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The immunostimulatory nature of mRNA lipid nanoparticles

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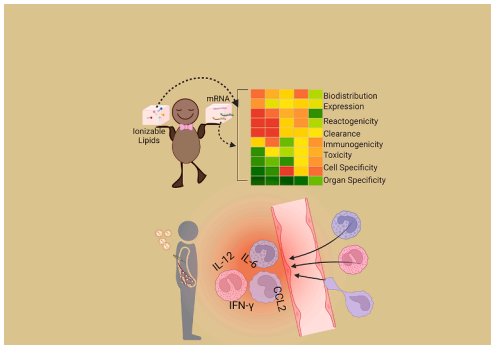
GRAPHICAL ABSTRACT



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ABSTRACT

mRNA-Lipid nanoparticles (LNPs) are at the forefront of global medical research. With the development of mRNA-LNP vaccines to combat the COVID-19 pandemic, the clinical potential of this platform was unleashed. Upon administering 16 billion doses that protected billions of people, it became clear that a fraction of them witnessed mild and in some cases even severe adverse effects. Therefore, it is paramount to define the safety along with the therapeutic efficacy of the mRNA-LNP platform for the successful translation of new genetic medicines based on this technology. While mRNA was the effector molecule of this platform, the ionizable lipid component of the LNPs played an indispensable role in its success. However, both of these components possess the ability to induce undesired immunostimulation, which is an area that needs to be addressed systematically. The immune cell agitation caused by this platform is a two-edged sword as it may prove beneficial for vaccination but detrimental to other applications. Therefore, a key challenge in advancing the mRNA-LNP drug delivery platform from bench to bedside is understanding the immunostimulatory behavior of these components. Herein, we provide a detailed overview of the structural modifications and immunogenicity of synthetic mRNA. We discuss the effect of ionizable lipid structure on LNP functionality and offer a mechanistic overview of the

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ability of LNPs to elicit an immune response. Finally, we shed some light on the current status of this technology in clinical trials and discuss a few challenges to be addressed to advance the field.

1. Introduction

Messenger RNA (mRNA) was discovered by pioneering studies in 1961 during attempts to unravel how the genetic message gets from DNA to produce proteins.[1,2] In the following years, researchers began to synthesize *in vitro*–transcribed mRNA (IVT mRNA). The newly synthesized IVT mRNA emerged as a new class of drug with the potential to revolutionize the field of gene therapy, a role that was being envisioned for DNA. However, a suitable delivery system was required for efficient treatment of patients. Fortuitously, a second class of biomolecules was investigated almost parallel to mRNA, *i.e.*, lipids.

Lipids were being investigated for their ability to form nanoparticles for usage as drug delivery systems. Liposomes, the first generation of LNPs, successfully made it to the clinic in 1995 in the form of Doxil™. Doxil™ (doxorubicin liposomes) is an anti-tumor agent used to treat ovarian cancer and is the earliest example of LNPs being rolled out in the market.[3,4] Over the next two decades, LNPs were comprehensively investigated, and many successfully entered the clinic. These include, *e.g.,* Doxil™ (1995; doxorubicin in polyethylene glycol (PEG) conjugated liposome), DaunoXome™ (1996, daunorubicin citrate liposome) DepoCyt™ (1999; cytarabine), AmBisome™ (2000, amphotericin B liposome), MyoCet™ (2000; doxorubicin liposome), Visudyne™ (2002, verteporfin liposome) DepoDur™ (2004; morphine), Mepact™ (2009; mifamurtide), Exparel™ (2012; bupivacaine), Marqibo™ (2013; vincristine), Onivyde™ (2015; irinotecan in PEGylated liposome), Vyxeos™ (2017; cytarabine and daunorubicin).[5] It should be noted that in all of the drugs mentioned above, the LNPs encapsulated small molecules, not nucleic acids.

The first approved drug using nucleic acids encapsulated in LNPs was Onpattro™, which received regulatory approval in August 2018. This LNP formulation carries short interfering RNA (siRNA), inhibiting the synthesis of the transthyretin (TTR) protein in the liver as a treatment for the polyneuropathies induced by hereditary transthyretin amyloidosis.[6] The success of Onpattro™ paved the way for the clinical development of many nucleic acid-based therapies enabled by LNP delivery.[7] Despite its great success, using LNPs to deliver mRNA was not highlighted until the COVID-19 pandemic.

It was during the COVID-19 pandemic that mRNA-LNPs received the global spotlight due to their role in the two approved mRNA vaccines against SARS-CoV-2 (Comirnaty™ and Spikevax™).[8,9] These LNP-based mRNA vaccines got approval for emergency use and became the fastest vaccines to be ever produced on a global scale. To many, it appeared to be an overnight success of the emerging technology, when in fact it was the culmination of decades of research leading to the most clinically advanced nucleic acid delivery system. LNP technology potentially enables all forms of gene therapy to interfere with harmful gene expression via gene silencing and gene editing and to enable gene expression to supplement the insufficient expression of endogenous genes [10–14]. Besides siRNA and mRNA, LNPs are also being used to deliver antisense oligonucleotides (ASOs), DNA and microRNA, potentially making it one of the most efficient drug delivery systems to date [15,16].

However, while the efficiency of the drug delivery system is essential, safety is paramount as it dictates the ultimate success of clinical trials. When nanoparticles carrying the payload enter the body, the innate immune system gets alerted as it senses the various foreign components of the drug delivery system. As a consequence, inflammation occurs, as observed in the phase 3 studies of Comirnaty™ and Spikevax™, in which more than 80 % of LNP-mRNA recipients reported local adverse events. However, the underlying causes are yet to be determined.[17–19] Safe and efficient vaccines must stimulate the cells of the immune system at the desired level by providing signals for priming the adaptive immune response while maintaining a balance with the reactogenicity [17,20].

Many reviews in the field of LNPs focus on how the payload executes the therapeutic effect via altering cellular biology. Relatively less attention has been given to discussing how various components of the delivery system itself can activate the immune system. Herein, we begin by providing a brief historical perspective of the development of mRNA and LNP technology. We describe various factors contributing to immunostimulation by synthetic mRNA. Our discussion then leads to the role of ionizable lipids, the component of paramount importance in LNP functionality, and their structure–activity relationship. Finally, we focus on the impact of the ionizable lipid on endowing immunostimulatory properties to the LNP and the challenges associated with mRNA-LNP technology that currently hinder successful implementation from the bench to the clinic.

Before proceeding to discuss this important topic, we feel it would be prudent to begin by diving deeper into the historical development of both mRNA and LNP technologies.

2. mRNA: The therapeutic messenger

2.1. mRNA as a therapeutic agent

messenger RNA (mRNA) was discovered in the 1960 s, but the concept of employing it as a therapy caught attention only a few decades ago. Until then, DNA-based therapies were intensively studied for applications requiring protein replacement therapies. Gradually, the advantages that mRNA offers over DNA-based therapies were clear.[21] First, while DNA-based therapeutics must be delivered to the nucleus to undergo transcription, mRNA only has to enter the cytosol, where it is translated instantly. In addition, unlike plasmid DNA and viral vectors, mRNA does not integrate into the genome and, therefore, does not pose the risk of insertional mutagenesis. Importantly, mRNA is only transitorily active in the physiological system and gets degraded via metabolic pathways of the cells, conferring safety over the long-term expression of plasmid or viral DNA-based therapies [21,22].

In a landmark study in 1990, Felgner and co-workers demonstrated that injecting pure mRNA directly into mouse skeletal muscle resulted in a significant expression of a reporter gene (chloramphenicol acetyltransferase) in the muscle cells within 18 h.[23] Following this, researchers began synthesizing RNA *in vitro* and engineering its structure to resemble the endogenously occurring post-transcriptionally modified mRNA more closely. Hence, the IVT mRNA is a single-stranded molecule with a 5′ cap and a 3′ poly (A) tail. Furthermore, it contains untranslated regions (UTRs) upstream and downstream of an open reading frame that encodes the protein of interest between a start codon and a stop codon. In 1992, almost 30 years after its discovery, mRNA was first described as a potential therapeutic agent when Bloom et al. gave an intrahypothalamic injection of synthetic vasopressin mRNA to Brattleboro rats, resulting in a temporary (for up to 5 days) reversal of diabetes insipidus.[24] Both of these early results highlighted the therapeutic potential of mRNA, especially when compared to DNA-based technology [22].

Despite the advantages mentioned, mRNA was rarely explored on the grounds that it is immunogenic.[25] Katalin Karikó and Drew Weissman successfully overcame this bottleneck by introducing modified nucleosides into the mRNA sequence. Their landmark discoveries concerning nucleoside base modifications enabled the development of effective mRNA vaccines against COVID-19, for which they were awarded the 2023 Nobel Prize in Physiology or Medicine.[26–29] In particular,

replacing the uridines of the mRNA with pseudouridines rendered the mRNA nonimmunogenic without interfering with translation efficiency. [28,29] Substantial efforts have also been made to increase the amount of protein per unit of mRNA and to prolong the time frame of expression. The structural elements of IVT mRNA, particularly the 5′ cap, 5′- and 3′-UTRs, the poly (A) tail, and the coding sequence itself have been optimized to systematically improve its intracellular stability and translational efficiency and reduce immunogenicity.[30] The following section describes common modifications performed on synthetic mRNA for their successful use in clinics (Fig. 1).

2.2. Modifications of synthetic mRNA

5′ cap: During the transcription process in mammalian cells, the first nucleotide of the eukaryotic mRNA gets linked to a 7-methylguanosine (m^7G) cap by a 5′-5′-triphosphate bond (ppp) (m^7GpppN structure). This 5′ cap is crucial as it binds to the eukaryotic translation initiation factor 4E (EIF4E), an essential step for efficient mRNA translation. In addition, the binding of the cap to the decapping enzymes leads to mRNA decay, affecting the level of translation.[31–33] Additionally, methylation also prevents the recognition of viral RNA by cytosolic sensors. To date, various approaches to cap the mRNA during or after *in vitro* transcription reactions have been employed. One approach involves subjecting the IVT mRNA to an additional reaction with recombinant vaccinia virus-derived capping enzymes.[34] Another strategy is to add a synthetic cap analogue into the *in vitro* transcription reaction.

While the majority of ongoing clinical trials use mRNAs with a m^7GpppG cap, a major limitation of the process is that the cap analogue

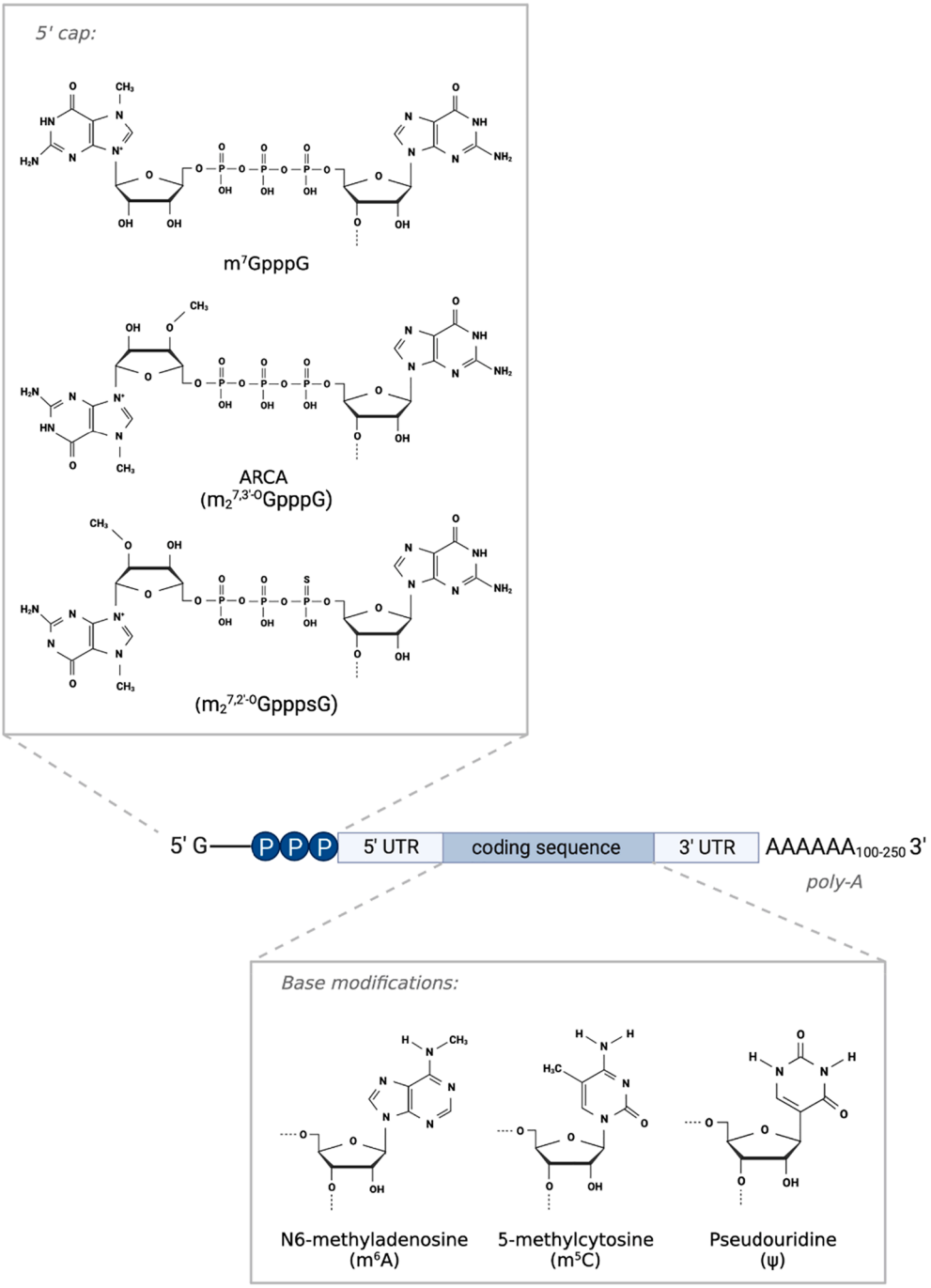

Fig. 1. Schematic overview of commonly employed mRNA modifications.

and the GTP nucleotide compete during *in vitro* transcription reaction, resulting in some of the mRNA remaining uncapped and translationally inactive. Another drawback of the m^7GpppG cap is that a substantial proportion (one-third to one-half) of the m^7GpppG analogue is incorporated in reverse orientation into the mRNA and, therefore, not recognized by the translational machinery, resulting in a lower translational activity.[35] Thus, in the quest for improving mRNA translation, anti-reverse cap analogues (ARCAs; $m_2^{7,3'-O}$ GpppG) were introduced, which are incapable of being incorporated in the reverse orientation, thus not compromising the translational efficiency of the mRNA. In fact, *in vitro* testing in mammalian cells showed that mRNAs capped with ARCAs are translated 2- to 2.5-fold more efficiently than those capped with m^7GpppG [36].

