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Authors

Claudia Augusta Di Trani[1,2,*], Myriam Fernandez-Sendin[1,2,*], Assunta Cirella[1,2,*], Aina Segués[3,4], Irene Olivera[1,2], Elixabet Bolaños[1,2], Ignacio Melero[1,2,5,6], Pedro Berraondo[1,2,5]

Affiliations

[1]Program of Immunology and Immunotherapy, Cima Universidad de Navarra, Pamplona, Spain.

[2]Navarra Institute for Health Research (IDISNA), Pamplona, Spain.

[3]Faculty of Veterinary Medicine, Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, The Netherlands.

[4]Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3FL, United Kingdom.

[5]Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Spain.

[6]Departments of Oncology and Immunology, Clínica Universidad de Navarra, Pamplona, Spain

*Contributed equally

Contact information for corresponding author:

Pedro Berraondo

Cima Universidad de Navarra

Avenida Pio XII 55

31008 Pamplona Spain

Tfln: +34948194700 e-mail: pberraondol@unav.es

Keywords

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Abstract

Introduction: Immune checkpoint inhibitors and adoptive T-cell therapy based on chimeric antigen receptors are the spearhead strategies to exploit the immune system to fight cancer. To take advantage of the full potential of the immune system, cancer immunotherapy must incorporate new biotechnologies such as mRNA technology that may synergize with already approved immunotherapies and act more effectively on immune targets.

Areas covered: This review describes the basics of mRNA biotechnology and provides insight into the recent advances in the use of mRNA for the local and systemic delivery of immunostimulatory antibodies, proinflammatory cytokines or for optimizing adoptive T-cell therapy.

Expert opinion: mRNA-based nanomedicines have great potential to expand the arsenal of immunotherapy tools due to their ability to simplify and accelerate drug development and their suitability for transient and local expression of immunostimulatory molecules, whose systemic and sustained expression would be toxic. The success of mRNA-based COVID-19 vaccines has highlighted the feasibility of this approach. Continuous advances in the delivery and construction of RNA-based vectors hold promise for improvements in clinical efficacy.

Article highlights

- Immunotherapy is a breakthrough in cancer treatment, and mRNA-based drugs can provide novel strategies to harness the immune system to fight cancer.
- mRNA technology has successfully been applied to the *in vivo* production of conventional or bispecific antibodies, as a strategy to reduce the toxicity associated with repeated and high doses of antibodies.
- Cytokine-based immunotherapy encoded by mRNAs aims to raise the concentration of cytokine/s in the tumor microenvironment, while minimizing the systemic exposure and toxicity associated with the administration of the recombinant proteins.
- *Ex vivo* transient engineering of T cells with mRNA molecules has been evaluated for adoptive cell transfer in pre-clinical and clinical studies. Impressively, advances in delivery technology have allowed *in vivo* targeting of the therapeutic RNAs to T lymphocytes.
- Development of mRNA-based immunotherapies has to overcome current limitations of this technology, but further refinements will expand the cancer immunotherapy applications.

1. Introduction

During oncogenesis, cancer cells accumulate mutations and epigenetic alterations that confer the capacity to overcome the numerous homeostatic constraints that oppose cancer development. Among these, the immune system plays a critical antitumor role, and evading the immune system is recognized as one of the hallmarks of cancer [1]. Therapeutic intervention in the complex interactions between the tumor and the immune system has proved to be more difficult than anticipated. Until the discovery of the PD-1/PD-L1 pathway, few immunotherapy strategies had had any real impact or only marginaly affected the survival of cancer patients. The clinical scenario has dramatically changed since the development of monoclonal antibodies targeting PD-1 or PD-L1 [2]. Treatments for more than 20 malignant conditions have been approved by the FDA using these antibodies, reflecting the essential role of the PD-1 molecule in the interplay between the tumor cells and the immune system [3]. Tremendous efforts are being devoted to extending the benefit of immunotherapy to those patients who do not respond or respond transiently to the anti-PD-1/PD-L1 antibodies as a result of primary and secondary resistances. New targets and novel biotechnology strategies are being evaluated both in pre-clinical research and clinical trials. Among the novel methods used to expand the arsenal of immunotherapy tools, retroviral/lentiviral gene transfer technologies have led to the development of potent cell therapy products that have received clinical approval to treat several hematological B-cell neoplasias [4]. An alternative for the *ex-vivo* gene transfer obtained with retroviral or lentiviral vector is the use of RNA-based non-viral vectors [5].

mRNAs are safe and well-tolerated molecules able to induce a peak expression of the encoded protein in hours. Expression decreases within few days, limiting the toxicity

associated with the corresponding recombinant protein. mRNAs are composed of different structural elements. From 5' to 3', the mRNA encompasses a cap, followed by a 5' untranslated region (UTR), the open reading frame, a 3' UTR, and a polyA. To design a drug in the form of an mRNA each element has to be optimized. Anti-reverse cap analogs are used to avoid the generation of RNA molecules that cannot be translated. The optimum size of the polyA has been defined as between 120 to 150 nucleotides. The rest of the mRNA sequence has to be engineered to maximize the expression yield and stability. The open reading frame must be codon-optimized, and UTRs are borrowed from naturally stable RNA molecules. These sequence-engineered mRNAs are potent inductors of type I interferons upon recognition by pathogen-associated pattern recognition receptors such as TLR-7 [6]. Although the activation of the innate system may be of interest in certain instances, type I interferons are potent suppressors of protein expression and thereby dramatically reduce the expression yield. Three main strategies have been developed to minimize this issue. Nucleoside-modified mRNAs are based on incorporating naturally modified nucleosides or synthetic nucleoside analogs that reduce the engagement by pathogen-associated pattern recognition receptors and improve the translational capacity and stability of the modified mRNA [6]. Interestingly, the non-inflammatory mRNA can also be exploited to induce immune tolerance for the treatment of autoimmune disease. For instance, Krienke *et al.* generated a 1 methylpseudouridine-modified mRNA vaccine that delivers Multiple Sclerosis autoantigens into lymphoid dendritic cells. Their presentation in a non-inflammatory context expands antigen-specific effector Treg cells, thereby enabling disease control [7]. A second strategy to deimmunize RNA vectors is the use of circular RNA. Circularization of the RNA can be efficiently achieved using a self-splicing intron, and highly pure circular RNA is not detected by pathogen-associated pattern receptors

[8,9]. Self-amplifying RNAs can also markedly increase protein yield. Such RNAs are derived from sequences in alphavirus genomes, which are engineered to replace the genes encoding the structural protein by the gene of interest, while the RNA replication genes are maintained so many copies of the mRNA encoding the therapeutic transgene appear in the transduced cell [10].

To deliver the optimized RNA to the cytoplasm of the target cells, several non-viral vectors have been employed. For *ex vivo* immunotherapy, naked RNA can be uptaken directly by phagocytic cells such as dendritic cells [11], or cellular entry can be facilitated by electroporation. This strategy has been used in adoptive transfer protocols with dendritic cells, T cells, and NK cells [12-14]. *In vivo* expression by injection of naked RNA has also been achieved by electroporation [15] or by direct intratumoral injection of the naked RNA in Ringer lactate. This simple strategy induced expression levels similar to those achieved by electroporation [16]. Moreover, cross-presenting dendritic cells (cDC1) express most of the RNA-encoded protein delivered intratumorally dissolved in Ringer lactate, with this being an interesting strategy to deliver immunostimulatory molecules that promote tumor antigen presentation [17].

In addition to electroporation, numerous non-viral vectors such as cationic nanoemulsions, liposomes, polyplexes, or polypeptidic-based systems have been evaluated for RNA delivery [18]. Among these methods, lipid nanoparticles led to the first regulatory approval of mRNA-based nanomedicines: the mRNA COVID-19 vaccines [19,20]. Lipid nanoparticles are composed of cholesterol, phospholipids, a polyethylene glycol-conjugated lipid, and an ionizable cationic lipid. The advantage of employing cationic lipids is that they maintain the nanoparticle neutral in electrostatic charge at physiological pH whereas

they undergo protonation at the acidic pH of the endosome following endocytosis. Ionization in the endosome leads to its disruption and the release of the vesicle cargo into the cytoplasm. This process is called the "proton sponge effect" and there are studies that propose that many other mechanisms could be involved in mRNA cytosolic release [21,22]. The cationic lipids used in the COVID-19 vaccines are SM-102 in the case of the Moderna vaccine and ALC-0315 in the Pfizer/BioNtech vaccine [23]. Delivery vectors can be optimized to provide tissue- or cell-specific expression, expanding the safety and efficacy of mRNA-based immunotherapy [24].

