[179]
Tumor cells	Lipid/PLGA/G0-14/DSPE-PEG/CTCE NPs	p53	110	negative	p53-null RIL-175 murine hepatocellular carcinoma	Promoting anti-tumor immune cells and decreasing pro-tumor immune cells	Suppressing tumor growth	i.v.	[35]
Tumor cells	liposome-protamine lipoplex	survivin-T34A	186.1 \pm 3.1	/	C26 murine colon cancer	Inducing caspase-dependent cell apoptosis, and modulation of tumor microenvironment	Suppressing tumor growth	i.p./i.t./i.v.	[187]

Note: i.v.: intravenous injection; i.m.: intramuscular injection; s.c.: subcutaneous injection; i.d.: intradermal injection; DSPE: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine.

Mn²⁺) can promote the production of reactive oxygen species (ROS) via biochemical reactions, causing ROS-mediated cell damage [189]. Increased accumulation of Mn²⁺ can also trigger nanotoxicity in central nervous system, such as Parkinson diseases [190]. To encapsulate negatively charged mRNA, nanoformulation delivery systems often contain cationic components, which cause their extensive interactions with cellular components (such as endosome membrane) and subsequent apoptotic cell death [191]. Beyond nanotoxicity, cationic nano-mRNA delivery systems potentially induce pro-inflammatory responses [192]. As discussed above, ionizable LNPs are one of the most promising candidates in clinical studies, which could potentially reduce nanotoxicity caused by cationic LNPs. The candidates with excellent safety profile and therapeutic efficacy have entered clinical trials, which will be discussed in next section.

5. Progress of clinical trials

The first clinical trial of mRNA therapeutics was conducted in patients with breast cancer (NCT00003432) in 1998, where patients received DC vaccines pulsed with naked mRNA expressing carcinoembryonic antigen for evaluating cancer immunotherapy efficacy. In the context of nano-mRNA immunotherapeutics, most clinical trials have focused on cancer vaccines, of which protamine-delivered mRNA vaccines were at advanced clinical development (Table 3). The early studies on mRNA vaccine delivery using protamine nanoformulations revealed that mRNA-protamine acted as adjuvants via activation of TLR7/8 pathway, inducing balanced and potent cellular immunity after easy intradermal administration [193], while the translation efficacy of tightly bounded mRNA was moderate. To overcome this hurdle, CureVac developed RActive® by combination of protamine-delivered mRNA and naked nucleoside modified mRNA with a ratio of 1:1. RActive® encoding multiple TAAs were clinically evaluated in patients with metastatic prostate cancer, stage IIIB/IV NSCLC (non-small-cell lung cancer) or metastatic melanoma [194–198] (Table 3). This vaccination strategy alone or in combination with radiation therapy was proven well tolerated and immunogenic in patients under phase I/II clinical studies. The development of RActive® was halted by current preference of LNPs delivered mRNA therapeutics in clinic.

Lipoplex nanoformulations developed by BioNTech are the most advanced LNP-mRNA cancer vaccines, of which BNT111 encoding four melanoma TAAs is being evaluated in phase I trial in melanoma patients (NCT02410733). The clinical results of BNT111 have been reported [23], showing that patients intravenously administrated with BNT111 vaccine induced potent and broad T cell response against TAAs. Antigen specific CTLs with a high magnitude and long durability were observed in some responders who experienced the treatment of anti-PD-1 mAbs. Patients vaccinated with BNT111 appeared with flu-like symptoms in mild to moderate levels, which were resolved within 1 day. Given the encouraging clinical safety profile and immunogenic responses, BNT111 now is under an active phase II study with or without anti-PD-1 mAbs to evaluate the efficacy as well as safety in patients with unresectable melanoma (NCT04526899).

Lipoplex cancer vaccine technology is expanding its clinical evaluations in patients with other types of malignancies (Table 3), including BNT112 vaccine encoding five prostate cancer antigens in prostate cancer patients (NCT04382898), BNT113 encoding viral neoantigen HPV16 E6/E7 in patients with HPV16 positive HNSCC (head and neck squamous cell carcinoma) (NCT04534205, NCT03418480), BNT114 encoding three breast cancer antigens in breast cancer patients (NCT023164570), BNT115 encoding mixed ovarian cancer TAAs in patients with ovarian cancer (NCT04163094), and BNT116 encoding mixed NSCLC TAAs in NSCLC patients (NCT05142189). BioNTech also developed lipoplex-based neoantigen mRNA vaccine of BNT122 for multiple cancer treatment. BNT122 encoding up to 20 neopeptides for individual patient is currently under clinical evaluation in four studies (Table 3). These vaccine product candidates are to augment the

production of antigen specific endogenous T cells, while the newly investigated BNT211 vaccine product encodes CLDN6 for in vivo expansion of adoptive CLDN6 CAR-T cells. The brief report on phase I/II clinical results of BNT211 (NCT04503278) demonstrated encouraging safety profile and efficacy in patients with CLDN6+ solid tumors [199], which might be a hallmark in CAR-T mediated solid tumor treatment.

Moderna developed an alternative LNP-based nanoformulation for delivery of personalised cancer mRNA vaccines. It is a key but remains a challenge to select appropriate immunogenic neoantigens. Moderna has developed different pipelines for identification and selections of neoantigens that can be recognised by functional tumor-infiltrating T cells in patients. For example, Moderna identified 15 human leukocyte antigen (HLA) class I neoantigens from driver gene mutations by exome sequencing of patient tumor samples, which were then encoded into a mRNA vaccine together with the defined antigens (named mRNA-4650). The personalized mRNA-4650 vaccine was administrated intramuscularly in patients with metastatic gastrointestinal cancer in a phase I/II study (NCT03480152) to evaluate the safety, tolerability, and immunogenic efficacy. The clinical results from 4 patients displayed no severe side effects, while this vaccine did not stimulate increased neoantigen specific T cell immunity [200]. Given that no clinical response was detected, Moderna decided to terminate this clinical trial. Instead, a closely related mRNA-4157 vaccine was developed via a different neoantigen selection protocol, where a single mRNA encoded up to 34 selected neoantigens. mRNA-4157 vaccine alone or in combination with pembrolizumab (anti-PD1 mAbs) currently is in a phase I study in patients with solid tumors (NCT03313778, starting from 2017) and phase II study in patients with complete resected cutaneous melanoma (NCT03897881). Moderna also developed TAA encoded mRNA vaccine, such as mRNA-5671 incorporating four mutated KRAS (kirsten rat sarcoma viral oncogene homolog) antigens. mRNA-5671 vaccine as monotherapy and combination therapy with pembrolizumab currently is evaluated in patients with multiple KRAS mutant advanced or metastatic cancers under a phase I study (NCT03948763). Another newly developed DOTAP liposome vaccine containing autologous total tumor mRNA and mRNA encoding lysosome membrane-associated protein (LAMP) is under a first human phase I study in patients with glioblastoma (NCT04573140).

Along with therapeutic nano-mRNA cancer vaccines, both BioNTech and Moderna recently are remarkably increasing investments on nano-mRNA development pipelines for the replacement of immunotherapeutic antibodies or cytokines and in vivo production of T/NK cells with CARs and TCRs. One such product, BNT142 encoding CD3 × CLDN6 bispecific antibodies, is just starting the first human phase I/II studies (NCT05262530) this year to evaluate its safety and immunogenetic efficacy in patients with CLDN6+ advanced solid tumors. Another type of products, BNT151 encoding optimized IL-2, BNT151 encoding IL-2 and BNT153 encoding IL-7, are under clinical trials (NCT04455620, NCT04710043) alone or combined with other therapeutic agents in solid tumor patients via intravenous administration. An ongoing study just started in 2022 is evaluating the safety and pharmacokinetics of nano-mRNA product (BNT141) encoding therapeutic mAbs against tumor antigen CLDN18.2 in patients with CLDN18.2+ tumor (NCT04683939). An intratumor administrated product (MEDI1191) that encodes fusion proteins of IL-12 α and IL-12 β is in a phase I study in patients with solid tumor (NCT03946800). Another two products, mRNA-2416 encoding OX40L and mRNA-2752 (NCT02872025, starting from 2017) incorporating encoded OX-40L, IL-36 γ and IL-23, are being evaluated in phase I studies (Table 3). The tumor samples from NSCLC patients in mRNA-2416 clinical trials were spatially mapped to evaluate the clinical significance of OX40/OX40L pathway [201]. The results demonstrated that local injection of mRNA-2416 increased the expression of OX40L in TME, which was associated with increased infiltrated CD4⁺ and CD8⁺ T cells and conventional type 1 DCs (cDC1s) in NSCLC. The preliminary clinical response data are encouraging.

Recent explosion and success of new immunotargets, genetically

Table 3
Summary of representative nano-mRNA therapeutics for targeted cancer immunotherapy under clinical trials.

Targeted immune cells	Nanoformulations	mRNA encoded molecules	Tumor types	Administration routes	Nano-mRNA name	Phase	Status (Start year)	NCT Number (Ref)
Splenic cDCs, pDCs, and macrophages	Lipoplex	Three TAAs	Triple-negative breast cancer	i.v.	BNT114	Phase-I	Active, not recruiting (2016)	NCT02316457
As above	Lipoplex	Three TAAs	Ovarian cancer	i.v.	BNT115 (W_ova1)	Phase-I	Recruiting (2019)	NCT04163094
As above	Lipoplex	TAAs	Non-small-cell lung cancer	i.v.	BNT116	Phase-I	Not yet recruiting (Estimated in 2022)	NCT05142189
As above	Lipoplex	Kallikrein-2 + Kallikrein-3 + ACP-Prostate + HOXB13 + NK3 homeobox 1	Metastatic castration-resistant prostate cancer	i.v.	BNT112 (W_pro1)	Phase-I/II	Recruiting (2019)	NCT04382898
As above	Lipoplex	NY-ESO-1 + MAGE-A3 + TPTE + Tyrosinase	melanoma	i.v.	BNT111	Phase-I/II	Recruiting (2021)/ Active, not recruiting (2015)	NCT04526899/ NCT02410733 ([23])
Ex vivo human DCs	Protamine	NY-ESO-1 + MAGE-C2 + MUC1	metastatic castration-resistant prostate cancer	i.n.	/	Phase-II	Completed (2015)	NCT02692976 ([194])
Skin-APCs (RNActive®)	Protamine	NY-ESO-1 + MAGE-C1 + 5T4 + MAGE-C2 + Survivin + MUC-1	Non-small-cell lung cancer	i.d.	CV9202 (BI-1361849)	Phase-I/II	Completed (2017)/ Terminated (2013)	NCT03164772/ NCT01915524 ([195,196])
As above	Protamine	NY-ESO-1 + MAGE-C1 + 5T4 + MAGE-C2 + Survivin	Stage IIIB/IV non-small-cell lung cancer	i.d.	CV9201	Phase-I/II	Completed (2009)	NCT00923312 ([197])
As above	Protamine	Melan-A + Mage-A1 + GP100 + Mage-A3 + Survivin + Tyrosinase	Stages III/IV metastatic melanoma	i.d.	/	Phase-I/II	Completed (2004)	NCT00204607
As above	Protamine	PSA + PSCA + PSMA + STEAP1	Hormone-resistant prostate cancer	i.d.	CV9103	Phase-I/II	Completed (2009)	NCT00831467 ([198])
As above	Protamine (RNActive®)	PSA + PSCA + PSMA + STEAP1 + PAP + Muc1	High/Intermediate risk prostate cancer	i.d.	CV9104	Phase-II	Terminated (2014)	NCT02140138
Splenic cDCs, pDCs, and macrophages	Lipoplex	E6 + E7	HPV16+ head and neck squamous cell carcinoma	i.v./i.d.	BNT113	Phase-I/II	Recruiting (2021)/ Recruiting (2017)	NCT04534205/ NCT03418480
As above	DOTAP Liposome	Total tumor mRNA + pp65 full length LAMP mRNA	Pediatric high-grade glioma/ glioblastoma	i.v.	RNA-LP	Phase-I	Recruiting (2021)	NCT04573140
dLN-APCs	LNP	Personalised cancer vaccine	Multiple cancers	i.m.	NCI-4650 (mRNA-4650)	Phase-I/II	Terminated (2018)	NCT03480152 ([200])
As above	LNP	As above	Solid tumor/ melanoma	i.m.	mRNA-4157	Phase-I/II	Recruiting (2017)/ Active, not recruiting (2019)	NCT03313778/ NCT03897881
As above	Lipopolyplex	As above	Multiple advanced digestive system cancers	s.c.	/	Not Applicable	Unknown (2018)	NCT03468244
APCs	Protamine	As above	Multiple digestive system cancers	/	PGV002	Not Applicable	Recruiting (Estimated in 2022)	NCT05192460
Splenic cDCs, pDCs, and macrophages	Lipoplex	As above	Multiple resected/ advanced cancers	i.v.	BNT122 (RO7198457)	Phase-I/II	Recruiting (2021)/ Active, not recruiting (2019)/ NCT03815058	NCT04486378/ NCT04161755/ NCT03815058

