RNA in Cancer Immunotherapy: Unlocking the Potential of the Immune System

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ABSTRACT

Recent advances in the manufacturing, modification, purification, and cellular delivery of ribonucleic acid (RNA) have enabled the development of RNA-based therapeutics for a broad array of applications. The approval of two SARS-CoV-2–targeting mRNA-based vaccines has highlighted the advances of this technology. Offering rapid and straightforward manufacturing, clinical safety, and versatility, this paves the way for RNA therapeutics to expand into cancer immunotherapy. Together with ongoing trials on RNA cancer vaccination and cellular therapy, RNA therapeutics could be introduced into clinical practice, possibly stewarding future personalized approaches. In the present review, we discuss recent advances in RNA-based immuno-oncology together with an update on ongoing clinical applications and their current challenges.

Introduction

RNA has become a widely popular tool for vaccination against infectious diseases following the SARS-CoV2 pandemic (1). Potentially, this molecule can be exploited for many more applications, especially in cancer immunotherapy, offering a broad range of RNA-based therapeutic strategies (2, 3). Since its discovery in 1961 by Brenner and colleagues, RNA has evolved from what was initially considered a mere intermediary between DNA and protein to a versatile molecule operating at multiple cellular levels, exploitable for immunotherapeutic applications (4). *In vitro* transcribed messenger RNA (*iV*T-mRNA), self-amplifying RNA (SAM), antisense oligonucleotides (ASO), aptamers, small interfering RNA (siRNA), and microRNA (miRNA) are the most studied RNA formats at present, which can be categorized into two main groups: (i) coding RNA (cRNA) translating into protein, including mRNA and SAM, and (ii) noncoding RNA (ncRNA) that does not translate into proteins but rather regulates cell physiology and functions, including among others ASOs, aptamers, siRNA, and miRNA. Based on their role related to cancer biology, ncRNA can function as oncogenes or tumor suppressor genes (**Fig. 1**; ref. 5) and therefore be implemented for therapeutic use. Initially, RNA was not considered a suitable therapeutic tool due to its unstable nature and susceptibility to rapid degradation by ubiquitous ribonucleases (RNases). Other concerns regarding potential toxicity, unspecific immune activation, and unknown effectiveness also needed further investigation (6). At present, many limitations have been overtaken, including chemical modification of the RNA structure, paralleled with the development of novel technologies for RNA delivery and protection. This allows fast, cost-effective, and versatile generation of mRNA suitable for clinical application in the context of cancer and infectious diseases (4, 7), introducing RNA as a promising pharmaceutical product (**Table 1**; refs. 8–12).

In vitro transcribed mRNA and self-amplifying RNA

Mature eukaryotic mRNA consists of highly conserved molecular features, including a cap structure at the 5′ terminus, two extended untranslated regions (UTR) at the 5′ and 3′ end of the open reading frame (ORF), and a poly-A tail at the 3′ terminus (13), all influencing mRNA degradation, stability, immunogenicity, and translatability (14). For the generation of *iV*T-mRNA, a DNA template is required, commonly derived from a linearized plasmid (15), from which mRNA is transcribed using RNA polymerase enzymes such as T7, SP6, or T3 (16). Other main components of the *iV*T reaction mix comprise a RNase inhibitor, pyrophosphatases, and a reaction buffer including the four ribonucleoside triphosphates (rNTP) and a capping reagent (ARCA, Clean Cap^{TM}). The final concentration of each component, the reaction temperature, and time will determine the final mRNA yield obtained (**Fig. 1**; refs. 16, 17).

After the *iV*T reaction, the DNA template, all enzymes used, organic and inorganic contaminants and secondary transcription products, such as incomplete/truncated RNA molecules and double-stranded RNA (dsRNA) have to be removed (18). A DNase digestion is performed to eliminate the DNA template, followed by a salt and ethanol precipitation or more accurately by high-pressure/performance liquid chromatography (HPLC) to obtain pure mRNA (19). The purification process reduces immune activation triggered by contaminants and therefore can improve mRNA translation upon delivery into the cell (19). In addition to manufacturing and purification, thorough quality controls are performed to assess the final RNA integrity, purity, concentration, capping efficiency, sequence, and poly-A tail length (20).

Poor mRNA uptake and *in vivo* expression are often observed due to extracellular RNase activity (15) and intrinsic mRNA immunogenicity (21), triggering interferon (IFN) pathway activation (7). Packaging mRNA into lipid nanoparticles (LNP) has proven an effective solution to ensure protection and successful RNA delivery into the cell (22). A multitude of nanoparticles have been tested and extensively reviewed by others (23–25). At present, the LNP-mRNA COVID-19 vaccines Tozinameran and Elasomeran, with LNPs composed of ionizable lipids, phospholipids, cholesterol, and PEGylated lipids, have been

