Review Article

Considerations on the Design of Lipid-based mRNA Vaccines Against Cancer

Sofie Meulewaeter [1,2], Yao Zhang [3,5], Abishek Wadhwa [4], Kevin Fox [5], Ine Lentacker [1,2], Kenneth W. Harder [4], Pieter R. Cullis [5], Stefaan C. De Smedt [1,2], Miffy H. Y. Cheng [5,†,*] and Rein Verbeke [1,2,*,†]

*1 - **Laboratory of General Biochemistry and Physical Pharmacy,** Faculty of Pharmaceutical Sciences, Ghent University, Ghent 9000, Belgium*

*2 - **Cancer Research Institute Ghent (CRIG),** Ghent University Hospital, Ghent University, Ghent 9000, Belgium*

*3 - **School of Biomedical Engineering,** University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada*

*4 - **Department of Microbiology and Immunology,** University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada*

*5 - **Department of Biochemistry and Molecular Biology,** University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada*

***Correspondence to Miffy H.Y. Cheng and Rein Verbeke:** Miffy.Cheng@ubc.ca (M.H.Y. Cheng), Rein.Verbeke@UGent.be (R. Verbeke)*
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Abstract

Throughout the last decades, mRNA vaccines have been developed as a cancer immunotherapeutic and the technology recently gained momentum during the COVID-19 pandemic. Recent promising results obtained from clinical trials investigating lipid-based mRNA vaccines in cancer therapy further highlighted the potential of this therapy. Interestingly, while the technologies being used in authorized mRNA vaccines for the prevention of COVID-19 are relatively similar, mRNA vaccines in clinical development for cancer vaccination show marked differences in mRNA modification, lipid carrier, and administration route. In this review, we describe findings on how these factors can impact the potency of mRNA vaccines in cancer therapy and provide insights into the complex interplay between them. We discuss how lipid carrier composition can affect passive targeting to immune cells to improve the efficacy and safety of mRNA vaccines. Finally, we summarize strategies that are established or still being explored to improve the efficacy of mRNA cancer vaccines and include next-generation vaccines that are on the horizon in clinical development.

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Introduction

Therapeutic cancer vaccines are designed to elicit an immune response that can recognize and selectively eliminate cancer cells in patients. To achieve this, cancer vaccines are generally focused on generating robust cellular immune responses. More specifically, cytotoxic $CD8^+$ T lymphocytes (CTLs) are able to directly attack cancer cells upon recognition of intracellular antigens presented in the context of major histocompatibility complex I (MHC-I). In addition, helper $CD4^+$ T cells recognize tumor epitopes in MHC-II molecules presented by antigen-presenting cells (APCs) in the tumor microenvironment and can provide multifaceted contributions to antitumor immunity, including support to $CD8^+$ T cells, as well as by having

direct and indirect killing capacities (Figure 1). Cancer vaccine approaches that evoke antibody responses against upregulated proteins in cancer could also be of interest.[1] However, given the importance of cellular immunity in cancer immunotherapy, this review will only focus on the cellular response to mRNA vaccination.

To elicit cellular immunity, two crucial elements, namely a tumor antigen (see BOX 1) and an immune stimulant, should be delivered to innate immune cells specialized in antigen presentation, e.g. dendritic cells (DCs). For this purpose, several vaccination strategies were clinically evaluated in the last decades.[2] Of them, lipid-based messenger RNA (mRNA) vaccines are of major interest as they have proven to be a safe, easy-to-produce, and powerful vaccine platform during the COVID-19 pandemic.[3] Multiple reports showed that the marketed mRNA vaccines elicit potent (memory) CTL- and T helper type 1 (Th1)-skewed T cell responses in addition to robust humoral immunity.[4–7] This is further highlighted by the encouraging results obtained in recent clinical trials studying mRNA-based cancer vaccines. Sequential vaccinations with Moderna's personalized vaccine mRNA-4157 (V590) in combination therapy with the immune checkpoint inhibitor (ICI) pembrolizumab (PD-1 inhibitor) lowered the risk of distant metastasis or death by 65% in patients with resected high-risk melanoma (stage III/IV) compared to patients who received Pembrolizumab treatment alone at approximately two years after treatment.[8] Similarly, to the race of developing a COVID vaccine, BioNTech is also leading the charge in the development of lipid-based mRNA vaccines for different types of cancer. To exemplify, BioNTech has published on the clinical potential of mRNA vaccination to elicit strong $CD4^+$- and $CD8^+$ T cell immunity against tumor antigens in pancreatic cancer (BNT122)[9] and melanoma cancer (BNT111).[10,11]

In contrast to the COVID vaccines, these mRNA cancer vaccines show remarkable differences in vaccine design and delivery approach, such as for the lipid carrier, the modifications or absence of modifications to the mRNA molecule, and route of immunization. Moreover, next-generation mRNA vaccines are on the horizon, including mRNA vaccine technologies that aim for prolonged antigen synthesis such as those making use of self-amplifying mRNA (saRNA) and circular mRNA (circRNA). In addition, further iterations to the lipid carriers are being made in order to induce more balanced immune responses by tuning LNP composition and lipid chemistry.

Therefore, in this review, we aim to elucidate the differences between most clinically advanced mRNA cancer vaccines and synthesize the most relevant findings obtained from recent preclinical studies. As such, we will discuss how choices in mRNA design, lipid carrier and administration route may impact the overall performance of mRNA cancer vaccines.

mRNA Cancer Vaccines in Clinical Development

In 2010, the first DC-based vaccine Provenge® (Sipuleucel-T) received FDA-approval for

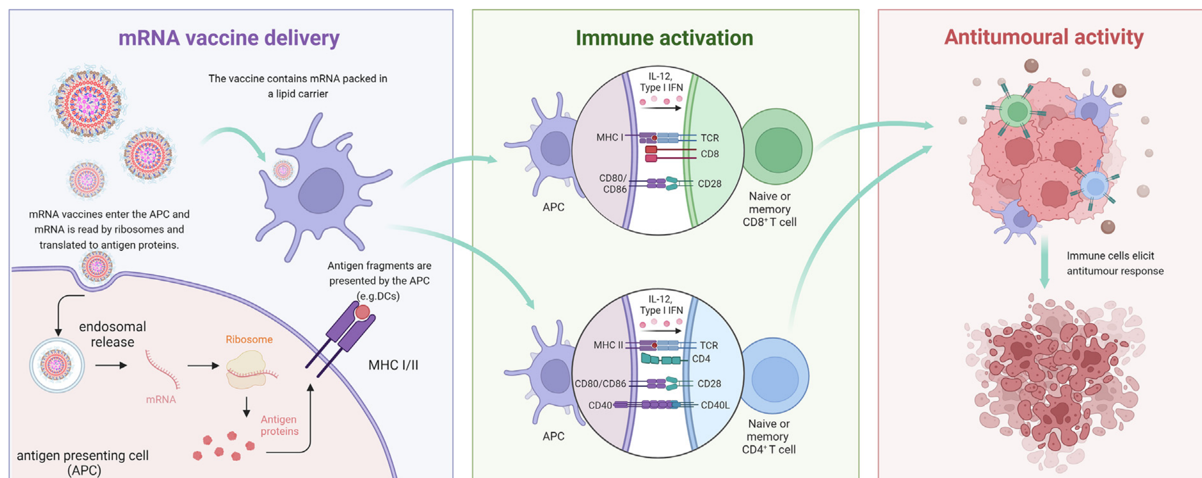

Figure 1. Mechanism of mRNA cancer vaccines. mRNA encoding TAAs or neoantigens is packed in lipid-based carriers and internalized by APCs. Upon endosomal release, mRNA is translated into tumor antigens which are subsequently presented in MHC molecules to $CD4^+$- and $CD8^+$ T cells. Stimulated APCs secrete pro-inflammatory cytokines and activate cellular immunity by presenting antigen fragments to T cells and by providing a co-stimulatory signal (via CD80/86 and/or CD40). The evoked T cells can selectively recognize and attack cancer cells. (Figure was created with biorender.com).

prostate cancer.[12] Despite this achievement, DC-based vaccines suffer from several important drawbacks such as the labor-intensive procedure of blood cell isolation and *ex vivo* manipulation of the collected cells, as well as the suboptimal quality of the DCs that are given back to the patient by infusion.[13] Therefore, *in situ* priming of DCs (or APCs in general) by delivery of tumor antigens (TAs) to APCs has been explored ranging from whole-tumor lysate, peptides, and proteins to DNA and mRNA.[14] Notably, the delivery of TAs encoded by mRNA enables the cytosolic expression of antigens, followed by a favourable presentation in MHC-I molecules, without the risk of insertional mutagenesis inherent to DNA. Moreover, the modular design of the mRNA platform also provides flexibility to easily change the encoded TA(s) or antigenic epitope(s), as well as the mRNA sequence which can be further optimized to enhance the cellular trafficking of antigens toward MHC molecules. As an example of the latter, the addition of an N-terminal leader peptide with an MHC class I trafficking signal in the mRNA construct allows simultaneously improved MHC-I and MHC-II presentation, and thereby enables enhanced expansion of antigen-specific $CD4^{+}$ and $CD8^{+}$ T cells.[15]

The first demonstration of delivery of mRNA inside eukaryotic cells dates from the late 1970s.[16,17] Two decades later, the feasibility of *in situ* protein expression upon mRNA delivery was demonstrated[18] but the further applicability and delivery of mRNA have been hampered by the biological instability, physicochemical characteristics (i.e. bulkiness and negative charge), and inflammatory nature of the molecule. Since then, many efforts have been invested to tackle these issues. The intracellular stability and translatability of the mRNA molecule have been improved by optimizing its molecular design, as excellently reviewed by others.[19,20] Moreover, a better understanding of the innate immune-stimulating activity of mRNA and the subsequent discovery that modification of nucleotides (modified mRNA) could circumvent immune recognition by several cellular receptors,[21] represented a milestone in the development of mRNA as a drug. Paradoxically, the immune-stimulating activity of IVT mRNA has also been studied for its self-adjuvant properties, with multiple studies advocating for the use of an unmodified mRNA format in vaccines, which will be discussed in detail below.

Although several (clinical) studies reported on vaccination using naked mRNA or protamine-complexed mRNA, degradation by the action of extracellular nucleases (RNase) and the limited cellular uptake due to the large size and negative charge of the mRNA molecule challenged the development of mRNA therapeutics. This is also evidenced by the administration routes that were applied for the delivery of naked mRNA vaccines, e.g. intranodal (i.n.)[11] or intradermal (i.d.)[22] injection, that are less prone to RNA degradation and are rich in APCs,[23] but are more difficult to administer than through subcutaneous (s.c.) or intramuscular (i.m.) injections. To address this need, several delivery vehicles were evaluated for RNA delivery, most of them based on either lipids or polymers.[24] Particularly lipid-based nanoparticles received major interest as carriers for RNA drugs, as highlighted by multiple clinical success stories in recent years.[25–27] The encapsulation of mRNA in a lipid carrier relies on a charge interaction between the negatively charged phosphate backbone of mRNA and the positively charged amino headgroups of cationic lipids. Depending on the number of substituents in the amino headgroup, the cationic lipids are either positively (permanent cationic lipids, e.g. DOTMA or DOTAP) or neutrally (ionizable lipids, e.g. DODMA, DODAP or DLin-MC3-DMA) charged at physiological pH.[28] Other lipids typically included in lipid-based nanoparticles are cholesterol, a phospholipid (e.g. DSPC, DOPE), and a polyethylene glycol (PEG) lipid (e.g. DMG-PEG2000). Moreover, besides aiding in the delivery of nucleic acids, lipid-based carriers can also have intrinsic innate immune-stimulating activities; a topic which recently gained a lot of interest. Notably, the type of carrier and lipid composition seem to drastically affect the innate immune response to mRNA vaccines, and thereby can play a critical role in the vaccine's performance and safety.

As a consequence of the pioneering work on both the mRNA chemistry and the delivery of mRNA, many mRNA-based drugs are currently being evaluated in clinical trials (e.g. for protein replacement therapy and prophylactic as well as therapeutic vaccines) which so far resulted in two authorized products: Spikevax (mRNA-1273) and Comirnaty (BNT162b2).[29] In Table 1, we only summarized the clinical trials focusing on mRNA lipid-based nanoparticles for therapeutic cancer vaccination. For an overview of clinical trials investigating mRNA vaccines based on carriers other than lipid carriers, we refer the reader to a recently published review by Lorentzen et al.[30] In the first sections of this review, we will explain the differences and similarities between these vaccine candidates and discuss how both the lipid-based nanocarrier and mRNA molecule can contribute to the antigenicity and immunogenicity of mRNA vaccines.

