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mRNA: A Versatile Molecule for Cancer Vaccines

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Abstract
mRNA vaccines are finally ready to assume their rightful place at the forefront of nucleic acid-based vaccines. Major achievements within the last two decades have turned this highly versatile molecule into a safe and very attractive pharmaceutical platform that combines many positive attributes able to address a broad range of diseases, including cancer. The simplicity of mRNA vaccines greatly reduces complications generally associated with the production of biological vaccines. Intrinsic costimulatory and inflammatory triggers in addition to the provision of the antigenic information makes mRNA an all-in-one molecule that does not need additional adjuvants and that does not pose the risk of genomic integration. Clinical studies in various cancer types are moving forward and promising results with favorable clinical outcome are awaited. This review will recapitulate conceptual, mechanistic and immune-related features of this highly versatile molecule, elucidate how these features have been addressed in the past, and how comprehensive understanding can foster further optimization for broad application possibilities in cancer treatment.

Box 1. List of abbreviations.

APC antigen presenting cell	MITD MHC class I trafficking domain
ARCA Anti-reverse cap analog	mTOR mechanistic target of rapamycin
CEA carcinoembryonic antigen	ORF open reading frame
CMV cytomegalovirus	OVA ovalbumin
CTL cytotoxic T lymphocyte	PABP poly(A) binding protein
DC dendritic cells	pDNA plasmid DNA
dsRNA double-stranded RNA	Poly(A) poly-adenosine
ER endoplasmic reticulum	PRR pattern recognition receptor
FDA Food and Drug Administration	RIG-I retinoic acid-inducible gene-I
FLT3L Fms-like tyrosine kinase 3 ligand	RNA-LPX RNA-lipoplex
GMP good manufacturing practice	RSV respiratory syncytial virus
HIV human immune deficiency virus	s.c. subcutaneous
HPV human papilloma virus	SP signal peptide
i.d. intradermal	ssRNA single-stranded RNA
i.m. intramuscular	TAA tumor-associated antigen
i.n. intranodal	TAP transporter associated with protein processing
IFN interferon	Th T helper
LAMP-1 lysosome-associated membrane protein-1	TLR Toll-like receptor
MART1 melanoma antigen recognized by T cells1	TRP2 tyrosinase-related protein 2
MCA methylcholanthrene	UTR untranslated region

Introduction

Despite early reports in the 1990s that mRNA-based vaccines were particularly suitable for the generation of potent cytotoxic T lymphocyte (CTL) responses as they enable expression of the encoded protein in the cytoplasm of antigen presenting cells (APC) (Conry et al., 1995; Martinon et al., 1993), research has mainly focused on the development of plasmid DNA (pDNA) and viral vector vaccines. For a long time, mRNA was erroneously perceived to be a fragile and unstable molecule that would be associated with unacceptably high cost and effort for the production of good manufacturing practice (GMP)-grade material. However, the limitations associated with classical pDNA approaches have led to a renewed interest in mRNA-based vaccination strategies. Major developments achieved within the last two decades have turned this highly versatile molecule into a safe and very attractive pharmaceutical platform that combines many positive attributes able to address a broad range of diseases.

All-in-one molecule: Simplicity, safety and immunogenicity

The simplicity of mRNA vaccines greatly reduces complications generally associated with the production of biological vaccines, such as handling of infectious agents, genetic variability or environmental risks. Despite initial concerns, mRNA-based vaccines can be easily and rapidly produced from pDNA templates, potentially within few days after provision of genome sequence information. Robust production and purification protocols paved the way for a highly flexible and scalable GMP-compatible production process at low costs, regardless of the encoded antigen (Kallen and Theß, 2014; Weide et al., 2008a) in contrast to peptide or protein formats facing challenges concerning manufacturing controls (e.g. expression, solubility, mixing of different peptides). Lyophilization greatly facilitates storage and resistance to thermal stress, obviating the need for a temperature-controlled transfer (Petsch et al., 2012). The prospect of being able to produce various mRNA vaccines within a very short amount of time with limited financial investments is of great importance for pandemic scenarios in infectious diseases and for personalized cancer vaccines against patient-specific mutated neo-antigens (Castle et al., 2012; Kreiter et al., 2012).