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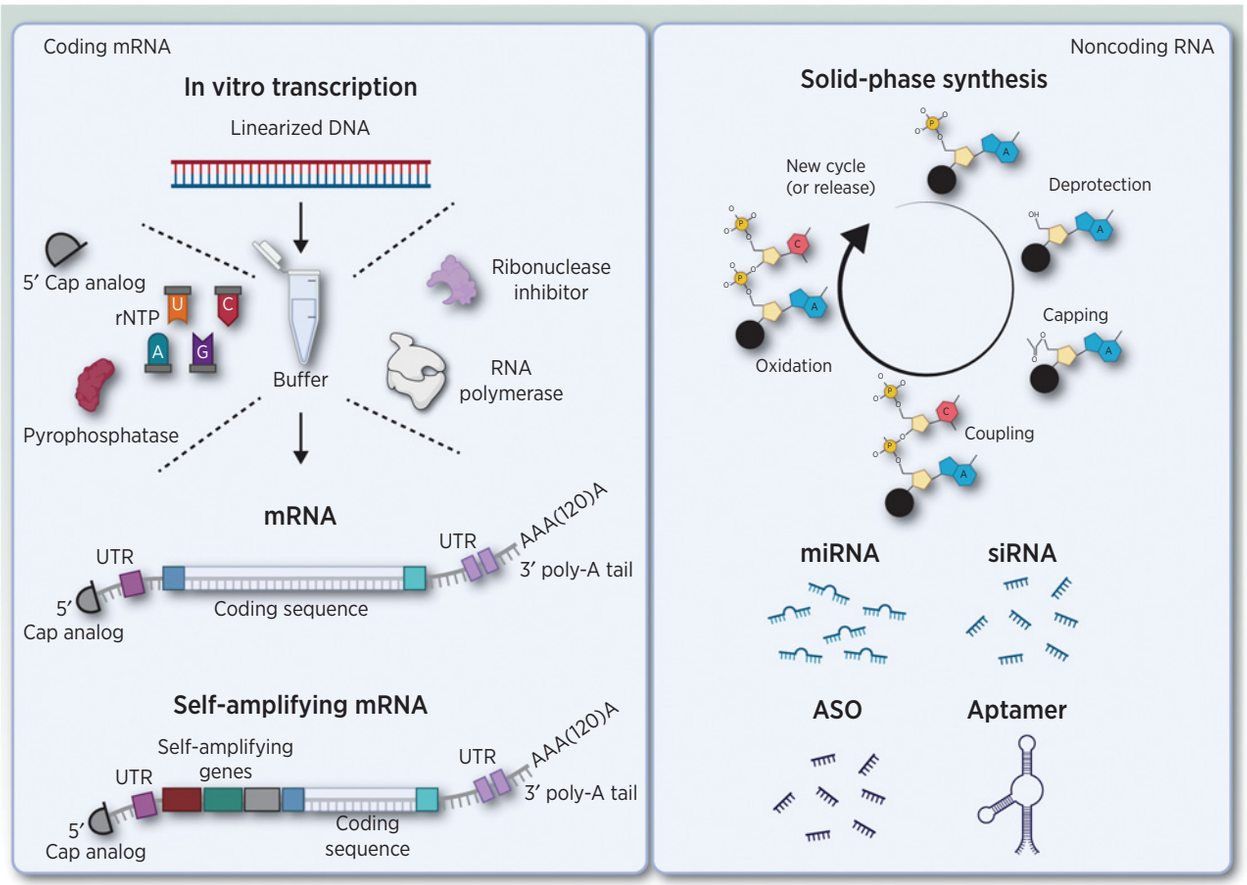

Figure 1.
Overview of coding and noncoding RNA structures. Left, *in vitro* transcription (iVT) of messenger RNA (mRNA). mRNA has several conserved features, including a 5′ cap structure, two extended untranslated regions (UTR) at the 5′ and 3′ end of the ORF, and a 3′ poly-A tail. The final iVT product can be mRNA or self-amplifying mRNA. Right, solid-phase synthesis of noncoding and antisense oligonucleotides. Abbreviations: ASO, antisense oligonucleotides; mRNA, messenger RNA; UTR, untranslated region; rNTP, ribonucleoside triphosphates; miRNA, microRNA; siRNA, small interfering RNA. Adapted from an image created with BioRender.com.

clinically approved (26). Moreover, RNA chemical modifications such as N1-methyladenosine, 5-methylcytidine, pseudouridine, N6-methyladenosine, 5-methyl deoxycytidine, and inosine pioneered by Kariko and colleagues (27–30) have been exploited to generate stable, nonimmunogenic, and high-translatable RNA molecules, including also modified base analogs as 5`cap structures (31, 32). Other work on the optimization of the poly-A tail length showed enhanced mRNA stability and lowered immunogenic profile (33). Additional optimization of UTRs and codon optimization paired with sequence design of the encoding sequence (34) aimed to improve RNA lifetime stability and expression (35–37).

Table 1. Advantages and disadvantages of RNA- compared with DNA- and protein-based therapeutics (data from references 8–12).

	RNA	DNA	Protein
Synthesis	+	+	−
Manufacture time[a]	+/−	+/−	−
Genome integration	−	+/−	−
Self-adjuvancy	+/−	+/−	−
Storage[b]	+/−	+/−	+
Administration	+	+	−

[a]RNA manufacture time is fast but depends on a DNA template.
[b]Storage requirements depend on the formulation used for the therapeutic product.

Noncoding and antisense oligonucleotides

Solid-phase synthesis is mostly used for manufacturing (antisense) oligonucleotides. The first nucleotide of the desired molecule is attached to a solid phase and elongated using a repetition of a four-step cycle, adding one nucleotide per cycle until the oligonucleotide is fully synthesized. The four-step cycle includes detritylation/deblocking, coupling, capping, and oxidation or thiolation. The synthesis is performed in a column reactor under programmed delivery of all reagents (**Fig. 1**). A 70% to 80% success synthesis rate is achieved of the desired oligonucleotide length, requiring a final HPLC purification step, removing all polymers showing incorrect length ($n \pm x$; ref. 4). Chemical modifications were implemented to this process, aiming for improved pharmacokinetics (38). The most used modifications include a substitution of the phosphodiester bond with a phosphorothioate, proving to increase resistance toward nuclease degradation

and reduce binding to plasma proteins, and decreasing renal clearance (39). However, reduced target affinity is also observed, and therefore 2′-methoxyethyl, 2′-deoxy-2′-fluoro, and 2′-O-methyl modifications were introduced on the ribose of the RNA to improve specificity (20).

A similar manufacturing approach is exploited for the synthesis of RNA aptamers. A widely used method to design aptamers is by Systematic Evolution of Ligands by Exponential enrichment (SELEX). In short, a high diversity library of single-stranded RNA (ssRNA) is synthetized by *in vitro* transcription (40). From the RNA library, a selective target binding ssRNA is isolated through repeated rounds of exposure, binding, selection, and amplification. Once the desired sequence/design of the RNA aptamers is obtained, the aptamers are manufactured using solid-phase synthesis (41).