(continued on next page)

Table 3 (continued)

Targeted immune cells	Nanoformulations	mRNA encoded molecules	Tumor types	Administration routes	Nano-mRNA name	Phase	Status (Start year)	NCT Number (Ref)
dLN-APCs	LNP	Four KRAS mutated proteins (G12D + G12V + G13D + G12C)	Multiple KRAS mutant advanced/metastatic cancers	i.m.	mRNA-5671	Phase-I	(2019)/Withdrawn (2021)/Active, not recruiting (2017) Active, not recruiting (2019)	NCT04267237/ NCT03289962 NCT03948763
Splenic cDCs, pDCs, and macrophages	Lipoplex	CDLN6	Solid Tumor	i.v.	BNT211	Phase-I/II	Recruiting (2020)	NCT04503278 ([199])
Tumor-T cells, Tumor cells	Cationic polymer/lipid formulation	CD3 × CLDN6 bispecific antibodies	Solid Tumor	i.v.	BNT142	Phase-I/II	Recruiting (2022)	NCT05262530
T cells	LNP	Optimized IL-2	Solid Tumor	i.v.	BNT151	Phase-I/II	Recruiting (2021)	NCT04455620
As above	LNP	IL-2 + IL-7	Solid Tumor	i.v.	BNT152 + BNT153	Phase-I	Recruiting (2021)	NCT04710043
Tumor-T cells, NK cells, macrophages	LNP	IL-12	Advanced Solid tumor	i.t.	MEDI1191	Phase-I	Recruiting (2019)	NCT03946800
Tumor - NK cells	LNP	Anti-Claudin 18.2 antibodies	Solid Tumor	i.v.	BNT141	Phase-I/II	Recruiting (2022)	NCT04683939
Tumor and TdLN - T cells, macrophages, monocytes, granulocytes, DCs, Tumor cells	LNP	OX40L	Recurrent/refractory solid malignant tumor or lymphoma	i.t.	mRNA-2416	Phase-I	Active, not recruiting (2017)	NCT03323398 ([201])
As above	LNP	OX40L + IL-23 + IL-36 γ	multiple advanced cancers	i.t.	mRNA-2752	Early Phase-I	Recruiting (2018)/ Recruiting (2017)	NCT03739931/ NCT02872025

Note: i.n.: intranodal injection; TdLN: Tumor draining lymph node.

engineered adoptive T cell therapy and mRNA COVID-19 vaccines have fuelled research interest and investment from biotechnology companies in the use of nano-mRNA therapeutics for targeted cancer immunotherapy. As seen from Table 3, organic nanoparticles, such as LNPs and polymer nanoparticles, are more prevalent in clinical studies compared with inorganic counterparts, contributing by the advantages of organic nanoparticles (listed in Table 1), such as high biocompatibility and simplicity in scale-up fabrication. Most LNP-mRNA therapeutics in cancer immunotherapy are at their early phase of clinical trials and have not yet employed in clinic. It had been a long journey for researchers to address the issues of mRNA instability and low transfection efficacy in vivo prior to their recent clinical trials. The preliminary results from current clinical trials are encouraging, while there are still some unsolved critical issues (discussed in the last section) that might impede their clinical translation in future.

6. Conclusions and future directions

The global recognition of the integrated technology of nano-mRNA in the rapid development of safe and effective COVID-19 vaccines has reignited the research interest and investment in mRNA therapeutics. The increasing knowledge in mRNA biology allows appropriate modulation on mRNA construct, inherent immunogenicity and production process and functional adaptability. Nanotechnologies that can encapsulate, protect, and deliver mRNA to specific sites of the body are indispensable for the extraordinary success of mRNA therapeutics in clinic. In parallel, immunotherapy has revolutionized the modalities of cancer treatment, such as ICIs and adoptive cell therapy. Given tumor heterogeneity, only 10–30% patients can benefit from cancer immunotherapy. Short half-life and autoimmune related adverse effects of

immuno-drugs are additional barriers for the broad implementation of cancer immunotherapy in clinical practices. The rapid progress of nano-mRNA promotes the promise of cancer immunotherapy by targeted delivery of encoded immuno-drugs to specific immune cells, enabling production of long-acting therapeutics and reducing off-target associated toxicities. As exemplified above, recent technological advances in delivery platforms and mRNA constructs have led to diverse promising paradigms in targeted cancer immunotherapy under preclinical and clinical studies. The flexibility of mRNA technology in encoding a great variety of immuno-molecules has created numerous clinical products, ranging from therapeutic cancer vaccines to immunostimulatory cytokines, antigen specific therapeutic antibodies, and in vivo genetically engineered T cells with CARs. Despite the stunning success of nano-mRNA technology in targeted cancer immunotherapy, there are still some challenges to be addressed for advancing the use of nano-mRNA as therapeutic immuno-medicines in clinic.

- (1) **Improved optimization of mRNA technology and understanding of molecular mechanisms of constructed mRNA.** The efforts in the past decade have driven the rapid development of transformative mRNA technologies to clinical reality. The incredible speed in mRNA vaccine deployment globally has proven the safety, efficacy, and adaptability of mRNA therapeutics in medicine. The discoveries in mRNA modifications are the key for the success of mRNA vaccines. Nevertheless, the remaining technical obstacles facing mRNA platforms are apparent, such as limited stability in vivo and low expression of encoded molecules. Continued development of mRNA technology with increased stability, immunogenicity and translatability could lead to the next generation of mRNA-based immuno-drugs,

fulfilling the medical potential of mRNA technology. To this end, it is essential to deepen the understanding of the relationship between the construct of modified mRNA and its stability/translation/immune modulation. High throughput methods are an appealing strategy to fast and accurately establish the correlations between the performance of mRNA and its massive structure variants. For example, the newly developed sequencing-based evaluation platform called PERSIST-seq enables systematic delineation of mRNA stability of a library of mRNA with diverse constructs [202]. Beyond conventional nonreplicating mRNA technology widely explored in cancer immunotherapy, self-amplify mRNAs have emerged as vaccine candidates for preventing infectious diseases [203]. Compared to conventional mRNA, self-amplified mRNA can copy itself and promote expression of encoded proteins under a low dose. However, it remains a challenge to precisely control the modification, transport, and strong immunogenicity-mediated side effects of large sized self-amplifying mRNAs. Very recently, a self-amplifying mRNA was reported inducing potent protective immunity in non-human primates against SARS-CoV-2 [204]. These encouraging preclinical data along with further technological discoveries might expand the applications of self-amplifying mRNA technology in therapeutic cancer vaccines and therapeutic protein-based immunotherapy (Fig. 17).

- (2) **Optimization of mRNA delivery platforms with a precise organ selectivity and high encapsulating capacity.** The stability of naked mRNA is substantially enhanced by mRNA delivery platforms. Currently ionizable LNPs are the preferred formulations to transport mRNA therapeutics in animal models and humans, with improved safety profile and therapeutic efficacy. Selective delivery of mRNA therapeutics to the action site in an organism is crucial to achieve the therapeutic outcome and reduce off-target associated toxicity. Targeting DCs in lymphoid organs is essential to initiate T cell immunity of delivered mRNA vaccines, thus spleen-target lipoplex products have been developed and applied in several cancer mRNA vaccine formulations that are under extensive clinical investigations. In addition to specific targeting efficacy, mRNA delivery platform is expected to have a high payload with multiple antigen candidates, which is normally comprised by the consideration of dose tolerability of LNPs. Thus, redesigning mRNA delivery platforms with increased loading capability and enhanced the translation efficacy of mRNA is a potential solution to balance the safety and efficacy. In the context of targeting immune cells at tumor sites, nano-mRNA encoding immune stimulatory proteins can be passively

delivered to tumors driven by the small nanosize-mediated EPR effect. Nonetheless, the accumulation efficacy is limited by the heterogenous physical barriers inside tumor [205]. Therefore, selective LNPs recently have been rationally engineered for active targeting delivery of mRNA to specific organs of lung, liver, or spleen [206,207]. This intriguing mRNA delivery system holds immense potential in targeted cancer immunotherapy. Alternatively, VLPs that have been approved as antigens in human use could potentially serve as promising delivery vehicles for mRNA therapeutics. The reengineering strategies are required to promote the translatability and immunogenicity of VLPs in the body.

- (3) **Improved understanding on the clinical response of nano-mRNA immunotherapeutics.** As reflected in Table 3, most LNP-mRNA modalities in the treatment of cancer patients are still under early phases of clinical trials. The positive therapeutic outcomes from these clinical studies will support the progress of promising candidates toward next evaluation stage. More importantly, the clinical data will expand our understanding on how and to what degree the patient response to the newly developed LNP-mRNA immuno-drug, which is extremely valuable in determining optimization directions for both mRNA and delivery technologies. The positive feedback loop will advance the translation of nano-mRNA technology in clinic.
- (4) **Exploration of uncovered mechanisms at the interface of nano-mRNA and tumor microenvironment.** Tumors are made up of heterogenous groups of malignant, stromal, and immune cells, with substantial levels of heterogeneity in each type of cells. The crosstalk among these cells creates an amalgam of signals that promote or suppress tumor growth and evolution. The bulk transcriptomic analysis tends to obscure specific subtype and status of cells that might be crucial for a therapeutic target. The integration of single-cell and bulk analyses is particularly vital in cancer immunotherapy, which can provide the insights into tumor or patient specific mechanisms driving a therapeutic response [208]. The emerging high-dimensional technologies, such as single-cell sequencing, single cell T cell receptor analysis, spatial transcriptomics, high dimensional flow cytometry, are revolutionary tools, which will advance our understanding of the interaction mechanisms between nano-mRNA and patient TME. The detailed molecular mechanisms that drive the response or resistance to nano-mRNA immunotherapy will yield informative results for the development of next generation of nano-mRNA technologies.
- (5) **Improved research collaborations across disciplines.** Multi-disciplinary research is vital to address research problems and

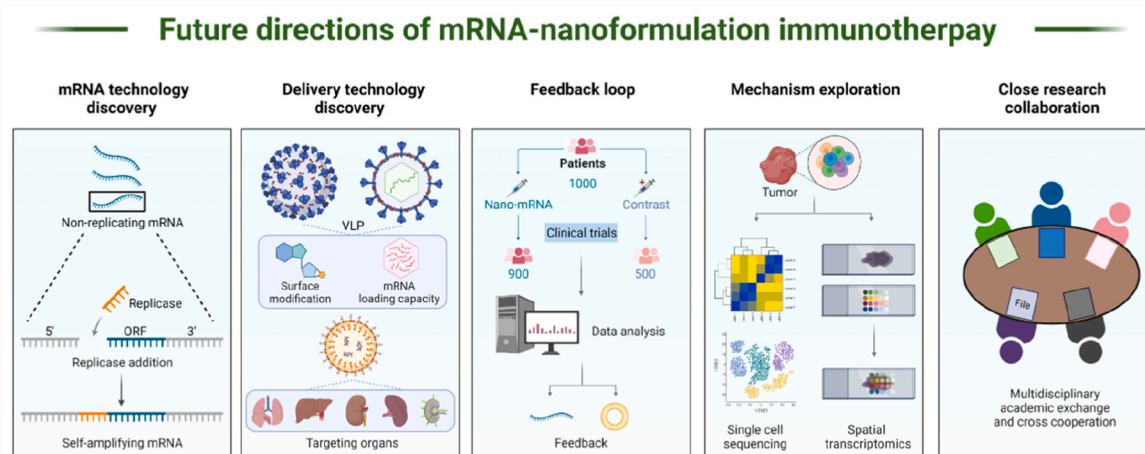


Fig. 17. An illustrated scheme showing the proposed future directions for implementation of nano-mRNA integrated technology in targeted cancer immunotherapy. Created with permission by BioRender.

society challenges. For example, the success of mRNA vaccines is a product of close and intensive collaboration with scientists at universities from different fields. Similarly, combined advances from nanotechnology, mRNA biology, immunology, and oncology, have furthered our knowledge of nano-mRNA technology in cancer immunotherapy. To advance the translation of nano-mRNA technology from basic science into medical products, it is indispensable to overcome the barriers caused by distinct technical vocabulary and commutation cultures, thereby fostering mutual understanding in research goals.