A major reason for the use of mRNA vaccines is their superior safety profile compared to pDNA or viral vectors. In the extracellular environment, mRNA cannot persist as it is rapidly degraded by ubiquitous RNases. mRNA represents the minimal genetic vector containing only the elements directly required for expression of the encoded protein. As opposed to recombinant viruses or bacteria, mRNA codes exclusively for the protein or epitope(s) of interest. Since additional sequences such as plasmid backbone and viral packing proteins are lacking in mRNA vaccines, pre-existing or induced antivector antibodies have not been observed. While the risks of insertional mutagenesis and long-term expression have hindered Food and Drug Administration (FDA) approval of DNA-based vaccines for human use and similarly of some viral vectors, these concerns are not applicable for mRNA vaccines. Firstly, mRNA does not integrate into the genome, excluding permanent genetic alteration, and secondly, self-limiting synthesis of the antigen due to rapid clearance of mRNA molecules in the cytoplasm by the endogenous mRNA degradation machinery ensures transient and controlled antigen exposure. In the case of therapeutic antitumor vaccination, the risk of tolerance induction associated with long-term antigen exposure is minimized.

Safety provided, the efficacy of a cancer vaccine is measured by its potency in triggering an adaptive immune response. Induction of antigen-specific T cell immunity is a complex, multifaceted process that for it to happen properly requires antigenic information presented by costimulating, mature dendritic cells (DC) in the presence of specific cytokines and chemokines that drive T cell expansion and determine differentiation. As a result of being a ligand for immunostimulatory receptors, mRNA is naturally equipped with costimulatory and inflammatory triggers in addition to the antigenic information. Together with its simple design and favorable safety profile, mRNA comes as an all-in-one solution.

mRNA can encode virtually any transcript-based protein and enables strong antigen expression without the need for crossing the nuclear membrane for transcription, which is a major obstacle for pDNA. While pDNA transfection is restricted to actively dividing cells as they depend on nuclear envelope breakdown, mRNA is also translated in non- or slowly dividing cells,

such as DCs, professional APCs required for initiation of the immune responses. The synthesis of native antigen in situ offers a great operative range, including natural or intended intracellular localization, membrane association, secretion, posttranslational modification, multi-protein complexes, or structural optimization of delivered antigen. In the case of cancer therapy or viral infections, mRNA can be designed to encode the whole antigen to ensure simultaneous delivery and presentation of all possible epitopes, without being restricted to a defined HLA type as in the case of peptide vaccines, rendering mRNA-based vaccines broadly applicable (Van Nuffel et al., 2012; Pascolo, 2004). Presentation of mRNA-encoded epitopes to T helper (Th) cells enables the induction of a combined immune response including CTL immunity as well as B cell-based humoral responses, as with live-attenuated vaccines (Amanna and Slifka, 2011).

In addition to delivery of the antigenic information, the interaction of costimulatory receptors and ligands on the surface of antigen-presenting DCs with their counterparts on antigen-specific T cells in the presence of a polarizing cytokine environment is crucial for T cell activation. Indeed, antigen presentation in the absence of these “danger” signals will promote T cell deletion, anergy or the induction of regulatory T cells. Adjuvants such as aluminum salts (alum), monophosphoryl lipid A or toll like receptor (TLR) agonists usually ensure immunostimulation when peptide or protein vaccines with low intrinsic immunogenicity are used. However, exogenous mRNA is recognized by specific pattern recognition receptors (PRRs), causing strong activation in both mouse and human APCs, and thus acts per se immunostimulatory (Diebold et al., 2004; Ishii and Akira, 2005; Karikó et al., 2004). In immune cells, ligation of endosomal TLR3, TLR7 or TLR8 with endocytosed exogenous mRNA initiates signaling cascades ultimately triggering the production of type I interferon (IFN), a master regulator of diverse inflammatory cytokines, Th cytokines, costimulatory molecules, chemokine ligands and receptors. While TLR3 is activated by double-stranded RNA (dsRNA) (Alexopoulou et al., 2001) and single-stranded RNA (ssRNA) forming double-stranded secondary structures (Karikó et al., 2004), TLR7 and 8 signal in response to ssRNA (Diebold et al., 2004; Heil et al., 2004). Broadly expressed by immune and non-immune cells, soluble cytoplasmic receptors can also recognize dsRNA derived from pathogens that do not rely on endocytosis for infection and trigger the expression of type I IFN and proinflammatory factors similar to TLRs (Yoneyama and Fujita, 2007). Although the natural ligand for the retinoic acid-inducible gene-I (RIG-I) is short RNA with blunt-ended double-stranded base pairing and an uncapped 5' triphosphate end present in viral genomes or replication intermediates (Hornung et al., 2006; Schlee et al., 2009), RIG-I has been reported to bind to various dsRNA ligands (Ablasser et al., 2009). Interestingly, activation of melanoma differentiation-associated antigen 5 (MDA5) has been reported to require much longer dsRNA than RIG-I (at least 2 kb) (Pichlmair et al., 2009), or viral mRNA lacking 2’-O-methylation (Züst et al., 2011).

