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The future of genetic medicines delivered via targeted lipid nanoparticles to leukocytes

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ABSTRACT

Genetic medicines hold vast therapeutic potential, offering the ability to silence or induce gene expression, knock out genes, and even edit DNA fragments. Applying these therapeutic modalities to leukocytes offers a promising path for treating various conditions yet overcoming the obstacles of specific and efficient delivery to leukocytes remains a major bottleneck in their clinical translation. Lipid nanoparticles (LNPs) have emerged as the leading delivery system for nucleic acids due to their remarkable versatility and ability to improve their in vivo stability, pharmacokinetics, and therapeutic benefits. Equipping LNPs with targeting moieties can promote their specific cellular uptake and internalization to leukocytes, making targeted LNPs (tLNPs) an inseparable part of developing leukocyte-targeted gene therapy. However, despite the significant advancements in research, genetic medicines for leukocytes using targeted delivery approaches have not been translated into the clinic yet. Herein, we discuss the important aspects of designing tLNPs and highlight the considerations for choosing an appropriate bioconjugation strategy and targeting moiety. Furthermore, we provide our insights on limiting challenges and identify key areas for further research to advance these exciting therapies for patient care.

1. The current landscape of targeted-based lipid nanoparticles

Genetic medicine is an exciting therapeutic arena, which utilizes DNA or RNA delivered into cells in the body. Lipid nanoparticles (LNPs) are the leading carriers in the field of nucleic acid delivery and have considerably advanced nanomedicine from preclinical research to patient care [1]. So far, genetic medicine has successfully translated into the clinic by using LNPs to encapsulate small interference RNA (siRNA) for treating a hepatic genetic disease [2] and messenger RNA (mRNA) for prophylactic vaccines against SARS-Cov-2 [3,4] and RSV [5] viruses. The encapsulation of nucleic acids by LNPs protects them from rapid degradation and activation of nucleic acid sensing mechanisms, alters their pharmacokinetic properties and biodistribution, and can enable their passage across the cell membrane [6,7]. Furthermore, LNPs can be used for delivering a variety of nucleic acid cargo (Box 1) and administered via different routes. In addition, LNPs are extremely adaptable to changes in the formulation composition and chemical or biological modification.

Recent advances in the gene therapy field have come up with outstanding novel developments. From the chimeric antigen receptor (CAR) [8,9] platform to gene editing and DNA writing [10,11]. The remarkable potential of these technologies and others necessitates the rapid development of precise delivery systems. Importantly, critical thinking should be applied when determining how to best align the treatment application, with nucleic acid cargo and the delivery vehicle.

Leukocytes are an appealing therapeutic target as they can often be the source of the problem, complication mediators, and solution, all at once. Accordingly, they are the focus of extensive research across

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various indications, even when they are not necessarily the primary site of action (Table 1). This includes diseases such as cancer, auto-inflammatory and inflammatory diseases and cardiac injury, as well as bacterial and viral infections [12,13]. (See Box 1.)

Targeting leukocytes can be used to manipulate immune responses, defend against threats that arise from both inside and outside the body, and modify disease conditions. Upon consideration of how to best employ genetic medicine for leukocytes, on the one hand, LNPs are an obvious companion. LNPs potentially reach all blood cells in the circulation upon intravenous injection or tissue-residing leukocytes depending on specialized injections. Indeed, LNPs are easily engulfed by members of the mononuclear phagocyte system (MPS) [14,15]. These cells include blood monocytes, lymph nodes, and spleen macrophages, as well as subtypes of endothelial cells, Kupffer cells in the liver, and osteoclasts in the bone marrow. On the other hand, targeting leukocytes with LNPs is quite challenging due to their dispersity throughout the body, inflammatory response to DNA and RNA by sensing receptors [16], and low transfection yields in some leukocytes, particularly lymphocytes [12,13]. Studies demonstrate that applying targeting moieties to LNPs enhances leukocyte transfection, making targeted LNPs (tLNPs) critical for achieving the therapeutic efficacy of genetic medicines [17,18]. Furthermore, applying tLNPs for nucleic acid delivery has several other difficulties such as avoiding renal exertion, non-specific uptake by the liver [19], and achieving sufficient endosomal escape in the target cells after their internalization [20,21]. These hurdles as well as overcoming the regulatory and good manufacturing practices (GMP) [22–24] processes remain a primary bottleneck in using tLNPs. Nevertheless, the potential and feasibility of clinically applying tLNPs for gene therapy in leukocytes remains unequivocal.

LNPs can be applied for delivery of nucleic acids to leukocytes by passive or active targeting [14,25,26]. Passive targeting is enforced when the surface of the LNP isn't subjected to any addition of a targeting moiety, and the targeting is obtained only by the physical-chemical characteristics of the LNP. This could be achieved either by local injection of the LNPs to the target site (like in the case of intratumoral administration to the tumor bed), or by optimizing the LNP formulation to reach specific tissues upon systemic administration (also known as endogenous targeting) [25]. Optimization of the LNP formulation can be accomplished either by incorporating unique ionizable cationic lipids to the LNPs that lead to specific accumulation in certain tissues [27–29], by selecting the type of helper lipid, or by adjustment of the lipid ratios of the LNP formulation [30,31]. This review will give less prominence to passive and endogenous targeting, which are extensively discussed elsewhere [32–34].

Active targeting is achieved by targeting moieties bound to the surface of the LNPs that facilitate specific internalization into the target cells [12,35,36]. Targeted LNPs (tLNPs) can be generated by the bioconjugation of a variety of targeting moieties ranging from aptamers, natural ligands, and peptides to antibody and antibody fragments (e.g, scFv, Fab, $F(ab)_2$, diabodies, nanobodies, etc.). The tLNPs are usually designed to bind overexpressed or clustered receptors on the target cells [37]. As for delivery to tissues beyond the liver, it is important to note that adding a targeting moiety to the LNPs does not necessarily change their biodistribution or overall tissue dispersion, unless the targeting moiety facilitates specific transcytosis [38–40], and sufficient arrival of the tLNPs to the target tissue by passive targeting is necessary. Instead, the targeting moiety enhances their specific cellular uptake (also known as cellular biodistribution), which is essential for the effectiveness of DNA and RNA therapies. Therefore, it is crucial to validate that enough LNPs reach the target site before diving into the targeting design and generating tLNPs. As well, the LNPs may be tailored to reduce uptake and translation in all cells in the absence of the targeting ligand. Finally, substantial thought should be invested in meticulously planning the targeting strategy and the mean to execute its production.

In this review, we will focus on the active targeting of genetic medicine to leukocytes achieved by targeted LNPs. We will detail how to rationally design and tailor tLNPs for specific needs. Furthermore, we will examine different bioconjugation strategies for generating tLNPs and critically discuss different targeting moieties and their success and obstacles for targeting leukocytes. Finally, we will offer our insights on the future of tLNPs and propose research focus areas that could accelerate the accomplishments of genetic medicine from dream to reality.

Table 1
Therapeutic application of genetic medicines using LNPs to specific leukocytes.

Target cell	Disease application	Aim	Ref.
Myeloid cells	Vaccinations	Anti-viral and anti-bacterial vaccines	[3–5,48]
		Cancer vaccines	[49–52]
	Cancer	CAR-macrophage cell therapy	[53]
		Induce anti-tumoral responses	[54,55]
	Inflammation	Treatment for inflammatory diseases	[56–60]
		Manage infectious diseases	[61,62]
	Transplantation	Reduce transplantation rejection	[63]
B lymphocytes	Cancer	Treatment for hematological malignancies	[17,64,65]
	Inflammation	Induce anti-inflammatory effects	[66]
	Cancer	CAR-T cell therapy	[67]
	Cardiac injury	CAR-T cell therapy	[68]
T lymphocytes	Autoimmunity	Reduce autoimmune responses	[69]
	Inflammation	Induce anti-inflammatory effects	[70]
Natural killer (NK) cells	Cancer	CAR-NK cell therapy	[71]
Hematopoietic stem cells (HSCs)	Autologous stem cell treatment	Stem cell reprogramming	[72,73]

2. Design principles for targeted lipid nanoparticles

Active cellular targeting can improve the specific uptake of tLNPs by the target cells and facilitate the intracellular crossing of nucleic acids [6,14,35]. Accordingly, tLNPs could be utilized for two purposes. Firstly, tLNPs can direct internalization towards a specific cell population while the general LNP architecture can be tailored to decrease uptake that would be harmful or unnecessary to bystander cells. For example, to target gut-homing activated leukocytes in colitis-bearing m[67]ice, tLNPs that specifically bind a high-affinity (HA) conformation of integrin $\alpha_4\beta_7$ were employed to avoid other leukocyte sub populations [59]. Secondly, active cellular targeting by tLNPs can enhance internalization into hard-to-transfect cells, such as lymphocytes, where passive targeting by LNPs would be otherwise inefficient [12,13]. For instance, in vivo production of CAR T cells by reprogramming T lymphocytes with tLNPs coated with an anti-CD5 antibody [68]. Designing tLNPs and tailoring them to a specific need requires balanced planning and consideration of many aspects. Herein we will elaborate on the factors involved in optimizing tLNPs (Fig. 1).