The advances in mRNA technology and nanoformulation strategies have demonstrated immense potential in cancer immunotherapy by targeted modulation of specific subsets of immune cells. The integrated nano-mRNA technology mediated immuno-therapeutics have been broadly studied for cancer treatment in preclinical and clinical studies. Continued research efforts and discoveries in this multidisciplinary field is expected to fulfil the far-reaching potential of nano-mRNA therapeutics in clinical practices for cancer treatment as well as other medical applications [209,210].

Ethics statement

This is a review-type manuscript. No ethics approval and consent to participate are required for this review-type manuscript.

CRediT authorship contribution statement

Wei Yang: Conceptualization, Literature research, Investigation, Writing – original draft. **Jianwei Cao:** Investigation, Writing – original draft. **Hui Cheng:** Investigation, Visualization. **Liang Chen:** Investigation, Visualization. **Meihua Yu:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition. **Yu Chen:** Conceptualization, Supervision, Writing – review & editing, Funding acquisition. **Xingang Cui:** Supervision, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare no competing financial interest.

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Abbreviations

DCs	dendritic cells
CTLs	cytotoxic T lymphocytes
TLRs	Toll-like receptors
APCs	antigen presenting cells
STING	intracellular stimulator of interferon genes
NK cells	natural killer cells
IVT	in vitro transcription
pDNA	plasmid DNA
UTR	untranslated region
ORF	open reading frame

PAMP	pathogen-associated molecular pattern
PRRs	pattern recognition receptors
IFN	interferon
TAA	tumor-associated antigen
HPLC	high performance liquid chromatography
LNP	lipid nanoparticle
DOTMA	1,2-di-O-octadecyl-3-trimethylammonium propane (chloride salt)
RIG-1	recombinant retinoic acid inducible gene 1 protein
MDA-5	melanoma differentiation-associated protein 5
m7G	7-methylguanosine;
eIF4F	eukaryotic initiation factor 4F
IRES	internal ribosome entry sites
miRNA	microRNA
GC	guanine cytosine;
U	uracil
dsRNA	double-stranded mRNA
RNase	ribonuclease
Da	dalton
MHC	major histocompatibility complex
ER	endoplasmic reticulum
GM-CSF	granulocyte-macrophage colony-stimulating factor
IL-12	interleukin-12
CPP	cationic cell-penetrating peptide;
VLPs	virus-like particles
HPV	human papillomavirus
PEG10	paternally expressed 10
PEG	polyethylene glycol
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine;
DOTAP	1,2-Dioleoyl-3-trimethylammonium-propane, chloride;
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine;
PEI	polyethylenimine;
PBAE	poly (β -amino ester)s
PLGA	poly(lactic-co-glycolic acid)
OMVs	outer membrane vesicles
dLNs	draining lymph nodes
TCRs	T-cell receptors
IONPs	iron oxide nanoparticles
IO-RNA-NPs	OVA-mRNA in the optimized liposomes
MRI	magnetic resonance imaging
OVA	ovalbumin
ICOSL	inducible costimulator ligand;
CD40	cluster of differentiation 40
Luc	luciferase labeling
CLDN6	claudin 6
CAR-T cells	chimeric antigen receptor-T cells
LPX	lipoplex
NSG	NOD scid gamma
PAMAM	poly(-amidoamine)
DiR	1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide;
EGFP	enhanced green fluorescent protein
R848	a TLR7/8 agonist
LPS	lipopolysaccharide;
FFL	firefly luciferase
SEM	scanning electron microscope
TEM	transmission electron microscope
IRF	interferon regulatory factor
dsDNA	double stranded DNA
NF- κ B	nuclear factor kappa-B;
ISRE	interferon stimulated response element
CLRs	C-type lectin receptors; Mann, mannan
Dex	dextran
CTLA-4	cytotoxic T lymphocyte-associated antigen-4
mAbs	monoclonal antibody; cy5.5, cyanine5.5
Fluc	firefly luciferin

ICIs	immune checkpoint inhibitors
PD-1	programmed death 1
α -GC	α -Glucosidase
iNKT cells	invariant natural killer T cells
NKT cells	natural killer T cells
PKR inhibitor	protein kinase inhibitor
Cre	cAMP response element
HER2	human epidermal growth factor receptor type 2
ADCC	antibody dependent cell mediated cytotoxicity
CCL5	C–C motif ligand 5
DLS	dynamic light scattering
NP	nanoparticle
PTEN	gene of phosphate and tension homology deleted on chromosome ten
CRT	calreticulin
CTCE	CXCR4 antagonist
HCC	hepatic cell carcinoma
pDCs	plasmacytoid dendritic cells
GO	graphite oxide;
PGA	polyglycolic acid
GM-CSF	granulocyte-macrophage colony-stimulating factor
Di	dialkylcarbocyanines
CART	charge-altering releasable transporter
DSPE	1,2-distearoyl-sn-glycero-3-phosphoethanolamine;
i.v.	intravenous injection
i.m.	intramuscular injection
s.c.	subcutaneous injection
i.d.	intra dermal injection
NSCLC	non-small-cell lung cancer
HNSCC	head and neck squamous cell carcinoma
HLA	human leukocyte antigen
KRAS	kirsten rat sarcoma viral oncogene homolog
PAP	prostatic acid phosphatase
TME	tumor microenvironment
DAMPs	damage associated molecular patterns

References

- [1] F.P. Polack, S.J. Thomas, N. Kitchin, J. Absalon, A. Gurtman, S. Lockhart, J. L. Perez, G.P. Marc, E.D. Moreira, C. Zerbini, R. Bailey, K.A. Swanson, S. Roychoudhury, K. Koury, P. Li, W.V. Kalina, D. Cooper, R.W. Frenc, L. L. Hammit, O. Tureci, H. Nell, A. Schaefer, S. Unal, D.B. Tresnan, S. Mather, P. R. Dormitzer, U. Sahin, K.U. Jansen, W.C. Gruber, C.C.T. Grp, Safety and efficacy of the BNT162b2 mRNA covid-19 vaccine, *N. Engl. J. Med.* 383 (2020) 2603–2615.
- [2] L.R. Baden, H.M. El Sahly, B. Essink, K. Kotloff, S. Frey, R. Novak, D. Diemert, S. A. Spector, N. Rouphael, C.B. Creech, J. McGettigan, S. Khetan, N. Segall, J. Solis, A. Brosz, C. Fierro, H. Schwartz, K. Neuzil, L. Corey, P. Gilbert, H. Janes, D. Follmann, M. Marovich, J. Mascola, L. Polakowski, J. Ledgerwood, B. S. Graham, H. Bennett, R. Pajon, C. Knightly, B. Leav, W.P. Deng, H.H. Zhou, S. Han, M. Ivarsson, J. Miller, T. Zaks, C.S. Grp, Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine, *N. Engl. J. Med.* 384 (2021) 403–416.
- [3] V.K. Morris, S. Kopetz, Don't Blame the Messenger: Lessons Learned for Cancer mRNA Vaccines during the COVID-19 Pandemic, *Nat Rev Cancer*, 2022.
- [4] R.W. Malone, P.L. Felgner, I.M. Verma, Cationic liposome-mediated RNA transfection, *Proc. Natl. Acad. Sci. U.S.A.* 86 (1989) 6077–6081.
- [5] J.A. Wolff, R.W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, P.L. Felgner, Direct gene transfer into mouse muscle in vivo, *Science (New York, N.Y.)* 247 (1990) 1465–1468.
- [6] G.F. Jirikowski, P.P. Sanna, D. Maciejewski-Lenoir, F.E. Bloom, Reversal of diabetes insipidus in Brattleboro rats: intrahypothalamic injection of vasopressin mRNA, *Science (New York, N.Y.)* 255 (1992) 996–998.
