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Review

Inflammatory and immune-mediated toxicity of lipid nanoparticles

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ABSTRACT

Lipid nanoparticles (LNPs) have emerged as promising drug delivery systems. However, side effects such as inflammation, hepatotoxicity, and excessive immune responses remain major challenges. A comprehensive understanding of the mechanisms underlying these adverse effects is crucial to improving the safety of LNP-based therapeutics. Ionizable lipids can activate the NLRP3 (NOD-, LRR-, and pyrin domain-containing protein 3) inflammasome, resulting in cellular damage and the release of pro-inflammatory cytokines. Polyethylene glycol (PEG)-lipids are known to trigger acute hypersensitivity reactions, including anaphylaxis and pseudo-allergies, via the induction of anti-PEG antibodies. In addition, phospholipids and cholesterol may also contribute to toxicity through oxidation or accumulation. To address these issues, a variety of strategies are actively being explored, such as replacing ionizable lipids with biodegradable materials and developing novel surface modification technologies to mitigate anti-PEG immune responses. This review aims to provide an in-depth overview of the mechanisms of toxicity and immune activation induced by the major lipid components of LNPs and to propose strategies for improving toxicity evaluation systems. These efforts are expected to contribute to the development of next-generation LNPs with enhanced safety profiles.

1. Introduction

The rapid global spread of SARS-CoV-2, which emerged in December 2019, led the World Health Organization (WHO) to declare a Public Health Emergency of International Concern (PHEIC). Among the COVID-19 vaccines approved for emergency use by the U.S. Food and Drug Administration (FDA), those developed using lipid nanoparticle (LNP)-based platforms, such as Moderna's Spikevax (mRNA-1273) and Pfizer-BioNTech's Comirnaty (BNT162b2), achieved remarkable success [1–3]. LNPs represent one of the most advanced drug delivery systems currently available. They protect nucleic acids, which are highly susceptible to degradation in extracellular environments, by shielding them from nucleases. Additionally, they enable efficient cellular delivery of negatively charged nucleic acids, which otherwise cannot readily cross cellular membranes, by facilitating endocytic uptake mechanisms [4].

The rational design of LNPs requires a systematic understanding of how their chemical structures and physicochemical properties govern biological outcomes (Fig. 1). LNPs are typically composed of four major components: ionizable lipids, phospholipids, cholesterol, and PEG-lipids (Fig. 1A). The pKa of ionizable lipids is a critical determinant of their ionization state. They remain neutral at physiological pH to ensure stability but become protonated in acidic endosomes to facilitate nucleic acid release. Phospholipids stabilize the lipid bilayer structure, assist membrane fusion, and contribute to endosomal disruption. Cholesterol increases particle rigidity and stability, while PEG-lipids regulate particle size and prevent aggregation by improving colloidal stability [5]. In addition, the PEG-lipid shedding rate and surface charge significantly influence the circulation time and interaction of LNPs with biological environment (Fig. 1B).

Upon administration, LNPs circulate through the bloodstream and are internalized by target cells, predominantly via endocytosis. The formation of a protein corona on the LNP surface mediates cellular

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uptake via receptor-mediated endocytosis (Fig. 1C-1). Within the cell, LNP-containing endosomes undergo maturation, during which the internal pH decreases progressively from approximately 6.8–4.5 [6]. In this acidic environment, ionizable lipids become protonated, destabilizing the endosomal membrane and releasing the encapsulated nucleic acids into the cytoplasm (Fig. 1C-2) [7]. These delivery processes are highly influenced by the chemical structure of the lipid components, particularly ionizable lipids, as well as particle size, surface charge, and other physicochemical properties [5,8]. Reflecting their increasing clinical relevance in nucleic acid-based therapies, various LNP formulations are being actively developed to enhance tissue-specific targeting and endosomal escape efficiency [9–12].

As non-viral vectors, LNPs offer several advantages over viral delivery systems, including scalable manufacturing, high reproducibility, lower risk of insertional mutagenesis, and greater flexibility for personalized medicine applications [13,14]. However, the widespread clinical use of LNP-based therapeutics has raised growing concerns regarding inflammatory and immune-mediated toxicity, hepatotoxicity, anaphylaxis, and pseudo allergic reactions [15–17]. In particular, repeated or high-dose administration of LNPs has been linked to both local and systemic inflammatory responses and tissue damage [18–20]. For instance, LNPs and their mRNA cargo can be recognized by endosomal (TLR7/8) or cytosolic sensors (RIG-I, MDA5), activating pro-inflammatory pathways such as NF-kB and IRF3/7 (Fig. 1C-3). These molecular interactions ultimately culminate in acute toxicity, such as injection site inflammation, anaphylaxis (CARPA), or myocarditis, as well as chronic toxicity including organ accumulation and potential fibrosis (Fig. 1C-4).

In this review, we summarize current knowledge regarding the toxicological mechanisms associated with LNPs, focusing on individual lipid components, effects of repeated administration, and biodistribution and accumulation in vivo. We also discuss emerging strategies to mitigate these toxicities and propose future directions to guide the development of safer and more effective LNP-based gene delivery platforms.

2. Toxicity mechanisms of major LNP components

Since the FDA authorized mRNA vaccines utilizing LNP delivery systems for COVID-19, various reports have described adverse events related to LNPs, including excessive inflammatory, immune, and allergic responses [15,21]. A recent study by Parhiz et al. showed that the inflammatory responses were primarily driven by the LNPs themselves rather than the modified mRNA cargo [22]. Specifically, while modified mRNA-LNPs were well tolerated in mice under normal conditions, administration in mice pretreated with lipopolysaccharide (LPS) resulted in significantly elevated levels of inflammatory cytokines, such as serum interleukin-6 (IL-6) and hepatic macrophage inflammatory protein 2 (MIP-2). Another study by Sellaturay et al. showed that PEG-lipids—a key component of LNPs—were responsible for anaphylactic reactions, which represent a major and frequently encountered concern in the clinical use of LNPs [23]. These findings strongly suggest that LNPs can induce unexpected and excessive immune responses.

As a result, regulatory authorities such as the FDA require comprehensive evaluation of the toxicity profiles of LNP-based therapeutics before market approval. In particular, assessment of the immunostimulatory potential of each LNP component is critical for applications involving patients with immune hypersensitivity or when repeated

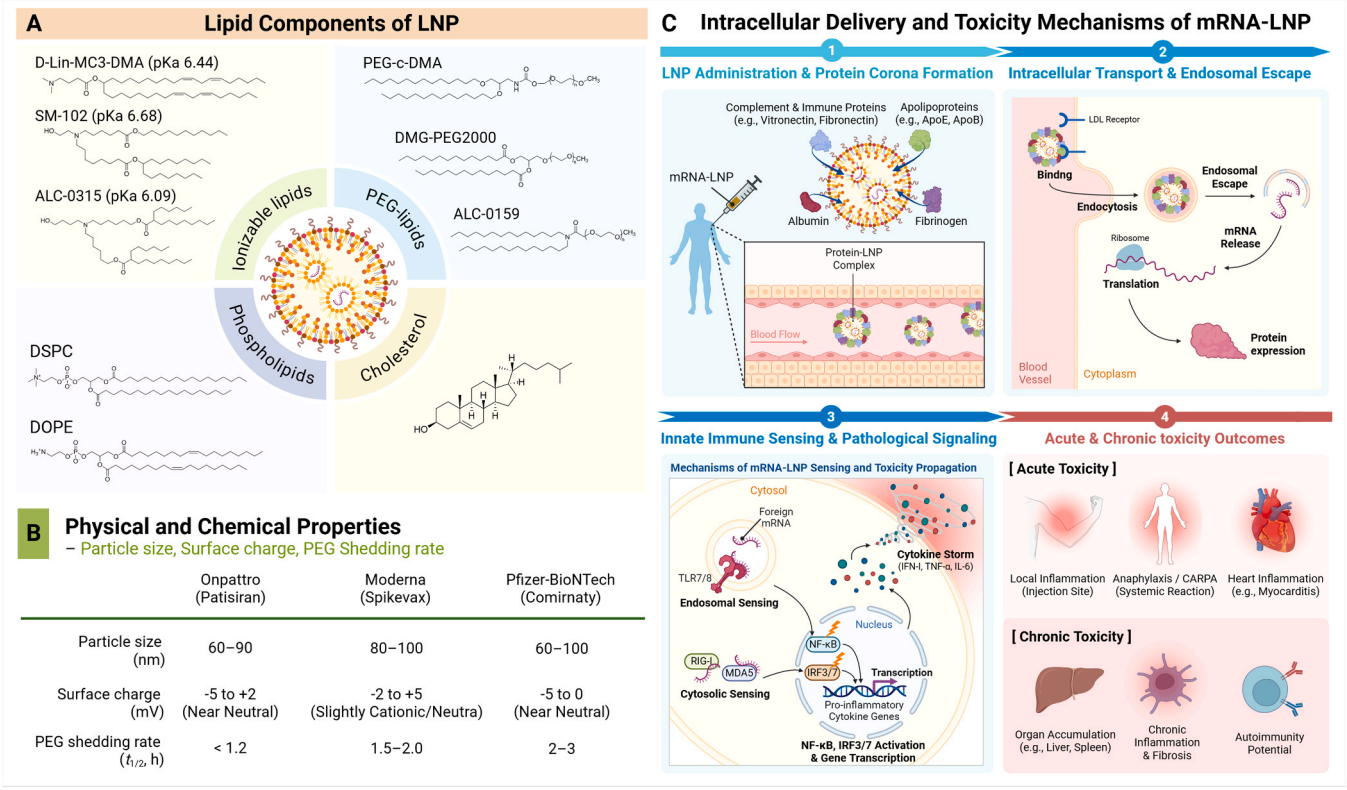


Fig. 1. Schematic overview of the mRNA-LNP life cycle and associated safety profile. (A) Representative chemical structures of the four major lipid components commonly used in LNPs formulations for nucleic acid delivery.: ionizable lipids (ALC-0315, SM-102, and DLin-MC3-DMA), PEG-lipids (ALC-0159, DMG-PEG2000, and PEG-c-DMA), cholesterol, and phospholipids (DOPE and DSPC). (B) Physical and chemical properties of mRNA-LNPs, including a comparison of particle size, surface, and PEG shedding rate across Onpattro, Moderna, and Pfizer-BioNTech LNP formulations. (C) Intracellular delivery pathway, encompassing receptor-mediated endocytosis, endosomal escape, and innate immune sensing via endosomal (TLR7/8) and cytosolic (RIG-I, MDA5) receptors. And toxicological outcomes, including acute toxicities (injection site inflammation, anaphylaxis, myocarditis) and chronic toxicities (organ accumulation, fibrosis and autoimmune potential) driven by pro-inflammatory cytokine signaling.

administration is necessary. Each lipid component of LNPs is associated with distinct intracellular toxicity mechanisms. In this section, we review the specific toxicity pathways linked to each major lipid component and highlight the key challenges that remain to be addressed.

