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Delivery strategies of RNA therapeutics to leukocytes

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ABSTRACT

Harnessing RNA-based therapeutics for cancer, inflammation, and viral diseases is hindered by poor delivery of therapeutic RNA molecules. Targeting leukocytes to treat these conditions holds great promise, as they are key participants in their initiation, drug response, and treatment. The various extra- and intra-cellular obstacles that impediment the clinical implementation of therapeutic RNA can be overcome by utilizing drug delivery systems. However, delivery of therapeutic RNA to leukocytes poses an even greater challenge as these cells are difficult to reach and transfect upon systemic administration. This review briefly describes the existing successful delivery strategies that efficiently target leukocytes *in vivo* and discuss their potential clinical applicability.

1. Current landscape of RNA delivery to leukocytes

Since Fire et al. first demonstrated the ability of dsRNA to manipulate the genetic expression of a particular gene in *C. Elegans* [1], the field of RNA therapy has progressed tremendously and raised the attention of both academia and the industry. In only four years, the US Food and Drug Administration (FDA) -approved drugs based on RNA therapy proven their effectiveness and relevance in both personalized medicine [2] and public health concerns [3,4]. The immense potential of RNA therapy stems from its versatility. While small interference RNA (siRNA) and micro-RNA (miRNA) can be utilized to inhibit the production of any disease-related proteins, messenger RNA (mRNA) can be used for protein expression [5,6]. The promise of RNA-based therapeutics intensifies when considering the therapeutic potential of this field to target "undruggable" proteins [7]. Moreover, the development of novel RNA molecules such as small-activating RNA (saRNA) [8] and CRISPR/Cas9 components [9], further expands the capabilities of this field.

Despite the potential of RNA-based therapeutics, there are still many limitations to overcome [10,11]. Chemical modifications advanced this field greatly as they reduce the immunogenicity and increase the stability of RNA molecules [12]. Yet, they are not enough to protect RNA molecules upon systemic delivery or facilitate their intracellular delivery. Other impediments in their clinical translation include avoiding renal exertion and the mononuclear phagocyte system (MPS), reaching the target tissue, crossing the cell membrane, and evading from the endosome to the cytoplasm ('endosomal escape'). This process is particularly challenging and considered to be the most significant bottleneck in delivering therapeutic RNA [13–15]. These significant obstacles have made drug delivery systems the key to the success of RNA-based therapeutics.

Delivery systems focus not only on protecting the RNA payload from exertion and degradation but most importantly on facilitating their specific delivery to the target tissues and across the cell membrane. To date, delivery of therapeutic RNA to the liver is successfully managed by various delivery systems [2,16], but reaching extrahepatic tissues remains a challenge [17–19].

Leukocytes pose an alluring target for genetic manipulation owing to their high involvement in cancer, inflammation, autoimmune responses, and viral infections [20,21]. Targeting leukocytes is a daunting task due to their dispersity throughout the body, their inflammatory responses to transfection with RNA cargo, and their variety of RNA-sensing receptors [22]. Moreover, lymphocytes, which constitute an integral part of the immune system, are considered notoriously hard to transfect *in vivo* [13,15]. Overcoming the barriers of RNA delivery to leukocytes requires intense research and further development of novel delivery strategies.

Herein, we will summarize the advantages and disadvantages of the

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distinct therapeutic RNA molecules (Fig. 1) and critically discuss the existing delivery strategies to leukocytes (summarized in Table 1). We will focus on achievements that demonstrate *in vivo* efficacy for cancer therapy, inflammatory-related diseases, viral infections, and autoimmune responses, and emphasize their clinical feasibility. Despite the advanced progress in the approval and evaluation of RNA-based approaches for vaccination and chimeric-antigen receptor T cell therapy (CAR-T), we will not cover those fields in this review since these topics are wildly covered elsewhere.

2. RNA therapeutics

Over the years, RNA-based therapeutics have been studied to treat various diseases and conditions, including cancer and inflammatory diseases. RNA molecules can modify the genetic expression of cells in a sequence-specific manner, and their design and production today are relatively fast and simple due to the use of in-vitro transcription (IVT) and chemical synthesis, making them an alluring therapeutic approach. Moreover, a single RNA molecule can alter the expression of several proteins or generate many copies of a protein, as opposed to small molecule drugs. These advantageous attributes can be used to generate quick and efficient treatments for personalized medicine, as well as quickly-evolving viral infections [23]. Choosing an appropriate therapeutic RNA depends on a variety of factors, such as the purpose of the treatment, the disease we wish to eradicate, the target cells, and funding, resources, and financial concerns.

2.1. Antisense oligonucleotides

Antisense oligonucleotides (ASOs) are short, synthetic, single-stranded oligonucleotides that can be either RNA or DNA-based [24].