Another process that required investigation and intervention by mRNA scientists was a committed step in eliminating mRNA from the cytosol involving decapping by the Dcp1/Dcp2 complex and degradation of the exposed transcript by exonuclease Xrn1. This process contributes to reducing the half-life of IVT mRNA inside the cells. To overcome this, addition of phosphorothioate moieties at either the α, β, or γ positions of the triphosphate chain ($m_2{}^{7,2'-O}Gppp_SG$, $m_2{}^{7,2'-O}Gpp_SpG$, and $m_2{}^{7,2'-O}Gp_SppG$) was performed and has been shown to render the synthetic mRNA resistant to Dcp2 hydrolysis *in vitro* and increase the stability of mRNA in cultured cells, leading to enhanced translational efficiency [37].

Poly (A) tail: It is a modification that occurs post-transcriptionally at the 3′ end of nascent mRNA. The primary role of the poly (A) tail is to protect the mRNA molecule from degradation. This protection ensures that the mRNA remains stable and intact for a longer period, allowing it to be translated into protein multiple times. The poly (A) tail is also crucial for the initiation of translation as it interacts with various translation initiation factors and ribosomal subunits, facilitating the assembly of the translation machinery at the mRNA's 5′ cap structure. [38] The length and composition of the poly (A) tail plays an important role in the translational efficiency and stability of the mRNA and aid in the export of mRNA from the nucleus to the cytosol. Similar to the 5′ cap addition, poly (A) tail can be added to mRNA during or after the *in vitro* mRNA synthesis by enzymatic reactions with Poly (A) Polymerase. In mammalian cells, a poly (A) tail typically comprises 100–250 adenosine residues. However, there is no consensus on the optimal length of the poly (A) tail for IVT mRNA.[39] Interestingly, other modifications can also be done to the poly (A) tail besides length optimization. For example, BioNTech uses a segmented poly (A) tail where two-tail structures are linked in tandem by a ten bp UGC linker sequence.[40] Interestingly, adding poly (A) tails reduces the relative U content and shields the uridines in the mRNA sequence, thus lowering the stimulation of the immune system by the mRNA [41].

UTRs: 5′ and 3′ UTR are the non-coding regions present upstream and downstream of the mRNA coding sequence. While UTRs are not translated, these regions interact with protein complexes to regulate mRNA stability, transport to the cytosol and translation. The 5′ UTR is recognized by ribosomes to initiate the translation at the start codon. To increase the translational efficiency of IVT mRNA, several modifications to UTRs have been proposed. For instance, 3′ UTRs are usually enriched with adenine/uridine (adenylate/uridylate rich elements or AREs), essential for their degradation in response to stress by ARE-binding proteins. Counteracting this, high GC content in 3′ UTRs of IVT mRNA can help evade degradation and stabilize the mRNA for a longer period. [42] Increasing the GC content of the mRNA transcript has been proven to improve protein expression and reduce immunostimulation. In another example of the effect of optimizing the non-coding region of mRNA, CureVac demonstrated that the inclusion of a 5′ UTR region based on the human hydroxysteroid 17-beta dehydrogenase 4 gene (GSD17B4) and 3′ UTR based on Homo sapiens proteasome 20S subunit beta 3 (PSMB3) greatly improved the immunogenicity of vaccines [43].

The 5′ end of the mRNA undergoes essential modifications for stability and translation initiation. Most synthetic mRNA is capped with the m^7GpppG cap. Cap analogues, including ARCA, $m_2^{7,3'-O}$GpppG, or caps with phosphorothioate moieties ($m_2{}^{7,2'-O}Gppp_SG$, $m_2{}^{7,2'-O}Gpp_SpG$, and $m_2{}^{7,2'-O}Gp_SppG$), can be introduced to optimize translational efficiency. The 3′ poly-A tail, typically comprising 100–250 adenosine residues, protects the mRNA molecule from degradation. Non-coding 5′ and 3′ untranslated regions (UTRs) are pivotal in post-transcriptional regulation. They interact with proteins to regulate mRNA translation, stability, and transport to the cytosol. The coding sequence can undergo various nucleoside modifications: N6-methyladenosine (m^6A) as an adenosine substituent, 5-methylcytosine (m^5C) as a cytosine substituent and pseudouridine (Ψ) as a uridine substituent. These modifications impact the stability and immunogenicity of *in vitro* transcribed (IVT) mRNA.

2.3. Immunostimulation by synthetic mRNA

Historically, one of the major challenges for therapeutic use of mRNA has been its inherent immunostimulatory nature. Immune cells are equipped with diverse surface and intracellular receptors to recognize foreign nucleic acids such as viral RNA. IVT-mRNA, if not modified, stimulates the immune system by being recognized by one or more intracellular pattern-recognition receptors (PRRs).[44–46] Specifically, Toll-like receptors (TLR) TLR3, TLR7 and TLR8, present on the endosomal membranes, act as cytoplasmic RNA sensors and induce an interferon response upon stimulation.[45,46] Different structural components of RNA trigger different pathways of immune cell activation (Fig. 2 and Table 1).[47–52] This recognition and the subsequent innate immune response impede the therapeutic efficacy of the mRNA by affecting the treatment safety because of aberrant immune cell responses and by reducing the translation efficiency as part of cellular stress responses.[53] Developments in the understanding of innate immunity have established the belief that the immunogenicity of the IVT mRNA predominantly arises because of impurities or byproducts of *in vitro* mRNA transcription reactions. Unfortunately, IVT yields not only the desired ssRNA but also several types of by-products, including dsRNA generated due to T7 RNAP's RNA-dependent RNAP activity and short oligonucleotides arising from abortive transcription. The dsRNA structures formed due to the annealing of complementary sequences intra- or intermolecularly are recognized by PRRs, including melanoma differentiation-associated protein 5 (MDA-5) and retinoic acid-inducible gene I (RIG-1).[54] Upon activation, the downstream signalling pathway leads to nuclear translocation of the transcription factors interferon regulatory factor 3 (IRF3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and the production of proinflammatory cytokines, chemokines and eventually to induction of hundreds of IFN-stimulated genes (ISGs).[54] While mild immune activation by mRNA proves propitious if the mRNA encodes for an antigen in vaccine applications, it may prove detrimental in applications requiring treatment of an auto-immune disease.

Therefore, it is critical to check IVT mRNA for the presence of impurities such as dsRNA and purify it to eliminate them. The presence of dsRNA can be detected by running the RNA on native PAGE followed by staining using acridine orange, which has been shown to distinguish between ssRNA and dsRNA.[55] In another widely used assay, treating the RNAs with different types of RNases can also provide information on the kinds of RNA present in the IVT mRNA. Furthermore, ELISA can be employed to detect dsRNA.[56] Following identification, column purification is performed to get rid of dsRNAs. For example, cellulose selectively binds to dsRNA in ethanol buffer and can be used to separate dsRNA from ssRNA using column chromatography. However, this method is not effective in its entirety as 10 % of the dsRNA contaminants still remain, which could cause unnecessary immunostimulation. [57] The gold standard for mRNA purification is high-performance liquid chromatography (HPLC), which removes both dsRNA and abortive transcripts [58].

Along with purified mRNA, nucleoside modifications are commonly employed to reduce mRNA immunogenicity. Thus far, vaccines

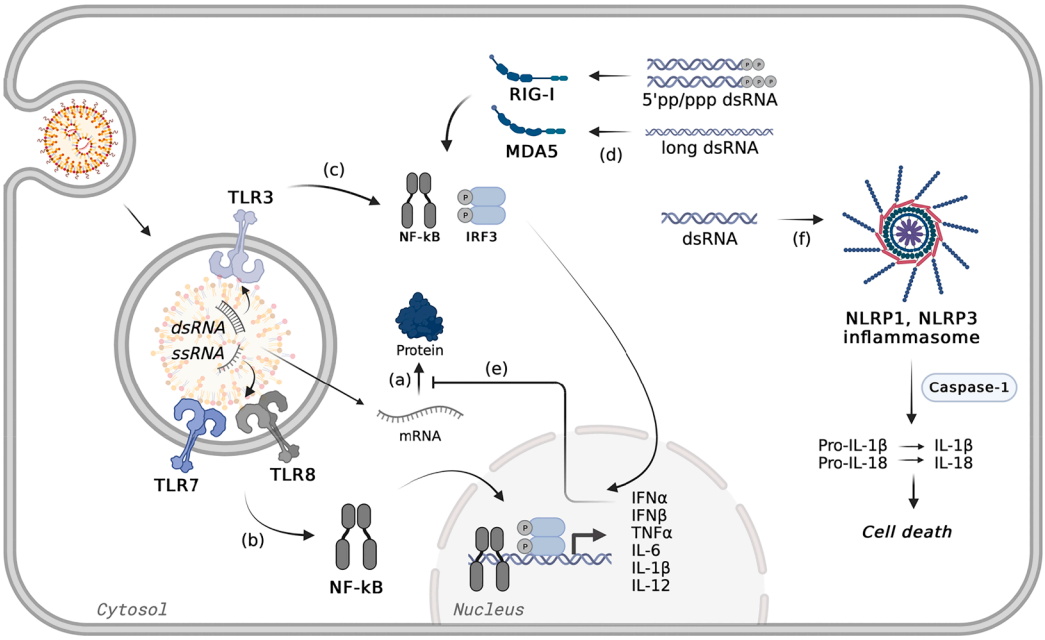

Fig. 2. Schematic overview of the innate immune cell response to IVT mRNA and the reaction byproducts.

featuring non-modified mRNA have not been successful. For example, CureVac's unmodified CureVac COVID-19 vaccine (CVnCOV) showed only 47 % protection against coronavirus infection in clinical trials. There are many opinions on the reasons for the failure of CVnCOV. Firstly, the dose of unmodified mRNA used by CureVac (12 μg) was much lower than what BioNTech (30 μg) and Moderna-Pfizer (100 μg) used, which may have been insufficient to induce an effective neutralizing Ab production. Secondly, some experts believe that unmodified mRNA has lower translational efficiency, resulting in lower levels of antigens.[59] However, CureVac pointed to the changing SARS-CoV-2 variant landscape during its trial as a reason for the lower efficacy. As mentioned previously, to improve the stability and to abate the immunogenicity of IVT mRNA, incorporation of naturally occurring modified nucleosides such as pseudouridines, 2-thiouridine, 5-methyluridine, 5-methylcytidine or N6-methyladenosine into the IVT mRNA has proven to be effective as these tags render the IVT mRNA to be undetectable by the receptors of the innate immune system.[26,27] Of note, a strategy that CureVac has utilized to avoid detection by PRRs uses sequence engineering and codon optimization to deplete uridines by boosting the GC content of the vaccine mRNA.

Yet, not all modifications provide a fit-for-all solution. A specific modification may be less immunogenic than the others but may lower the translation efficiency of the transcript. Further, different cell types may exhibit varying sensitivities to the same modification, adding to the challenge of selecting the appropriate modification. Nevertheless, the evaluation of immunogenicity of mRNA is critical to define the safety profile of mRNA therapeutics before the formulation is taken into clinical trials. For this, experiments with systems such as whole blood, which address the complexity of human immune cells, should be performed. Human whole blood assays have been extensively used in clinical research for various investigations, including immunogenicity and antigen reactivity.[60,61] Alternatively, mouse models may be utilized to assess the immunogenicity of IVT-mRNA. However, significant differences exist between mice and humans, which must be accounted for. For example, although TLR1–TLR9 are being conserved in both species, mouse TLR10 is not functional because of a retrovirus insertion, and TLR11-TLR13 have been lost from the human genome.[62] In addition to mice, other animals have been used as models to test immunostimulation. For instance, pigs are a naturally oversensitive model to study complement activation-related pseudo allergy (CARPA).[63] Finally, primate models are the closest model to humans and can be employed for immunostimulation studies [64].

a) After internalization of the mRNA-LNP and endosomal escape of the encapsulated mRNA, the mRNA is translated into the desired protein product. b) Inside the endosome, ssRNA interacts with TLR7 or TLR8, activating the MYD88 pathway, resulting in the translocation of NF-kB to the nucleus, leading to the secretion of multiple pro-inflammatory cytokines and chemokines. c) TLR3 senses dsRNA and leads to the activation of transcription factors NF-kB and IRF3. d) Phosphorylated and long dsRNA are recognized by RLRs RIG-1 and MDA5, respectively, leading to the activation of NF-kB and IRF3. e) The produced pro-inflammatory cytokines and chemokines inhibit the translation of mRNA into protein, resulting in decreased protein product. f) dsRNA can also activate the NLRP1 and NLRP3 inflammasomes, contributing to activation of IL-1β and IL-18, which induce cell death.

