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Review article

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Strategies to reduce the risks of mRNA drug and vaccine toxicity

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Abstract

mRNA formulated with lipid nanoparticles is a transformative technology that has enabled the rapid development and administration of billions of coronavirus disease 2019 (COVID-19) vaccine doses worldwide. However, avoiding unacceptable toxicity with mRNA drugs and vaccines presents challenges. Lipid nanoparticle structural components, production methods, route of administration and proteins produced from complexed mRNAs all present toxicity concerns. Here, we discuss these concerns, specifically how cell tropism and tissue distribution of mRNA and lipid nanoparticles can lead to toxicity, and their possible reactogenicity. We focus on adverse events from mRNA applications for protein replacement and gene editing therapies as well as vaccines, tracing common biochemical and cellular pathways. The potential and limitations of existing models and tools used to screen for on-target efficacy and de-risk off-target toxicity, including in vivo and next-generation in vitro models, are also discussed.

Sections

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Introduction

mRNA has proven to be a disruptive biomedical technology, with billions of doses of mRNA vaccines successfully used worldwide for the prevention of coronavirus disease 2019 (COVID-19) and to reduce the risk of development of its most severe symptoms[1–4]. The use of mRNA technology offers many advantages over conventional vaccine and drug development, including short manufacturing time and diversity of applications through simple changes in mRNA sequence. The rapid development of bivalent COVID-19 mRNA vaccines to target both the ancestral Wuhan-Hu-1 and omicron B.1.1.529 spike in under a year demonstrates the rapid timeline for modifications with mRNA technology in the clinic. Moreover, these bivalent vaccines elicit superior neutralizing antibody responses against omicron compared with the original vaccine, which targeted just the ancestral strain[5].

The clinical use of mRNA in vaccines and drugs is relatively new, which raises important safety concerns that need to be addressed. Dozens of drugs and vaccines that use mRNA technology are in late-stage development and in clinical trials for respiratory and latent viruses, rare diseases, cancer, autoimmune diseases and many other human health conditions[4,6] (Box 1). A major challenge now is how to efficiently de-risk potential toxicities associated with mRNA technology as its clinical applications rapidly expand beyond immunization purposes.

Application of mRNA drugs and vaccines to research and development pipelines has strong similarities to biologics as well as some aspects of more traditional small-molecule drugs. This includes the initial identification of therapeutic areas with unmet need and assessment of whether an mRNA can be developed to target those needs. Once a target area is defined, mRNA drug and vaccine early development occurs with preclinical pharmacology assessments of delivery to appropriate sites and validation of responses at levels that would be expected to be sufficient to provide clinical benefit. Toxicology assessments are performed either simultaneously or subsequently to identify no-observed-adverse-effect levels, maximum tolerated dose or a maximum feasible dose in preclinical animal models to help anticipate human exposure scenarios. With the US Senate passage of the Food and Drug Administration Modernization Act 2.0, S.5002 (ref. 7), alternatives to animal models may be more frequently used to assess toxicity risks in the future[8], but, at present, toxicity assessments are performed in relevant animal models. At the nonclinical stage, toxicological pathology informs potential human risk of adverse effects associated with exposure to mRNA drugs and vaccines. Additionally, nonclinical studies provide toxicology profiles with sufficient duration and dose exposure to support the movement of these investigational mRNA drugs and vaccines into clinical trials.

Like some biologics, mRNAs used to generate functional proteins might lead to toxicities[9,10]. Additionally, mRNA drugs and vaccines are mostly delivered in lipid nanoparticles (LNPs) that contain many buffer and small-molecule lipid components that are also capable of inducing toxicities[9,11,12]. This complexity of current mRNA drug and vaccine delivery formulations raises toxicity risks from various factors. Aside from some single-dose vaccines or gene editing purposes, mRNA drugs and vaccines may require repeat dosing, with additional toxicological concerns[13,14].

As the field of nucleic acid therapeutics is rapidly expanding (Box 1), several reviews have focused on the mechanisms behind the desirable immunogenicity of mRNA-based COVID-19 vaccines[15], highlighted advances in lipid engineering for nucleic acid delivery[16] and summarized potential therapeutic applications of mRNA[17]. At the same time, there is a need to comprehensively summarize safety concerns associated with the mRNA platform and the tools that can be used to interrogate them at the preclinical drug development phase. In this Review, we focus on the toxicities and pathogenicities that have been identified during the preclinical development of novel mRNA drugs and vaccines. We discuss how these may be linked to their components and highlight some emerging toxicological concerns. Studies on clinically approved mRNA vaccines and their post-marketing surveillance relate to pharmacovigilance and are therefore not covered in this article. As ionizable lipids have become the staple mRNA vector, most published data considered here are on LNP-formulated mRNA. Finally, we discuss advances in next-generation, nonclinical models and how they could be leveraged to de-risk the preclinical development of novel mRNA formulations.

Translating mRNA therapeutics

Once a protein target is identified, its mRNA sequence can be synthesized by in vitro transcription (IVT) in a cell-free environment: a linearized, plasmid DNA molecule is combined with ribonucleotides in the presence of bacteriophage RNA polymerase (of which T7 is the most widely used); the polymerases then recognize the promoter region in the DNA template and synthesize the RNA transcripts in the presence of ribonucleotides[18]. However, unmodified IVT mRNA is fraught with aberrant immunogenicity and inefficient intracellular delivery. The successful clinical translation of mRNA therapeutics was catalysed by two scientific milestones: the mitigation of the aberrant immunogenicity of IVT mRNA and the engineering of LNPs that can deliver it to the cell cytoplasm.

Ablating the immunogenicity of IVT mRNA

The immunogenicity of double-stranded RNA (dsRNA) was first shown when poly(I:C) (a synthetic analogue of dsRNA) triggered Toll-like receptor 3 (TLR3) in human embryonic kidney 293 (HEK293) cells, which activated nuclear factor κB (NF-κB) and led to increased IL-6, IL-12 and tumour necrosis factor (TNF) production[19]. It was later demonstrated that unmodified IVT mRNA may contain double-stranded regions (for example, a hairpin), which can bind to and modulate the expression of TLR3, leading to NF-κB activation (Fig. 1a) and increased intracellular mRNA levels of TNF (in dendritic cells) and IL-8 secretion (from HEK293 cells)[20] (Fig. 1b). IVT mRNA activated the interferon regulatory factor 1 (IRF1) in dendritic cells and increased the mRNA levels of IL-1 receptor-associated kinase M (IRAK-M; also known as IRAK3). These events are all associated with TLR signalling: IRF1 positively regulates TLR3 expression[21], IRAK-M is induced by and negatively regulates TLR stimulation[22], and TNF can be expressed downstream of TLR3 activation in the presence of intact[23] or damaged non-coding regions of dsRNA[24].

Unmodified IVT mRNA can also trigger TLR7 or TLR8 and result in the production of pro-inflammatory cytokines[25]. In early studies with single-stranded RNA (ssRNA), it was shown that unmodified HIV *gag*-encoded IVT mRNA provides a maturation signal to primary human dendritic cells, which can then induce $CD4^+$ and $CD8^+$ T cell primary immune responses[26]. Similarly, unmodified ssRNA led to a marked increase in IL-12 secretion by dendritic cells and T cell polarization[27]. Subsequent studies showed that ssRNA can trigger TLR7-dependent production of interferon-α (IFNα)[28]. At the same time, uridine (U)- and guanosine (G)-rich ssRNAs were identified to trigger TLR7 or TLR8 and increase the secretion of TNF, IL-12p40 and IL-6 from dendritic cells and macrophages[29]. Interestingly, the crystal structure of TLR7 revealed distinct binding sites for U- or G-rich ssRNA[30], whereas TLR8

can sense enzymatic degradation products of ssRNA[31]. Finally, it is worth noting that in a recent study, an IVT ssRNA molecule with good homology to a well-studied bacterial oligodeoxynucleotide TLR9 ligand caused the strongest immune response by means of increased IL-6 and TNF production in murine macrophages and mice[32].

Immune responses to unmodified IVT mRNA may implicate pattern recognition receptors (PRRs) other than TLRs, such as RIG-I-like receptors (RLRs). In HEK293 cells, it was shown that IVT mRNA could be transcribed from its promoter-less end to produce fully complementary antisense RNA; the produced dsRNA stimulated melanoma differentiation-associated protein 5 (MDA5; also known as IFIH1, a cytosolic PRR), which upregulated the production of intracellular IFNβ[33]. In murine macrophages, IVT mRNA caused the phosphorylation of IκBα protein, NF-κB activation and increased intercellular adhesion molecule 1 (ICAM1)[32], thus promoting an inflammation-resolving M2 phenotype (although these events could also be downstream of TLR signalling)[34]. RNA immunogenicity is now more than ever an area of active research, and interested readers are referred to recent reviews that provide a comprehensive summary of the field[35–38].

Ablating the aberrant immunogenicity of IVT RNA was arguably indispensable to its clinical translation. This was marked by a seminal work that showed that nucleoside methylation and pseudouridine incorporation dampen the innate immune recognition of IVT ssRNA[39]. In particular, TNF or IL-12 secretion from dendritic cells was diminished when exposed to ssRNA synthesized with pseudouridine, N^6-methyladenosine, 5-methylcytidine or 2-thiouridine. Furthermore, the modified ssRNA did not stimulate TLR3, TLR7 or TLR8 in stably TLR-expressing HEK293 cells, which produced less IL-8 than when treated with unmodified ssRNA. Nucleoside modification has since been instrumental in the development of immunotolerant and effective therapeutic mRNA applications. For example, 5-methylcytidine and 2-thiouridine nucleoside-containing mRNAs encoding mouse erythropoietin (mEPO) effectively increased the serum mEPO and haematocrit levels in the injected mice without eliciting strong immune responses[40]. Purification of IVT mRNA from dsRNA impurities by means of reverse-phase high-performance liquid chromatography (RP-HPLC) further dampened its immunogenicity while improving its translation efficiency in human dendritic cells for human erythropoietin (hEPO)[41]. In vivo, dsRNA purification in combination with nucleoside modification of mouse *Epo* or rhesus *EPO* IVT mRNA increased and maintained higher EPO serum levels when compared with unmodified mRNA, following a single intraperitoneal injection in mice or macaques, respectively[42].

For therapeutic applications, in vitro synthesized mRNAs usually undergo dsRNA purification and feature some sort of nucleotide modification. Other design modifications are also applied. Encoding a 3′ poly(A) tail in the template adds 120–150 adenines, which facilitates initiation of translation and enhances the stability of the mRNA strand[43,44]. Equally important, modifications to include a 5′ N^7-methylguanosine triphosphate structure on the 2′-O-methylated ribose of the first and sometimes second nucleotide constitute a cap that marks IVT mRNA as 'self' and abrogates recognition by interferon-induced protein with tetratricopeptide repeats 1 (IFIT1)[45,46]. In fact, nucleoside modification and purification techniques are constantly being optimized. For example, N^1-methylpseudouridine (1mψ) provides better transfection efficiency and further reduces immunogenicity over pseudouridine[47], and cellulose fibres retain dsRNA impurities at levels comparable to those achieved by RP-HPLC[48]. More recently, a mutant of T7 RNA polymerase was computationally engineered to minimize dsRNA impurities during IVT mRNA, which suppressed immune stimulation in vitro[49]. Currently, 1mψ modification and combination of resin and RP-HPLC for dsRNA removal return low immune stimulation and achieve high protein expression levels, both in vitro and in vivo[50]. A thorough review of nucleic acid engineering techniques and how they have facilitated mRNA therapeutic applications has recently been published[51].