- [7] F. Martinon, S. Krishnan, G. Lenzen, R. Magné, E. Gomard, J.-G. Guillet, J.-P. Lévy, P. Meulien, Induction of virus-specific cytotoxic T lymphocytes in vivo by liposome-entrapped mRNA, *Eur. J. Immunol.* 23 (1993) 1719–1722.
- [8] R.M. Conry, A.F. LoBuglio, M. Wright, L. Sumerel, M.J. Pike, F. Johanning, R. Benjamin, D. Lu, D.T. Curiel, Characterization of a messenger RNA polynucleotide vaccine vector, *Cancer Res.* 55 (1995) 1397–1400.
- [9] R. Medzhitov, Toll-like receptors and innate immunity, *Nat. Rev. Immunol.* 1 (2001) 135–145.
- [10] L. Alexopoulou, A.C. Holt, R. Medzhitov, R.A. Flavell, Recognition of double-stranded RNA and activation of NF-kappa B by Toll-like receptor 3, *Nature* 413 (2001) 732–738.
- [11] S.S. Diebold, T. Kaisho, H. Hemmi, S. Akira, C.R.E. Sousa, Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA, *Science* 303 (2004) 1529–1531.
- [12] F. Heil, H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, S. Bauer, Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8, *Science* 303 (2004) 1526–1529.
- [13] K. Kariko, M. Buckstein, H.P. Ni, D. Weissman, Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA, *Immunity* 23 (2005) 165–175.
- [14] N. Pardi, M.J. Hogan, F.W. Porter, D. Weissman, mRNA vaccines - a new era in vaccinology, *Nat. Rev. Drug Discov.* 17 (2018) 261–279.
- [15] Y.F. Xiao, Z.M. Tang, X.G. Huang, W. Chen, J. Zhou, H.J. Liu, C. Liu, N. Kong, W. Tao, Emerging mRNA technologies: delivery strategies and biomedical applications, *Chem. Soc. Rev.* 51 (2022) 3828–3845.
- [16] X.C. Hou, T. Zaks, R. Langer, Y.Z. Dong, Lipid nanoparticles for mRNA delivery, *Nat. Rev. Mater.* 6 (2021) 1078–1094.
- [17] N. Chaudhary, D. Weissman, K.A. Whitehead, mRNA vaccines for infectious diseases: principles, delivery and clinical translation, *Nat. Rev. Drug Discov.* 20 (2021) 817–838.
- [18] A.L. Huff, E.M. Jaffee, N. Zaidi, Messenger RNA vaccines for cancer immunotherapy: progress promotes promise, *J. Clin. Invest.* 132 (2022), e156211.
- [19] A. Cagigi, K. Lore, Immune responses induced by mRNA vaccination in mice, monkeys and humans, *Vaccines* 9 (61–14) (2021) 61.
- [20] M.J. Bevan, Helping the CD8(+) T-cell response, *Nat. Rev. Immunol.* 4 (2004) 595–602.
- [21] L.M. Kranz, M. Diken, H. Haas, S. Kreiter, C. Loquai, K.C. Reuter, M. Meng, D. Fritz, F. Vascotto, H. Hefesha, C. Grunwitz, M. Vormehr, Y. Husemann, A. Selmi, A.N. Kuhn, J. Buck, E. Derhovanessian, R. Rae, S. Attig, J. Diekmann, R. A. Jabulowsky, S. Heesch, J. Hassel, P. Langguth, S. Grabbe, C. Huber, O. Tureci, U. Sahin, Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy, *Nature* 534 (2016) 396–401.
- [22] L. Miao, L.X. Li, Y.X. Huang, D. Delcassian, J. Chahal, J.S. Han, Y.H. Shi, K. Sadtler, W.T. Gao, J.Q. Lin, J.C. Doloff, R. Langer, D.G. Anderson, Delivery of mRNA vaccines with heterocyclic lipids increases anti-tumor efficacy by STING-mediated immune cell activation, *Nat. Biotechnol.* 37 (2019) 1174–1185.
- [23] U. Sahin, P. Oehm, E. Derhovanessian, R.A. Jabulowsky, M. Vormehr, M. Gold, D. Maurus, D. Schwarck-Kokarakis, A.N. Kuhn, T. Omokoko, L.M. Kranz, M. Diken, S. Kreiter, H. Haas, S. Attig, R. Rae, K. Cuk, A. Kemmer-Bruck, A. Breitkreuz, C. Tolliver, J. Caspar, J. Quinkhardt, L. Hebich, M. Stein, A. Hohberger, I. Vogler, I. Liebig, S. Renken, J. Sikorski, M. Leierer, V. Muller, H. Mittel-Rink, M. Miederer, C. Huber, S. Grabbe, J. Utikal, A. Pinter, R. Kaufmann, J.C. Hassel, C. Loquai, O. Tureci, An RNA vaccine drives immunity in checkpoint-inhibitor-treated melanoma, *Nature* 585 (2020) 107.
- [24] Y. Li, X.T. Ma, Y.L. Yue, K.Y. Zhang, K.M. Cheng, Q.Q. Feng, N.N. Ma, J. Liang, T. J. Zhang, L.Z. Zhang, Z.Q. Chen, X.W. Wang, L. Ren, X. Zhao, G.J. Nie, Rapid surface display of mRNA antigens by bacteria-derived outer membrane vesicles for a personalized tumor vaccine, *Adv. Mater.* 34 (2022), e2109984.
- [25] H.X. Zhang, X.R. You, X.J. Wang, L. Cui, Z.N. Wang, F.F. Xu, M.Y. Li, Z.G. Yang, J. Y. Liu, P. Huang, Y. Kang, J. Wu, X.J. Xia, Delivery of mRNA vaccine with a lipid-like material potentiates antitumor efficacy through Toll-like receptor 4 signaling, *P Natl Acad Sci USA* 118 (2021), e2005191118.
- [26] J.Q. Liu, C.X. Zhang, X.F. Zhang, J.Y. Yan, C.X. Zeng, F. Talebian, K. Lynch, W. Y. Zhao, X.C. Hou, S. Du, D.D. Kang, B.B. Deng, D.W. McComb, X.F. Bai, Y. Z. Dong, Intratumoral delivery of IL-12 and IL-27 mRNA using lipid nanoparticles for cancer immunotherapy, *J. Contr. Release* 345 (2022) 306–313.
- [27] A.Y. Hu, J.M. Zhu, C.X. Zeng, C.H. Lin, J.Y. Yu, J.Q. Liu, K. Lynch, F. Talebian, X. L. Pan, J.Y. Yan, Y.Z. Dong, Z.H. Li, X.F. Bai, IL-27 induces CCL5 production by T lymphocytes, which contributes to antitumor activity, *J. Immunol.* 208 (2022) 2239–2245.
- [28] C.R. Stadler, H. Bahr-Mahmud, L. Celik, B. Hebich, A.S. Roth, R.P. Roth, K. Kariko, O. Tureci, U. Sahin, Elimination of large tumors in mice by mRNA-encoded bispecific antibodies, *Nat. Med.* 23 (2017) 815–817.
- [29] L.P. Wu, W.W. Wang, J.L. Tian, C.R. Qi, Z.X. Cai, W.H. Yan, S.A. Xuan, A. Q. Shang, Intravenous delivery of RNA encoding anti-PD-1 human monoclonal antibody for treating intestinal cancer, *J. Cancer* 13 (2022) 579–588.
- [30] Y. Rybakova, P.S. Kowalski, Y.X. Huang, J.T. Gonzalez, M.W. Heartlein, F. DeRosa, D. Delcassian, D.G. Anderson, mRNA delivery for therapeutic anti-HER2 antibody expression in vivo, *Mol. Ther.* 27 (2019) 1415–1423.
- [31] F. Zhang, N.N. Parayath, C.I. Ene, S.B. Stephan, A.L. Koehne, M.E. Coon, E. C. Holland, M.T. Stephan, Genetic programming of macrophages to perform anti-tumor functions using targeted mRNA nanocarriers, *Nat. Commun.* 10 (2019) 3974.
- [32] N.N. Parayath, S.B. Stephan, A.L. Koehne, P.S. Nelson, M.T. Stephan, In vitro-transcribed antigen receptor mRNA nanocarriers for transient expression in circulating T cells in vivo, *Nat. Commun.* 11 (2020) 6080.
- [33] H.F. Moffett, M.E. Coon, S. Radtke, S.B. Stephan, L. McKnight, A. Lambert, B. L. Stoddard, H.P. Kiem, M.T. Stephan, Hit-and-run programming of therapeutic cytoreagents using mRNA nanocarriers, *Nat. Commun.* 8 (2017).
- [34] K. Reinhard, B. Rengstl, P. Oehm, K. Michel, A. Billmeier, N. Hayduk, O. Klein, K. Kuna, Y. Ouchan, S. Woll, E. Christ, D. Weber, M. Suchan, T. Bukur, M. Birtel, V. Jahndel, K. Mroz, K. Hobohm, L. Kranz, M. Diken, K. Kuhlcke, O. Tureci, U. Sahin, An RNA vaccine drives expansion and efficacy of claudin-CAR-T cells against solid tumors, *Science* 367 (2020) 446–453.
- [35] Y.L. Xiao, J. Chen, H. Zhou, X.D. Zeng, Z.P. Ruan, Z.Y. Pu, X.Y. Jiang, A. Matsui, L.L. Zhu, Z. Amoozgar, D.S. Chen, X.F. Han, D.G. Duda, J.J. Shi, Combining p53