The unprecedented success of the RNA-based COVID-19 vaccines has demonstrated the feasibility and advantages of this technology for fast drug development. The previous knowledge acquired during the development of prophylactic vaccines against other infectious diseases and personalized therapeutic vaccines against cancer provided instrumental experience towards the success of the COVID-19 vaccines [25-27]. But the application of the mRNA biotechnology goes beyond vaccination. This review will focus on three mRNA applications that will significantly impact the cancer immunotherapy: expression of immunostimulatory antibodies, cytokines, and transient immune cell engineering.

2. Expression of immunostimulatory antibody-based moieties by mRNA

Monoclonal antibodies (mAbs) have revolutionized the cancer immunotherapy field [28]. The production of monoclonal antibodies was first developed thanks to the hybridoma techniques, and the discovery of innovative biotechnology approaches has dramatically

expanded the applications of mAbs [29]. To date, a wide variety of mAbs has been approved in the treatment of several diseases. A significant fraction of the approved mAbs is indicated for cancer treatment due to their ability to directly or indirectly modulate tumor biology [30,31]. However, despite the fact that mAbs have become crucial players in the cancer immunotherapy field, the whole production and purification process can be expensive, complex, and challenging. Moreover, purified antibodies can undergo protein aggregation. Such aggregates can affect the functionality of the monomeric protein and can generate agent-neutralizing immune responses [32-34].

To overcome these limitations, alternative strategies to produce mAbs *in vivo* have been widely investigated. Adeno-associated viruses (AAVs) have had a leading role in the development of gene therapy strategies aiming at the production of monoclonal antibodies for the treatment of several disorders, including cancer [35-39]. However, the sustained transgene expression achieved using this viral vector may not be adequate for the production of immunostimulatory monoclonal antibodies due to their often narrow therapeutic windows [40].

To overcome the problems related to the AAV vectors, the production of monoclonal antibodies using non-viral DNA or RNA vectors has attracted much interest. Such molecules lack the disadvantages of virus-associated approaches and allow the production of the target protein directly *in vivo*. Weiner *et al*. [41-44] have investigated the *in vivo* production of mAbs using electroporation of DNA plasmids, leading to the production and characterization of several mAbs to treat infectious diseases and for cancer immunotherapy. Regarding infectious diseases, the DNA-encoded monoclonal antibody (DMab) technology has been applied to Ebola virus, HIV, Zika virus, and *Pseudomonas aeruginosa* infections

[41-44]. The pre-clinical results obtained in the *in vivo* delivery of an anti-Zika virus DMab led to the start of the first, still ongoing, open-label clinical study to test the safety and tolerability of INO-A002 to prevent Zika virus infection (ClinicalTrials.gov Identifier: NCT03831503).

More recently, Weiner *et al*. [45,46] applied the previous experience in the field of DMab to the production of DMab-based immunotherapies such as αCTLA-4 and αPSMA antibodies. Both strategies were able to control tumor growth and improve survival of mice after an intramuscular injection of 100 µg of PSMA-DMAb or 400 µg of αCTLA-4-DMAb followed by electroporation [45,46].

Therefore, DNA electroporation may represent an efficient technology to induce consistent expression levels of several proteins, including antibodies. However, electroporation is, for the time being, limited to accessible tumoral lesions. To extend the DNA technology to other organs or non-accessible tumor lesions, lipid formulations of diverse DNA constructs are currently under investigation by different biotech companies.

An innovative way to deliver the genetic information for the *in vivo* production of monoclonal antibodies is the use of RNA-containing non-viral vectors (Figure 1). Various lipid-complexed antibody-encoding mRNAs have been developed to protect from HIV, influenza, rabies, and chikungunya virus upon intravenous injection [47-49]. The mRNA encoding an anti-chikungunya antibody is currently under evaluation in a clinical trial (ClinicalTrials.gov Identifier: NCT03829384).

This mRNA technology has also been developed in the cancer immunotherapy field. Thran *et al.* [48] produced an mRNA-encoded rituximab antibody and evaluated it in mice bearing

human Burkitt's lymphoma. RNA complexed in lipid nanoparticles (LNP-RNA) was administered by intravenous injections at doses of 10 or 50 µg of RNA. The RNAs encoding the heavy and light chains were injected at a molar ratio of 1.5:1 twice a week for a total of five injections. Both doses induced a significant antitumor effect with the highest dose being even more effective than repeated administration of the recombinant mAb [48]. Further evidence of the functional *in vivo* delivery of RNA-encoded mAbs came from Rybakova *et al.,* who applied this technology for the production of trastuzumab, an anti-HER2 antibody [50]. Likewise, the heavy and light chains of the mAbs are encoded by different RNAs. Injection of 2 mg/kg of mRNAs in a ratio of heavy chain to light chain RNAs of 2:1 was sufficient to generate 40 µg/ml of the circulating antibody after 24 hours, with expression lasting around one week. Efficacy studies were performed using xenograft-bearing mice. Subcutaneous injection of MDA-MB-231 cells expressing or not the HER2 receptor was used to establish the tumor model. Mice received 2 mg/kg of intravenous LNP-RNA treatment once a week for four weeks, resulting in a significant reduction of the HER2-expressing tumor growth and in increased survival of the mice [50].

In spite of the many successful examples of mAbs in oncology, the full antibodies have limited capacity to homogenously distribute through the tumor mass because of poor tissue penetration [51]. Hence, modifications of the antibody structure have been designed, leading to the generation of new types of antibody fragments endowed with better tissue prenetration [52]. However, small antibody fragments lack the Fc domain, which deprives them from the binding to the neonatal Fc receptor and, therefore, from the antibody recycling Fc-dependent pathway. Hence, the half-lives in circulation are greatly diminished. mRNA technology has been applied to overcome these limitations. Stadler *et*

al. used engineered mRNA to produce *in vivo* a bispecific single-chain fragment variable (scFv) molecule. They formulated several RNA-encoded bispecific constructions called RiboMABs, able to bind CD3 and to a tumor-associated antigen. The proof-of-concept has been achieved targeting CLDN6, CLDN18.2, and EpCAM as model tumor-associated antigens. These RNA-based formulations were injected intravenously, and the of 5µg dose was able to give a peak expression of 6 and 8 µg/ml in serum at 6 hours. The kinetics were considered more favorable than the kinetics of the recombinant protein counterpart, since the proteins were cleared more rapidly from circulation. The CD3xCLDN6 and CD3xEpCAM exerted antitumor activities as tested in OV-90 tumor-bearing NOD scid gamma (NGS) mice treated with 3µg of the LNP-RNA. Importantly, complete eradications of the tumors were observed. CD3xCLDN6 effectiveness was further confirmed on NGS mice bearing subcutaneous tumors expressing the CLDN6 protein, and antitumor effects were also detected on tumor cells that lacked CLDN6 [53].

3. mRNA-based expression of cytokines in the tumor microenvironment.

Cytokines are secreted proteins that play an essential role in the regulation of the immune system by controlling the proliferation, differentiation, effector functions, and survival of immune cells [54-56]. In some instances, cytokines can operate adsorbed into plasma membranes. Due to their pleiotropic effects on immune cells, cytokines have been explored since their initial discovery in the field of cancer immunotherapy, and several studies have demonstrated their antitumor activity, which has been translated to the clinic (Table 1) [56,57].

Numerous cytokines in the form of recombinant proteins, including interferon alpha (IFN-α), granulocyte macrophage colony-stimulating factor (GM-CSF), and interleukin (IL)- 2, IL-15, IL-12, and IL-18, among others, have been evaluated in clinical trials for patients with diverse types of cancer [57]. Despite the interest in cytokine-based approaches, to date only IL-2 and IFN-α have received approval by the FDA. This is because cytokine-based therapies have some shortcomings that needs to be overcome, such as their short half-life, low availability at the tumor metastatic site, and potential toxicities when intravenously delivered [56]. These drawbacks are exemplified by IL-2 therapy, where high doses are needed to provide a significant response rate but, at the same time, these high doses cause life-threatening toxicities [55]. Therefore, it is crucial to find strategies to enhance the therapeutic effects in the tumor while minimizing systemic toxicity [56]. Hence, several studies have explored new strategies based on mRNA as the vehicle of choice to direct deliver cytokines to the tumor microenvironment (TME) (Figure 2).