2.1. Mechanisms of toxicity caused by ionizable lipids

Ionizable lipids, which constitute the largest proportion of the lipid components in LNPs, play a critical role in determining their toxicity profile [24]. These pH-sensitive lipids remain neutral at physiological pH but become protonated in acidic environments such as endosomes. The protonation of the lipid head group facilitates endosomal membrane disruption, thereby enabling the release of nucleic acids into cytoplasm. While this process is essential for gene delivery, it can also cause cellular stress and contribute to toxicity [25].

Endosomal damage induced by ionized lipids can lead to the generation of reactive oxygen species (ROS) and initiate cellular stress responses such as endosome-lysosome rupture. These events activate the intracellular NOD-like receptor protein 3 (NLRP3) inflammasome, triggering the release of pro-inflammatory cytokines including interleukin-1β (IL-1β) and interleukin-18 (IL-18). In addition, LNPs have been shown to stimulate Toll-like receptors (TLRs), including TLR3, TLR4, TLR7, TLR8, and TLR9, leading to the activation of downstream signaling pathways such as AP-1, CREB, C/EBP, IRF3, and NF-κB [26]. These pathways further amplify cytokine and chemokine production, as well as type I interferon responses. In the specific context of vaccination, these immune responses serve as a potent intrinsic adjuvant mechanism. By promoting the maturation of antigen-presenting cells (APCs) and enhancing the recruitment of immune cells to the injection site, the ionizable lipid-induced inflammasome pathway significantly boosts the magnitude and quality of the adaptive immune response against the encoded antigen. Recent studies have shown that chemical structures of ionizable lipids play a key role in specific immune responses such as Th1-biased responses that enhance vaccine efficacy; additionally, cholesterol, another component, also has the ability to regulate the adjuvant activity of LNPs [27,28]. However, excessive immune stimulation can be detrimental, particularly in patients with pre-existing inflammatory conditions or compromised immune function [29] (Fig. 2A).

A study by Ndeupen et al. provided compelling evidence that ionizable lipids are the primary drivers of acute inflammation induced by LNPs [32]. Following subcutaneous injection of LNPs, mice exhibited localized erythema and swelling, as well as neutrophil infiltration and weight loss. These effects were abolished when ionizable lipids were removed from the formulation, highlighting their central role in inflammation. Furthermore, high expression levels of chemokines (e.g., CCL2, CCL3, CCL4, CCL7, CCL12, CXCL1, CXCL2) and cytokines (IL-1β, IL-6, GM-CSF) were observed, indicating robust recruitment and activation of immune cells. In this study, the inflammatory responses elicited by lipid nanoparticles (LNPs) were evaluated based on the presence or absence of ionizable lipids. Wild-type C57BL/6 J mice were intradermal (ID) injected with 10 μg (2.5 μg/spot) mRNA-LNP at four sites on the back skin. Twenty-four hours post-injection, skin samples were collected and visually examined for signs of inflammation. Injection sites treated with ionizable LNPs (iLNPs) showed signs of inflammation, whereas injection sites with LNP without ionizable lipids (nLNP) did not. Flow cytometric analysis of skin samples collected from the injection sites revealed leukocytic infiltration in iLNP-injected tissues, whereas no such infiltration was observed in the nLNP. Collectively, these results indicate that ionizable lipid component represents the primary determinant of LNP associated inflammatory responses (Fig. 2B).

Kedmi et al. also reported that LNPs with a net positive surface charge induced systemic toxicity and hepatic inflammation [31]. In this study, all nanoparticles (NPs) were formulated using hydrogenated soy phosphatidylcholine (HSPC) and cholesterol as the lipid components. To modulate surface charge, positively charged NPs [(+)NPs] contained 1, 2-dioleoyl-3-trimethylammonium-propane (DOTAP), and negatively charged NPs [(−)NPs] contained 1,2-distearoyl-sn-glycero-3-phospho -(1′-rac-glycerol) (DSPG). C57BL/6 mice were intravenous (IV) injected with 10 mg/kg neutral charged NPs, (−)NPs, (+)NPs, or saline (mock-treatment). The (+)NPs exhibited elevated levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), suggesting hepatocellular injury. Changes in body weight revealed no significant weight loss in mice injected with neutral charged NPs or (−)NPs. However, mice injected with (+)NPs showed bodyweight loss (Fig. 2C). These findings support the conclusion that ionizable lipids can accumulate within endosomes and elicit both local and systemic toxic effects.

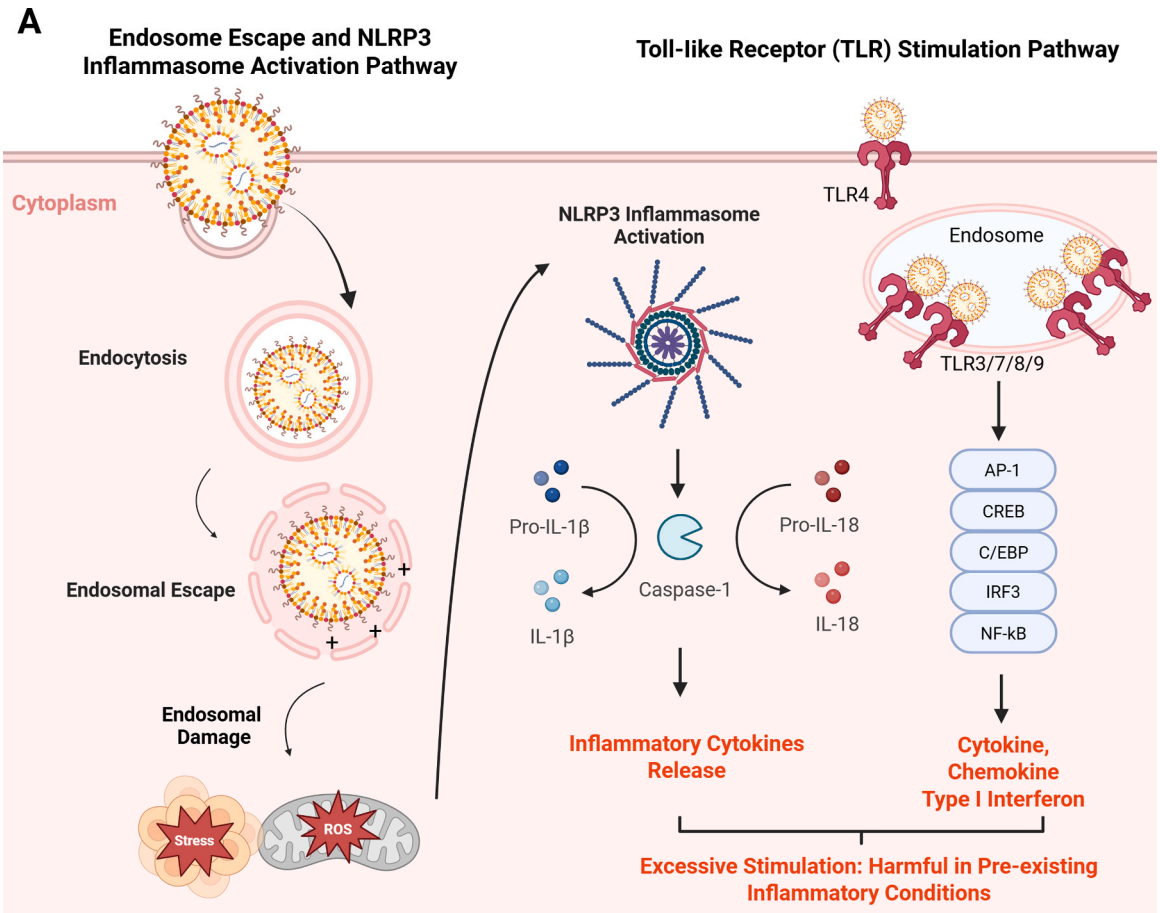
2.2. Toxicity mechanisms of PEG-lipids

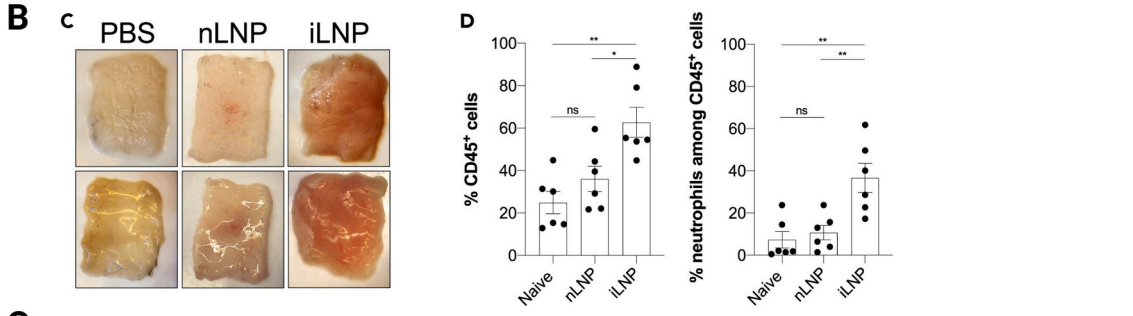
PEG-lipids are commonly used in LNP formulations to prevent particle aggregation and to regulate particle size and circulation time. Despite these pharmacokinetic advantages, PEG-lipids have been implicated in unintended immune activation, including the induction of anti-PEG antibodies and complement-mediated hypersensitivity, which may compromise both safety and therapeutic efficacy. Repeated exposure to PEG-lipids has been shown to increase anti-PEG IgM levels in vivo, which can trigger anaphylaxis, acute hypersensitivity reactions, and unwanted immune responses [33,34]. Recent studies have reported that the chemical structure and molar ratio of PEG-lipids used in LNPs significantly influence their immunogenicity and toxicity profiles [35]. For example, LNPs formulated with DMG-PEG 2000 or C16-PEG 2000 ceramide were compared, and the latter induced significantly higher levels of anti-PEG IgM. This formulation also led to greater complement activation and triggered the complement activation-related pseudo-allergy (CARPA) upon repeated administration (Fig. 3A).