Table 1 **Overview of clinical trials involving lipid-based mRNA therapeutic cancer vaccines.** Included trials were found on ClinicalTrials.gov searching for active or completed trials using search terms 'RNA vaccine' or 'neoantigen vaccine' and 'cancer' and which are also listed on the web page of the developer. Abbreviations: RA: route of administration, LPX: lipoplex, LNP: lipid nanoparticle, HPV16: human papillomavirus 16, NSCLC: non-small cell lung cancer, CLDN6: claudin 6, CAR: chimeric antigen receptor, TAA: tumor-associated antigen, PD-(L)1: programmed death-(ligand) 1, IDO1: indoleamine 2,3-dioxygenase, KRAS: Kirsten rat sarcoma virus, EBV: Epstein–Barr virus.

Lipid carrier	RNA	RA	Tumor antigen	Indication	Combination therapy	Name	Phase	Status	Sponsor (Developer)	ClinicalTrials.gov Identifier
LPX (DOTAP)	autologous mRNA	i.v.	tumor mRNA + viral antigen	glioblastoma	monotherapy	PNOC020	I	recruiting	University of Florida	NCT04573140
LPX (DOTAP)	autologous mRNA	i.v.	tumor mRNA	pulmonary osteosarcoma	monotherapy	RNA-LP vaccine	I/II	not yet recruiting	University of Florida	NCT05660408
LPX (DOTAP)	autologous mRNA	i.v.	tumor mRNA	melanoma	monotherapy	RNA-LP vaccine	I	not yet recruiting	University of Florida	NCT05264974
LPX (DOTMA)	unmodified mRNA	i.v.	TAA	advanced melanoma	cemiplimab	BNT111[32]	II	recruiting	BioNTech SE	NCT04526899
LPX (DOTMA)	unmodified mRNA	i.v.	TAA	prostate cancer	cemiplimab	BNT112[141]	I/II	recruiting	BioNTech SE	NCT04382898
LPX (DOTMA)	unmodified mRNA	i.v.	viral antigen	HPV16-positive head and neck cancer	pembrolizumab	BNT113	II	recruiting	BioNTech SE	NCT04534205
LPX (DOTMA)	unmodified mRNA	i.d.	viral antigen	HPV16-positive cancer	monotherapy	BNT113	I/II	suspended	University of Southampton (BioNTech SE)	NCT03418480
LPX (DOTMA)	unmodified mRNA	i.v.	TAA	NSCLC	cemiplimab or docetaxel	BNT116	I	recruiting	BioNTech SE	NCT05142189
LPX (DOTMA)	unmodified mRNA	i.v.	TAA	CLDN6-positive solid tumor	BNT211 (CAR-T cell therapy)	CARVac[142]	I/II	recruiting	BioNTech SE	NCT04503278
LPX (DOTMA)	unmodified mRNA	i.v.	neoantigen	advanced melanoma	pembrolizumab	BNT 122	II	active, not recruiting	Genentech, Inc. (BioNTech SE)	NCT03815058
LPX (DOTMA)	unmodified mRNA	i.v.	neoantigen	pancreatic cancer	atezolizumab and mFOLFIRINOX	BNT 122[9]	I	active, not recruiting	Memorial Sloan Kettering Cancer Center (BioNTech SE)	NCT04161755
LPX (DOTMA)	unmodified mRNA	i.v.	neoantigen	colorectal cancer	monotherapy	BNT 122[143]	II	recruiting	BioNTech SE	NCT04486378
LPX (DOTMA)	unmodified mRNA	i.v.	neoantigen	solid tumor	atezolizumab	BNT 122	I	active, not recruiting	Genentech, Inc. (BioNTech SE)	NCT03289962
Lipid carrier	**RNA**	**RA**	**Tumor antigen**	**Indication**	**Combination therapy**	**Name**	**Phase**	**Status**	**Sponsor (Developer)**	**ClinicalTrials.gov Identifier**
LNP	unmodified mRNA	i.m.	TAA	glioblastoma	monotherapy	CVGBM[144]	I	recruiting	CureVac	NCT05938387
LNP	modified mRNA	i.m.	neoantigen	melanoma	pembrolizumab	mRNA-4157	III	recruiting	Merck Sharp & Dohme LLC (ModernaTX, Inc.)	NCT05933577

Table 1 (*continued*)

Lipid carrier	RNA	RA	Tumor antigen	Indication	Combination therapy	Name	Phase	Status	Sponsor (Developer)	ClinicalTrials.gov Identifier
LNP	undisclosed	i.m.	PD-L1 and IDO1	advanced solid tumor	pembrolizumab	mRNA-4359[145]	I/II	recruiting	ModernaTX, Inc.	NCT05533697
LNP	undisclosed	i.m.	TAA	KRAS-mutated advanced solid tumor	pembrolizumab	mRNA-5671	I	completed	Merck Sharp & Dohme LLC	NCT03948763
LNP	undisclosed	i.m.	viral antigen	EBV-positive tumor	monotherapy	EBV mRNA vaccine	I	recruiting	West China Hospital	NCT05714748
LNP	undisclosed	i.d.	neoantigen	gastric, esophageal and liver cancer	PD-1/PD-L1 inhibition	NeoCura Ag-IND	I	recruiting	jianming xu (NeoCura)	NCT05192460
LNP (Genevant)	saRNA	i.m.	shared neoantigen	NSCLC, colorectal and						
pancreatic cancer	nivolumab and ipilimumab	GRT-R904 (boost vaccination)	I/II	active, not recruiting	Gritstone bio, Inc.	NCT03953235				
LNP	saRNA	i.m.	neoantigen	colorectal cancer	atezolizumab and ipilimumab	GRT-R902 (boost vaccination)[146]	II/III	recruiting	Gritstone bio, Inc.	NCT05141721
LNP	saRNA	undis-closed	neoantigen	advanced solid tumor	monotherapy	JCXH-212	I	recruiting	Peking University Cancer Hospital & Institute (Immorna)	NCT05579275

Box 1 Tumor antigens in mRNA cancer vaccines. In contrast to pathogens, cancer cells have a high similarity with the patient's own cells. To develop a successful cancer vaccine, it is therefore critical to define tumor antigens that are (i) specifically expressed in the tumor and that are (ii) sufficiently immunogenic (i.e. non-self and presented by MHC molecules) to activate CTLs. Tumor antigens are typically divided into two classes, namely tumor-associated antigens (TAAs) and neoantigens. While Moderna focuses on neoantigen-based vaccines in their cancer vaccine portfolio, BioNTech cancer vaccines have candidates targeting both shared TAAs or individualized neoantigens, i.e. the FixVac technology platform or the iNeST platform, respectively.[30] TAAs are non-mutated self-antigens that are overexpressed in cancer cells and therefore have a relatively specific expression in the tumor. Although encouraging CTL responses were observed after TAA-vaccination,[31,32] TAAs are considered weak antigens as they need to overcome the central T cell tolerance to self-antigens.[33] Another disadvantage of targeting TAAs is that patients who do not express the target antigen(s) are not eligible for this approach, as illustrated by the exclusion of this patient population in the Phase 1/2 clinical trial of BNT111 (NCT02410733).[32] As a response to the challenges of TAAs, neoantigens have emerged as a new class of antigen targets. Neoantigens are tumor-specific antigens that arise from genetic alterations in the tumor which can be caused by mutations, insertion of viral open reading frames upon viral infections, and many other events.[34] Neoepitopes are typically predicted through DNA and RNA sequencing and subsequent analysis using bioinformatic tools that detect mutations in tumor genetic material. Aberrant peptides that are retrieved via this analysis are screened for their predicted binding affinity to MHC molecules to ensure presentation of the epitopes. A complete overview of this process is not within the scope of this review and the reader is referred to excellent reviews on this topic.[35,36] In conclusion, because of the tumor-specific and aberrant (non-self) nature, neoantigens tackle drawbacks associated with TAA. However, this approach is less suited for off-the-shelf vaccine production, which implies that more time and resources need to be invested compared to TAA-based vaccines, as evidenced by Moderna's statement that it takes a few weeks from tumor biopsy to vaccination with the personalized mRNA-4157 vaccine.[37]

Lipid Carriers for mRNA Vaccine Delivery

Based on their different morphologies and biological effects, the lipid carriers for gene delivery are typically separated into two classes; (cationic) lipoplexes and (ionizable) lipid nanoparticles (LNPs) as shown in Figure 2.

Lipoplexes are multilayered structures that are formed upon the complexation of nucleic acids with a liposomal carrier based on a permanent cationic lipid. DOTMA and DOTAP are the most commonly used permanent cationic lipids and differ only by their linker groups (linking the positively charged quaternary ammonium head group with the lipid tail). More specifically, DOTMA contains two ether linkages whereas DOTAP contains two ester linkages that aid in the biodegradability of the lipid.[28] Several elements of their lipid chemistry, such as the linker group, were shown to affect the gene expression of permanent cationic DNA lipoplexes upon i.v. injection in mice, with DOTMA outperforming DOTAP.[38] To improve the release of the nucleic acid payload into the cytosol upon endocytosis, helper lipids (mostly DOPE or cholesterol) are added to the cationic lipid.[39] These lipids favor the phase transition from bilayer to inverted micelle or hexagonal (H_{II}) phase when cationic lipids interact with the anionic phospholipids of the endosomal membrane, which results in destabilization of the lipoplex and release of nucleic acids from the endosomal compartment into the cytosol.[40]

Several studies have shown that mRNA lipoplexes containing permanent cationic lipids can induce antitumor immunity in mice,[41,42] but this delivery platform suffers from several issues for clinical use. First, the positive charge of the liposomal carrier poses several safety risks which are associated with cell necrosis and inflammation.[43,44] Another drawback of positively charged mRNA lipoplexes is their immediate disposition in the lungs after systemic administration,[45] limiting their usefulness on grounds of safety. It was recently reported that both cationic mRNA-LNPs and mRNA lipoplexes with tropism to the lungs induce blood clotting shortly after i.v. injection.[46] To solve these issues, BioNTech has optimized the net charge of DOTMA/DOPE lipoplexes by using an excess of mRNA molecules to lipids. This modification renders slightly negative particles which were found to be efficiently shifting the passive targeting of mRNA lipoplexes from lungs to dendritic cells in the spleen.[10] Notably, this optimized lipoplex formulation is currently being used in BioNTech's Lipo-MERIT and FixVac mRNA cancer vaccine pipeline and has shown acceptable tolerability in several clinical trials.[9,10]

LNPs are generally composed of four lipid components: an ionizable lipid to encapsulate genetic payloads, cholesterol, a phospholipid, and a PEG-conjugated lipid. To exemplify, the LNP carrier of BioNTech's BNT162b2 is composed of ALC-0315, cholesterol, DSPC and ALC-0159 respectively.[46] Moderna's Spikevax is composed of SM-102, cholesterol, DSPC and PEG2000-DMG.[47] Note here that LNPs are typically PEGylated to ensure their colloidal stability whereas lipoplexes, such as BioNTech's liposomal platform, are mostly not PEGylated and rely on charge repulsion to obtain colloidal stability. As discussed later in this review, the PEG-lipid can influence the biodistribution of the mRNA vaccine and therefore represents an important difference between both lipid carriers used for mRNA cancer vaccination.

The ionizable lipid is an amino lipid with an acid dissociation constant (pKa) optimally between 6 and

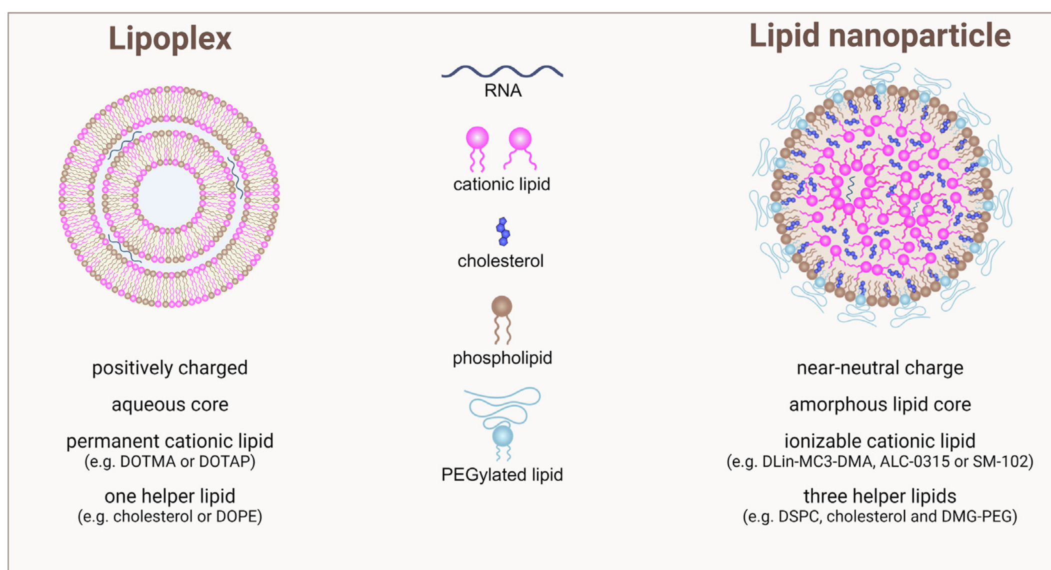

Figure 2. Clinically used cancer vaccine lipid carriers for encapsulation of mRNA. Lipoplexes have a multilayered structure and are formed upon complexation of cationic liposomes with mRNA and usually consist of a permanent cationic lipid and a helper lipid. Lipid nanoparticles (LNPs) have an amorphous structure and are formed by mixing an ionizable cationic lipid and three helper lipids (cholesterol, a phospholipid and a PEG-conjugated lipid) with mRNA. (Figure was created with biorender.com).