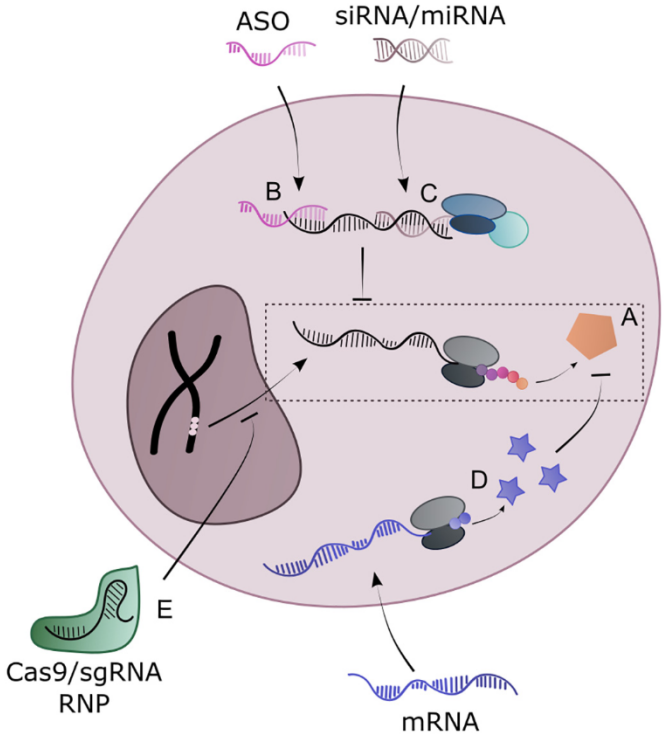

Fig. 1. Overview of therapeutic RNA molecules and their mechanism of action. A) Translation of a pathogenic protein. B) ASOs hybridize with the target mRNA, while the C) siRNA/miRNA utilize the RISC complex to inhibit translation of target mRNA. D) Expression of a therapeutic protein to inhibit the function of the pathogenic protein by delivering the mRNA of the therapeutic protein. E) Complete gene knockout of the pathogenic protein using Cas9 and sgRNA ribonucleoprotein (RNP) complexes.

These sequences are complementary to their target mRNA and can thus hybridize and modulate protein expression (Fig. 1B). In the last decade, many ASOs are have gone through clinical evaluation, and several were FDA-approved ASOs for different disorders [25,26]. However, so far, the successful *in vivo* delivery of ASOs to leukocytes, has not yet been accomplished.

2.2. Small interference RNA and micro-RNA

Since its discovery in mammalian cells, RNA interference (RNAi) has become an important tool in understanding gene function and expression in many cell types [27]. RNAi is a conserved cellular mechanism that induces post-transcriptional suppression of gene expression by blocking or inhibiting translation of mRNA in a sequence-specific manner [10,11,18] (Fig. 1C). The siRNA and miRNA can be recycled in after blocking protein production, and thus result in highly efficient gene silencing [28–31]. The potentially unlimited ability of RNAi to silence any gene can be used to target proteins that are considered undruggable and are commonly expressed cancer and inflammatory conditions, such as translocated, overexpressed, and mutated genes [17–19,32–35]. Moreover, many disease are characterized by an altered expression profile of miRNAs [36]. In case of downregulation of a certain miRNA, miRNA mimics can be designed to as replacement therapy. On the other hand, an abnormal expression of miRNA can be treated with miRNA antagonists (anti-miRs) that bind to their complementary target miRNA and lead to its inhibition [36,37]. Therefore, RNAi-based therapeutics may be utilized as the future of targeted therapeutic approach for cancer and inflammation.

The locked nucleic acid (LNA) anti-miR cobomarsen (MRG-106), that targets the oncogenic miR-155, was evaluated in a phase I clinical trial (NCT02580552) in 2016 for lymphoma and leukemia patients [38,39]. The preclinical data revealed cobomarsen improved the cutaneous lesions without any observable adverse effects and therefore, a year later, the FDA and the European Medicines Agency (EMA) granted Orphan Drug Designation to cobomarsen for the treatment of mycosis fungoides (MF), the most common form of cutaneous T-cell lymphoma (CTCL). In 2018, a phase II clinical trial (NCT03713320) was continued to evaluate the efficacy of cobomarsen for the treatment of MF [39]. Although the study was terminated in December 2020 due to business reasons, it encouraged the development of miRNAs as cancer therapy, and cobomarsen was again granted Orphan Drug Designation for the treatment of T-cell lymphoma.