Mechanism of action

mRNA tumor vaccination is a complex, multi-step process (Figure 1). Exogenous mRNA needs to overcome tissue-dependent physical barriers and evade extracellular enzymatic degradation by ubiquitous RNases. mRNA that succeeds in escaping degradation needs to be actively endocytosed as passive diffusion is severely hindered by its negative charge and size. Endocytosis can occur by a large range of cells by cell-specific uptake mechanisms, such as macropinocytosis in immature DCs (Diken et al., 2011), and complexing agents can protect mRNA from extracellular degradation and enhance tissue- or cell type-specific uptake (see below). Uptake of mRNA in DCs is a saturable process (Probst et al., 2007), and accompanied by danger signals conveyed by RNA-recognizing TLRs in endosomes leads to the differentiation of DCs from endocytic scavengers to antigen-presenters. Escape of mRNA from endosomal compartments (the mechanism of which is not yet fully understood) makes synthetic mRNA eligible for the same mechanisms that regulate the stability and translation of endogenous mRNA. Translation is initiated within minutes (Diken et al., 2011; Lorenz et al., 2011; Selmi et al., 2016), and is controlled by mRNA decay processes involving decapping enzymes DCP1, DCP2 and DCPS (Li and Kiledjian, 2010), 5'-3' exoribonuclease 1 (XRN1), and exosomal endonucleolytic cleavage (Li et al., 2010; Tomecki and Dziembowski, 2010; Wilusz, 2009). Post-translational modification and proteasomal degradation are prerequisites for immunothera-