7.[48] In an acidic environment, these ionizable lipids carry a positive charge upon protonation of the tertiary amine headgroup, allowing encapsulation of mRNA and release of mRNA in the cytosol.[49] However, at physiological pH, these lipids are neutrally charged thus potentially minimizing the toxicity associated with positively charged nanoparticles. Another important advantage of LNPs over lipoplexes is that LNPs allow high mRNA expression upon local administration,[50] which is in contrast to non-PEGylated lipoplexes which only show moderate expression levels after i.v. administration.[51,52] Note here that different results might be obtained in case of PEGylated lipoplexes.[53] Nevertheless, it was shown that mRNA expression of LNPs based on the ionizable lipids DLin-MC3-DMA and SM-102 is strongly enhanced compared to DOTAP-based lipoplexes, both upon i.v. and i.m. delivery.[54] Of note, similarly to permanent cationic lipids, the transfection efficiency and biodegradability of LNPs can be improved by optimizing the lipid chemistry of the ionizable lipid (e.g. by introducing double bonds and ester groups in the lipid tail respectively).[55] Assuming that increased mRNA expression results in enhanced antigen presentation and cellular immunity, the mRNA delivery efficiency associated with LNPs could be a highly appealing feature of this lipid carrier.

Impact of mRNA Modalities and Modifications on T Cell Response

A key aspect of mRNA medicinal products is the innate immune-stimulating activity of *in vitro* transcribed (IVT) mRNA. Several elements of IVT mRNA, more specifically the 5′ cap, uridine-containing sequences, and double-stranded RNA (dsRNA) byproducts are sensed by endosomal pathogen recognition receptors (PRRs) (toll-like receptor (TLR) 3/7/8) and several cytosolic receptors (e.g. retinoic acid-inducible gene-I (RIG-I)-like receptors). An overview of how these structures are recognized and the respective signaling cascades that are subsequently activated is excellently summarized elsewhere.[56–59] In short, these signaling cascades can result in degradation of mRNA (e.g. activation of RNase L), inhibition of translation (e.g. inactivation of the eukaryotic translation initiation factor 2α (eIF2α)), and production of pro-inflammatory cytokines, most notably type I interferons (IFNs), which in turn will further promote inhibitory mechanisms. It should however be noted that it was shown that $CD169^+$ macrophages in the spleen acquire resistance to type I IFNs through upregulation of ubiquitin-specific peptidase 18 (USP18), which allows them to translate viral antigens despite secretion of type I IFNs.[60]

Karikó and colleagues were the first to describe that by modification of nucleotides in the mRNA molecule (e.g. replacement of uridine by pseudouridine or cytosine by 5-methylcytidine), sensing of the mRNA molecule by several PRRs was greatly reduced.[21] Later, it was reported that particularly substitution of uridine with pseudouridine (ψ) resulted in decreased immune stimulation combined with a higher translatability.[61] In 2015, Andries et al. showed that N^1-methylation of

ψ (N^1-methylpseudouridine, 1mψ) could further reduce immune stimulation and improve protein expression over pseudouridine.[62] While modifications such as ψ or 1mψ could (depending on formulation and injection route) contribute to the translatability and safety profile of mRNA products, this capacity of mRNA to stimulate antiviral innate immune responses might also be exploited as a potential powerful adjuvant for eliciting B cell and T cell responses. This paradigm has created a lot of debate about the pros and cons of using unmodified versus uridine-modified mRNA for vaccine development, as evidenced by the different strategies of companies developing mRNA COVID vaccines. More specifically, Pfizer/BioNTech and Moderna used 1mψ-modified mRNA,[63,64] whereas CureVac advanced with an unmodified mRNA vaccine candidate, all given via i.m. injection.[65] After disappointing results of the Phase 3 study of the unmodified CvnCoV vaccine (48.2% overall efficacy),[65] the company first worked on an unmodified COVID vaccine with optimized non-coding regions (CV2CoV),[66] but recently also made the switch towards an 1mψ-modified mRNA vaccine (CV0501) based on superiority of this platform in Phase I clinical trials.[67] Interestingly, mRNA doses up to 200 μg are being clinically evaluated for CV0501, whereas 12 μg was the maximal dose for the unmodified CvnCoV vaccine candidate.[68] This seems to confirm the hypothesis that the dose-limiting toxicity of CvnCoV interfered with administering an effective dose, at least upon i.m. delivery.[69] Notwithstanding that this safety-related information is also of high relevance for cancer vaccination, the inherent immunogenicity of unmodified mRNA may play a beneficial role in the induction of anti-tumor responses, in particular to elicit cellular immunity. Besides unmodified nucleotides, other aspects of the mRNA molecule are known to stimulate the innate immune system. As an outstanding example, a study on the mechanisms of BNT162b2 (mRNA COVID vaccine developed by Pfizer/BioNTech) revealed that the melanoma differentiation-associated protein 5 (MDA5)–IFN-I signaling pathway was crucial for the vaccine's capacity to elicit $CD8^+$ T cells.[70] As it has been described that long-dsRNA is sensed by MDA5,[71] it is likely that dsRNA contaminants that might be present in the BNT162b2 vaccine might trigger the MDA5-IFN pathway, but this has yet to be proven. These findings suggest that a mere modification of the uridine nucleotide may not be sufficient to entirely eliminate the inflammatory nature of mRNA.

How to deal with the pro-inflammatory capacity of mRNA is not as black and white, as several preclinical studies have shown that the outcome/benefits of type I IFN signaling on the T cell response can heavily depend on the type of lipid carrier and the route of administration. In a lipoplex format, Broos et al. found that mice lacking the IFN-α/β receptor ($IFNAR1^{-/-}$) showed strongly decreased antigen-specific cytotoxicity compared with wild-type mice following intravenous (i.v.) delivery of unmodified mRNA.[51] Similarly, Kranz et al. observed that spleen-directed DOTMA/DOPE lipoplexes with unmodified mRNA induced type I IFN secretion in humans and that maturation and activation of APCs and T cells respectively, was impaired in $IFNAR1^{-/-}$ mice.[10] In another study by BioNTech, the same DOTMA/DOPE delivery approach was evaluated for the systemic delivery of 1mψ-modified mRNA purified from dsRNA contaminants.[72] In line with Kranz et al., immunization with unmodified mRNA lipoplexes resulted in a type I IFN-driven immune activation, while an opposing immune outcome of effector T cell depletion and expansion of regulatory $CD4^+$ T cells was achieved with 1mψ-modified mRNA lipoplexes.

In sharp contrast with these studies, De Beuckelaer et al. found upon local administration (i.e. intradermal (i.d.), i.m. or subcutaneous (s.c.) delivery) of DOTAP/DOPE lipoplexes with an unmodified mRNA format, that cellular immunity was stronger in $IFNAR1^{-/-}$ mice compared to vaccination in the wild-type group.[73] Moreover, in a follow-up study by this group, the authors discovered that the opposing effects of type I IFN induction by this mRNA vaccine platform upon local versus systemic administration were mediated through type I IFN signaling on T cells, rather than by exerting its effects at the level of DCs. More specifically, T cells deficient in IFNAR showed robust cell proliferation and killing capacity upon local administration, whereas the T cell response was enhanced by type I IFN signaling on T cells when the systemic route was applied. Therefore, the authors hypothesized that antigen presentation (i.e. T cell receptor (TCR) signaling) should precede the type I IFN signaling on T cells in order to optimally activate T cells, which is likely not the case upon local administration of mRNA lipoplexes. Possibly, the insufficient translational efficiency of (unmodified) mRNA lipoplexes upon local administration through type I IFN signaling might also in part explain these results, as IFN-signaling interferes with mRNA translation and it is known that there is a sharp threshold of TCR signaling that is required for T cells to proliferate.[74] Another possible explanation could be that USP18-expressing cells are targeted upon i.v. administration (i.e. $CD169^+$ macrophages in the spleen),[60] but not upon local administration of lipoplexes. However, since it was also reported that dendritic cells in the spleen and lymph nodes also express USP18, this mechanism could also play a role upon local administration.[75] Taken together, type I IFNs plays a dual but decisive role in the potency of unmodified mRNA lipoplex vaccines, with delivery of unmodified mRNA following intravenous administration showing to be a feasible and promising strategy to elicit tumor specific T cell responses in preclinical models, as well as in cancer patients.

With the delivery of mRNA (cancer) vaccines by LNPs containing ionizable lipids, it remains puzzling which mRNA modality can offer the best levels of cellular immunity. Sittplangkoon and colleagues recently evaluated the impact of mRNA 1mψ-modifications and type I IFNs in the context of cancer vaccination with mRNA-LNPs following i.m. administration in mice.[76] They observed that vaccination with unmodified mRNA-LNPs elicited strong T cell responses upon local administration driven by type I IFNs. Moreover, 1mψ-modified mRNA seemed to perform inferior to unmodified mRNA, as evidenced by the lowered T cell responses and decreased survival in a B16 melanoma model. Strikingly, these findings were challenged in a comparative viral vaccination study using LNPs that were similar to the LNP formulation of BNT162b2. Here, Knudson et al. found that i.d. immunization with 1mψ-modified mRNA induced robust epitope-specific $CD44^+$ $CD8^+$ T cell responses similar to unmodified mRNA, but at a lower grade of reactogenicity.[77] Another interesting finding of this study was that unmodified mRNA was superior in evoking short-lived effector T cells, whereas the opposite trend was observed for memory precursor effector cells (MPECs). Another recent study by da Silva et al. found that i.m. administration of a 1mψ-modified mRNA-LNP yielded more CTLs in TC-1 tumor-bearing mice as compared with unmodified mRNA.[78] When looking into the survival of the tumor-bearing mice, 1mψ-modified and unmodified mRNA vaccines eradicated tumors in a relatively comparable manner when the tumor was subcutaneously implanted. But, interestingly, unmodified mRNA-LNPs were superior in eliciting tissue-resident memory T cells (T_{RM}) and showed better therapeutic effects in mucosal growing tumors than their modified counterparts. Notably, this long-lived memory phenotype of T lymphocytes that resides in epithelial and mucosal tissues, recently gained great interest as they are associated with a favorable prognosis in different types of cancer.[79,80]

Until now, our discussion was mainly focused on the modification of mRNA, i.e. unmodified versus uridine-modified, but other RNA modalities are also being explored for cancer vaccine development. Self-amplifying RNA (saRNA) and circular RNA (circRNA) are two variants of mRNA (Figure 3) that can theoretically induce higher and prolonged protein expression, either through introducing a sequence encoding for a replicon (saRNA) or through improving the stability of the molecule by creating a closed-ring structure that is thereby protected from exonuclease-mediated degradation (circRNA). De Alwis et al. evaluated single immunization against SARS-CoV-2 with either a saRNA-based vaccine (ARCT-021) or an