Tumor-infiltrating cytotoxic T lymphocytes display a dampened antitumoral activity due to the influence of a hostile tumor environment characterized by the presence of immunosuppressive elements [58]. Over the years, researchers have sought to modulate the TME, either through reactivation of immune cells or neutralizing the immunosuppressive environment. Interestingly, synergistic effects can be achieved when both such strategies are combined. Recombinant bifunctional molecules such as immunocytokines or bispecific antibodies can exert this dual activity, but their large-scale production can be challenging. *In vivo* production of these complex molecules can be achieved by mRNA-encoded constructs. Van der Jeught *et al.* evaluated $F\beta^2$ [59], a new mRNA-encoded fusokine composed of interferon beta (IFN-β) and the ectodomain of the TGF-β receptor II. This

fusokine has a dual role: (i) it increases immune reactivity by potentiating the antigen-presenting function of dendritic cells while (ii) decreasing TGF-β mediated immunosuppression. Treating E.G7-OVA or TC-1-bearing mice with three intratumoral injections in a three-day interval resulted in increased long-term survival. This effect is mediated by $CD8^+$ T cells since their depletion abrogated the observed therapeutic effect. Further, combining $F\beta^2$ with anti-PD-1 monoclonal antibody delayed tumor growth.

A second approach that can been therapeutically exploited thanks to mRNA technology is the intratumoral expression of cytokines. Local expression of cytokines increase their concentration in the target tissue, minimizing systemic toxicity. The potential of this strategy is best exemplified with interleukin 12 (IL-12). IL-12 is a heterodimeric cytokine that exerts potent effects on T cells and natural killer cells. IL-12 recombinant protein clinical trials have shown severe side effects due to systemic induction of IFNγ [12,60,61]. It has been shown that intratumoral injections of mRNA encoding IL-12 increased IFNγ expression, promoting a T helper 1 (Th1) transformation of the TME and, importantly, with no treatment-associated toxicities [60,62]. After a single intratumoral injection of IL-2 mRNA, robust tumor regression was observed in both injected and non-injected tumors. Furthermore, this work demonstrated that the combination of immune checkpoint inhibitors and IL-12 mRNA enhances the antitumor response improving overall survival and tumor regression in mouse tumor models resistant to checkpoint inhibitors [62]. These pre-clinical studies led to the development of MEDI119, a lipid nanoparticle (LNP) formulation with human IL-12 mRNA. This mRNA is currently being evaluated in a phase I clinical trial (NCT03946800) for intratumoral injection in combination with the anti-PD-L1 mAb durvalumab [62]. Similarly, a recent study investigated the therapeutic activity of IL-12

mRNA with intravenous injection in an MYC-driven transgenic mouse model of hepatocellular carcinoma (HCC). This study demonstrated that weekly intravenous injections reduced tumor burden due to the increased infiltration of $CD3^+$ $CD4^+$ $CD44^+$ T cells without altering MYC protein levels and apparently without setting off safety signals in the mice [63].

Another advantage of the mRNA technology is the possibility of expressing simultaneously synergistic combinations of cytokines. Hewitt and colleges [24] proposed combining T cell costimulatory molecules and inflammatory cytokines by the intratumoral injection of LPN-formulated mRNAs encoding OX40L, IL-23, and IL-36γ. Whereas these cytokines/costimulatory ligands in monotherapies exhibited little therapeutic effects, an increase in the efficacy was observed with the mRNA mixture, that resulted in complete responses after repeated administrations without notable adverse reactions. Remarkably, the triplet mRNA has not only a local but also a distant effect. After a single intratumoral injection, there was a 100% complete control of both treated and distal untreated tumors. This strategy is being evaluated in a clinical trial (NCT03739931). The mRNAs encapsulated in lipid nanoparticles are injected intratumorally, and the feasibility and safety are being evaluated alone or in combination with durvalumab, an anti-PD-L1 monoclonal antibody. Another mRNA combination that is being tested in clinical trials is SAR44100 (BNT131). This is a mixture of mRNAs encoding IL-12 single chain (IL-12sc), IL-15 with the sushi domain of the IL15Rα, IFNα, and GM-CSF. Pre-clinical studies determined that local administration of SAR44100 mediates successful antitumor immunity leading to tumor eradication in both treated and non-treated tumors. The combination of the mRNA mixture with immune checkpoint inhibitors improved the overall survival of mice because

of enhanced antitumor immune reactivity. Based on these pre-clinical studies, a phase I clinical trial (NCT03871348) was initiated in which SAR44100 is being administered intratumorally as monotherapy or in combination with cemiplimab, an anti-PD-1 monoclonal antibody, to patients with metastatic neoplasms [60].

4. RNA-engineered T lymphocytes

Adoptive T-cell therapy is currently the focus of well-deserved research and clinical attention. Adoptive T-cell transfer for cancer patients dates back to 1985 when Rosenberg *et al.* administered autologous T cells, *ex-vivo* expanded with IL-2, together with recombinant IL-2 inducing objective cancer regressions in 25 patients [64]. In the last decade, research on adoptive T-cell transfer has experienced an exponential growth, culminating in the recent regulatory approvals of chimeric antigen receptor (CAR) T cells for certain types of non-Hodgkin lymphoma, acute lymphoblastic leukemia, relapsed/refractory mantle cell lymphoma, and advanced multiple myeloma [65-69]. Retroviral/lentiviral transduction has been the method employed to genetically engineer therapeutic T lymphocytes. In spite of the fact that this strategy led to impressive clinical results, there are some limitations. The most relevant shortcomings are the potential risk of insertional mutagenesis and the possibility of off-target toxicities. One of the alternative strategies to develop safer T cell products is the transfection of T cells with messenger RNA because it is transient and non-integrative. Accordingly, there is no risks of insertional mutagenesis, and the potential side effects will be self-limiting. The transient nature of mRNA has been exploited to more safely express immunostimulatory molecules

to increase the antitumor effect of tumor-specific T lymphocytes or to confer naïve T cells with tumor-specificity by expressing transgenic T cell receptors (TCR) or CARs (Figure 3).

The first studies demonstrating the possibilities of inserting *in-vitro* transcribed mRNAs into T cells date back to the beginning of the 21st century when Smits *et al.* [70] electroporated GFP mRNA into T lymphocytes. They observed that only stimulated T cells could translate the electroporated mRNA, whereas the non-stimulated cells were not capable of this. The following year, Yangbing Zhao *et al.* [71] electroporated OKT3-stimulated peripheral blood lymphocytes with mRNAs encoding the α and β chains of HLA-A2-restricted TCRs recognizing NY-ESO-1, MART-1- and p53, and such T lymphocytes were able to secrete IFNγ upon antigen recognition. The cytotoxic capacity of electroporated T cells with mRNAs encoding TCRs was demonstrated for the first time with a TCR specific for HLA-A2/gp100 [72]. This strategy has been further refined by Campillo-Davo *et al.* [73]. They described a double sequential electroporation platform where Dicer-substrate small interfering RNAs (DsiRNA) are first introduced to attenuate endogenous TCR α and β expression. 24 hours later, a second electroporation is performed with a codon-optimized mRNA, that encodes a TCR against the Wilms' tumor protein 1 (WT1) and is made resistant to the previously applied siRNA silencing. This technology prevents TCR mispairing resulting in enhanced functionality of engineered T cells able to lyse the target cells at least *in vitro*.

RNA technology has also been evaluated to improve CAR T cell therapy. The research on transiently-engineered RNA-CAR T cells against hematological tumors has mainly focused on targeting CD19, and antitumoral efficacy has been shown in pre-clinical models against Daudi lymphoma [74] and in human leukemia xenografts [75,76]. One study by Almåsbak

et al. [77] demonstrated how co-electroporation of CD19-specific RNA-CAR and RNAs coding for CCR7 and CXCR4 improve T-cell chemotaxis *in vitro*. Interestingly, Liu *et al.* in 2016 engineered human T cells with an RNA coding for a CD19xCD3-bispecific T cell engager (BiTE). BiTEs were secreted to the milieu and enabled both the electroporated and bystander T cells to specifically recognize and kill $CD19^+$ cell lines. Proliferation, persistence, and increased cytokine production were observed, thus resulting in a more potent anti-leukemia growth effect as compared to CD19-RNA-CAR T cells [78].

The promising results in pre-clinical models led the University of Pennsylvania to start the first clinical trial using non-viral CAR T cell therapy with an RNA-CAR targeting CD19 for relapsed classical Hodgkin's lymphoma (registered as NCT02277522 for adults and NCT02624258 for children) (Table 2). The four patients infused with the specified cell dose showed transient clinical responses with no severe toxicities [79].