RNA as a Vaccination Strategy

Therapeutic cancer vaccines aim to generate antigen-specific T-cell responses targeting tumor cells and potentially achieve long-term clinical benefits (42, 43). The approval of two mRNA vaccines for COVID-19 prevention has highlighted the potential of mRNA technology (44). Two main RNA-based approaches have been extensively explored for cancer vaccination: *ex vivo* mRNA-loaded dendritic cell (DC) vaccines (45, 46) and mRNA-LNP vaccines (ref. 47; **Fig. 2**). In both strategies, mRNA is used to deliver the tumor-associated antigen (TAA) or tumor-specific antigen to elicit an antitumor immune response. Upon cellular entry followed by translation of the mRNA, the proteasome processes the mRNA-encoded protein into peptides that ultimately will be processed and presented by human leucocyte antigen (HLA) class I molecules to $CD8^+$ T cells (48). $CD4^+$ T-cell stimulation is also recommended to support the $CD8^+$ T-cell response (49). In this regard, coupling an HLA class II sorting signal, such as the signal sequence of the invariant chain, lysosomal-associated membrane protein (LAMP), or DC-LAMP, to the antigen sequence is required to ensure that antigen-derived peptides enter the HLA class II presentation pathway, despite the protein being synthesized in the cytosol of the cell (50, 51).

Cancer vaccines, mostly targeting cancer–testis and differentiation antigens, as monotherapy, have not shown significant activity thus far. Most clinical trials have been ineffective, and the induced immune reactivity was insufficient, limited in time, and narrow. The lack of clinical efficacy from vaccine treatment alone can be attributed to weak antigen delivery modalities that induced low T-cell titers as well as immune checkpoints remaining intact, which ultimately prevented tumor cell killing. It is now thought that neoantigens generated by somatic alterations could be differentially recognized by the immune system as these proteins/peptides would be unique to the tumor and the T-cell repertoire recognizing such neoantigens would not have been subjected to central tolerance mechanisms, as is the case for cancer–testis- and differentiation antigen-specific T cells (8, 52).

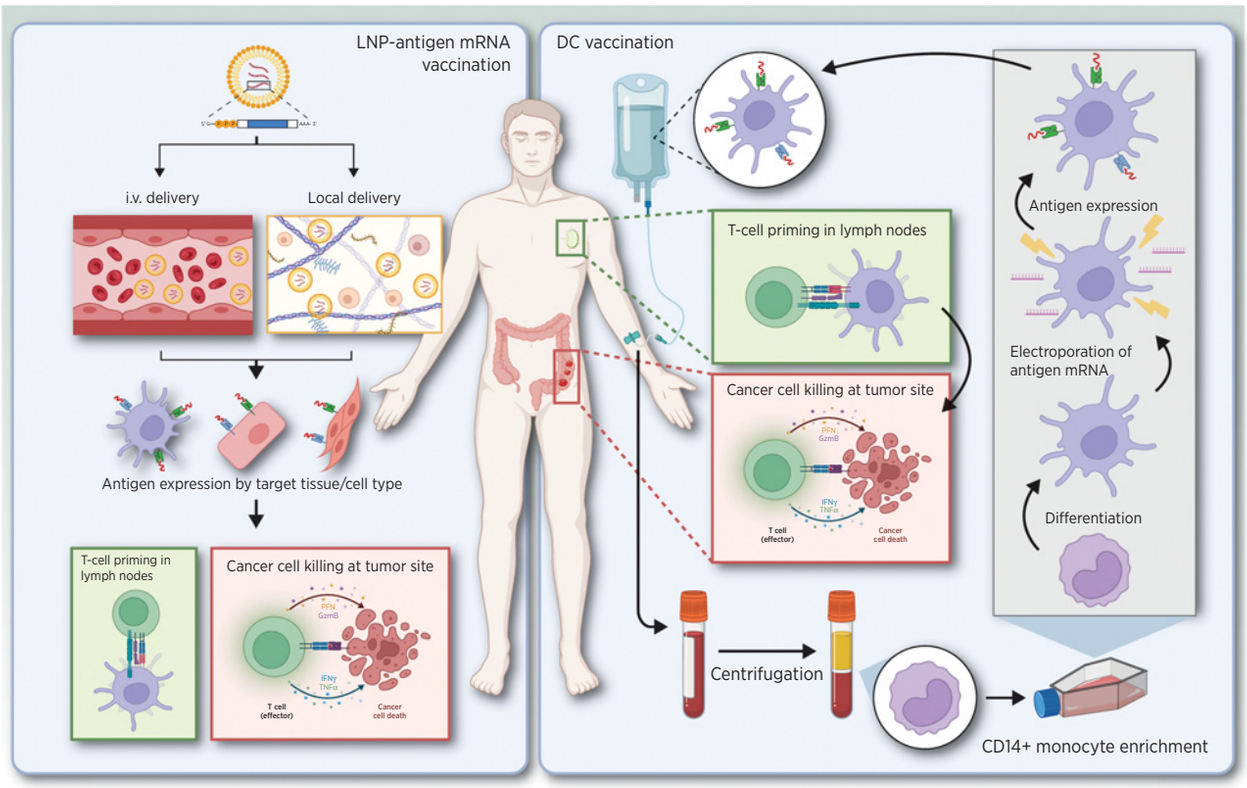

Figure 2.
Overview of active mRNA-based immunotherapeutic strategies. Left, LNP-antigen mRNA vaccine delivered systemic or locally, followed by antigen expression resulting in T-cell priming and eventually cancer cell killing. Right, monocytes or hematopoietic progenitor cells are isolated from blood, further cultured, and differentiated into DCs. mRNA is then used to load the DCs *ex vivo* with tumor antigens. The modified DCs are administered to patients, where they will prime T cells, eventually resulting in the killing of cancer cells. Adapted from an image created with BioRender.com.