Altogether, the tremendous advances made in the fields of mRNA engineering and in understanding the mechanism behind its immunostimulatory effects indicate that this technology is viable and effective for multiple treatments. Therefore, the next step is understanding the immunostimulatory effects of various components of the delivery system, LNPs.

3. Lipid nanoparticles

3.1. Three decades of evolution in the clinics

When phospholipids are dispersed in an aqueous medium, self-assembled closed vesicles with concentric lipid bilayers and hydrophilic cores are spontaneously formed. This was first observed in 1965 by A. D. Bangham, who coined the term "liposome" for these vesicles.[65] Following this, the physical and chemical characteristics of liposomes and various methods of preparing the liposomes were evaluated.[65] The interaction of liposomes with the cells and *in vivo* behaviour was also examined. With advancements in the field of nanotechnology during the 1990 s, the term "lipid nanoparticles" was put in use.[66] Various classes of lipid nanoparticles used for drug delivery include liposomes, solid lipid nanoparticles, nanoemulsions, nanostructured lipid carriers, lipoplexes and lipid nanocapsules. The ability of LNPs to act as drug carriers was immediately appreciated. It was observed that the amphipathic nature of the phospholipid bilayer of LNPs is similar to the

Table 1
Immune cell pathways activated by IVT mRNA and reaction byproducts.

	Type	Pathway activated	Agonist	Ref.
RIG-1 (cytosolic)	RIG-1-like receptor (RLR)	IFN signaling	5′-di/triphosphate dsRNA with + 20 base pairs (bp), with optimal signal transduction efficiency at 40–150 bp	47, 48,50
MDA5 (cytosolic)	RLR	IFN signaling	Long dsRNA molecules (>1000 bp), independent of the cap structure	47, 48,50
LGP2 (cytosolic)	RLR	IFN and NF-κB signaling	dsRNA recognized by RIG-1 and MDA5	47, 48,50
TLR3 (endosomal)	Toll-like receptor (TLR)	IFN and NF-κB signaling	dsRNA of +/- 40 bp	48
TLR7 & TLR8 (endosomal)	TLR	IFN and NF-κB signaling	ssRNA	48
NLRP1 & NLRP3	NOD-like receptor (NLR)	Inflammasome formation, together with ASC and caspase 1	dsRNA	50,52
Protein Kinase R (PKR)	dsRNA binding protein	NF-κB mediated apoptosis and blocking of global translation	RNA > 33 bp	49,50
2′,5′-Oligoadenylatetase (OAS)	dsRNA binding protein	Global translation arrest and activation of RNase L	dsRNA	50

mammalian cell membrane, enabling fusion between LNP and cell membrane for uptake by the cells. Moreover, ligands can be attached to LNPs to improve the specificity of targeting and reduce off-target side effects.[67] In the beginning, LNPs were utilized for the encapsulation of water-insoluble drugs for the treatment of cancer and infectious diseases. Eventually, it was realized that these lipid vesicles also possess the ability to bind and condense nucleic acids through electrostatic interactions and can deliver the payload across the cellular membrane into the cytoplasm of target cells.[16,68] With this, efforts for encapsulating mRNA in nanoparticles began in the early 1970 s when polymeric particles were used to entrap and deliver nucleic acids.[69] Eventually, in 1978, a successful translation of rabbit globin mRNA was demonstrated in mouse lymphocytes when the mRNA was encapsulated in liposomes [70].

The standard structure of a LNP typically consists of four components: ionizable lipid, cholesterol, helper lipid and PEG-lipid conjugate. The structural and biological properties of LNPs are attributed to not just one component but rather the combination of lipids.[71] We focus our discussion on the role of ionizable lipids, the most critical excipient, and how their structure dictates the activity of LNPs.

3.2. Ionizable lipids and their structure–activity relationship

mRNA is anionic in nature, and thus, it cannot pass through the negatively charged lipid bilayer of the cell membrane without assistance. In addition, the hydrophilic nature and molecular weight of mRNA also pose a challenge for intracellular delivery. If unallied, it is degraded by nucleases post-engulfment by cells belonging to the innate arm of the immune system. Therefore, mRNA transport requires delivery systems that can safely escort the mRNA intracellularly to the cytosol to be transcribed into the protein of interest. LNPs provide a suitable drug delivery platform for mRNA delivery. After cellular uptake, LNPs enter endosomes where the cationic ionizable lipid becomes protonated because of decreasing pH and acquires a positive charge. This cationic nature allows the interaction with negatively charged phospholipids in the inner leaflet of the endosomal membrane, facilitating the endosomal escape of the mRNA into the cytosol, where it gets translated.[72,73]

3.2.1. Cationic ionizable lipids

Before the ideation of ionizable lipids by Peter Cullis and the team, cationic or permanently positively charged lipids were used in LNP formulations. The positive charge of cationic lipids interacts with the negatively charged mRNA to encapsulate the nucleic acid in the vesicle. The use of cationic lipid was demonstrated for the first time in 1987 by Felgner et al., who used liposomes made of N-[1-(2,3-dioleyloxy) propyl]-N, N, N-trimethylammonium chloride (DOTMA) to encapsulate DNA. These unilamellar liposomes were internalized, and DNA was efficiently expressed in various cell lines, establishing the efficiency of cationic lipids for nucleic acid delivery.[74] Unfortunately, due to this positive charge, the cationic lipids demonstrated toxic effects mediated via uncontrolled activation of pro-apoptotic and pro-inflammatory cellular pathways.[75–77] For instance, LNPs composed of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl (**DOTAP**), a commonly used cationic lipid, induce the production of mitochondrial reactive oxygen species (ROS) which triggers an influx of calcium leading to nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3 (NLRP3) inflammasome activation and the release of the pro-inflammatory cytokine IL-1β.[78] Several chemokine genes, including CCL2, CCL3 and CCL4, also get activated in immune cells upon interaction with cationic liposomes.[79] Additionally, cationic lipids also activate TLRs, leading to the expression of pro-inflammatory cytokines such as IL-2, IFN γ and TNF α [76].

Therefore, to minimize the toxicity caused by cationic lipids, cationic ionizable lipids were introduced to deliver nucleic acids. 1,2-dioleyloxy-3-dimethylaminopropane (**DODMA**) was the first ionizable lipid for nucleic acid delivery. The chemical structure of **DODMA** evolved through rational design and led to the creation of **D-Lin-DMA** and, finally, **D-Lin-MC3-DMA** [80], which has been used in Onpattro™ for the delivery of siRNA. Ionizable lipids carry a positive charge under acidic pH but remain neutral at body pH to retain electrical neutrality in the bloodstream. Once internalized by cells, the acidic milieu of the endosomes facilitates the protonation of ionizable lipids, which then engage with the negatively charged phospholipids of the endosomal membrane, destabilizing the membrane to enable the escape of the mRNA into the cytosol. Thus, the ionizable lipid not only governs the encapsulation of mRNA but also its escape from the endosomes, an important event required for the successful translation of the mRNA to the protein of interest.[81,82]

A typical ionizable lipid has three structural components: head group, linker, and tail. **(**Fig. 3**)** The head group usually consists of amine groups, the linker is designed to be stable yet biodegradable, and the tail consists of carbon atoms of variable lengths, symmetries, and degrees of saturation. Collectively, the structural features impact the expression,

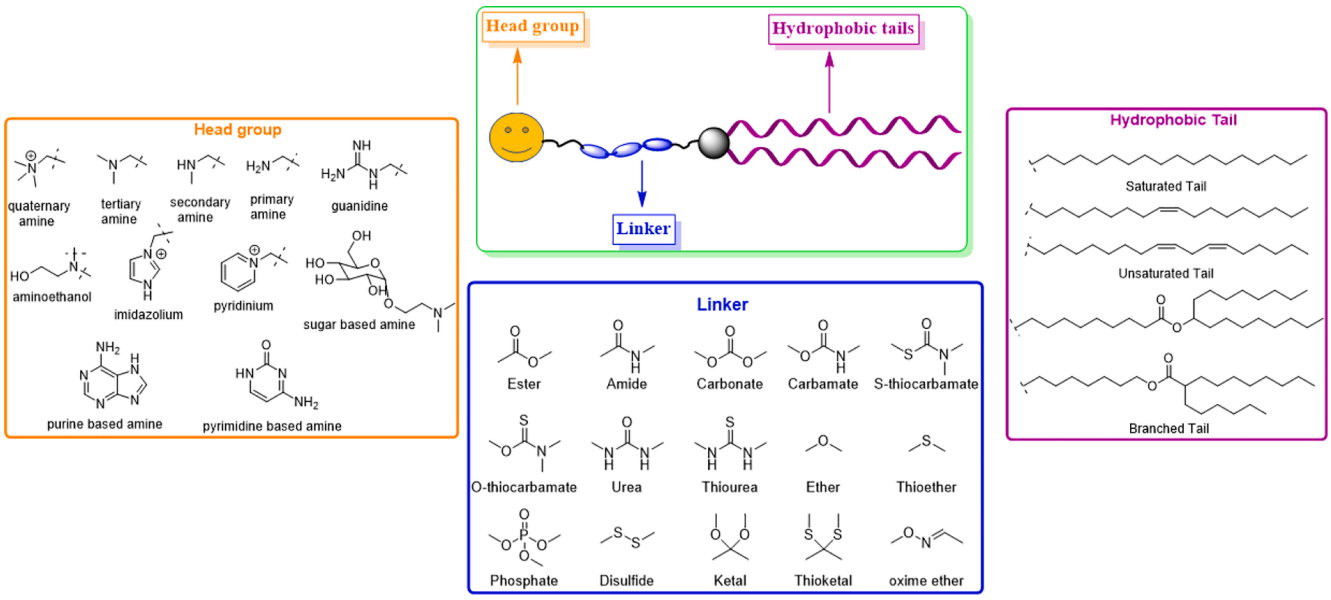

Fig. 3. Commonly used head groups, linkers, and hydrophobic tails in the ionizable lipid structure.

biodegradability, and immunogenicity of the mRNA-LNP. The structural diversity of ionizable lipids that research groups use is growing rapidly, and to accelerate the discovery of novel formulations capable of efficiently delivering the mRNA *in vivo*, research groups in academia and industry have harnessed combinatorial and high throughput approaches to synthesize large libraries and evaluate them *in vivo*. [83,84] In the next section, we discuss some important structural and functional features of ionizable lipids and their impact on LNP performance.