Several targets other than CD19 have been investigated for RNA-CAR T cell therapy (Table 2). In the case of CD20, the cellular product was well tolerated and led to a modest and transient antitumor activity in a dog with relapsed B cell lymphoma [80]. The RNA-CAR strategy was particularly useful in the case of CD33. The transient nature of RNA in this strategy was able to overcome the toxicity caused by stably transduced T cells with a CD33 CAR [81]. Moreover, in the case of the CD123-directed CAR, the transiently engineered T cells outperformed the conventional CAR T cells in acute myeloid leukemia, and were much less toxic given the fact that CD123 is also expressed by normal hematopoietic cells and their progeny, as well as on some endothelial cells [82]. The University of Pennsylvania sought to test the feasibility and toxicity of T cells electroporated with anti-CD123 CAR mRNA in 7 patients with relapsed/refractory acute

myeloid leukemia (NCT02623582). The several cell infusions were safe, but the approach showed limitations because of the poor quality of patients' T cells that led to technical problems manufacturing the planned number of doses and the lack of CAR T cell persistence. B-cell maturation antigen (BCMA)-specific mRNA CAR-T cells have been developed in multiple myeloma. Two versions have been tested in clinical trials: Descartes-08 and Descartes-11, an optimized/humanized version of the first one. Descartes-08 showed durable clinical responses without significant toxicity (NCT03448978). A phase I study (NCT03994705) of 18 patients has already been undertaken with Descartes-11, and results showed no evidence of cytokine release syndrome or neurotoxicity, and a phase IIa study (NCT04436029) aims to enroll 30 patients in a single-arm multicenter study.

In 2020, Parayath *et al.* reported a different approach in the RNA CAR field. They showed that transient reprogramming of host T cells directly *in vivo* was feasible. For this purpose, investigators used the systemic administration of T cell-directed biodegradable nanoparticles containing either CD19-specific or ROR1-specific RNA-CAR that were able to control established leukemia tumors and prostate cancers in NSG mice, respectively. *In vivo* engineered T cells with this technology are able to control tumor growth with an efficiency comparable to stably transduced T cells. If translated to humans, this strategy has the outstanding potential to make T cell reprogramming more accessible to patients by overcoming the elaborate and expensive expansion and modification of T cells *ex vivo*. Still, this approach has limitations because its success depends on the presence of a sufficient number of T cells in the patient, and its efficacy could be blunted by the generation of anti-drug antibodies [83].

The success of CAR T cells for hematological neoplasias has sparked interest in translating the benefit of these advanced therapies to solid tumors. RNA-CAR modified T cells have been evaluated in solid tumors targeting Her-2/neu, ErbB2, CEA, mesothelin, GD2, FRα, MCSP, VEGFR2, EPCAM, EGFR, and c-MET. In all cases, the RNA-CAR T cells demonstrated to have an anti-tumor effect in pre-clinical murine models against murine or human tumors expressing the cognate targets [84-93].

Particularly promising pre-clinical results have been obtained by administering T cells engineered with mRNA coding for CAR specific for mesothelin, a protein overexpressed in mesothelioma (meso-RNA-CAR-T cells) [86]. This led the University of Pennsylvania to conduct two first-in-human studies to test the safety of multiple infusions of meso-RNA-CAR-T cells (NCT01355965 with subjects with malignant pleural mesothelioma and NCT01897415 with subjects with metastatic pancreatic ductal adenocarcinoma). Out of 21 infusions of meso-RNA-CAR-T cells (given to 4 patients), one infusion caused anaphylaxis and cardiac arrest within minutes of completing the third infusion (given four weeks after the second one) [94]. This dismal result forced the sponsor to modify the ongoing clinical trials to prohibit infusion breaks lasting more than ten days to avoid the IgE class switching that can occur during this time interval. In a subsequent follow-up study, Beatty *et al.* [95] reported that the treatment mediated partial antitumor activity *in vivo*, including the induction of epitope-spreading and the generation of antitumor IgG antibodies against the tumor that were not present before starting therapy. Such a phenomenon was also observed also in another follow-up study in 6 patients with chemotherapy-refractory metastatic pancreatic ductal adenocarcinoma [96].

Another RNA-CAR T cell product evaluated in clinical trials is RNA-CAR T cells against c-MET, a tyrosine kinase receptor up-regulated in various solid tumors, notably in approximately 50% of breast cancers. Intratumoral injection in s.c. established human ovarian cancer in NSG mice controlled tumor growth. Given the promising results, the University of Pennsylvania initiated an open-label phase 0 clinical trial (NCT01837602) to evaluate the safety and feasibility of mRNA c-Met-CAR T cells in treating metastatic c-Met^{+} breast cancer with a single intratumoral injection. The treatment was well tolerated, evoked an inflammatory response with extensive tumor necrosis at the injection site and recruitment of macrophages. Nonetheless, no clinical responses were observed in these patients. A phase I trial (NCT03060356) was conducted and consisted of intravenously administering autologous cMET-directed RNA-CAR T cells in patients with melanoma and breast carcinoma. The treatment was safe and feasible and stabilized the disease in 4 patients out of 77 [97].

In addition to synthetic TCR or CAR generation, T cell effector functions can be augmented upon transfection with immune enhancers, such as cytokines, immunotoxins, and ligands of activating cytotoxic receptors. In 2012, Lehner *et al.* transiently modified T cells with an mRNA coding for NKG2D-L, which triggered the elimination of Ewing's sarcoma tumor cell lines *in vitro* [98]. Pato and colleagues in 2015 electroporated tumor-infiltrating T lymphocytes (TILs), prepared from melanoma patients, with a constitutively active TLR-4, which enhanced their cytolytic activity and increased the secretion of IFN-γ, TNF, and GM-CSF for at least four days post-transfection [99]. One year later, the same group demonstrated that short-term cultured, unselected “young” TILs could be electroporated with an efficiency comparable to conventionally selected TILs. These

investigators aimed at potentiating the immune functions of T cells by electroporating them with mRNAs encoding membrane-anchored IL-2, IL-12, and IL-15. The strategy of anchoring cytokines to the membrane was designed to avoid systemic toxicity and to avoid expanding regulatory T cells. Moreover, the constructs were designed to activate their receptor *in cis*, maximizing the availability of the expressed cytokines to the therapeutic T cells [100].

Another strategy to increase the performance of T cells was to electroporate them with mRNA encoding constitutively active CD40 [101]. This optimized CD40 could markedly upregulate the activation of T cells and costimulatory molecules such as CD137, OX40, CD28, CD25, and CD127 in anti-melanoma selected TILs, young TILs, $CD8^+$, and $CD4^+$ T cells isolated from PBLs.

Recently, tumor-specific T cells modified with mRNA encoding GM-CSF were evaluated to treat orthotopic murine brain tumors. Specifically, systemic injection of RNA-electroporated OTI T cells significantly enhanced the survival of mice bearing B16F10-OVA intracranial tumors. Such responses were associated with increased secretion of IFN-γ in the tumor microenvironment and systemic antigen-specific T cell expansion [102]. The strategy of using mRNA encoding immunostimulatory cytokines in tumor-specific T cells was exploited also for IL-12 [12]. Tumor-specific T cells were engineered with mRNA encoding the murine single-chain IL-12. Interestingly, administration of engineered OTI T cells injected intratumorally, but not intravenously, led to complete rejections of both treated B16-OVA tumors and distant contralateral tumors. Therapeutic efficacy was further enhanced by co-injection of agonist anti-CD137 mAb or by co-electroporation of mRNA coding for CD137L. The IL-12 OTI treatment induced epitope spreading of the endogenous

$CD8^+$ T cell population in a cDC1-dependent manner, as BAFT3-KO mice showed a significantly reduced percentage of tumor-specific endogenous $CD8^+$ T cells compared to wild type mice. This strategy could be clinically feasible since the authors successfully electroporated patient-derived TILs and intratumorally treated the related patient-derived xenograft, achieving the tumor growth control compared to mock electroporated cells.