Moreover, LNPs containing 1.5% DMG-PEG exhibited higher levels of complement activation than those with 1.1%, indicating a dose-dependent effect. In a study by Ju et al., two doses of SARS-CoV-2 mRNA vaccines (BNT162b2 and mRNA-1273) resulted in increased anti-PEG antibody levels in human recipients. The mRNA-1273 vaccine, which contains a higher PEG-lipids dose, was associated with more pronounced antibody induction [36]. These antibodies can enhance immune recognition of PEGylated therapeutics during subsequent treatments, increasing the risk of adverse reactions.

Importantly, Chen et al. reported that a significant proportion of the population already has circulating anti-PEG antibodies. In their analysis of 1504 individuals, 44.3% tested positive for anti-PEG IgG or IgM, and 8.3% were positive for both [37]. To investigate the ability of binding between pre-existing anti-PEG antibodies and PEGylated medicines, plasma samples from donors positive for anti-PEG lgG, lgM, or both were analyzed via ELISA. Plasma samples from donors who were positive for anti-PEG IgG (G1, G4, G5, and G6), anti-PEG IgM (M1, M4, M5, and M6), and both anti-PEG IgG and IgM (GM2 and GM3) bound strongly to Pegasys (PEG-interferon alpha-2a) [(a, b)] and Lipodox (PEG-liposomal-doxorubicin) [(c, d)] (Fig. 3B). Given this high prevalence, PEG-containing formulations, including mRNA-LNP vaccines and PEGylated drugs, may elicit unexpected immune responses in a substantial portion of the population. Anti-PEG antibodies can bind to PEGylated drugs to form immune complexes and activate the complement system, secreting anaphylatoxins such as C3a and C5a, which in turn result in hypersensitivity reactions including complement activation-related pseudo-allergy (CARPA) [38].

The mechanisms underlying these hypersensitivity reactions have been demonstrated in both animal and human studies. For instance, Chen et al. showed that the presence of anti-PEG IgG enhanced the secretion of inflammatory mediators, such as platelet-activating factor (PAF) and histamine via Fcγ receptor signaling in innate immune cells, leading to hypersensitivity upon administration of PEGylated liposomal doxorubicin (PLD) [39]. Similarly, Kozma et al. demonstrated that injecting PEGylated liposomes into pigs with induced anti-PEG IgM led





C

A

	Mock		NPs		(-)NPs		(+)NPs	
Liver enzymes	4h	24h	4h	24h	4h	24h	4h	24h
ALT (U/L)	55 ± 7	62 ± 9	71 ± 15	108 ± 19	66 ± 8	97 ± 12	102 ± 21	**347 ± 31**
AST (U/L)	110 ± 17	127 ± 31	189 ± 37	174 ± 23	129 ± 18	156 ± 27	223 ± 21	**694 ± 54**
ALP (U/L)	100 ± 9	96 ± 8	123 ± 12	128 ± 21	97 ± 8	103 ± 10	156 ± 23	**276 ± 22**

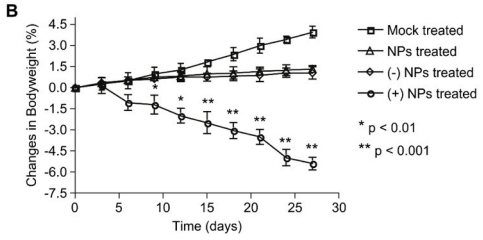


Fig. 2. Ionizable lipids in triggering Local and systemic immune toxicity. **(**A) Mechanisms of toxicity caused by ionizable lipids. Toxic induction by ionizing lipids in the process of endosome escape. When LNPs are delivered to cells, endocytosis occurs, and the acidic conditions of the endosome cause the ionizable lipids to become positively charged, thereby facilitating endosomal escape. In this process, endosomal damage, such as the generation of ROS and cellular stress, can occur. This damage triggers the NLRP3 inflammasome, which leads to the release of pro-inflammatory cytokines via caspase-1. LNP activates TLR3/7/8/9 located on the endosome and TRL4 located on the cell surface. This induces pathways such as AP-1, CREB, C/EBP, IRF3, and NF-kB, leading to the release of cytokines, chemokines and type 1 interferon. An excessive immune response can be triggered by these two processes. (B) While iLNPs caused visible inflammatory signs, nLNPs did not. Analysis of skin samples for leukocytic infiltration demonstrated that the absence of the ionizable lipid component prevented the leukocytic infiltration. Adapted with permission from Nogueira et al. [30]. Copyright 2021 Elsevier. (C) In contrast to NPs and (−)NPs, (+)NPs induced significant elevations in ALT, AST and ALP levels and marked loss bodyweight. Adapted with permission from Kedmi et al. [31]. Copyright 2010 Elsevier.

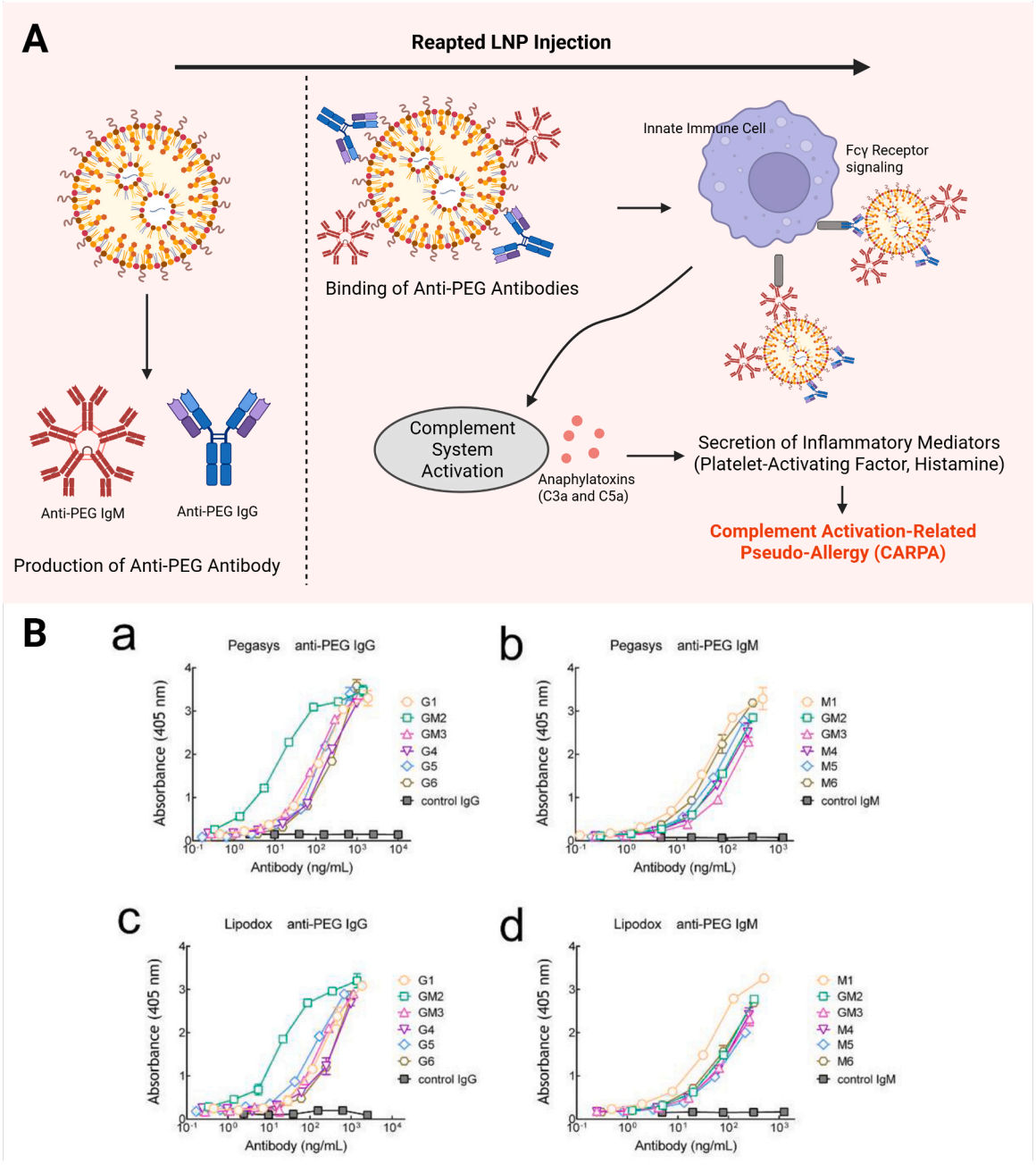


Fig. 3. Anti-PEG antibody mediated CARRA and Binging with PEGylated drugs. (A) Repeated administration of PEG-lipids can induce the production of anti-PEG antibodies. Upon subsequent injection, PEG-lipids bind to these antibodies, and immune cells recognizing these complexes activate the complement system. In this process, anaphylatoxin such as C3a and C5a and inflammatory mediators such as histamine and PAG (platelet-activating factor) are secreted, resulting in component activation-related pseudo-allergy (CARPA). (B) Plasma samples positive for anti-PEG antibodies were assayed for binding to Pegasys or Lipodox. Adapted with permission from Chen et al. Copyright 2016, American Chemical Society.

to complement activation and pseudo-anaphylaxis [40].

Another concern is the persistence of PEG-lipids in the body even after successful delivery of the cargo. Studies have shown that PEG-lipids can remain in circulation and bind to anti-PEG antibodies during repeated injections [41,42]. This persistence may increase the likelihood of immune cell interaction and result in further hypersensitivity reactions or systemic toxicity.