3.2.2. *Effect of linker on biodegradability*

The biodegradability of lipids is an important feature of ionizable lipids to prevent their accumulation in the body and reduce the potential side effects. Biodegradation is much more pertinent when the application requires repeated dosing at frequent intervals. In addition, increased biodegradability leads to a reduction in injection site inflammation.[85] The structural component of the ionizable lipid that substantially affects biodegradability is the linker. Therefore, ester-bonds are usually incorporated in the ionizable lipids, as evident from the structure of **SM-102** (Spikevax™), **D-Lin-MC3-DMA** (Onpattro™) and **ALC-0315** (Comirnaty™) (Fig. 4), all being ionizable lipids that have been used in FDA-approved LNPs. Ester bonds are enzymatically hydrolyzed by intracellular and intratissue esterases, readily breaking

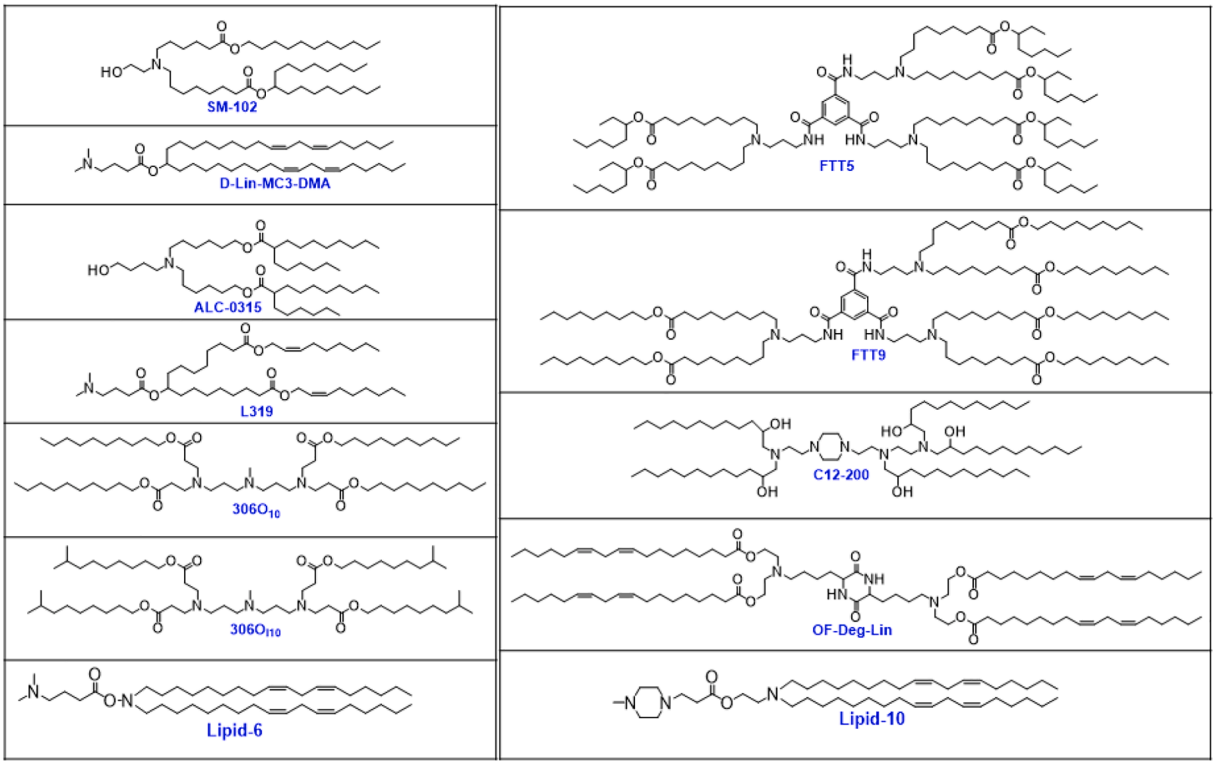

Fig. 4. Structures of Ionizable lipids reported herein.

down the ionizable lipid. The presence of ester bonds in the tail region has also been shown to affect the clearance of LNPs. For example, by replacing one of the double bonds in each tail with a primary ester in the MC3 lipids, the elimination of the lipid (**L319**) was accelerated while efficacy was retained.[86] Notably, this may not always hold true, as shown by experiments performed by Sabnis et al. on a library of amino lipids constructed using an ethanolamine head group and di-linoleic-based lipid tails. Substitution of a double bond with an ester linkage enabled faster clearance of lipids from mice but poor *in vivo* delivery of mRNA. Interestingly, a significant improvement in protein expression could be seen when a secondary ester was introduced in the lipid tails.[82] Further on, changing the position of the ester in the lipid tail by placing the ester closer to the amine group of the head slowed down the clearance of the lipid from the liver. Overall, combining the ethanolamine head group, a primary ester at the C8 position in one lipid tail, and a secondary ester in the second lipid tail provided the ideal balance of *in vivo* lipid clearance and protein expression. These studies indicate that an activity-degradability tradeoff needs to be balanced to harness the full potential of LNPs. In addition to ester, other commonly used linkages for the conjugation of building blocks of ionizable lipids include amide, carbonate, carbamate, urea, phosphate, and disulfide. Moreover, the recent change in the design of ionizable lipids is to include hydrolyzable bonds to facilitate faster clearance. However, these bonds may compromise the stability of formulations, thus adding to an already existing challenge in the field. An interesting example is Tilstra et al., who conducted an iterative study to find an ionizable lipid candidate for intramuscular delivery. Increasing the linker length by just one carbon led to enhanced mRNA expression while maintaining high encapsulation and desired size distribution, though the apparent pKa increased by one unit. [41] These studies highlight how minor changes to the chemical structure can dictate the biodegradability of the LNPs and impact the *in vivo* behaviour of mRNA-LNPs.

The discussion on biodegradability will be completed by highlighting the differences in the biodegradability and clearance profiles of ionizable lipids **ALC-0315** and **SM-102**. **ALC-0315** has a longer half-life.[87] Based on an early pharmacokinetics investigation in rats, the half-life of **ALC-0135** in humans was extrapolated, and it was calculated that it takes 30–40 days after injection for 95 % of **ALC–0315** to be eliminated. Contrary to this, **SM-102** and its degradation products were predicted to be rapidly eliminated.[88] For **SM-102**, efficient metabolization via ester hydrolysis and rapid elimination of the remaining aliphatic acid head group via biliary and renal clearance were reported. Upon LNP administration, the persistence of the **SM-102** lipid component in any tissue beyond 168 h was not expected from the existing data on lipids with similar structures. The difference in the clearance rate of **ALC-0315** and **SM-102** lipids can be explained by the linear fatty alcoholic tail of **SM-102**, which allows for higher accessibility for enzymatic cleavage of the first ester bond and subsequently also the second due to reduced steric hindrance.

3.2.3. Lipid tails

The hydrophobic tail of the ionizable lipid is crucial in determining the geometry, self-assembly and fusogenicity with the endosomal membrane. Efforts have been put to dissect the optimal length of the lipid tail most effective for the delivery of mRNA. In addition to the length, branching of the fatty acid tail significantly alters the geometry of the ionizable lipid, playing a key role in the functionality of LNPs. Hajj et al. examined a library of 11 lipidoids (lipid-like structures containing tertiary amines) with one-carbon differences in their tail structures.[81] The 11 lipidoids studied were all generated from the amine 3,3′-diamino-N-methyldipropylamine (**amine 306**) using Michael addition chemistry and demonstrated efficient siRNA delivery in mice. Interestingly, upon adding a branch to one of the lipid tails, the corresponding LNP was 10-fold more effective than all other lipids with linear fatty acid tails. Mechanistically, the branching of the tail (**306O$_{i10}$**) led to a stronger ionization at pH 5, which is the pH of the late endosomes, the compartment where the release of the mRNA is believed to occur. Importantly, even after changing the head groups to a different amine moiety, the lipidoids with a branched tail were superior in delivering mRNA *in vivo*, underlining the importance of branching.

The improved delivery efficiency of branched tail lipids was also highlighted in a recent study by Dong et al. The lipid-like nanomaterials with branched ester chain **(FTT5)** were superior in delivering FVIII mRNA than their linear chain **(FTT9)** analogs.[89] In another screening study, Qiu et al. compared the mRNA delivery efficiency of lipids containing amide **306-N16B** (N-series) bonds in their tails to those having an ester **306-O12B** (O-series) bond. While the N-series lipids were previously shown to deliver mRNA to the lungs, the O-series lipids previously demonstrated delivery to the liver.[90] The authors employed proteomics and hypothesized that the predominant reason for lung tropism was the presence of unique proteins in the protein corona of the N-series lipid, which may direct the transport of LNPs to the liver.[91]

Another tail structure-associated factor that impacts the transfection efficacy of ionizable lipids is the degree of saturation. The presence of double bonds influences the fluidity of the lipid layer and, hence, its interaction with the endosomal membrane. For example, the addition of 2 *cis*-double bonds increases the tendency of the lipids to form a non-bilayer phase, which means enhanced disruption of the endosomal membrane and greater payload release. Therefore, the first ionizable lipid, **D-Lin-MC3-DMA**, was structurally optimized to have a linoleyl tail. Overall, the lipid tail length, branching and saturation contribute to the packaging of the mRNA and delivery efficiency. A critical and iterative assessment should be performed when designing novel ionizable lipid candidates.

3.2.4. Head group and the pKa of ionizable lipids

The chemical composition of the head group gives the ionizable lipid its pKa value. The apparent pKa of an ionizable lipid is a critical parameter that dictates the delivery of nucleic acids. The optimal pKa value of the head group of an ionizable lipid provides an uncharged and inert surface when the LNPs circulate in the blood. In contrast, at a lower pH it allows the interaction of the LNP with the endosomal membrane. The standard 2(p-toluidino)-6-napthalene sulfonic acid (TNS) binding assay is commonly used to assess the apparent pKa of LNPs.[92] A pKa value of 6.2–7 was initially identified as a crucial parameter for successful nucleic acid delivery. Onpattro™ contains ionizable lipid **D-Lin-MC3-DMA,** which contains a tertiary amine head group. These LNPs intended to deliver siRNA to the liver have an apparent pKa of 6.44. On the other hand, the apparent pKa of **SM-102** and **ALC-0315** is determined to be 6.68. and 6.2, respectively. Interestingly, both **SM-102** and **ALC-0315** have amino alcohol lipid head groups and are highly effective for intramuscular mRNA delivery, which suggests that the pKa is not the only determinant of the effectiveness of ionizable lipids and also that the chemical properties of LNP formulations optimized for siRNA delivery may not be similar to those required to deliver mRNA. Nevertheless, there is no consensus on the optimal pKa required for effective mRNA delivery [93,94].

Interestingly, a few studies highlight the differences in the pKa of LNPs targeted to different organs. The pKa of spleen-specific ionizable lipid **OF-Deg-Lin** LNPs was lower (~5.7) than the pKa observed for liver-targeting mRNA LNPs (~7.0).[95] Similarly, Siegwart laboratory's selective organ-targeted (SORT) LNPs corroborated these results. While the liver-targeting SORT LNPs had an apparent pKa ranging between 6 and 7, spleen-targeting LNPs had a lower pKa between 2 and 6.[96] Contrastingly, in a study using a combinatorial library of ionizable lipids, our laboratory showed that there is a very low correlation between pKa value and splenic mRNA expression.[92] Our lab demonstrated that LNPs with a piperazine head group **(Lipid 10)** accumulated more in the spleen and liver, contrary to lipids with a tertiary amine **(Lipid 6)** head group, which got more in the liver when LNPs were injected intravenously in mice.[83] Another study observed that LNPs with imidazole heads are effective for the *in vivo* mRNA delivery to T

cells.[97] In an interesting finding, Sanofi R&D discovered that by using imidazole as a head group **(DOG-IM4)**, the stability of mRNA-LNPs could be improved at 4 degrees while promoting robust mRNA expression as tested in mice and non-human primates. [98]This could be an impactful finding since thermostability is one of the limitations of the mRNA-LNP technology. Ultracold distribution chains were needed for both Comirnaty™ and Spikevax™ [98].

Another noteworthy aspect to investigate is the effect of the pKa of the ionizable lipid on the immunogenicity of LNPs. In this context, Hassett and colleagues evaluated a set of ionizable lipids for their immunogenicity post-intramuscular administration. In their rodent study, they observed that the pKa was a strong determinant of immunogenicity, with a range of 6.6–6.9 being optimal. However, the pKa was not the sole determinant of immunogenicity since many lipids that fell within this range were less immunogenic.[85] Alongside the head group, the choice of linker can also affect the pKa of the ionizable lipid. By screening an ionizable lipid library consisting of different linkers, our lab showed that hydrazine linkers impart a higher pKa to ionizable lipids than hydroxyl amine or ethanolamine linkers [83].

On a different note, naturally occurring ionizable lipids have also been leveraged for nucleic acid delivery. In this regard, ionizable lipids based on alkenyl amino alcohols (AAA), a functional group combination found in several bioactive molecules, including sphingosine, were shown to promote high levels of *in vivo* protein expression when formulated into mRNA LNPs.[99] Recently, the effect of stereochemistry of ionizable lipids has also come to light. LNPs formulated with S enantiomer of ionizable lipid **C12-200** delivered up to 2.8-fold and 6.1-fold more mRNA *in vivo* than its racemic and R enantiomer controls, respectively.[100] While stereochemistry did not alter the biophysical traits of the LNP, the stereo pure LNPs displayed improved immunotolerance in mice.

3.2.5. Cholesterol, phospholipid and PEG lipids

Cholesterol is often incorporated in the LNPs to enhance stability and regulate membrane fluidity. Since cholesterol occurs naturally in cell membranes, incorporating cholesterol improves the biocompatibility of LNPs and promotes endocytosis. On the other hand, PEG imparts stealth properties to the LNPs, making them less prone to recognition and clearance by the immune system. This, in turn, allows the LNPs to circulate in the bloodstream longer. Phospholipids such as 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) are used as structural lipids as they spontaneously organize into lipid bilayers. Additionally, their high phase transition temperatures confer membrane stability to the LNP.[101] We direct the reader to refer to other review articles for details on the helper lipids.

By changing the helper lipid chemistry, delivery to organs beyond the liver can be achieved. While anionic helper lipids are shown to shift the tropism to the spleen, cationic helper lipids shift it to the lungs.[102] Specifically, cationic helper lipids were shown to effectively deliver mRNA to the endothelial cells of the lungs.[103] Cholesterol stereochemistry also impacts the *in vivo* delivery of mRNA-LNPs. Recently, Hatit et al. demonstrated that LNPs containing stereo pure 20α-hydroxycholesterol increase the delivery of mRNA to liver cells by 3-fold as opposed to LNPs containing a mixture of both 20α- and 20β-hydroxycholesterols.[104] Recently, the Siegwart laboratory developed a SORT strategy to engineer LNPs to achieve targeting beyond the liver to the lung and the spleen. This was achieved by the addition of an extra excipient in the LNP formulation. They reported that live SORT LNPs predominantly adsorbed ApoE proteins; spleen SORT LNPs mainly adsorbed β2-GPI proteins, and lung SORT LNPs mostly adsorbed Vtn proteins. While emphasizing the role of excipients in targeting LNPs, this study also highlights how the protein corona endows a new biological identity to LNPs, governing their biodistribution.[96] On a different note, the crucial role of the protein corona is also demonstrated by the fact that **D-Lin-MC3-DMA** LNPs leverage the physiological function of ApoE for siRNA delivery to the liver.[96] This is because ApoE endogenously targets the LDL-R, which is highly expressed by liver hepatocytes.