Novel in vitro RNA engineering techniques such as circular mRNA (Box 2) have recently been proposed to improve on the relatively short intracellular half-life of linear mRNA.

Delivering mRNA to cells

Cytosolic delivery is a prerequisite for efficient mRNA drugs and vaccines, but negatively charged, long mRNA molecules diffuse very slowly across phospholipid bilayers because of their size and negative charge, [52]. Another hurdle is that mRNA undergoes fast degradation by intracellular ribonucleases (RNases) as well as RNases in the blood[53]. These challenges were overcome by complexing mRNA with lipids to form LNPs, which, to date, are the major delivery vehicle for mRNA medicines.

Box 1

The landscape of mRNA drugs and vaccines

Nucleic acid therapeutics introduced a paradigm shift in the design of novel drugs and vaccines that treat diseases at the DNA or RNA level[245]. The mRNA platform has the advantage of using cytoplasmic cellular apparatuses for protein translation towards protein and enzyme replacement therapies, immunization against pathogens, cancer immunotherapy and gene editing[54,246]. The FDA-approved mRNA-based vaccines against coronavirus disease 2019 (COVID-19) are excellent examples of how this platform can provide fast, efficient and safe vaccines against pathogens. Moreover, mRNA provides a promising platform to treat diseases caused by malfunctioning or missing proteins or enzymes[18]. In situ protein expression enables their subcellular transfer, which also improves therapeutic efficacy[43]. Natural mRNA decay and protein turnover rates limit the duration of their therapeutic effect[247,248] but their efficacy and longevity still outperform those of DNA or traditional protein-based therapies[249]. In comparison with gene therapies, mRNA activity is not hindered by the permeability of the nuclear membrane and, as such, there is no risk of mutagenesis owing to off-target addition into DNA segments[250].

Owing to the versatility of the mRNA platform, drug and vaccine candidates have been synthesized to treat many different diseases and immunize against many pathogens, respectively[198,251]. These are usually coupled with existing or novel lipid nanoparticle constructs to enhance efficacy and avoid adverse effects[64,182]. By mid-2023, there were 41 mRNA-based vaccines for infectious diseases and ten for cancer immunotherapy in phase II or more advanced clinical trials, as well as 21 mRNA formulations for other indications (for example, protein replacement therapies)[17].

mRNA formulated in lipid nanoparticles (LNP–mRNA) is protected from biodegradation and exhibits improved half-life, increased cellular uptake and protein translation compared with naked mRNA delivery[54]. Preparation of LNP–mRNA particles is achieved by the chaotic mixing of lipids dissolved in ethanol and mRNA molecules dissolved in low pH aqueous solutions[55,56]. Typically, lipids in LNP formulations include an ionizable (amino) lipid, a polyethylene glycol-linked (PEGylated), cholesterol and a helper lipid, all of which may impact the biological activity, biodegradability and structural stability of the LNP[57]. Specifically, the ionizable lipid improves endosomal escape and transfection efficiency for the delivered mRNA[58], PEGylated lipids improve aqueous colloidal stability and impede LNP uptake by the mononuclear phagocyte system (MPS)[59], cholesterol imparts flexibility and allows fusion with cellular membranes[60], and helper lipids provide structural integrity and improve encapsulation efficiency[61,62]. LNP self-assembly is a physical, bottom-up process that leads to a thermodynamically favourable arrangement of the mixed components, driven by electrostatic interactions (between negatively charged mRNA and positively charged ionized lipids) and the amphiphilicity of other lipids[63]. The size, surface charge and composition of the final LNP largely depend on the lipid ratio during formulation and on the individual properties of lipids (for example, pK_a, alkyl chain length, branching of ionizable lipid and ester bonds, among others)[64].

Advancements in our understanding of RNA biology[4] and LNP engineering[57] have led to crucial improvements in the pharmacokinetic and pharmacodynamic profile of mRNA vaccines that enabled the safe and effective immunization of hundreds of millions of people against COVID-19. The pharmaceutical and biotech industry has since invested in the proven capacity of the platform for rapid synthesis of RNAs for therapeutic or preventive applications[65] (Box 1).

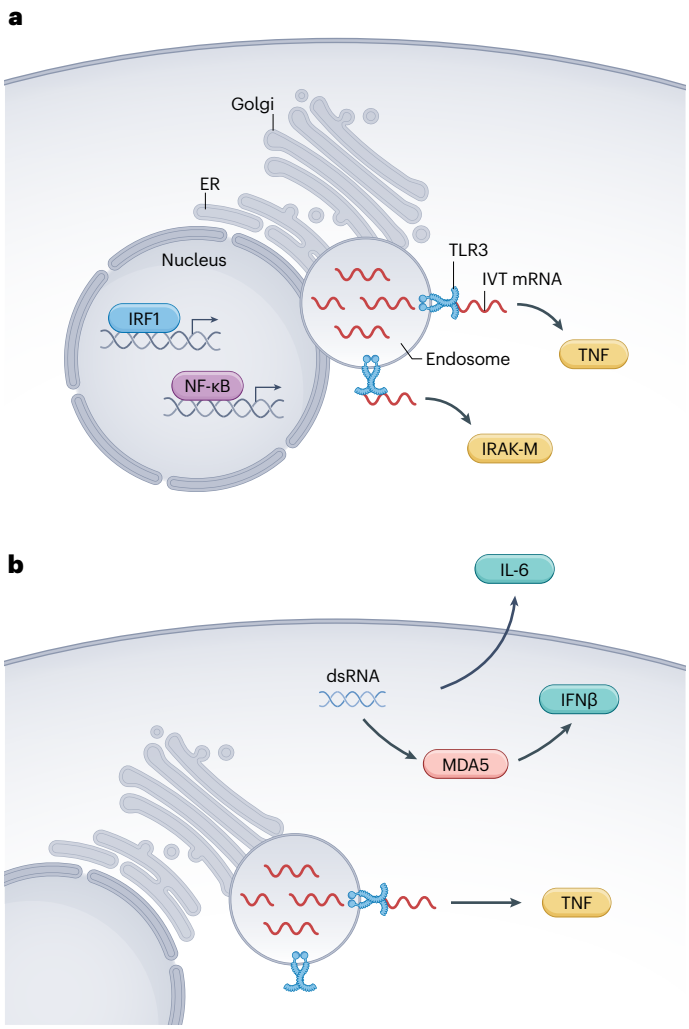

Fig. 1 | Innate immune responses to unmodified IVT mRNA. a, Endosomal signalling of Toll-like receptor 3 (TLR3) upregulates IL-1 receptor-associated kinase M (IRAK-M) and tumour necrosis factor (TNF), and nuclear transcription factors interferon regulatory factor 1 (IRF1) and nuclear factor κB (NF-κB) are activated. **b**, Endosomal signalling of TLR3 leads to increased intracellular levels of TNF. Adventitious, double-stranded RNA can activate cytoplasmic melanoma differentiation-associated protein 5 (MDA5) and lead to increased intracellular interferon-β (IFNβ) and extracellular IL-6. ER, endoplasmic reticulum; IVT, in vitro transcription.

Routes of administration

The route of administration for mRNA medical products depends on the intended application (for example, therapy versus immunization) and disease pathophysiology. Depending on the route of administration, formulation parameters such as size and lipid composition need to be adjusted to improve the biopharmaceutical profile of mRNA, including specific organ tropism[66]. To date, most mRNA vaccines in advanced stages of development are designed for intramuscular administration. In addition, the platform has great prospects for protein replacement therapies, capitalizing on the natural hepatic tropism of LNP–mRNA following intravenous administration. Nonetheless, per os and other routes of administration need further development to overcome physical limitations and physiological barriers[67].

Intramuscular and intratumoural administration

Intramuscular and intratumoural administration dominate clinical trials. The development of intramuscularly administered vaccines is particularly promising as only a small amount of antigen production suffices to elicit innate immune responses that can also initiate cell-based adaptive immunity[4]. Indeed, of the LNP–mRNA formulations in clinical trials that are administered intramuscularly, most target infectious diseases[68]. Immune cell interception of the LNP–mRNA construct, antigen production and access to the lymphatic system are required for successful immunization[69]. The size and surface properties of the LNP–mRNA particles can impact opsonization (that is, tagging of particles with antibodies and complement fragment for uptake by phagocytic cells) and thus uptake by antigen-presenting cells such as macrophages[70]. In fact, mRNA-based antigen production allows for the natural presentation of any transmembrane domains and formation of multimeric complexes, leading to strong T cell responses[71,72].

Recent studies have shed light on the key immunological events that follow intramuscular administration of mRNA-based vaccines, including those against COVID-19 (ref. 15). In summary, following intramuscular injection of the LNP–mRNA construct, dendritic cells, monocytes and neutrophils infiltrate the injection site, resulting in local inflammation[73]. This initial local inflammation is transient and less severe when compared with subcutaneous administration[74], presumably owing to better muscle tissue vascularization and lymphatic drainage. Of the infiltrating immune cells, neutrophils are the least efficient in translating the mRNA product[73], therefore ample

presence of monocytes and dendritic cells is necessary for efficient immunization[75]. In the case of the BioNTech–Pfizer BNT162b2 vaccine, activated monocytes, dendritic cells and macrophages are responsible for antigen presentation in the draining lymph nodes of mice[76], but it is important to mention that free LNP–mRNA can also diffuse to the draining lymph nodes independently of its transport there by endocytic immune cells[77]. Activation of $CD4^+$ T cells to follicular helper T cells (T_{FH} cells) supports the formation of germinal centres, activation of B cells and eventual production of plasma and memory B cells[78]. The mRNA construct and the encoded spike protein for both the Moderna mRNA-1273 and BNT162b2 vaccines have been detected in the draining axillary lymph nodes of vaccinated humans up to 60 days after second-dose vaccination[79]; further, antigen-specific T_{FH} cells have been detected in the circulation 6 months after boost vaccination, which suggests ongoing germinal centre activity in the lymph nodes or spleen[80].

Of particular note is the adjuvanticity of ionizable lipids in LNP–mRNA vaccines. Given the suppressed immunogenicity of the modified mRNA, it was not clear until recently which component, if any, assumes the adjuvant role in mRNA-based vaccines. Ionizable lipids in the LNPs were demonstrated to trigger the production of IL-6, antigen-specific $CD4^+$ T_{FH} cells and the differentiation of B cells in the germinal centre, following a single intramuscular administration of the LNP–mRNA construct in mice[81]. This work suggested that the ionizable lipid component alone (and not the mRNA) provides adjuvanticity by stimulating the innate immune system outside the TLR or RLR pathways associated with RNA sensing. Conversely, another study showed that the mRNA payloads of both mRNA-1273 and BNT162b2 vaccines do confer adjuvanticity by interacting with MDA5, producing type I interferons and thus inducing strong $CD8^+$ T cell production in mice[76]. So far, the adjuvanticity and immunogenicity of LNP–mRNA vaccines seem to be contingent on both the lipid and nucleic acid components, while any potential immunostimulatory synergy has not been adequately addressed.