mRNA-based dendritic cell vaccines

mRNA-based DC cancer vaccination was introduced more than two decades ago (53). At present, more than 30 clinical trials have been published, recently reviewed by Dorrie and colleagues (46). For DC generation, monocytes or hematopoietic progenitor cells are isolated from blood and further cultured and differentiated into DCs (54). mRNA is then used to load the DCs *ex vivo* with tumor antigens. The modified DCs are administered to patients (**Fig. 2**) via intravenous, intradermal, subcutaneous, or intranodal injections (55). DC maturation is most often induced using a cytokine cocktail (56), feasible with both protein and mRNA delivery (57). Examples of mRNA-induced functional manipulation of DCs include TriMix mRNA, a mixture consisting of CD40 Ligand (CD40L), CD70, and constitutively active Toll-like receptor 4 (TLR4) encoding mRNA. Several studies showed that TriMix-DC vaccination induces robust, TAA-specific T-cell responses in the majority of analyzed patients (58–60). Other strategies to improve the activation of T cells by mRNA-modified DC have been studied and show promising results (61–65).

DC-based vaccines have shown to induce adaptive immune responses, and RNA transfection is emerging as an ideal method for antigen-loading and functional manipulation of the applied cells. DC vaccination rarely produces adverse events and has a highly safe profile (46). However, challenges that call for imperative improvements such as the manufacturing process, the optimal choice of DC subset or their *in vitro* generation, the antigen choice, the route of administration, and vaccination schedule still need to be addressed (66).

mRNA-based vaccines

First clinical results were observed with intradermal or intranodal naked mRNA administration, resulting in mRNA uptake by antigen-presenting cells in the dermis or lymph nodes followed by antigen presentation and T-cell stimulation (67). Even though it has a positive clinical outcome and favorable safety profile, the many limitations, such as limited mRNA uptake, resulting in low bioavailability of antigens and lowered immune responses, hampered its implementation (15).

Recent attempts opted for intramuscular or intravenous administration of mRNA encapsulated by delivery carriers (24). Four recent clinical trials, using mRNA packaged in LNPs, have shown promising clinical and immunologic results in patients with solid tumors. In the Lipo-Merit trial (NCT02410733; **Table 2**), a vaccine consisting of nonmodified lipoplexed mRNA targeting a variety of TAAs (NY-ESO-1, MAGE-A3, tyrosinase, and TPTE) was administered intravenously to advanced melanoma patients. The adverse events (pyrexia, chills, and flu-like symptoms) were mild to moderate and transient. Expansion and activation of antigen-specific T cells with cytolytic activity against tumor cells could be documented. Continuous vaccination resulted in the persistence of antigen-specific memory T cells. Encouraging clinical responses have been reported (68, 69).

In the R07198457-trial (NCT03815058; **Table 2**), the administration of nonmodified mRNA encoding up to 20 patient-specific neoantigens has been studied as monotherapy and in combination with a PD-L1 inhibitor, in patients with advanced solid tumors (breast cancer, prostate cancer, ovarian cancer, melanoma, non–small cell lung cancer, bladder cancer, and colorectal cancer; refs. 70, 71). Also, in this trial, the adverse events were mostly of grade 1–2 and transient. Neoantigen-specific T-cell responses were observed in most of the patients. Promising clinical results in these often heavily pretreated patients were noted.

In both the KEYNOTE-603 trial (NCT03313778; **Table 2**) and the KEYNOTE-942 trial (NCT03897881; **Table 2**; Moderna and Merck), the safety and immunogenicity of intramuscularly administered lipid-protected modified mRNA encoding neoantigens were evaluated, either as monotherapy or in combination with anti–PD-1 monoclonal antibodies (mAb, pembrolizumab) in patients with solid tumors (72–74). This mRNA-based personalized cancer vaccine has an acceptable safety profile along with observed clinical responses in combination with pembrolizumab. Preliminary efficacy analysis from checkpoint inhibition-naïve relapsed/refractory human papillomavirus (HPV) negative head and neck squamous cell carcinoma (cohort suggests activity of this combination (73).

An alternative mRNA-based approach uses SAM, originating from positive ssRNA alphaviruses, consisting of the RNA replication machinery of the alphavirus (self-assembly genes; **Fig. 1**) and replacing other genetic regions with the gene sequence encoding the antigen(s) of interest. SAM amplifies over time (up to 2 months) and consequently induces more potent and persistent immune responses (75, 76). Clinical applications using SAM have been promising in preventing infectious diseases (77) and are transitioning into the cancer immunotherapy field. Gritstone, a California-based company, is performing clinical studies, where a viral prime and a SAM boost are used to induce immune responses against private or shared neoantigens (78).

RNA in Passive Immunotherapy

Passive immunotherapy is used as an umbrella term to describe any strategy designed to help a patient to fight disease by administration of immune system components that have been generated in the laboratory, including delivery of proinflammatory cytokines, immune-modulatory mAbs, or *ex vivo* manipulated autologous effector immune cells (79, 80). As with active immunotherapies, passive strategies can also benefit from the implementation of not only *iV*T-mRNA but also ncRNA, as passive immunotherapy covers a broader range of strategies (**Fig. 3**).

mRNA for protein therapy *in vivo*

In vivo delivery of antibody encoding mRNA

Since the development of the hybridoma technique in 1975 by Milstein and Köhler, therapeutic mAbs have been introduced for numerous indications. The mAb-mediated blockade of immune checkpoints, such as programmed cell death-1 (PD-1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4), has revolutionized cancer treatment (81–83). Besides full-sized mAb, antibody fragments, such as single-chain variable fragments (scFv) and heavy-chain V_H domains, have been heavily studied. In parallel, a variety of tumor-associated targets, such as TAAs, vascular, and stromal cells, have been explored as targets (10, 80, 84, 85).

Although mRNA-based antibody therapies have yet to face technical and clinical challenges (e.g., frequency and route of administration; ref. 86), the use of mRNA for *in vivo* production of therapeutic antibodies remains a promising approach (10).