4. The role of LNPs in vaccine immunogenicity

An appreciable advantage that mRNA-LNP vaccines offer is that they effectively generate a robust protective immunity without requiring the addition of an adjuvant. The adjuvant activity of modified mRNA-LNPs can stem from the mRNA, LNP, or both. The mRNA used in the COVID-19 vaccines has been designed to reduce its immunogenicity by using various modifications, as previously discussed. Although the respective contribution of mRNA and LNP components has not been ascertained, there are studies that demonstrate the important role of LNPs in strong Tfh and GC B cell responses upon vaccine administration. There is accumulating proof that LNPs are clearly not immunosorbent. In this section, we focus on various reports that uncovered the adjuvant activity of either LNPs or the ionizable lipid component on its own.

4.1. Adjuvant activity of LNPs

To begin with, it is still being determined how, if at all, the LNPs are sensed by immune cells. Still, the amalgamation of LNPs and modified mRNA has been demonstrated to induce innate immune signals required to drive T and B cell responses in humans. [105,106] In recent years, different research groups have studied the adjuvant activity of LNPs. Alameh et al. compared the adjuvant activity of LNPs to Adda-Vax by intramuscularly/intradermally administrating rHA mixed with either empty LNPs (LNPs not encapsulating mRNA) or adjuvanted with Adda-Vax. Influenza HA proteins induced much more robust immune responses in mice compared to AddaVax-adjuvanted protein vaccines. [107] Even before the clinical use of mRNA-LNP vaccines, researchers used empty LNPs as adjuvant to vaccines containing the hepatitis B virus and the dengue virus. These experiments showed that LNPs could enhance the B-cell responses to levels comparable to the classical vaccine adjuvants IMO (aluminum-based adjuvant) and the TLR4 agonist, 3-O-deacetylated monophosphoryl lipid A (MPL).[108] In another study, Pardi et al. revealed that nucleoside-modified mRNA-LNP vaccines outperform adjuvanted protein and inactivated virus vaccines. [109] Taken together, these works demonstrated that LNPs are more than just the delivery agent.

Recent reports showed that the LNP component responsible for the adjuvant activity is the ionizable lipid. Alameh et al., who immunized mice with recombinant hemagglutinin (rHA) mixed with LNPs formulated without ionizable lipids, reported that these mice failed to generate hemagglutination inhibition (HAI) activity in sera. Contrastingly, as expected, when LNPs were formulated with ionizable lipids, they showed HAI titers 4 and 8 weeks post-vaccine administration in mice. [107] Furthermore, increasing the molar ratio of ionizable lipids in the formulations accentuated the adjuvant activity, validating the role of ionizable lipids as adjuvants. On further investigation, it was observed that empty LNPs trigger the production of IL-6 in mice following intradermal administration, which was found to be critical for the induction of Tfh cells in response to LNP-based vaccines. However, the mechanism of how the LNPs triggered IL-6 secretion could not be determined, although the authors proved that TLRs or RIG-1/MDA5 did not mediate it.

4.2. LNPs trigger innate immune cell activation

While the mechanism underlying the sensing of LNPs by immune cells is yet to be understood, their ability to trigger the activation of various innate immune system components is clearly evident from several recent studies. Secretion of cytokines is a key event for communication between cells of the innate and adaptive arms of the immune system. Alameh et al. observed the production of pro-inflammatory cytokine IL-6 in mice following LNP administration. To

get mechanistic insights, it was shown that when LNPs were injected in IL-6-deficient (Il6$^{-/-}$) mice, they failed to elicit optimal Tfh cell and GC B cell responses. It has been previously demonstrated that IL-6 is indeed required for the differentiation of T cells to Tfh cells.[110] IL-6 release was also observed by Connors et al., who evaluated the effect of empty LNPs on antigen-presenting immune cells. LNPs formulated with ionizable lipids (proprietary to Acuitas Therapeutics) induced human monocyte-derived dendritic cells to not only secrete IL-6 but also pro-Tfh cytokines, including IL-21. In addition, these LNPs induced the maturation of monocyte-derived dendritic cells and upregulated CD40 expression. [111] They went one step further to see if the presence of empty LNPs affects the phagocytic function of PBMCs. It was observed that empty LNPs acted as a stimulator of phagocytosis. Empty LNP treatment was also linked to the phosphorylation of IRF7 and TBK-1, key signalling molecules in the antiviral response.

Furthermore, COVID-19 mRNA vaccine recipients presented side effects, most often pain, fever and swelling. These side effects are characteristic symptoms linked with inflammation initiated by pyrogenic cytokines such as IL-1β and IL-6. In fact, Ndeupen et al. demonstrated that intradermal and intramuscular injection of LNPs (formulated with proprietary to Acuitas Therapeutics; US patent US10,221,127) leads to secretion of major and minor pyrogens, IL-1β/IL-6 and CCL3 and CCL4, respectively. In addition, they also observed the upregulation of NLRP3 and genes involved in necroptosis.[112] Necroptosis could cause the release of damage-associated molecular patterns (DAMPs), which could be one of the possible mechanisms of LNP-driven immune cell activation.

Zamani et al. also investigated the immunogenicity of an mRNA-LNP vaccine against COVID-19 in non-human primates by formulating LNPs using an ionizable lipid based on **D-Lin-DMA** and administering them intramuscularly. Interestingly, significantly higher production of IFN-γ, IL-2 and TNF cytokines was detected in the control group of mice that received empty LNPs as compared to the PBS control group.[113] Another study highlighted the ability of ionizable lipids to stimulate innate immunity using **SM-102**-formulated LNPs. Here, Tahtinen et al. showed that these mRNA-LNPs induced the release of cytokines IL-6, TNF-α and IL-1 from human peripheral blood mononuclear cells.[114] Interestingly, upon treatment with NLRP3 inhibitor MCC950, IL-1β secretion reduced, signifying the involvement of NLRP3 inflammasome and corroborating studies performed in mice.[112,114] The above studies have been performed with empty LNPs; it is important to note that a significant portion of any mRNA vaccine formulation contains empty LNPs, emphasizing the need to include empty LNPs as a control group in the experiments.[115] Nevertheless, these studies indicate the ability of ionizable lipids to agitate innate immune cells. To unleash the full potential of ionizable lipids, the underlying mechanisms must be investigated, and we will discuss some of this in the next section.

4.3. *The mechanisms behind LNP-induced immunostimulation*

As mentioned, the mechanism underlying the sensing of LNPs by immune cells remains a mystery. (Fig. 5) In one possibility, the LNPs as a whole or the ionizable lipid component can be sensed by one or more of the PRRs, as shown by studies in various knockout models of mice. For instance, lipids with cyclic amino head groups, when injected intramuscularly or subcutaneously, were shown to activate the MYD88 (TLR7 or 8)/RLR (RIG-I and MDA5)-independent STING pathway, resulting in dendritic cell maturation.[65] In another interesting study to delineate the mechanism of LNP performance, Li et al. observed that the CD8 + T cell responses to Spikevax™ (administered intramuscularly or subcutaneously) were dependent on type I interferon-dependent MDA5 signalling, corroborating with the previous study. Using knockout mice, they demonstrated that response to BNT162b2 remained unaffected in TLR-2, 4, and 5 knockout mice or NLRP3, cGAS/STING, or

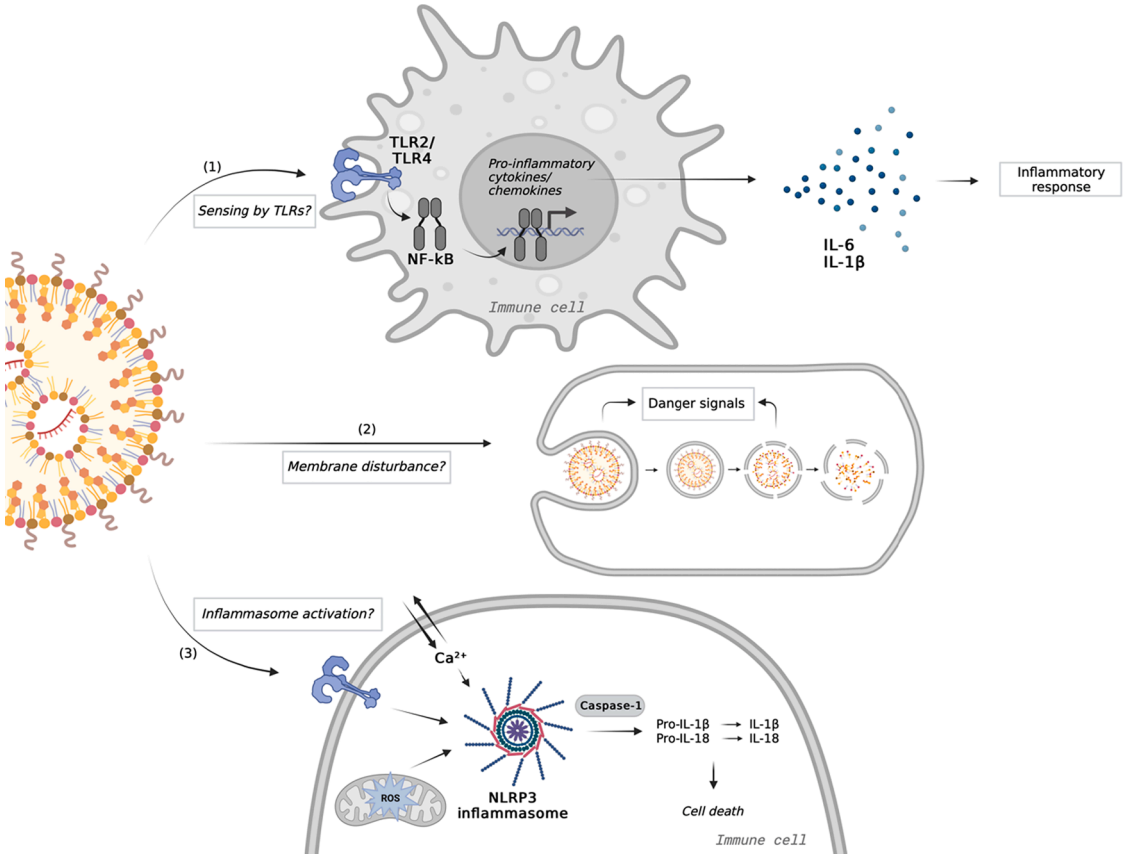

Fig. 5. Schematic overview of probable mechanisms contributing to the immune response to LNPs.

RIPK3/GSDMD knockout mice models, indicating the complexity of the signalling events involved in the mode of action initiated by vaccines. [66]

In another possible scenario, LNPs could mediate their adjuvant effect by delivering the mRNA to compartments such as lymph nodes, where the mRNA is processed into antigens by antigen-presenting cells. The rationale for this observation is the presence of translated protein in lymph nodes nearest to the injection site.[116,117]

1) LNP interacts with TLRs on the immune cell membrane, activating the MYD88 pathway, leading to the nuclear localization of NF-kB and secretion of pro-inflammatory cytokines and chemokines, such as IL-6 or IL-1β. 2) Membrane disturbance and endosomal membrane disruption could lead to the production of danger signals by the cells leading to inflammatory response in the cell. 3) LNPs or their components could induce reactive oxygen species (ROS) production by mitochondria in the cell, leading to the activation of the NLRP3-inflammasome, which in collaboration with caspase-1 activates IL-1β and IL-18 secretion, leading to cell cycle arrest and cell death.

Finally, delivering the mRNA to cells involves LNP internalization at the cell membrane and fusion with the endosomal membrane. Therefore, the immunostimulatory effect of cationic ionizable lipids could also arise due to their tendency to disrupt cell and endosomal membranes and the subsequent sensing of this "membrane patch" as a danger signal by immune cell sensors. This has been demonstrated in the context of virus-immune cell fusion.[118] Finally, it is also likely that the LNPs might induce cytokine production indirectly by causing cytotoxicity mediated by the generation of cellular ROS, mitochondrial dysfunction, activation of caspases and cell cycle arrest [119].

While activation of the innate immune system contributes to the development of adaptive immunity, excessive stimulation might lead to immunotoxicity and aberrant immune cell behavior. As the mRNA-LNP field is making swift progress, it is necessary to strike a balance between the adjuvant activity and the undesirable inflammatory properties of ionizable lipids. As previously mentioned, the ionizable lipid structure dictates the biological functionality of LNPs. Likewise, there are correlations between ionizable lipid structure and immunotoxicity. The head group of the lipid provides the positive charge to the ionizable lipid. Commonly included head groups are quaternary ammonium, amine, guanidinium and heterocyclic head groups. Among these, quaternary ammonium head groups are observed to be more toxic than their tertiary amine counterparts as these structures interact with cellular enzymes such as protein kinase C (PKC) to a tenfold greater extent, which may prove deleterious to the cellular function.[120] In the context of linkers used in the ionizable lipid structure, biodegradable linkages, such as ester or amide, are cleaved *in vivo* and cleared from the body faster than non-biodegradable structures, such as ether bonds. Due to faster elimination, biodegradable linkages are generally associated with lower toxicity [86].