Intramuscular delivery is also the route of choice for cancer immunotherapies[82]. mRNA-based cancer immunotherapies are translated into tumour-associated antigens or neoantigens. Tumour-associated antigens are proteins and enzymes overexpressed in cancer cells, whereas neoantigens are proteins expressed uniquely by cancer cells. The therapeutic effect relies on the translation and presentation of these antigens, which, if successful and adequate, recruits $CD8^+$ and $CD4^+$ T cells against the cancer cells that bear them[83]. One such formulation is in a phase I dose escalation study to assess its safety when administered as a monotherapy against resected solid tumours or in combination with pembrolizumab (an anti-PD1 monoclonal antibody) against unresectable solid tumours (NCT03313778). Here, it is worth mentioning that intratumoural administration is also used in cancer immunotherapy. In one phase I study, the delivered mRNA encodes the cytokines IL-23 and IL-36γ and the antitumour stimulatory OX40 pathway on effector T lymphocytes to boost the patient's immune response within the microenvironment of solid tumours (NCT03739931).

Intradermal and subcutaneous administration

Intradermal and subcutaneous administration represent promising alternative routes, as demonstrated in several in vitro and in vivo studies. Following intradermal delivery, Langerhans cells and dendritic cells in the dermis and epidermis provide efficient antigen presentation and immune response[84] for vaccination against pathogens or for cancer immunotherapy[85]. In vivo, intradermal delivery of self-amplifying mRNA has outperformed electroporation in terms of transfection efficiency[86]; in another study, single intradermal administration of mRNA-LNPs encoding vascular endothelial growth factor C could support lymphatic growth and alleviate experimental lymphoedema in mice[87]. Finally, it is important to mention that subcutaneous administration (that is, injection into the adipose tissue under the dermis) can also be used for immunization purposes[88] and has been shown to induce strong cytotoxic T cell immunity against a melanoma model in mice[89].

Intravenous administration

The intravenous administration of mRNA therapeutics faces several challenges. Following intravenous administration, LNP–mRNA constructs mainly distribute to the spleen and liver. The high splenic biodistribution is due to the opsonization of LNP–mRNA, which facilitates uptake by splenic macrophages of the MPS[90], as well as the propensity of LNP–mRNA to drift through the large endothelial fenestrations of spleen sinusoidal capillaries. Hepatic biodistribution is also pronounced for similar reasons, that is, uptake by resident macrophages and tissue extravasation. But ligand–receptor interactions with hepatocytes amplifies hepatic LNP–mRNA biodistribution. Specifically, the

Box 2

Circular mRNA is a promising alternative to linear mRNA

Circular mRNA (circRNA) is a single-stranded mRNA molecule that has joined 3′ and 5′ ends, first discovered in plant viroids[252]. Since then, it has been detected in mammalian cells and is postulated to have various functions[253]. Exogenous circRNA triggers potent RIG-I responses regardless of codon optimization, double-stranded RNA purification or 5′ cap modifications[254]. Suppressed immunogenicity for in vitro transcription-synthesized circRNA was instead achieved by using human introns that allow various RNA-binding proteins to mark it as 'self'[254], as well as by N^6-methyladenosine RNA modification[255]. Alternatively[256], it has been shown that unmodified circRNA is immunosilent as long as it has been sufficiently purified of linear RNA. In the same study, it was shown how lipid nanoparticles can be used to efficiently deliver circRNA to the adipose tissue of mice where protein expression was achieved for longer periods than with linear mRNA. This was attributed to the excellent stability of circRNA, which avoids the major degradation pathway of linear mRNA initiated by the exonuclease-mediated shortening of the 3′-poly(A) tail[257].

Since the coronavirus disease 2019 (COVID-19) pandemic, interest in the clinical use of circRNA has been reinvigorated, with many vaccine applications having been proposed in recent years[257]. Although circRNA enjoys better stability, and, potentially, improved pharmacokinetics compared with linear mRNA, there are some hurdles before it enjoys widespread use in drug development. Briefly, these relate to the circularization yield during in vitro transcription and the need for multiple purification techniques to avoid immunogenicity[258].

protein- and lipid-based layer (biomolecular corona) around LNPs as soon as they enter the bloodstream is rich in apolipoprotein E (ApoE)[91–93], a natural ligand for the low-density lipoprotein receptors (LDLRs) on hepatocytes[92,94]. Of note, other corona ligand–cell receptor interactions have also been shown to promote LNP–mRNA uptake in the liver[95] and, although LDLR–ApoE interactions are not exclusive to the liver, they are further enabled by liver physiology: 10–15% of the total blood volume reaches the liver through the hepatic artery and portal vein, of which 60% is in small capacitance sinusoids[96]. There, decreased blood flow slows down the transport of nanoparticles[97] and allows them to interface with and be taken up by hepatocytes and non-parenchymal cells (for example, Kupffer cells, stellate cells and endothelial cells)[98]. Taken together, hepatic physiology and biomolecular corona-mediated cellular uptake of LNP–mRNA particles enable the passive targeting of the liver at the expense of extrahepatic biodistribution.

Challenges with repeat intravenous administration. Because of the transient nature of mRNA, repeat administration may be necessary to maintain a therapeutic outcome with chronic enzyme replacement therapies or when antibodies or cytokines need to be regularly produced[68]. Despite the potential immunogenicity of LNP–mRNA formulations, most published studies focus on their therapeutic application[99–103]. If innate immune responses cannot be avoided or suppressed with anti-inflammatory drugs, the challenge then becomes to keep them transient, tolerable and sparse enough to avoid chronic inflammation[86].

Apart from adverse effects, another well-described pitfall with repeat administration of PEGylated nanomedicine formulations can be impaired pharmacokinetics due to accelerated blood clearance (ABC)[104]. ABC has been described as an immunological response and its putative mechanism involves the production of IgM to a PEG component after the first dose[105]. Upon subsequent doses, anti-PEG IgM-mediated complement activation leads to particle opsonization and pronounced particle uptake by the MPS (mainly in the liver)[106–108]. Because of ABC, the repeat administration of PEGylated lipid-based formulations such as liposomal doxorubicin hydrochloride (DOXIL) returns reduced half-life and pronounced hepatic biodistribution[109]. Ultimately, the PEG component in LNP–mRNA formulations can be problematic if misaligned with the intended application and dosing scheme, as described in a later section of this review.

Oral administration

Oral administration is underdeveloped in the LNP–mRNA field despite enjoying the highest patient compliance. A major problem with oral administration is the acidic pH of the gastric phase, which would protonate ionizable lipids and prematurely release mRNA content in the gastrointestinal tract[110], where nucleases will rapidly degrade it[111]. Even if structural integrity could be attained despite the low pH of the gastric phase or the lipases and esterases across the gastrointestinal tract, the bioaccessibility of LNP–mRNA particles would be limited by the layer of mucin glycoproteins that line the gut epithelium[111].

Preclinical toxicity

Several clinical trials of mRNA-based drugs and vaccines have not managed to progress past phase I or II (Table 1). The reasons behind these discontinuations are diverse, including low efficacy and unexpected changes in the risk–benefit profile of the drug candidates. Preclinical safety assessments aim to identify well-tolerated and efficient LNP–mRNA formulations. When toxicities are observed, investigative in vivo, in vitro and ex vivo experiments aim to understand the underlying mechanisms and ideally improve the design of formulations in development. This section discusses studies on the immunopathogenicity and hepatic or splenic toxicity of LNP-encapsulated IVT mRNA as the main safety concerns with mRNA-based drugs and vaccines in preclinical development[15,98,112]. Unless otherwise noted, only studies on modified and/or dsRNA-purified mRNA are considered.

Liver and spleen toxicity

Owing to pronounced hepatic and splenic biodistribution of LNP–mRNA, microscopic observations and histopathology of the liver and spleen are standard practice during preclinical development. Acute drug-induced liver injury is routinely evaluated by measuring blood plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (APT)[98,113]. Expectedly, most publicly available studies promoting LNP–mRNA therapeutic applications report only minor pathological findings. In one such study, modified mRNA that encoded human methylmalonyl-CoA mutase (hMUT) was synthesized to treat a hypomorphic mouse model of methylmalonic acidaemia/aciduria (MMA, an ultra-rare metabolic disorder). At the highest intravenously administered dose of LNP–mRNA formulation, there were no clinical chemistry findings, but 80% of mice presented with a mild decrease in lymphocytes next to the central splenic arteries[113]. This effect was attributed to the LNPs and not the hMUT mRNA or its expression, as it was also observed in LNPs carrying mRNA for enhanced green fluorescent protein (EGFP). In another study, modified mRNA encoding human arginase was used in a mouse model of arginase deficiency[114]. Although there were no biochemical or histopathological findings, electron microscopy on liver sections from the group that received a single control formulation of firefly luciferase mRNA presented with sub-micrometre-sized lipid droplets. Finally, a single intramuscular injection of LNP–mRNA formulation encoding the influenza haemagglutinin H3 antigen showed increased AST, ALT and C-reactive protein levels in rabbits[115]. Liver histopathological findings included focal subscapular vacuolation, inflammatory cell and erythrocyte infiltration, and increased cellularity (lymphocyte expansion) was observed in the germinal centres of the spleen. In summary, hepatic or splenic pathogenicity can arise after intravenous as well as intramuscular administration of LNP–mRNA, and hepatocyte uptake of the lipid component seems capable of disturbing fatty acid and lipid management.

Immune responses

Adverse immunological responses to nanomedicines include reactogenicity following vaccinations, hypersensitivity reactions, systemic complement immune responses and cytokine-mediated responses, among others[116]. The mechanism of some rare immunological adverse effects after mRNA-based COVID-19 vaccination are gradually being clarified[3,15,116]. For LNP–mRNA-based therapeutics in preclinical development, such events might compromise their safety profile and downgrade therapeutic efficiency[117,118]. Because of their inherent compositional complexity, identifying which component of a given LNP–mRNA complex are responsible for unwanted innate immune responses or what conditions might exacerbate them (dose, route of administration, pre-existing inflammation, etc.) is not trivial. In this section, the available literature on the immunopathogenicity of LNP-formulated IVT mRNA is discussed grouped by measured outcomes, namely TLR activation and cytokine secretion, inflammasome activation and complement activation.