2.3. *Potential toxicity from phospholipid metabolites*

Phospholipids are amphiphilic molecules consisting of a hydrophilic head and a hydrophobic tail. They are categorized based on the structure of their head groups. Among these, distearoylphosphatidylcholine (DSPC), a type of phosphatidylcholine (PC), and dioleoylphosphatidylethanolamine (DOPE), a type of phosphatidylethanolamine (PE), are commonly used in LNP formulations (Fig. 1). The amphiphilic nature of phospholipids stabilizes LNP structures and facilitates endosomal escape of nucleic acid cargo [43]. Despite their widespread use, the potential toxicity of phospholipids has been less extensively studied [44]. Some studies have reported that DOPE, when combined with cationic lipids, may enhance toxicity toward macrophages [45].

Phospholipid-induced toxicity is primarily mediated by their diverse metabolic byproducts. Phospholipids are hydrolyzed by various

phospholipases under physiological conditions, generating metabolites such as oxidized phospholipids (OxPLs) and lysophospholipids (LPLs). These metabolites have been shown to modulate immune and inflammatory responses [46]. Among the OxPLs, oxidized phosphocholines can function as damage-associated molecular patterns (DAMPs), activating CD14, TLR4, and the NLRP3 inflammasome. These pathways influence cellular processes including phagocytosis, endocytosis, and inflammasome activation. Depending on the context, the resulting immune responses may be either pro-inflammatory or anti-inflammatory, although the precise outcomes remain variable and not yet fully understood [47,48]. In scenarios where pro-inflammatory responses dominate, oxidized phospholipids may promote cytokine production and systemic inflammation, thereby contributing to toxicity (Fig. 4A).

Lysophosphatidylcholine (LPC), a major LPL species, can also induce

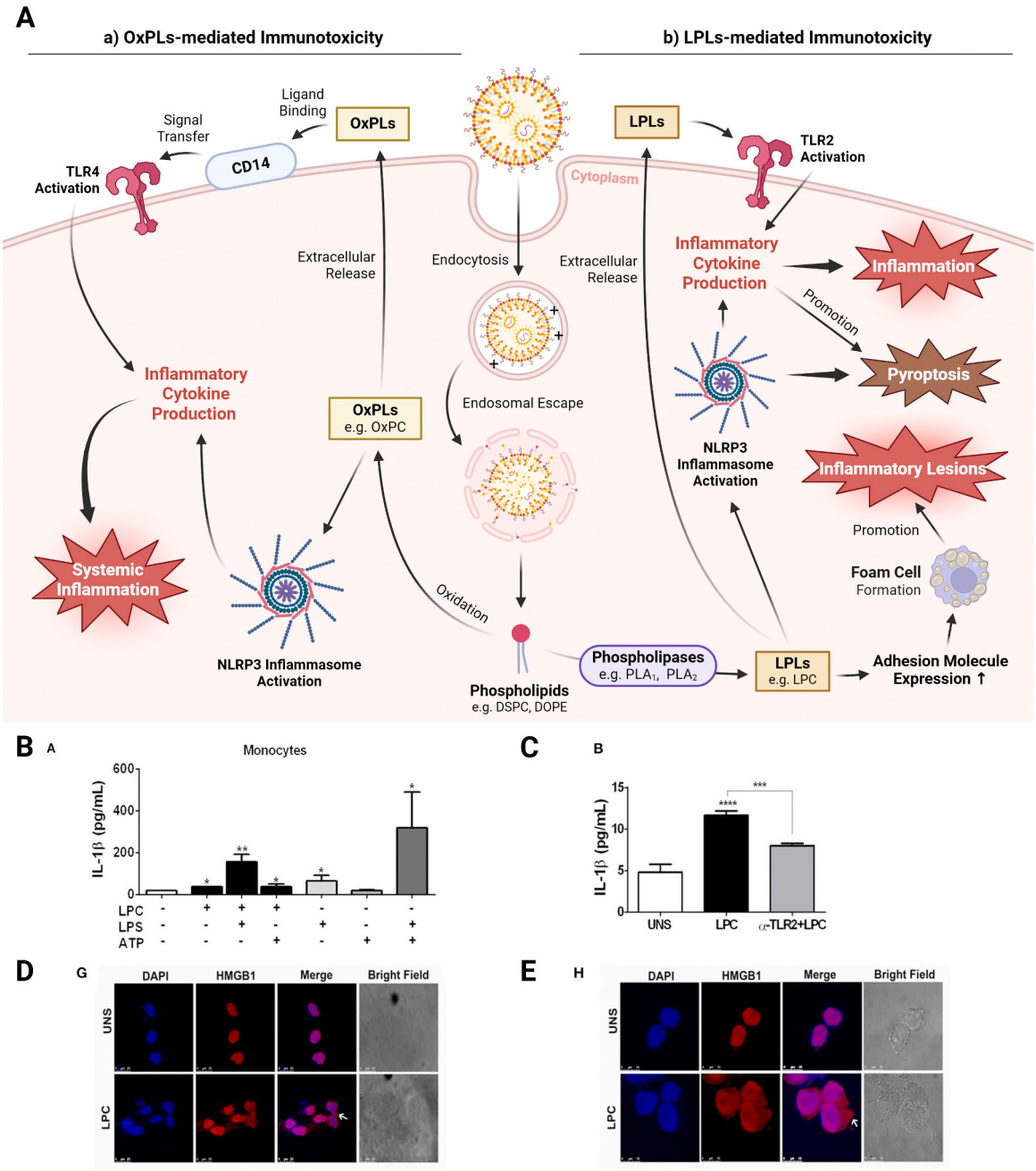


Fig. 4. Immunotoxic mechanisms of OxPLs/LPLs and experimental evidence of LPC-induced pyroptosis. (A) Toxicity pathways mediated by oxidized phospholipids (OxPLs) and lysophospholipids (LPLs). Phospholipids can be oxidized to generate OxPLs and LPLs. OxPLs-mediated immunotoxicity. Oxidized phosphatidylcholine (OxPC), a representative OxPLs species, activates the NLRP3 inflammasome or binds CD14 to trigger TLR4 signaling, leading to pro-inflammatory cytokine release and systemic inflammation. LPLs-mediated immunotoxicity. Lysophosphatidylcholine (LPC), a representative LPLs species, activate TLR2 and the NLRP3 inflammasome, promoting inflammatory cytokine production and contributing to inflammatory responses and pyroptosis. Furthermore, LPC increases the expression of adhesion molecules, which facilitates foam cell formation and promotes the development of inflammatory lesions. (B, C) LPC induces IL-1β secretion in human monocytes via a TLR2-dependent pathway. (B) IL-1β release from monocytes stimulated with LPC alone, or in combination with LPS and ATP. (C) IL-1β levels in monocytes pre-treated with a TLR2-neutralizing antibody (α-TLR2) prior to LPC stimulation. (D) Human endothelial cells and (E) human monocytes showed distinct translocation of HMGB1 from the nucleus to the cytoplasm following LPC stimulation, serving as a key indicator of pyroptosis in both cell types. This figure is reproduced from Corrêa et al. [49] under the Creative Commons Attribution License (CC BY).

immune and inflammatory responses through multiple mechanisms. Corrêa et al. reported that LPC promotes foam cell formation in monocytes and endothelial cells by activating the NLRP3 inflammasome [49]. Foam cells, characterized by cytoplasmic accumulation of lipid droplets, play a central role in the progression of atherosclerosis and in leukocyte recruitment to inflammatory sites. In the same study, LPC was shown to activate TLR2 in monocytes and induce the production of pro-inflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor-alpha (TNF-α) in endothelial cells. To examine whether LPC induces inflammasome activation in human monocytes, THP-1 cells were stimulated under three conditions: (I) LPS (500 ng/ml) followed by LPC (1 μg/ml); (II) LPC alone (1 μg/ml); or (III) LPC (1 μg/ml) followed by ATP (1 mM). LPC alone (1 μg/ml) significantly increased IL-1β release from human monocytes (THP-1 cells), and this effect was further amplified by LPS pre-stimulation. LPC also functioned as a priming signal when combined with ATP, confirming its ability to activate the inflammasome (Fig. 4B). Also, to elucidate the mechanisms underlying LPC-induced IL-1β secretion, human monocytes were pre-treated with a TLR2-neutralizing antibody for 1 h, followed by LPC stimulation (1 μg/ml) for 24 h. IL-1β levels were quantified by ELISA. Pre-treatment with the TLR2-neutralizing antibody significantly attenuated LPC-induced IL-1β secretion, demonstrating that TLR2 recognition is a key upstream event in LPC-mediated inflammasome activation (Fig. 4C). Beyond cytokine secretion, inflammasome activation by LPC was also shown to promote pyroptotic cell death in both human monocytes and endothelial cells. HMGB1 is a non-histone nuclear protein that translocates to the cytoplasm and is secreted extracellularly upon cellular stress or death, functioning as a key DAMP (damage-associated molecular pattern). Its cytoplasmic translocation occurs downstream of caspase-1 activation and serves as an indicator of pyroptosis. Extracellular HMGB1 can further amplify inflammatory responses in neighboring cells. Following LPC stimulation (10 μg/ml, 18 h), confocal microscopy revealed nuclear-to-cytoplasmic translocation of HMGB1 in both human endothelial cells (Fig. 4D) and monocytes (Fig. 4E), indicating that LPC promotes pyroptotic cell death through HMGB1 release. Additionally, Kume et al. demonstrated that LPC promotes monocyte recruitment and vascular endothelial activation, further contributing to chronic vascular inflammation and atherosclerosis [50]. LPC was found to stimulate endothelial cells to express adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), thereby enhancing monocyte adhesion and infiltration. These monocytes can subsequently differentiate into foam cells within the vascular intima, promoting the formation of inflammatory lesions.

Collectively, these findings suggest that phospholipid metabolites may provoke excessive immune responses and contribute to systemic inflammation. Given that phospholipids are essential components in current LNP formulations, further research is warranted to clarify their potential toxicological effects and to guide safer LNP design.