2.1. Construction of LNPs

The structure and composition of LNPs can dramatically shape their physiochemical properties, pharmacokinetic behavior, organ distribution, cellular uptake, intracellular trafficking, and toxicity. Size, shape, rigidness, charge, stability, and PEGylation (adding polyethylene glycol to lipids) are only some of the characteristics of LNPs that define their ability to overcome delivery barriers, whether they are designated for passive or active targeting (Table 2) [30,74–76]. Furthermore, reaching

Box 1
Types of genetic medicines for leukocyte-related applications.

pDNA: Plasmid DNA has been utilized as an expression vector for gene therapy for over 30 years [41]. Using pDNA for genetic medicines offers several advantages, including high stability, ease of production and manipulation, and the ability to carry large DNA inserts. However, its immunogenicity and difficulty in encapsulating large pDNA hinder its clinical translation. Additionally, the intracellular localization of the pDNA within the nucleus can limit its effectiveness. Non-plasmid DNA-based platforms, such as minicircle DNA, offer an alternative gene therapy approach by blocking gene expression [42].

siRNA and miRNA: Small interference RNA (siRNA) and microRNA (miRNA) are short (20–25 nucleotides) and double-stranded RNA molecules [43,44]. In the cytoplasm, the RNA interference (RNAi) machinery is harnessed to degrade mRNA in a sequence-specific targeted manner. Argonaute 2 (AGO2) cleaves the sense strand of the siRNA or miRNA, and the antisense strand is loaded to into the RNA-induced silencing complex (RISC). The RISC is then guided by the antisense strand to recognize and cleave the target mRNA, and lead to gene silencing.

mRNA: Messenger RNA payloads are synthesized via in vitro transcription reactions based on a DNA template encoding the desired protein. Furthermore, mRNA can incorporate modified nucleosides, such as N1-methylpseudouridine, to avoid recognition by intracellular innate immune sensors [45,46]. Upon reaching the cytoplasm, mRNA is translated and can be used for protein replacement therapy, antigen presentation, or in vivo production of proteins such as antibodies. Despite its susceptibility to degradation and the challenges of regulating gene expression, mRNA is a powerful tool in the arsenal of genetic medicines.

CRISPR/Cas9: The CRISPR/Cas9 gene editing platform offers great potential to permanently disrupt gene expression [11,47]. The Cas9 nuclease is directed by a short single-guide (sgRNA) to modify a specific region in the chromosomal DNA by inducing a sequence-specific double-strand break (DSB). Exploiting the CRISPR/Cas9 system for genetic medicines entails co-delivering the Cas9 mRNA (~4300 bases) along with the sgRNA (~130 bases) to the cytoplasm, expressing the Cas9 protein, and co-localization of the Cas9 protein with the sgRNA and following by their nuclear internalization. The large size of the Cas9 mRNA and possible on-target and off-target activity pose a major hurdle in their clinical implementation and require efficient delivery systems or limiting their use to ex vivo applications only.

Base and prime editors: Base editors involve either a catalytically dead Cas9 (dCas9), which cannot cut the DNA, or a Cas9 nickase (nCas9), which nicks one DNA strand with a deaminase enzyme to achieve a single nucleotide polymorphism (SNP) without inducing DSBs [10]. Prime editors combine nCas9 with a prime editing guide RNA (pegRNA). The pegRNA is comprised of a spacer sequence to locate the target site and a template sequence, which is used as a template for the reverse transcriptase, resulting in the incorporation of a new sequence to the DNA. Similarly to CRISPR/Cas9 system, the activity site of base and prime editors is in the nucleus, which poses a delivery challenge.

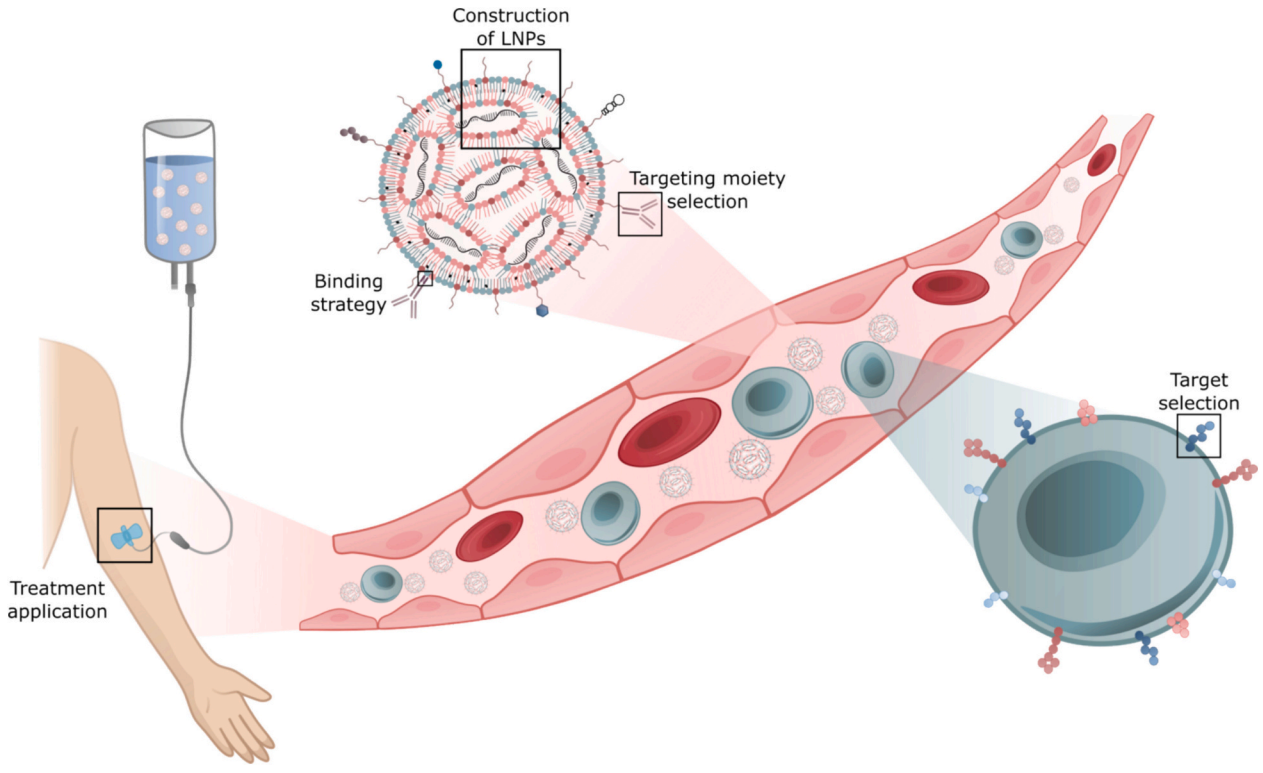

Fig. 1. Adjustable parameters for optimizing targeted LNPs delivery of genetic medicines.

extra-hepatic tissues in which leukocytes reside, such as the lymph nodes, bone marrow, or circulating leukocytes may entail screening of different LNPs to improve their endogenous targeting [77].

There are many physiological and cellular obstacles that LNPs need to overcome to achieve a therapeutic effect [14]. Before reaching the target site, LNPs are subjected to shear stress in the circulation, encounter endothelial barriers in the process of extravasation, followed by extracellular matrix restrains, and face clearance by members of the

Table 2
Delivery barriers for genetic medicine to overcome and limitations that specifically concern leukocyte-related delivery.

1. Systemic risks	2. Target site challenges	3. Intracellular Barriers	4. Leukocyte-related limitations
Avoid clearance by members of the MPS	Extravasation/ localization to the target site	Avoid lysosomal pathway	Dispersity throughout the body
Avoid excretion	Endothelial and extracellular matrix (ECM) barriers	Endosomal release	Localization in extra-hepatic tissues
Avoid nuclease degradation	Internalization to the target cell	Reach intracellular activity site	Inflammatory response to transfection with RNA and DNA cargo
Shear stress in circulation			Lymphocytes are hard-to-transfect cells
Protein corona			

MPS, hepatocytes, and Kupffer cells [19,43]. Moreover, serum proteins can coat the LNPs (also known as protein corona) and affect the internalization of the LNPs, their therapeutic efficacy, immunogenicity, organ biodistribution, and serum stability [78,79]. For tLNPs, protein corona can greatly affect the targeting efficacy and can mask the targeting moieties, therefore decreasing their specific recognition, or increase or alter their target specificity [80,81]. More research should be devoted to understanding the influence of the protein corona on the biodistribution of LNPs and its relationship with targeting moieties. For instance, Lian X. et al. recently demonstrated that protein corona could be harnessed to improve endogenous targeting of LNPs to the bone marrow (BM) [82]. A library of covalent-bond-forming lipids and crosslinkers was screened and incorporated as a supplement to a conventional base-4 lipid formulation to achieve successful targeting of BM-residing cells. Lead formulations demonstrated efficacious delivery to hematopoietic stem cells and activity of mRNA, CRISPR/Cas9, and base editor in healthy mice and sickle cell and acute myeloid leukemia (AML) disease mouse models. They hypothesized that the incorporation of unique functional groups on the surface of the LNPs changes the organ tropism. Indeed, proteomics analysis of the absorbed proteins has shown that the BM tropism was dependent on apolipoprotein E (ApoE) enrichment. Further investigation of these LNPs is required to advance their utilization for leukocyte-related diseases. Moreover, it is important to evaluate the delivery of these LNPs to the liver, especially due to the significant role ApoE plays in endogenous liver targeting [75,83,84], and consequences of CRISPR/Cas9 and base editor off-target activity [11].