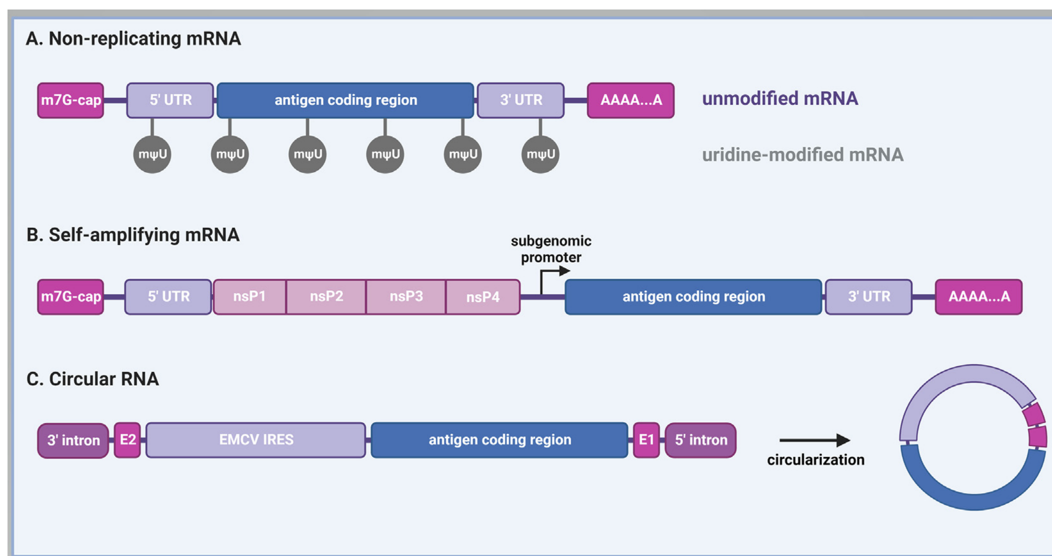

Figure 3. Schematic illustration of RNA modalities that are being explored for cancer vaccination. (A) Non-replicating mRNA consists of a cap structure, a 5′ and 3′ untranslated region (UTR), the antigen coding region, a poly (A) tail. **(B)** Self-amplifying RNA consists of the same building blocks as non-replicating mRNA, but also contains sequences of viral non-structural proteins (nsP1-4) and a subgenomic promoter (SGP) which allow replication of the original saRNA-molecule and, to a greater degree, of subgenomic RNA that is formed by recognition of the SGP. **(C)** Many in vitro synthesis methods and designs of circular RNA are currently under investigation, here a permuted intron–exon (PIE) splicing strategy is shown. A linear RNA precursor consisting of a 3′ and 5′ intron, exon fragment 1 (E1) and 2 (E2), the encephalomyocarditis virus (ECMV) internal ribosome entry site (IRES) which allows initiation of translation and the antigen coding region. PIE splicing yields a circular RNA product that consists of the IRES, antigen coding region, E1 and E2. (Figure was created with biorender.com).

unmodified mRNA vaccine and found that saRNA elicited higher numbers of spike-specific CD8$^+$ T cells in mice.[81] Da Silva et al. also evaluated tumor growth and survival after a single-dose administration of non-replicating mRNA-LNPs (unmodified or modified) or saRNA LNPs and reported robust antitumor responses with saRNA, also showing this modality's potential to induce T_{RM} immune responses.[78] With respect to the potential of circRNA, Li et al. recently published a proof-of-concept study, in which it was shown that circRNA LNPs inhibited tumor growth in an MC38 and B16 tumor model, however, circRNA did not outperform 1mψ-modified mRNA.[82] Of note, as mentioned in Table 1, one saRNA cancer vaccine (GRT-R902) was evaluated in a recently completed Phase I clinical trial as a booster vaccine following prime vaccination with an adenoviral vector (GRT-C901). It was observed that the vaccination strategy induced long-lasting T cell responses and several patients had improved overall survival.[83] A second saRNA cancer vaccine is also currently evaluated (NCT05579275), but no results have been disclosed yet.

The Contribution of the Lipid Carrier to the Reactogenicity and Immunogenicity of mRNA Vaccines

As already introduced in the previous section, the type of lipid carrier, seems to heavily impact the immune responses to mRNA vaccines, pointing out the need for a better understanding of the intrinsic adjuvant properties of the carrier.[58] Recently, Tahtinen et al. described that both mRNA lipoplexes and mRNA-LNPs can trigger IL-1 responses, predominantly IL-1β.[84] The authors demonstrated that IL-1β initiates the pro-inflammatory cytokine response to mRNA vaccines. This was evidenced by a significant reduction in a broad spectrum of cytokines, including IL-6, tumor necrosis factor (TNF), IFNα1 and IL-10, when unmodified mRNA lipoplexes were systemically injected in mice deficient of IL-1 receptor type 1. This observation could also be confirmed in an *in vitro* cell model of human peripheral blood mononuclear cells (PBMCs), when anti-IL-1β antibodies were co-delivered. Interestingly, the authors discovered that these effects of IL-1β were strongly counteracted in mice by a robust secretion of IL-1 receptor antagonist (IL-1ra), as well as after exposing non-human primate (NHP) PBMCs to these mRNA lipoplexes. However, in blood samples collected from the cancer patients being treated with these materials, IL-1ra levels were found to be much lower over IL-1β. Note that this difference in IL-1ra production might be relevant to explain the huge differences in the maximum tolerable doses of these products in humans versus what has been observed in mice and NHPs. Moreover, Tahtinen and colleagues showed that IL-1 responses induced by mRNA vaccination to a certain extent contribute to the adjuvanticity of mRNA vaccines since mice models who lack IL-1ra (Il1rn$^{-/-}$ mice) displayed enhanced cellular immunity upon vaccination with unmodified mRNA lipoplexes.[84]

With respect to the type of lipid carrier, Tahtinen et al. observed that for BioNTech's cancer vaccine technology both the DOTMA/DOPE liposomal carrier and unmodified mRNA were needed to induce IL-1β secretion in human PBMCs.[84] In contrast, SM-102 LNPs containing 1mψ-modified mRNA and even empty SM-102 LNPs already provoked strong IL-1β secretion in human PBMCs, suggesting a higher inflammatory activity of LNPs over cationic lipoplexes. Consistent with these findings, Alameh et al. showed that empty LNPs outperformed the FDA-approved AddaVax as a vaccine adjuvant in eliciting humoral immunity upon vaccination with a subunit protein vaccine, demonstrating adjuvant activity of LNPs.[85] Additionally, it was found that particularly the ionizable lipid, in this case a lipid proprietary to Acuitas, was critical for the adjuvant activity of the LNP and, interestingly, that LNPs based on the permanent cationic lipid DOTAP did not contribute to humoral responses. Of note, IL-6$^{-/-}$ mice showed impaired humoral immunity upon vaccination with both mRNA-LNPs and a protein subunit vaccine mixed with LNPs, indicating an important role of IL-6. Note, however, that this study evaluated the contribution of the adjuvant properties of empty LNPs for humoral immunity, but did not study the adjuvant effects on cellular immunity. To identify the role of the lipid carrier in the reactogenicity occurring upon vaccination with mRNA-LNPs, Ndeupen et al. locally administered empty LNPs based on an ionizable lipid proprietary to Acuitas and evaluated the inflammation by measuring the immune cell infiltration.[86] The authors observed that the injection of empty LNPs prompted an influx of neutrophils and other innate immune cells to the injection site. Importantly, this inflammatory response was hardly observed for empty LNPs devoid of ionizable lipids, demonstrating a prominent role of the ionizable lipid in the reactogenicity toward mRNA-LNPs. In line with observations discussed in the previous paragraph, Ndeupen et al. observed robust release of IL-6 and IL-1β which are known to elicit inflammatory reactions and therefore also possibly mediate the reactogenicity of LNPs.

While the molecular mechanisms on how ionizable lipids are sensed by the immune system remain to be deciphered, it should be noted that the reactogenicity of ionizable lipids varies and can be reduced by optimizing the lipid chemistry. Moderna Therapeutics screened a collection of ionizable lipids which contained ester groups in the lipid tails.[54] Of them, lipid H (SM-102) was reported to elicit increased antibody titers (in mice, not in NHPs) and to degrade more rapidly compared to DLin-MC3-DMA and several other lipids

that were tested. Furthermore, the reactogenicity of this lipid was clearly reduced as evidenced by improved local tolerance and lowered secretion of chemokines and cytokines such as IL-6, which could partially be attributed to the shortened half-life of the ionizable lipid. As another example, Genevant, the company that developed the proprietary LNP-platform for Gritstone's neoantigen-based saRNA cancer vaccine candidate,[87] recently published a study screening several ionizable lipids for i.v. delivery of small interfering RNA (siRNA) and mRNA-LNPs. These lipids all contained a third lipid tail (trialkyl ionizable lipids) and other optimizations, such as unsaturated bonds, that favor endosomal escape.[88] Based on the screening, lipid 10 was selected and compared to DLin-MC3-DMA, SM-102, and ALC-0315 for reactogenicity (measured by the secretion of chemokine monocyte chemoattractant protein-1 (MCP-1)) and humoral immunity. Interestingly, they demonstrated that higher MCP-1 levels were measured upon i.m. immunization with ALC-0315 mRNA-LNPs at a high mRNA dose compared to LNPs with other ionizable lipids, suggesting that ALC-0315 has a relatively higher reactogenicity than the other ionizable lipids tested.

Importantly, the translational efficiency and potency of uridine-modified mRNA were also found to be affected by the type of ionizable lipid included in the lipid carrier. In a comparative study evaluating the *in vivo* mRNA expression upon i.v. delivery using several LNPs, Melamed and colleagues observed that the extent to which modified nucleotides affected mRNA expression in mice was dependent on the ionizable lipid, with a strong increase observed for C12-200 and $200O_{i10}$ LNPs when modified mRNA was delivered, while a moderate increase for ZA3-Ep10 LNPs and only slightly increased expression for cKK-E12 LNPs were seen.[89] Moreover, they found that the spleen, and particularly myeloid cells in the spleen, showed enhanced expression in response to mRNA modifications. Likewise, Sanofi recently reported on the role of the ionizable lipid in the potency of unmodified versus modified mRNA vaccines and also found that, depending on the ionizable lipid, modification of mRNA had a neutral or positive impact on humoral immunity and the induction of IFN-γ-secreting $CD8^+$ T cells in NHPs.[90] Interestingly, LNPs based on KC2 and MC3 that were found to elicit improved immune responses upon mRNA modification also showed a high production of IFN-α when unmodified mRNA was included in the formulation, whereas this was not the case for the LNP formulation containing the ionizable lipid L319. Moreover, with the L319 LNPs, there was also a faster induction of IL-1RA detected. Note here that MC3 and L319 both are comprised of the same dimethyl amino ionizable head group with similar pKa but L319 has different lipid tails with hydrolyzable ester bonds, which leads to improved biodegradation.[91] It remains unclear if the observed effects of L319 LNPs could be attributed to the rapid clearance of the lipid or are due to other factors such as the increased size of L319 LNPs compared to MC3 LNPs.

Taken together, both the performance and reactogenicity profile of mRNA vaccines seem to be heavily impacted by the type of lipid carrier, where current research seems to be focused on optimizing the ionizable lipid chemistry. While for now these studies are based on a screening approach, we can expect that further insights into the immune sensing pathways of LNPs will result in a knowledge-based selection of ionizable lipids, specifically tailored for the desired therapeutic outcome and most optimal safety profile.[58]

The Use of Adjuvants and Combination Therapies to Empower the Antitumor Potential of mRNA Vaccines

To further improve the adjuvant activity and anti-tumor properties of mRNA vaccines, an alternative approach can be to include additional immune-stimulants inside the formulation. One of the first was the inclusion of the hydrophobic TLR4 agonist monophosphoryl lipid A (MPLA) inside DOTAP/Cholesterol liposomal carriers which were complexed with 5-methylcytidine (5mC)- and ψ-modified mRNA.[41] Addition of this adjuvant did not interfere with the translational capacity of nucleoside-modified mRNA upon systemic administration and was able to elicit antigen-specific T cell responses at the same level as observed for unmodified mRNA, but without having the inhibitory translational effects of type I IFNs. In a similar approach, Pan et al. found that addition of MPLA in unmodified mRNA MC3 LNPs further increased antigen-specific CD8 T cells and effector memory T cells which translated in improved survival in EG7.OVA tumor-bearing mice.[92]

We and others have also identified alpha-galactosylceramide (α-GC) as a powerful adjuvant to improve the anti-tumor immune responses to mRNA vaccines.[40] This is a glycolipid antigen that activates natural killer T (NKT) cells and it was observed that, due to the engagement of this cell type, not only cellular immunity was empowered, but also a more broadened antitumor response could be obtained. This was evidenced by the bystander activation of NK cells and the indirect immunomodulatory effects of NKT cells on suppressive myeloid cells present in the tumor microenvironment. Moreover, in the context of an mRNA vaccine against malaria, the inclusion of NKT cell agonists inside mRNA lipoplexes resulted in significant generation of liver T_{RM} cells and effective protection.[93] Other examples of immune adjuvants that have been tested for mRNA vaccination, include stimulator of interferon genes (STING)-agonists,[94]

mRNA encoding constitutively active STING,[95] and short dsRNA.[95]

Instead of adding the adjuvant to the formulation, the ionizable lipid can also be chemically modified to have inbuilt immune-stimulatory properties. Han et al. recently synthesized an ionizable lipidoid by covalently binding a lipid tail to an amine-containing TLR7/8 agonist.[97] The resulting ionizable cationic lipid was then used to partially replace the ionizable lipid in the LNP. The adjuvanted mRNA-LNPs were found superior to their unmodified (ionizable lipid without conjugated TLR7/8 agonist) LNP counterparts in generating cellular immunity. Interestingly, this approach also improved cellular immunity and antibody responses upon s.c. vaccination in case the adjuvanted lipidoid was added to SM-102 LNPs. Miao et al. synthesized a library of ionizable lipids with various chemical modifications and found that ionizable lipids with a cyclic amino head group induced the STING-pathway.[98] The authors showed that this property provoked potent cellular immunity and anti-tumor effects in several tumor models. Similarly, Zhang et al. synthesized a lipid-like material, named C1, for delivery of modified mRNA as a cancer vaccine with affinity for the TLR4 receptor.[99] Although the inclusion of additional adjuvants in mRNA-LNP formulation is promising as it can strengthen the cellular immune response, caution should be taken to avoid excessive reactogenicity and endanger the safety of the vaccine.