In 2019, Rybakova and colleagues demonstrated the delivery of mRNA encoding the humanized anti-human epidermal growth factor receptor 2 (HER2) antibody, trastuzumab, via LNP in tumor-bearing mice. The reported serum–antibody concentrations were detectable up to 14 days after LNP injection, demonstrating more favorable pharmacodynamics compared with the recombinant mAbs. In general, the mRNA transcribed antibody retained its cell toxicity properties *in vivo*,

Table 2. Overview of active and recruiting clinical trials using mRNA-based therapeutics.

IT type	Cancer	Study	Phase	Target	Formulation	Combination
CAR	Solid tumors	NCT04981691	Phase I	MESO	EP autologous T cells	NA
Vaccine	NSCLC, CRC, PDAC	NCT03948763	Phase I	KRAS	V941 mRNA	Pembrolizumab
siRNA (CAS3/SS3)	B-cell NHL	NCT04995536	Phase I	TLR9, STAT3	siRNA linked to CpG oligonucleotide	Radiotherapy
Synthetic naked mRNA	BC	NCT03788083	Phase I	TriMix	NA	NA
Vaccine	OC	NCT04163094	Phase I	3 OC TAA	LNP	Carboplatin/paclitaxel
Vaccine	Esophageal, NSCLC	NCT03908671	Pilot study	Neoantigen	Undisclosed	NA
Vaccine	Melanoma	NCT03897881	Phase II	Neoantigen	LNP	Pembrolizumab
Vaccine	Solid tumors	NCT03313778	Phase I	Neoantigen	LNP	Pembrolizumab
Vaccine	Melanoma	NCT03815058	Phase II	Neoantigen	RNA-LPX	Pembrolizumab
Vaccine	Melanoma	NCT02410733	Phase I	4 TAA	RNA-LPX	NA
Vaccine	Prostate	NCT04382898	Phase I/II	5 PC TAA	LNP	Cemiplimab
Vaccine	Esophageal, gastric cancer, CRC, PC	NCT03468244	Pilot study	Neoantigen	Undisclosed	NA
Vaccine	GBM	NCT03688178	Phase II	CMV pp65	mRNA-Loaded autologous DC	Varlilumab
Vaccine	GBM	NCT02649582	Phase I/II	WT-1	mRNA-Loaded autologous DC	Temozolomide
Vaccine	NSCLC	NCT03164772	Phase I/II	6 NSCLC TAA	BI 1361849	Durvalumab, tremelimumab
Vaccine	Melanoma	NCT01456104	Phase I	Melanoma TAA	Autologous LC EP with TAA mRNA	NA
Vaccine	GBM	NCT03927222	Phase II	CMV	Pp65-LAMP mRNA-loaded autologous DC	Temozolomide, GM-CSF Tetanus-diphteria, toxoid
Vaccine	GMB	NCT02465268	Phase II	CMV	Pp65-shLAMP mRNA-loaded DC	GM-CSF
Vaccine	Brain metastasis	NCT02808416	Phase II	Neoantigen	mRNA tumor antigen pulsed DC	NA
Vaccine	GBM	NCT00639639	Phase I	CMV	Pp65-LAMP mRNA-loaded autologous DC	Tetanus toxoid
Vaccine	PC	NCT01197625	Phase I/II	Tumor antigen	Tumor mRNA-loaded DC	hTERT Survivin
Vaccine	AML	NCT01686334	Phase I/II	WT1 antigen	mRNA EP autologous DC	(Potentially) low-dose chemotherapy
Vaccine	MM	NCT01995708	Phase I	CT7, MAGE-A3, WT1	mRNA EP autologous LC	Standard of care
Vaccine	AML, HRMS	NCT03083054	Phase I/II	WT1 antigen	mRNA EP autologous DC	NA
Vaccine	NSCLC, GEA, mUC, MSS-CRC	NCT03639714	Phase I/II	Neoantigen	SAM	ChAd, nivolumab, ipilimumab
Vaccine	MSS-CRC, NSCLC, PDAC	NCT03953235	Phase I/II	Shared neoantigen Kras	SAM	ChAd, nivolumab, ipilimumab
Vaccine	CRC	NCT05141721	Phase II/III	Neoantigen	SAM	ChAd, standard of care atezolizumab, ipilimumab
RNAi	PDAC	NCT01676259	Phase II	KRAS	siG12D-LODER	Gemcitabine, paclitaxel, FOLFIRINOX
RNAi	Advanced malignant solid neoplasms	NCT01591356	Phase I	EPHA2	DOPC encapsulation	NA
Vaccine	Melanoma	NCT02410733	Phase I	NYESO-1, MAGE A3 tyrosinase, TPTE	Lipo-MERIT	NA
Vaccine	Melanoma	NCT03815058	Phase II	Neoantigen	Lipoplex	Pembrolizumab
mAB	Solid tumors	NCT04683939	Phase I/II	CLDN18.2	Undisclosed	Paclitaxel, gemcitabine
Immune inducers	Solid tumors, lymphoma	NCT03739931	Phase I/II	NA	OX40L, IL23, IL36y coding mRNA	Durvalumab

Note: Data from Meisel et al. (74) and Wang et al. (76).

Abbreviations: BC, breast cancer; ChAd, chimpanzee adenovirus; CMV, cytomegalovirus; EP, electroporated; GBM, glioblastoma multiforme; GEA, gastroesophageal adenocarcinoma; HRMS, high-risk myelodysplastic syndrome; IT, immunotherapy; LCs, Langerhans cells; AML, acute myeloid leukemia; MESO, mesothelin; MSS-CRC, microsatellite stable colorectal cancer; mUC, metastatic urothelial carcinoma; NA, not applicable; NHL, non-Hodgkin lymphoma; NSCLC, non–small cell lung carcinoma; OC, ovarian cancer; PC, prostate cancer; PDAC, pancreatic ductal adenocarcinoma; pHGG, pediatric high-grade glioma; STAT3, signal transducer and activator of transcription 3; TAA, tumor-associated antigens; TLR9, Toll-like receptor 3.

which contributed to a significant delay in HER2-positive tumor growth when administered weekly (87).