Table 1 | Discontinued clinical trials of investigational mRNA drugs and vaccines

Company/ Investigator — collaborators	mRNA drug or vaccine name	Target condition or disease	Formulation	Route of administration	Reason for discontinuation[a]	Phase	Clinical trial identifier
CureVac	CV9104	Prostate cancer	Protamine–mRNA complex	Intradermal	Recruitment was terminated after enrolment of 35 instead of 36 evaluable patients for administrative reasons	II	NCT02140138
Dr R. A Gruters — multiple collaborators[b]	HIVACAT-TriMix	HIV infection	mRNA alone	Intranodal	Interim analysis did not show sufficient immunogenicity of investigational medicinal product compared with placebo	IIa	NCT02888756
ModernaTX, Inc.	mRNA-2416	Advanced relapsed or refractory solid tumour malignancies or lymphoma	LNP-formulated mRNA with or without durvalumab	Intratumoural	This study was halted prematurely because the efficacy end points were not met for either treatment arm	I/II	NCT03323398
eTheRNA immunotherapies	ECI-006	Melanoma	mRNA alone	Intranodal	Expiry of study medication	I	NCT03394937
National Cancer Institute	NCI-4650	Metastatic melanoma or epithelial cancer	LNP-formulated mRNA	Intramuscular	Slow accrual	I/II	NCT03480152
Translate Bio, Inc.	MRT5201	Ornithine transcarbamylase deficiency	LNP-formulated mRNA	Intravenous	Programme was discontinued	I/II	NCT03767270
ModernaTX, Inc.	mRNA-3704	Methylmalonic acidaemia	LNP-formulated mRNA	Intravenous	Study was terminated before the start of dosing owing to a business decision and not owing to safety or efficacy reasons	I/II	NCT03810690
CureVac	CVnCoV	SARS-CoV-2	LNP-formulated mRNA	Intramuscular	The study was withdrawn based on an assessment of immunogenicity in elderly adults	III	NCT04838847
Bayer — CureVac	CVnCoV	SARS-CoV-2	LNP-formulated mRNA with or without quadrivalent influenza vaccine	Intramuscular	The study cannot be conducted	III	NCT04848467
CureVac	CVnCoV	SARS-CoV-2	LNP-formulated mRNA	Intramuscular	The principal investigators and CureVac decided to terminate the trial early following a change to the risk–benefit profile	III	NCT04860258
Gritstone bio, Inc.	GRT-R902	Colon cancer	LNP-formulated self-amplifying mRNA co-administered with adenoviral tumour-specific neoantigen priming vaccine and small-molecule immune checkpoint inhibitors	Intramuscular	Terminated owing to reprioritization	II	NCT05456165
University Medical Center Groningen — BioNTech SE	W_ova1	Ovarian cancer	Liposome-formulated mRNA that encodes three ovarian cancer tumour-associated antigens	Intravenous	'the target number of evaluable patients defined in the study protocol could not be reached...'	I	NCT04163094
BioNTech SE	BNT141	Advanced unresectable or metastatic Claudin 18.2-positive solid tumours	Two LNP-formulated pseudouridine-modified mRNAs	Intravenous	Sponsor decision	I/IIa	NCT04683939
Ultragenyx Pharmaceutical, Inc.	UX053	Glycogen storage disease type III	LNP-formulated mRNA	Intravenous	Sponsor decision not related to safety concerns	I/II	NCT04990388

Table 1 (Continued) | Discontinued clinical trials of investigational mRNA drugs and vaccines

Company/ Investigator — collaborators	mRNA drug or vaccine name	Target condition or disease	Formulation	Route of administration	Reason for discontinuation[a]	Phase	Clinical trial identifier
Wuhan Recogen Biotechnology Co. Ltd	RH109	SARS-CoV-2	LNP-formulated mRNA	Intramuscular	Business strategy	I	NCT05366296
RVAC Medicines (US), Inc.	RVM-V001	SARS-CoV-2	RNA based — unknown formulation	Intramuscular	The landscape of the study area changed, making it impossible to continue the study	I	NCT05420077
GreenLight Biosciences, Inc.	GLB-COV2-043 (booster)	SARS-CoV-2	LNP-formulated mRNA[244]	Not mentioned, presumed intramuscular	To prioritize other programmes	I/II	NCT05602961
Wuhan Rhegen Biotechnology Co. Ltd — Shenzhen Rhegen Biotechnology Co. Ltd, Wuhan Recogen Biotechnology Co. Ltd	RH109	SARS-CoV-2	LNP-formulated mRNA	Local administration, presumed intramuscular	Business strategy adjustment	I	NCT05609045
RVAC Medicines (US), Inc.	RVM-V001, RVM-V002	SARS-CoV-2	RNA based — unknown formulation	Intramuscular	Changes in COVID-19 pandemic landscape	Ib	NCT05788185
ModernaTX, Inc.	mRNA-2736	Relapsed or refractory multiple myeloma	LNP-formulated mRNA	Intravenous	The sponsor decided to discontinue development of mRNA-2736 for strategic business reasons	I	NCT05918250

Sourced from clinicatrials.gov. Studies with cells transfected with mRNA ex vivo have been excluded. Data collection date: 12 November 2023. COVID-19, coronaviurs disease 2019; LNP, lipid nanoparticle; SARS-Co-V-2, severe acute respiratory syndrome coronavirus 2. [a]Reported verbatim from clinicaltrials.gov. [b]Institut d'Investigacions Biomèdiques August Pi i Sunyer, IrsiCaixa, Institute of Tropical Medicine, Belgium, Vrije Universiteit Brussel, Synapse bv, Asphalion, eTheRNA immunotherapies, CR2O, Hospital Clinic of Barcelona, Germans Trias i Pujol Hospital, Universitair Ziekenhuis Brussel.

TLR activation and cytokine secretion. Stimulation of TLRs by LNP–mRNA has been suggested to be upstream of cytokine production (Fig. 2a). In a relevant study, mice having first been challenged with lipopolysaccharide (LPS) and then having received a single intravenous administration of LNP–mRNA, exhibited elevated levels of IL-6, C-C motif chemokine ligand 2 (CCL2) and other pro-inflammatory cytokines (in the serum) as well as C-X-C motif chemokine ligand 2 (CXCL2, in liver homogenates)[119]. Similar immune responses were observed when mice were administered empty LNPs, pointing to the ionizable cationic lipid (proprietary to Acuitas) as the immunostimulatory component. The inflammatory phenotype was ablated in macrophage-depleted mice as well as in a *Tlr4*$^{-/-}$ mouse model. Interestingly, mRNA formulations using two other ionizable cationic lipids (DLin-MC3-DMA or C12-200) produced similar findings. These results raise questions about which other components of the LNP–mRNA complex or its biomolecular corona may stimulate membrane-associated TLRs, assuming the ionizable lipids are not abundantly displayed on the outermost layer of the particles[120,121].

In an independent study, endosomal colocalization of TLR4 and LNP–mRNA following LPS priming of murine macrophages ex vivo corroborated the hypothesis that LNP–mRNA triggers the innate immune system through TLR4 when there is pre-existing inflammation[122] (Fig. 2b). Under these conditions, endosomal escape was impaired, while phosphorylation of protein kinase R (PKR) downstream of TLR4 was at least partially responsible for decreased cytosolic mRNA translation. The cationic ionizable lipid used in this study was cKK-E12, which, like C12-200, also features a pyrimidine group substituted with long hydrocarbon chains. Although an LNP-alone control was not included, these findings in relation to TLR4 point to a lipid-mediated biological effect. Intradermal administration of an ionizable cationic lipid-based (proprietary to Acuitas) LNP-formulated, non-coding, poly-cytosine mRNA had similar, albeit local, pro-inflammatory effects[123]. A single injection led to the upregulation of genes encoding pro-inflammatory IL-1β, IL-6 and CXCL and CCL proteins. RNA sequencing (RNA-seq) and gene set enrichment analyses pointed to RLR and TLR stimulation as well as inflammasome activation. Empty LNP controls returned the same pro-inflammatory phenotype, so it was suggested that the ionizable lipid might be triggering the observed inflammation. In a similar experiment, intradermal injection of self-amplifying luciferase mRNA in ionizable, cationic lipid-based (of unspecified chemical structure) LNPs led to strong IFNβ production[86]. Further, luciferase expression kinetics appeared limited by the concurrent inflammatory response, demonstrating how the latter can severely impact the therapeutic index of mRNA-based drugs (Fig. 2b). Other ionizable cationic lipids have been shown to elicit milder innate immune responses. Using yet another proprietary ionizable cationic lipid, repeat intravenous administration of LNP-formulated mRNA encoding factor IX did not lead to elevated levels of TNF or IFNγ in the blood plasma of mice[99]. Still, other inflammatory cytokines, such as CCL2, IL-6, CCL4, granulocyte–macrophage colony-stimulating factor (GM-CSF) and chemokines such as RANTES (regulated on activation, normal T cell expressed and secreted) were transiently increased[99].

In some studies, pro-inflammatory responses have been more clearly attributed to the mRNA. Intradermal electroporation of modified EGFP mRNA elicited IFNβ production in mice around the site of injection, but it is unclear whether the mRNA was dsRNA purified[86].

Contradicting the implication of the ionizable lipid, a study found strong pro-inflammatory cytokine response in mouse serum following intravenous administration of C12-200-based, LNP-formulated, nucleoside-modified, scrambled mouse *Epo* mRNA[124]. IL-6, IL-5, CXCL2 and CCL2, among other cytokines, were considerably more elevated compared with an empty LNP control, although the mRNA was not dsRNA purified.

Overall, TLR activation and release of pro-inflammatory cytokines emerges as a frequent LNP–mRNA effect on the innate immune system that can elicit sometimes strong adverse effects and compromise protein translation. TLR4 activation by ionizable cationic lipids is a probable initiating event, although an exact molecular mechanism has yet to be described. Other factors may also be required for, or exacerbate, said inflammatory effect, which might be payload, dose or route of administration dependent.

Inflammasome activation. Inflammasome activation has recently been identified as a distinct innate immune system effect of LNP–mRNA (Fig. 3). The canonical pathway to pyroptosis requires a priming signal to activate NF-κB and start the transcription of NOD leucine-rich repeat and pyrin-containing protein 3 (NLRP3) and pro-IL-1β, which can originate from TLR activation[125]. The secondary signal initiates NLRP3 inflammasome assembly and activation[126]. In one in vitro study, NLRP3 inflammasome activation led to reduced mRNA transfection efficiency following in vitro administration of DLin-MC3-DMA-based LNP–mRNA to bone marrow-derived macrophages[127]. LPS was used to provide the priming signal, but mRNA could also have contributed to priming via TLR activation, as it is unclear whether it was modified or purified. Lysosomal rupture upon LNP–mRNA escape and release of damage-associated molecular patterns was proposed as the secondary signal. NLRP3 activation was then inferred from IL-1β release, cleaved gasdermin D and caspase 1 expression, and cathepsin B maturation[126,128].

Either DLin-MC3-DMA or SM-102 (the ionizable cationic lipid in the Moderna COVID-19 vaccine) was used to formulate LNPs loaded with EGFP[129]. When human peripheral blood mononuclear cells (PBMCs) were treated with either formulation, there was a sharp increase in IL-1β release, along with other pro-inflammatory cytokines, such as IL-6, CCL2, CCL4 and TNF, among others. Notably, empty SM-102-based formulations also elicited strong IL-1β secretion, suggesting that these lipids provide both priming and activating signals for inflammasome activation. The mRNA was synthesized with 1mψ, bore a low immunogenicity 5′ cap modification and was purified from dsRNA impurities with cellulose treatment and so was assumed to be immunosilent. The priming signal was suggested to originate from LNP–PRR interactions, with LNP-induced damage-associated molecular patterns providing the secondary signal. This is a valid hypothesis given the propensity of ionizable lipid LNP–mRNA formulations to activate TLRs and release IL-1 cytokines; IL-1β release could mediate the phosphorylation of MYD88 and thus provide an autocrine or paracrine priming signal[130].