Finally, once the LNPs eventually reach their target organ, their specific uptake by the target cells followed by the escape of the nucleic acid cargo from the *endo*-lysosomal system into nucleic acid payload into the cytoplasm, also known as endosomal escape, is largely thought to be the bottleneck of genetic medicines [18]. Little is known about this ineffective and crucial process, and different release processes take place for different nucleic acid payloads and LNP compositions. Endocytosis of LNPs can occur via clathrin-dependent or clathrin-independent mechanisms, such as macropinocytosis [21,85]. Regardless, the intracellular pathway they will go through after their internalization and the decomplexation of the nucleic acids from the cationic lipids [86], will determine their ultimate therapeutic efficacy. In addition, even today, the relationship between the structure and function of the ionizable lipid that can increase endosomal release is not fully understood. Nevertheless, a great deal of research is devoted to understanding the structure-activity relationship (SAR) of ionizable lipids as well as neutral and PEGylated lipids to enhance endosomal escape [87–89]. Furthermore, considerable efforts are being made to improve methods for detection of endosomal escape, by direct imaging or indirect analysis processes [20]. Lastly, improving the design of LNP formulations to achieve endosomal release and overcome other delivery challenges can be improved using machine learning [90]. Therefore, screening various ionizable lipids and optimizing a lead LNP formulation, both in vitro and in vivo, is an unavoidable step to ensure there is sufficient endocytosis of therapeutic RNA or DNA to the cytosol of the target cells.

2.2. Target selection

The design of tLNPs towards a specific target should be one of the earliest steps. Poor target selection and incomplete research of the target biology may have dire consequences on the targeting efficiency [91]. Furthermore, choosing a good target for delivery of tLNPs may entail different factors to acknowledge than for selecting a target for other therapeutics. For example, a target that would be ideal for mAb treatment could be ineffective for specific delivery of nucleic acids. Primarily, tLNPs facilitate the internalization of the nucleic acids and the targeting moieties should encourage a fast internalization and endosomal escape [92]. Receptors that can overall bypass the destructive *endo*-lysosomal pathway upon internalization would be substantially preferable to target and a close examination of the kinetics and internalization capabilities into the target cells is necessary [14]. It is important to investigate the internalization of the entire tLNP, as it may be different for the targeting moiety alone compared with when it is conjugated to LNPs [17]. Secondly, it is important to research the biology of the chosen target upon the target cells. Receptors with high recycling rates [85], receptors that undergo downregulation during different stages of the disease or upon binding to their target [93], and receptors that upon binding have unfavorable downstream activity, may be problematic targets for tLNPs. For example, the transferrin (Tf) receptor undergoes rapid recycling and often prevents its trafficking to the lysosome [94]. Furthermore, the endocytic uptake and recycling of the Tf receptor is transiently increased upon activation of T cells, making it an appealing target (see section 4.4) [95]. Other factors that should be considered upon selection of an appropriate target are interactions with other receptors or glycosylations that may interfere with the binding efficiency of tLNPs to their target [96–98]. Finally, a heterogenous expression of the target may lead to suboptimal delivery efficiency, and ubiquitously expressed targets would have beneficial therapeutic outcomes [99]. Particularly for cancer, targeting a heterogeneously expressed target may induce the tumor to decrease its expression levels, as can occur with monoclonal antibody therapy [100]. Therefore, profound biological knowledge is a fundamental prerequisite for target selection.

Certainly, there are many considerations to take into account when choosing a favorable target, and for most inductions, it is almost impossible to select a perfect target. Ideally, the preferred target should be as specific as possible and would be minimally expressed on non-related cells. Upon preclinical observation, and especially for xenograft models, this would be a non-issue, and proof of concept for tLNPs can be easily achieved. However, moving forward to clinical settings and translating mouse and even non-human primates (NHP) targets to human targets, may not be as trivial. For instance, lymphocyte antigen 6 family member C1 (Ly6C) is a commonly used target for specific subsets of monocytes in murine models [57,58,101], however, applying it to humans is problematic as there is no human equivalent to Ly6C [102]. Furthermore, the treatment timing and combination or pre-medication with other therapeutics may have dramatic effects on target selection and should be considered as well.

2.3. Targeting moiety selection

Upon designing genetic medicines for leukocytes, harnessing a targeting moiety to LNPs can increase their selectivity. Specifically for lymphocytes, which are particularly challenging to transfect with passive LNPs. Combining a targeting moiety with LNPs may be crucial as they can reduce bystander cell toxicity, enhance the therapeutic index of

drugs, prevent premature cargo release, and surpass biological barriers [12,36]. Different targeting moieties can be adopted for directing tLNPs to target cells and facilitate their internalization. Choosing a targeting moiety should, therefore, fit the selected target and treatment application. Regardless of whether a peptide, aptamer, natural ligand, or antibody is chosen, it is generally preferable for the targeting moiety to possess high binding affinity to the target, be easily manufactured, and exhibit maximum selectivity for the chosen target. Yet, exceptions may arise to these requirements, such as when selecting mannose as a targeting moiety [103]. The size of the targeting moiety can be a factor to consider when designing tLNPs for dense tissues, such as solid tumors, where a smaller targeting moiety may have an advantage over a larger one that would increase the overall size of the tLNPs [39]. A detailed overview of different targeting moieties appears in Section 4.

2.4. Binding strategy of the selected moiety

The bioconjugation strategy of the targeting moiety to the LNP is an important aspect of designing tLNPs, as it can affect the toxicity, stability, and functionality upon the tLNPs, manufacturing process, and regulatory supervision. A conjugation method that would produce tLNPs as consistently as possible would be preferential as it would decrease batch-to-batch variations and increase the homogeneity of the tLNPs, making them more applicable and appealing for clinical use [91]. A key parameter for choosing the binding strategy is the chosen targeting moiety and its requirements. For instance, the conjugation orientation of antibodies is crucial for their functionality and can greatly affect the immunogenicity of tLNPs [101]. The fragment antigen-binding region (Fab) needs to face the outside of the tLNPs to attach to its target, and an exposure of the fragment crystallizable region (Fc) can lead to recognition by Fc-receptors and initiation of immune responses. Therefore, the appropriate attachment of antibodies to the LNPs is significant for their activity as targeting moieties. Furthermore, whether conjugating antibodies or other targeting moieties to the tLNPs, optimizing their density on the tLNPs surface is a crucial step [104]. The density of the targeting moiety could affect transfection efficiency, receptor clustering on the target cells, and their immunogenicity [104–106]. For instance, Kappel C. et al. demonstrated that the density of the coating antibody upon nanoparticles affects uptake by liver sinusoidal endothelial cells via Fc receptors [104]. Adjusting the conjugation density of targeting ligands to tLNPs should be individually evaluated and calibrated as different targeting moieties may differ in their optimal density ratio. This step should not be overlooked, and examining the conjugation strategy should go hand in hand with the calibration of the targeting moiety density. The existing bioconjugation strategies for generating tLNPs are elaborated in Section 3 and Table 3.

Table 3
Binding strategies for generation of targeted lipid nanoparticles for delivery of genetics medicines.

Conjugation strategy	Functional groups involved	Advantages	Disadvantages	Commonly used for	Ref.
Chemical linkage of targeting moieties					
Thiol-maleimide reaction	A thiol group forms a thioether bind with a maleimide group	Performed in aqueous environment Rapid kinetics Stable product Simple to perform Can be performed in physiological pH	Reduction of the targeting moiety is necessary Requires high amounts of the targeting moiety Uncontrolled conjugation orientation Lacks specificity	Antibodies and antibody fragments	[17,18,59,64,67,68,70,73,141–143]
Carbodiimide coupling	Carboxyl groups and primary amines form amide bonds	Simple to perform Doesn't require modifying the targeting moiety	Uncontrolled conjugation orientation Sensitive to hydrolysis May result in cross-linking of the targeting moieties	Peptides, ligands and antibodies	[60,144]
Strain-promoted alkyne-azide cycloaddition (SPAAC)	Alkyne groups interact with azide groups	A click chemistry reaction A bioorthogonal reaction with reduced toxicity Suitable for physiological conditions Stable binding	Requires modifying the targeting moiety with an azide group Potential limitations of cell penetration, conjugation specificity, scalability, and possible toxicities should be evaluated	Antibodies and ligands	[137,145]
Biological linkage of targeting moieties					
ASSET/LAND	A lipidated scFv binds the targeting moiety (ASSET) or directly binds to the target (LAND)	Controlled orientation of the targeting moiety Doesn't require modifying the targeting moiety Requires low amounts of the targeting moiety Modular Doesn't require purifying the tLNPs Simple to perform	ASSET is currently restricted to antibodies of Rat IgG2a LAND is currently under evaluation for delivery to leukocytes	Antibodies	[56–58,140]

2.5. Treatment applications

Leukocyte genetic medicines can be applied for healthy individuals, such as with the COVID-19 mRNA-LNP vaccines, or for the treatment of different diseases. Before designing tLNPs, their treatment application should be as clearly defined as possible, with the guiding principle being 'fit to need'. The choice to utilize tLNPs for a certain therapeutic purpose should be closely associated with the choice of an appropriate route of administration and evaluation model. Importantly, since the LNP formulation is the primary determinant of the overall organ distribution, not the targeting moiety, the tLNPs should be specifically tailored to the treatment application.