2.3. Messenger RNA

mRNA-based therapeutics have emerged as powerful and promising alternatives to DNA-based methods for different therapeutic purposes, such as protein replacement therapy, vaccines, and cellular reprogramming [40–46]. Like RNAi, mRNA-based therapeutics rely on supplementation of mRNA molecules that, upon successful delivery, utilizes the cellular machinery for the expression of a specific protein (Fig. 1D). This approach is highly favorable, as the mRNA degrades quickly, thus reducing the risk of mutagenesis and potential transient effects from long-lasting expression or genomic integration. However, the application of mRNA-based therapy has been hindered by several barriers, such as problematic large-scale synthesis of mRNA molecules, *in vivo* instability, and potential immunogenicity. Also, the regulation on mRNA expression is more difficult than that of DNA expression, in terms of expression levels, location, and timing. Hence, mRNA expression at excessive levels or at off-target sites can lead to unwanted protein expression and potential toxicity. This has been shown previously for factor VIII, factor IX, and interleukin-12 [47]. Recent insights into mRNA structure and function, alongside the improvements in IVT technology, sophisticated regulation systems for mRNA expression, and the development of modified nucleotides, advanced mRNA dramatically for clinical use [40,48,49].

Table 1
RNA delivery systems available for leukocytes.

	RNA delivery vehicle			In-vivo experiments		
	Drug	Vehicle	RNA content	Targeting moiety	Target cells	Ref
Peptide, protein or antibody conjugates	Protamine-scFv fusion protein	Antibody-siRNA conjugate	Ku70	anti-LFA1 mAB	Activated leukocyte population	[55]
	scFvCD7-9R	Antibody-siRNA conjugate	siRNA CD4, CCR5, Tat and Vif	anti-CD7 mAB	HIV infected T cells	[56]
	RVG-9R	Peptide-siRNA conjugate	siRNA TNF-a	RVG-9dR	Activated leukocyte population	[57]
	CpG-siSTAT3	CpG-siRNA conjugate	siRNA (STAT3)	CpG	TLR9-expressing cells	[59,64,65]
Aptamer conjugates	Ch A-1	Aptamer-siRNA conjugate	siRNA Tat/Rev	Anti-gp120 aptamer	HIV infected CD4 T cells	[69]
	CD4-AsiCs	Aptamer-siRNA conjugate	siRNA Tat/Rev. and CCR5	Anti-CD4 aptamer	HIV infected CD4 T cells	[70]
	Aptamer-stick-cocktailed DsiRNA	Aptamer-siRNA conjugate	siRNA Tat/Rev., CD4, and TNPO3	Anti-gp120 aptamer	HIV infected CD4 T cells	[71]
	4-1BB-AsiCs	Aptamer-siRNA conjugate	siRNA mTOR complex 1	Anti- 4-1BB aptamer	CD8 T cells	[73]
	CTLA4-AsiCs	Aptamer-siRNA conjugate	siRNA STAT3	Anti-CTLA4 aptamer	CD8 T cells and Tregs	[74]
	CD40Apt-SMG1-shRNA	Aptamer-shRNA conjugate	shRNA SMG1	Anti CD40 aptamer	Malignany B lymphocytes	[126]
	4-1BB apt-CD25 siRNA	Aptamer-siRNA conjugate	siRNA CD25, Axin1	Anti-4-1BB aptamer	CD8 T cells	[75]
	A-1-stick-LTR-362 27-mer siRNA	Aptamer-siRNA conjugate	DsiRNA LTR-362	Anti-gp120 aptamer	HIV infected CD4 T cells	[72]
Polymer-based Nanoparticles without a targeting moiety	GeRP nanoparticles	Glucan/PEI-based nanoparticles	siRNA TNF-α, Map4k4	None	Macrophages	[94]
	Endo-Porter GeRP nanoparticles	Glucan/PEI-based nanoparticles with Endo-Porter peptide	siRNA Map4k4, CD45	None	Macrophages	[127]
	BG34-10-Re-I/siRNA	Glucan-based nanoparticles	siRNA MIF	None	Macrophages	[96]
	GP-EP14	Glucan/PEI-based nanoparticles with peptide modifications	siRNA F4/80, osteopontin	None	Peritoneal macrophages	[95]
	β1,3-d-glucan-encapsulated siRNA particle (GeRP)	Glucan-based nanoparticles	siRNA S1PR2	None	Macrophages	[97]
	Cationic PAMAM Dendrimer-DsiRNA	Triethanolamine-core PAMAM dendrimer	siRNA tat/rev, CD4, and TNPO3	None	HIV infected CD4 T cells	[83]
	ANTP-NP anti miR-155	PLGA-based nanoparticles	Anti-miR (miR155)	Penetratin cell-penetrating peptide	Malignany B lymphocytes	[84]
	SNSO1-T	PEI-based nanoparticles	siRNA eIF5A and plasmid NH mutant of eIF5A	None	Malignany B lymphocytes	[85,86]
	miR34a nanoplexes	Chitosan/PLGA-based nanoparticles	miR-34a	None	Malignany B lymphocytes	[87]
	miR155-loaded sPEG/GLC	Galactose polypeptide with sheddable PEG copolymer	miR-115	None	Tumor associated macrophages	[88]
	siRNA/PEI-αLA	Linoleic acid/PEI-based nanoparticles	siRNA BCL-ABL	None	Malignany CML cells	[89]
Polymer-based Nanoparticles with a targeting moiety	RONDEL™ system	Cyclodextrin polymer-based system with transferrin	siRNA Bim, Puma	Transferrin	CD4 T lymphocytes and B lymphocytes	[93]
	Tf-PEI polyplex	PEI-based nanoparticles with tranferrin	siRNA conjuated to A647	Tranferrin	Activated T cells	[92]
	S2P-conjugated siRNA NPs	Cationic lipid-like G0-C14/PLGA with maleimide-based conjugation of S2P peptide	siRNA CaMKIIγ	S2P peptide	Macrophages	[90]
	PbAE/PGA-anti-CD8 nanoparticles	PbAE/PGA-based nanoparticles incorporated with mAb	mRNA CAR, TCR	Anti-CD8 mAb	T lymphocytes	[98]
Liposomes with a targeting moiety	I-tsNPs	HA-covered liposomes conjugated to mAb	siRNA CyclinD1, CCR5	Anti-integrin β7 mAb, anti-LFA1 mAb	Leukocytes	[31,100]
	M2NP-siCD115	Liposomes incorporarated with fusion peptide	siRNA CFS-1R	α-peptide and M2pep fusion peptide (α-M2pep)	Tumor associated macrophages	[102]
	CaP/miR@pMNPs	Lipid-coated calcium phosphonate nanoparticles conjugated to mannose	miR-115	Mannose	Tumor associated macrophages	[105]
	siRNA/HMG/OR micelles	Oligoarginine micelles incorporated with HMG peptide	siRNA Chil3, Chil4	HMG peptide	Macrophages	[103]
	ST-AS&Si	mPEG-phe-DBCO-based micelleplex	siRNA IKKβ	M2 peptide	M2 macrophages	[104]
	siCCR2-LNP	C12-200-based LNPs	siRNA CCR2	None		[106]