5. Conclusions

Messenger RNA technology has undergone a great development in the field of vaccination. This technology has been validated in the field of prophylactic vaccines against COVID-19 disease, leading to the first approvals of RNA-based drugs. Therapeutic vaccination against cancer is being pursued in the clinic with shared tumor antigens and personalized neoantigen-based mRNA vaccines and other applications of this technology in the field of cancer immunotherapy have been evaluated preclinically and in clinical trials (Table 1 and 2). In the case of antibody-based immunotherapies, mRNA allows the production of different formats of antibodies directly in the target organ or in a distant tissue, that is used as a factory to produce the therapeutic antibody. Proof-of-principle preclinical studies have demonstrated the potential of this approach, but there are not yet clinical trials evaluating the feasibility in humans of the mRNA-based antibody expression for cancer immunotherapy, even though trials with antiviral antibodies are in progress. The second type of immunotherapy that can benefit from RNA technologies involves the use of cytokines. Preclinical data have demonstrated the interest of mRNA in attaining local expression in the tumor microenvironment of cytokines, cytokine combinations, or immunocytokines. The use of mRNA-based strategies has shown improved antitumor

efficacy with limited systemic toxicity. These preclinical data have supported the quick translation to clinical trials of different strategies based on the mRNA-mediated expression of cytokines (Table 1). Finally, mRNA technology is also of interest in the field of adoptive T-cell transfer. T-lymphocyte engineering has first been approved for clinical use in the case of retrovirus or lentivirus transduced T lymphocytes expressing chimeric antigen receptors against CD19 or BCMA. Persistence of the transduced T lymphocytes is often associated with long-term control of the disease. In spite of this, the transient expression offered by mRNA is sufficient to engineer potent immunotherapies that otherwise may lead to unacceptable toxicity upon chronic exposure. Moreover, transient mRNA transfer of antigens recognized by CAR T cells is being tested in the clinic to boost therapeutic efficacy (NCT04503278). In addition to the *ex vivo* protocols that are being evaluated in the clinic (Table 2), non-viral vectors can also be used for *in vivo* delivery of mRNA to T lymphocytes.

6. Expert opinion

The great potential of cancer immunotherapy is well demonstrated by checkpoint inhibitors and by adoptive cell therapy with CAR T cells. Currently approved immunotherapies represent the backbone on which to build combination strategies that extend the therapeutic benefit to patients with primary and secondary resistance to immunotherapy. New targets and new technologies to exploit the full potential of these mechanisms are being quickly translated from pre-clinical research to clinical evaluation. mRNA-based strategies can play a key role in coming years. mRNA is a safe and well-tolerated vehicle able to deliver and induce expression of relevant proteins such as monoclonal antibodies, cytokines, and

chimeric immune receptors in cancer immunotherapy. Synthetic mRNA molecules can be quickly designed, modified and cost-effectively produced on a large scale [103]. Reduction of the manufacturing costs is considered advantageous when compared to recombinant protein-based molecules. This is in part due to the fact that mRNAs encoding for different therapeutic proteins chemically differ only in their nucleotide sequences, and therefore, the physicochemical properties of different therapeutic mRNAs are highly similar, thereby allowing standardization of the production methods. Thus, this emerging technology may overcome manufacturing-related limitations and creates opportunities for local and transient expression of molecules that may otherwise be prohibitively toxic. The approval of COVID-19 mRNA vaccines represents a milestone, but the technology can be greatly optimized since certain limitations are yet to be overcome. First, it is a pharmaceutical technology to which regulatory agencies have to adapt. The inclusion of mRNA-based agents in the guidelines of these agencies will allow smoothing of these regulatory processes [104]. Second, the *in situ* production of the therapeutic protein can give rise to interpatient variability depending on the particular characteristics of the target tissue. This variability can lead to toxic concentrations of the protein of interest or expression levels below the therapeutic threshold in some of the patients. Other limitations that must be taken into account are possible toxicities due to accumulation of the components of the LNP. Development of safer and more effective formulations will be key for a broader success of this technology. Finally, transient expression is an inherent feature of this technology. Although this may be an advantage for the expression of highly potent (and toxic) molecules, the stable expression that can be achieved by gene therapy vectors such as lentiviruses are generally considered an advantage in the field of adoptive transfer of T lymphocytes.

All in all, in our opinion, the technology is ready for primetime, but further refinement in the delivery vectors will foster industrial interest and standardize feasible applications in cancer immunotherapy. In the case of antibodies and cytokines, mRNA technology will be especially suitable for: i) overcoming the pharmacokinetic limitations of cytokines or bispecific antibody fragments devoid of the Fc domain, ii) restraining the expression to the tumor tissue, limiting the systemic toxicity, and iii) allowing expression of synergistic combinations. In the field of adoptive T cell transfer, RNA-reprogramming overcomes some therapy-related limitations but intrinsically brings others. On the one hand, the use of RNA avoids the risk of insertional mutagenesis, and its transient nature blunts some of the toxicities. On the other hand, the mRNA expression half-life is not sufficient to induce durable effects in most instances. Thus, an optimal treatment regimen remains to be determined, including dose selection and number of T cell infusions, since multiple injections of CAR T cells may induce anaphylaxis, as learned from the meso-RNA-CAR-T cell clinical trial [94]. Moreover, the cost and regulatory constraints associated with manufacturing T cells *ex vivo* can be excessive, so transiently programming T cells *in vivo* may be an advantageous alternative.

mRNA therapy is in its early infancy but offers room for improvements, sophistications, and discoveries that might make it the biotechnology of choice for novel approaches, including synergistic combinations.

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7. References

Papers of special note have been highlighted as either of interest (●) or of considerable interest (●●) to readers.

1. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011 Mar 4;144(5):646-74.
2. Sanmamed MF, Chen L. A Paradigm Shift in Cancer Immunotherapy: From Enhancement to Normalization. Cell. 2018 Oct 4;175(2):313-326.
3. Upadhaya S, Neftelino ST, Hodge JP, et al. Combinations take centre stage in PD1/PDL1 inhibitor clinical trials. Nature reviews Drug discovery. 2021 Mar;20(3):168-169.
4. Rafiq S, Hackett CS, Brentjens RJ. Engineering strategies to overcome the current roadblocks in CAR T cell therapy. Nature reviews Clinical oncology. 2020 Mar;17(3):147-167.
5. Pastor F, Berraondo P, Etxeberria I, et al. An RNA toolbox for cancer immunotherapy. Nature reviews Drug discovery. 2018 Oct;17(10):751-767.

6. Kariko K, Muramatsu H, Welsh FA, et al. Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. Molecular therapy : the journal of the American Society of Gene Therapy. 2008 Nov;16(11):1833-40. ●● First strategy to deimmunize mRNA based on the incorporation aturally modified nucleotides.

7. Krienke C, Kolb L, Diken E, et al. A noninflammatory mRNA vaccine for treatment of experimental autoimmune encephalomyelitis. Science. 2021 Jan 8;371(6525):145-153.

8. Wesselhoeft RA, Kowalski PS, Anderson DG. Engineering circular RNA for potent and stable translation in eukaryotic cells. Nature communications. 2018 Jul 6;9(1):2629. ●● First description of a methodology to produce circular RNAs.

9. Wesselhoeft RA, Kowalski PS, Parker-Hale FC, et al. RNA Circularization Diminishes Immunogenicity and Can Extend Translation Duration In Vivo. Molecular cell. 2019 May 2;74(3):508-520 e4.

10. Geall AJ, Verma A, Otten GR, et al. Nonviral delivery of self-amplifying RNA vaccines. Proceedings of the National Academy of Sciences of the United States of America. 2012 Sep 4;109(36):14604-9.

11. Morse MA, Nair SK, Boczkowski D, et al. The feasibility and safety of immunotherapy with dendritic cells loaded with CEA mRNA following neoadjuvant chemoradiotherapy and resection of pancreatic cancer. International journal of gastrointestinal cancer. 2002;32(1):1-6.

12. Etxeberria I, Bolanos E, Quetglas JI, et al. Intratumor Adoptive Transfer of IL-12 mRNA Transiently Engineered Antitumor CD8(+) T Cells. Cancer cell. 2019 Dec 9;36(6):613-629 e7.

13. Van Tendeloo VF, Ponsaerts P, Lardon F, et al. Highly efficient gene delivery by mRNA electroporation in human hematopoietic cells: superiority to lipofection and passive pulsing of mRNA and to electroporation of plasmid cDNA for tumor antigen loading of dendritic cells. Blood. 2001 Jul 1;98(1):49-56.

14. Boissel L, Betancur M, Wels WS, et al. Transfection with mRNA for CD19 specific chimeric antigen receptor restores NK cell mediated killing of CLL cells. Leukemia research. 2009 Sep;33(9):1255-9.

15. Golombek S, Pilz M, Steinle H, et al. Intradermal Delivery of Synthetic mRNA Using Hollow Microneedles for Efficient and Rapid Production of Exogenous Proteins in Skin. Molecular therapy Nucleic acids. 2018 Jun 1;11:382-392.