Besides the structure, the composition of LNPs might also contribute to the agitation of immune cells. Tahtinen et al. demonstrated that the amount of IL-1β secretion is significantly higher when human monocytes are treated with **SM-102** as opposed to **D-Lin-MC3-DMA** LNPs.[114] Using **D-Lin-MC3-DMA** as ionizable lipids, Forster III et al. highlighted the ability of LNPs to activate the NLRP3 inflammasome and trigger the release of pro-inflammatory cytokine IL-1β. Upon varying the percentage of various components of LNPs, **D-Lin-MC3-DMA** in tandem with high cationic lipid **DPTAP** and low cholesterol concentration induced the greatest activation of mouse bone marrow-derived macrophages (BMDMs). Underlying mechanisms involved mitochondrial ROS production and calcium influx. In addition, the strongest activating LNPs also caused lysosomal rupture [119].

While we highlight the immunostimulatory properties of LNPs, Krienke et al. designed lipoplexes that lacked adjuvant activity to treat multiple sclerosis (MS). These LNPs were loaded with nucleoside-modified mRNA coding for MS autoantigens, and their administration resulted in antigen presentation on CD11c + antigen-presenting cells in the spleen, however, without costimulatory signals. The absence of costimulatory signals led to the reduction of effector T cells and the development of T_{reg} cell populations, causing immunosuppression and reduced disease severity.[121] In conclusion, more studies are needed to determine how LNPs are sensed by the immune system. Recognition of molecular patterns for the activation of immune cell responses at the molecular level is a crucial aspect to be investigated for helping researchers design improved drug carriers to treat various modalities.

4.4. Adverse events

Alongside strong immunogenic response, side effects, ranging from mild reactogenicity to rare severe diseases, have also been reported following mRNA-LNP administration. Local side effects such as pain, swelling and redness at the injection site and systemic side effects such as fever, headache, myalgia, and arthralgia were commonly observed in recipients of COVID-19 mRNA vaccines. **(**Fig. 6**)** Notably, systemic side effects were more common in younger individuals (16–55 years old) than in the older population (>55 years of age) [20].

A balance between vaccine immunogenicity and reactogenicity is the key to mRNA-LNP-based vaccines. Immunogenicity is measured by the levels of neutralizing Ab against the target (coronavirus in case of COVID-19 mRNA vaccines) and the antigenic protein-specific T cell responses, whereas reactogenicity refers to physical manifestations of the inflammatory response to vaccination that occur shortly after vaccine administration (pain, redness, and swelling). In some rare cases severe adverse events have been reported, which include acute myocardial infarction (3.7 per million doses), Guillain–Barré syndrome (1 per million doses), Bell's palsy (6.4 per million doses), coagulopathy (14.5 per million doses), stroke (6.5 per million doses).[122,123] Many factors, including the age and gender of the host, injection technique, route of administration and dose number, can influence the reactogenicity. [124] In this regard, an important aspect of interest is the correlation of the effectiveness/immunogenicity of vaccines to the reactogenicity/adverse events. In an interesting report, 735 individuals who received the Comirnaty™ vaccine were studied to establish a correlation between the severity of adverse reactions (reactogenicity) and humoral and cellular response (immunogenicity). It was observed that males with severe adverse reactions to Comirnaty™ also had high IgG titers and neutralization activity when compared to men with minor or no injection side symptoms. However, findings are inconsistent across studies from different population groups from different regions of the world, making it challenging to reach a consensus [125–127].

1) Complement activation related pseudo allergy (CARPA) resulting in local, systemic, and rare severe side effects [123]. 2) Accelerated blood clearance (ABC) was observed upon repeated injections of PEG-containing therapeutics and attributed to the formation of anti-PEG antibodies. This may also result in decreased functional effect after the 2nd injection of the therapy.

Lipid-based nanoparticles have been reported to trigger an innate immune response causing an acute hypersensitivity syndrome, also known as complement activation-related pseudoallergy (CARPA), which occurs immediately after injection. The symptoms include swelling, chills, and anaphylaxis.[124,128] In fact, Doxil™ has also been reported to cause CARP; thereforee, the patients need to be strictly monitored both during and immediately after the administration.[129] A slow infusion rate is used to mitigate CARPA in clinics, and a corticosteroid is administered prior to infusion. Similarly, It should be pointed out that prior to infusion of Onpattro™ (administered intravenously to patients at a dose of 0.3 mg/kg once in three weeks) [6], patients receive immunosuppressants such as dexamethasone to alleviate the risk of adverse events. The underlying cause of CARPA has been believed to be activation of the complement system and generation of anaphylatoxins. Analysis of adverse events in individuals receiving mRNA COVID-19 vaccines from Dec 14, 2020, to June 14, 2021, indicated 5.5 anaphylaxis events per million doses administered. [123] While several experts

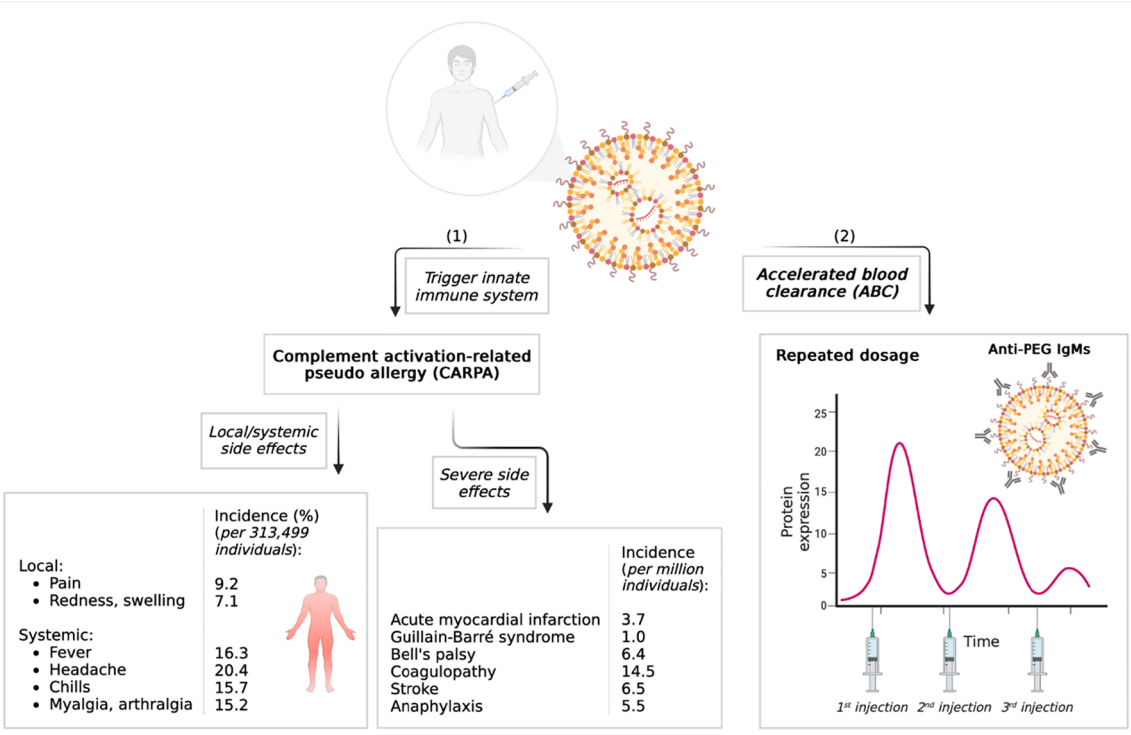

Fig. 6. Schematic overview of adverse events following mRNA-LNP injection.

have blamed PEG for the anaphylactic shock, the associated mechanisms are yet to be determined.[130] Identifying the sources of mRNA-LNP reactogenicity is difficult since animal models do not accurately recapitulate human symptoms. In addition, the severity of symptoms is highly variable across individuals, adding to the challenge. Importantly, adverse side effects and intolerability of mRNA-LNPs observed during clinical trials have also hindered the successful translation of many of the potential candidates. Nevertheless, the benefits of mRNA vaccines outweigh the risks associated with them.

4.5. Curious case of PEG lipids

PEG is widely used in drug delivery as a "stealth" polymer.[131] The presence of PEG on the nanoparticle shields the surface from opsonization and reduces phagocytosis by immune cells, which allows the particles to circulate for longer times. In addition, PEG plays a crucial role in determining the size and dispersity of nanoparticles, imparts stability and prevents aggregation during storage.[71] While PEGylation imparts stealth properties to LNPs, the increasing hydrophilicity can diminish the interaction of LNPs with cells and biomolecules of interest, which may cause a significant loss in the efficacy of the delivery system. This phenomenon is commonly known as the "PEG dilemma."[132] Therefore, the percentage of PEG is optimized to be low. For example, in Spikevax™, PEG-lipids constitute the smallest molar percentage (1.5 %) of the lipid components in LNPs. [133].

PEG-coating blocks the binding and recognition by opsonin proteins and immune cells. However, this notion is challenged by many studies. In fact, in many instances, PEGylated formulations are rapidly cleared from the body upon second and subsequent administration, a phenomenon termed accelerated blood clearance (ABC) observed across multiple animal species. The predominant reason for the clearance of PEGylated drugs is antibody production in response to the drug delivery system. **(**Fig. 6**)** Besin et al. investigated the mechanism behind the ABC of LNPs in mice. Upon first intravenous administration, LNPs activated B-1 lymphocytes, resulting in the production of antiphosphorylcholine IgM Abs. Upon subsequent injections, B-2 lymphocytes also activate to induce an anti–PEG adaptive humoral response.[134] In the context of humans, studies indicate that many of us have pre-existing anti-PEG antibodies. Both pre-existing and induced anti-PEG antibodies pose a challenge to the efficacy of putative mRNA-LNP therapeutics, specifically the ones that need to be administered at frequent intervals.[135] Antibodies specific to PEG_{2000}-C-DMG (anti-drug antibody) were also detected in patients receiving Onpattro™, though the occurrence was transient and observed at a low frequency of 3.4 % (5 of 145 patients). [136]The existence of anti-PEG responses for routes of administration other than IV is yet to be determined.

4.6. Effect of route of administration

There are several routes for administering mRNA-LNPs. While Onpattro™ was optimized to be injected intravenously (IV), Spikevax™ and Comirnaty™ were injected intramuscularly (IM). Other administration routes, such as intradermal (ID), subcutaneous (SC), and intranasal (IN), are also being investigated for efficacy and tolerability in several applications. Careful selection of administration route is crucial since factors such as absorption kinetics, anatomical and physiological properties and the injection site's local cellular environment influence the mRNA-LNPs' performance.[112] For instance, when injected IV, mRNA-LNPs tend to accumulate in the liver, an active site for protein synthesis, making the IV route suitable for applications such as protein replacement for inherited metabolic disorders.[137] Further, the immune cells encountered by mRNA-LNPs differ with the injection site; thus, different administration routes can give different immunogenic and reactogenic outcomes. For instance, skin tissue is infiltrated with antigen-presenting cells, which facilitate the direct capture of antigens to produce robust immunological responses upon ID injection of vaccines. Similarly, muscles have good vasculature that recruits circulating immune cells to the injection site. Combined with the ease of administration, IM is the most preferred administration route for vaccines. [85,116,138] Similarly, intranasal vaccination can stimulate mucosal immune responses and is a viable way to deliver mRNA-LNP vaccines against respiratory diseases [139].

Several studies compare the effectiveness of mRNA-LNP vaccines between different routes of administration. For example, Moderna's mRNA vaccine candidates against the influenza virus, H10N8 and H7N9, were evaluated for intradermal (ID) and intramuscular (IM) administration at varying dose ranges. A 25 μg ID dose induced HAI titers > 1:40 in 64.7 % of participants, whereas IM administration led to similar titers in only 34.5 %. However, ID vaccination was also associated with high rates of solicited adverse events.[140] In comparing the IM vs SC route, when Comirnaty™ was administered via SC instead of the IM route in a mouse model, the systemic adverse events were reduced without compromising the humoral immune response.[138] The intranasal route is also being investigated in several clinical trials. For example, in the phase II clinical trial by Translate Bio/Sanofi, adults with cystic fibrosis were given CFTR-mRNA (MRT5005) encapsulated in LNPs intranasally by nebulization. However, many individuals reported febrile and hypersensitivity reactions. [139] It should also be noted that the mRNA used in this study was unmodified and, thus, could also contribute to the observed reactogenicity.

Overall, the route of administration of choice determines the cell types available to interact with the LNPs, influencing the expression of mRNA, immune response, and tolerance. The mRNA-LNP-based vaccines and therapeutics should be examined in light of the route of administration and therapeutic goal. Also, investigations on the ionizable lipid structures suitable for different routes should be performed.