For example, cancer treatment for solid tumors with intravenous injection of tLNPs would be a poor choice due to their high dependence on the enhanced permeability and retention (EPR) effect [107,108]. While systemic administration of tLNPs has shown promise in treating solid tumors in mouse models, these successes rarely translate to clinical use. Human tumors exhibit a much weaker EPR effect compared to animal models due to their significantly higher complexity, hindering the infiltration of tLNPs [108]. However, a local administration of tLNPs to target infiltrated leukocytes in the tumor bed can benefit from this treatment. Similarly, a systemic administration of tLNPs for immune modulation of dispersed tumor-associated leukocytes, or treatment of hematological malignancies, e.g., multiple myeloma (MM), lymphoma, and leukemia, is a much more agreeable fit for cancer treatment with tLNPs. The same administration mode applies for harnessing tLNPs for non-malignant indications, such as: gene editing of leukocytes, in vivo generation of CAR T, NK, or macrophage cells, and prophylactic vaccines.

The route of administration can significantly influence the therapeutic efficacy and duration of gene therapy. For instance, Elia U. et al. compared the ability of different LNPs, composed of various ionizable lipids, to facilitate protein expression of mRNA upon using different routes of administration [109]. Primarily, a luciferase-based screen was used to evaluate the efficiency and kinetics of protein expression following an intramuscular, subcutaneous and intradermal injection of mRNA-LNPs. Later, the effects of different ionizable lipids and routes of administration were evaluated by immunization of mice with an mRNA vaccine composed of a SARS-CoV2 hFc conjugated receptor binding domain (RBD) construct. They observed that not only the LNP composition, but also the route of administration plays an important role and affects the induction of IgG antibody titer and cellular responses.

Finally, employing an appropriate model to evaluate the therapeutic effects and targeting capabilities of the tLNPs is essential. Especially for preclinical evaluation studies, the quality of the evaluation model and the translational gap that results from differences between these models and humans, are a considerable barrier. Discrepancies between animal models and human disease often result in false disease location, inaccurate immune responses, dissimilar disease kinetics and intensity, and different dosages and treatment regimens. For instance, Hatit M. Z. C et al. demonstrated that mRNA delivery and cellular responses change among species, as they compared delivery across murine, non-human primates and human hepatocytes in vivo. Resultingly, often more than one model is necessary to test distinct hypotheses. Disease location in preclinical models is particularly important for evaluation of systemically administered tLNPs for genetic medicines. For example, while there are many preclinical murine models for MM, no known model reproduces exactly the human form of the disease [110,111]. Xenograft MM mouse models best resemble the human disease signaling pathways and drug resistance mechanisms, and are, accordingly, mostly used for evaluating the therapeutic efficiency of novel drugs. However, generating these models requires immunodeficient mice, and the engraftment of MM cells to the bone marrow, as in human disease, is very poor. To evaluate the biodistribution of tLNPs coated with an anti-CD38 antibody, Tarab-Ravski D. et al. established a novel xenograft MM mouse model. This model demonstrates advanced homing of MM cells to the bone marrow and many clinical similarities to human disease [64]. Otherwise, assessment of tLNPs in an animal model with negligible presence of MM cells in the bone marrow would have been irrelevant from a clinical standpoint. Still, as this model was constructed in immunodeficient mice, a full preclinical evaluation of the tLNPs requires including another model with a fully active immune system. It is, therefore, incredibly important to invest in choosing an appropriate evaluation model and consider combining several models.

3. Bioconjugation strategies for generating targeted lipid nanoparticles

Various chemical approaches can be employed to covalently link the targeting moiety and generate tLNPs [112–114]. Alternatively, biological methods could be harnessed to coat LNPs with antibodies or fusion proteins [101].

Bioconjugation of targeting moieties to LNPs can be done as part of their preparation or by modifying them later on [112]. Production of tLNPs in one-step assembly during particle formulation requires generating a targeting moiety-lipid conjugate that would endure the preparation conditions (Fig. 2A). Therefore, targeting ligands like proteins are less favorable for this preparation method due to their high molecular weight. On the other hand, targeting ligands such as mannose are preferable due to their excellent water solubility and stability under the low pH conditions involved in LNP synthesis [115]. Although this preparation method yields high conjugation efficiency, its effectiveness can be limited if the accessibility of the targeting moiety restricted on the surface of the LNPs or if it faces the interior cavity of the LNPs.

Generation of tLNPs post-preparation can be done in one of two ways; either by surface modification of the LNPs (Fig. 2B) or by post insertion of micelles composed of targeting moiety-lipid conjugates (Fig. 2C) [112]. While these production methods are more suitable for large and fragile targeting moieties like antibodies and antibody fragments, the conjugation efficiencies can be quite low. Surface modification of LNPs requires adding a 'chemical handle' to one of the lipids and conjugating the targeting moiety directly to the LNPs, mostly via PEGylated lipids. This preparation process usually entails further purification processes by size exclusion columns to separate between the tLNPs and the targeting moiety. Post-insertion of targeting moiety-lipid micelles with LNPs requires thoroughly mixing these two components after their preparation by vortex. The micelles can be created from a targeting moiety-lipid conjugate, or by surface modification of the micelles with a targeting moiety before being added to the LNPs. Bioconjugation to micelles prior to their addition to LNPs can increase the uniformity of the tLNPs as the conjugation reaction and purification processes are much more contained. Furthermore, post-insertion preparation yields modular tLNPs, enabling an efficient screening of targeting moieties or LNP libraries [101,116,117]. This approach also offers the advantage of easily optimizing the targeting moiety density upon the tLNPs. This section details the various advantages and limitations of chemical and biological approaches currently used for bioconjugating targeting moieties.

3.1. Chemical linkage of targeting moieties

3.1.1. Thiol-maleimide reaction

The thiol-maleimide reaction (Fig. 3A) is a specific type of Michael addition and is one of the most common methods for conjugating drugs to macromolecules. It is performed in aqueous environments, characterized by rapid kinetics, and the product of this reaction is rather stable [118]. This chemistry can be used to conjugate targeting moieties to LNPs for generation of tLNPs and is currently applied to form a non-cleavable linker in many of the clinically approved antibody-drug conjugates (ADCs) [119,120]. It relies on the thiol group in cysteine residues of the targeting moiety, most commonly antibodies, due to their relatively low abundance and high nucleophilicity of the sulfhydryl