16. Probst J, Weide B, Scheel B, et al. Spontaneous cellular uptake of exogenous messenger RNA in vivo is nucleic acid-specific, saturable and ion dependent. Gene therapy. 2007 Aug;14(15):1175-80.

17. Van Lint S, Renmans D, Broos K, et al. Intratumoral Delivery of TriMix mRNA Results in T-cell Activation by Cross-Presenting Dendritic Cells. Cancer immunology research. 2016 Feb;4(2):146-56.

18. Gomez-Aguado I, Rodriguez-Castejon J, Vicente-Pascual M, et al. Nanomedicines to Deliver mRNA: State of the Art and Future Perspectives. Nanomaterials. 2020 Feb 20;10(2).

19. Polack FP, Thomas SJ, Kitchin N, et al. Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. The New England journal of medicine. 2020 Dec 31;383(27):2603-2615. ●● Clinical trial that lead to the approval of one of the first mRNA-based vaccines.

20. Baden LR, El Sahly HM, Essink B, et al. Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine. The New England journal of medicine. 2021 Feb 4;384(5):403-416. ●● Clinical trial that lead to the approval of one of the first mRNA-based vaccines.

21. Berraondo P, Martini PGV, Avila MA, et al. Messenger RNA therapy for rare genetic metabolic diseases. Gut. 2019 Jul;68(7):1323-1330.

22. Vermeulen LMP, Brans T, Samal SK, et al. Endosomal Size and Membrane Leakiness Influence Proton Sponge-Based Rupture of Endosomal Vesicles. ACS nano. 2018 Mar 27;12(3):2332-2345.

23. Buschmann MD, Carrasco MJ, Alishetty S, et al. Nanomaterial Delivery Systems for mRNA Vaccines. Vaccines. 2021 Jan 19;9(1).

24. Hewitt SL, Bai A, Bailey D, et al. Durable anticancer immunity from intratumoral administration of IL-23, IL-36gamma, and OX40L mRNAs. Science translational medicine. 2019 Jan 30;11(477). ● First demonstration of the feasibility of intratumoral administration of a mixture of mRNAs that encode proinflammatory cytokines

25. Sahin U, Derhovanessian E, Miller M, et al. Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. Nature. 2017 Jul 13;547(7662):222-226. ●● Clinical trial that demonstrated the feasibiltity of the personalized antitumor vaccines.

26. Kranz LM, Diken M, Haas H, et al. Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy. Nature. 2016 Jun 16;534(7607):396-401.

27. Van Lint S, Goyvaerts C, Maenhout S, et al. Preclinical evaluation of TriMix and antigen mRNA-based antitumor therapy. Cancer research. 2012 Apr 1;72(7):1661-71.

28. Weiner LM, Surana R, Wang S. Monoclonal antibodies: versatile platforms for cancer immunotherapy. Nature reviews Immunology. 2010 May;10(5):317-27.

29. Kunert R, Reinhart D. Advances in recombinant antibody manufacturing. Applied microbiology and biotechnology. 2016 Apr;100(8):3451-61.

30. Lu RM, Hwang YC, Liu IJ, et al. Development of therapeutic antibodies for the treatment of diseases. Journal of biomedical science. 2020 Jan 2;27(1):1.

31. Scott AM, Allison JP, Wolchok JD. Monoclonal antibodies in cancer therapy. Cancer immunity. 2012;12:14.

32. Vazquez-Rey M, Lang DA. Aggregates in monoclonal antibody manufacturing processes. Biotechnology and bioengineering. 2011 Jul;108(7):1494-508.

33. Xu Z, Li J, Zhou JX. Process development for robust removal of aggregates using cation exchange chromatography in monoclonal antibody purification with implementation of quality by design. Preparative biochemistry & biotechnology. 2012;42(2):183-202.

34. van der Kant R, Karow-Zwick AR, Van Durme J, et al. Prediction and Reduction of the Aggregation of Monoclonal Antibodies. Journal of molecular biology. 2017 Apr 21;429(8):1244-1261.

35. Johnson PR, Schnepp BC, Zhang J, et al. Vector-mediated gene transfer engenders long-lived neutralizing activity and protection against SIV infection in monkeys. Nature medicine. 2009 Aug;15(8):901-6.

36. Lewis AD, Chen R, Montefiori DC, et al. Generation of neutralizing activity against human immunodeficiency virus type 1 in serum by antibody gene transfer. Journal of virology. 2002 Sep;76(17):8769-75.

37. Balazs AB, Chen J, Hong CM, et al. Antibody-based protection against HIV infection by vectored immunoprophylaxis. Nature. 2011 Nov 30;481(7379):81-4.

38. Reul J, Frisch J, Engeland CE, et al. Tumor-Specific Delivery of Immune Checkpoint Inhibitors by Engineered AAV Vectors. Frontiers in oncology. 2019;9:52.

39. Silva-Pilipich N, Martisova E, Ballesteros-Briones MC, et al. Long-Term Systemic Expression of a Novel PD-1 Blocking Nanobody from an AAV Vector Provides Antitumor Activity without Toxicity. Biomedicines. 2020 Dec 2;8(12).

40. Colella P, Ronzitti G, Mingozzi F. Emerging Issues in AAV-Mediated In Vivo Gene Therapy. Molecular therapy Methods & clinical development. 2018 Mar 16;8:87-104.

41. Wise MC, Xu Z, Tello-Ruiz E, et al. In vivo delivery of synthetic DNA-encoded antibodies induces broad HIV-1-neutralizing activity. The Journal of clinical investigation. 2020 Feb 3;130(2):827-837.

42. Patel A, Park DH, Davis CW, et al. In Vivo Delivery of Synthetic Human DNA-Encoded Monoclonal Antibodies Protect against Ebolavirus Infection in a Mouse Model. Cell reports. 2018 Nov 13;25(7):1982-1993 e4.

43. Esquivel RN, Patel A, Kudchodkar SB, et al. In Vivo Delivery of a DNA-Encoded Monoclonal Antibody Protects Non-human Primates against Zika Virus. Molecular therapy : the journal of the American Society of Gene Therapy. 2019 May 8;27(5):974-985.

44. Patel A, DiGiandomenico A, Keller AE, et al. An engineered bispecific DNA-encoded IgG antibody protects against Pseudomonas aeruginosa in a pneumonia challenge model. Nature communications. 2017 Sep 21;8(1):637.

45. Muthumani K, Marnin L, Kudchodkar SB, et al. Novel prostate cancer immunotherapy with a DNA-encoded anti-prostate-specific membrane antigen monoclonal antibody. Cancer immunology, immunotherapy : CII. 2017 Dec;66(12):1577-1588.

46. Duperret EK, Trautz A, Stoltz R, et al. Synthetic DNA-Encoded Monoclonal Antibody Delivery of Anti-CTLA-4 Antibodies Induces Tumor Shrinkage In Vivo. Cancer research. 2018 Nov 15;78(22):6363-6370.

47. Pardi N, Secreto AJ, Shan X, et al. Administration of nucleoside-modified mRNA encoding broadly neutralizing antibody protects humanized mice from HIV-1 challenge. Nature communications. 2017 Mar 2;8:14630. ● First evidence of immune protection against virus conferred by in vivo RNA-encoded antibodies.

48. Thran M, Mukherjee J, Ponisch M, et al. mRNA mediates passive vaccination against infectious agents, toxins, and tumors. EMBO molecular medicine. 2017 Oct;9(10):1434-1447.

49. Kose N, Fox JM, Sapparapu G, et al. A lipid-encapsulated mRNA encoding a potently neutralizing human monoclonal antibody protects against chikungunya infection. Science immunology. 2019 May 17;4(35).

50. Rybakova Y, Kowalski PS, Huang Y, et al. mRNA Delivery for Therapeutic Anti-HER2 Antibody Expression In Vivo. Molecular therapy : the journal of the American Society of Gene Therapy. 2019 Aug 7;27(8):1415-1423.

51. Yokota T, Milenic DE, Whitlow M, et al. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. Cancer research. 1992 Jun 15;52(12):3402-8.

52. Nelson AL. Antibody fragments: hope and hype. mAbs. 2010 Jan-Feb;2(1):77-83.

53. Stadler CR, Bahr-Mahmud H, Celik L, et al. Elimination of large tumors in mice by mRNA-encoded bispecific antibodies. Nature medicine. 2017 Jul;23(7):815-817. ●● First evidence of in vivo RNA-encoded single-chain bispecific T-cell engagers

54. Waldmann TA. Cytokines in Cancer Immunotherapy. Cold Spring Harbor perspectives in biology. 2018 Dec 3;10(12).

55. Jiang T, Zhou C, Ren S. Role of IL-2 in cancer immunotherapy. Oncoimmunology. 2016 Jun;5(6):e1163462.

56. Berraondo P, Sanmamed MF, Ochoa MC, et al. Cytokines in clinical cancer immunotherapy. British journal of cancer. 2019 Jan;120(1):6-15.