(*continued on next page*)

Table 1 (*continued*)

	RNA delivery vehicle			In-vivo experiments		
	Drug	Vehicle	RNA content	Targeting moiety	Target cells	Ref
Lipid-based Nanoparticles without a targeting moiety	siCCR2-LNP	C12-200 and KC2-based LNPs	siRNA CD45, CD11b, TNFa	None	Inflammatory monocytes Myeloid cells in non-human primates	[107]
	Lipid emulsion-formulated miR-34a	Neutral lipid emulsion	Synthetic miR-34a	None	Malignany B lymphocytes	[111]
	SNALPs miR-34a	DODAP-based LNPs	Synthetic miR-34a	None	Malignany B lymphocytes	[112]
	OF-Deg-Lin mRNA LNPs	OF-Deg-Lin-based LNPs	Fluc mRNA	None	Splenic B lymphocytes	[116]
	LNP/CD45 si	Novel cationic lipid-based LNPs	siRNA CD45	None	Macrophages and dendritic cells	[109]
	LNP-BCR-ABL siRNA	MC3-based LNPs	siRNA BCR-ABL	None	Malignany CML cells	[108]
	CLANmCas9/gCD40	PEG-b-PLGA cationic lipid-assisted nanoparticles	mRNA Cas9, gRNA CD40	None	Dendritic cells	[114]
	Constrained LNPs	LNP library	siRNA GFP-based barcoding and guide RNA	None	T cells	[110]
	SORT LNPs	A variety of LNPs	mRNA Luc, IL-10, hEPO, mKL ECD, Cre and Cas9/sgRNA, and Cas9/sgRNA RNPs	None	B and T lymphocytes	[115]
	miR-146a-loaded nanoparticles	Empty DOTAP/Linoleic acid LNPs complexed with PEI/mRNA polyplex	miR-146a	None	Alveolar macrophages	[113]
	anti CD4-tLNPs-siRNA	MC3-based LNPs with maleimide-based conjugation	siRNA CD45	Anti-CD4 mAb	CD4 T lymphocytes	[119]
Lipid-based Nanoparticles with a targeting moiety	anti CD38-LNPs-siRNA	MC3-based LNPs with maleimide-based conjugation	siRNA CyclinD1	Anti-CD38 mAb	Malignany B lymphocytes	[33]
	CD38-NP-miRs	DOPE/Linoleic acid-based LNPs with meleimide-based conjugation	miR-26a, miR-130a, anti-miR-155	Anti-CD38 mAb	Malignany B lymphocytes	[120]
	ASSEt-tLNPs	MC3-based LNPs with ASSET targeting platform	siRNA PLK1, IRF8 and mRNA IL-10	Anti-CD29 mAb, anti-Ly6C mAb	Malignany B lymphocytes, inflammatory leukocytes	[32,34,122]
	2A2-miR29b-ILP	PEI/Linoleic acid LNPs with postinsertion-based addition of targeting moiety	Synthetic miR-29b	Anti-ROR1 mAb	Malignany B lymphocytes	[118]
	siIFN-γ-D1D2 LNPs	MC3-based LNPs with maleimide-based conjugation	siRNA IFN-γ, CD45	Anti α4β7 integrin conformation-senstivie fusion protein	Activated gut-homing leukocytes	[32]
Exosomes hybridyzed with polymers	MicroRNA-DC-Exosomes	MSCs-derived exosomes encapsulating miRNA, coated with PEI/PEG polyplex and aptamer/siRNA chimera	Various miRNA and siRNA mTOR	An aptamer specific for binding DCs	Dendritic cells	[124]
	EM-PLGA@Dnmt3aossmart silencer	PLGA nanoparticles encapsulating Dnmt3aossmart, coated with PEI polymer and M2-derived exosome membrane	Dnmt3aossmart silencer (siRNA)	None	M2 macrophages	[125]