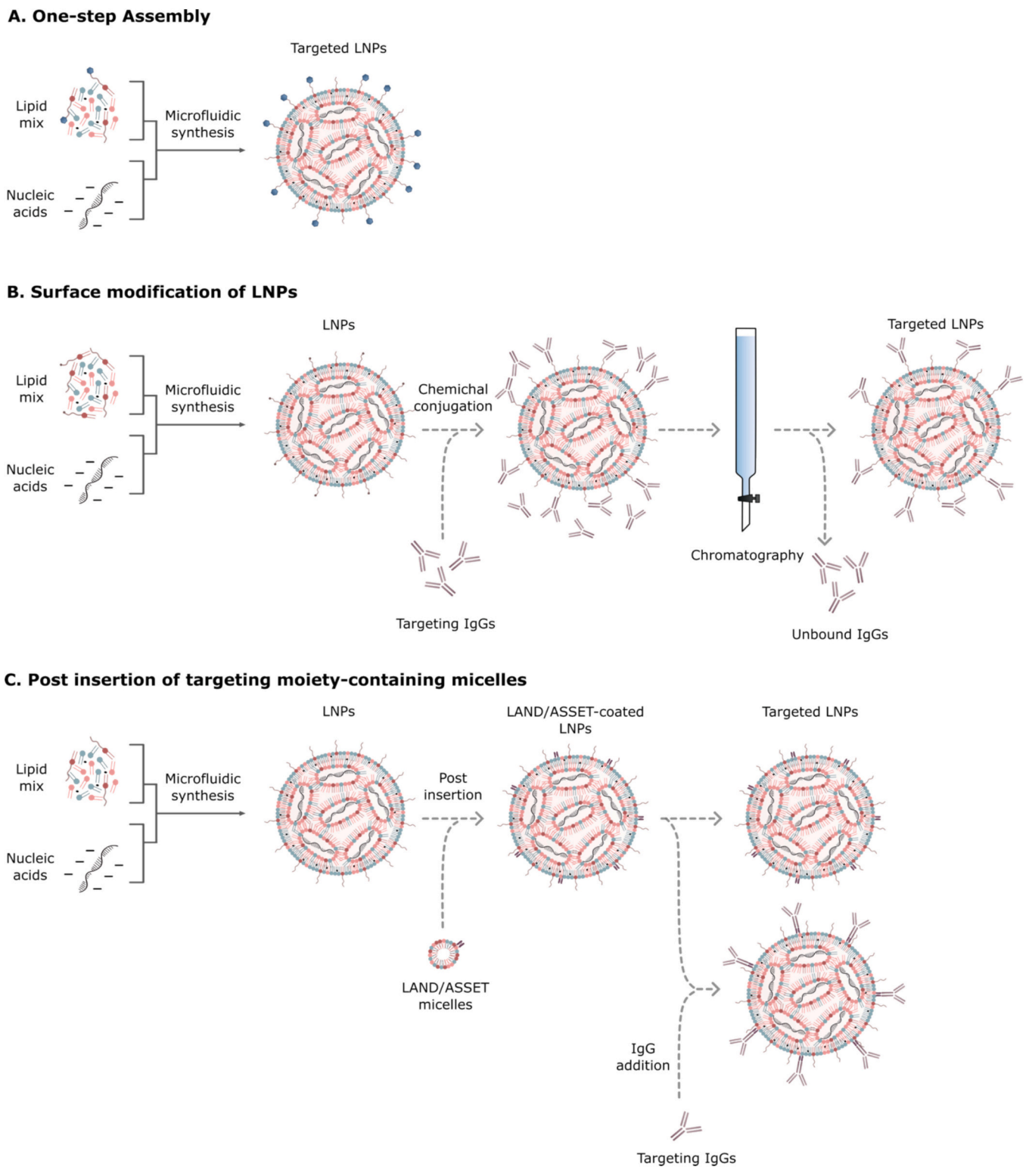

Fig. 2. ***Preparation strategies of targeted lipid nanoparticles (tLNPs).*** **(A)** One-step assembly involves production of tLNPs composed of a targeting moiety-lipid conjugate during LNP preparation. **(B)** Surface modification of LNPs entails incorporation of lipids w with a reactive chemical group. The targeting moiety, here, a full IgG antibody, is then chemically conjugated to the surface of the LNPs, and the tLNPs are purified by chromatography from the unbound targeting moiety. **(C)** Post insertion of targeting moiety-containing micelles requires separate preparation of the LNPs and micelles. The targeting moiety-containing micelles are generated either by a lipidated targeting moiety or by surface modification of the micelles composed of a lipid with a reactive group. Here, LAND micelles can be added to pre-prepared LNPs as a lipidated targeting moiety to form tLNPs by post insertion that directly bind to target cells through their scFv domain. Alternatively, ASSET micelles can be post-inserted to the pre-prepared LNPs, and the ASSET-LNPs are then coated with full IgG antibodies while maintaining the proper orientation of the IgGs. ASSET- Anchored Secondary scFv Enabling Targeting. LAND- Lipidated Antibody Nanoparticle Delivery.

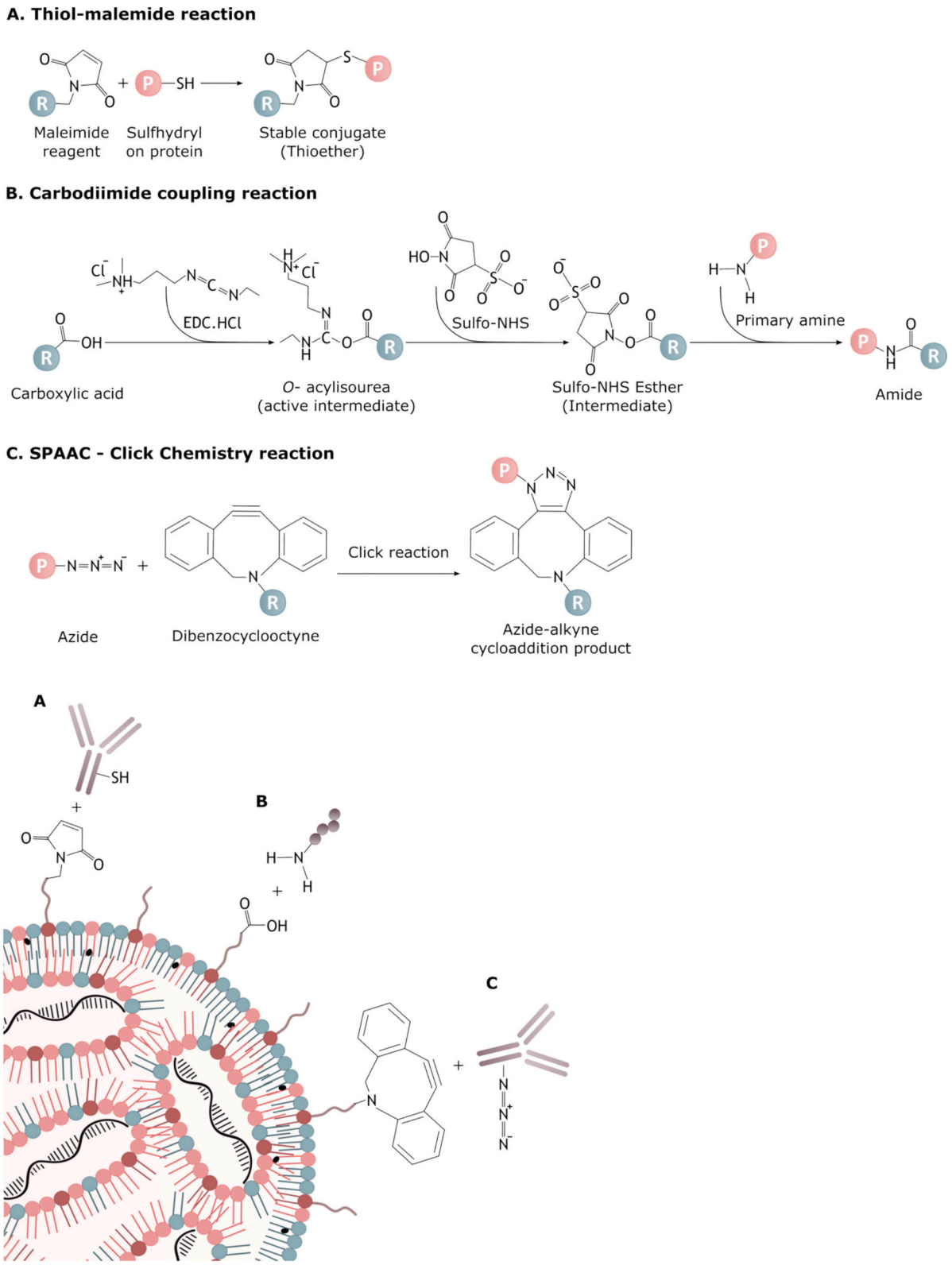

Fig. 3. ***Chemical linkage methods of targeting moieties to LNPs.*** **(A)** Thiol-maleimide reaction involves a maleimide reagent, usually upon PEGylated lipids, interacting with a sulfhydryl group on protein, such as full IgG antibody. **(B)** Carbodiimide coupling reactions can be performed with EDC Sulfo-NHC coupling to covalently bind a carboxylic group originated from the LNPs with a primary amine on the targeting moiety, for instance, a short peptide, to form an amide bond. **(C)** Strain-promoted alkyne-azide cycloaddition (SPAAC), a click chemistry reaction, can be utilized to bind a targeting moiety with an azide group, here a full IgG antibody, to a cyclooctyne molecule like DBCO. PEG- polyethylene glycol, EDC- 1-Ethyl-3-(3-dimeethylaminopropyl)carbodiimide, Sulfo-NHS- Sulfo-N-hydroxysuccinimide, DBCO- dibenzocyclooctyne.

group. The first step requires a partial reduction of the targeting moiety to introduce reactive sulfhydryl residues by interaction with a reducing agent, such as tris-(2-carboxyethyl)-phosphine (TCEP), dithiothreitol (DTT) or N-succinimidyl *S*-acetylthioacetate (SATA). Next, the reducing agent is removed, and the reactive endogenous cysteines are exploited to form a stable thioether bond with a maleimide group, which is usually linked to PEGylated lipids.

While this conjugation chemistry is relatively easy to perform, there are some limitations in using it for the generation of tLNPs. Primarily, it is difficult to control the partial reduction of the targeting moiety, which can lead to bioconjugation in an improper orientation and increase batch-to-batch variations [101,121,122]. Furthermore, of antibodies may impact their safety and binding efficiency [123]. A selective reduction of the cysteine groups can be achieved by insertion of engineered cysteines, enzymatic conjugations via peptide tags, or incorporation of unnatural amino acids [124,125]. For example: engineering C-terminal cysteines of antibodies that upon calibration will be solely reduced while leaving the other cysteines intact [126]. Generation of site-specific covalent attachments to maleimide groups will ensure a correct attachment of the targeting moiety, decrease harming the functionality of the targeting moiety, and increase the homogeneity of the tLNPs [36,101]. Today, while technologies for site-specific reduction are wildly used for the generation of ADCs, they haven't been clinically translated yet for tLNPs. A possible explanation for this could be those current solutions for inducing an efficient and site-specific reduction focus on modifying the targeting moiety, rather than changing the reaction chemistry. A successful combination of modified targeting moieties for site-specific conjugation and bioconjugation to tLNPs remains a significant challenge to overcome.

Another limitation of the thiol-maleimide reaction is the retro-Michael reaction which can lead to a premature release of the targeting moiety [118]. Hydrolyzation of the thiol-maleimide conjugate to their ring-opened counterpart before in vivo application may improve their stability and decrease de-conjugation rates [127,128].