57. Lee S, Margolin K. Cytokines in cancer immunotherapy. Cancers. 2011 Oct 13;3(4):3856-93.

58. Chulpanova DS, Kitaeva KV, Green AR, et al. Molecular Aspects and Future Perspectives of Cytokine-Based Anti-cancer Immunotherapy. Frontiers in cell and developmental biology. 2020;8:402.

59. Van der Jeught K, Joe PT, Bialkowski L, et al. Intratumoral administration of mRNA encoding a fusokine consisting of IFN-beta and the ectodomain of the TGF-beta receptor II potentiates antitumor immunity. Oncotarget. 2014 Oct 30;5(20):10100-13.

60. Nguyen KG, Vrabel MR, Mantooth SM, et al. Localized Interleukin-12 for Cancer Immunotherapy. Frontiers in immunology. 2020;11:575597.

61. Rakhit A, Yeon MM, Ferrante J, et al. Down-regulation of the pharmacokinetic-pharmacodynamic response to interleukin-12 during long-term administration to patients with renal cell carcinoma and evaluation of the mechanism of this "adaptive response" in mice. Clinical pharmacology and therapeutics. 1999 Jun;65(6):615-29.

62. Hewitt SL, Bailey D, Zielinski J, et al. Intratumoral IL12 mRNA Therapy Promotes TH1 Transformation of the Tumor Microenvironment. Clinical cancer research : an official journal of the American Association for Cancer Research. 2020 Dec 1;26(23):6284-6298. ● Preclinical data that supports the first clinical trial on intratumoral administration of an mRNA encoding IL-12.

63. Lai I, Swaminathan S, Baylot V, et al. Lipid nanoparticles that deliver IL-12 messenger RNA suppress tumorigenesis in MYC oncogene-driven hepatocellular carcinoma. Journal for immunotherapy of cancer. 2018 Nov 20;6(1):125.

64. Rosenberg SA, Lotze MT, Muul LM, et al. Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. The New England journal of medicine. 1985 Dec 5;313(23):1485-92.

65. Munshi NC, Anderson LD, Jr., Shah N, et al. Idecabtagene Vicleucel in Relapsed and Refractory Multiple Myeloma. The New England journal of medicine. 2021 Feb 25;384(8):705-716.

66. Wang M, Munoz J, Goy A, et al. KTE-X19 CAR T-Cell Therapy in Relapsed or Refractory Mantle-Cell Lymphoma. The New England journal of medicine. 2020 Apr 2;382(14):1331-1342.

67. Schuster SJ, Bishop MR, Tam CS, et al. Tisagenlecleucel in Adult Relapsed or Refractory Diffuse Large B-Cell Lymphoma. The New England journal of medicine. 2019 Jan 3;380(1):45-56.

68. Park JH, Riviere I, Gonen M, et al. Long-Term Follow-up of CD19 CAR Therapy in Acute Lymphoblastic Leukemia. The New England journal of medicine. 2018 Feb 1;378(5):449-459.

69. Neelapu SS, Locke FL, Bartlett NL, et al. Axicabtagene Ciloleucel CAR T-Cell Therapy in Refractory Large B-Cell Lymphoma. The New England journal of medicine. 2017 Dec 28;377(26):2531-2544.

70. Smits E, Ponsaerts P, Lenjou M, et al. RNA-based gene transfer for adult stem cells and T cells. Leukemia. 2004 Nov;18(11):1898-902.

71. Zhao Y, Zheng Z, Cohen CJ, et al. High-efficiency transfection of primary human and mouse T lymphocytes using RNA electroporation. Molecular therapy : the journal of the American Society of Gene Therapy. 2006 Jan;13(1):151-9.

72. Schaft N, Dorrie J, Muller I, et al. A new way to generate cytolytic tumor-specific T cells: electroporation of RNA coding for a T cell receptor into T lymphocytes. Cancer immunology, immunotherapy : CII. 2006 Sep;55(9):1132-41.

73. Campillo-Davo D, Fujiki F, Van den Bergh JMJ, et al. Efficient and Non-genotoxic RNA-Based Engineering of Human T Cells Using Tumor-Specific T Cell Receptors With Minimal TCR Mispairing. Frontiers in immunology. 2018;9:2503.

74. Rabinovich PM, Komarovskaya ME, Wrzesinski SH, et al. Chimeric receptor mRNA transfection as a tool to generate antineoplastic lymphocytes. Human gene therapy. 2009 Jan;20(1):51-61.

75. Barrett DM, Liu X, Jiang S, et al. Regimen-specific effects of RNA-modified chimeric antigen receptor T cells in mice with advanced leukemia. Human gene therapy. 2013 Aug;24(8):717-27.

76. Foster JB, Choudhari N, Perazzelli J, et al. Purification of mRNA Encoding Chimeric Antigen Receptor Is Critical for Generation of a Robust T-Cell Response. Human gene therapy. 2019 Feb;30(2):168-178.

77. Almasbak H, Rian E, Hoel HJ, et al. Transiently redirected T cells for adoptive transfer. Cytotherapy. 2011 May;13(5):629-40.

78. Liu X, Barrett DM, Jiang S, et al. Improved anti-leukemia activities of adoptively transferred T cells expressing bispecific T-cell engager in mice. Blood cancer journal. 2016 Jun 3;6(6):e430. ● This is the first published paper where transferred T cells are electroporated with a mRNA coding for an antibody, with a BITE format particularly.

79. Svoboda J, Rheingold SR, Gill SI, et al. Nonviral RNA chimeric antigen receptor-modified T cells in patients with Hodgkin lymphoma. Blood. 2018 Sep 6;132(10):1022-1026.

80. Panjwani MK, Smith JB, Schutsky K, et al. Feasibility and Safety of RNA-transfected CD20-specific Chimeric Antigen Receptor T Cells in Dogs with Spontaneous B Cell Lymphoma. Molecular therapy : the journal of the American Society of Gene Therapy. 2016 Sep;24(9):1602-14.

81. Kenderian SS, Ruella M, Shestova O, et al. CD33-specific chimeric antigen receptor T cells exhibit potent preclinical activity against human acute myeloid leukemia. Leukemia. 2015 Aug;29(8):1637-47.

82. Tasian SK, Kenderian SS, Shen F, et al. Optimized depletion of chimeric antigen receptor T cells in murine xenograft models of human acute myeloid leukemia. Blood. 2017 Apr 27;129(17):2395-2407.

83. Parayath NN, Stephan SB, Koehne AL, et al. In vitro-transcribed antigen receptor mRNA nanocarriers for transient expression in circulating T cells in vivo. Nature communications. 2020 Nov 27;11(1):6080. ●● First demonstration of the feasibility of T cell transient engineering in vivo with T cell-directed RNA-nanoparticles, in murine models. .

84. Yoon SH, Lee JM, Cho HI, et al. Adoptive immunotherapy using human peripheral blood lymphocytes transferred with RNA encoding Her-2/neu-specific chimeric immune receptor in ovarian cancer xenograft model. Cancer gene therapy. 2009 Jun;16(6):489-97.

85. Birkholz K, Hombach A, Krug C, et al. Transfer of mRNA encoding recombinant immunoreceptors reprograms CD4+ and CD8+ T cells for use in the adoptive immunotherapy of cancer. Gene therapy. 2009 May;16(5):596-604.

86. Zhao Y, Moon E, Carpenito C, et al. Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor. Cancer research. 2010 Nov 15;70(22):9053-61.

87. Hung CF, Xu X, Li L, et al. Development of Anti-Human Mesothelin-Targeted Chimeric Antigen Receptor Messenger RNA-Transfected Peripheral Blood Lymphocytes for Ovarian Cancer Therapy. Human gene therapy. 2018 May;29(5):614-625.

88. Singh N, Liu X, Hulitt J, et al. Nature of tumor control by permanently and transiently modified GD2 chimeric antigen receptor T cells in xenograft models of neuroblastoma. Cancer immunology research. 2014 Nov;2(11):1059-70.