The potential of bispecific T-cell–engaging antibodies is high, but their manufacturing is often challenging. Stadler and colleagues tested the *in vivo* production of bispecific antibodies by treating mice with pharmacologically optimized, nucleoside-modified *i*VT-mRNA encoding the bispecific antibody. Sustained endogenous synthesis of the bispecific antibody was achieved, eliminating advanced tumors as

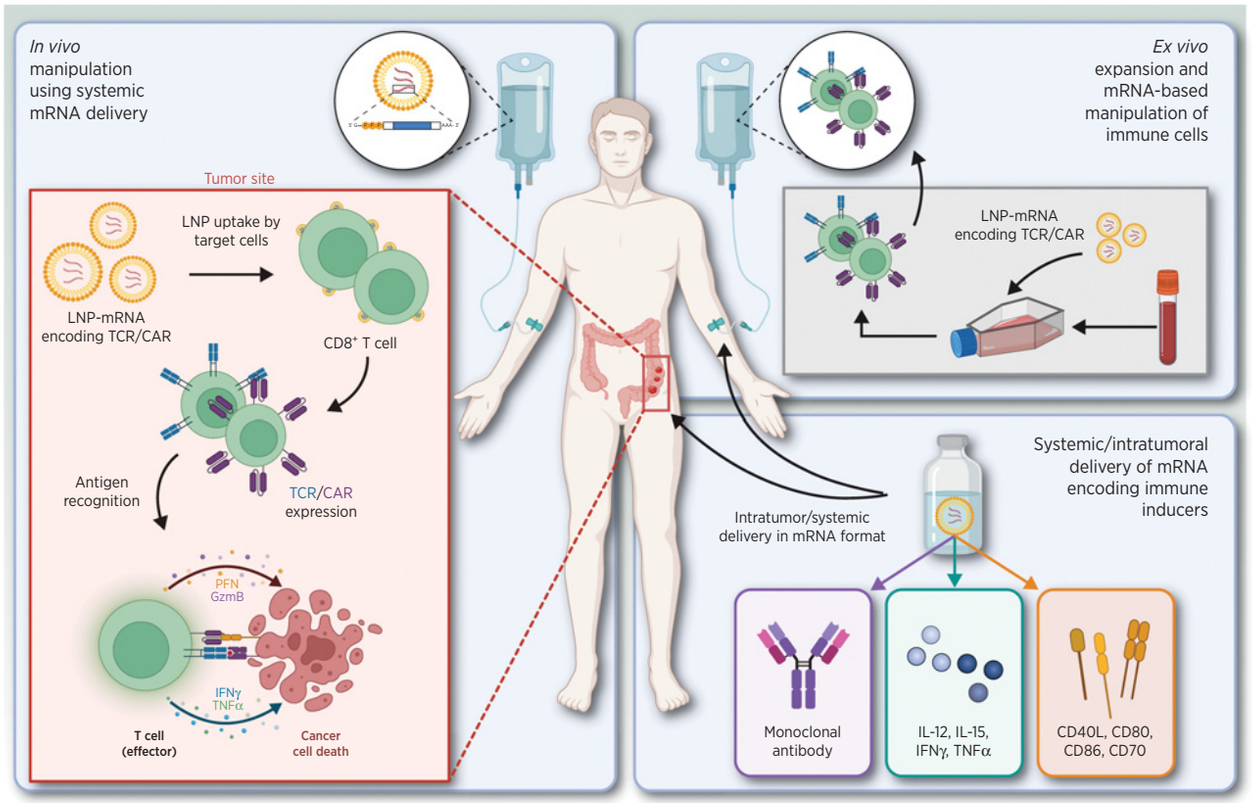

Figure 3.

Overview of passive immunotherapeutic strategies. Left, systemic administration of TCR and CAR LNP-mRNA causes specific uptake by $CD8^+$ T cells, followed by expression and antigen recognition, resulting in cancer cell death. Top right, *ex vivo* TCR/CAR LNP-mRNA manipulation of T cells, followed by systemic administration. Bottom right, intratumoral or systemic delivery of LNP-mRNA encoding monoclonal antibodies (mAbs), immune-inducing cytokines, or stimulatory receptors. Abbreviations: TCR, T-cell receptor; CAR, chimeric antigen receptor. Adapted from an image created with BioRender.com.

effectively as the corresponding purified bispecific antibody was achieved. This approach could accelerate the clinical development of novel bispecific antibodies (88).

A clinical trial by BioNTech is investigating the safety and pharmacokinetics of BNT141 (NCT04683939; **Table 2**) and BNT142, both mRNA encoding antibodies targeting CLDN18.2 and $CD3^-$CLDN6, respectively, in unresectable or metastatic claudin 18.2-positive solid tumors for which no available standard therapy is likely to confer clinical benefits.

mRNA-engineered adoptive cell therapies

Although immune-checkpoint blocking and mAb-based methods have shown promising results in breast cancer (89), melanoma (90), and non–small cell lung carcinoma (91, 92), among others, many patients still develop disease progression after these therapies, requiring additional treatment options (80, 93, 94). This demand was met with the introduction of adoptive cell therapy (ACT), pioneered by Steven A. Rosenberg, who demonstrated the *in vivo* antitumor activity of tumor-infiltrating lymphocytes (TIL; refs. 80, 88, 94). Over the years, ACT moved beyond the use of TILs. New strategies include *ex vivo* expansion and modification of tumor-residing or peripheral T cells with TCRs or CARs that convey specificity for the cancer cells (94, 95). Although ACT mainly focuses on the introduction of T cells expressing a TCR or CAR, Exteberria and colleagues showcased the therapeutic effect of the intratumoral administration of T cells transiently expressing IL12 in combination with transient CD137 ligand expression resulting in antitumor toxicity (96).