Nevertheless, thiol-maleimide reactions have been employed in many studies and succeeded in delivering siRNA and mRNA to specific populations of leukocytes (see section 4), and despite the current limitations of thiol-maleimide reactions, there is little doubt that advancements will be made to address them. Consequently, the potential for clinical evaluation of these reactions can be expected to materialize in the not-too-distant future.

3.1.2. Carbodiimide coupling

Cross-linking of proteins can be used as a bioconjugation strategy for generating tLNPs. 1-Ethyl-3-(3-dimeethylaminopropyl)carbodiimide (EDC) is a water-soluble carbodiimide coupling agent commonly employed for protein labeling and cross-linking reactions [60,129]. It works by activating carboxyl groups for direct conjugation with primary amines to form amide bonds (Fig. 3B). Once the EDC reacts with the carboxyl group, an unstable intermediate named O-acylisourea is formed, and that intermediate could easily be displaced by an amino group through a nucleophilic attack. *N*-hydroxysuccinimide (NHS) or Sulfo-*N*-hydroxysuccinimide (Sulfo-NHS) esters are not a requirement to perform carbodiimide cross-linking but may act as stabilizers [130]. They enhance conjugation efficiencies by creating a semi-stable amine-reactive ester intermediate, and following a nucleophilic attack by the primary amine, the NHS/Sulfo-NHS groups are released. Furthermore, NHS/Sulfo-NHS allows to perform this reaction in a physiological pH. Sulfo-NHS is a water-soluble analog of NHS and would be preferentially used for bioconjugation to tLNPs [131].

Applying carbodiimide coupling for the bioconjugation of peptides and proteins has a few limitations. Primarily, this reaction lacks specificity, as any primary amine present on the LNPs or other targeting moieties could be attached, and may result in a heterogeneous population of tLNPs [132]. Targeting moieties can also react with each other, and conjugation orientation is uncontrollable using this chemistry. Secondly, the pH range in which an effective EDC NHS coupling would occur is very restricted and might not be suitable for all tLNPs [133]. Lastly, hydrolysis can break the amide bond formed by this reaction, therefore risking the stability of the targeting moiety upon the tLNPs [134]. Overall, given the drawbacks of the EDC NHS coupling, this bioconjugation strategy may be less favorable for tLNPs generation but could be considered for shorter peptides. Nevertheless, incorporating other carboxylic activators, such as 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium (DMTMM), can be used to increase the efficiency of this conjugation method and reduce side-reactions [135].

3.1.3. Strain-promoted alkyne-azide cycloaddition (SPAAC)

Click chemistry was first articulated by Sharpless in 2001 and refers to any chemical reaction that shares these key features: simplicity, modularity, readily available reagents, high yields, easy product isolation, and stability under physiological conditions [136]. These characteristics of click chemistry reactions have made them a significant tool for drug discovery and bioconjugation. While several click chemistry reactions can be used for conjugating a variety of ligands to nanoparticles of diverse types [113], only strain-promoted alkyne-azide cycloaddition (SPAAC) was utilized to generate tLNPs with nucleic acid cargo (Fig. 3C). Sakurai et al. covalently attached an antibody with an azide group located on the Fc region to dibenzocyclooctyne (DBCO) groups on PEGylated DSPE for specifically delivering siRNA to lymphatic endothelial cells [137]. Being bioorthogonal, this reaction forms a covalent bond without a metal catalyst, which reduces toxicity compared to copper-catalyzed azide-alkyne cycloaddition (CuAAC) and occurs in physiological conditions [138]. Moreover, additionally to the advantages of using click chemistry, by mediating a direct covalent coupling of alkyne groups to azide groups, SPAAC offers a specific bioconjugation with decreased aggregation of LNPs or cross-linking between targeting moieties. Further research into click chemistry for generating tLNPs is necessary to progress it for clinical evaluation and address potential limitations such as cell penetration issues that may result from low water solubility of click reactive groups [139], conjugation specificity and unwanted side reactions, production scalability, and possible cytotoxicity.

3.2. Biological linkage of targeting moieties

While chemical bioconjugation strategies form covalent bonds between the LNPs and the targeting moieties, ensuring strong and stable binding, these processes can damage the functionality of the targeting moiety. This damage can manifest in two ways: improper orientation conjugation, which can limit the binding site of the targeting moiety, and the need for introducing a reactive group for the chemical reaction, which can alter the stability and effectivity of the targeting moiety [101,114]. Furthermore, applying thiol-maleimide reaction and carbodiimide coupling for bioconjugation of antibodies can be highly inefficient and require large amounts of antibodies, further increasing the cost of tLNPs production [101,129].

We have developed a self-assembled modular platform for bioconjugation of antibodies to LNPs named ASSET, Anchored Secondary scFv Enabling Targeting. The ASSET system utilizes a biological approach by employing a lipidated scFv produced in *E. coli*, and following a purification process, is incorporated into LNPs by post-insertion. The ASSET-LNPs are then coated with an IgG antibody, and after a short incubation at room temperature, tLNPs are obtained. The scFv fragment of the ASSET specifically binds the Fc region of antibodies, therefore enabling the assembly of a theoretically infinite repertoire of mAbs to generate tLNPs (Fig. 2C). Although the ASSET system successfully bypasses the limitations of chemical conjugation and demonstrated specific delivery capabilities of siRNA [57,101,140] and mRNA [58] to leukocytes, it is currently restricted to binding only antibodies of Rat IgG2a isotype. Alternatively, by simply changing the scFv of the ASSET system to another scFv that directly binds to the target cells

instead of the Fc region of antibodies, we can post-insert these Lipidated Antibody Nanoparticle Delivery (LAND) micelles into LNPs. Production of LAND-tLNPs can altogether bypass the addition of an antibody as a targeting moiety, therefore significantly reducing production cost and complexity of tLNPs. Further development of these bioconjugation strategies is necessary to increase their ability to bind more targets and advance it for clinical evaluation.

4. Types of targeting moieties

Targeting moieties can be added to the surface of LNPs to recognize and specifically bind to target cells. Targeting molecules are categorized into four main types: (i) antibodies and antibody fragments, (ii) peptides, (iii) oligonucleotide aptamers, and (iv) other ligands (e.g. proteins and saccharides).

Antibodies provide the highest affinity and specificity for their targets but are costly and may cause immunogenicity issues. In contrast, oligonucleotide aptamers and peptides, while having lower affinities, are gaining attention due to their lower production costs and simpler methods for achieving functional chemical modifications. They are generally more stable and less susceptible to proteases compared to antibodies. Peptides, in particular, have the advantage of being smaller than antibodies and oligonucleotides, making their in-silico selection processes faster and more reliable. The ease of peptide synthesis allows them to be readily incorporated into carriers through both non-covalent and covalent methods.

Other molecules, such as serum proteins (e.g., transferrin or cytokines), can also be used in nanoparticle functionalization. However, their poor stability, high vulnerability to intracellular proteases, and challenges in achieving optimal orientation for targeting hinder their transition from experimental stages to clinical application. Here, we explore various delivery strategies that demonstrate the effective transport of therapeutic nucleic acids to leukocytes using tLNPs.

4.1. Antibodies and antibody fragments

Antibodies and antibody fragments are part of the immunoglobulin family and are key protein components of the adaptive immune system. Advancements in the field of antibody engineering and the outstanding success of the therapeutic antibody industry for the treatment of various diseases, including cancer and autoimmune disorders, have positioned antibodies as leading players in the field of nanomedicine [72,73]. The primary functional property of antibodies is their inherent binding specificity, which develops as the antibody matures. Therefore, antibodies and antibody fragments can be utilized as targeting moieties to improve the specificity of tLNPs.

Upon designing antibody-targeted LNPs (Ab-tLNPs), the binding efficiency, specificity, and ability to induce internalization need to be validated [146]. These features may change significantly between different antibodies or antibody fragments, even if they bind to the same target, due to changes in binding affinity and avidity. Antibodies can be incorporated into LNPs using chemical and biological methods of bioconjugation, each with distinct advantages and limitations (see section 3). The most used chemical bioconjugation method for antibodies is a thiol-maleimide reaction, however, biological bioconjugation methods have also been used. Kedmi R. and Veiga N. et al. demonstrated that ASSET-tLNPs coated with different antibodies bind to their target immune cells in a highly specific manner. Using this platform, the authors successfully deliver siRNA-PLK1 (polo-lke kinase 1) to Mantle cell lymphoma (MCL) cells, and siRNA-IRF8 (interferon regulatory factor 8), siRNA-TNFα (tumor necrosis factor alpha), or mRNA-IL-10 (interleukin 10) to Ly6C-expressing cells, in DSS colitis mouse model, and improve therapeutic outcomes [14].