4.7. Impact of underlying health condition

Vaccine-mediated immunity depends on the generation of protective antibodies and memory B cells. A key question regarding the mRNA-LNP platform is whether it evokes antigen-specific immune responses equally well in healthy individuals and individuals lacking a fully functional immune system, such as those receiving immunosuppressants or individuals with pre-existing inflammatory conditions. A handful of studies investigate the functionality and impact of mRNA-LNPs on diseased individuals. For instance, immune response profiling of kidney transplant recipients indicated blunted SARS-CoV-2-specific B and T cell responses and critically impeded neutralizing antibody responses.[141] A reduced magnitude of vaccine-induced response is also seen in patients with inflammatory bowel disease (IBD) receiving anti-TNF-α therapy. The lower response in IBD patients over healthy individuals is linked to increased baseline inflammation and lack of activated cTfh1 cell expansion, leading to impaired memory B cell formation.[142] Similarly, the seroconversion rate was lower in immunocompromised patients with human immunodeficiency virus infection, allogeneic hematopoietic stem cell transplantation/CAR T cell therapy, solid organ transplantation, or chronic lymphocytic leukemia.[143] Contrary to this, LNPs may augment the pre-existing inflammation, as shown in studies on mice where the administration of LNPs to mice with pre-existing LPS-induced inflammation led to enhanced inflammatory cytokine (IL-6 and MCP-1) responses. This exacerbation was found to be independent of the mRNA cargo. As expected, mRNA-LNP formulated without ionizable lipid could not initiate the inflammation exacerbation effect, substantiating the contribution of ionizable lipid component in LNP-induced immune cell activation [144].

Clinical trials for the COVID-19 vaccine had exclusion criteria removing some of the high-risk populations and diseased individuals from the group and, therefore, did not reflect the impact of mRNA-LNPs. There is a shortage of information on this topic, which must be examined since the underlying health conditions can hinder the successful translation of the application. Nevertheless, the studies described here indicate that patient-tailored vaccination dosage/schedules may be required to make the mRNA-LNP platform successful. In addition, ionizable lipids should be evaluated in disease models to understand and discover the most suitable candidates for a particular health indication. Understanding the impact of mRNA-LNP in the setting of ongoing immune activation in various health indications can greatly benefit the clinical implementation of these therapeutics.

5. Status of clinical developments

While mRNA vaccines have been successfully used in the clinic, mRNA therapeutics are yet to reach this milestone, with many in clinical trials. While, in theory, mRNA therapeutics can be designed to produce any protein of interest, non-hepatic delivery and tolerability are two significant obstacles. Typically, delivering mRNA systemically via LNPs inherently results in LNP accumulation in the liver. The ability to target mRNA-LNP to other organs opens the door to new therapeutic applications and is a major focus of mRNA-LNP research. Although challenging, there are some observations on how changing LNP structural components can lead to organ tropism, as previously discussed. Thus, the applications of LNPs are expanded beyond the liver. Over the past three years multiple promising clinical trials have been started, and we shall discuss a few of them in this section. Excitingly, mRNA-LNPs are being researched for applications other than vaccines, including the treatment of cancer, genetic disorders, and autoimmunity. Prenatal delivery of mRNA, which can treat disease before the patient is born, has also been investigated [145].

5.1. Infectious disease

Historically, live attenuated viruses have successfully been eradicating fatal diseases such as smallpox (cowpox vaccine 1796). Other examples of vaccines that use live attenuated viruses include those against measles, mumps, and rubella (MMR) and the vaccine against varicella (chickenpox). Later, researchers developed subunit vaccines that use only part of a target pathogen to provoke a response from the immune system. These include polysaccharide vaccines, conjugate vaccines, and protein-based vaccines. Another class of vaccines used against bacteria is toxoid vaccines, in which, rather than targeting the bacteria itself, inactivated toxins are used to target the toxic activity created by the bacteria. Examples include the Tetanus vaccine and the diphtheria vaccine. The latest in the list are viral vector vaccines that deliver the genetic code for the antigen against which the immune response needs to be generated. The Ebola and COVID-19 vaccines by AstraZeneca and Johnson & Johnson are examples. Many of these vaccines require the addition of adjuvants to enhance the induction of durable protective immune responses.[146].

Despite this versatility, there are many diseases, such as tuberculosis, malaria, and AIDS, where the causative agent evades immune surveillance, making it challenging to design an effective vaccine. In this scenario, mRNA-LNPs have emerged as an important tool in the arsenal for fighting infectious diseases caused by deadly viruses and bacteria. [147,148] The mRNA-LNP vaccine platform offers several advantages over traditional vaccine technologies. Firstly, mRNA-LNPs can be rapidly manufactured using a cell-free system and are intrinsically versatile, leading to an accelerated vaccine development and market rollout process. The accelerable nature was evident from the fact that it took only 63 days for the mRNA-LNP COVID-19 vaccine to reach its first clinical trial, demonstrating this platform's importance in emergency preparedness. As reported, a five-litre bioreactor could produce almost a million mRNA vaccine doses per reaction, highlighting the scalability of the production process, which is a boon in situations of public health crises like a pandemic.[149] Secondly, mRNA-LNP vaccines induce both humoral and cellular immune responses, and the inherent immunostimulatory nature of both mRNA and LNP formulation improves vaccine efficacy.[150] Moreover, a single mRNA vaccine can encode multiple antigens, strengthening the immune response against pathogens. We briefly discuss a few examples of ongoing clinical trials of various indications [151].

Zika: Zika virus infection is spread among humans by Aedes mosquitoes and is associated with fetal and placental dysfunction and congenital disabilities during pregnancy. During the Zika virus outbreak

2016, Moderna developed mRNA-1325 and mRNA-1893 Zika virus vaccines and published the results of Phase I clinical trials early this year. While mRNA-1325 failed to induce effective Ab production, mRNA-1893 caused strong Zika virus-specific serum-neutralizing Ab responses after two doses.[152] Notably, the enhanced immunogenicity of mRNA-1893 over mRNA-1325 is attributed to a single amino acid residue difference, indicating the importance of mRNA sequence optimization.[153]

Influenza: Influenza is a global public health concern. Influenza viruses are highly mutating and ever evolving, posing a challenge to vaccine design. mRNA-1010 is a quadrivalent messenger RNA (mRNA) vaccine against seasonal influenza developed by Moderna. A single dose of mRNA-1010 (50 μg, 100 μg, or 200 μg) elicited HAI titers against vaccine-matched strains.[154] Moderna announced the interim Phase 3 safety and immunogenicity data early this year.[155] Notably, 70 % of mRNA-1010 recipients reported solicited adverse reactions (SARs), compared to 48 % of participants in the active comparator group receiving a licensed influenza vaccine. Moderna is expanding its influenza vaccine program and developing a portfolio of five influenza vaccine candidates, two of which (mRNA-1011 and mRNA-1012) incorporate additional HA antigens for broader coverage of circulating influenza A strains and the other two (mRNA-1020 and mRNA-1030) contain both HA and neuraminidase (NA) antigens to target multiple proteins involved in the influenza virus lifecycle and thus lessen the risk of viral antigenic escape. In May 2023, CureVac also announced the dosing of the first participant for a combined Phase 1/2 study, which will evaluate mRNA-based, modified, multivalent influenza vaccine candidates for safety, reactogenicity and immune responses [156].

Respiratory syncytial virus (RSV): RSV is the most common cause of lower respiratory tract infections, which spreads through contact with respiratory droplets from an infected person or touching surfaces contaminated with the virus. Although the symptoms are mild, RSV may cause severe illness in some people, including infants and older adults. Moderna's phase 3 efficacy trial of the mRNA 1345 vaccine demonstrated a vaccine efficacy of 83.7 % against RSV lower respiratory tract disease in adults 60 years of age and older. mRNA-1345 encodes for a stabilized perfusion S glycoprotein. The study was conducted on 37,000 adults 60 or older in 22 countries. Furthermore, there is an ongoing Phase 1 trial in pediatric populations.[157]

Chikungunya virus (CHIKV): CHIKV is mosquito-borne and causes fever, rash and arthralgia and sometimes progresses to arthritis in up to 50 % of the cases. Currently, there are no effective anti-viral drugs or vaccines to prevent infection with CHIKV. LNP encapsulating mRNA-1944 encoding the heavy and light chains of a CHIKV-specific monoclonal neutralizing antibody, targeting the CHIKV E2 glycoprotein, has completed human Phase I trials.[158] Administration of mRNA-LNP led to high levels of functional neutralizing antibody, which persisted for several months at levels above the predicted protective titers considered necessary to prevent CHIKV infection. However, three of the four participants experienced infusion-related reactions. While pretreatment with steroids prevented the occurrence of adverse effects, it also lowered the levels of neutralizing Ab.[158] Nevertheless, using an mRNA encoding a neutralizing antibody is a promising approach since monoclonal antibody therapies have been impeded by factors such as production challenges, subtherapeutic neutralizing effects and high manufacturing costs of recombinant antibody proteins.

The application of mRNA-LNP formulations is not limited to viral infections; some recently published reports demonstrate its effectiveness against lethal bacterial pathogens. Contrary to viral pathogens, bacteria typically express several thousand proteins, making it challenging to choose the right protein antigens for vaccination. Our lab successfully demonstrated the use of mRNA-LNP vaccine for protection against *Y. pestis*, a highly virulent bacterial pathogen.[159] Considering the emerging global antibiotic resistance crisis, the mRNA-LNP vaccines could potentially serve as promising tools to combat bacterial infections.

5.2. Cancer

The only approved immunotherapeutic cancer vaccine, Sipuleucel-T (Provenge), is an autologous dendritic cell therapy for the treatment of asymptomatic or minimally symptomatic metastatic prostate cancer. It was approved in 2010; however, it never gained widespread use owing to its limited accessibility, high cost and low clinical efficacy.[160] mRNA-LNPs offer an exciting opportunity to develop cancer treatment modalities and, more specifically, personalized treatment in oncology. Broadly classified, mRNA-LNPs can mediate immunotherapeutic effects in four major ways, including (1) tumor-associated antigen (TAA) mRNA, (2) neoantigen mRNA, (3) antibody mRNA, and (4) immunomodulator mRNA [12].

A well-known example of candidates for a novel TAA mRNA-based vaccine is KRAS, a proto-oncogene (mutated in 25 % of all tumors) which is currently being evaluated for the treatment of metastatic non-small cell lung cancer, metastatic colorectal cancer, and metastatic adenocarcinoma of the pancreas. mRNA-5671 is a cancer vaccine candidate developed by Moderna that targets the four most prevalent mutations in KRAS. The modified mRNA-LNP is administered through the intramuscular route and elicits the T-cell responses required for curative cancer therapy. Phase 1 trials of mRNA-5671 were recently completed as a monotherapy or in combination with pembrolizumab, an antibody against immune checkpoint inhibitor PD-1. In June 2023, a different cancer vaccine trial started when CureVac announced the dosing of the first patient with a cancer vaccine for glioblastoma in a Phase I study. Interestingly, CureVac used unmodified mRNA, which encodes for a single fusion protein comprising eight epitopes derived from tumor-associated antigens (TAA) with relevance in glioblastoma, including HLA class I and class II epitopes [154].

Contrary to TAA, neoantigens are unique to cancer cells and appear as important targets for personalized tumor immunotherapy. Patient-specific neoantigens can be selected, their sequences encoded *in vitro* transcribed mRNA that can be used as a vaccine against the tumor. Multiple neoantigens can be encoded in a single mRNA to target a spectrum of cancer mutations. For example, BNT121, a vaccine encoding ten neoantigens, is under development for treating malignant melanoma. It involves repeated administration in the inguinal lymph nodes and is under Phase I trial (13 melanoma patients) with encouraging results on immunological responses. A second clinical trial is BNT122, which contains 20 patient-specific neoantigens for treating pancreatic cancer. Phase I of BNT122 in combination therapy for pancreatic cancer showed recurrence-free disease activity in 16 patients. However, as with other mRNA-LNP vaccines, the patients experienced transient adverse events such as fever and chill. Moderna developed mRNA-4157 that comprises up to 34 neoantigens. In combination with pembrolizumab, it has shown promising results for adjuvant treatment of melanoma patients.[161]

In an exclusive approach, BioNTech designed BNT111, an intravenously administered cancer vaccine against melanoma that utilizes a negatively charged lipid nanoparticle formulation to increase uptake by antigen-presenting cells. It is currently in Phase II clinical trials, and patients receiving these RNA-LPX showed robust clinical responses with solid induction of antigen-specific $CD4^+$ and $CD8^+$ responses.[162]

The clinical trials mentioned above show great interest in using mRNA-LNPs for cancer vaccines. However, other research avenues for anti-cancer therapies are also under development.

5.3. Beyond vaccines

An area of renewed interest is using mRNA to generate therapeutic levels of immunomodulatory proteins or the replacement of defective or missing proteins. For successful use of mRNA-LNPs as protein immunotherapies, the treatment must lead to higher protein expression to reach the therapeutic effect compared to infectious disease and cancer vaccines, probably requiring lifelong treatment with recurrent dosage

[163]. Some examples of protein-replacement applications of mRNA-LNP include rare metabolic disorders such as propionic acidemia [164], methylmalonic acidemia [165] and glycogen storage disease type 1a[166] and hematological disorders including hemophilia A (factor VIII deficiency) and hemophilia B (factor IX deficiency).[137,167] The standard treatment for hemophilia A and B involves systemic injection of factor VIII or factor IX recombinant proteins three to seven times a week because of the relatively short half-life of the proteins. However, when factor VIII mRNA carrying LNPs were administered in mice, a single weekly systemic injection maintained the protein above therapeutic levels up to 5–7 days post-injection.[137] In another recent study, daily intraperitoneal administration of ARG1-mRNA-LNPs in constitutive knockout mice during the neonatal period was shown to prevent abnormalities in myelination and restore normal oligodendrocyte function associated with arginase deficiency.[168] Similarly, for treatment of Ornithine transcarbamylase (OTC) deficiency, intravenous administration of human OTC mRNA-LNPs was shown to restore urea cycle and a dose-dependent amelioration of survival rate in mice [169].