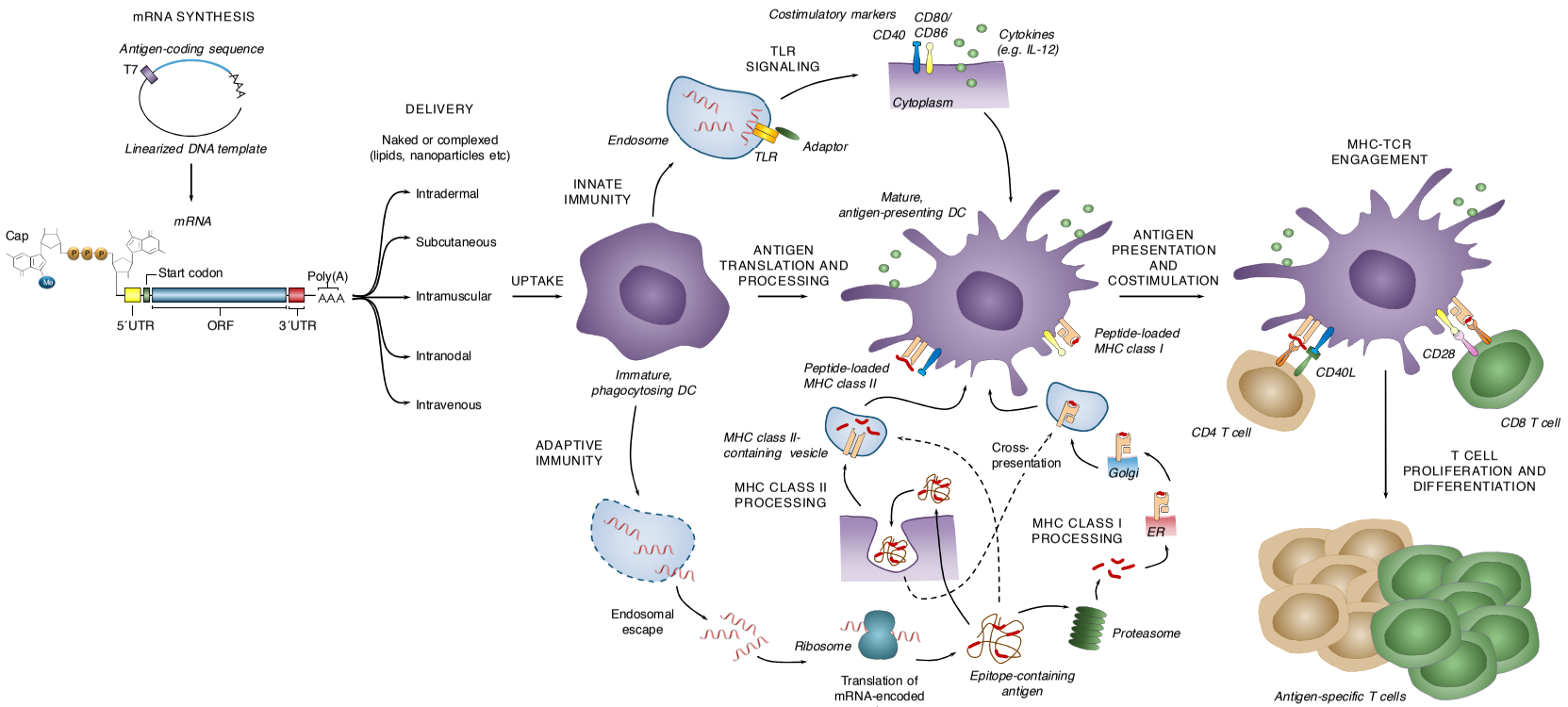

Figure 1. Mechanism of immune induction by antigen-coding mRNA

peutic purposes and ensure processing into antigenic peptides. Cytoplasmic peptides are routed into the endoplasmic reticulum (ER) to be loaded onto MHC class I molecules for surface presentation to CD8 T cells via the secretory pathway. In order to include MHC class II presentation for cognate CD4 T cell priming, routing signals of endosomal or lysosomal proteins residing in MHC class II antigen processing compartments [such as invariant chain (Bonehill et al., 2003), lysosome-associated membrane protein-1 (LAMP-1) (Bonehill et al., 2004; Bonini et al., 2001; Su et al., 2002), ER chaperone calreticulin, and human immune deficiency virus (HIV) TAT protein transduction domain (Kim et al., 2008)] can be incorporated into the open reading frame (ORF). In another approach, the MHC class I signal peptide (SP) was added to the N terminus and the MHC class I trafficking domain (MITD) to the C terminus of the encoded antigen. DCs transfected with RNA encoding such SP-antigen-MITD fusion proteins showed a significantly higher stimulatory capacity of both CD8 and CD4 T cell proliferation (Kreiter et al., 2007, 2008). In addition to routing the protein antigen to the extracellular space where it can be reinternalized and enter the MHC class II presentation pathway, the secretion signal might improve antigen processing through a better interplay of protein degradation by ER-adjacent proteasomes and access to transporter associated with protein processing (TAP) molecules. Moreover, CD8α DCs are able to present internalized exogenous antigens on MHC class I molecules for CD8 T cell priming by a mechanism that is known as cross-presentation (Bevan, 2006).

Optimization of stability and translation for improved T cell priming

mRNA is one of the easiest, most versatile, and theoretically safest technologies to induce antigen-specific immunity. Usually, mRNA contains five basic elements: (i) A cap structure consisting of methyl-7-guanine followed by three phosphate groups at the 5' end (m^7Gp_3N; N, any nucleotide), (ii) a 5' untranslated region (UTR), (iii) the ORF flanked by a start codon in Kozak surrounding and a stop codon, (iv) a 3' UTR and (v) a poly-adenosine (poly(A)) tail at the 3' end (Banerjee, 1980; Jackson, 1993). Extensive efforts have been undertaken to increase the stability and translation efficiency of mRNA vaccines through structure and sequence modifications of these elements.

Capping of mRNA species facilitates recognition of mature mRNA by the translation initiation factor eIF4E and as a consequence improves translation initiation and RNA stability during protein synthesis (Sonenberg and Hinnebusch, 2009). Conventional cap analogs (m^7Gp_3N) are incorporated into mRNA in both forward and reverse orientations leading to two isomeric RNA populations (Pasquinelli et al., 1995), and mRNAs capped with reverse 5' caps are not translated. Anti-reverse cap analogs (ARCA), $m_2^{7,3'O}Gp_3G$ and $m^73'dGp_3G$, in which a 3' OH group of the normal cap is removed or replaced with OCH_3, ensure insertion only in the functional, translation-competent orientation (Stepinski et al., 2001). Mockey et al. found that ARCA-capped luciferase mRNA showed a 25-50-fold higher luciferase activity than mRNA exhibiting a standard cap in DCs (Mockey et al., 2006). Improving the cap structure further, Grudzien-Nogalska et al. reported phosphorothioate analogs of ARCA (S-ARCA), which stabilized and increased the efficiency of translation (Grudzien-Nogalska et al., 2007) [most likely due to resistance to hydrolysis by the decapping enzyme DCP2 (Grudzien et al., 2006)]. Vaccination with S-ARCA-capped mRNA boosted antigen production in immature DCs and induced superior antigen-specific T cell responses (Kuhn et al., 2010).

mRNA stability can be further enhanced by the choice of 5' and 3' UTR. So far UTRs of naturally occurring α- and β-globin have been most widely incorporated, and systematic screening revealed that two copies instead of one of the β-globin 3' UTR synergistically increased mRNA stability and functional half-life (Holtkamp et al., 2006). Structural elements found in viral mRNAs also serve to positively affect translational efficacy (Bergman et al., 2007; Chiu et al., 2005; Garneau et al., 2008; Pogue et al., 1993; Sjöberg et al., 1994).