2.4. Considerations for the use of cholesterol

Cholesterol is the second most abundant lipid component in LNPs. It plays a crucial role in enhancing particle stability, promoting membrane fusion, and maintaining the overall structural integrity of the nanoparticle [5]. As shown in Table 1, FDA-approved clinical LNP formulations, such as Patisiran, Comirnaty, and Spikevax, consistently incorporate high molar ratios of cholesterol, ranging from approximately 38.5–42.7% [51,52]. Under normal physiological conditions, cholesterol is generally considered non-immunogenic and does not elicit significant immune responses. However, the risk of cholesterol-mediated toxicity can vary significantly depending on the specific clinical formulation, the type of nucleic acid cargo, and the required administration route. For instance, prophylactic mRNA vaccines (e.g., Comirnaty and Spikevax) are typically delivered via intramuscular (IM) injection. In contrast, siRNA therapeutics like Patisiran are administered via intravenous (IV) injection and often require repeated dosing. When the cholesterol presents in excess or in its oxidized form, cholesterol may acquire immunologically active properties, potentially leading to inflammatory responses.

LNPs are readily taken up by macrophages, where cholesterol can be enzymatically converted to oxysterols by cholesterol hydroxylases. Oxysterols can also be formed through non-enzymatic auto-oxidation during storage or under oxidative stress conditions [53]. These oxysterols exhibit strong immunomodulatory activity and have been implicated in the initiation of inflammation and immune dysregulation. Studies have shown that oxysterols may contribute to the pathogenesis of atherosclerosis and cancer by modulating immune cell behavior [54].

Cholesterol homeostasis is tightly regulated through biosynthesis, transport, and excretion pathways. However, when this balance is disrupted, cholesterol may accumulate within cells and tissues. Such accumulation is associated with hypercholesterolemia and can lead to the formation of foam cells, particularly in macrophages. Foam cell formation is a hallmark of atherosclerosis and a trigger for chronic inflammation [55]. In the liver, excessive cholesterol can accumulate in lipid droplets within Kupffer cells, which are the liver-resident macrophages. These cholesterol-laden foam cells can activate the NLRP3 inflammasome, thereby initiating a cycle of inflammation and promoting the development of chronic liver diseases [56]. The formation of cholesterol crystals within foam cells can further amplify inflammatory signaling pathways, contributing to tissue damage (Fig. 5).

These observations underscore the need for caution when using LNPs with high cholesterol content or administering them repeatedly. Excess cholesterol may accumulate in target or off-target tissues, potentially triggering inflammation and toxicity. Additional studies are required to better understand the immunological and toxicological risks associated with cholesterol in LNP formulations, particularly in the context of long-term or high-dose use in clinical applications.

2.5. Immune responses mediated by mRNA cargo

Both the LNP and the nucleic acid cargo can independently trigger innate immune responses. While ionizable lipids predominantly activate the NLRP3 inflammasome and specific Toll-like receptors (TLRs), the mRNA cargo interacts with distinct pattern recognition receptors (PRRs). Specifically, the RNA can stimulate endosomal sensors, such as TLR3 and TLR7/8, as well as cytosolic sensors, including retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated

Table 1
Comparison of lipid compositions in FDA-approved clinical LNP formulations. Molar ratios of lipid components in FDA-approved LNP formulations demonstrate consistently high cholesterol content across formulations: Patisiran/Onpattro(38.5 mol%), Comirnaty/BNT162b2(42.7%), Spikevax/mRNA-1273(38.5%).

Drug Name	Nucleic Acid Cargo	Ionizable Lipid (mol %)	Phospholipid (mol %)	Cholesterol (mol %)	PEG-lipid (mol%)	Administration Route
Patisiran / Onpattro (siRNA therapy)	siRNA	DLin-MC3-DMA (50%)	DSPC (10%)	38.5%	PEG2000-C-DMG (1.5%)	Intravenous (IV)
Comirnaty / BNT162b2 (COVID-19 Vaccine)	mRNA	ALC-0315 (46.3%)	DSPC (9.4%)	42.7%	ALC-0159 (1.6%)	Intramuscular (IM)
Spikevax / mRNA-1273 (COVID-19 Vaccine)	mRNA	SM-102 (50%)	DSPC (10%)	38.5%	PEG2000-DMG (1.5%)	Intramuscular (IM)

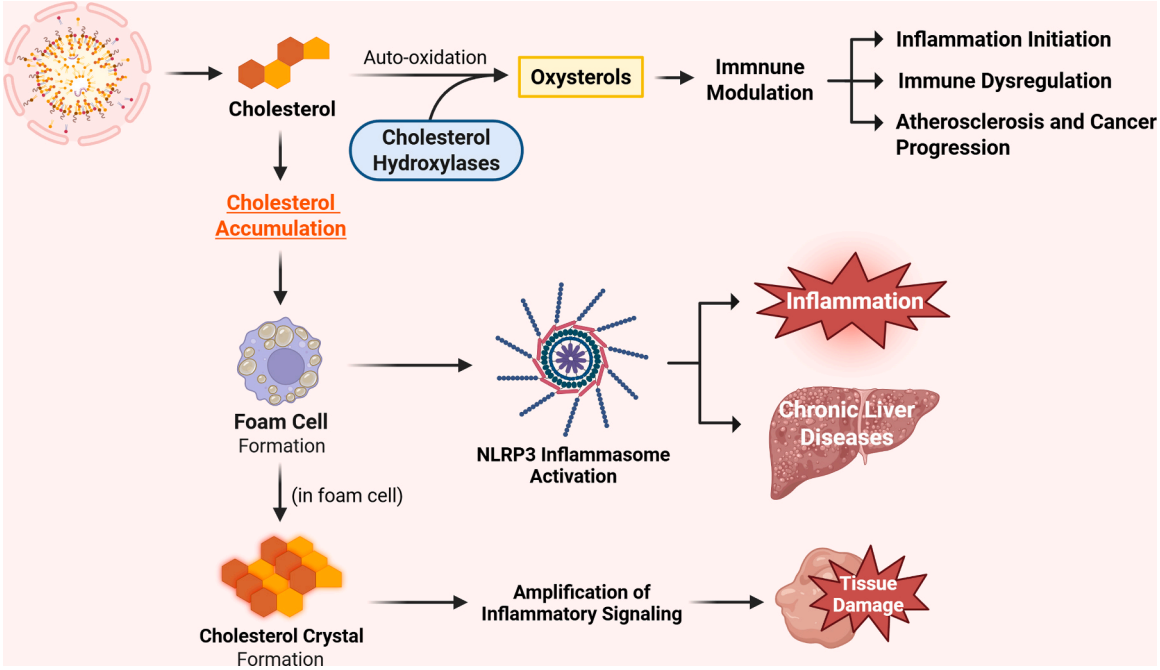


Fig. 5. Toxicity pathways mediated by oxysterols and cholesterol accumulation. Cholesterol can contribute to toxicity through oxidation and intracellular accumulation. Oxysterols, the oxidized derivatives of cholesterol, regulate the activation and differentiation of immune cells, thereby contributing to inflammation, immune dysregulation, and the pathogenesis of diseases. Excessive intracellular cholesterol accumulation promotes foam cell formation, which activates the NLRP3 inflammasome and triggers inflammation and chronic liver diseases. Cholesterol crystals within foam cells further amplify inflammatory signals, causing tissue damage.

protein 5 (MDA5). As highlighted by a study by Li C et al., the interaction of mRNA with the intracellular sensor MDA5 plays a critical role in driving immune recognition and initiating robust antiviral-like responses [57]. The activation of these cytosolic sensors by the mRNA cargo induces the production of type I interferons (IFN-α) and other pro-inflammatory cytokines such as IL-1β. This synergistic immune activation functions as a potent intrinsic adjuvant mechanism, which is highly beneficial for enhancing the magnitude and quality of the adaptive immune response against the encoded antigen in vaccine applications. However, this synergy also implies that the specific sequence, structural modifications (e.g., nucleoside modifications), and immunostimulatory potential of the mRNA cargo must be carefully evaluated alongside the LNP vehicle. Excessive activation of cytosolic sensors by mRNA can exacerbate systemic inflammation or cause unwanted reactogenicity, complicating the toxicity profile of the therapeutic. Therefore, a comprehensive understanding of LNP-based systems requires evaluating not only the lipid-mediated toxicological effects but also the immunomodulatory contributions of the mRNA cargo, ensuring a safe balance between sufficient adjuvant effect and minimal adverse inflammation.

3. Biodistribution and accumulation of LNP

The biodistribution and accumulation of LNPs are influenced by multiple factors, including their lipid composition, the chemical structure of individual lipid components (particularly ionizable lipids), particle size and charge, and the route of administration [58]. Among various organs, the liver is the primary site of LNP accumulation. After entering the bloodstream, LNPs interact with apolipoprotein E (ApoE), which facilitates their uptake by hepatocytes via low-density lipoprotein (LDL) receptors. The pKa and chemical structure of ionizable lipids are often optimized to promote hepatic delivery [59].

LNPs are typically administered by either intramuscular (IM) or intravenous (IV) injection, depending on the therapeutic objective [60,61]. For example, prophylactic mRNA vaccines are usually delivered via the IM route. Regardless of the route, LNPs tend to accumulate predominantly in the liver. This hepatic tropism has raised concerns regarding liver-specific toxicity. In a study conducted by Ahn et al., repeated administration of LNPs resulted in route-dependent adverse effects [62]. Specifically, mice treated with IV injections showed elevated levels of AST and ALT, indicating hepatic stress. In contrast, IM injections were associated with acute local inflammation at the injection site (Fig. 6). The study also found that IV administration induced more severe systemic toxicity than IM delivery. Moreover, repeated dosing led to cumulative tissue damage, suggesting that administration route significantly affects the nature and severity of LNP-induced inflammatory responses. These findings highlight the importance of carefully selecting the administration route to minimize toxicity while maintaining therapeutic efficacy.

4. Toxicity evaluation methods for LNP

The FDA has established a risk-based framework for evaluating the safety of LNP-based drug products, articulated primarily across three guidance documents: "Drug Products, Including Biological Products, that Contain Nanomaterials" (2022) [63], "Liposome Drug Products" (2018) [64], and "Immunogenicity Assessment for Therapeutic Protein Products" (2014) [65]. These documents define the critical toxicity evaluation domains that must be addressed throughout LNP development, from physicochemical characterization to clinical immunogenicity monitoring.