- mRNA nanotherapy with immune checkpoint blockade reprograms the immune microenvironment for effective cancer therapy, *Nat. Commun.* 13 (2022).
- [36] S. Brenner, F. Jacob, M. Meselson, An Unstable Intermediate Carrying Information from Genes to Ribosomes for Protein Synthesis 190 (1961) 576–581.
- [37] R.E. Lockard, J.B. Lingrel, The synthesis of mouse hemoglobin beta-chains in a rabbit reticulocyte cell-free system programmed with mouse reticulocyte 9S RNA, *Biochem. Biophys. Res. Commun.* 37 (1969) 204–212.
- [38] J.B. Gurdon, C.D. Lane, H.R. Woodland, G. Marbaix, Use of Frog Eggs and Oocytes for the Study of Messenger RNA and its Translation in Living Cells, 233, 1971, pp. 177–182.
- [39] P.A. Krieg, D.A. Melton, Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs, *Nucleic Acids Res.* 12 (1984) 7057–7070.
- [40] G. Krupp, RNA synthesis: strategies for the use of bacteriophage RNA polymerases, *Gene* 72 (1988) 75–89.
- [41] B.M. Paschal, L.A. McReynolds, C.J. Noren, N.M. Nichols, RNA Polymerases, *Current Protocols in Molecular Biology* 8, 2008 (Chapter 3).
- [42] S. Muthukrishnan, G.W. Both, Y. Furuchi, A.J. Shatkin, 5'-Terminal 7-methyl-guanosine in Eukaryotic mRNA Is Required for Translation 255, 1975, pp. 33–37.
- [43] A. Isaacs, R.A. Cox, Z. Rotem, Foreign nucleic acids as the stimulus to make interferon, *Lancet (London, England)* 2 (1963) 113–116.
- [44] M. Yoneyama, M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira, T. Fujita, The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses, *Nat. Immunol.* 5 (2004) 730–737.
- [45] A.C. Gingras, B. Raught, N. Sonenberg, eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation, *Annu. Rev. Biochem.* 68 (1999) 913–963.
- [46] F. Muttach, N. Muthmann, A. Rentmeister, Synthetic mRNA capping, *Beilstein J. Org. Chem.* 13 (2017) 2819–2832.
- [47] J. Stepinski, C. Waddell, R. Stolarski, E. Darzynkiewicz, R.E. Rhoads, Synthesis and properties of mRNAs containing the novel "anti-reverse" cap analogs 7-methyl(3'-O-methyl)GpppG and 7-methyl (3'-deoxy)GpppG, *RNA* 7 (2001) 1486–1495.
- [48] N. Elango, S. Elango, P. Shivshankar, M.S. Katz, Optimized transfection of mRNA transcribed from a d(A/T)(100) tail-containing vector, *Biochem Bioph Res Co* 330 (2005) 958–966.
- [49] J. Peng, D.R. Schoenberg, mRNA with a <20-nt poly(A) tail imparted by the poly(A)-limiting element is translated as efficiently *in vivo* as long poly(A) mRNA, *RNA* 11 (2005) 1131–1140.
- [50] S. Holtkamp, S. Kreiter, A. Selmi, P. Simon, M. Koslowski, C. Huber, O. Tureci, U. Sahin, Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells, *Blood* 108 (2006) 4009–4017.
- [51] A. Heine, S. Juranek, P. Brossart, Clinical and immunological effects of mRNA vaccines in malignant diseases, *Mol. Cancer* 20 (2021) 52.
- [52] D. Weissman, mRNA transcript therapy, *Expert Rev. Vaccines* 14 (2015) 265–281.
- [53] S. Vagner, B. Galy, S. Pyronnet, Irresistible IRES - attracting the translation machinery to internal ribosome entry sites, *EMBO Rep.* 2 (2001) 893–898.
- [54] K. Leppke, R. Das, M. Barna, Functional 5' UTR mRNA structures in eukaryotic translation regulation and how to find them, *Nat. Rev. Mol. Cell Biol.* 19 (2018) 158–174.
- [55] P. Gaspar, G. Moura, M.A.S. Santos, J.L. Oliveira, mRNA secondary structure optimization using a correlated stem-loop prediction, *Nucleic Acids Res.* 41 (2013) e73.
- [56] S. Linares-Fernandez, C. Lacroix, J.Y. Exposito, B. Verrier, Tailoring mRNA vaccine to balance innate/adaptive immune response, *Trends Mol. Med.* 26 (2020) 311–323.
- [57] J. Guhaniyogi, G. Brewer, Regulation of mRNA stability in mammalian cells, *Gene* 265 (2001) 11–23.
- [58] Y. Jiang, X.S. Xu, J.E. Russell, A nucleolin-binding 3' untranslated region element stabilizes beta-globin mRNA *in vivo*, *Mol. Cell Biol.* 26 (2006) 2419–2429.
- [59] G. Kudla, L. Lipinski, F. Cuffin, A. Helwak, M. Zyllicz, High guanine and cytosine content increases mRNA levels in mammalian cells, *PLoS Biol.* 4 (2006) 933–942.
- [60] A.B. Al-Hawash, X.Y. Zhang, F.Y. Ma, Strategies of codon optimization for high-level heterologous protein expression in microbial expression systems, *Gene Rep* 9 (2017) 46–53.
- [61] X.W. Yan, T.A. Hoek, R.D. Vale, M.E. Tanenbaum, Dynamics of translation of single mRNA molecules *in vivo*, *Cell* 165 (2016) 976–989.
- [62] P.S. Spencer, E. Siller, J.F. Anderson, J.M. Barral, Silent substitutions predictably alter translation elongation rates and protein folding efficiencies, *J. Mol. Biol.* 422 (2012), 328–335.
- [63] A.F. Durbin, C. Wang, J. Marcotrigiano, L. Gehrke, RNAs containing modified nucleotides fail to trigger RIG-I conformational changes for innate immune signaling, *mBio* 7 (2016) e00833-00816.
- [64] K. Kariko, H. Muramatsu, F.A. Welsh, J. Ludwig, H. Kato, S. Akira, D. Weissman, Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability, *Mol. Ther.* 16 (2008) 1833–1840.
- [65] K. Kariko, H. Muramatsu, J.M. Keller, D. Weissman, Increased erythropoiesis in mice injected with submicrogram quantities of pseudouridine-containing mRNA encoding erythropoietin, *Mol. Ther.* 20 (2012) 948–953.
- [66] O. Andries, S. Mc Cafferty, S.C. De Smedt, R. Weiss, N.N. Sanders, T. Kitada, N-1-methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice, *J. Contr. Release* 217 (2015) 337–344.
- [67] Y.V. Svitkin, Y.M. Cheng, T. Chakraborty, V. Presnyak, M. John, N. Sonenberg, N1-methyl-pseudouridine in mRNA enhances translation through eIF2 alpha-dependent and independent mechanisms by increasing ribosome density, *Nucleic Acids Res.* 45 (2017) 6023–6036.
- [68] K. Kariko, H. Muramatsu, J. Ludwig, D. Weissman, Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA, *Nucleic Acids Res.* 39 (2011) e142.
- [69] A. Thess, S. Grund, B.L. Mui, M.J. Hope, P. Baumhof, M. Fotin-Mleczek, T. Schlake, Sequence-engineered mRNA without chemical nucleoside modifications enables an effective protein therapy in large animals, *Mol. Ther.* 23 (2015) 1456–1464.
- [70] M. Baiersdorfer, G. Boros, H. Muramatsu, A. Mahiny, I. Vlatkovic, U. Sahin, K. Kariko, A facile method for the removal of dsRNA contaminant from *in vitro* transcribed mRNA, *Mol. Ther. Nucleic Acids* 15 (2019) 26–35.
- [71] J.B. Foster, N. Choudhari, J. Perazzelli, J. Storm, T.J. Hofmann, P. Jain, P. B. Storm, N. Pardi, D. Weissman, A.J. Waanders, S.A. Grupp, K. Karik, A. C. Resnick, D.M. Barrett, Purification of mRNA encoding chimeric antigen receptor is critical for generation of a robust T-cell response, *Hum. Gene Ther.* 30 (2019) 168–178.
- [72] I. Hoerr, R. Obst, H.G. Rammensee, G. Jung, *In vivo* application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies, *Eur. J. Immunol.* 30 (2000) 1–7.
- [73] Y.H. Weng, C.H. Li, T.R. Yang, B. Hu, M.J. Zhang, S. Guo, H.H. Xiao, X.J. Liang, Y. Y. Huang, The challenge and prospect of mRNA therapeutics landscape, *Biotechnol. Adv.* 40 (2020), 107534.
- [74] L. Van Hoecke, S. Van Lint, K. Roose, A. Van Parys, P. Vandenebee, J. Grooten, J. Tavernier, S. De Koker, X. Saelens, Treatment with mRNA coding for the necroptosis mediator MLKL induces antitumor immunity directed against neoepitopes, *Nat. Commun.* 9 (2018) 3417.
- [75] Y. Bai, S.F. Kan, S.X. Zhou, Y.T. Wang, J. Xu, J.P. Cooke, J.H. Wen, H.K. Deng, Enhancement of the *in vivo* persistence and antitumor efficacy of CD19 chimeric antigen receptor T cells through the delivery of modified TERT mRNA, *Cell Discov* 1 (2015), 15040.
- [76] P. Qiu, P. Ziegelhoffer, J. Sun, N.S. Yang, Gene gun delivery of mRNA *in situ* results in efficient transgene expression and genetic immunization, *Gene Ther.* 3 (1996) 262–268.
- [77] L. Raes, S. Stremersch, J.C. Fraire, T. Brans, G. Goetgeluk, S. De Munter, L. Van Hoecke, R. Verbeke, J. Van Hoek, R.H. Xiong, X. Saelens, B. Vandekerckhove, S. De Smedt, K. Raemdonck, K. Braeckmans, Intracellular delivery of mRNA in adherent and suspension cells by vapor nanobubble photoporation, *Nano-Micro Lett.* 12 (2020) 185.
- [78] C. Hotz, T.R. Wagenaar, F. Gieseke, D.S. Bangari, M. Callahan, H. Cao, J. Diekmann, M. Diken, C. Grunwitz, A. Hebert, K. Hsu, M. Bernardo, K. Kariko, S. Kreiter, A.N. Kuhn, M. Levit, N. Malkova, S. Masciari, J. Pollard, H. Qu, S. Ryan, A. Selmi, J. Schlereth, K. Singh, F.X. Sun, B. Tillmann, T. Tolstykh, W. Weber, L. Wicke, S. Witzel, Q.Y. Yu, Y.A. Zhang, G. Zheng, J. Lager, G. J. Nabel, U. Sahin, D. Wiederschain, Local delivery of mRNA-encoding cytokines promotes antitumor immunity and tumor eradication across multiple preclinical tumor models, *Sci. Transl. Med.* 13 (2021), eabc7804.
- [79] X.J. Liu, S.G. Jiang, C.Y. Fang, H. Li, X.H. Zhang, F.Q. Zhang, C.H. June, Y. B. Zhao, Novel T cells with improved *in vivo* anti-tumor activity generated by RNA electroporation, *Protein Cell* 8 (2017) 514–526.
- [80] A.M.T. Van Nuffel, D. Bentejn, S. Wilgenhof, L. Pierret, J. Corthals, C. Heirman, P. van der Bruggen, P.G. Coulié, B. Neyns, K. Thielemans, A. Bonehill, Dendritic cells loaded with mRNA encoding full-length tumor antigens prime CD4(+) and CD8(+) T cells in melanoma patients, *Mol. Ther.* 20 (2012) 1063–1074.
- [81] G.L. Beatty, M.H. O'Hara, S.F. Lacey, D.A. Torigian, F. Nazimuddin, F. Chen, I. M. Kulikovskaya, M.C. Soulen, M. McGarvey, A.M. Nelson, W.L. Gladney, B. L. Levine, J.J. Melenhorst, G. Plesa, C.H. June, Activity of mesothelin-specific chimeric antigen receptor T cells against pancreatic carcinoma metastases in a phase 1 trial, *Gastroenterology* 155 (2018) 29–32.
- [82] V.B. Vassilev, L.H.V.G. Gil, R.O. Donis, Microparticle-mediated RNA immunization against bovine viral diarrhea virus, *Vaccine* 19 (2001) 2012–2019.
- [83] U.K. Tirlapur, K. Konig, Cell biology - targeted transfection by femtosecond laser, *Nature* 418 (2002) 290–291.
- [84] S. Van Lint, C. Goyvaerts, S. Maenhout, L. Goethals, A. Disy, D. Bentejn, J. Pen, A. Bonehill, C. Heirman, K. Breckpot, K. Thielemans, Preclinical evaluation of TriMix and antigen mRNA-based antitumor therapy, *Cancer Res.* 72 (2012) 1661–1671.
- [85] B. Weide, J.P. Carralot, A. Reese, B. Scheel, T.K. Eigentler, I. Hoerr, H. G. Rammensee, C. Garbe, S. Pascolo, Results of the first phase I/II clinical vaccination trial with direct injection of mRNA, *J. Immunother.* 31 (2008) 180–188.
- [86] L.J. Kubiatowicz, A. Mohapatra, N. Krishnan, R.H. Fang, L. Zhang, mRNA Nanomedicine: Design and Recent Applications, *Exploration*, 2022 n/a, 20210217.
- [87] M. Segel, B. Lash, J.W. Song, A. Ladha, C.C. Liu, X. Jin, S.L. Mekhedov, R. K. Macrae, E.V. Koonin, F. Zhang, Mammalian retrovirus-like protein PEG10 packages its own mRNA and can be pseudotyped for mRNA delivery, *Science* 373 (2021) 882–889.
- [88] J.C. Kaczmarek, A.K. Patel, K.J. Kauffman, O.S. Fenton, M.J. Webber, M. W. Heartlein, F. DeRosa, D.G. Anderson, Polymer-lipid nanoparticles for systemic delivery of mRNA to the lungs, *Angew. Chem., Int. Ed.* 55 (2016) 13808–13812.