Another key challenge is to ensure that the engaged CTLs reach the tumor and perform their effector functions. Depending on the T cell abundance in the tumor, tumors can be classified into inflamed (high abundance of (exhausted) T cells), immune excluded (T cells are not able to reach cancer cells), and immune desert tumors (low abundance of T cells). For each of these tumor types, several strategies exist to improve the anti-tumor T cell reactivity as excellently reviewed by Zhang et al.[100] Of these strategies, blockade of programmed death-1 (PD-1) or its ligand PD-L1 is of special interest as it has shown to reinvigorate dysfunctional, exhausted T cells and is particularly effective in patients with inflamed tumors.[101] Therefore, the combination of cancer vaccination and blockade of PD-L1/PD-1 holds tremendous potential, as vaccination aims to increase T cell infiltration in tumors. This is demonstrated by the 65% reduced risk of distant metastasis or death upon combination therapy of Moderna's personalized vaccine (mRNA 4157) and pembrolizumab (anti-PD-1) in comparison with pembrolizumab alone.[8] Apart from PD-L1/PD-1, combination therapies with other ICIs, more specifically against cytotoxic T lymphocyte-associated protein 4 (CTLA-4) involved in the priming of T cells, are also clinically evaluated.[102] Another clinically evaluated strategy is the combination of cancer vaccines and antiangiogenic drugs, which can improve infiltration of T cells through the tumor vasculature in immune-excluded tumors. However, the results of the Phase III clinical trial testing the combination therapy of a DC vaccine and sunitinib (NCT01582672) did not show benefit of the combinatorial approach.[103] Lastly, in this context, it should also be noted that type I IFNs also hold potential to further improve the outcome of vaccination as it was shown that MHC class I expression and infiltration of tissues by T-cells is promoted by type I IFNs.[104]

Route of Administration of lipid Carrier-based mRNA Vaccines

Besides the mRNA platform and lipid carrier, the administration route is also known to affect the immune response and vaccination outcome. Administration through different injection sites can result in altered kinetics of mRNA expression. Additionally, the type, number, and quality of APCs (especially DCs) that are available for vaccine uptake can vary between administration sites (Figure 4). In this regard, the dermis and lymph nodes are rich in immune cells and therefore interesting targets for vaccine delivery. However, both delivery routes are technically challenging, with intranodal (i.n.) injection requiring ultrasound guidance and i.d. injection by Mantoux technique, often resulting in inaccurate administrations. Therefore, the s.c. and i.m injection are considered more practical modes of administration. This results in a localized inflammation reaction characterized by immune cell infiltration and vaccine dissemination from the injection site into draining lymph nodes; with the latter being the major compartment(s) for antigen presentation and stimulation of T cells.[105,105] Similar to the COVID-19 mRNA-LNP vaccines, most mRNA-LNP cancer vaccine candidates are also administered through i.m. injection, but typically at much higher mRNA doses and with multiple administrations. For example, Moderna's most advanced cancer vaccine candidate, mRNA-4157, was given in nine doses with a three-week interval at a dose of 1 mg mRNA versus a 100 μg dose of mRNA-1273 (Moderna's COVID-19 mRNA vaccine). Note that, in contrast to LNP-based mRNA vaccines, lipoplex-based mRNA vaccines currently evaluated in clinical trials (Table 1) are exclusively administered intravenously, except for one clinical study that has tested i.d. administration (NCT03418480).

Whether the choice of i.m. administration of mRNA-LNP vaccines was driven by (preclinical) experimental data or rather was motivated by clinical practicality remains unclear. Interestingly, Friedensohn et al. reported on the immunogenicity of BNT162b2 (Pfizer/BioNTech COVID-19 vaccine) in military personnel, who accidentally received the first vaccine dose using a needle intended for s.c. administration and found high seroconversion rates, similar to what was reported

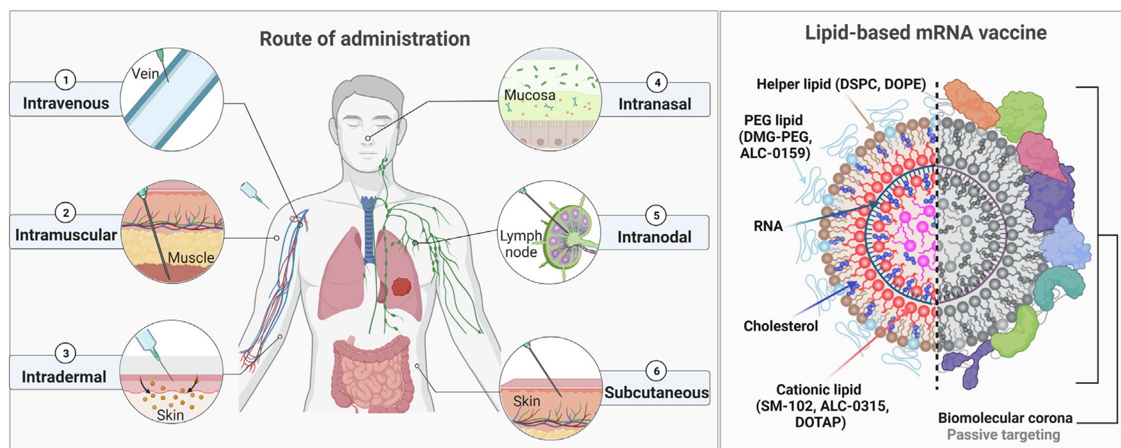

Figure 4. Administration route and fate of lipid-based mRNA vaccines. Several administration routes have been considered for the administration of mRNA vaccines, namely intravenous, intramuscular, intradermal, intranodal, intranasal and subcutaneous administration. To further optimize targeting toward immune-rich tissues, the physicochemical properties and composition of the lipid carrier can be altered to drive the formation of a favorable biomolecular corona. (Figure was created with biorender.com).

after a single i.m. administered dose.[107] In another clinical study, i.d. administration of BNT162b2 was evaluated as a potential strategy of dose sparing.[108] Compared with i.m. administration of a full vaccine dose (30 μg), it was found that i.d. administration of a fractional dose of BNT162b2 (6 μg) resulted in slightly lower anti-spike IgG titers. Nonetheless, the two administration routes provided effective seroconversion for all participants. Local adverse effects, such as swelling and redness, occurred more frequently in the i.d. group, while some of the systemic adverse reactions (headache, emesis, and myalgia) were less frequently reported than in the i.m. group. Moderna has also reported on data from a Phase I clinical trial evaluating an influenza mRNA vaccine following i.m. and i.d. administration. Importantly, during this study, enrollment in the i.d. route was halted because of local side effects, which made i.m. injection the preferred choice for vaccine administration.[109]

Comparison of administration routes in preclinical animal models can provide some insights on how the administration route impacts the mRNA vaccine's performance and biodistribution, but with caution that the translation to humans can be hampered due to differences between species in skins' physiological properties such as thickness, compactness and connection to adjacent tissues, etc. that can potentially affect the biological fate of the administered vaccines. In mice, Pardi et al. demonstrated that mRNA-LNPs can be efficiently delivered via different administration routes, but with variable duration of expression and translation kinetics.[50] Local administration following i.m. and i.d. injection achieved the longest duration of protein expression up to 7 days (dose of 1 μg mRNA), while the s.c. route resulted in detectable protein expression for 4 days. The i.m. route also resulted in higher systemic exposure to mRNA-LNPs indicated by significant expression levels in the liver, whereas expression upon i.d. and s.c. delivery remained localized to the injection site. Interestingly, the i.v. route had the highest levels of protein expression at 4 hours post-injection (mainly dominated by liver expression), but this route had the shortest duration of expression. The long expression and duration of mRNA-translated protein from local administration might have potential benefits for vaccine applications as prolonged antigenic stimulus in the lymphoid organs has been indicated to dictate the generation of strong adaptive immune responses.[110]

With respect to vaccine performance between administration routes, altering the administration of BNT162b2 from i.m. to the s.c. route in mice resulted in a lower pro-inflammatory response and correspondingly the extent of reactogenicity. Interestingly, the humoral response to BNT162b2 was similar between both administration routes, while the s.c. route could significantly improve T cell responses against the spike antigen.[111] Some other studies have advocated against the s.c. route for mRNA vaccination. Yavuz et al. demonstrated that mRNA-LNPs based on DLin-MC3-DMA induced a Th1-biased immune response upon i.m. injection as measured by IgG1/IgG2a ratios, but not when administered s.c.[112] In addition, Münter et al. studied the induction of antibody responses against the LNP carrier when administered by the i.m., s.c., or i.v. route; a potential concern that limits the long-term use of LNPs and is associated with allergic reactions. I.m. injections in mice were found to generate low and dose-independent levels of anti-LNP antibodies, while both i.v. and s.c. LNP

injections generated a substantial and highly dose-dependent amount of anti-LNP antibodies.[113]

Other routes of administration and/or approaches currently being evaluated with different degrees of success, include the exploration of mucosal immunization routes and microneedle patch systems[114,115] for i.d. delivery of mRNA vaccines. Moderna reported on an intranasal COVID-19 mRNA-LNP vaccine in Syrian golden hamsters.[115] While a 2-dose regimen of intranasally-administered mRNA-LNPs resulted in successful immunogenicity and protection, this vaccination approach required much higher mRNA doses than the i.m. controls and did not result in higher titers of IgA antibodies. In line with this report, Blakney et al. found that intranasal delivery of a saRNA LNP formulation achieved lower immunogenicity than after i.m. administration (both measured for humoral- and cellular immunity).[117] Ndeupen et al. showed even more dramatically that intranasal delivery of mRNA-LNPs leads to massive inflammation in the lungs and a high mortality rate in mice. These reports clearly warrant the need for further optimization of the lipid carrier or exploration of other delivery systems to achieve safe and effective mucosal delivery of mRNA vaccines.[85]

Modification of Lipid-based Nanoparticles to Enhance APC Targeting

In addition to the administration route, uptake of mRNA vaccines in APCs is also determined by the lipid carrier encapsulating the mRNA payload. Like most nanoparticles, lipid-based nanoparticles exhibit inherent liver tropism, especially when administered intravenously. A clinical example hereof is Onpattro™, an FDA-approved siRNA LNP drug for the treatment of a genetic liver disease, which is passively targeted to hepatocytes upon i.v. administration.[27] Lipid-based nanoparticles adopt a biological identity after administration, as endogenous biomolecules such as proteins adsorb to their surface, as such forming a biomolecular corona (Figure 4). The biomolecular corona is composed of specific proteins that can significantly alter the surface chemistry of the LNPs and dictates preferential cellular uptake through receptor-mediated endocytosis.[118,119] In the case of Onpattro, the uptake in hepatocytes has been attributed to the surface adsorption of apolipoprotein E (ApoE), thereby enabling uptake through low-density lipoprotein receptor (LDLR)-mediated endocytosis.[120] Although liver tropism of lipid-based nanoparticles is desired in the treatment of liver diseases, uptake in the liver is in most cases unfavorable in the context of cancer vaccination where the aim is to target organs rich in APCs (such as spleen and lymph nodes) and to optimally activate anti-tumor T cells. Nevertheless, it should be noted that targeting mRNA vaccines to the liver could be advantageous for the treatment of liver cancer. Nakamae et al. found that mRNA-LNPs targeting the liver expanded the amount of OVA-specific $CD8^+$ T cells of OT-I mice (OVA TCR transgenic mice) in the liver, more than in the spleen.[121] Interestingly, they also observed a drastically higher presence of antigen-specific T cells with a T_{RM} phenotype in the liver compared to the spleen. Therefore, liver-targeting mRNA vaccines that evoke protective T_{RM} immunity could hold potential for the treatment of liver cancer.[122] It is possible that other localized tumors could also be considered for this approach.