4.1. Physicochemical characterization

Physicochemical attributes, such as particle size, surface charge (zeta potential), morphology, and encapsulation efficiency are the primary determinants of LNP biological fate and toxicity. Particle size governs biodistribution and mononuclear phagocyte system (MPS)-mediated organ clearance, with aggregated or large particles accumulating in the liver and spleen and increasing hepatotoxicity risk. Cationic surface

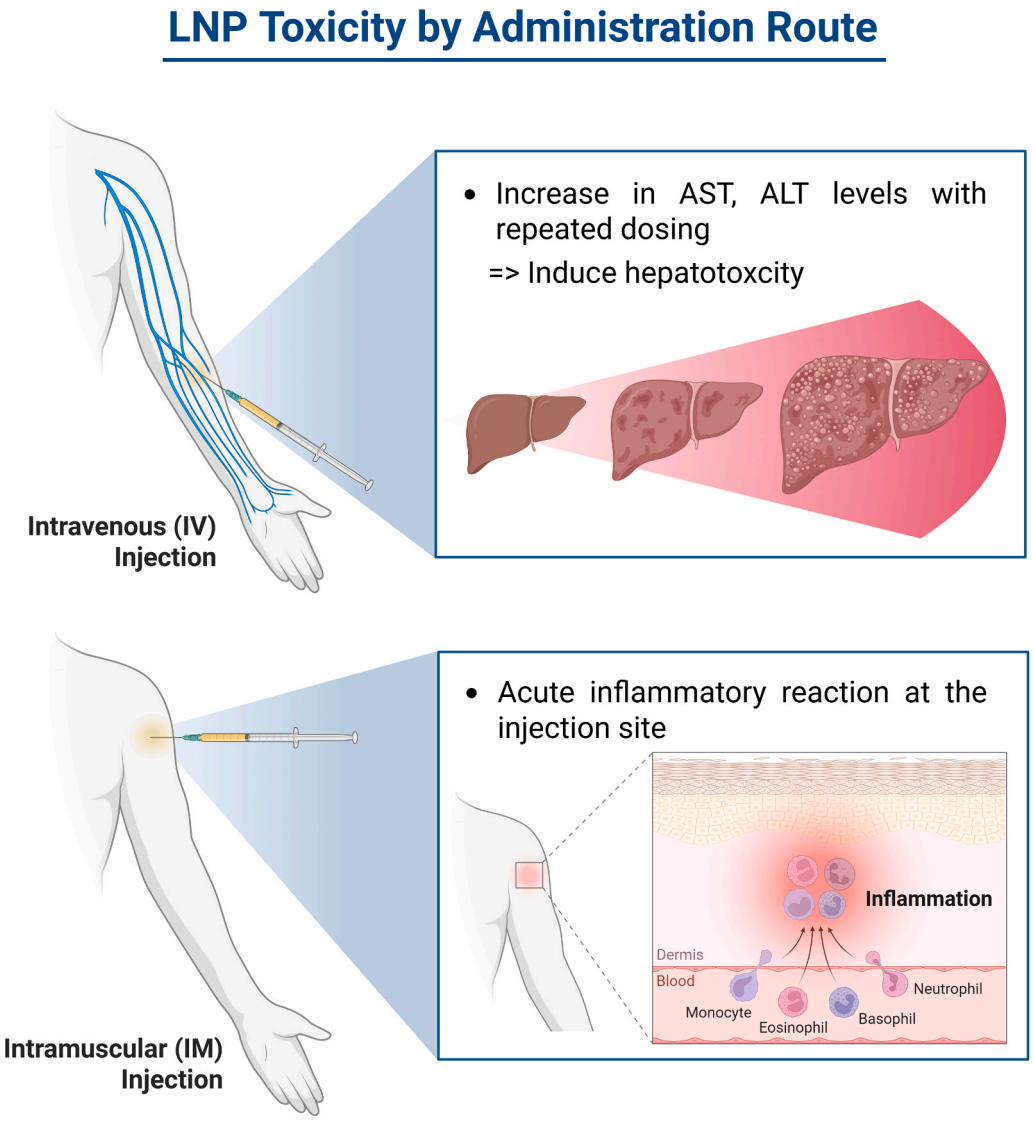


Fig. 6. Toxicological effects of lipid nanoparticles (LNPs) according to administration route. Intravenous (IV) injection: Repeated systemic administration can elevate serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels, indicating potential hepatotoxicity. Intramuscular (IM) injection: Local delivery can induce acute inflammatory reactions at the injection site, characterized by the recruitment and activation of neutrophils, monocytes, eosinophils, and basophils within the dermis and surrounding tissue.

charge promotes non-specific membrane binding and cytotoxicity, while unencapsulated free drug contributes independently to systemic toxicity. The FDA recommends complementary measurement methods including dynamic light scattering (DLS) for particle size and electrophoretic light scattering for zeta potential.

4.2. Lipid purity, stability, and degradation products

Lipid degradation generates cytotoxic impurities requiring active monitoring. Oxidation of unsaturated lipids produces lipid peroxides, malondialdehyde (MDA), and 4-hydroxynonenal (4-HNE), causing oxidative stress and DNA damage. The FDA guidance for liposome products requires identity, assay, and impurity profiling for each lipid component, with stability studies. Chemical stability is assessed using validated stability-indicating HPLC methods, and physical stability testing monitors particle size, aggregation, and drug leakage over the shelf life.

4.3. Nonclinical safety: In Vivo study design, biodistribution, and systemic toxicology

The FDA requires evaluation of the biological fate of the LNP independently from the encapsulated RNA, as repeated MPS-mediated clearance can cause lipid accumulation in the liver and spleen, producing organ toxicity not predicted by the free drug profile. Biodistribution is assessed using radiolabeled or fluorescently labeled LNPs in rodent models to quantify tissue exposure kinetics. Repeat-dose toxicity studies must include hepatic enzyme panels, hematology, organ weight assessment, and histopathology with emphasis on the liver, spleen, and lungs. For novel ionizable lipids, genotoxicity testing (Ames assay, in vitro chromosomal aberration, and in vivo micronucleus assay) is required.

Single-dose acute toxicity studies establish the maximum tolerated dose (MTD), identify the primary target organs of acute toxicity, and characterize the dose-mortality relationship. The study design should incorporate at minimum three dose levels spanning the intended clinical dose and at least one level at 10–50 × the clinical dose, administered via the intended clinical route. Animals (commonly Sprague-Dawley rats

and C57BL/6 mice for rodent; cynomolgus macaques for non-rodent if warranted) are observed for 14 days post-dose with daily clinical observation, body weight measurement, and food consumption monitoring. Terminal endpoints include full necropsy, organ weight measurements (liver, spleen, kidneys, lungs, heart), and histopathological examination. Blood sampling at 4–6 h and 24 h post-dose for clinical chemistry (ALT, AST, bilirubin, creatinine, BUN) and hematology provides early biochemical evidence of organ-specific injury before gross pathology is detectable. For ionizable lipid-based LNPs, transient elevation of hepatic enzymes within the first 24 h must be distinguished from persistent hepatocellular damage through kinetic sampling.

Repeated-dose sub-chronic studies are essential for characterizing cumulative organ toxicity arising from MPS saturation, lipid accumulation, and progressive inflammatory responses not apparent after a single administration. Study design should include at least three dose groups plus a vehicle control, using the intended clinical dosing interval and route of administration. Endpoint assessments should include weekly body weight and clinical observations, periodic clinical chemistry and hematology (with attention to hepatic enzyme trends, thrombocytopenia, and neutrophil/lymphocyte ratios), and comprehensive histopathology of all major organs at necropsy. Vacuolar changes in hepatocytes and Kupffer cells (lipid vacuolation), spleen follicular hyperplasia, and pulmonary macrophage aggregates are characteristic findings commonly observed following repeated LNP administration that require systematic scoring. The no-observed-adverse-effect level (NOAEL) established from the 90-day study forms the quantitative basis for calculation of the human equivalent dose and the initial clinical safety margins.

4.4. Hemocompatibility and protein corona analysis

Upon intravenous injection, LNPs are immediately exposed to blood, necessitating hemocompatibility testing including hemolysis, platelet activation, and complement activation assays. PEGylated LNPs are prone to triggering complement activation-related pseudoallergy (CARPA) via anti-PEG antibodies; complement fragment levels (C3a, C5a, SC5b-9) are measured by ELISA after LNP incubation in human serum. The FDA also recommends characterizing plasma protein corona formation by proteomics (nanoLC-MS/MS), as opsonin enrichment accelerates MPS clearance and amplifies inflammatory responses.

Total complement consumption (CH50 hemolytic assay) or the terminal complement complex SC5b-9 by ELISA in serum serves as the recommended first-tier screening approach, followed by a second-tier panel quantifying C3a, C4a, and C5a to further characterize complement activation. For hemolysis, washed human erythrocytes are incubated with LNP at concentrations spanning the anticipated clinical Cmax for 1–3 h at 37°C and lysis is quantified spectrophotometrically (absorbance at 541 nm) relative to 100% lysis controls. For platelet assessment, P-selectin (CD62P) surface expression and glycoprotein IIb/IIIa activation state by flow cytometry constitute the standard readouts; prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin generation assays in platelet-poor plasma further characterize coagulation effects.

4.5. Immunogenicity and Innate Immune Activation

Both the LNP and the nucleic acid cargo can independently trigger innate immune responses. Ionizable lipids activate the NLRP3 inflammasome, while unmodified RNA stimulates endosomal TLRs (TLR3, TLR7/8) and cytosolic sensors (RIG-I/MDA5), inducing IL-1β, IFN-α, and pro-inflammatory cytokines. Innate immunostimulatory potential is assessed by cytokine release assays using human PBMCs or whole blood. For adaptive immunogenicity, the FDA recommends a three-tier anti-drug antibody (ADA) testing strategy in clinical studies including screening, confirmatory, and neutralizing antibody characterization with pre-dose assessment of pre-existing anti-PEG antibodies.