2.4. gRNA for CRISPR-Cas9 genome editing

The development of CRISPR-Cas9 system has led to significant progress in the field of RNA therapeutics, bringing their development to the forefront. The CRISPR-Cas9 system was first discovered in bacteria and can selectively edit a target DNA sequence using a Cas nuclease protein and a gRNA molecule, complementary to the target sequence [50] (Fig. 1E). Since CRISPR-Cas9 technology was first applied in mammalian cells for genome editing in 2013 [51,52], this platform rapidly expanded its applications in gene expression modulation, including genomic sequence disruption, correction, alteration, and even epigenetic and transcriptional modifications. However, the translation of this promising technology into clinical settings remains hindered by the need of efficient and targeted delivery system [53].

3. Delivery of RNA therapeutics to leukocytes

Systemic delivery of naked RNA is considered inefficient due to the large molecular weight and negative charge of the phosphate backbone of RNA, thus requiring the administration of high doses to overcome the cellular barriers [5]. On the other hand, while local administration of naked therapeutic RNA has been successfully performed to tumor tissues, the eye, brain, and heart, this route of administration is not suitable for targeting leukocytes. As a result, developing drug delivery systems has become an integral part of RNA-based therapeutics and is especially necessary for the genetic manipulation of leukocytes. Conjugates and nanocarriers can change the pharmacodynamic and pharmacokinetic properties of the RNA and facilitate its delivery to the target cells.

The therapeutic efficacy of “passively” targeted nanocarrier-based delivery systems is based on the enhanced permeability and retention (EPR) effect and allows their accumulation in leaky organs and tumors with leaky vasculature [13]. Passive nanocarriers or naked RNA

molecules could be decorated with active targeting moieties such as monoclonal antibodies, peptides, aptamers, or carbohydrates to improve tissue localization and increase their targeting efficiency to specific cell types. The hardship of developing active targeting systems is added to the relatively complex scalability and laborious manufacturing process of those systems, which poses another significant regulatory challenge in translating them into the clinic. Below, we examine several delivery strategies that demonstrated the safe and successful delivery of therapeutic RNA to leukocytes.

3.1. Conjugates

Bioconjugates are biogenic molecules, covalently bound to the RNA, that can specifically bind to receptors on the surface of the target cells and were developed to deliver therapeutic RNA safely and efficiently. The attachment of an active targeting moiety to the RNA molecule can reduce renal clearance, improve their stability, direct the RNA specifically to the target cells, and facilitate its internalization. A recent successful example of an RNA conjugate used in the clinic is Givosiran, the first FDA-approved drug that uses an RNA conjugated to N-acetylgalactosamine (GalNAc) for delivering siRNA to hepatocytes [16]. However, reaching immune cells is a far more difficult task.

3.1.1. Peptides, proteins, and antibodies

Antibodies are an attractive choice as a targeting strategy since they are highly specific, have high affinity to their target, their structure is well defined, and they have a prolonged *in vivo* circulation time [54]. The first antibody-siRNA conjugate was a fusion protein of a single-chain variable region fragment (scFv) with the positive peptide protamine to bind the negatively charged siRNA, and demonstrated successful systemic targeting of immune cells in leukemia-bearing mice [55]. An additional fusion protein, consisting of another scFv and the nona-d-arginine (9R) peptide (scFvCD7-9R) was developed for systemic targeting of T lymphocytes in humanized mice challenged with HIV [56]. Another strategy utilized the 9R peptide for siRNA delivery and combined it with the rabies virus glycoprotein (RVG) for macrophage/microglia targeting in an LPS-induced neuroinflammation mouse model [57]. However, these fusion proteins platforms are less suitable for clinical use as they are expensive to manufacture, require laborious protein engineering methods, and were found to be highly toxic [58].