Complement activation and hypersensitivity reactions. Complement proteins in the plasma and cell surfaces constitute a fundamental part of the innate immune system[131]. Complement activation, that is, a series of proteolytic events, supports the phagocytic clearance of substances or particles identified as pathogenic. Identification of pathogenicity is the event that initiates complement activation, of which there are three pathways: the classical pathway, initiated by pattern recognition by IgG or IgM; the alternative pathway, initiated by the hydrolysis of the thioester bond in complement protein C3; finally, the lectin pathway, initiated upon carbohydrate pathogen-associated molecular pattern recognition by mannose-binding lectins (MBLs) and ficolin PRRs[132]. The advent of biologics and nucleic acid therapies quickly elevated complement-activation-related immunotoxicological investigations to routine practice in drug development pipelines. In support of these efforts, complement component knockout models[133] or

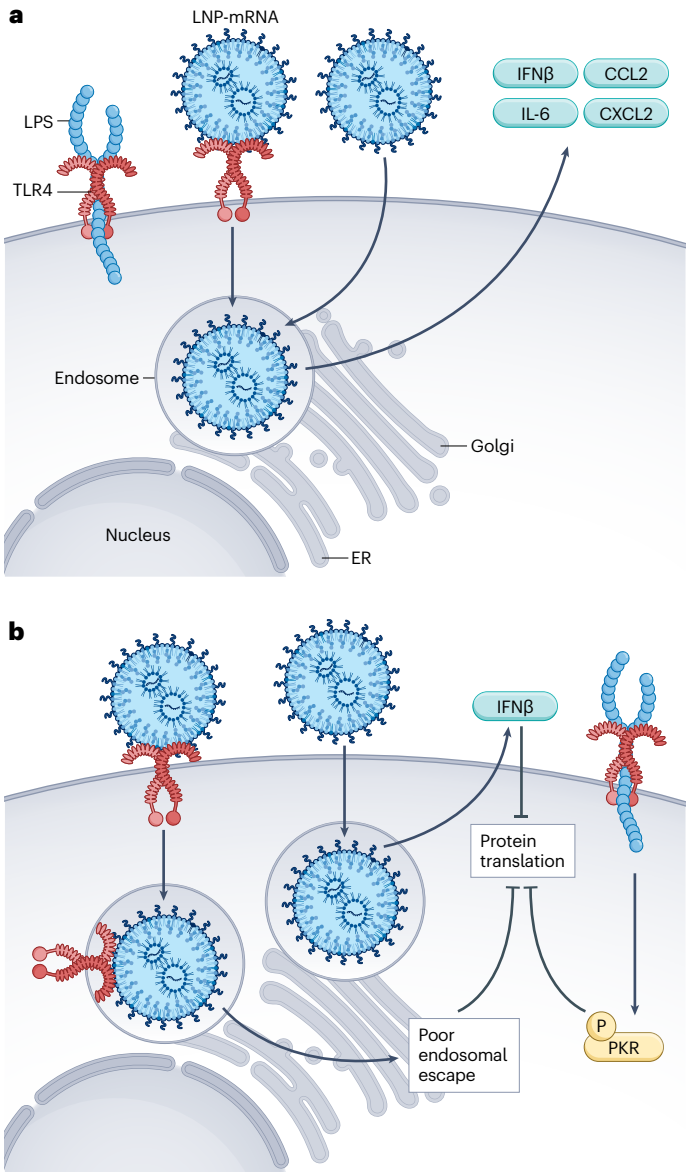

Fig. 2 | Cytokine release as a response to LNP-formulated modified IVT mRNA. a, With prior lipopolysaccharide (LPS) challenge, mRNA formulated in lipid nanoparticles (LNP–mRNA) triggers Toll-like receptor 4 (TLR4). C-C motif chemokine ligand 2 (CCL2), C-X-C motif chemokine ligand 2 (CXCL2) and IL-6 can then be detected in serum or tissue homogenate samples. **b**, Interferon-β (IFNβ) release, LPS challenge or TLR4 triggering can independently inhibit transfection efficiency. In one case, decreased transfection efficiency was attributed to poor endosomal escape and protein kinase R (PKR) phosphorylation (P), but the relative contributions of these events remain unclear. Please see main text for more details. ER, endoplasmic reticulum.

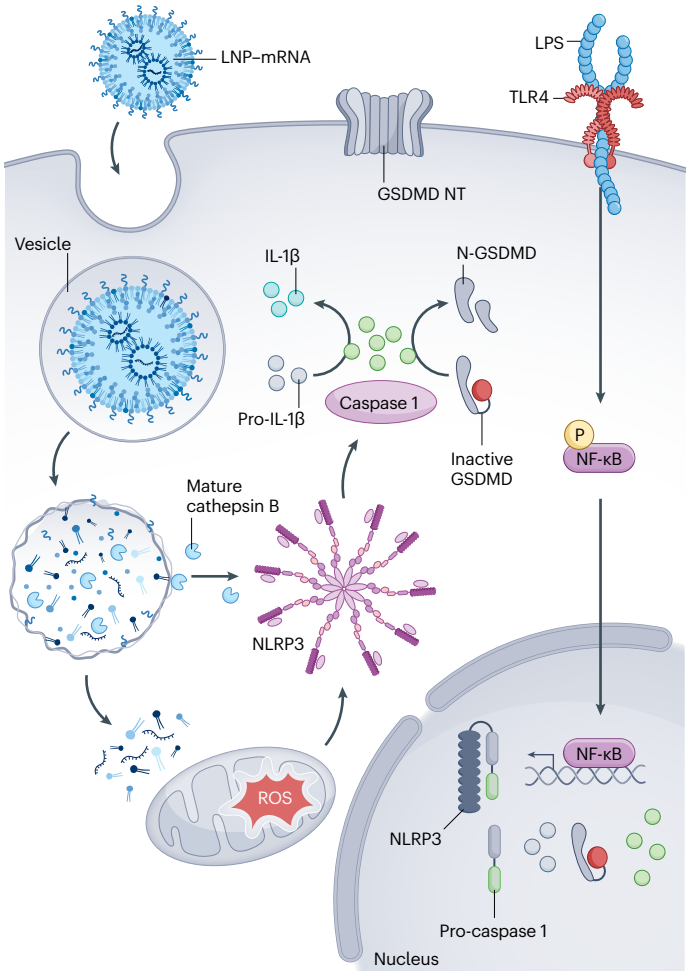

Fig. 3 | LNP–mRNA activates the inflammasome. Macrophages primed with lipopolysaccharide (LPS) or peripheral blood mononuclear cells (PBMCs) without prior challenge activate nuclear factor-κB (NF-κB) and induce the production of pro-inflammatory IL-1 cytokines, along with other molecules that are present in the canonical inflammasome activation pathway. These include caspase 1, cathepsin B, adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) specks, N-gasdermin D (N-GSDMD) and mitochondrial reactive oxygen species (ROS). LNP–mRNA, mRNA formulated in lipid nanoparticles; GSDMD NT, gasdermin D N-terminal cleavage product; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; TLR4, Toll-like receptor 4.

complement-depleted sera are useful tools, and pathway-specific[134] or inducible models[135] have also been described in the literature.

LNP–mRNA formulations have been shown to activate the complement pathway. In an in vivo study, intravenous administration of LNP–mRNA translating for hEPO resulted in mild and reversible elevation of blood plasma levels of C3a and C5b–9 in cynomolgus monkeys[100]. In a different study, after LNP–mRNA translating for CD40L was incubated in complement-active human serum, C3b/c and soluble C5b–9 were also elevated[108]. Anti-PEG IgM was necessary for complement activation and was linked to loss of LNP integrity. These findings are in line with previous reports of accelerated immune cell-mediated clearance of nanomedicine formulations owing to immunoglobulin opsonization and complement activation[136–138]. Complement activation may also result in rare anaphylactic reactions. After the first doses of the BNT162b2 or SM-102 COVID-19 vaccine a few cases of anaphylaxis were reported[139]. The affected patients had high levels of C5a, but not IgE[140], suggesting that mast cell degranulation (and onset of anaphylaxis) did not require prior sensitization and antigen-specific IgE production, as a true allergic reaction would[141]. These findings point to complement-activation-related pseudo-allergy (CARPA) by which mast cell degranulation does not necessitate immunoglobulin mediation, at either the complement activation or the mast cell degranulation level[107]. To date, it is unclear which vaccine component may have led to the release of C5a. It is possible that pre-existing anti-PEG IgG or IgM triggered the classical pathway[105] and led to CARPA[142]; alternatively, the classical pathway may have been activated upon intracellular ionization of the lipids[143].

Hypersensitivity reactions (HSRs) include adverse immunological events following the intravenous infusion of drugs, including nanomedicines[144,145]. HSRs are rare, but potentially dangerous as they may manifest with severe anaphylaxis, myocarditis, swelling of the throat, respiratory failure or haemodynamic changes, among other symptoms[116]. Any LNP–mRNA component can theoretically elicit an HSR, but historical data point to the PEGylated lipid as the most potentially reactogenic component so far[112,146]. When a PEG moiety binds to antigen-specific IgE on the surface of primed mast cells, the latter degranulate and cause anaphylaxis in what is called type I immediate-type HSR[141]. In contrast to type I HSR, CARPA, as described above, is a more severe type of HSR that stems from direct mast cell degranulation and anaphylaxis-like symptoms. Beyond CARPA and type I HSR, there are more types of HSR (II–IV) for which no LNP–mRNA-specific reports are available in the public domain to the best of our knowledge.

Knowledge gaps in LNP–mRNA toxicities

Regulatory agencies recognize the complicated relationship between the absorption, distribution, metabolism and excretion (ADME) profile of nanoscale materials that contain drug products and their physicochemical properties at the molecular, particulate and formulation levels[147]. The toxicity and reactogenicity, and toxicodynamics of LNP–mRNA formulations seem confounded by multiple factors among which are particle size, ionizable lipid chemistry, PEG length and surface density, and payload reactogenicity (even in the case of modified IVT mRNA)[15,112]. Not least, the enzymatic and biochemical signature of the administration route and ability of tissues to manage the metabolic byproducts of LNP could also be determining factors of the safety profile of LNP–mRNA drugs and vaccines[15,112].

Toxicology of lipids and their metabolites

Ionizable lipids. Lipids and their metabolites can be signalling molecules that participate in energy metabolism and immunity[148]. Therefore, novel ionizable lipids should not only be tested in terms of their transfection efficiency, but also scrutinized to exclude potential toxicities tied to their specific biochemical properties. In the section ‘TLR activation and cytokine secretion’ above, we describe how some ionizable cationic lipids seem to engage with PRRs. Other unwanted outcomes need also to be excluded. For example, monounsaturated fatty acids may have a protective effect against ferroptosis[149], but their susceptibility to desaturation (for example, by FADS2) and production of potentially harmful unsaturated fatty acids is still under investigation in health and disease[150,151]. Moreover, intracellular esterified fatty acid accumulation may lend itself to lipid peroxidation[152], which

can be pathogenic unless the peroxidized products are metabolically managed[153]. Finally, fatty acids may directly stimulate inflammatory pathways through peroxisome proliferator-activated receptors (PPARs)[154,155].