Successful clinical outcome has been reported with CAR-T therapies in hematologic malignancies. For instance, CAR-T therapy targeting CD19 in chronic lymphocytic and acute lymphoblastic leukemia (97), CD33 and CD123 CAR targeting in acute myeloid leukemia (AML; refs. 97, 98), and anti-BCMA CAR-T cell therapy in multiple myeloma (MM; ref. 99). This success was unmet when translated to solid malignancies as it is among others more challenging to identify the right target molecules.

At present, the clinical implementation of engineered T cells still raises many questions, including cross reactivity, controllability of permanently modified cellular products, and safety assessments (100). Because of these safety concerns, there has been an increasing interest for mRNA-based T-cell manipulation as transient expression is ensured in both TCR and CAR approaches (97, 100). However, despite this major advantage, other disadvantages such as insufficient longevity of mRNA-encoded CAR or TCR expression need to be addressed. This drawback also results in higher T-cell demands as repeated administration would be necessary to compensate the reduced half-life of CAR or TCR expression (97).

In 2020, Parayath and colleagues reported on the use of an injectable nanocarrier to deliver CAR or TCR encoding mRNA directly to circulating T cells, eliminating the need for *ex vivo* T-cell expansion (101). In this study, using leukemia and prostate cancer mouse

models, the nanoparticles were manufactured using poly β-amino ester, which self-assembles into nanocomplexes when interacting with anionic nucleic acids. These nanoparticles specifically targeted $CD8^+$ T cells by incorporation of an anti-CD8–linked polyglutamic acid. For CAR therapy, the nanoparticles were administered weekly, as the CAR expression lasted up to 8 days. A similar duration of TCR expression was achieved. The authors demonstrated that the use of injectable nanocarrier for mRNA delivery was sufficient to bring disease regression (101). More recently, the successful *in vivo* generation of CAR-T cells by delivery of modified mRNA packaged into T-cell targeted LNPs was reported. Transient expression and functionality of the CAR were observed (102).

Phase I trials in mesothelioma (NCT01355965) and pancreatic cancer (NCT01897415) have been initiated using autologous T cells transfected with mRNA encoding a mesothelin targeting CAR. The use of such mRNA-engineered T cells appeared to be feasible and safe. Early signs of antitumor activity and absence of overt off-tumor on-target toxicity were observed (103). A phase I clinical trial using autologous cMet-redirected T cells administered intratumorally in patients with breast cancer (NCT01837602) has shown that cMet-CAR-T cell injections were well tolerated, as no patients experienced above grade 1 adverse events, whereas tumor necrosis, and a consequential inflammatory response, was present when IHC was performed on tumor resections (104).

mRNA-based modulation of the tumor microenvironment

The intratumoral delivery of therapeutic compounds is an attractive option to increase the *in situ* bioavailability and, thus, the efficacy of immunotherapies. This applies to compounds targeted to tumor tissue as well as for compounds targeting immune cells that play an important role in immune evasion, such as regulatory T cells, tumor-associated macrophages (TAM), neutrophils (TAN), and immature DCs (105–109). Therefore, delivery of mRNA encoding such compounds can contribute to antitumor immunity, as shown before by delivery of mRNA encoding a fusokine consisting of IFNβ and the ectodomain of the TGFβ type III receptor (110). In a study reported by Haabeth and colleagues, charge-altering releasable transporters were used for the intratumoral delivery of mRNA encoding immune modulators (111). In this study, a monotherapy with mRNA encoding for IFNγ, IL12, CD70, CD80, CD86, and CD40L was investigated, and in particular a significant tumor growth delay was observed for CD40L, CD80, and CD86, as confirmed also by Van Lint and colleagues through intratumoral delivery of TriMix mRNA (112). More recent studies have published similar results with mRNA formulated in saline solution (113). mRNA encoding IFNα, IL12 single chain, granulocyte–monocyte colony stimulation factor (GM-CSF), and IL15 sushi was administered intratumorally, resulting in an increase of immune cell populations accompanied by intratumoral IFNγ induction, systemic antigen-specific T-cell expansion, increased granzyme B^+ T-cell infiltration, and formation of immune memory (113). In another preclinical study, *i*VT-mRNA encoding IL15 was administered *in vivo*. This mRNA was complexed using a protamine/liposome system. In both local and systemic administration, the CLLP/IL15 mRNA resulted in significant tumor-inhibitory effects in subcutaneous, abdominal cavity, and pulmonary metastasis models (114).

Several clinical trials using intratumoral delivery of mRNA are ongoing. A phase I study (NCT03739931; **Table 2**) is evaluating the intratumoral delivery of LNP-encapsulated mRNA encoding human OX40L, IL23, and IL36γ, either as monotherapy or in combination with immune-checkpoint blockade in patients with advanced malignancies. BioNTech is testing the intratumoral delivery of BNT131 or a mRNA mixture encoding IL12 single chain, IFN-alpha2b, GM-CSF, and IL15 sushi as monotherapy and in combination with PD-1 targeting cemiplimab in advanced solid tumors. Another phase I study by eTheRNA in collaboration with VUB-UZ aims to deliver synthetic naked mRNA encoding TriMix intratumorally in early-stage breast cancer (NCT03788083; **Table 2**).

ncRNA for the reduction of expression

RNA interference–based therapeutic interventions

All the above-mentioned applications involve the use of mRNA to mediate the expression of immune-boosting proteins. Notably, progressively more studies have focused on RNA as an intermediary, not only for expression but also for the regulation of expression, since RNA interference (RNAi) has been discovered in 1998 (115). The starting feature of RNAi is siRNA, short hairpin RNA (shRNA), or miRNA (116, 117), which after cleavage by the Dicer enzyme and after association with the RISC/ago2 enzyme complex has the capacity to hybridize with a complementary mRNA strand and results in the cleaving of that strand (118–120). This was successfully applied in immuno-oncology by Li and colleagues, where siRNA-PD-L1 (siPD-L1) was codelivered with imatinib in liposomal nanoparticles, resulting in the reduced expression of PD-L1, synergistically causing a tumor delay more significant than the monotherapies (121). Similar results were achieved in a mouse melanoma model by Wang and colleagues (122), indicating that, in combination with chemotherapy, or extracellular targeting, RNAi-mediated cell disruption can significantly promote the antitumor effects of already clinically available cancer treatment (116). Moreover, RNAi can as well be exploited beyond the alleviation of inhibitory pathways, but also for TME remodeling on TAMs, TANs, and immature DCs (123, 124) and in conjugation with mRNA for DC vaccination (61, 125). Although RNAi holds promise, important improvements regarding clinical applications, such as pharmacodynamics and pharmacokinetics, as well as toxicity need to be addressed (120). However, improvements regarding toxicity have already been booked with LNP formulations, as toxicity is often due to unintended and on-target off-tissue RNAi activity.