3.1.2. CpG-RNAi conjugates

Conjugation of siRNA to single-stranded unmethylated cytosine-phosphate-guanine (CpG) oligodeoxynucleotide, the natural ligand of Toll-like Receptors 9 (TLR9), can be utilized to deliver therapeutic RNA to dendritic cells, macrophages, and B-cells [59,60]. Since TLR9 activation initiates pro-inflammatory reactions, this conjugate can be utilized to treat multiple diseases, including cancer, autoimmune diseases, and allergy [61,62]. Kortylewski M. et al. was the first to demonstrate the delivery of siRNA using CpG-siRNA conjugate to tumor-associated myeloid and B-cells in several mouse models [59,60,63–65]. The CpG-siRNA triggered an antitumoral immunomodulation both by the therapeutic siRNA and through TLR9 activation. Their application for systemic administration is limited due to their short half-life, resulting in fast clearance and rapid serum degradation, unless the RNA is chemically modified [12]. However, because CpG-siRNA conjugate trigger and activate TLR9, they induce internalization and endosomal escape of the construct and bridge one of the major bottlenecks of the RNA-based therapeutics.

The clinical relevance of CpG oligodeoxynucleotides is evaluated for numerous applications. Recently, a new phase I clinical trial has started recruiting participants for the determination of optimal CpG-Stat3 siRNA dose and evaluation of the safety and feasibility of combined treatment with localized radiation therapy for the treatment of relapsed cases of non-Hodgkin's lymphoma B-cell malignancies (NCT04995536).

3.1.3. Aptamer-RNAi conjugates

Aptamers are short single-stranded RNA or DNA oligonucleotides that fold into defined architectures and can specifically bind a large variety of target molecules [66,67]. Aptamers are comparable to antibodies in terms of specificity and affinity but are smaller, more stable, and easier to generate [68]. The first aptamer-siRNA conjugates targeted gp120 (the HIV envelope glycoprotein) or CD4 [69–72] on HIV-1 infected primary T cells and were able to prevent HIV infection in mice. Aptamer-siRNA platform was also utilized for cancer immunotherapy applications in several studies [73–75], and overall these immune-aptamers managed to promote anti-tumoral immune responses, thus highlighting their potential as a new clinically feasible therapeutic platform for cancer immunotherapy. To date, several aptamers are evaluated for clinical use in oncology, inflammatory, and hematology indications, however, none of them are used in combination with RNAi or as a delivery strategy in general [76].

3.2. Nanocarrier-based

As opposed to conjugation-mediated delivery strategies, nanocarrier-based delivery strategies encapsulate the therapeutic RNA and protect it from renal clearance and degradation by nucleases [77]. Also, they don't require using complex protein engineering and purification methods. Furthermore, the nanocarriers can improve the stability, biodistribution, and therapeutic potential of the RNA payload, and allow co-encapsulation with small molecule drugs. More importantly, the nanocarriers can be easily modified in size, charge, shape, and be subjected to surface modification. The clinical potential of nanocarrier-based delivery for leukocyte-related diseases holds great promise, but still has many struggles to overcome.

3.2.1. Polymer-based

Polymer-based delivery for RNA therapeutics usually utilizes cationic polymers such as polyethylenimine (PEI) and chitosan due to their enormous chemical diversity and potential for functionalization. Polymers can be linear or branched, and can consist of many branched repeats such as in the case of dendrimers [78]. Their positive charge allows better encapsulation of the RNA molecules, as well as enhancement of endosomal release [79,80]. However, cationic polymers have been shown to induce cytotoxicity and unwanted immune responses [81]. Thus, alternative polymers are being developed as well as polymer-based nanoparticles (NPs) for drug delivery.

3.2.1.1. Polymer-based nanocarriers without a targeting ligand. Dendrimers are applicable for RNA delivery due to their tunable structure, uniformity, and effective dendrimer-nucleic acid condensation [82]. Zhou et el. Demonstrated the efficacy of a dendrimer-mediated DsiRNA delivery into HIV infected T-cells in humanized mice [83], and resulted in complete inhibition of HIV-1 titers. Other polymers were also used to deliver RNAi for cancer immunotherapy [84–89]. SNS01-T is the most advanced polymer-based delivery system that has reached clinical trials for the treatment of B-cell malignancies (NCT 01435720) after it demonstrated a significant inhibition of tumor growth in multiple animal models. Liu et al. was able to generate a redox/pH dual-responsive nanovectors and showed it could be a promising strategy to reprogram tumor-associated macrophages (TAMs) for cancer immunotherapy [88].