The examples mentioned above involve encapsulation of single mRNA inside the LNPs. However, in many studies multiple mRNAs have been encapsulated in a single LNP for immunomodulation applications to induce a desired therapeutic outcome. One such example is mRNA-2752, developed by Moderna, which consists of LNPs containing three different mRNAs (OX40 ligand, IL-23, IL-36γ) and is designed to inject directly into tumors. Together, these mRNAs boost the expansion of CD4 and CD8 T cells and enhance priming and maturation of dendritic cells. [170] Finally, LNP-mediated delivery of mRNA is now being proposed for treating inflammatory conditions like ulcerative colitis [171] and myocardial infarction [172].

A summary of the various clinical trials mentioned in this section can be found in Table 2. As seen, mRNA-LNPs are currently undergoing clinical testing for multiple applications. However, as more data is collected, new challenges arise that need to be addressed to open the path for the technology to expand to further applications.

6. Challenges, outlook, and future perspective

Thanks to the unprecedented speed at which mRNA-LNP vaccines were produced and administered to millions of people to curb the spread of coronavirus, the potential of this platform was validated. An optimal mRNA-LNP drug candidate should induce a high expression of the desired protein, be chemically stable and non-toxic, not accumulate in the body and can be repeatedly administered without causing unwanted elicitation of the immune system. There are several significant concerns about this technology that, when addressed, will speed up the transition of discoveries into clinical practice to reduce the incidence, morbidity, and mortality of several human diseases.

Firstly, it is essential to optimize the IVT reactions to synthesize mRNA with reduced impurities and couple this with purification steps to produce transcripts with high translation efficiency and decreased innate immune cell activation. The use of chemically modified nucleotides has been successfully employed; however, the mechanisms underlying this need to be investigated to further advance the design strategies for IVT mRNA.

The surging interest in mRNA as a drug modality is attributed to several factors, including the ability of LNPs to transport the mRNA intracellularly, the advantage of rapid production at a mass scale during emergencies and the strong immunogenicity without using an adjuvant. Therefore, LNPs are on double duty, both as a carrier and adjuvant. To improve the role of the carrier, a better understanding of LNP internalization, fusion with the endosomal membrane, and the event of endosomal escape is required [72,173]. Since the two mRNA-LNP vaccines received emergency approval, much remains to be learned about the immune responses elicited by the platform. The mechanisms by which LNPs exert their adjuvant effects are not fully known, and investigations on this will broaden the spectrum of applications of the mRNA-LNP platform.

The recipients of the mRNA vaccine reported adverse effects, symbolizing some inflammation.[174] Although it is proposed and even practiced to use immunosuppressive agents before the drug administration, this intervention can lead to decreased protein expression.[158] Therefore, there is a scope for improvement in the design of ionizable lipid candidates which give effective mRNA translation without jeopardizing safety and tolerability. However, this also raises an important question: is there a correlation between inflammation and mRNA translation? The story gets more complicated when the application involves repeated dosing at frequent intervals. It is documented that a more significant number of adverse events were reported following the second dose of mRNA-LNP COVID-19 vaccines compared to the first dose.[154] Along these lines, an important question is what will be the tolerability upon repeated administration of mRNA, which is challenging to predict since it depends upon multiple factors, including the route of administration, the components of LNPs and the payload. It is yet to be determined if the structure of ionizable lipids also impacts the behaviour of mRNA-LNP when dosed multiple times. Besides tolerability, another pertinent question is regarding the accumulation of lipid metabolites, as their buildup may lead to toxicity and pose a safety risk. There needs to be more data on eliminating the lipid and its metabolites. As mentioned, **ALC-0315** was speculated to be cleared more slowly than **SM-102**.[175] Therefore, the effects of the long-term presence of ionizable lipids and their metabolites also need to be determined. Other components of LNPs could also induce changes in immune cells, which is another aspect that requires testing. For example, cholesterol-rich LNPs were shown to induce tolerogenic dendritic cell maturation, mediated by intracellular accumulation of cholesterol and activation of liver X receptor (LXR) pathway [176].

As highlighted in this review, the ionizable lipid is the most critical component that dictates the functionality of LNPs. There is no explanatory data on the immunogenicity of the ionizable lipid components used in the COVID-19 vaccine formulations. However, it seems that identifying and selecting an appropriate lipid formulation significantly impacts how quickly it enters clinical trials. From the ongoing clinical trials, LNP formulation with regulatory precedence has been used in different vaccine candidates. mRNA-1273 (Comirnaty™) employs the same LNP as mRNA-1647 (a vaccine against the CMV virus) and mRNA-1653 (a vaccine against the hMPV-PIV3 virus).[17,158,177] Since there is a strong correlation between the structure of ionizable lipids and the activity of LNPs, we believe that the hunt for potent ionizable lipids must continue. As shown by the data on the lower efficacy of clinically approved ionizable lipids on individuals with underlying medical conditions, there is room for rational design of novel lipid structures. The placebo used during the mRNA-LNP vaccine or therapeutics clinical trials is 0.9 % saline. We propose to use empty LNPs as a control group to elucidate the contribution of the LNP components to inflammation. However, it should be noted that empty LNPs might have different physicochemical properties when they are complexed with negatively charged mRNA.

Next, the impurities arising from synthesizing ionizable lipids can also negatively impact the performance of the mRNA-LNPs. For example, oxidation and subsequent hydrolysis of the tertiary amine head group of ionizable lipids can generate complex chemical groups with the mRNA payload and render it non-translatable. It is critical to monitor and control the formation of mRNA-Lipid adducts during the design of formulations since these impurities often remain undetectable.[178] Techniques such as reversed-phase ion pair high performance liquid chromatography (RP-IP HPLC) can detect even single adduct events on intact mRNA.

Finally, the stability of mRNA-LNPs is another area with ample space for advancement. Despite tremendous success, mRNA-LNP vaccines require storage at deep freeze temperatures, which restricts their availability and has proved to be one of the significant challenges during the COVID-19 pandemic. mRNA molecules are unstable at room

Table 2
Current clinical trials of mRNA-LNP vaccines and therapeutics.

Modality	Type	Program	Company	Clinical stage
COVID-19 vaccines	*COVID-19*	Mono- and bivalent constructs (modified mRNA)	CureVac*Collaboration with GSK*	Phase 2
		CV0501 (modified mRNA)	CureVac*Collaboration with GSK*	Phase 1
		CV2CoV (unmodified mRNA)	CureVac*Collaboration with GSK*	Phase 1
		LUNAR(R) - COV19 (partnered with CSL)	Arcturus therapeutics	Phase 3
		SARS-COV-2 mRNA vaccine (variants)	Walvax Biotechnology	Phase 3
		DS-5670 (JP):		
		• mutant strain, primary vaccination, 5 to 11 aged children		Phase 2
		• original strain, primary vaccination, 12 to 17 aged children	Daiichi Sankyo	Phase 3
		• mutant strain, booster vaccination, 12-year-old and over		Phase 3
		• mutant strain, booster vaccination, 5 to 11 aged children		Phase 3
		• original strain, booster vaccination		Registration
		COVID-19 vaccine (next generation - 2-5 °C) (mRNA-1283)	Moderna	Phase 3
		Prophylactic COVID-19 mRNA vaccine:		
		• EU-children 6 months - 4 years of age		Registration
		• U.S. - 5-11 years of age	Pfizer	Registration
		• U.S. - 6 months - 4 years of age		Registration
		• Omicron variant, U.S. - 6 months and older		Registration
	COVID-19 combinatorial	mRNA COVID-19 (GSK4396687)	GSK	Phase 1
		COVID-19 + Flu vaccine (mRNA-1073)	Moderna	Phase 1
		COVID-19 + Flu vaccine (mRNA-1083)	Moderna	Phase 1
		Prophylactic COVID-19 + Influenza mRNA vaccine (fast track – U.S.)	Pfizer	Phase 1
		COVID-19 + Flu + RSV vaccine (mRNA-1230)	Moderna	Phase 1
Other infectious diseases (viral/bacterial)	*Viral infections*	Multivalent constructs (Influenza) (modified mRNA)	CureVac*Collaboration with GSK*	Phase 1
		Flu-SV mRNA (Influenza) (modified mRNA)	CureVac*Collaboration with GSK*	Phase 1
		CVSQIV (Influenza) (unmodified mRNA)	CureVac*Collaboration with GSK*	Phase 1
		CV7202 Rabies vaccine candidate	CureVac	End phase 1
		Flu vaccine:		
		• mRNA-1010	Moderna	Phase 3
		• mRNA-1020		Phase 2
		• mRNA-1030		Phase 2
		• mRNA-1011		Phase 2
		• mRNA-1012		Phase 2
		Older adults RSV vaccine (mRNA-1345)	Moderna	Phase 3
		Flu + RSV vaccine (mRNA-1045)	Moderna	Phase 1
		Pandemic Flu (mRNA-1018)	Moderna	Phase 1
		Pediatric RSV vaccine (mRNA-1345)	Moderna	Phase 1
		Pediatric hMPV + PIV3 vaccine (mRNA-1653)	Moderna	Phase 1
		Pediatric RSV + hMPV vaccine (mRNA-1365)	Moderna	Phase 1
		Prophylactic mRNA vaccine:	Pfizer	
		• Varicella		Phase 1
		• Influenza (adults)		Phase 3
		SP0273 Influenza (mRNA QIV)	Sanofi	Phase 1
		SP0256 RSV older adults (mRNA RSV)	Sanofi	Phase 1
		Seasonal Flu (GSK4382276)	GSK	Phase 1
	Public health	CMV vaccine (mRNA-1647)	Moderna	Phase 3
		EBV vaccine to prevent infectious mononucleosis (mRNA-1189)	Moderna	Phase 1
		EBV vaccine to prevent long term EBV sequelae (mRNA-1195)	Moderna	Phase 1
		VZV vaccine (mRNA-1468)	Moderna	Phase 1
		HIV vaccine:		
		• mRNA-1644	Moderna	Phase 1
		• mRNA-1574		Phase 1
	Bacterial infections	Zika vaccine (mRNA-1893) (BANDA funded)	Moderna	Phase 2
		Nipah vaccine (mRNA-1215) (NIH funded)	Moderna	Phase 1
		Lyme disease vaccine:	Moderna	
		• mRNA-1975		Phase 1
		• mRNA-1982		Phase 1
		GVGBM multi-epitope mRNA cancer vaccine candidate for surgically resected glioblastoma	CureVac	Phase 1
Disease/deficiency vaccines and therapies	*Cancer/immuno-oncologyvaccines and therapies*	CV8102 non-coding RNA oncology candidate for solid tumors	CureVac	End phase 1
		Individualized neoantigen therapy (INT) (mRNA-4157) (50/50 global profit sharing with Merck)	Moderna	Phase 3
		KRAS vaccine (mRNA-5671)	Moderna	Phase 1
		Checkpoint vaccine (mRNA-4359)	Moderna	Phase 1

(*continued on next page*)

Table 2 (*continued*)

Modality	Type	Program	Company	Clinical stage
		OX40L/IL-23/IL-36γ (triplet) for solid tumors/ lymphoma (mRNA-2752)	Moderna	Phase 1
		IL-12 solid tumors (MEDI1191)	Moderna	Phase 1
		BNT142 (RiboMab) multiple solid tumors (CD3xCLDN6)	BioNTech	End Phase 1
		BNT151 (RiboCytokine) multiple solid tumors (IL-2 variant)	BioNTech	End Phase 1
		BNT152 + BNT153 (RiboCytokine) multiple solid tumors (IL-7, IL-2)	BioNTech	Phase 1
	Other disease/deficiency vaccines and therapies	LUNAR® OTC (Ornithine Transcarbamylase Deficiency)	Arcturus therapeutics	Phase 2
		LUNAR® CF (Cystic Fibrosis)	Arcturus therapeutics	Phase 1
		Relaxin (mRNA-0184)	Moderna	Phase 1
		VEGF-A myocardial Ischemia (AZD8601)	Moderna	Phase 2
		Propionic acidemia (PA) (mRNA-3927)	Moderna	Phase 2
		Methylmalonic acidemia (MMA) (mRNA-3705)	Moderna	Phase 1
		Glycogen storage disease type 1a (GSD1a) (mRNA-3745)	Moderna	Phase 1
		Cystic fibrosis (CF) (VX-522)	Moderna	Phase 1

temperature, resulting in the need for cold (−20 °C) or ultracold (−70 °C) shipping and storage for mRNA-LNP vaccines. Encouragingly, the lyophilization technique has been shown to prolong the shelf-life of mRNA vaccines stored at room temperature and 4 degrees without compromising the efficacy.[179,180] In this regard, Pfizer has initiated a phase 3 study to compare the safety and tolerability of the lyophilized Spikevax™ formulation with the frozen counterpart.[181].

In conclusion, as we mitigate these challenges and expand our knowledge of mechanisms of the functionality of various components of the mRNA-LNP platform, we will be empowered to tailor and design a variety of mRNA-LNP vaccines and therapeutics. The future of the mRNA-LNP platform is bright.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

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

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