- [89] W. Zhang, Y. Liu, J.M. Chin, K.K.L. Phua, Sustained release of PKR inhibitor C16 from mesoporous silica nanoparticles significantly enhances mRNA translation and anti-tumor vaccination, *Eur. J. Pharm. Biopharm.* 163 (2021) 179–187.
- [90] H. Amos, Protamine enhancement of RNA uptake by cultured chick cells 5 (1961) 1–4.
- [91] B. Weide, S. Pascolo, B. Scheel, E. Derhovanessian, A. Pflugfelder, T.K. Eigentler, G. Pawelec, I. Hoerr, H.G. Rammensee, C. Garbe, Direct injection of protamine-protected mRNA: results of a phase 1/2 vaccination trial in metastatic melanoma patients, *Journal of immunotherapy (Hagerstown, Md)* 32 (1997) 498–507, 2009.
- [92] P. Lundin, H. Johansson, P. Guterstam, T. Holm, M. Hansen, U. Langel, S.E. L. Andaloussi, Distinct uptake routes of cell-penetrating peptide conjugates, *Bioconjugate Chem.* 19 (2008) 2535–2542.
- [93] V.K. Udhayakumar, A. De Beuckelaer, J. McCaffrey, C.M. McCrudden, J. L. Kirschman, D. Vanover, L. Van Hoecke, K. Roose, K. Deswarte, B.G. De Geest, S. Lienenklaus, P.J. Santangelo, J. Grooten, H.O. McCarthy, S. De Koker, Arginine-rich peptide-based mRNA nanocomplexes efficiently instigate cytotoxic T cell immunity dependent on the amphipathic organization of the peptide, *Adv Healthc Mater* 6 (2017), 1601412.
- [94] I. Ruseska, A. Zimmer, Internalization mechanisms of cell-penetrating peptides, *Beilstein J. Nanotechnol.* 11 (2020) 101–123.
- [95] V. Rodriguez, J. Lascani, J.A. Asenjo, B.A. Andrews, Production of cell-penetrating peptides in *Escherichia coli* using an intein-mediated system, *Appl. Biochem. Biotechnol.* 175 (2015) 3025–3037.
- [96] M.O. Mohsen, L.S. Zha, G. Cabral-Miranda, M.F. Bachmann, Major findings and recent advances in virus like particle (VLP)-based vaccines, *Semin. Immunol.* 34 (2017) 123–132.
- [97] S. Jekhmene, R. de Haas, O.P.D.S. Filho, A.H. van Asbeck, M.E. Favretto, A. H. Garcia, R. Brock, R. de Vries, Virus-like particles of mRNA with artificial minimal coat proteins: particle formation, stability, and transfection efficiency, *Nucleic Acid Therapeut.* 27 (2017) 159–167.
- [98] W.Q. Li, X.F. Zhang, C.X. Zhang, J.Y. Yan, X.C. Hou, S. Du, C.X. Zeng, W.Y. Zhao, B.B. Deng, D.W. McComb, Y.B. Zhang, D.D. Kang, J.N. Li, W.E. Carson, Y.Z. Dong, Biomimetic nanoparticles deliver mRNAs encoding costimulatory receptors and enhance T cell mediated cancer immunotherapy, *Nat. Commun.* 12 (2021) 7264.
- [99] I. Yildiz, K.L. Lee, K. Chen, S. Shukla, N.F. Steinmetz, Infusion of imaging and therapeutic molecules into the plant virus-based carrier cowpea mosaic virus: cargo-loading and delivery, *J. Contr. Release* 172 (2013) 568–578.
- [100] J.M. Li, Y.L. Sun, T.T. Jia, R. Zhang, K. Zhang, L.N. Wang, Messenger RNA vaccine based on recombinant MS2 virus-like particles against prostate cancer, *Int. J. Cancer* 134 (2014) 1683–1694.
- [101] M.O. Mohsen, A.C. Gomes, M. Vogel, M.F. Bachmann, Interaction of viral capsid-derived virus-like particles (VLPs) with the innate immune system, *Vaccines-Basel* 6 (2018) 37.
- [102] G.J. Dimitriadis, Translation of Rabbit Globin mRNA Introduced by Liposomes into Mouse Lymphocytes 274, 1978, pp. 923–924.
- [103] M.J. Ostro, D. Giacomoni, D.O.N. Lavelle, W. Paxton, S. Dray, Evidence for Translation of Rabbit Globin mRNA after Liposomemediated Insertion into a Human Cell Line 274, 1978, pp. 921–923.
- [104] A.J. Geall, A. Verma, G.R. Otten, C.A. Shaw, A. Hekele, K. Banerjee, Y. Cu, C. W. Beard, L.A. Brito, T. Krucker, D.T. O'Hagan, M. Singh, P.W. Mason, N. M. Valiante, P.R. Dormitzer, S.W. Barnett, R. Rappuoli, J.B. Ulmer, C.W. Mandl, Nonviral delivery of self-amplifying RNA vaccines, *Proc. Natl. Acad. Sci. U.S.A.* 109 (2012) 14604–14609.
- [105] P.S. Kowalski, A. Rudra, L. Miao, D.G. Anderson, Delivering the messenger: advances in technologies for therapeutic mRNA delivery, *Mol. Ther.* 27 (2019), 710–728.
- [106] S. Kreiter, M. Vormehr, N. van de Roemer, M. Diken, M. Lower, J. Diekmann, S. Boegel, B. Schrors, F. Vascotto, J.C. Castle, A.D. Tadmor, S.P. Schoenberger, C. Huber, O. Tureci, U. Sahin, Mutant MHC class II epitopes drive therapeutic immune responses to cancer, *Nature* 520 (2015), 692–U269.
- [107] K.J. Kauffman, J.R. Dorkin, J.H. Yang, M.W. Heartlein, F. DeRosa, F.F. Mir, O. S. Fenton, D.G. Anderson, Optimization of lipid nanoparticle formulations for mRNA delivery in vivo with fractional factorial and definitive screening designs, *Nano Lett.* 15 (2015) 7300–7306.
- [108] M.J. Mitchell, M.M. Billingsley, R.M. Haley, M.E. Wechsler, N.A. Peppas, R. Langer, Engineering precision nanoparticles for drug delivery, *Nat. Rev. Drug Discov.* 20 (2021) 101–124.
- [109] A. Vaheri, J.S. Pagano, Infectious poliovirus RNA: a sensitive method of assay, *Virology* 27 (1965) 434–436.
- [110] T. Bus, A. Traeger, U.S. Schubert, The great escape: how cationic polyplexes overcome the endosomal barrier, *J. Mater. Chem. B* 6 (2018) 6904–6918.
- [111] S.M. Moghimi, P. Symonds, J.C. Murray, A.C. Hunter, G. Debska, A. Szczyzyk, A two-stage poly(ethylenimine)-mediated cytotoxicity: implications for gene transfer/therapy, *Mol. Ther.* 11 (2005) 990–995.
- [112] X.Y. Ke, L. Shelton, Y.Z. Hu, Y.N. Zhu, E. Chow, H.Y. Tang, J.L. Santos, H.Q. Mao, Surface-functionalized PEGylated nanoparticles deliver messenger RNA to pulmonary immune cells, *ACS Appl Mater Inter* 12 (2020) 35835–35844.
- [113] M. Breunig, U. Lungwitz, R. Liebl, A. Goepferich, Breaking up the correlation between efficacy and toxicity for nonviral gene delivery, *P Natl Acad Sci USA* 104 (2007) 14454–14459.
- [114] J.C. Kaczmarek, K.J. Kauffman, O.S. Fenton, K. Sadler, A.K. Patel, M. W. Heartlein, F. DeRosa, D.G. Anderson, Optimization of a degradable polymer-lipid nanoparticle for potent systemic delivery of mRNA to the lung endothelium and immune cells, *Nano Lett.* 18 (2018) 6449–6454.
- [115] A.K. Patel, J.C. Kaczmarek, S. Bose, K.J. Kauffman, F. Mir, M.W. Heartlein, F. DeRosa, R. Langer, D.G. Anderson, Inhaled nanoformulated mRNA polyplexes for protein production in lung epithelium, *Adv. Mater.* 31 (2019), e1805116.
- [116] H.J. Kim, S. Ogura, T. Otabe, R. Kamegawa, M. Sato, K. Kataoka, K. Miyata, Fine-tuning of hydrophobicity in amphiphilic polyaspartamide derivatives for rapid and transient expression of messenger RNA directed toward genome engineering in brain, *Acs Central Sci* 5 (2019) 1866–1875.
- [117] Y.H. Wang, H.H. Su, Y. Yang, Y.X. Hu, L. Zhang, P. Blancafort, L. Huang, Systemic delivery of modified mRNA encoding herpes simplex virus 1 thymidine kinase for targeted cancer gene therapy, *Mol. Ther.* 21 (2013) 358–367.
- [118] N. Kong, R.N. Zhang, G.W. Wu, X.B. Sui, J.Q. Wang, N.Y. Kim, S. Blake, D.B. De, T. Xie, Y.H. Cao, W. Tao, Intravesical delivery of KDM6A-mRNA via mucoadhesive nanoparticles inhibits the metastasis of bladder cancer, *P Natl Acad Sci USA* 119 (2022), e2112696119.
- [119] J.N. Yang, S. Arya, P.S. Lung, Q.B. Lin, J.D. Huang, Q. Li, Hybrid nanovaccine for the co-delivery of the mRNA antigen and adjuvant, *Nanoscale* 11 (2019) 21782–21789.
- [120] S.B. Lei, X.Y. Zhang, K. Men, Y. Gao, X.J. Yang, S.S. Wu, X.M. Duan, Y.Q. Wei, R. S. Tong, Efficient colorectal cancer gene therapy with IL-15 mRNA nanoformulation, *Mol. Pharm.* 17 (2020) 3378–3391.
- [121] R. Zhang, K. Men, X.Y. Zhang, R. Huang, Y.M. Tian, B.L. Zhou, C.H. Yu, Y. T. Wang, X. Ji, Q.Y. Hu, L. Yang, Delivery of a modified mRNA encoding IL-22 binding protein (IL-22BP) for colon cancer gene therapy, *J. Biomed. Nanotechnol.* 14 (2018) 1239–1251.
- [122] F. Maiyo, M. Singh, Folate-targeted mRNA delivery using chitosan-functionalized selenium nanoparticles: potential in cancer immunotherapy, *Pharmaceut. Biol.* 12 (2019) 164.
- [123] L.S. Mbatha, F. Maiyo, A. Daniels, M. Singh, Dendrimer-coated gold nanoparticles for efficient folate-targeted mRNA delivery in vitro, *Pharmaceutics* 13 (2021) 900.
- [124] L.N. Liu, Y.H. Wang, L. Miao, Q. Liu, S. Musetti, J. Li, L. Huang, Combination immunotherapy of MUC1 mRNA nano-vaccine and CTLA-4 blockade effectively inhibits growth of triple negative breast cancer, *Mol. Ther.* 26 (2018) 45–55.
- [125] Z. Zou, L.B. He, X.X. Deng, H.X. Wang, Z.Y. Huang, Q. Xue, Z.H. Qing, Y.L. Lei, R. H. Yang, J.W. Liu, Zn²⁺-Coordination-Driven RNA assembly with retained integrity and biological functions, *Angew. Chem., Int. Ed.* 60 (2021) 22970–22976.
- [126] M. Saxena, S.H. van der Burg, C.J.M. Melief, N. Bhardwaj, Therapeutic cancer vaccines 21 (2021) 360–378.
- [127] S.H. van der Burg, R. Arens, F. Ossendorp, T. van Hall, A.J.M. Melief, Vaccines for established cancer: overcoming the challenges posed by immune evasion, *Nat. Rev. Cancer* 16 (2016) 219–233.
- [128] N. Pishesha, T.J. Harmand, H.L. Ploegh, A guide to antigen processing and presentation, *Nat. Rev. Immunol.* (2022), <https://doi.org/10.1038/s41577-41022-00707-41572>.
- [129] S.K. Wculek, F.J. Cueto, A.M. Mujal, I. Melero, M.F. Krummel, D. Sancho, Dendritic cells in cancer immunology and immunotherapy, *Nat. Rev. Immunol.* 20 (2020) 7–24.
- [130] A. Bonehill, C. Heirman, S. Tuyaerts, A. Michiels, K. Breckpot, F. Brasseur, Y. Zhang, P. van der Bruggen, K. Thielemans, Messenger RNA-electroporated dendritic cells presenting MAGE-A3 simultaneously in HLA class I and class II molecules, *J. Immunol.* 172 (2004) 6649–6657.
- [131] F. Pastor, P. Berraondo, I. Etxeberria, J. Frederick, U. Sahin, E. Gilboa, I. Melero, An RNA toolbox for cancer immunotherapy, *Nat. Rev. Drug Discov.* 17 (2018) 751–767.
- [132] K. Van der Jeught, S. De Koker, L. Bialkowski, C. Heirman, P.T. Joe, F. Perche, S. Menaout, S. Bevers, K. Broos, K. Deswarte, V. Malard, H. Hamad, P. Baril, T. Benvegnu, P.A. Jaffres, S.A.A. Kooijmans, R. Schifflers, S. Lienenklaus, P. Midoux, C. Pichon, K. Breckpot, K. Thielemans, Dendritic cell targeting mRNA lipopolyplexes combine strong antitumor T-cell immunity with improved inflammatory safety, *ACS Nano* 12 (2018) 9815–9829.
- [133] S.L. Hewitt, D. Bailey, J. Zielinski, A. Apte, F. Musenge, R. Karp, S. Burke, F. Garcon, A. Mishra, S. Gurumurthy, A. Watkins, K. Arnold, J. Moynihan, E. Clancy-Thompson, K. Mulgrew, G. Adjei, K. Deschler, D. Potz, G. Moody, D. A. Leinster, S. Novick, M. Sulikowski, C. Bagnall, P. Martin, J.M. Lapointe, H. Si, C. Morehouse, M. Sedic, R.W. Wilkinson, R. Herbst, J.P. Frederick, N. Luheshi, Intratumoral IL12 mRNA therapy promotes TH1 transformation of the tumor microenvironment, *Clin. Cancer Res.* 26 (2020) 6284–6298.
- [134] K.F. Bol, G. Schreibelt, W.R. Gerritsen, I.J.M. de Vries, C.G. Figdor, Dendritic cell-based immunotherapy: state of the art and beyond, *Clin. Cancer Res.* 22 (2016) 1897–1906.
- [135] A. Harari, M. Graciotti, M. Bassani-Sternberg, L.E. Kandalaf, Antitumor dendritic cell vaccination in a priming and boosting approach, *Nat. Rev. Drug Discov.* 19 (2020) 635–652.
- [136] D. Boczkowski, S.K. Nair, D. Snyder, E. Gilboa, Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo, *J. Exp. Med.* 184 (1996) 465–472.
- [137] A.J. Grippin, B. Wummer, T. Wildes, K. Dyson, V. Triyedi, C.L. Yang, M. Sebastian, H.R. Mendez-Gomez, S. Padala, M. Grubb, M. Fillingim, A. Monsalve, E.J. Sayour, J. Dobson, D.A. Mitchell, Dendritic cell-activating magnetic nanoparticles enable early prediction of antitumor response with magnetic resonance imaging, *ACS Nano* 13 (2019) 13884–13898.
- [138] N. Tateshita, N. Miura, H. Tanaka, T. Masuda, S. Ohtsuki, K. Tange, Y. Nakai, H. Yoshioka, H. Akita, Development of a lipoplex-type mRNA carrier composed of an ionizable lipid with a vitamin E scaffold and the KALA peptide for use as an ex vivo dendritic cell-based cancer vaccine, *J. Contr. Release* 310 (2019) 36–46.