For a rigorous cytokine release assessment, freshly isolated human PBMCs or whole blood from a multi-donor panel should be incubated with LNP formulations for 24–48 h, followed by quantification of a panel of pro-inflammatory mediators including TNF-α, IL-1β, IL-6, IFN-γ, IFN-α, and IL-8 using multiplex bead-based immunoassays (e.g., Luminex) or single-plex ELISA. Dose-response evaluation across at least three LNP concentrations, together with appropriate positive controls (e.g., LPS for TLR4 activation, R848 for TLR7/8 activation) and negative controls, allows calculation of a stimulation index and characterization of concentration-dependent immunostimulation. For mRNA-LNP products, the contribution of the nucleic acid cargo, which can be distinguishable by comparing empty LNPs versus loaded LNPs, must be assessed separately to attribute cytokine induction to either the lipid carrier or the RNA sequence.

4.6. Species differences and translational considerations

Interspecies differences in complement activation, MPS phagocytic capacity, TLR expression profiles, and lipid metabolism create significant barriers to direct extrapolation of rodent toxicology data to humans. Rodents differ from humans in both the abundance and function of natural killer T (NKT) cells, which are functionally and immunologically distinct from their rodent counterparts and have been implicated in LNP-induced hepatic inflammation; these differences mean that rodent models systematically underestimate both immunological and hepatotoxic risk for many LNP formulations [66,67]. Non-human primates (NHP; cynomolgus macaques) more closely approximate human complement biology, platelet reactivity, and hepatic lipid processing, and are therefore the preferred species for bridging studies when rodent data raise safety signals or when the intended clinical indication involves IV bolus administration at high doses.

4.7. Clinical biomarkers for translational safety monitoring

The selection of clinically relevant biomarkers for LNP safety monitoring is a critical step in translating nonclinical findings into actionable clinical stopping rules and dose-modification criteria. A tiered biomarker strategy including encompassing markers of hepatotoxicity, inflammation, hematological toxicity, and kidney function should be pre-specified in the clinical protocol and monitored at defined intervals aligned with the expected pharmacokinetic half-life of the LNP.

For hepatotoxicity, the conventional panel of ALT, AST, alkaline phosphatase (ALP), and total bilirubin remains the regulatory standard. However, given the lipid-loading mechanism specific to LNPs, emerging novel biomarkers offer improved sensitivity and specificity: microRNA-122 (miR-122) is a highly liver-specific serum biomarker that rises earlier than ALT in hepatocellular injury; glutamate dehydrogenase (GLDH) indicates mitochondrial injury and is less susceptible to muscle-derived confounding than ALT; and osteopontin is elevated in Kupffer cell activation and hepatic fibrosis [68]. These exploratory biomarkers should be co-collected with the standard panel in Phase I/II trials to build qualification datasets.

5. LNP toxicity mitigation strategies

In recent years, considerable efforts have been directed toward optimizing the composition of LNPs to reduce their toxicity. This includes strategies to mitigate excessive immune activation and inflammation through the structural modification of lipid components. Researchers are exploring various approaches, such as replacing ionizable lipids and PEG-lipids, which are primary contributors to toxicity, with safer alternatives. In addition to modifying lipid composition, optimizing administration routes is also under investigation as a means to reduce systemic and tissue-specific adverse effects.

5.1. Optimizing LNP composition

One major strategy to reduce LNP-induced toxicity involves incorporating functional components with improved safety profiles. High doses of LNPs often accumulate in liver sinusoidal endothelial cells (LSECs), leading to the production of inflammatory cytokines and the activation of neutrophil-mediated responses, which in turn cause hepatic injury. To address this, Sato et al. designed LNPs incorporating N-acetyl-D-galactosamine (GalNAc) conjugated to PEG [69]. GalNAc is a high-affinity ligand for the asialoglycoprotein receptor (ASGPR), which is specifically expressed on hepatocytes, allowing selective delivery of LNPs to these cells [70]. This hepatocyte-specific targeting not only improves delivery efficiency but also reduces off-target accumulation in LSECs and minimizes liver inflammation (Fig. 7A). To minimize ApoE/LDLR pathway-mediated uptake by LSECs, the LNPs were formulated with mPEG2k-DSG and GalNAc3-PEG2k-DSG (GalNAc/-PEG-LNPs) to provide steric shielding. This optimized design significantly reduced body weight loss and plasma ALT and AST levels compared to GalNAc-LNPs alone, with body weight change and AST levels showing no significant difference from the PBS control group, suggesting effective mitigation of lipid-associated hepatotoxicity (Fig. 7B–D). These results suggest that limiting LNP uptake by non-targeted cell types, such as LSECs, can significantly mitigate liver toxicity and systemic immune activation.

To prevent liver accumulation and increase the non-hepatic tissue targetability of LNPs especially to tissues or cells critical for vaccine efficacy, such as professional antigen-presenting cells (APCs) including dendritic cells and macrophages in lymphoid tissues, active redirection

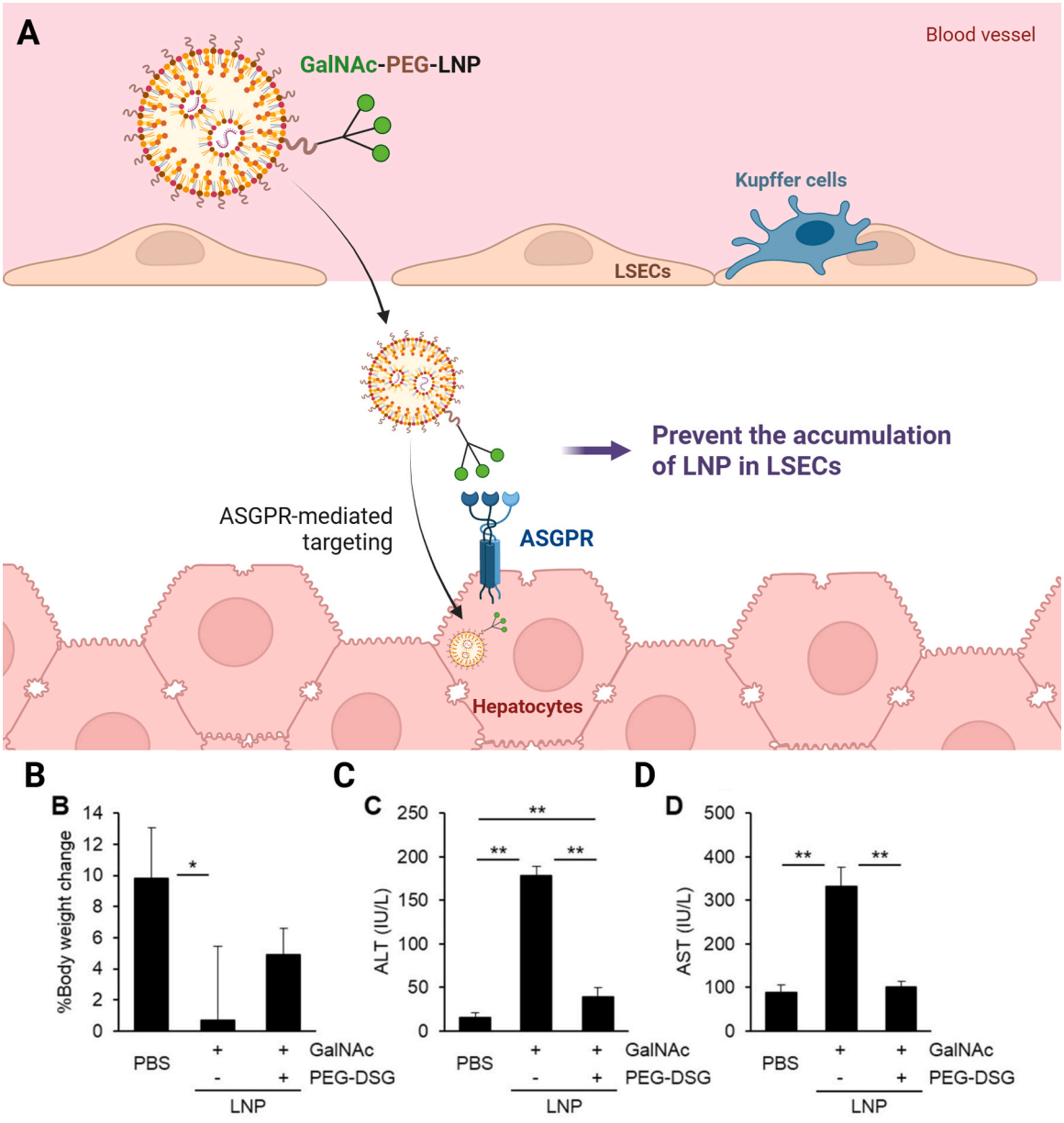


Fig. 7. Targeted delivery mechanism and in vivo safety profile of GalNAc/PEG-LNPs. (A) Schematic illustration of N-acetylgalactosamine (GalNAc)-PEG–modified lipid nanoparticles (LNPs) for targeted hepatic delivery. GalNAc ligands on the LNP surface selectively bind to the asialoglycoprotein receptor (ASGPR) expressed on hepatocytes, facilitating receptor-mediated uptake and enhancing delivery specificity. This targeting strategy reduces nonspecific accumulation of LNPs in liver sinusoidal endothelial cells (LSECs) and minimizes clearance by Kupffer cells, thereby improving delivery efficiency to hepatocytes. Evaluation of (B) body weight change, (C) plasma ALT levels, and (D) plasma AST levels at 24 h post-injection. The formulated GalNAc/PEG-LNPs significantly reduced systemic and hepatic toxicity, showing comparable safety profiles to the PBS control group. Adapted with permission from Sato et al. [18]. Copyright 2017 Elsevier.

strategies have been extensively explored. For effective immunization, LNPs must be specifically directed toward these professional APCs to facilitate robust antigen presentation and subsequent T-cell activation. Conventional LNPs are often sequestered by the liver via apolipoprotein E (ApoE)-mediated pathways, which not only diverts the cargo from its intended lymphoid targets but also increases the risk of hepatotoxicity through off-target accumulation. Consequently, modulating the biodistribution of LNPs toward the lymphatic system is essential for enhancing the therapeutic window. Strategies such as the incorporation of mannosylated lipids to target mannose receptors on DCs or the utilization of Selective Organ Targeting (SORT) technology have shown promise in bypassing hepatic uptake and promoting spleen- or lymph node-specific delivery. By optimizing these formulations to favor lymphoid uptake over liver sequestration, it is possible to achieve potent immune responses while significantly minimizing systemic inflammatory responses and liver injury.