Ab-tLNPs can be used for targeting lymphocytes, which are notoriously hard to transfect and are of great interest for many therapeutic applications. For instance, T helper cells are essential players in immune responses during inflammation, cancer, and infectious diseases [57,58,101]. Targeted LNPs coated with an anti-CD4 antibody were independently developed by several research groups to target CD4-expressing T cells while avoiding CD8-expressing T cells or non-T lymphocytes [147]. Another example of harnessing Ab-tLNPs to transfect lymphocytes is for the treatment of B-cell malignancies [18,142]. Employment of an anti-CD38 antibody to tLNPs (αCD38-tLNPs) containing siRNA, significantly improved their biodistribution to malignant B cells residing inside the BM of tumor-bearing mice, compared to the isotype control-tLNPs (isotLNPs). Weinstein S. et al. demonstrated a 6-fold increase in targeting MCL cells using αCD38-tLNPs, and significantly improved therapeutic outcome upon encapsulation of siRNA to silence the expression of cyclin D1. Tarab-Ravski D. et al. showed a 3-fold increase in targeting BM-residing multiple myeloma cells, and upon encapsulation of αCD38-tLNPs with siRNA against cytoskeleton-associated protein 5 (CKAP5) considerably improved disease burden.

Many therapeutic approaches for treating leukocyte-related conditions involve ex vivo manipulation, which may include complex, expensive, and time-consuming processes [17,64]. CAR T cell therapy and the gene editing of hematopoietic stem cells (HSCs) are examples for this approach. The ability to specifically transfect T lymphocytes and HSCs in vivo can potentially simplify and expedite these therapeutic modalities, as it will allow for the adaptation of current ex vivo strategies to in vivo settings. Rurik J.G. et al. demonstrated that αCD5-tLNPs, encapsulated with mRNA encoding the chimeric antigen receptor, can be used to transiently express receptors that recognize fibrotic cells in the heart [148,149]. This approach enables the creation of therapeutic CAR T cells entirely in vivo, presenting a promising strategy for treating cardiac fibrosis. Billingsley M.M. et al. further evaluated different targets expressed on the surface of circulating T cells to enhance CAR mRNA expression mediated by tLNPs in vivo [68]. This study showed that among CD5, CD7, and CD3 as T lymphocyte targets, αCD3-tLNPs were most efficient in CAR and the induction of IL-6, GM-CSF, and TNF-α expression. Additionally, Zhou J.E. et al. generated αCD3-tLNPs for in vivo production of CAR T cells against CD19-expressing cells, a highly expressed target in B-cell malignancies [141].

Recently, αCD117-tLNPs encapsulated with Cre recombinase protein mRNA were generated to target hematopoietic stem cells (HSCs) in vivo in tdTomato mouse model. These authors demonstrated approximately 95 % transfection efficiency of the BM-HSCs and hematopoietic stem and progenitor cells (HSPCs) [67]. Moreover, this led to the expression of tdTomato across all the blood cell lineages derived from the transfected cells, thereby providing evidence of successful in vivo editing of HSCs.

Lastly, antibody fragments can be used as well as targeting moieties for LNPs. Coating LNPs with antibody fragments instead of full IgGs may result in smaller LNPs, which can provide better tissue penetration. Additionally, from an antibody engineering perspective, the manufacturing process of certain antibody fragments, such as scFv and Fab, is simpler than of full IgG [72,73].

While removing the Fc region of antibodies can prevent their rapid uptake by circulating Fc receptors-expressing cells [32], adding an Fc region to small targeting moieties as a fusion protein can be used as an advantage. The use of Fc-fusion proteins as targeting moieties of LNPs provides relatively easy chemical conjugations that are usually applied for antibodies. For example, Dammes N. et al. generated a conformation-sensitive recombinant Fc-fusion protein as a targeting moiety and chemically conjugated it by thiol-maleimide reaction to LNPs [150]. These tLNPs were used to target gut-homing activated leukocytes in a colitis mouse model showing improvement in their therapeutic outcome.

4.2. Peptides

Peptides are short chains of amino acids that can be created through experimental methods or computer simulations (in silico methods) [59].

The use of in silico approaches is becoming more common for discovering new peptides or enhancing the properties of existing ones identified by other methods. This approach helps to speed up the experimental process and refine the selection of peptides. Currently, numerous peptide sequences have been identified for various targets, and different chemical modifications, including peptide cyclization, are utilized to enhance their selection, binding affinity, stability, and specificity.

Decorating LNPs with external peptides is a common technique to confer or increase targeting capacities. Effective strategies involved using peptides that specifically bind to membrane proteins or receptors uniquely expressed on the target cells. For instance, an elegant study by Herrera-Barrera M. et al. recently showed an in vivo phage display, biopanning, screen for peptides specifically bind to the retinal photoreceptors [151]. The selected peptides were conjugated to LNPs by thiol-maleimide reaction or EDC NHS coupling. Intravitreally injection of peptide-conjugated LNPs delivered mRNA to the neural retina photoreceptors in mice and nonhuman primate eyes. Peptides designed as LNPs targeting moieties not only need to be highly specific for the target cells but also to mediate the internalization of the nanoparticle into the cell cytoplasm and the release of its nucleic acid cargo. Likewise, peptide-decorating LNPs mediate mRNA expression or siRNA silencing in various cell types [144].

Cell-penetrating peptides (CPPs) are short peptides that can traverse cell membranes and facilitate the transport of various bioactive substances, including nucleic acids, into cells [144,152,153]. When combined with LNPs composed of pH-responsive ionizable lipids, CPPs can enable efficient cellular entry followed by cargo release in response to the acidic environment of the endosome. However, CPP-conjugated nanoparticles tend to enter multiple cell types through a non-specific mechanism, without requiring specific binding to a ligand−receptor. As a result, they are primarily utilized for local or intratumoral administration [154]. These characteristics are likely to limit their application in mediating specific lymphocyte transfection.

Recently, Su F.Y. et al. demonstrated in vivo mRNA delivery to specific clones of CD8 T cells using peptide-MHCI-presenting mRNA-LNPs [154]. They developed MHCI molecules refolded with photocleavable peptides to enable rapid peptide exchange. These molecules were site-specifically conjugated with a lipid tail for postinsertion into preformed mRNA lipid nanoparticles. In mice infected with PR8 influenza, multiplexed delivery of UV-exchanged antigen-presenting nanoparticles against three immunodominant epitopes resulted in approximately 50 % transfection of a VHH mRNA reporter in cognate antigen-specific CD8+ T cells. This platform allows the presentation of variable peptide-antigens on MHCI-LNPs and delivers mRNA cargo specifically to distinct populations of antigen-specific T cells.

4.3. Aptamers

Aptamers are short, single-stranded DNA or RNA (ssDNA or ssRNA) oligonucleotides capable of selectively binding proteins or other cellular targets with high affinity [70]. Their ability to form helices and single-stranded loops contributes to a diverse array of secondary and tertiary structures. Generally, aptamers are more stable than antibodies and have a longer shelf life. The binding affinity of constrained aptamers can be as much as 1000 times higher than the free peptide [155]. They are produced through a simple and inexpensive process, and the time required to generate aptamers is comparatively short. Since their discovery, aptamers have attracted considerable interest in medical applications. They are used for the targeted delivery of nucleic acids to CD4+ and CD8+ T-cells, in aptamer-conjugated systems, where aptamers are directly conjugated to the RNA molecules and deliver them [156], and in aptamer-nanoparticle systems, where nanoparticles function together with aptamers as targeted drug delivery [157]. For instance, the decoration of LNPs encapsulating siRNA by the CH6 aptamer improved their in vivo delivery to bone osteoclasts. Thiol-modified CH6 aptamer was pre-conjugated to DSPE-PEG2000-Maleimide in the form of micelles. Then, the aptamer-PEG200-DSPE micelles were post-inserted into the surface of the LNPs. A systemic administration of CH6 aptamer-targeted-LNPs encapsulating with osteogenic pleckstrin homology domain-containing family O member 1 (Plekho1) siRNA boosted in vivo silencing of Plekho1 gene in osteoclasts. Consequently, this treatment promoted bone formation, improved bone microarchitecture, increased bone mass, and enhanced mechanical properties in both osteopenic and healthy rodents [143].

4.4. Other ligands

4.4.1. Mannose ligand

The mannose receptor (MR) is part of the C-type lectin family and can bind and internalize various endogenous and pathogen-associated ligands, including the monosaccharide mannose [143]. The MR is predominantly expressed by certain macrophage subpopulations, immature dendritic cells, and endothelial cells. Due to these characteristics, mannose is often used as a ligand to coat nanoparticles and mediate the targeting of tissue-residing macrophages. LNPs decorated by covalently attached mannose to the PEG-lipid component and loaded by High mobility group box 1 (HMGB1)-siRNA were designed to target liver macrophages [158]. A one-step assembly of mannose-tLNPs was performed using DSPE-PEG-Mannose. The authors have shown that mannose-tLNPs-siRNA-HMGB1 were capable of targeting macrophages in vitro and in vivo through the mediation of mannose receptors. As a result, they induced silencing of the HMGB1 gene, reduced the release of inflammatory factors, improved liver function, and decreased steatosis of mice suffering from non-alcoholic steatohepatitis disease. Furthermore, lipid nanoparticles coated with hyaluronic acid (HA) and mannose were used as dual-targeting for lung tumor cells and inflammatory macrophages, respectively. Inhalation of the mannose-HA-coated-mRNA-LNPs efficiently expressed the mRNA-coded protein in the lung tissues [115].