3.2.1.2. Polymer-based nanocarriers with a targeting ligand. Different targeting moieties have been used to direct polymer-based delivery of siRNA and mRNA into immune cells for cancer immunotherapy, inflammation, and atherosclerosis [90,91]. Existing targeted polymer-based nanocarriers demonstrated efficient delivery to monocytes and macrophages by utilizing transferrin, glucans, and mannose as targeting moieties in asthmatic, inflammation, and cancer mice models [92–97]. Moreover, Parayath et al. demonstrated successful systemic *in vivo*

delivery to T lymphocytes of chimeric antigen receptors (CAR) mRNA for transiently reprogramming of circulating T-cells to recognize disease-relevant antigens [98], and led to disease regression in mouse models of human leukemia and prostate cancer. The possible toxicity and problematic scalability of those systems hinder the clinical approval of them, yet the successful delivery and therapeutic effects they induce hold great promise for this delivery strategy.

3.2.2. Lipid-based nanocarriers

The first nanocarrier ever to reach the clinic was the passively targeted PEGylated liposomal doxorubicin (DOXIL™) in 1995 [99]. Since then, lipid-based nanocarriers received FDA approvals for the delivery of other small molecules, siRNA, and mRNA, and today are the lead delivery system in the clinic for RNA-based therapeutics [5,77]. They are biocompatible and biodegradable, and their production today is a robust, scalable, and cost-effective process.

3.2.2.1. Liposomes. Liposomal delivery systems were initially developed for small molecules and are spherical, self-closed structures composed of a lipid bilayer and an aqueous core, allowing the encapsulation of the drug payload inside.

The first efficient liposomal delivery of siRNA to leukocytes *in vivo* utilized integrin-targeted and stable liposome-based nanoparticles (I-tsNPs) that resulted in reversing the experimentally-induced colitis of mice [31]. The potential of this same delivery platform was also demonstrated in humanized mice challenged with HIV and successfully delivered siRNA to T lymphocytes and macrophages by simply switching the monoclonal antibody (mAb) that was conjugated to the tsNPs [100]. In the past years, other versatile actively targeted liposomal delivery systems decorated with mAbs [101], peptides [102–104], and mannose [105] were developed for the efficient delivery of siRNA and miRNA to macrophages and lymphocytes in tumor-bearing mice and asthma mice model. Despite the significant therapeutic efficiency those distinct targeted liposomes achieved *in vivo*, there is no liposomal delivery system for RNA therapy under evaluation for clinical use. Also, mRNA hasn't been used with liposomal delivery systems, probably due to its inefficient encapsulation within the liposome. We believe the difficulties in clinically translating those delivery systems are high batch-to-batch variations, the ineffective processes of conjugating targeting moieties, and the possible toxicity of those liposomes. Moreover, the lack of untargeted liposomal delivery systems to leukocytes indicates liposomes are ineffective in targeting leukocytes without a targeting moiety. Therefore, while actively targeted liposomes demonstrated effective delivery of therapeutic RNA to leukocytes in mice, the road to clinically applying those systems still bears many challenges.

3.2.2.2. Lipid nanoparticles. In 2018, Onpattro® was the first FDA-approved drug to utilize lipid nanoparticles (LNPs) for the efficient delivery of therapeutic siRNA to hepatocytes [2]. Today, LNPs are the most advanced delivery system for RNA therapy due to their high encapsulation efficiencies and low immunotoxicity. LNPs are formed by combining a mixture of lipids with the RNA payload, usually with a microfluidic mixing technology. Preparation methods of LNPs are favorable over the preparation of liposomes as they are robust, scalable, cost-effective, less laborious, and decrease batch-to-batch variations.

LNPs are composed of cationic lipids, cholesterol, neutral lipids, and polyethylene glycol-conjugated lipids (PEGylated) that together form a nanoparticle which encapsulates the RNA payload [17,77]. The generation of pH-sensitive ionizable cationic lipids enables potent encapsulation of therapeutic RNA in low pH due to electrostatic interactions with the RNA payload, while maintaining a neutral charge of the LNPs in the circulation. Moreover, once the LNPs reach the endosome, due to the low pH, the ionizable lipids are protonated and trigger the endosomal release of the RNA payload to the cytoplasm. The existing clinically approved LNPs tend to accumulate in the liver upon systemic administration. Therefore, reaching leukocytes requires both avoiding liver accumulation and successfully distributing and internalizing into leukocytes.