In most cases, however, it is desired to deliver mRNA extrahepatic, therefore, researchers are investigating the modification of lipid compositions in hopes of achieving higher organ- and cell-specificity. Many recent efforts have focused on including a charged lipid in the LNP-formulation by introducing an additional lipid component. An example hereof involves the use of a selective organ targeting (SORT) molecule to assess the influence of LNP charge for the delivery of mRNA to specific organs as demonstrated by Cheng et al.[44] More specifically, it was found that the addition of an anionic lipid (e.g. 18PA, 14PA and 18BMP) to several ionizable lipid LNPs (based on 5A2-SC8, DLin-MC3-DMA or C12-200) yielded selective transfection of spleen tissue, including transfection of spleen B cells, T cells, and macrophages. Note, however, that 18PA-SORT mRNA-LNPs also were taken up in the liver but mRNA expression was limited to the spleen.[123] Using a similar strategy, Luozhong et al. demonstrated that the addition of the negatively charged 1,2-dioleoyl-sn-glycero-3-phospho-l-serine (DOPS) to conventional mRNA MC3-LNPs led to a more selective and potent transfection of the spleen and lymph nodes post i.v. administration in comparison with 18-PA SORT MC3-LNPs.[124] Although it should be noted that both formulations had different molar compositions, the increased transfection and specificity of DOPS MC3-LNPs could be attributed to the phosphatidylserine (PS) lipid, which is a potential target for scavenger or PS receptors on APCs, thereby promoting endocytosis and leading to improved transfection in these cell types.[125] DOPS-LNPs were successfully taken up by dendritic cells, neutrophils, lymphocytes, eosinophils, and macrophages. Note that, contrary to the findings of Cheng et al., this study suggests that spleen and lymph node targeting also depends on the anionic lipid choice. Interestingly, it was found that the addition of spleen-targeting SORT molecules, such as 18PA, shifted the pKa of the corresponding LNP from 6-7 (optimal for hepatic delivery) to 2-6.[123] Additionally, the authors reported that spleen-targeting SORT LNPs established a predominant β2-glycoprotein I (β2-GPI) biomolecular corona, as opposed to the liver-SORT LNPs that have an ApoE-enriched biomolecular corona. It was hypoth-

esized by the authors that this adsorption was induced by the chemical structure and charge of the head group of the SORT lipid.

Besides spleen-targeting SORT-strategies, it should also be mentioned that Cheng et al. demonstrated that, using DOTAP as a positively charged SORT lipid, mRNA-LNPs can be targeted toward the lung and are able to transfect 20% of the immune cells in the lung upon i.v. administration. As discussed earlier in this review, although cationic LNPs can enable selective lung targeting, there are still many safety concerns to address such as thrombosis[45] or elevated levels of cytotoxicity[125] manifesting from the LNP's positive charge.

The relative composition of the constituting lipids of the carrier and the type of lipids included in the lipid formulation were also found to drive mRNA vaccines to immune-rich tissues. Bevers et al. found that the chemistry of the PEG-conjugated lipid in the LNP-formulation affected the T cell response of an i.v. administered mRNA-LNP vaccine.[127] It was observed that increasing the molar ratios of ionizable cationic lipid and lowering the ratios of phospholipid (DOPE) and PEG-conjugated lipid resulted in larger LNP sizes (>100 nm), and further improved the CD8 T cell response. Optimized LNPs containing DSG-PEG_{2000} were found to transfect APCs (macrophages, DCs and B cells) in the spleen. The authors hypothesize that the preferential spleen expression upon repeated administration is due to the long acyl chain of the PEG-conjugated lipid, which increases immunoglobulin opsonization (binding of anti-PEG antibodies) and potentially alters cell tropism. Alternatively, optimization of the ionizable lipid chemistry has also been reported to improve increased specificity toward the spleen and lymph nodes.[128,129] Additionally, the ionizable lipid structures included in mRNA-LNPs have been found to drive tropism to other organs.[130] For instance, lipidoids with varied tail chemistry (N-series LNPs) have demonstrated mRNA delivery to the lung while targeting pulmonary subcellular populations following systemic administration. This selective delivery was suggested to be indicative of the relative differences in its protein corona, which is mostly comprised of serum albumin, fibrinogen beta chain, and fibrinogen gamma chain, which may have improved endothelial cell adhesion and internalization. Despite the differences between the protein corona profiles, no significant differences emerged between the zeta potential of the two LNPs.[131]

Finally, it should be noted that LNP size also contributes to the passive targeting of mRNA-LNPs, but these studies are often challenging to conduct. One way to control LNP size is by titrating the molar ratio of PEG-lipid within the formulation,[132] but this can alter *in vivo* performance as the ratio of the diffusible PEG-lipid is crucial for preventing LNP aggregation during formation, providing LNP stability upon administration and for enhancing endosomal escape *in vivo*.[133,134] Other studies have induced changes in particle size through the addition of salt[135,136] or lipid choice.[137] However, it is unclear what the effects of ionic strength or lipid type might have on biodistribution.

Although the simple design of a passive targeting mRNA lipid system is preferable for clinical translation by avoiding the complex synthesis, purification, and upscaling of GMP manufacturing which is associated with approaches using active targeting moieties, optimal targeting of mRNA cancer immunotherapeutics remains an ongoing topic of research.[138] It is still difficult to contextualize and understand the structure–activity relationships between LNP and immune cell interaction. More research with systematic and rational design is needed to better clarify the role of different functional lipid components and selective targeting of LNPs as the current observations are often contradictory and incomplete. This would clearly contribute to our understanding of how we can improve delivery to specific APCs and highlight their fundamental role in next-generation cancer vaccines.

Conclusions

In the past few years, tremendous progress has been made in the field of mRNA vaccination using lipid-based carriers. Ongoing clinical trials by Moderna/Merck and BioNTech/Roche have both successfully demonstrated the utility of mRNA as a cancer vaccine against melanoma (mRNA-4157, BNT111) and pancreatic cancer (BNT122). While the technologies being used in authorized mRNA vaccines for the prevention of COVID-19 have high similarities, mRNA vaccines in clinical development for cancer vaccination show marked differences. We discussed that the outcome of lipid-based mRNA platforms for cancer vaccination depends on the complex interplay of the vaccine's components, the administration route, and the capacity of the vaccine to target relevant cell types. mRNA packaged in lipid carriers not only enables the synthesis of tumor antigens, but both components can also stimulate the innate immune system. The innate immune-activity of the mRNA vaccine should be carefully balanced to optimally stimulate APCs and T cells and studies focusing on cellular immunity in response to both vaccine's components could help to fully capitalize on the potential of mRNA cancer vaccines. In addition, a better understanding of the nano-bio interaction and the structure–activity relationship of LNPs will benefit the rational design of these carrier systems, which also plays a critical part in safely and efficiently delivering mRNA to elicit a strong anti-tumor immune response while avoiding adverse events. While comparative preclinical studies have

informed our understanding of how different parameters (e.g. lipid carrier, type of mRNA, and mode of administration) can dictate the potency and safety of mRNA cancer vaccines, there is also the danger of overestimating or generalizing the conclusions of these studies. Certainly, since other variables besides the design of the vaccine may also impact the overall performance of mRNA vaccines, such as several manufacturing parameters,[139] e.g., microfluidic conditions,[140] purification steps, excipients[141] and/or other currently unknown factors.

To conclude, there is still a large playground to further advance and optimize mRNA vaccines for therapeutic use in cancer patients. Further improvement of the anti-tumor immune response could be obtained by the inclusion of adjuvants and the synergy with immune checkpoint inhibitors (ICIs), while remaining alert for immune related adverse events. The majority of currently clinically evaluated mRNA cancer vaccines are applied as a synergistic approach with ICIs. Given the encouraging results of mRNA cancer vaccines evaluated in this setting, we expect mRNA cancer vaccines to show their first benefit in improving the therapeutic outcome in response to ICIs.

CRediT authorship contribution statement

Sofie Meulewaeter: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Yao Zhang:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Abishek Wadhwa:** Writing – original draft, Writing – review & editing. **Kevin Fox:** . **Ine Lentacker:** . **Kenneth W. Harder:** Funding acquisition, Supervision, Writing – review & editing. **Pieter R. Cullis:** Funding acquisition, Supervision. **Stefaan C. De Smedt:** Funding acquisition, Supervision. **Miffy H. Y. Cheng:** Conceptualization, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Rein Verbeke:** Conceptualization, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

DATA AVAILABILITY

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

DECLARATION OF COMPETING INTEREST

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: R.V., I.L., and S.D.S. are contributors to patent applications no. WO2020058239A1; Therapeutic nanoparticles and methods of use thereof, and no. EP22170845.6; Vaccine Compositions against Listeria Infection. K.W.H. has a financial interest and is a director of Myeloid Enhancement Therapeutics Inc. P.R.C. has a financial interest in Acuitas Therapeutics and NanoVation Therapeutics as well as being Chair of NanoVation Therapeutics.

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† Last authors contributed equally to this work.

References

1. Vega, Y.I.F. et al, (2023). Survival of NSCLC patients treated with Cimavax-EGF as switch maintenance in the real-world scenario. *J. Cancer* **14**, 874.
2. Liu, J. et al, (2022). Cancer vaccines as promising immuno-therapeutics: platforms and current progress. *J. Hematol. Oncol.* **15**, 1–26.
3. Verbeke, R., Lentacker, I., De Smedt, S.C., Dewitte, H., (2021). The dawn of mRNA vaccines: The COVID-19 case. *J. Control Release* **333**, 511–520.
4. Tarke, A. et al, (2022). SARS-CoV-2 vaccination induces immunological T cell memory able to cross-recognize variants from Alpha to Omicron. *Cell* **185**, 847–859.e11.
5. Reinscheid, M. et al, (2022). COVID-19 mRNA booster vaccine induces transient CD8+ T effector cell responses