- [139] S. Daneshmandi, A.A. Pourfathollah, M. Forouzandeh-Moghaddam, Enhanced CD40 and ICOSL expression on dendritic cells surface improve anti-tumor immune responses; effectiveness of mRNA/chitosan nanoparticles, *Immunopharmacol. Immunotoxicol.* 40 (2018) 375–386.
- [140] S.S. Neelapu, F.L. Locke, N.L. Bartlett, L.J. Lekakis, D.B. Miklos, C.A. Jacobson, I. Braunschweig, O.O. Oluwole, T. Siddiqi, Y. Lin, J.M. Timmerman, P.J. Stiff, J. W. Friedberg, I.W. Flinn, A. Goy, B.T. Hill, M.R. Smith, A. Deol, U. Farooq, P. McSweeney, J. Munoz, I. Avivi, J.E. Castro, J.R. Westin, J.C. Chavez, A. Ghobadi, K.V. Komanduri, R. Levy, E.D. Jacobsen, T.E. Witzig, P. Reagan, A. Bot, J. Rossi, L. Navale, Y. Jiang, J. Aycock, M. Elias, D. Chang, J. Wieszorek, W. Y. Go, Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma, *N. Engl. J. Med.* 377 (2017) 2531–2544.
- [141] S.L. Maude, T.W. Laetsch, J. Buechner, S. Rives, M. Boyer, H. Bittencourt, P. Bader, M.R. Verneris, H.E. Stefanski, G.D. Myers, M. Qayed, B. De Moerloose, H. Hiramatsu, K. Schlis, K.L. Davis, P.L. Martin, E.R. Nemecek, G.A. Yanik, C. Peters, A. Baruchel, N. Boissel, F. Mechinaud, A. Balduzzi, J. Krueger, C. H. June, B.L. Levine, P. Wood, T. Taran, M. Leung, K.T. Mueller, Y. Zhang, K. Sen, D. Lebowitz, M.A. Pulsipher, S.A. Grupp, Tisagenlecleucel in children and Young adults with B-cell lymphoblastic leukemia, *N. Engl. J. Med.* 378 (2018) 439–448.
- [142] I. Scarfo, M.V. Maus, Current approaches to increase CAR T cell potency in solid tumors: targeting the tumor microenvironment, *J Immunother Cancer* 5 (2017) 28.
- [143] C. Schwachheimer, M.J. Kuehn, Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions, *Nat. Rev. Microbiol.* 13 (2015) 605–619.
- [144] O.Y. Kim, H.T. Park, N.T.H. Dinh, S.J. Choi, J. Lee, J.H. Kim, S.W. Lee, Y.S. Gho, Bacterial outer membrane vesicles suppress tumor by interferon-gamma-mediated antitumor response, *Nat. Commun.* 8 (2017) 626.
- [145] Y. Yin, X.Y. Li, H.X. Ma, J. Zhang, D. Yu, R.F. Zhao, S.J. Yu, G.J. Nie, H. Wang, In situ transforming RNA nanovaccines from polyethyleneimine functionalized graphene oxide hydrogel for durable cancer immunotherapy, *Nano Lett.* 21 (2021) 2224–2231.
- [146] M.A. Oberli, A.M. Reichmuth, J.R. Dorkin, M.J. Mitchell, O.S. Fenton, A. Jaklencic, D.G. Anderson, R. Langer, D. Blankschtein, Lipid nanoparticle assisted mRNA delivery for potent cancer immunotherapy, *Nano Lett.* 17 (2017) 1326–1335.
- [147] M.A. Islam, J. Rice, E. Reesor, H. Zope, W. Tao, M. Lim, J.X. Ding, Y.H. Chen, D. Aduloso, B.R. Zetter, O.C. Farokhzad, J.J. Shi, Adjuvant-pulsed mRNA vaccine nanoparticle for immunoprophylactic and therapeutic tumor suppression in mice, *Biomaterials* 266 (2021), 120431.
- [148] S. Jensen, A.R. Thomsen, Sensing of RNA viruses: a review of innate immune receptors involved in recognizing RNA virus invasion, *J. Virol.* 86 (2012) 2900–2910.
- [149] U. Sahin, K. Kariko, O. Tureci, mRNA-based therapeutics - developing a new class of drugs, *Nat. Rev. Drug Discov.* 13 (2014) 759–780.
- [150] A. De Beuckelaer, J. Grooten, S. De Koker, Type I interferons modulate CD8+, T Cell Immunity to mRNA Vaccines 23 (2017) 216–226.
- [151] A. Gutjahr, G. Tiraby, E. Perouzel, B. Verrier, S. Paul, Triggering intracellular receptors for vaccine adjuvantation (vol 37, pg 573, 2016), *Trends Immunol.* 37 (2016), 716–716.
- [152] D.B. Stetson, R. Medzhitov, Recognition of cytosolic DNA activates an IRF3-dependent innate immune response, *Immunity* 24 (2006) 93–103.
- [153] S. Crunkhorn, Strengthening the sting of immunotherapy, *Nat. Rev. Drug Discov.* 19 (2020), 669–669.
- [154] N. Jeremiah, B. Neven, M. Gentili, I. Callebaut, S. Maschalidi, M.C. Stolzenberg, N. Goudin, M.L. Fremont, P. Nitschke, T.J. Molina, S. Blanche, C. Picard, G. I. Rice, Y.J. Crow, N. Manel, A. Fischer, B. Bader-Meunier, F. Rieux-Laucat, Inherited STING-activating mutation underlies a familial inflammatory syndrome with lupus-like manifestations, *J. Clin. Invest.* 124 (2014) 5516–5520.
- [155] S.W. Tse, K. McKinney, W. Walker, M. Nguyen, J. Iacovelli, C. Small, K. Hopson, T. Zaks, E. Huang, mRNA-encoded, constitutively active STING(V155M) is a potent genetic adjuvant of antigen-specific CD8(+) T cell response, *Mol. Ther.* 29 (2021) 2227–2238.
- [156] F. Borriello, V. Poli, E. Shrock, R. Spreafico, X. Liu, N. Pishesha, C. Carpenet, J. Chou, M. Di Gioia, M.E. McGrath, C.A. Dillen, N.A. Barrett, L. Lacanfora, M. E. Franco, L. Marongiu, Y. Iwakura, F. Pucci, M.D. Kruppa, Z.C. Ma, D. W. Lowman, H.E. Ensley, E. Nanishi, Y. Saito, T.R. O'Meara, H.S. Seo, S. Dhe-Paganon, D.J. Dowling, M. Frieman, S.J. Elledge, O. Levy, D.J. Irvine, H. L. Ploegh, D.L. Williams, I. Zanoni, An adjuvant strategy enabled by modulation of the physical properties of microbial ligands expands antigen immunogenicity, *Cell* 185 (2022) 614–629.
- [157] C.J. Qi, Y.H. Cai, L. Gunn, C.L. Ding, B. Li, G. Kloecker, K.Q. Qian, J. Vasilakos, S. Saijo, Y. Iwakura, J.R. Yannelli, J. Yan, Differential pathways regulating innate and adaptive antitumor immune responses by particulate and soluble yeast-derived beta-glucans, *Blood* 117 (2011) 6825–6836.
- [158] S. Son, J. Nam, I. Zenkov, L.J. Ochyil, Y. Xu, L. Scheetz, J.J. Shi, O.C. Farokhzad, J. Moon, Sugar-nanocapsules imprinted with microbial molecular patterns for mRNA vaccination, *Nano Lett.* 20 (2020) 1499–1509.
- [159] R. Verbeke, I. Lentacker, K. Breckpot, J. Janssens, S. Van Calenbergh, S.C. De Smedt, H. Dewitte, Broadening the message: a nanovaccine Co-loaded with messenger RNA and alpha-GalCer induces antitumor immunity through conventional and natural killer T cells, *ACS Nano* 13 (2019) 1655–1669.
- [160] E.J. Sayour, A. Grippin, G. De Leon, B. Stoyer, M. Rahman, A. Karachi, B. Wummer, G. Moore, P. Castillo-Caro, K. Fredenburg, M.R. Sarkisian, J. P. Huang, L.P. Deleyrolle, B. Sahay, S. Carrera-Justiz, H.R. Mendez-Gomez, D. A. Mitchell, Personalized tumor RNA loaded lipid-nanoparticles prime the systemic and intratumoral milieu for response to cancer immunotherapy, *Nano Lett.* 18 (2018) 6195–6206.
- [161] N. Salomon, F. Vascotto, A. Selmi, M. Vormehr, J. Quinkhardt, T. Bukur, B. Schrors, M. Loewer, M. Diken, O. Tureci, U. Sahin, S. Kreiter, A liposomal RNA vaccine inducing neoantigen-specific CD4(+)T cells augments the antitumor activity of local radiotherapy in mice, *Oncolimmunology* 9 (2020), 1771925.
- [162] N. Salomon, A. Selmi, C. Grunwicz, A. Kong, E. Stanganello, J. Neumaier, J. Petschenka, M. Diken, S. Kreiter, O. Tureci, U. Sahin, F. Vascotto, Local radiotherapy and E7 RNA-LPX vaccination show enhanced therapeutic efficacy in preclinical models of HPV16(+) cancer, *Cancer Immunol. Immunother.* 71 (2022) 1975–1988.
- [163] L. Basler, A. Kowalczyk, R. Heidenreich, M. Fotin-Mleczek, S. Tseitsekidis, D. Zips, F. Eckert, S.M. Huber, Abscopal effects of radiotherapy and combined mRNA-based immunotherapy in a syngeneic, OVA-expressing thymoma mouse model, *Cancer Immunol. Immunother.* 67 (2018) 653–662.
- [164] C. Grunwicz, N. Salomon, F. Vascotto, A. Selmi, T. Bukur, M. Diken, S. Kreiter, O. Tureci, U. Sahin, HPV16 RNA-LPX vaccine mediates complete regression of aggressively growing HPV-positive mouse tumors and establishes protective T cell memory, *Oncolimmunology* 8 (2019), e1629259.
- [165] A.D. Waldman, J.M. Fritz, M.J. Lenardo, A guide to cancer immunotherapy: from T cell basic science to clinical practice, *Nat. Rev. Immunol.* 20 (2020) 651–668.
- [166] G.Z. Xie, H. Dong, Y. Liang, J.D. Ham, R. Rizwan, J.Z. Chen, CAR-NK cells: a promising cellular immunotherapy for cancer, *EBioMedicine* 59 (2020), 102975.
- [167] L.E. Budde, S. Assouline, L.H. Sehn, S.J. Schuster, S.S. Yoon, D.H. Yoon, M. J. Matasar, F. Bosch, W.S. Kim, L.J. Nastoupil, I.W. Flinn, M. Shadman, C. Diefenbach, C. O'Hear, H. Huang, A. Kwan, C.C. Li, E.C. Piccione, M.C. Wei, S. Yin, N.L. Bartlett, Single-agent mosunetuzumab shows durable complete responses in patients with relapsed or refractory B-cell lymphomas: phase I dose-escalation study, *J. Clin. Oncol. : official journal of the American Society of Clinical Oncology* 40 (2022) 481–491.
- [168] E. Seung, Z. Xing, L. Wu, E.R. Rao, V. Cortez-Retamozo, B. Ospina, L.Q. Chen, C. Beil, Z.L. Song, B.L. Zhang, M. Levit, G.J. Deng, A. Hebert, P. Kirby, A.Q. Li, E. J. Poulton, R. Vicente, A. Garrigou, P. Piephagen, G. Ulinski, M. Sanicola-Nadel, D.S. Bangari, H.W. Qiu, L. Pao, D. Wiederschain, R.N. Wei, Z.Y. Yang, G. J. Nabel, A trispecific antibody targeting HER2 and T cells inhibits breast cancer growth via CD4 cells, *Nature* 603 (2022) 328–334.
- [169] I. Melero, W.W. Shuford, S.A. Newby, A. Aruffo, J.A. Ledbetter, K.E. Hellstrom, R. S. Mittler, L.P. Chen, Monoclonal antibodies against the 4-1BB/T cell activation molecule eradicate established tumors, *Nat. Med.* 3 (1997) 682–685.
- [170] A.W. Tolcher, M. Sznol, S. Hu-Lieskovan, K.P. Papadopoulos, A. Patnaik, D. Rasco, D. Di Gravio, B. Huang, D. Gambhire, Y. Chen, A.D. Thall, N. Pathan, E. V. Schmidt, L.Q.M. Chow, Phase Ib study of Utomilumab (PF-05082566), a 4-1BB/CD137 agonist, in combination with pembrolizumab (MK-3475) in patients with advanced solid tumors, *Clin. Cancer Res.* 23 (2017) 5349–5357.
- [171] N.H. Segal, T.F. Logan, F.S. Hodi, D. McDermott, I. Melero, O. Hamid, H. Schmidt, C. Robert, V. Chiarion-Sileni, P.A. Ascierto, M. Maio, W.J. Urba, T.C. Gangadhar, S. Suryawanshi, J. Neely, M. Jure-Kunkel, S. Krishnan, H. Kohrt, M. Sznol, R. Levy, Results from an integrated safety analysis of Urelumab, an agonist anti-CD137 monoclonal antibody, *Clin. Cancer Res.* 23 (2017) 1929–1936.
- [172] K. Garber, Immune agonist antibodies face critical test, *Nat. Rev. Drug Discov.* 19 (2020) 3–5.
- [173] S.L. Hewitt, A. Bai, D. Bailey, K. Ichikawa, J. Zielinski, R. Karp, A. Apte, K. Arnold, S.J. Zacharek, M.S. Iliou, K. Bhatt, M. Garnaas, F. Musenge, A. Davis, N. Khatwani, S.V. Su, G. MacLean, S.J. Farlow, K. Burke, J.P. Frederick, Durable anticancer immunity from intratumoral administration of IL-23, IL-36 gamma, and OX40L mRNAs, *Sci. Transl. Med.* 11 (2019), eaat9143.
- [174] C.A. Hudis, Drug therapy: trastuzumab - Mechanism of action and use in clinical practice, *N. Engl. J. Med.* 357 (2007) 39–51.
- [175] M. Thran, J. Mukherjee, M. Ponisch, K. Fiedler, A. Thess, B.L. Mui, M.J. Hope, Y. K. Tam, N. Horscroft, R. Heidenreich, M. Fotin-Mleczek, C.B. Shoemaker, T. Schlake, mRNA mediates passive vaccination against infectious agents, toxins, and tumors, *EMBO Mol. Med.* 9 (2017) 1434–1447.
- [176] P. Berraondo, M.F. Sanmamed, M.C. Ochoa, I. Etxeberria, M.A. Aznar, J.L. Perez-Gracia, M.E. Rodriguez-Ruiz, M. Ponz-Sarvisse, E. Castanon, I. Melero, Cytokines in clinical cancer immunotherapy, *Br. J. Cancer* 120 (2019) 6–15.
- [177] J.P. Leonard, M.L. Sherman, G.L. Fisher, L.J. Buchanan, G. Larsen, M.B. Atkins, J. A. Sosman, J.P. Dutcher, N.J. Vogelzang, J.L. Ryan, Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production, *Blood* 90 (1997) 2541–2548.
- [178] D.R. Coman, R.P. deLong, M. McCutcheon, Studies on the mechanisms of metastasis. The distribution of tumors in various organs in relation to the distribution of arterial emboli, *Cancer Res.* 11 (1951) 648–651.
- [179] Y.X. Lin, Y. Wang, J.X. Ding, A.P. Jiang, J. Wang, M.A. Yu, S. Blake, S.S. Liu, C. J. Bieberich, O.C. Farokhzad, L. Mei, H. Wang, J.J. Shi, Reactivation of the tumor suppressor PTEN by mRNA nanoparticles enhances antitumor immunity in preclinical models, *Sci. Transl. Med.* 13 (2021), eaba9772.
- [180] R.A. Meyer, S.Y. Neshat, J.J. Green, J.L. Santos, A.D. Tuesca, Targeting strategies for mRNA delivery 14 (2022), 100240.
- [181] F. Barbero, L. Russo, M. Vitali, J. Piella, I. Salvo, M.L. Borrajo, M. Busquets-Fité, R. Grandori, N.G. Bastús, E. Casals, V. Puentes, formation of the protein corona: the interface between nanoparticles and the immune system, *Semin. Immunol.* 34 (2017) 52–60.
- [182] P.R. Cullis, M.J. Hope, Lipid nanoparticle systems for enabling gene therapies, *Mol. Ther.* 25 (2017) 1467–1475.
- [183] S. Patel, J. Kim, M. Herrera, A. Mukherjee, A.V. Kabanov, G. Sahay, Brief update on endocytosis of nanomedicines, *Adv. Drug Deliv. Rev.* 144 (2019) 90–111.