3.2.2.3. Lipid nanoparticles without a targeting ligand. LNPs have proven to be successful in reaching different sub-populations of leukocytes for the delivery of siRNA [106–110], miRNA [111–113], mRNA [114–116], and CRISPR/Cas9 in mice. Because the effectiveness of those systems depends on their ability to avoid liver accumulation, a large portion of generating potent LNPs relies on the development of novel ionizable lipids that target leukocytes or better reach organs in which leukocytes reside, such as the spleen. For example, Fenton et al. designed a novel ionizable lipid that demonstrated efficient delivery of mRNA to B lymphocytes in the spleen [116]. However, the clinical implementation of such LNPs is hindered by (1) the lack of predictability between *in vitro* and *in vivo* targeting abilities requires high throughput screening (HTS) will be done in animal models, (2) the differences between animal models and human diseases, (3) and our poor understanding of the relationship between lipid chemistry and formulations, and the effectiveness of the LNPs in reaching their target cells. Moreover, internalization of LNPs is one of the major bottlenecks in the field of RNA therapy [13–15], and while LNPs can internalize relatively easy to dendritic cells, macrophages, and monocytes, internalization into lymphocytes, is challenging with "naked" LNPs and may require a targeting moiety. Nevertheless, we believe that the design of new ionizable lipids, rational-driven modifications of lipid formulations, and advances in HTS methods would lead to improvements in this delivery field and hopefully to the initiation of clinical trials soon.

3.2.2.4. Lipid nanoparticles with a targeting ligand. The use of a targeting moiety to improve the specificity of LNPs is a promising delivery strategy. Targeted LNPs (tLNPs) don't accumulate less in the liver but maintain their same biodistribution pattern, with the added benefit of retention of the tLNPs in the immediate proximity of the target cells [6]. Moreover, the targeting moiety can facilitate the internalization and endosomal release processes in hard-to-transfect cells [13]. Monoclonal antibodies are commonly used as a targeting moiety due to their wide availability and selectivity, and although methods of chemical conjugation that covalently bind mAbs to the LNPs are inefficient, some studies were able to reach lymphocytes in disease-bearing mice [117–120]. Dammes et al. recently described a novel conformation-sensitive recombinant fusion protein as a targeting moiety to generate tLNPs encapsulating siRNA, which selectively target active leukocytes that home to the intestinal tissues during colitis, and demonstrated potent targeting abilities despite the limitations of chemical conjugation methods [121]. Another limitation of this process is the difficulty of utilizing this method to produce chemically conjugated tLNPs that encapsulate mRNA, due to its instability. ASSET, Anchored Secondary scFv Enabling Targeting, is a self-assembly modular platform that allows binding of mAbs to LNPs by utilizing a biological approach [32]. We recently demonstrated the ability tLNPs that bind potentially any mAbs with the ASSET platform to efficiently deliver siRNA [32,34] and we were the first to show systemic, cell specific delivery of mRNA to leukocytes in animals that induce an immunomodulatory effect [122].

3.2.2.5. Hybrid exosome nanoparticles. Exosomes are microvesicles with a diameter of 30-100 nm. They contain RNA, proteins, and other components, and play a pivotal role in cellular interactions during cancer, inflammation, and homeostasis [123]. Exosomes could be exploited to transport therapeutic RNA as they are biocompatible, stable, and have tunable targeting properties. Li et al. and Pei et al. recently constructed novel exosome-based targeted delivery systems of therapeutic RNA by hybridizing exosomes with polymers and demonstrated therapeutic efficacy in mice [124,125]. Although this delivery strategy succeeded *in vivo*, there are still many questions regarding the characteristics,

production and purification methods of exosomes before evaluating them for clinical use as a delivery platform.

4. Future outlook and conclusions

RNA-based therapeutics developed profoundly in the past years and their path to approval seems closer than ever. In this review, we covered the successful attempts of delivering therapeutic RNA, highlighted the importance of developing drug delivery systems, and discussed the limitations and advantages of clinically implementing each delivery strategy. There is still much work to be done to utilize other therapeutic RNA molecules, such as genome editing components (based on genome editing, prime editing or base editing), saRNAs, and circular RNA (circRNA) and to improve the existing delivery systems to leukocytes by study receptor-ligand interaction under sheer-flow conditions. Also, the limited understanding we have concerning the major bottlenecks of RNA expression, hinder our ability to improve the therapeutic efficacy of those systems. Nevertheless, the recent approval of prophylactic RNA vaccines and gene silencing for clinical use and the broad research and resources that are devoted to their acceleration in both academic institutions and almost in every major pharma company, proved that RNA-based therapeutics are no longer a fantasy but a real life-changing technology that could benefit us all, both in the field of vaccines, therapeutics and even personalized medicine.

Credit Author Statement

Dana Tarab-Ravski, Lior Stotsky-Oterin and Dan Peer – design and wrote the manuscript.

Declaration of Competing Interest

D.P. declares the following competing financial interest(s): 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) for, lectured (and received a fee) or conducts sponsored research at TAU for the following entities: ART Biosciences, BioNtech SE, EPM Inc., Earli Inc., lmpetis Biosciences, Kernal Biologics, Newphase Ltd., NLC Pharma Ltd., NeoVac Ltd., Roche, SirTLabs Corporation, Teva Pharmaceuticals Inc.,

All other authors declare no competing financial interests.

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