while conserving the memory pool for subsequent reactivation. *Nature Commun.* **13**, 1–11.
6. Oberhardt, V. et al, (2021). Rapid and stable mobilization of CD8+ T cells by SARS-CoV-2 mRNA vaccine. *Nature* **597**, 268–273.
7. Guerrera, G. et al, (2021). BNT162b2 vaccination induces durable SARS-CoV-2-specific T cells with a stem cell memory phenotype. *Sci. Immunol.* **6**
8. Moderna and Merck Announce mRNA-4157 (V940) in Combination With KEYTRUDA(R) (pembrolizumab) Demonstrated a Statistically Significant and Clinically Meaningful Improvement in Distant Metastasis-Free Survival in Patients with High-Risk Stage III/IV Melanoma Following Complete Resection Versus KEYTRUDA. https://investors.modernatx.com/news/news-details/2023/Moderna-and-Merck-Announce-mRNA-4157-V940-in-Combination-With-KEYTRUDAR-pembrolizumab-Demonstrated-a-Statistically-Significant-and-Clinically-Meaningful-Improvement-in-Distant-Metastasis-Free-Survival-in-Patients-with-High-Risk-Stage-IIIIV-Melanoma-F/default.aspx
9. Rojas, L.A. et al, (2023). Personalized RNA neoantigen vaccines stimulate T cells in pancreatic cancer. *Nature* **618**, 144–150.
10. Kranz, L.M. et al, (2016). Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy. *Nature* **534**, 396–401.
11. Sahin, U. et al, (2017). Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature* **547**, 222–226.
12. Small, E.J. et al, (2006). Placebo-controlled phase III trial of immunologic therapy with sipuleucel-T (APC8015) in patients with metastatic, asymptomatic hormone refractory prostate cancer. *J. Clin. Oncol.* **24**, 3089–3094.
13. Dewitte, H., Verbeke, R., Breckpot, K., De Smedt, S.C., Lentacker, I., (2014). Nanoparticle design to induce tumor immunity and challenge the suppressive tumor microenvironment. *Nano Today* **9**, 743–758.
14. Igarashi, Y., Sasada, T., (2020). Cancer vaccines: toward the next breakthrough in cancer immunotherapy. *J. Immunol. Res.* **2020**
15. Kreiter, S. et al, (2008). Increased antigen presentation efficiency by coupling antigens to MHC class I trafficking signals. *J. Immunol.* **180**, 309–318.
16. Dimitriadis, G.J., (1978). Translation of rabbit globin mRNA introduced by liposomes into mouse lymphocytes. *Nature* **274**, 923–924.
17. Ostro, M.J., Giacomoni, D., Lavelle, D., Paxton, W., Dray, S., (1978). Evidence for translation of rabbit globin mRNA after liposome-mediated insertion into a human cell line. *Nature* **274**, 921–923.
18. Wolff, J.A. et al, (1990). Direct gene transfer into mouse muscle in vivo. *Science* **1979** (247), 1465–1468.
19. Sahin, U., Karikó, K., Türeci, Ö., (2014). mRNA-based therapeutics — developing a new class of drugs. *Nature Rev. Drug Discov.* **13**, 759–780.
20. Chaudhary, N., Weissman, D., Whitehead, K.A., (2021). mRNA vaccines for infectious diseases: principles, delivery and clinical translation. *Nature Rev. Drug Discov.* **20**, 817–838.
21. Karikó, K., Buckstein, M., Ni, H., Weissman, D., (2005). Suppression of RNA recognition by toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity* **23**, 165–175.
22. Weide, B. et al, (2008). Results of the first phase I/II clinical vaccination trial with direct injection of mRNA. *J. Immunother.* **31**, 180–188.
23. Zeng, C., Zhang, C., Walker, P.G., Dong, Y., (2022). Formulation and delivery technologies for mRNA vaccines. *Curr. Top. Microbiol. Immunol.* **440**, 71.
24. Paunovska, K., Loughrey, D., Dahlman, J.E., (2022). Drug delivery systems for RNA therapeutics. *Nature Rev. Genet.* **23**, 265–280.
25. Polack, F.P. et al, (2020). Safety and efficacy of the BNT162b2 mRNA covid-19 vaccine. *N. Engl. J. Med.* **383**, 2603–2615.
26. Baden, L.R. et al, (2021). Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. *N. Engl. J. Med.* **384**, 403–416.
27. Adams, D. et al, (2018). Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis. *N. Engl. J. Med.* **379**, 11–21.
28. Sun, D., Lu, Z.R., (2023). Structure and function of cationic and ionizable lipids for nucleic acid delivery. *Pharm. Res.* **40**, 27.
29. Barbier, A.J., Jiang, A.Y., Zhang, P., Wooster, R., Anderson, D.G., (2022). The clinical progress of mRNA vaccines and immunotherapies. *Nature Biotechnol.* **40**, 840–854.
30. Lorentzen, C.L., Haanen, J.B., Met, Ö., Svane, I.M., (2022). Clinical advances and ongoing trials on mRNA vaccines for cancer treatment. *Lancet Oncol.* **23**, e450–e458.
31. Rosenberg, S.A. et al, (2005). Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. *J. Immunol.* **175**, 6169–6176.
32. Sahin, U. et al, (2020). An RNA vaccine drives immunity in checkpoint-inhibitor-treated melanoma. *Nature* **585**, 107–112.
33. Kyewski, B., Derbinski, J., (2004). Self-representation in the thymus: an extended view. *Nature Rev. Immunol.* **4**, 688–698.
34. Xie, N. et al, (2023). Neoantigens: promising targets for cancer therapy. *Signal Trans. Target. Therapy* **8**, 1–38.
35. Lang, F., Schrörs, B., Löwer, M., Türeci, Ö., Sahin, U., (2022). Identification of neoantigens for individualized therapeutic cancer vaccines. *Nature Rev. Drug Discov.* **21**, 261–282.
36. White P. Moderna Science and Technology Day. (2022).
37. Ren, T., Song, Y.K., Zhang, G., Liu, D., (2000). Structural basis of DOTMA for its high intravenous transfection activity in mouse. *Gene Therapy* **7**, 764–768.
38. Regelin, A.E. et al, (2000). Biophysical and lipofection studies of DOTAP analogs. *Biochim. Biophys. Acta (BBA) – Biomembr.* **1464**, 151–164.
39. Hafez, I.M., Maurer, N., Cullis, P.R., (2001). On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. *Gene Therapy* **8**, 1188–1196.
40. Verbeke, R. et al, (2019). Broadening the message: a nanovaccine co-loaded with messenger RNA and α-GalCer induces antitumor immunity through conventional and natural killer T cells acsnano.8b07660 *ACS Nano*. https://doi.org/10.1021/acsnano.8b07660.
41. Verbeke, R. et al, (2017). Co-delivery of nucleoside-modified mRNA and TLR agonists for cancer

immunotherapy: restoring the immunogenicity of immunosilent mRNA. *J. Control. Release* **266**, 287–300.
42. Wei, X. et al, (2015). Cationic nanocarriers induce cell necrosis through impairment of Na+/K+-ATPase and cause subsequent inflammatory response. *Cell Res.* **25**, 237–253.
43. Lv, H., Zhang, S., Wang, B., Cui, S., Yan, J., (2006). Toxicity of cationic lipids and cationic polymers in gene delivery. *J. Control. Release* **114**, 100–109.
44. Cheng, Q. et al, (2020). Selective organ targeting (SORT) nanoparticles for tissue-specific mRNA delivery and CRISPR–Cas gene editing. *Nature Nanotechnol.* **15**, 313–320.
45. Omo-Lamai, S. *et al.* Physicochemical targeting of lipid nanoparticles to the lungs induces clotting: mechanisms and solutions. *bioRxiv* 2023.07.21.550080 (2023) doi:10.1101/2023.07.21.550080.
46. Comirnaty 30 micrograms/dose concentrate for dispersion for injection 12+ years COVID-19 mRNA Vaccine (nucleoside modified) - Summary of Product Characteristics (SmPC) - (emc). https://www.medicines.org.uk/emc/product/12740/smpc#SHELF_LIFE
47. ANNEX I SUMMARY OF PRODUCT CHARACTERISTICS..
48. Patel, P., Ibrahim, N.M., Cheng, K., (2021). The importance of apparent pka in the development of nanoparticles encapsulating siRNA and mRNA. *Trends Pharmacol. Sci.* **42**, 448–460.
49. Schlich, M. et al, (2021). Cytosolic delivery of nucleic acids: the case of ionizable lipid nanoparticles. *Bioeng. Transl. Med.* **6**
50. Pardi, N. et al, (2015). Expression kinetics of nucleoside-modified mRNA delivered in lipid nanoparticles to mice by various routes. *J. Control. Release* **217**, 345–351.
51. Broos, K. et al, (2016). Particle-mediated intravenous delivery of antigen mRNA results in strong antigen-specific T-cell responses despite the induction of type I interferon. *Mol. Ther. Nucleic Acids* **5**, e326.
52. Van Hoecke, L. et al, (2020). The opposing effect of type I IFN on the T cell response by non-modified mRNA-lipoplex vaccines is determined by the route of administration. *Mol. Ther. Nucleic Acids* **22**, 373.
53. Tang, M. et al, (2023). Efficient mRNA delivery with mRNA lipoplexes prepared using a modified ethanol injection method. *Pharmaceutics* **15**
54. Hassett, K.J. et al, (2019). Optimization of lipid nanoparticles for intramuscular administration of mRNA vaccines. *Mol. Ther. Nucleic Acids* **15**, 1–11.
55. Han, X. et al, (2021). An ionizable lipid toolbox for RNA delivery. *Nature Commun.* **12**, 1–6.
56. Alameh, M.-G., Weissman, D., (2022). *Nucleoside modifications of in vitro transcribed mRNA to reduce immunogenicity and improve translation of prophylactic and therapeutic antigens.*. https://doi.org/10.1016/B978-0-12-821595-1.00014-2.
57. Minnaert, A.K. et al, (2021). Strategies for controlling the innate immune activity of conventional and self-amplifying mRNA therapeutics: Getting the message across. *Adv. Drug Deliv. Rev.* **176**, 113900
58. Verbeke, R., Hogan, M.J., Loré, K., Pardi, N., (2022). Innate immune mechanisms of mRNA vaccines. *Immunity* **55**, 1993–2005.
59. Verbeke, R., Lentacker, I., De Smedt, S.C., Dewitte, H., (2019). Three decades of messenger RNA vaccine development. *Nano Today* **28**, 100766
60. Honke, N. et al, (2011). Enforced viral replication activates adaptive immunity and is essential for the control of a cytopathic virus. *Nature Immunol.* **13**, 51–57.
61. Karikó, K. et al, (2008). Incorporation of pseudouridine into mrna yields superior nonimmunogenic vector with increased translational capacity and biological stability. *Mol. Ther.* **16**, 1833–1840.
62. Andries, O. et al, (2015). N(1)-methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice. *J. Control. Release* **217**, 337–344.
63. Sahin, U. et al, (2021). BNT162b2 vaccine induces neutralizing antibodies and poly-specific T cells in humans. *Nature* **595**, 572–577.
64. Corbett, K.S. et al, (2020). SARS-CoV-2 mRNA vaccine design enabled by prototype pathogen preparedness. *Nature* **586**, 567–571.
65. Kremsner, P.G. et al, (2022). Efficacy and safety of the CVnCoV SARS-CoV-2 mRNA vaccine candidate in ten countries in Europe and Latin America (HERALD): a randomised, observer-blinded, placebo-controlled, phase 2b/3 trial. *Lancet Infect. Dis.* **22**, 329–340.
66. Gebre, M.S. et al, (2022). Optimization of non-coding regions for a non-modified mRNA COVID-19 vaccine. *Nature* **601**, 410–414.
67. CureVac Announces Positive Data on Joint COVID-19 and Flu mRNA Vaccine Development Programs - CureVac. https://www.curevac.com/en/curevac-announces-positive-data-on-joint-covid-19-and-flu-mrna-vaccine-development-programs/
68. Kremsner, P.G. et al, (2021). Safety and immunogenicity of an mRNA-lipid nanoparticle vaccine candidate against SARS-CoV-2: A phase 1 randomized clinical trial. *Wien. Klin. Wochenschr.* **133**, 931.
69. Morais, P., Adachi, H., Yu, Y.T., (2021). The critical contribution of pseudouridine to mRNA COVID-19 vaccines. *Front. Cell Dev. Biol.* **9**, 3187.
70. Li, C. et al, (2022). Mechanisms of innate and adaptive immunity to the Pfizer-BioNTech BNT162b2 vaccine. *Nature Immunol.* **23**, 543–555.
71. Wu, B. et al, (2013). Structural basis for dsRNA recognition, filament formation, and antiviral signal activation by MDA5. *Cell* **152**, 276–289.
72. Krienke, C. et al, (2021). A noninflammatory mRNA vaccine for treatment of experimental autoimmune encephalomyelitis. *Science* **1979** (371), 145–153.
73. De Beuckelaer, A. et al, (2016). Type I Interferons Interfere with the Capacity of mRNA Lipoplex Vaccines to Elicit Cytolytic T Cell Responses. *Mol. Ther.* **24**, 2012–2020.
74. Au-Yeung, B.B. et al, (2014). A sharp T-cell antigen receptor signaling threshold for T-cell proliferation. *PNAS* **111**, E3679–E3688.
75. Honke, N. et al, (2013). Usp18 driven enforced viral replication in dendritic cells contributes to break of immunological tolerance in autoimmune diabetes. *PLoS Pathog.* **9**, e1003650
76. Sittplangkoon, C. et al, (2022). mRNA vaccine with unmodified uridine induces robust type I interferon-