- [184] I. Lai, S. Swaminathan, V. Baylot, A. Mosley, R. Dhanasekaran, M. Gabay, D. W. Felsher, Lipid nanoparticles that deliver IL-12 messenger RNA suppress tumorigenesis in MYC oncogene-driven hepatocellular carcinoma, *J Immunother Cancer* 6 (2018) 125.
- [185] O.A.W. Haabeth, T.R. Blake, C.J. McKinlay, A.A. Tveita, A. Sallets, R. M. Waymouth, P.A. Wender, R. Levy, Local delivery of Ox40L, Cd80, and Cd86 mRNA kindles global anticancer immunity, *Cancer Res.* 79 (2019) 1624–1634.
- [186] S. George, D. Miao, G.D. Demetri, D. Adeegbe, S.J. Rodig, S. Shukla, M. Lipschitz, A. Amin-Mansour, C.P. Raut, S.L. Carter, P. Hammerman, G.J. Freeman, C.J. Wu, P.A. Ott, K.K. Wong, E.M. Van Allen, Loss of PTEN is associated with resistance to anti-PD-1 checkpoint blockade therapy in metastatic uterine leiomyosarcoma, *Immunity* 46 (2017) 197–204.
- [187] X.Y. Zhang, K. Men, Y.F. Zhang, R. Zhang, L. Yang, X.M. Duan, Local and systemic delivery of mRNA encoding survivin-T34A by lipoplex for efficient colon cancer gene therapy, *Int. J. Nanomed.* 14 (2019) 2733–2751.
- [188] D.B. Johnson, C.A. Nebhan, J.J. Moslehi, J.M. Balko, Immune-checkpoint inhibitors: long-term implications of toxicity, *Nat. Rev. Clin. Oncol.* 19 (2022) 254–267.
- [189] S.J. Soenen, P. Rivera-Gil, J.M. Montenegro, W.J. Parak, S.C. De Smedt, K. Braeckmans, Cellular toxicity of inorganic nanoparticles: common aspects and guidelines for improved nanotoxicity evaluation, *Nano Today* 6 (2011) 446–465.
- [190] T.V. Peres, M.R.C. Schettinger, P. Chen, F. Carvalho, D.S. Avila, A.B. Bowman, M. Aschner, Manganese-induced neurotoxicity: a review of its behavioral consequences and neuroprotective strategies, *Bmc Pharmacol Toxicol* 17 (2016) 57.
- [191] O. Zabirnyk, M. Yezhelyev, O. Seleverstov, Nanoparticles as a novel class of autophagy activators, *Autophagy* 3 (2007) 278–281.
- [192] S.H. Cui, Y.Y. Wang, Y. Gong, X. Lin, Y.N. Zhao, D.F. Zhi, Q. Zhou, S.B. Zhang, Correlation of the cytotoxic effects of cationic lipids with their headgroups, *Toxicol Res-Uk* 7 (2018) 473–479.
- [193] B. Scheel, R. Teufel, J. Probst, J.P. Carralot, J. Geginat, M. Radsak, D. Jarrossay, H. Wagner, G. Jung, H.G. Rammensee, I. Hoerr, S. Pascolo, Toll-like receptor-dependent activation of several human blood cell types by protamine-condensed mRNA, *Eur. J. Immunol.* 35 (2005) 1557–1566.
- [194] H. Westdorp, J.H.A. Creemers, I.M. van Oort, G. Schreibelt, M.A.J. Gorris, N. Mehra, M. Simons, A.L. de Goede, M.M. van Rossum, A.J. Croockewit, C. G. Figdor, J.A. Witjes, E.H.J.G. Aarntzen, R.D.M. Mus, M. Bruning, K. Petry, M. Gotthardt, J.O. Barentsz, I.J.M. de Vries, W.R. Gerritsen, Blood-derived dendritic cell vaccinations induce immune responses that correlate with clinical outcome in patients with chemo-naive castration-resistant prostate cancer, *J Immunother Cancer* 7 (2019) 302.
- [195] A. Papachristofilou, M.M. Hipp, U. Klinkhardt, M. Fruh, M. Sebastian, C. Weiss, M. Pless, R. Cathomas, W. Hilbe, G. Pall, T. Wehler, J. Alt, H. Bischoff, M. Geissler, F. Griesinger, K.J. Kallen, M. Fotin-Mleczeck, A. Schroder, B. Scheel, A. Muth, T. Seibel, C. Stosnach, F. Doener, H.S. Hong, S.D. Koch, U. Gnad-Vogt, A. Zippelius, Phase Ib evaluation of a self-adjuvanted protamine formulated mRNA-based active cancer immunotherapy, BI1361849 (CV9202), combined with local radiation treatment in patients with stage IV non-small cell lung cancer, *J Immunother Cancer* 7 (2019) 38.
- [196] M. Sebastian, A. Papachristofilou, C. Weiss, M. Fruh, R. Cathomas, W. Hilbe, T. Wehler, G. Rippin, S.D. Koch, B. Scheel, M. Fotin-Mleczeck, R. Heidenreich, K. J. Kallen, U. Gnad-Vogt, A. Zippelius, Phase Ib study evaluating a self-adjuvanted mRNA cancer vaccine (RNAActive (R)) combined with local radiation as consolidation and maintenance treatment for patients with stage IV non-small cell lung cancer, *BMC Cancer* 14 (2014).
- [197] H.S. Hong, S.D. Koch, B. Scheel, U. Gnad-Vogt, A. Schroder, K.J. Kallen, V. Wiegand, L. Backert, O. Kohlbacher, I. Hoerr, M. Fotin-Mleczeck, J. M. Billingsley, Distinct transcriptional changes in non-small cell lung cancer patients associated with multi-antigenic RNAActive (R) CV9201 immunotherapy, *Oncol Immunology* 5 (2016), e1249560.
- [198] H. Kubler, B. Scheel, U. Gnad-Vogt, K. Miller, W. Schultze-Seemann, F. vom Dorp, G. Parmiani, C. Hampel, S. Wedel, L. Trojan, D. Jocham, T. Maurer, G. Rippin, M. Fotin-Mleczeck, F. von der Mulbe, J. Probst, I. Hoerr, K.J. Kallen, T. Lander, A. Stenzl, Self-adjuvanted mRNA vaccination in advanced prostate cancer patients: a first-in-man phase I/IIa study, *J Immunother Cancer* 3 (2015) 26.
- [199] A. Mackensen, C. Koenecke, J. Haanen, W. Alsdorf, A. Desuki, E. Wagner-Drouet, D. Heudobler, P. Borchmann, E. Wiegert, C. Schulz, B. Rengstl, L. Preussner, O. Tuereci, U. Sahin, Bnt211: a phase I/II trial to evaluate safety and efficacy of Cldn6 car-T cells and vaccine-mediated in vivo expansion in patients with cldn6-positive advanced solid tumors, *J Immunother Cancer* 9 (2021). A1008-A1008.
- [200] G. Cafri, J.J. Gartner, T. Zaks, K. Hopson, N. Levin, B.C. Paria, M.R. Parkhurst, R. Yossef, F.J. Lowery, M.S. Jafferji, T.D. Prickett, S.L. Goff, C.T. McGowan, S. Seitter, M.L. Shindorf, A. Parikh, P.D. Chatani, P.F. Robbins, S.A. Rosenberg, mRNA vaccine-induced neoantigen-specific T cell immunity in patients with gastrointestinal cancer, *J. Clin. Invest.* 130 (2020) 5976–5988.
- [201] A. Porciuncula, M. Morgado, R. Gupta, K. Syrigos, R. Meehan, S.J. Zacharek, J. P. Frederick, K.A. Schalper, Spatial mapping and immunomodulatory role of the OX40/OX40L pathway in human non-small cell lung cancer, *Clin. Cancer Res.* 27 (2021) 6174–6183.
- [202] K. Leppik, G.W. Byeon, W. Kladwang, H.K. Wayment-Steele, C.H. Kerr, A.D.F. Xu, D. Kim, V.V. Topkar, C. Choe, D. Rothschild, G.C. Tiu, R. Wellington-Oguri, K. Fujii, E. Sharma, A.M. Watkins, J.J. Nicol, J. Romano, B. Tunguz, F. Diaz, H. Cai, P.B. Guo, J.W. Wu, F.Y. Meng, S. Shi, E. Participants, P.R. Dormitzer, A. Solorzano, M. Barna, R. Das, Combinatorial optimization of mRNA structure, stability, and translation for RNA-based therapeutics, *Nat. Commun.* 13 (2022) 1536.
- [203] K. Bloom, F. van den Berg, P. Arbutnot, Self-amplifying RNA vaccines for infectious diseases, *Gene Ther.* 28 (2021) 117–129.
- [204] A.R. Rappaport, S.J. Hong, C.D. Scallan, L. Gitlin, A. Akoopie, G.R. Boucher, M. Egorova, J.A. Espinosa, M. Fidanza, M.A. Kachura, A.N. Shen, G. Sivko, A. Van Abbema, R.L. Veres, K. Jooss, Low-dose self-amplifying mRNA COVID-19 vaccine drives strong protective immunity in non-human primates against SARS-CoV-2 infection, *Nat. Commun.* 13 (2022) 3289.
- [205] R.K. Jain, Physiological barriers to delivery of monoclonal antibodies and other macromolecules in tumors, *Cancer Res.* 50 (1990) 814s–819s.
- [206] Q. Cheng, T. Wei, L. Farbiak, L.T. Johnson, S.A. Dilliard, D.J. Siegwart, Selective organ targeting (SORT) nanoparticles for tissue-specific mRNA delivery and CRISPR-Cas gene editing, *Nat. Nanotechnol.* 15 (2020) 313–320.
- [207] M. Qiu, Y. Tang, J.J. Chen, R. Murip, Z.F. Ye, C.F. Huang, J. Evans, E.P. Henske, Q.B. Xu, Lung-selective mRNA delivery of synthetic lipid nanoparticles for the treatment of pulmonary lymphangioleiomyomatosis, *P Natl Acad Sci USA* 119 (2022) 119.
- [208] S.H. Gohil, J.B. Iorgulescu, D.A. Braun, D.B. Keskin, K.J. Livak, Applying high-dimensional single-cell technologies to the analysis of cancer immunotherapy, *Nat. Rev. Clin. Oncol.* 18 (2021) 244–256.
- [209] C. Xu, Z. Lu, Y. Luo, Y. Liu, Z. Cao, S. Shen, H. Li, J. Liu, K. Chen, Z. Chen, X. Yang, Z. Gu, J. Wang, Targeting of NLRP3 inflammasome with gene editing for the amelioration of inflammatory diseases, *Nat. Commun.* 9 (2018) 4092.
- [210] Y. Zhang, S. Shen, G. Zhao, C.F. Xu, H.B. Zhang, Y.L. Luo, Z.T. Cao, J. Shi, Z. B. Zhao, Z.X. Lian, J. Wang, In situ repurposing of dendritic cells with CRISPR/Cas9-based nanomedicine to induce transplant tolerance, *Biomaterials* 217 (2019), 119302.