Testing empty LNPs (without a nucleic acid) at lipid concentrations equimolar to those used for nucleic acid delivery can be useful to identify which toxicities may arise from the ionizable lipid. One such study showed that empty LNPs carrying the ionizable lipid YSK13 can elevate plasma ALT and AST when administered intravenously at elevated doses[156]. These changes were attributed to hepatic neutrophil infiltration, as Kupffer cells were not implicated in hepatic injury. Importantly, empty LNPs had the same effect as those loaded with small interfering RNA (siRNA), suggesting that the carrier alone is capable of driving hepatic toxicity[156]. Although other parameters may also be driving toxicity (for example, dose, route of administration, etc.), testing empty LNPs decouples lipid biocompatibility issues from toxicities attributable to the mRNA or its translation product.

PEGylated lipids. PEGylation inhibits capture of the LNP–mRNA by the MPS, increases its half-life and, with that, its chances of extravasating to other tissues[59]. The PEG–lipid molar ratio also affects the size of LNPs[157] and with that their biodistribution profile[158]. Having said that, the balance between the ADME-modulating capacity and toxicodynamics of PEG is under scrutiny[159], with some reports raising concerns about its long-term safety[136]. Other complications include low transfection efficiency for LNP-siRNA complexes because of problematic cellular uptake[160] or alteration of the endocytic pathways necessary for the therapeutic translation of lipid–DNA particles[161]. Where repeat dosing may be required, PEGylation has been shown to weaken the pharmacokinetics of LNP–mRNA after the initial administration[108]. Notwithstanding these concerns, PEG has already been successfully translated to products such as the COVID vaccines, and PEGylated lipids can be administered via all routes, including the oral one.

In the specific case of LNPs with phosphorylcholine (PC), natural anti-PC IgM and B-1a cells with heightened PC specificity in the spleen of mice were shown to activate B2 cells to produce anti-PEG IgM[162] (Fig. 4). This study provides a plausible explanation for the accelerated blood clearance (ABC) effect of LNP–mRNA without prior exposure to PEG and the declining protein expression sometimes observed with repeat administration. Equally important, it proposes how B-1a cells could bridge innate and adaptive immune responses to LNP–mRNA, similarly to their role in controlling bacterial and viral growth[163]. Another proposed mechanism for ABC is that anti-PEG IgM (and possibly IgG) antibodies (because of past PEG exposure), can opsonize PEGylated formulations and mediate their rapid MPS clearance from systemic circulation, mainly by Kupffer cell uptake[164]. Overall, several PEG physicochemical properties (for example, length, architecture, terminal group) and their incorporation in a LNP–mRNA complex (for example, PEG–lipid molar ratio, hydrophobic anchoring group, shedding rate) may impact their immunogenicity[112,141]. As such, the preclinical development of LNP–mRNA drugs and vaccines bearing PEGylated lipids should be accompanied by rigorous immunotoxicological assessments.

Emerging additional components. Traditional LNPs are composed of ionizable lipids, cholesterol, helper phospholipids and PEGylated lipids at various molar ratios. Recently, additional components have been explored in an attempt to modulate the biodistribution and improve cell transfection of LNP–mRNA formulations. Notable examples include the addition of a fifth, permanently charged (anionic or cationic) lipid[165], sphingomyelin[166] or cholesterol derivatives[60,167]. Increasing molar ratios of 1,2-dioleoyl-3-trimethylammonium propane (DOTAP, which carries a positively charged quaternary ammonium group), shifted protein expression of DLin-MC3-DMA-based LNP-formulated luciferase mRNA to the lungs following intravenous administration to mice[165]. Incorporation of permanently anionic lipids also changed the biodistribution of LNP–mRNA formulations. Certain cationic lipids can trigger PRRs[168], are pro-inflammatory[169], and their toxicity has been posing problems for the clinical translation of liposomes[170]. Therefore, high molar percentage incorporation in LNPs should be accompanied by stringent immunotoxicological screening. Cholesterol analogues (for example, β-sitosterol) and cholesterol oxidation products have been used to improve the transfection efficiency of LNP–mRNA in vitro[167] and improve mRNA delivery to liver endothelial cells in vivo[60], respectively. Anticipating repeat dosing and improved cell tropism, it is important to rule out lipotoxicity or disturbances in cell metabolism, similar to what has been observed for cholesterol and its oxidation products[171,172].

LNP–mRNA engineering complexity will probably increase in an attempt to fine-tune their pharmacokinetic and pharmacodynamic properties. In response, safety assessments should anticipate undesired effects from changes in size, chemistry and related biotransformations (for example, changes in biomolecular corona). Some aspects of LNP–mRNA toxicity have been seemingly neglected by the research community (Box 3).

Current approaches for LNP–mRNA risk avoidance

Preclinical studies that demonstrate the pharmaceutical potential of the mRNA platform are now being published at an unprecedented rate. The safety and tolerability of novel mRNA therapeutics can be sought through various means. For example, microneedles for intradermal administration can be dose sparing[173], protect the mRNA from RNases, and minimize local and systemic adverse effects[174]. In two relevant

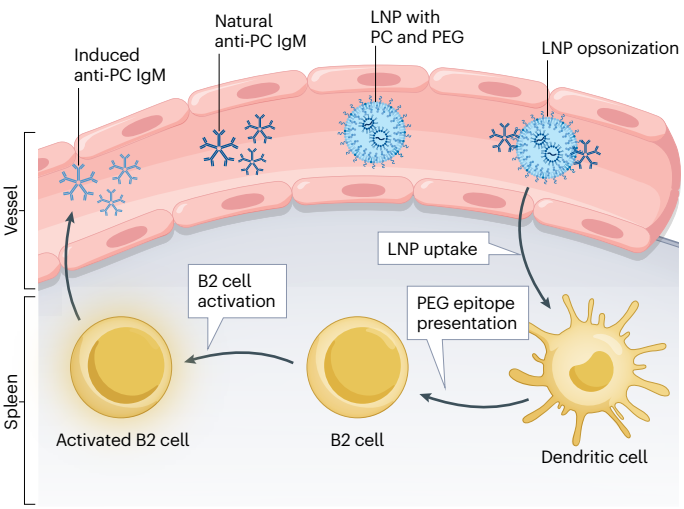

Fig. 4 | PEGylated LNP–mRNAs raise adaptive immune responses in the spleen. Natural anti-phosphorylcholine (PC) IgM antibodies promote the opsonization of mRNA formulated in lipid nanoparticles (LNP–mRNA) by dendritic cells in the spleen. There, polyethylene glycol (PEG) epitopes are presented to B2 cells, which are activated and produce anti-PEG IgM.

Box 3

Understudied areas of LNP–mRNA toxicity

Toxicodynamics of extracellular LNP–mRNA

In vitro, only 1–2% of lipid nanoparticle (LNP)-mediated nucleic acid delivery leads to successful cell transfection[259]. However, the pathogenic potential of the remaining 98% is understudied. On that front, it is known that innate immunological responses can be triggered by adventitiously released mRNA or LNP-mediated Toll-like receptor 4 (TLR4) stimulation[122], but there may be more causes for concern. Nanoparticles can trigger the complement cascade both directly[141] and by surface adsorption of immunoglobulins[108]. In the bloodstream, this increases particle opsonization and clearance, but the organ-specific toxicological impact of complement activation induced by mRNA formulated in LNPs (LNP–mRNA) is not clear. This knowledge gap is particularly pertinent as LNP–mRNA formulations have increased hepatic biodistribution where complement proteins are synthesized, while hepatocytes and non-parenchymal cells are equipped with complement receptors[260,261]. Indeed, C5a release and binding to C5R1 have been linked to chemoattractive, profibrogenic and pro-inflammatory signals in the human liver[262], which can severely hamper the tolerability of mRNA drugs. Extracellular disintegration of LNP–mRNA is another event with mostly unknown consequences. Beyond polyethylene glycol (PEG) shedding — which concerns only surface-bound lipids — LNP–mRNAs are susceptible to complete disintegration mediated by the formation of a biomolecular corona[263]. Moreover, it has been shown that lipids with ester bonds are vulnerable to extracellular endothelial and lipoprotein lipases[201]. The eventual release of ionizable lipids with tertiary amines in their polar headgroup, which are susceptible to N-oxidation (for example, from endogenous amine oxidases), could generate toxic aldehydes[264].

Particulate LNP–mRNA toxicity

To date, two LNP-based mRNA vaccines have received emergency authorization from the FDA for clinical use[265]. However the same ionizable lipids may not be translatable to other LNP–mRNA drugs or vaccines. That is because the unique properties of each mRNA cargo (length, negatively charged phosphate groups) influence the self-assembly of the LNP–mRNA complex. Its final structure is also affected by the biological milieu, as the formation of the biomolecular corona leads to internal restructuring and compositional rearrangement[266]. The biophysical properties of LNP–mRNA particles can thus impact their pharmacodynamic profile: small changes in particle curvature and surface chemistry have been shown to affect the composition of the particles' biomolecular corona, and, with that, their immunoglobulin-mediated complement activation[267], opsonization[138] and cell tropism in vivo[268]. When tested in vitro, the biophysical properties of LNP–mRNA particles may determine their deposition to cells and their cell binding affinity, as has been shown for other colloidally stable nanoparticles[269]. Overall, the particulate nature and biophysical properties of LNP–mRNAs have to be taken into account when assessing their toxicity and pharmacodynamics, especially within the drug development field where these parameters are not usually considered for small-molecule development.

studies, polymeric dissolvable microneedles loaded with mRNA[175] or hollow microneedles loaded with either naked mRNA or LNP–mRNA[176] efficiently induced immunogenicity for vaccine applications, as demonstrated in ex vivo or in vivo models, respectively. Polysarcosine (a polymer of repeating N-substituted glycine monomers) has recently emerged as a promising PEG alternative that can curtail some of the unwanted immunological responses to PEGylated lipids. In a patent filing, polysarcosine–lipid conjugates could form a complex with RNA to form nanoparticles with precise size control and surface characteristics, including various end-groups to adjust charge[177]. Indeed, polysarcosine–lipid conjugates were demonstrated to efficiently complex with mEPO or luciferase mRNA and achieve protein expression at levels comparable to those from PEGylated LNP–mRNA formulations. Crucially, the polysarcosine formulations had a better safety profile with reduced inflammogenicity in vitro or liver toxicity in vivo[178].