89. Schutsky K, Song DG, Lynn R, et al. Rigorous optimization and validation of potent RNA CAR T cell therapy for the treatment of common epithelial cancers expressing folate receptor. Oncotarget. 2015 Oct 6;6(30):28911-28.

90. Krug C, Birkholz K, Paulus A, et al. Stability and activity of MCSP-specific chimeric antigen receptors (CARs) depend on the scFv antigen-binding domain and the protein backbone. Cancer immunology, immunotherapy : CII. 2015 Dec;64(12):1623-35.

91. Inoo K, Inagaki R, Fujiwara K, et al. Immunological quality and performance of tumor vessel-targeting CAR-T cells prepared by mRNA-EP for clinical research. Molecular therapy oncolytics. 2016;3:16024.

92. Ang WX, Li Z, Chi Z, et al. Intraperitoneal immunotherapy with T cells stably and transiently expressing anti-EpCAM CAR in xenograft models of peritoneal carcinomatosis. Oncotarget. 2017 Feb 21;8(8):13545-13559.

93. Caruso HG, Torikai H, Zhang L, et al. Redirecting T-Cell Specificity to EGFR Using mRNA to Self-limit Expression of Chimeric Antigen Receptor. Journal of immunotherapy. 2016 Jun;39(5):205-17.

94. Maus MV, Haas AR, Beatty GL, et al. T cells expressing chimeric antigen receptors can cause anaphylaxis in humans. Cancer immunology research. 2013 Jul;1(1):26-31.

95. Beatty GL, Haas AR, Maus MV, et al. Mesothelin-specific chimeric antigen receptor mRNA-engineered T cells induce anti-tumor activity in solid malignancies. Cancer immunology research. 2014 Feb;2(2):112-20.

96. Beatty GL, O'Hara MH, Lacey SF, et al. Activity of Mesothelin-Specific Chimeric Antigen Receptor T Cells Against Pancreatic Carcinoma Metastases in a Phase 1 Trial. Gastroenterology. 2018 Jul;155(1):29-32.

97. Tchou J, Zhao Y, Levine BL, et al. Safety and Efficacy of Intratumoral Injections of Chimeric Antigen Receptor (CAR) T Cells in Metastatic Breast Cancer. Cancer immunology research. 2017 Dec;5(12):1152-1161.

98. Lehner M, Gotz G, Proff J, et al. Redirecting T cells to Ewing's sarcoma family of tumors by a chimeric NKG2D receptor expressed by lentiviral transduction or mRNA transfection. PloS one. 2012;7(2):e31210.

99. Pato A, Eisenberg G, Machlenkin A, et al. Messenger RNA encoding constitutively active Toll-like receptor 4 enhances effector functions of human T cells. Clinical and experimental immunology. 2015 Nov;182(2):220-9.

100. Weinstein-Marom H, Pato A, Levin N, et al. Membrane-attached Cytokines Expressed by mRNA Electroporation Act as Potent T-Cell Adjuvants. Journal of immunotherapy. 2016 Feb-Mar;39(2):60-70.

101. Levin N, Weinstein-Marom H, Pato A, et al. Potent Activation of Human T Cells by mRNA Encoding Constitutively Active CD40. Journal of immunology. 2018 Nov 15;201(10):2959-2968.

102. Pohl-Guimaraes F, Yang C, Dyson KA, et al. RNA-Modified T Cells Mediate Effective Delivery of Immunomodulatory Cytokines to Brain Tumors. Molecular

therapy : the journal of the American Society of Gene Therapy. 2019 Apr 10;27(4):837-849.

103. Jackson NAC, Kester KE, Casimiro D, et al. The promise of mRNA vaccines: a biotech and industrial perspective. NPJ vaccines. 2020;5:11.

104. Hinz T, Kallen K, Britten CM, et al. The European Regulatory Environment of RNA-Based Vaccines. Methods in molecular biology. 2017;1499:203-222.

8. Figure legends

Figure 1. *In vivo* production of therapeutic antibodies by mRNA complexed in lipid nanoparticles. Systemic or local administration of lipid nanoparticles containing mRNA encoding different formats induces the expression of therapeutic levels of the antibodies. The lipid nanoparticles are internalized by endocytosis in the target cells. The lipid nanoparticles allow mRNA release to the cytosol, where it will be translated into the ribosome. The therapeutic antibody is secreted into the circulation, thus gaining access to the tumor microenvironment.

Figure 2. Strategies to potentiate cytokine-based immunotherapy by intratumoral administration of mRNAs. Local injection of mRNA encoding cytokines, mixtures of cytokines, or fusion protein with attached cytokines have been evaluated for expression in the tumor microenvironment. The mRNA technology allows local expression, extending tumor exposure to the cytokine and minimizing systemic adverse effects.

Figure 3. RNA-based adoptive T cell transfer. T cells can be engineered *in vivo* by intravenous administration of T cell-directed nanoparticles loaded with *in vitro* transcribed mRNA. Once the cargo is released into the cytoplasm, it is translated into a CD19-specific or a ROR1-specific chimeric antigen receptor, reprogramming circulating T cells against leukemia and prostate tumors, respectively (left panel). Alternatively, T cells can be engineered *ex vivo* with RNA. T cells isolated from the patients by leukapheresis are expanded *in vitro,* and the mRNA is transfected. Modified T cells are then infused into the patient. Mb= membrane bound, ca= constitutively active.

Table 1. mRNA-based cytokines in clinical trials.

Table 2. mRNA-based adoptive transfer strategies in clinical trials.

Table 1. mRNA-based cytokines in clinical trials.

Trademark	Therapy (delivery route)	Indication(s)	NCT	Reference
MEDI1119	LNP with IL-12 mRNA (i.t.)	Solid tumors	NCT03946800 (I)	Hewitt et al., 2020 [62]
mRNA-2752 (Triplet)	LNP with OX40L, IL23 and IL-36γ mRNA (i.t.)	Relapsed/refractory solid tumor malignancies or lymphoma, TNBC, HNSCC, Non-Hodgkin lymphoma, urothelial cancer	NCT03739931 (I)	Patel et al., 2020 [104]
SAR44100 (BNT131)	scIL-12, IL-15sushi, IFNα and GM-CSF mRNA (i.t.)	Metastatic neoplasms	NCT03871348 (II, non-randomized)	Nguyen et al., 2020 [60]

Abbreviations: i.t., intratumoral; LNP, lipid nanoparticle; IL, interleukin; OX40L, OX40 ligand; TNBC, triple-negative breast cancer; HNSCC, head and neck squamous cell carcinoma; sc, single-chain; IFN, interferon; GM-CSF, granulocyte-macrophage colony-stimulating factor.

Table 2. mRNA-based adoptive transfer strategies in clinical trials.

Trademark	Therapy (delivery route)	Indication(s)	NCT	Reference
NA	CD19 CAR-T cells (i.v.)	Hodgkin lymphoma	NCT02277522 (I) NCT02624258 (I)	Svoboda et al., 2018 [79]
NA	CD123 CAR-T cells (i.v.)	Relapsed or refractory AML	NCT02623582 (I)	Cummins et al., 2018 [105]
NA	Mesothelin CAR-T cells (i.v.)	Malignant pleural mesothelioma	NCT01355965 (I)	Zhao et al, 2010 [86]
NA	Mesothelin CAR-T cells (i.v.)	Metastatic pancreatic ductal adenocarcinoma	NCT01897415 (I)	Beatty et al., 2019 [96]
NA	c-MET CAR-T cells (i.t.)	Metastatic breast cancer, TNBC	NCT01837602 (I)	Tchou et al., 2017 [97]
NA	c-MET CAR-T cells (i.v.)	Melanoma, breast cancer	NCT03060356 (I)	Tchou et al., 2017 [97]

Descartes08	BCMA CAR-T cells (NA)	Multiple myeloma	NCT03448978 (I)	Lin et al., 2021 [106]
Descartes11	BCMA CAR-T cells (NA)	Multiple myeloma	NCT04436029 (II) NCT03994705 (I/II)	Lin et al., 2021 [106]
NA	TGFβII CAR-T cells (i.v.)	Colorectal cancer	NCT03431311 (I/II)	Inderberg et al., 2017 [107]

Abbreviations: NA, not available; i.v., intravenous; i.t., intratumoral; TNBC, triple-negative breast cancer; CAR-T, Chimeric antigen receptor T; AML, acute myeloid leukemia; c-MET, protein kinase Met; BCMA, B cell maturation antigen; CLL, B-cell chronic lymphocytic leukemia; NHL, B-cell non-Hodgkin's lymphoma; TGFβII, transforming growth factor-beta receptor Type II.