Beyond RNAi

Besides their role in RNAi, small ncRNA, such as miRNA, could act not only as tumor suppressor miRNA (TS-miR), but also as oncogenes (oncomiR), depending on the target (126). miRNA mimics, classifiable as ASOs can be implemented for anticancer therapy. Around 20–25 bases long, ASOs bind to their miRNA targets, preventing interaction of that miRNA with its target mRNA, and resulting in RNase H–mediated degradation (127). The use of ASOs has already been demonstrated in a preclinical setting for glioma using anti-miR-21 with miR-21 being on oncomiR, suppressing IL12 (126) for anticancer therapy. Around 20–25 bases long, ASOs bind to their miRNA targets, preventing the interaction of that miRNA with its target mRNA and resulting in RNase H mediated degradation (127). The use of ASOs has already been demonstrated in a preclinical setting for glioma using anti-miR-21 with miR-21 being on oncomiR, suppressing IL12 (126). Next to ASOs, aptamers have also entered the scope of RNA-mediated immunotherapy. Aptamers possess a small molecular weight, making them suitable for TME entry. Moreover, their longer shelf-life and low immunogenicity, combined with their possibility of cell-free manufacturing, give them advantageous features for clinical applicability (128). In a study by Gao and colleagues, aptamers targeting PD-L1 were developed and validated (129). Besides this, aptamers targeting CXCL12 (NOX-A12) and CCL-2 (NOX-E36) have been tested in clinical trials (130), from which monotherapy of NOX-A12

showed induction of T helper 1 cytokines and resulted in prolonged time on treatment versus prior therapy in 35% of patients with metastatic microsatellite stable colorectal or pancreatic cancer in combination with pembrolizumab (131). These studies concluded that aptamers can be considered a valid alternative compared with mAbs, as the production costs are significantly lower and similar tumor inhibition and binding affinity as for mAbs was obtained (128).

Conclusion and Perspectives

The SARS-CoV-2 pandemic has unlocked the great potential of mRNA as a therapeutic agent, due to the extreme need for a prompt development of an effective COVID-19 vaccine. This rapid progress was possible only because of the preexisting long-term experience and already developed mRNA technology of the past three decades. Next, the mRNA format's high versatility could push further the implementation of mRNA-based personalized cancer therapies into the clinic, relying on an easily convertible manufacturing process (132). Nevertheless, personalized therapies still require the identification of novel, cancer-specific targets (including neoantigens) for which abundance and immunogenicity studies remain the main challenge. However, improvements in *in silico* prediction algorithms and next-generation sequencing (are expected to address this implementation; refs. 132, 133).

Despite high versatility, reduced costs, and quick manufacturing of mRNA vaccines, further insights are still required, especially regarding the mechanisms of action and therefore understanding the contribution of the innate immunogenicity of mRNA (134). The two SARS-CoV-2 mRNA-based vaccines, BNT162b2 (Tozinameran) and mRNA-1273 (Elasomeran), showed that mRNA chemical modifications and purity play an important role in reducing intrinsic immunogenicity, and this is key for intramuscular injected prophylactic vaccines (28). Low intrinsic immunogenicity is also necessary for other RNA therapeutics, where the protein level needs to be as high as possible including antibody encoding mRNA and mRNA-based modulation of the TME (28). Furthermore, the use of adjuvants and even the mRNA self-adjuvancy level has not yet been extensively evaluated in terms of potential benefits or adverse effects in mRNA cancer vaccine studies (21, 135).

The prompt optimization of LNP for clinical formulations contributed to the success of RNA as a therapeutic agent. However, many parameters need further investigation, such as biodegradability, tissue and cell tropism, long-term side effects, route of administration and delivery, all having a major effect on the overall cost, efficacy, and safety profile of LNP (23, 136).

Regarding RNAi, hereditary transthyretin amyloidosis and acute hepatic porphyria can already benefit from treatment options (137). Nevertheless, for the treatment of cancer, RNAi and ncRNA formulations have not yet been approved. The main challenge here is the scarce delivery of effector molecules in tumor cells to induce a clinically significant response. Two clinical trials (NCT01676259 and NCT01591356) are ongoing, using RNAi targeting KRAS and EPHA2, respectively. Positive results from this work could further accelerate the implementation of RNAi into the clinic (**Table 2**).

The use of RNA in passive immunotherapeutic approaches is catalyzed by current results from ongoing clinical trials (**Table 2**; ref. 2). More preclinical studies are necessary to thoroughly investigate RNA kinetics and dosage (80).

If, on the one hand, RNA therapeutics boast of a high safety profile (138) due to a transient dwelling time, on the other hand, more frequent administrations are required, which in terms of ACT might hamper the manufacturing process, as T cells are limited. In addition to this, injected T cells might also fail to induce a potent response as expression could be lost before reaching the tumor site. Local administrations could work as an efficient alternative, but to date have been unsuccessful in human clinical trials (98).

Altogether, the mentioned developments in the RNA field indicate its potential as an ideal candidate anticancer therapeutic agent, expanding on its current use for antiviral vaccination. Because of its manufacturing benefits, RNA therapeutics could establish a general presence in the drug development industry, going even beyond implementation for cancer immunotherapy.

Authors' Disclosures

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