Physiological idiosyncrasies of pathological conditions can also be harnessed to improve the ADME profile of LNP–mRNA formulations. In a relevant study, it was hypothesized that LNP–mRNA distribution to ischaemic cardiac tissue upon reperfusion could be increased because of its pronounced endothelial fenestration (akin to the enhanced permeation and retention effect)[179]. It was indeed shown that intravenously administered LNP–mRNA translating for Cre showed increased targeting of cardiac fibroblasts, cardiomyocytes and macrophages in the infarct area of Cre-reporter mice. Based on the same premise, another study showed that myocardial infarction remission and cardiac regeneration could be promoted with mRNA therapeutics that are translated into cell cycle regulators, such as pyruvate kinase muscle isoenzyme 2 (PKM2)[180]. microRNAs.

Other practices for risk avoidance include the systematic synthesis and screening of ionizable lipids, combination therapies, minimization of off-target effects and the development of circular mRNA (Box 2).

Lipid libraries and quantitative structure–activity relationship

Ionizable lipids are indispensable for the therapeutic efficacy of LNP–mRNA formulations: the low pK_a of their head group promotes the disassembly of the LNP–mRNA construct in the endosomes[181], and the stereochemistry of their hydrocarbon tail enables the release of the mRNA into the cytosol[182]. Novel lipids from combinatorial chemistry can be synthesized relatively quickly and then screened for their transfection efficiency and in vitro toxicity to identify lead candidates[183] or even perform quantitative structure–activity relationship studies[184]. Several rounds of optimization have improved the transfection efficiency of LNP-encapsulated mRNA. Nevertheless, unforeseen toxicities and low metabolite clearance call for improvements to the safety and tolerability profile[58,185]. Two recent examples show how rational design approaches can improve the PK and pharmacodynamics of LNP–mRNA formulations: orthogonal experimental design was used to identify LNP–mRNA formulations with optimal ex vivo stability in the amniotic fluid of mice[186]; and molecular dynamics was elsewhere

applied to describe how LNP–mRNA particles with squaramide ionizable lipids may improve target protein expression in the serum of non-human primates[64].

Combination therapies

Risks associated with mRNA drugs and vaccines can be reduced by increasing therapeutic efficacy through the co-administration of other drugs or adjuvants. In multiple early-phase clinical trials, mRNA is co-administered with immune checkpoint modulators or cytokines for enhanced antitumoural action[187]. Recently, results from a phase II clinical trial (NCT03897881) of Moderna's mRNA-4157, which encodes mutant KRAS (a tumour-associated antigen), administered in combination with pembrolizumab (an immune checkpoint inhibitor monoclonal antibody to PD1) showed a significant and considerable decrease in recurrence or death compared with monotherapy in patients with stage III or IV melanoma. At the preclinical stage, intratumoural injection of LNP–mRNA that encodes IL-12, IL-27 and GM-CSF in combination with naked *IL27* mRNA had a ten times synergistic effect in inhibiting tumour growth in a mouse melanoma model compared with the LNP–mRNA alone[188]. In another example, LNP–mRNA encoding mucin 1 (a cell-surface-associated transmembrane glycoprotein overexpressed in triple-negative breast cancer tumours) was expressed in mouse lymph nodes and activated tumour-specific T cells[189]. Intravenous co-administration with an anti-cytotoxic T lymphocyte-associated protein 4 (CTLA4) monoclonal antibody, which can further promote activation of cytotoxic T lymphocytes, inhibited tumour growth more than the LNP–mRNA treatment alone.

Administration of immunosuppressants is common practice for chemotherapeutics and could also be an integral part of nucleic acid therapeutics. In a proof-of-concept study, dexamethasone (dex) was covalently linked to acyl or alkyl moieties and incorporated in an LNP loaded with IVT mRNA encoding firefly luciferase[190]. In the absence of acute in vitro cytotoxicity, the dex–LNP–mRNA formulation showed considerably lower cytokine production than the LNP–mRNA alone after dose-matched intravenous administration in mice. Glucocorticoids and antihistaminic drugs were administered to patients with transthyretin amyloidosis before infusion of an LNP-enabled CRISPR–Cas9 gene editing therapy formulation to dampen possible systemic innate immune responses[191]. Other small molecules have also been found to be effective in mitigating unwanted immunogenicity related to lipids, such as Janus kinase inhibitors[192] and the putative antioxidant edaravone[193].

Minimization of off-target effects

To lower the risk of off-target effects in the development of mRNA therapeutics, it is possible to inhibit mRNA expression in a cell-specific manner or improve organ-specific uptake of LNP–mRNA. The former strategy is enabled by endogenous microRNAs (miRs), which are short, non-coding RNA molecules that naturally inhibit the translation of mRNA and thus regulate gene expression in a cell- and disease-specific manner[194]. Incorporation of a miR binding site in the 3′ untranslated region of an IVT mRNA diminishes its translation in cells in which that miR population is abundant. miR-122 is abundant in healthy hepatocytes but not in hepatocellular carcinoma cells[195]. Binding to miR-122 can inhibit IVT mRNA translation of pro-apoptotic or toxic proteins, minimizing off-target effects of mRNA-based cancer therapeutics. This has been demonstrated in vitro with mRNA translating for the non-structural protein on the S segment gene from the Rift Valley fever virus, which induced p53 apoptosis in a miR-122-positive but not in a miR-122-negative liver cell line[196]. In mice with high human hepatocyte engraftment bearing a Hep3b tumour xenograft, liver toxicity was suppressed when IVT mRNA encoding pro-apoptotic PUMA sequences or reversed caspase 6 were administered intravenously, while tumour cells were damaged[197]. Although the passive targeting of the liver following intravenous administration benefits protein and enzyme replacement therapies, extrahepatic targeting of LNP–mRNA presents an ongoing challenge.

Recent advances in LNP–mRNA engineering hold promise for the targeting of organs other than the liver and for improving cell tropism within the liver[198]. Lowering hepatic biodistribution and controlling non-parenchymal distribution are expected to minimize off-target effects in the liver, which currently bears the brunt of LNP–mRNA toxicodynamics. In one notable example, permanently charged lipids (cationic or anionic) were added to novel or clinically relevant LNPs (for example, DLin-MC3-DMA) to control their biodistribution in a molar ratio- and charge-dependent manner[165]. Three different cationic or anionic lipids were tested with reproducible results, and for each formulation there were clear molar ratio thresholds for preferential and predictable lung, spleen or liver biodistribution[199]. Specifically, luciferase protein expression could gradually shift from the liver to the spleen before presenting uniquely in the lungs. It was also demonstrated that the presence of ionizable lipids was necessary for successful mRNA translation. In vivo toxicity assessments of LNP–mRNA formulations of three therapeutic proteins (hEPO, IL-10 and mKL) did not present any histopathological alterations to the examined organs or reveal any increase in the measured serum cytokines. A more recent study used egg sphingomyelin (ESM, *N*-(hexadecanoyl)-sphing-4-enine-1-phosphocholine) to modulate the biodistribution of LNP–mRNA. ESM, a naturally occurring, biocompatible lipid[200], was added to DLin-MC3-DMA-based LNP-formulated luciferase mRNA. After intravenous administration in mice, these nanoparticles exhibited extended circulation times, which allowed improved extrahepatic biodistribution[166].

Chemical engineering of lipids has been used to modulate intrahepatic cell tropism of LNP-encapsulated siRNA. The pK_a of the ionizable lipid was shown to predictably shift uptake from the hepatocytes to liver sinusoidal endothelial cells[201]. The same study provided evidence that ester bonds in the linker between the acyl chain and polar headgroup of the ionizable lipid are differentially sensitive to enzymatic hydrolysis before the disintegration of the LNP structure in the endosome. Specifically, the PEGylated LNPs used in this study appeared sensitive to endothelial and lipoprotein lipases on the surface of liver sinusoidal endothelial cells, but not to hepatic esterases on hepatocytes.

Models to de-risk LNP–mRNA formulations

Risk management within the preclinical development of new drugs and vaccines is crucial for the safe transition to clinical trials and to achieve a favourable therapeutic index. Traditionally, animal models have been used to acquire a breadth of toxicological and pharmacological data, but they are rife with limitations (for example, costs, turnaround time) and bioethical concerns. In vitro and ex vivo models as well as instrumentation that can probe the nano-bio interactions of LNP–mRNA with cells and tissues, are complementary to animal models by providing more room for mechanistic investigations. As a result, failing early in preclinical development can be an opportunity to engineer safer LNP–mRNA drugs and vaccines and avoid redundant in vivo studies.

Glossary

Adjuvanticity
The property of certain substances to enhance the immune response against an antigen, thereby improving the effectiveness of vaccines.

Biomolecular corona
A layer of proteins, lipids and small molecules that forms on the surface of nanoparticles when they interact with biological fluids, influencing their biological identity and activity.

Drug-induced liver injury
Liver damage caused by medications or other xenobiotics, which ranges from small abnormalities in liver tests to severe liver dysfunction or failure.

Ionizable lipids
Lipids that remain neutral at physiological pH but are protonated at low pH and are commonly used in the formulation of lipid nanoparticles for RNA delivery.

Lipid nanoparticles
Nanoparticles made of ionizable and other types of lipid, often used as delivery vehicles for genetic material.

MicroRNAs
Small, non-coding, endogenous RNA molecules that regulate protein synthesis by binding to and destroying specific mRNA, thus inhibiting its translation.

Pattern recognition receptors
Proteins that recognize molecules found in pathogens or released because of cellular damage and that can regulate the innate immune response of cells.

Limitations of animal models

In the preclinical phase of LNP–mRNA drug and vaccine development, de-risking means to screen for and assess toxicities that could narrow the therapeutic index and potentially result in clinical trial failures[202]. Traditionally, animal models have been used to identify drug candidates with adequate therapeutic efficacy and acceptable adverse effects that could be prioritized for translation to the clinic. These models offer the means to test for systemic and local toxicity and determine how these responses depend on sex, age, dose regimen and route of administration, among other parameters. Moreover, clinical biomarkers, behavioural signs, histopathology and omics data can be collected before, during and after drug administration. Recent progress in gene editing and gene expression technologies such as CRISPR–Cas9 and siRNA, respectively, in combination with efficient human genome sequencing, have facilitated the production of mouse models that simulate many aspects of human diseases to test the efficacy and safety of new drugs[203]. In one example, exon 3 deletion on MMUT – which is thought to encode the ligand-binding cavity on the human methylmalonyl-CoA mutase (hMUT) – produced a mouse model of severe MMA[113]. Intravenous administration of LNP–mRNA encoding hMUT supported the safety and efficiency of mRNA therapy for this metabolic disorder. Rat and non-human primates are also used in mRNA safety and tolerability studies. mRNA encoding hEPO was formulated in LNPs and administered intravenously in both species[100]. Toxicological findings were associated with supratherapeutic levels of hEPO and LNP-driven inflammatory effects. In the studies mentioned above, there were no major interspecies differences, but species-dependent variability should be taken into consideration during preclinical development.