dependent anti-tumor immunity in a melanoma model. *Front. Immunol.* **13**, 983000
77. Knudson, C.J. et al, (2021). Lipid-nanoparticle-encapsulated mRNA vaccines induce protective memory CD8 T cells against a lethal viral infection. *Mol. Ther.* **29**, 2769–2781.
78. Ramos da Silva, J. et al, (2023). Single immunizations of self-amplifying or non-replicating mRNA-LNP vaccines control HPV-associated tumors in mice. *Sci. Transl. Med.* **15**, eabn3464
79. Zhang, H., Zhu, Z., Modrak, S., Little, A., (2022). Tissue-resident memory CD4+ T cells play a dominant role in the initiation of antitumor immunity. *J. Immunol.* **208**, 2837–2846.
80. Djenidi, F. et al, (2015). CD8+CD103+ tumor-infiltrating lymphocytes are tumor-specific tissue-resident memory T cells and a prognostic factor for survival in lung cancer patients. *J. Immunol.* **194**, 3475–3486.
81. de Alwis, R. et al, (2021). A single dose of self-transcribing and replicating RNA-based SARS-CoV-2 vaccine produces protective adaptive immunity in mice. *Mol. Ther.* **29**, 1970–1983.
82. Li, H. et al, (2022). Circular RNA cancer vaccines drive immunity in hard-to-treat malignancies. *Theranostics* **12**, 6422.
83. Palmer, C.D. et al, (2022). Individualized, heterologous chimpanzee adenovirus and self-amplifying mRNA neoantigen vaccine for advanced metastatic solid tumors: phase 1 trial interim results. *Nature Med.* **28**, 1619–1629.
84. Tahtinen, S. et al, (2022). IL-1 and IL-1ra are key regulators of the inflammatory response to RNA vaccines. *Nature Immunol.* **23**, 532–542.
85. Alameh, M.G. et al, (2021). Lipid nanoparticles enhance the efficacy of mRNA and protein subunit vaccines by inducing robust T follicular helper cell and humoral responses. *Immunity* **54**, 2877–2892.e7.
86. Ndeupen, S. et al, (2021). The mRNA-LNP platform's lipid nanoparticle component used in preclinical vaccine studies is highly inflammatory. *iScience* **24**, 103479
87. Gritstone and Genevant Sciences Announce License Agreement for COVID-19 Vaccine - Genevant Sciences Corporation. https://www.genevant.com/gritstone-and-genevant-sciences-announce-license-agreement-for-covid-19-vaccine/.
88. Lam, K. et al, (2023). Unsaturated, trialkyl ionizable lipids are versatile lipid-nanoparticle components for therapeutic and vaccine applications. *Adv. Mater.*. https://doi.org/10.1002/ADMA.202209624.
89. Melamed, J.R. et al, (2022). Lipid nanoparticle chemistry determines how nucleoside base modifications alter mRNA delivery. *J. Control. Release* **341**, 206–214.
90. Bernard, M.C. et al, (2023). The impact of nucleoside base modification in mRNA vaccine is influenced by the chemistry of its lipid nanoparticle delivery system. *Mol. Ther. Nucleic Acids* **32**, 794–806.
91. Maier, M.A. et al, (2013). Biodegradable lipids enabling rapidly eliminated lipid nanoparticles for systemic delivery of RNAi therapeutics. *Mol. Ther.* **21**, 1570.
92. Pan, L. et al, (2023). Spleen-selective co-delivery of mRNA and TLR4 agonist-loaded LNPs for synergistic immunostimulation and Th1 immune responses. *J. Control. Release* **357**, 133–148.
93. Ganley, M. et al, (2023). mRNA vaccine against malaria tailored for liver-resident memory T cells. *Nature Immunol.* **2023** (3), 1–12.
94. Fan, N. et al, (2022). Manganese-coordinated mRNA vaccines with enhanced mRNA expression and immunogenicity induce robust immune responses against SARS-CoV-2 variants. *Sci. Adv.* **8**
95. Tse, S.W. et al, (2021). mRNA-encoded, constitutively active STINGV155M is a potent genetic adjuvant of antigen-specific CD8+ T cell response. *Mol. Ther.* **29**, 2227–2238.
96. Tockary, T.A. et al, (2023). Comb-structured mRNA vaccine tethered with short double-stranded RNA adjuvants maximizes cellular immunity for cancer treatment. *PNAS* **120**
97. Han, X. et al, (2023). Adjuvant lipidoid-substituted lipid nanoparticles augment the immunogenicity of SARS-CoV-2 mRNA vaccines. *Nature Nanotechnol.* **2023** (12), 1–10.
98. Miao, L. et al, (2019). Delivery of mRNA vaccines with heterocyclic lipids increases anti-tumor efficacy by STING-mediated immune cell activation. *Nature Biotechnol.* **37**, 1174–1185.
99. Zhang, H. et al, (2021). Delivery of mRNA vaccine with a lipid-like material potentiates antitumor efficacy through Toll-like receptor 4 signaling. *PNAS* **118**
100. Zhang, J., Huang, D., Saw, P.E., Song, E., (2022). Turning cold tumors hot: from molecular mechanisms to clinical applications. *Trends Immunol.* **43**, 523–545.
101. Herbst, R.S. et al, (2014). Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* **515**, 563–567Rini, B.I. et al, (2016). IMA901, a multipeptide cancer vaccine, plus sunitinib versus sunitinib alone, as first-line therapy for advanced or metastatic renal cell carcinoma (IMPRINT): a multicentre, open-label, randomised, controlled, phase 3 trial. *Lancet Oncol.* **17**, 1599–1611.
102. Hassouneh, A. et al, (2022). Immune checkpoint inhibitors for vaccine improvements: current status and new approaches. *Pharmaceutics* **14**, 1721.
103. .
104. Lang, K.S. et al, (2005). Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. *Nature Med.* **11**, 138–145.
105. Liang, F. et al, (2017). Efficient targeting and activation of antigen-presenting cells in vivo after modified mRNA vaccine administration in rhesus macaques. *Mol. Ther.* **25**, 2635–2647.
106. Liang, F. et al, (2017). Vaccine priming is restricted to draining lymph nodes and controlled by adjuvant-mediated antigen uptake. *Sci. Transl. Med.* **9**
107. .
108. Temtanakitpaisan, Y. et al, (2022). Reactogenicity and immunogenicity of the intradermal administration of BNT162b2 mRNA vaccine in healthy adults who were primed with an inactivated SARS-CoV-2 vaccine. *Vaccine X* **12**, 100242
109. Feldman, R.A. et al, (2019). mRNA vaccines against H10N8 and H7N9 influenza viruses of pandemic potential are immunogenic and well tolerated in healthy adults in phase 1 randomized clinical trials. *Vaccine* **37**, 3326–3334.

110. Blair, D.A. et al, (2011). Duration of antigen availability influences the expansion and memory differentiation of T cells. *J. Immunol.* **187**, 2310.
111. Syenina, A. et al, (2022). Adverse effects following anti-COVID-19 vaccination with mRNA-based BNT162b2 are alleviated by altering the route of administration and correlate with baseline enrichment of T and NK cell genes. *PLoS Biol.* **20**
112. Yavuz, A. et al, (2023). DLin-MC3-containing mRNA lipid nanoparticles induce an antibody Th2-Biased immune response polarization in a delivery route-dependent manner in mice. *Pharmaceutics* **15**
113. Münter, R., Christensen, E., Andresen, T.L., Larsen, J.B., (2023). Studying how administration route and dose regulates antibody generation against LNPs for mRNA delivery with single-particle resolution. *Mol. Ther. Methods Clin. Dev.* **29**, 450–459.
114. vander Straeten, A. et al, (2023). A microneedle vaccine printer for thermostable COVID-19 mRNA vaccines. *Nature Biotechnol.* **1–8** https://doi.org/10.1038/s41587-023-01774-z.
115. Prins, M.L.M., Prins, C., de Vries, J.J.C., Visser, L.G., Roukens, A.H.E., (2023). Establishing immunogenicity and safety of needle-free intradermal delivery by nanoporous ceramic skin patch of mRNA SARS-CoV-2 vaccine as a revaccination strategy in healthy volunteers. *Virus Res.* **334**, 199175
116. Vaca, G.B. et al, (2023). Intranasal mRNA-LNP vaccination protects hamsters from SARS-CoV-2 infection. *Sci. Adv.* **9**
117. Blakney, A.K. et al, (2021). Polymeric and lipid nanoparticles for delivery of self-amplifying RNA vaccines. *J. Control. Release* **338**, 201–210.
118. Akinc, A. et al, (2019). The Onpattro story and the clinical translation of nanomedicines containing nucleic acid-based drugs. *Nature Nanotechnol.* **14**, 1084–1087.
119. Francia, V., Schiffelers, R.M., Cullis, P.R., Witzigmann, D., (2020). The biomolecular corona of lipid nanoparticles for gene therapy. *Bioconjug. Chem.* **31**, 2046–2059.
120. Akinc, A. et al, (2010). Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms. *Mol. Ther.* **18**, 1357–1364.
121. Nakamae, S. et al, (2023). Induction of liver-resident memory T cells and protection at liver-stage malaria by mRNA-containing lipid nanoparticles. *Front. Immunol.* **14**, 1116299.
122. Pallett, L.J., Maini, M.K., (2022). Liver-resident memory T cells: life in lockdown. *Sem. Immunopathol.* **44**, 813–825.
123. Dilliard, S.A., Cheng, Q., Siegwart, D.J., (2021). On the mechanism of tissue-specific mRNA delivery by selective organ targeting nanoparticles. *Proc. Natl. Acad. Sci. USA* **118**, e2109256118
124. Luozhong, S. et al, (2022). Phosphatidylserine lipid nanoparticles promote systemic RNA delivery to secondary lymphoid organs. *Nano Letter* **22**, 8304–8311.
125. Segawa, K., Nagata, S., (2015). An apoptotic 'eat me' signal: phosphatidylserine exposure. *Trends Cell Biol.* **25**, 639–650.
126. Cui, S. et al, (2018). Correlation of the cytotoxic effects of cationic lipids with their headgroups. *Toxicol Res (Camb)* **7**, 473–479.
127. Bevers, S. et al, (2022). mRNA-LNP vaccines tuned for systemic immunization induce strong antitumor immunity by engaging splenic immune cells. *Mol. Ther.* **30**, 3078–3094.
128. Naidu, G.S. et al, (2023). A combinatorial library of lipid nanoparticles for Cell type-specific mRNA delivery. *Adv. Sci.* **10**
129. Chen, J. et al, (2022). Lipid nanoparticle-mediated lymph node–targeting delivery of mRNA cancer vaccine elicits robust CD8+ T cell response. *Proc. Natl. Acad. Sci. USA* **119**
130. Ni, H. et al, (2022). Piperazine-derived lipid nanoparticles deliver mRNA to immune cells in vivo. *Nature Commun.* **13**, 1–9.
131. Qiu, M. et al, (2022). Lung-selective mRNA delivery of synthetic lipid nanoparticles for the treatment of pulmonary lymphangioleiomyomatosis. *PNAS* **119**
132. Nakamura, T. et al, (2020). The effect of size and charge of lipid nanoparticles prepared by microfluidic mixing on their lymph node transitivity and distribution. *Mol. Pharm.* **17**, 944–953.
133. Kulkarni, J.A. et al, (2021). The current landscape of nucleic acid therapeutics. *Nature Nanotechnol.* **16**, 630–643.
134. Witzigmann, D. et al, (2020). Lipid nanoparticle technology for therapeutic gene regulation in the liver. *Adv. Drug Deliv. Rev.* **159**, 344–363.
135. Cheng, M.H.Y. et al, (2023). Induction of Bleb Structures in Lipid Nanoparticle Formulations of mRNA Leads to Improved Transfection Potency. *Adv. Mater.* **35**, 2303370.
136. Okuda, K. et al, (2022). On the size-regulation of RNA-loaded lipid nanoparticles synthesized by microfluidic device. *J. Control. Release* **348**, 648–659.
137. Sasaki, K., Sato, Y., Okuda, K., Iwakawa, K., Harashima, H., (2022). mRNA-Loaded Lipid Nanoparticles Targeting Dendritic Cells for Cancer Immunotherapy. *Pharmaceutics* **14**
138. Kon, E., Elia, U., Peer, D., (2022). Principles for designing an optimal mRNA lipid nanoparticle vaccine. *Curr. Opin. Biotechnol.* **73**, 329–336.
139. Daniel, S., Kis, Z., Kontoravdi, C., Shah, N., (2022). Quality by design for enabling RNA platform production processes. *Trends Biotechnol.* **40**, 1213–1228.
140. Strelkova Petersen, D.M., Chaudhary, N., Arral, M.L., Weiss, R.M., Whitehead, K.A., (2023). The mixing method used to formulate lipid nanoparticles affects mRNA delivery efficacy and organ tropism. *Eur. J. Pharm. Biopharm.* **192**, 126–135.
141. Meulewaeter, S. et al, (2023). Continuous freeze-drying of messenger RNA lipid nanoparticles enables storage at higher temperatures. *J. Control. Release* **357**, 149–160.
142. Linch, M. et al, (2021). 421 A first-in-human (FIH) phase I/IIa clinical trial assessing a ribonucleic acid lipoplex (RNA-LPX) encoding shared tumor antigens for immunotherapy of prostate cancer; preliminary analysis of PRO-MERIT. *J. Immunother. Cancer* **9**, A451–A.
143. Mackensen, A. et al, (2021). 958 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**, A1008–A.
144. Kopetz, S. et al.A phase 2 multicenter, open-label, randomized, controlled trial in patients with stage II/III colorectal cancer who are ctDNA positive following resection to compare efficacy of autogene cevumeran

versus watchful waiting. https://doi.org/10.1200/JCO.2022.40.16_suppl.TPS364140, TPS3641–TPS3641 (2022).

145. CureVac Doses First Patient in Phase 1 Study of Cancer Vaccine Candidate for Surgically Resected Glioblastoma - CureVac. https://www.curevac.com/en/curevac-doses-first-patient-in-phase-1-study-of-cancer-vaccine-candidate-for-surgically-resected-glioblastoma/.

146. Powderly, J. D. et al. Phase 1/2 study of mRNA-4359 administered alone and in combination with immune checkpoint blockade in adult participants with advanced solid tumors. https://doi.org/10.1200/JCO.2023.41.16_suppl.TPS267641, TPS2676–TPS2676 (2023).