Other studies have focused on modifying ionizable lipid content. Bae et al. reported that partially substituting ionizable lipids with trehalose glycolipids, such as 6,6′-trehalose dioleate (TDO), reduced toxicity in the liver and heart while preserving immunogenicity and transgene expression [71]. LNPs formulated with this lipid (LNP S050L) contained only 25% ionizable lipid, compared to 50% in conventional LNPs, yet exhibited comparable luciferase expression and vaccine-induced immune responses. Mice treated with LNP S050L showed reduced inflammatory tissue damage, including myocardial injury, hepatocellular swelling, and splenic leukocyte infiltration. Similarly, Yoo et al. developed a vitamin B5-derived ionizable lipid to formulate LNP 5097, which targeted lymphoid tissues with reduced off-target toxicity [72]. LNP 5097 induced a balanced Th1/Th2 immune response and robust neutralizing antibody production, while minimizing toxicity in major organs such as the liver, kidney, and pancreas. These findings suggest that the incorporation of biodegradable or less immunogenic lipids into LNP formulations can significantly enhance their safety profile without compromising efficacy.

PEG, though widely utilized, has been associated with safety-related challenges, including immunogenicity and hypersensitivity reactions [73,74]. Given the immunogenicity and hypersensitivity reactions associated with PEG, alternative surface-modifying polymers have also been investigated. Nogueira et al. developed polysarcosine (pSar)-based LNPs by replacing PEG-lipids with pSar-lipids [30]. These pSar-LNPs demonstrated high mRNA delivery efficiency both in vitro and in vivo, and induced lower levels of complement activation proteins such as C3 and C5b-9 when incubated with human serum. In mouse models, pSar-LNPs elicited significantly lower pro-inflammatory cytokine production compared to PEG-LNPs, indicating improved biocompatibility and reduced immunotoxicity. These results support the use of pSar as a promising alternative to PEG in LNP systems. Beyond polysarcosine (pSar), other hydrophilic polymers such as poly(oxazoline) (POx), poly (vinyl alcohol) (PVA), and polyglycerols are being actively investigated as non-immunogenic PEG alternatives [75,76].

Furthermore, the toxicity and biodistribution of LNPs are fundamentally dictated by the protein corona which is the layer of plasma proteins that rapidly coats the nanoparticles upon intravenous injection. Enrichment of opsonins in the protein corona accelerates Mononuclear Phagocyte System (MPS) clearance and amplifies inflammatory responses. Consequently, contemporary safe LNP design has expanded into ‘protein corona engineering’. By precisely modulating the surface chemistry, charge, and stealth coating of LNPs, the binding of pro-inflammatory opsonins (such as complement factors and immunoglobulins) can be prevented [77,78]. This strategic regulation of the protein corona not only shields the LNP from immune recognition but also mitigates off-target toxicity and unintended immune cell activation.

5.2. Optimizing administration routes

The route of administration plays a critical role in determining the tissue distribution, delivery efficiency, and immune response associated with LNPs. Adjusting the administration route can significantly reduce inflammation, toxicity, and undesired immune activation. In a murine study, intramuscular administration of mRNA-LNP vaccines induced lower complement activation and inflammatory responses than intravenous injection, while maintaining vaccine efficacy [79]. Tailoring the route of delivery to the target tissue can further enhance safety. For example, pulmonary diseases may benefit from direct inhalation-based delivery, which improves tissue specificity and reduces systemic exposure compared to IV administration. To address the challenge of effective pulmonary delivery, Liu et al. developed charge-assisted stabilized (CAS) LNPs designed specifically for inhalation. These particles delivered mRNA exclusively to the lungs, minimizing off-target expression and toxicity [80]. Similarly, Bai et al. formulated LNPs optimized for inhalation-based antibody therapy for idiopathic pulmonary fibrosis. Their formulation showed selective lung accumulation without detectable expression in other organs, and did not induce significant elevations in liver enzymes such as ALT and AST [81]. These findings emphasize the importance of both LNP composition and administration route in reducing systemic and local toxicity, and highlight the value of tailoring delivery strategies to specific therapeutic contexts.

5.3. Biodegradable ionizable lipids with cleavable motifs

A broader contemporary direction for reducing toxicity of LNPs involves the rational design of biodegradable ionizable lipids with cleavable motifs. Traditional ionizable lipids can exhibit prolonged tissue half-lives, leading to long-term accumulation and subsequent chronic inflammation. To address this, recent lipid designs incorporate biocompatible cleavable linkages like ester bonds within the hydrophobic lipid tails [82]. These cleavable motifs are designed to remain stable during systemic circulation but undergo rapid enzymatic hydrolysis by intracellular esterases once the LNP escapes the endosome. This rapid degradation facilitates the fast clearance of lipid metabolites from the liver and other tissues, thereby significantly reducing hepatocellular stress, minimizing the persistent activation of the NLRP3 inflammasome, and lowering the risk of cumulative toxicity upon repeated administration.

Furthermore, the incorporation of sulfur units into the hydrophobic tails of ionizable lipids has emerged as a highly effective contemporary strategy to accelerate their in vivo clearance [83,84]. By strategically introducing sulfur atoms, the biodegradability and excretion rate of these lipids can be significantly enhanced after they facilitate endosomal escape. This rapid elimination prevents the prolonged accumulation of lipid metabolites in the liver and other tissues. Consequently, the addition of sulfur units drastically reduces the risk of hepatocellular toxicity, cumulative inflammation, and long-term systemic adverse effects associated with repeated dosing, further optimizing the safety profile of LNP therapeutics.

6. Conclusions

The rapid development and emergency approval of mRNA vaccines during the COVID-19 pandemic have brought unprecedented attention to LNPs as advanced drug delivery platforms. As their use expands beyond infectious disease vaccines to include gene therapy, cancer treatment, and rare disease applications, improving the safety profile of LNPs has become an essential goal for translational research.

In this review, we summarized the mechanisms of toxicity associated with the four major lipid components of LNPs. Ionizable lipids can induce endosomal disruption but also cause excessive immune activation, inflammation, and hepatotoxicity. PEG-lipids, although useful for enhancing circulation time and colloidal stability, have been linked to the generation of anti-PEG antibodies, hypersensitivity reactions, and complement activation. In contrast, the roles of phospholipids and cholesterol in LNP-associated toxicity are less well defined, although

emerging evidence suggests that their metabolic byproducts can contribute to inflammation and immune dysregulation. Furthermore, as highlighted, the overall reactogenicity of LNP is driven not only by these lipid components but also by their synergistic immune activation with the mRNA cargo via cytosolic sensors like RIG-I and MDA5.

To address these challenges, ongoing efforts are focusing on the structural optimization of LNPs. Strategies include substituting immunogenic lipids with biodegradable alternatives, modifying surface coatings to avoid anti-PEG responses, and tailoring administration routes to minimize off-target effects. Additional strategies involve engineering the protein corona to prevent opsonin binding, and applying Selective Organ Targeting (SORT) to bypass hepatic accumulation and redirect LNPs to specific lymphoid tissues. Comprehensive toxicity evaluation frameworks, encompassing physicochemical characterization, nonclinical biodistribution, and hemocompatibility assays, have also been standardized to assess these new formulations.

Despite progress in mitigating the toxicity of ionizable lipids and PEG-lipids, further investigation is needed to fully understand the immunological impact of phospholipids and cholesterol to address unpredictable toxicity issues. First, while alternative polymers like pSar effectively reduce complement activation in animal models, they lack the decades of long-term clinical safety data. Future directions should explore completely "sheddable" stealth coatings that dynamically detach upon reaching target tissues, or polymer-free "invisible" LNPs achieved entirely through protein corona engineering. Second, while the introduction of rapidly cleavable ester and sulfur motifs successfully accelerates lipid clearance to reduce hepatotoxicity, this high reactivity often entails a critical trade-off with the physical stability of the LNP and the encapsulation efficiency of the mRNA. To overcome this, future research must leverage artificial intelligence (AI) and machine learning (ML) to perform high-throughput screening of massive lipid libraries, discovering optimal lipid structures that perfectly balance systemic stability during circulation with rapid intracellular biodegradation.

Furthermore, current structural optimization efforts predominantly focus on ionizable and PEG-lipids, leaving the intrinsic toxicities of phospholipids and cholesterol largely unaddressed. To provide definitive solutions to the presented problems, future structural engineering must expand to these structural lipids. This includes the development of oxidation-resistant sterol analogs (e.g., synthetic phytosterols) that maintain LNP membrane fluidity without generating pro-inflammatory oxysterols under oxidative stress. Additionally, exploring synthetic phospholipid variants designed to resist degradation by specific phospholipases could prevent the accumulation of toxic LPLs and OxPLs.

Solving LNP-induced toxicity requires a critical reassessment of current preclinical evaluation models. While robust regulatory frameworks exist, animal models often fail to accurately replicate the complexity of the human immune system, particularly regarding pre-existing anti-PEG antibody prevalence, species-specific complement activation cascades, and human-specific protein corona dynamics. For instance, while SORT technology successfully redirects LNPs in murine models, translating these biodistribution profiles to humans requires cautious validation due to interspecies differences in plasma proteins. Therefore, future directions must mandate the integration of highly predictive in vitro models, such as human immune-organ-on-a-chip systems and personalized multidimensional immune profiling

A deeper understanding of the molecular mechanisms underlying LNP-induced toxicity will guide the development of safer and more effective delivery systems. These advances are expected to facilitate the broader clinical translation of LNP-based therapeutics across diverse biomedical applications.

CRediT authorship contribution statement

Kyuri Lee: Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Hyukjin Lee:** Writing – review & editing, Supervision, Conceptualization. **Kunwoo Lee:** Writing – review & editing. **Yeji Lee:** Writing – review & editing. **Choeun Park:** Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Soyeon Kim:** Writing – review & editing, Writing – original draft, Visualization, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT (OpenAI) in order to assist with grammar checking and language editing of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Declaration of Competing Interest

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

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Data availability

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

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