Animal studies can generate a vast breadth of actionable data for the transition of drug candidates to clinical trials. However, unaccounted for differences in human and animal physiology may impact the predictability of safety and efficacy data, especially for multi-component, nanoparticulate formulations[204,205]. In a very pertinent example, the IL-1 pathway was shown to assume a crucial function in initiating innate signalling associated with RNA vaccines, but its function in mice differs from that in humans: murine leukocytes upregulate the anti-inflammatory IL-1ra more than IL-1α in response to RNA vaccines, which protect the mice from cytokine-induced toxic effects[129]. Another study proposed a method to account for such differences by the simultaneous screening of multiple LNP–mRNA formulations, each one carrying a distinct DNA barcode, in mice with humanized, primatized or wild-type livers[206]. All formulations encoded a cell-surface-expressed glycosylphosphatidylinositol-anchored camelid VHH antibody, which could be fluorescently traced. Colocalization of the antibody with DNA barcodes and the integration of RNA sequencing-based transcriptomic data from hepatocytes helped to identify safe and efficient LNP carriers for mRNA translation in non-human primate or human hepatocytes. Nevertheless, interspecies physiological differences are system-wide, so that interplay of the metabolic and immune networks may impact drug tolerability. Overlapping models of the rat and human metabolic networks could account for known species differences (for example, de novo vitamin C synthesis in rats)[207]; this computational approach was used to calculate relative predisposition for the production of drug metabolites, which was in accordance with available pharmacological data. Integration of such computational approaches in preclinical development could support the meaningful interpretation of biomarker data collected from animal studies.

Advanced in vitro and ex vivo models

In vitro and ex vivo assays are used to validate the pharmacological activity of drug candidates[208] and refine them based on their cytotoxicity and impact on cell viability[209]. Besides their relative ease of use and low cost, human cell-based in vitro and ex vivo assays are needed to assess toxicity concerns due to differences in the genome and immune system between humans and all other animals. For example, LNP-based Cas9 mRNA delivery has several hurdles to overcome, including cell specificity and potentially detrimental off-target effects[210,211]. Interspecies genetic sequence differences could render animal models irrelevant for on-target and off-target genotoxicity. Innate and adaptive immune responses are also difficult to reconcile between species. For example, only human B cells and plasmacytoid dendritic cells express TLR9, whereas it is expressed in most other dendritic cells and macrophages in mice[32]. It is thus crucial to utilize and improve upon in vitro and ex vivo human systems in the context of comprehensive toxicological screening.

The advent of techniques that include the use of induced pluripotent stem cell (iPSC) organoids, spheroids and microfluidics has elevated the physiological relevance and variety of data that can be sourced from in vitro studies. Indeed, it is now possible to recapitulate in vitro pathophysiological conditions with high transcriptomic and functional fidelity and thus trace the molecular pathways of observed drug toxicities. Of the various alternatives to 'traditional' 2D cell culture, microfluidic organ-on-chip technology recreates mechanical and spatial attributes of various tissues while stem cell-derived organoids can accurately recapitulate organ (patho)physiology at the molecular and phenotypic levels. These technologies could be leveraged for the development of safe and efficient mRNA therapeutics. Finally, ex vivo models are iteratively becoming more high throughput, while machine learning algorithms can use omics and other types of data (for example,

imaging) to identify drug structures with optimal pharmacodynamics, predict their efficacy and anticipate their toxicity[212].

These advantages are reflected in recent legislation that authorizes, "alternatives to animal testing, including cell-based approaches and computer models", for a drug to enter clinical trials[7]. In tandem with ethical issues on the use of animals, animal shortage and the recent decision by the FDA to accept in vitro data in support of investigational new drug (IND) filings, there is a strong case to be made for using and developing physiologically relevant in vitro models for de-risking purposes during preclinical development.

iPSC-derived organoids. iPSCs are de-differentiated somatic cells that have been reprogrammed to follow alternative lineages by directed differentiation[213]. iPSCs can be directed to form various types of cell (for example, hepatocytes[214], cardiomyocytes[215], enterocytes[216] and tissue-resident macrophages[217], among others) and have been used to provide a near-inexhaustible cell bank for patient-specific in vitro disease modelling and tailored drug development[218].

The main advantage of iPSCs over primary cells is their ability to self-assemble in microscopic, 3D masses termed organoids. iPSC-derived organoids can present parenchymal and non-parenchymal cells and recapitulate species-, patient-, and organ-specific transcriptomic, metabolomic, histological and phenotypic traits[219]. There are now reports of human organoids that simulate human organs and even organ-specific diseases, such as hepatocellular cholangiocarcinoma[220] or alveoli that mimic the pathophysiology of cystic fibrosis[221]. Organoid-based assays are inherently more involved than 2D cell cultures, but progress in microfabrication, bioprinting and robotics can help to accelerate associated workflows[222]. Despite being a relatively new technology, iPSC-derived organoids are being increasingly incorporated into exploratory drug pipelines as they are expected to provide more sensitive and accurate toxicological and pharmacological data and thus help to promote viable drug candidates to clinical trials[223].

At the time of writing, iPSC-derived human hepatocytes have been used for the transfection optimization of LNP–mRNA products in preclinical development[224]. Nonetheless, iPSC organoids have not been used for mRNA drug and vaccine development. This could be attributed to the lack of vascularization and the inability of LNP-RNA to penetrate the entire organoid volume, thus limiting transfection to the outer few cell layers. Microfluidics-based approaches can provide continuous oxygen and drug perfusion for sufficiently thin tissue sections[225], but it is unclear whether continuous perfusion alone would suffice for the diffusion of LNP–mRNA throughout the organoid volume. These problems could be overcome by the combination of multiple iPSCs for the development of micro-vessels that would allow physiologically relevant organoid perfusion, similar to a work in which iPSCs were introduced to form the intestinal enteric nervous system within a human intestinal organoid[226].

Organs-on-chips. Improved microfabrication techniques and advances in tissue engineering have made possible the manufacture of devices (chips) that can sustain cell culture in microscopic growth medium-filled channels of various architectures. In these chips, cells can interact while embedded in an extracellular matrix to form 3D tissues (organs). Moreover, microfluidic channels can provide highly controllable cell perfusion, and flexible substrates can provide mechanical stimuli. Seeding of organ-specific human primary cells that grow under appropriate biochemical milieus and mechanical cues has enabled the in vitro reconstruction of tissues that present with the phenotype, biological functionality and transcriptomic profiles of their respective organ[8,227].

Many chip-based organ and tissue models have been proposed in the literature, including liver-, heart-, gut-, placenta-, kidney-, lung- and lymphoid-on-chip[228–234]. This technology has been lauded as a useful tool in drug development[235]. For example, small-drug accessibility to tumours has been studied using a tumour-vasculature-on-chip that recapitulates the leaky endothelial fenestration and dense extracellular matrix of solid tumours[236]. Its potential for de-risking purposes has also been established in multiple liver-on-chip drug toxicity studies[228,237,238]. A recent study aptly demonstrated that a liver-on-chip consisting of hepatocytes and non-parenchymal cells (Kupffer, stellate and sinusoidal endothelial cells) could generate drug-specific biomarkers and metabolites and reproduce drug-induced cell death[239]. Of note, more nuanced hepatic alterations could also be recreated, such as lipid accumulation from methotrexate-induced steatosis. Curiously, there are no pharmacological or toxicological studies of mRNA-based therapeutics in the published domain at the time of writing, despite their natural targeting of the liver or the usability of the platform to test nanoparticle toxicity[240].

As with every technology, there is always room for improvement. For example, human primary cells are necessary for physiological relevance, but also introduce donor-to-donor variability in the genetic background of the studied organ[8]. On another front, adequate perfusion is difficult to achieve when moving from single endothelial layers to 3D tissues in vitro. Towards this direction, the blueprint for vascularized breast tumour on a microfluidic chip has been presented[236]. Finally, circulation of PBMCs would introduce a much needed level of physiological complexity when studying the reactogenicity of nanoparticulate drugs, but there has been success only in embedding tissue-specific resident macrophages[241] or stationary lymphocytes[234].

Outlook

The rapid design of safe and efficient vaccines against COVID-19 was an inflexion point in biotherapeutics. Although mRNA therapeutics had been decades in the making, the successful COVID-19 vaccines from BioNTech–Pfizer and Moderna illustrated the efficiency of the platform. As a result, today, the once-waning nanomedicine field is reinvigorated. In academia, this is evident from an ever-increasing number of publications and heightened academic interest in RNA biology. In parallel, the pharmaceutical industry is allocating more resources towards the development of mRNA modalities for the treatment of rare diseases, many forms of cancer and infections. Arguably, the successful establishment of this new technology in the drug development space is largely dependent on its safety. A lethal case of systemic inflammation after the intravenous administration of an adenovirus vector during a phase I trial was a tragic event and halted progress in the entire gene therapy field for more than a decade. It serves as a reminder of how serious concerns about the safety of any given mRNA-based drug could affect the entire platform.

As outlined in this Review, de-risking mRNA drugs and vaccines is complicated. LNP-encapsulated IVT mRNA is a multi-component entity with bioactivity that might be more than the sum of its parts. The pharmacodynamics and potential toxicity of LNP–mRNA formulations are linked to their particulate properties, but also lipid chemistry and the mRNA translation product. Importantly, adjusting one parameter (for example, lipid chemistry) independently of the rest (for example, LNP–mRNA size) presents a formidable engineering challenge. Some obstacles remain in the path to safe and efficient

LNP–mRNA development. For example, biotechnology firms move towards in-house and patent-protected ionizable lipid synthesis, which may delay research efforts that could otherwise promote its safe application. Moreover, the complexity of LNP–mRNA formulations calls for a multidisciplinary approach during preclinical development that combines nanoparticle characterization, biochemistry, pharmacology and tissue engineering. Inevitably, relying on multiple techniques can lead to error propagation and uncertainty in the measured outcomes.

Preclinical screening of compositionally complex mRNA drugs and vaccines should transcend what is applied in small-molecule drug development. Primarily, there is a need for de-risking models that better recapitulate human physiology and pathology. Ideally, artificial intelligence-informed pharmacodynamic models and toxicogenomic networks should also be consulted for better data interpretation. A special note has to be made for single-cell techniques, which have massively expanded the volume of information that can be collected on the heterogeneity of single-cell responses to drugs in vitro and ex vivo[242]. Towards safe and effective LNP–mRNA development, a combination of next-generation sequencing, DNA barcoding and single-cell RNA-seq was demonstrated to identify which lipid-specific LNP–mRNA compositions would preferentially target specific liver cell subtypes in vivo[243]. As different LNP–mRNA modalities may share the same components (for example, ionizable lipids), single-cell techniques offer an opportunity to promote LNP–mRNA formulations with the desired pharmacodynamic profile and identify whether adverse effects are tissue- or immune system-specific. Early corrective engineering could be implemented, and animal use would be spared.

The safe development of mRNA-based drugs and vaccines demands a multidisciplinary approach that combines advanced in vitro toxicity screening methods, omics datasets for the early identification of risks and close monitoring of the ever-changing landscape of LNP and mRNA engineering. Although complicated, successful de-risking holds the key to low attrition rates and cost avoidance and represents a worthy investment for the budding field of mRNA therapeutics.

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Competing interests

E.J. is employed by Moderna, Inc. D.B. is a Northeastern University post-doctoral fellow with a Moderna, Inc.-sponsored fellowship. M.A.R. is currently affiliated with Intellia Therapeutics, but completed this review while working at Moderna.

Additional information

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