4.4.2. Transferrin ligand

Transferrin (Tf) is a blood plasma glycoprotein that plays a central role in iron metabolism and transports iron throughout the blood to various tissues, such as the liver, spleen, and bone marrow [159]. The expression of the transferrin receptor is upregulated on the surface of CD4 T-cells during T-cell activation. Jürgens D.C. et al. improved the ex vivo transfection of primary CD4 cells by introducing a transferrin ligand on the surface of LNPs via SPACC chemical conjugation of Tf to PEGylated lipids [160]. While this study opens the way for future research aimed at optimizing tLNPs for the treatment of T-cell-mediated diseases, Tf receptor is ubiquitously expressed on other cells and tissues and has not been used in vivo yet.

5. Bridging the gap from research to industry and clinic

Advancing genetic medicines delivered by tLNPs to clinical application involves many considerations. Despite years of extensive research and a wealth of published manuscripts, the number of clinical trials is strikingly low. The impasse that exists between scientific research and the practical application of tLNPs reveals fundamental unresolved challenges [145]. Comprehensively addressing all parameters for constructing tLNPs, along with potential scale-up issues that may arise from the different RNA or DNA technologies, is necessary for the successful development of these therapeutic modalities.

The pharmaceutical development of tLNPs is far more complicated than for passive LNPs, owing to their advanced critical chemistry, manufacturing, and control (CMC) aspects [161]. An additional targeting moiety and linker for bioconjugation increases the complexity level of this delivery strategy and the number of entities to regulate. So far, decreasing batch-to-batch variations that result from non-homogenous bioconjugation of targeting moieties remains a major obstacle for their clinical translation. Improving conjugation orientation strategies,

optimizing targeting moiety density, and a deep biological understanding of the chosen target, are important steps on the way of progressing tLNPs to clinical trials.

In particular, producing tLNPs under GMP conditions, scale-up manufacturing, and selection of appropriate analytical methods for their characterization is challenging., and these challenges progressively pile up as development progresses towards later clinical stages and licensing. Therefore, the specific requirements which are associated with pharmaceutical development of tLNPs should best be considered prior to their design [114,162]. One key limiting factor for GMP manufacturing of tLNPs lies in of the lack of appropriate analytical and functional assays. It will be necessary, for instance, to determine the lateral density, distribution, orientation and functionality of the targeting moiety at the surface of tLNPs. As well, fractions of free ligands and unsaturated coupling moieties, or (unintended) release of the liganded moieties can be relevant. So far, no generally accepted panel of controls for these items is available, and for many parameters, even the assays are still to be developed. This becomes even more difficult because the molar fractions of these moieties is typically very low, and reach the limit of quantification/detection of potentially eligible assays. Establishing appropriate analytical methods can be time-consuming and resource-intensive, but it is a critical step, and as written, should best be done as early as possible, as a prerequisite for entering into GMP production [14]. Furthermore, although the laborious production and purification of tLNPs are not the limiting factors in advancing them from research to patient care, they certainly require a great deal. Lastly, LNPs are incredibly sensitive to changes during production and even slight modifications can alter their physiochemical properties, ultimately affecting their immunogenicity, interaction with protein corona, therapeutic effectiveness, and organ distribution [163]. Given these considerations, tLNPs must undergo extensive quality control, are more expensive to produce, and their evaluation and manufacturing take longer. To ensure all the necessary data for the regulatory agencies and a robust and uniform production of tLNPs are obtained, Pharmaceutical Quality by Design (QbD) is a recommended concept to adopt for managing all tLNPs properties and successfully translating genetic medicines for leukocytes to the clinic [6,16,74,77].

Beyond the challenges mentioned above, additional limitations, inherent to nanomedicines, further impede the clinical translation of tLNPs. The need for better animal models, high cost of personalized medicine, and challenges in encapsulation of large DNA or RNA molecules often employed for gene-based therapeutics, such as CAR construct or Cas9 nuclease protein, are all limiting obstacles [164]. So far, systemically administered LNP-based genetic medicines FDA-approved or under clinical evaluation, including those containing siRNA, mRNA, and CRISPR/Cas9 components, are all targeted towards liver diseases [10,165]. Furthermore, clinically approved and evaluated genetic medicines in leukocyte-related applications have utilized so far only passively targeted LNPs. These include intramuscularly delivered antiviral mRNA-LNP vaccines and intratumorally or intravenously delivered anti-cancer mRNA-LNP vaccines [26].

To advance these promising therapies for leukocytes beyond the liver and with systemic administration, more accurate and specific tLNPs will be essential. Nevertheless, as good as systemically administered tLNPs may be, it is impossible to expect a perfect delivery and possible off-target effects should always be considered. A possibility for increasing specificity and decreasing off-target effects employs trigger-responsive elements on the tLNPs so their cargo release would be activated by an external or endogenous signal [25,166]. Such trigger-activated delivery systems are usually employed for liposomes or polyplexes but haven't been used for generation of tLNPs yet.

Lastly, the clinical implementation of tLNPs will not be complete without addressing the limitations of genetic medicine technologies for leukocytes, such as protein replacement therapy, CAR technology, gene editing, and DNA writing. While mRNA has been successfully translated into the clinic by mRNA-LNPs vaccines, the immense potential of protein replacement therapy for the treatment of genetic diseases and cancer is still under clinical evaluation [156]. It is difficult to say what will be the effectiveness of these therapeutic modalities upon repeated dosing to induce a long-term expression or upon incorporation of self-replicating elements. Nevertheless, these remain unresolved issues that need to be closely evaluated over time. As for CAR technology, clinically approved CAR T cell therapy has demonstrated its tremendous therapeutic effects for treatment of hematological malignancies [167]. However, applying it to solid tumors is troublesome due to the low infiltration of T cells and the complexity of the extracellular matrix. Furthermore, an immunosuppressive tumor microenvironment might limit their therapeutic effects and treatment application. Advancements in CAR T cell therapies and promising data from CAR NK and CAR macrophage clinical trials mitigate some of these limitations [8]. However, further research is necessary to realize their full potential. Finally, harnessing tLNPs for gene editing with the CRISPR/Cas9 system and DNA writing technologies with base and prime editors is an appealing goal [168,169]. Yielding these powerful therapies for clinical use poses some notable challenges before translating into the clinic. To ensure the safety and efficacy of CRISPR/Cas9 systems, minimizing off-target and on-target/off-site activities is crucial. The development of high-fidelity Cas9 variants, non-nuclease Cas9 variants, and conditional Cas9 expression strategies offer promising avenues to curtail these potentially harmful effects. Nevertheless, significant growth is still required for these approaches, alongside prime and base editing technologies, before clinical approval can be achieved.

Despite formidable challenges, the promise of tLNPs for leukocyte-based genetic medicine remains a towering summit in the landscape of nanomedicine. By expanding our biological understanding, utilizing accurate analytics tools, and fostering fruitful collaborations, we can pave the way for the clinical application of these exceptional therapies in the near future.

6. Future Outlook

Much like winning a chess game, employing tLNPs for leukocyte-related conditions requires careful planning and strategizing. We need to anticipate potential challenges and consider the distinct requirements for each treatment application. By meticulously tailoring the tLNPs to an adequate target with a suitable bioconjugation strategy, targeting moiety, and optimal LNP formulation, we can ensure the successful application of genetic medicines to the clinic.

Building on the promise of past and present successes of genetic medicines, significant limitations remain to be overcome. Bridging the considerable industrial and technological gaps should go hand in hand, as neither can succeed without the other. Developing better preclinical models and analytical tools to resolve scalability and GMP production issues are necessary steps. Furthermore, minimizing the academia-to-industry gap by encouraging multidisciplinary research, collaborations, and adoption of standardization protocols can further accelerate the transition of novel discoveries from bench to bedside. Acceleration of delivery by tLNPs for genetic medicines to target leukocytes may also be achieved by presenting incentives by regulatory agencies or investors due to their long and costly development and production process. We anticipate the ongoing research and the ever-increasing capabilities of tLNPs will usher in a future where genetic medicines are closer than ever to becoming a reality.

CRediT authorship contribution statement

Dana Tarab-Ravski: Writing – review & editing, Writing – original draft. **Lior Stotsky-Oterin:** Writing – review & editing, Writing – original draft. **Aviad Elisha:** Writing – review & editing, Writing – original draft. **Govinda Reddy Kundoor:** Methodology, Resources. **Srinivas Ramishetti:** Writing – review & editing. **Inbal Hazan-Halevy:** Writing – review & editing, Writing – original draft, Supervision. **Heinrich**

Haas: Writing – review & editing. **Dan Peer:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

D.P. receives licensing fees (to patents on which he was an inventor) from, invested in, consults (or on scientific advisory boards or boards of directors) or Founder and hold shares or conducts sponsored research at TAU for the following entities: ART Biosciences, BioNTech SE, Earli Inc., Geneditor Biologics Inc., Kernal Biologics, Merck KGaA, Newphase Ltd., NeoVac Ltd., RiboX Therapeutics, Roche, SirTLabs Corporation, Teva Pharmaceuticals Inc.

Srinivas Ramishetti and Heinrich Haas are employees of NeoVac Ltd. and hold shares in the company. All other authors declare no competing financial interests.

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Data availability

Data will be made available on request.

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