Codon optimization is another way of increasing translational efficiency. Species-specific codon optimization takes into account the abundance of specific tRNAs in the cytoplasm and the predicted structure of the mRNA, thereby avoiding non-favorable rare codons and minimizing secondary structures. However, altering codons should be carefully considered for each antigen, as this may eliminate important sources of immunogenic peptides such as cryptic T cell epitopes generated by ribosomal frame-shifting,

or internal initiation of transcription (Saulquin et al., 2002; Schwab et al., 2003).

The length of the poly(A) tail is critical for the inhibition of decapping and deadenylating enzymes through the formation of a circular structure with the help of poly(A) binding proteins (PABP) (Bernstein et al., 1989; Sachs et al., 1987). Poly(A) tails can be introduced either by direct implementation of the coding region in the vector template, or by enzymatic polyadenylation of the transcribed mRNA using poly(A) polymerase. As discovered by Holtkamp et al. only template-encoded poly(A) regions lead to reproducible lengths between different reactions, the importance of which should not be underestimated as protein expression has been reported to increase with the length of the poly(A) tail until around 120 adenosines (Holtkamp et al., 2006). An unmasked 3' end is essential for maximizing translational efficacy in DCs (Holtkamp et al., 2006). Moreover, the addition of a poly(A) tail in the range of 15–600 residues resulted in a 700-fold increase of expression from an ARCA-A100 mRNA compared to an mRNA with a conventional cap analog and an A65 tail (Mockey et al., 2006). Hence, expression in DCs is greatly improved by a long poly(A) tail combined with an optimized cap structure and UTR.

Replicons are yet another elegant strategy to enhance *in vivo* expression of RNA vaccines. The target antigen is translated from a bicistronic replicative RNA which codes both for the antigen and an RNA replicase (Herweijer et al., 1995; Zhou et al., 1994). Utilizing the self-amplifying characteristics of alphaviruses such as semliki forest virus (SFV) to produce large amounts of viral mRNA (Schlesinger, 2001), the structural genes of such RNA viruses can be replaced by the genes of interest while the nonstructural proteins are left intact to ensure replication and powerful protein translation. RNA is amplified by the replicase complex which synthesizes a genomic negative strand that itself represents the template for the synthesis of many genomic RNA positive-strands by the RNA replicase. Although mainly exploited for combatting infectious diseases, RNA replicons have also been preclinically tested as vaccines against cancer, and have been entering clinical testing [reviewed in (Pushko and Tretyakova, 2014)].

Delivery route and immunity in the preclinical and clinical settings

In addition to the administered antigen and adequate costimulation, the mode of delivery is a decisive factor for vaccine efficacy. The most studied and used mRNA-based vaccination approach relies on *ex vivo* transfection of mRNA into autologous DCs to be readministered to the patient, as initially described by Boczkowski et al. as early as 1996 (Boczkowski et al., 1996). Numerous studies have shown that DCs transfected with mRNA coding for tumor-associated antigens (TAAs) are able to induce potent antigen- and tumor-specific T cell responses and support the potential of this vaccine concept (Benteyn et al., 2015). Despite promising results in preclinical studies and phase I clinical trials, the *ex vivo* engineering of DCs has been hampered by high costs due to GMP cell culture, complicated logistics, and complex personalized vaccination procedures, thereby forfeiting major advantages of mRNA (see above) and strongly impeding its application to larger numbers of patients. Additionally, the pharmacokinetics, i.e. migration of DCs to draining lymph nodes, are complex and uncontrollable, and transferred antigen-presenting DCs are prone to T cell-mediated killing, especially after repetitive immunization (Hermans et al., 2000; Yang et al., 2006).

Based on the work of Conry et al. who were among the first to show that intramuscular (i.m.) injection of naked human β-globin UTR-stabilized mRNA coding for carcinoembryonic antigen (CEA) was able to elicit CEA-specific antibody responses (Conry et al., 1995), mRNA for direct application *in vivo* has been exploited as a versatile tool. Several years after the discovery that high molecular weight cationic polymers such as protamine can enhance the transfection efficiency of DNA complexed with cationic liposomes *in vitro* and *in vivo* by rendering the DNA nuclease-resistant (Gao and Huang, 1996), it was demonstrated that humoral and antigen-specific cytotoxic cellular immune responses were induced by injection of naked or protamine-protected mRNA into the ear pinna of mice (Hoerr et al., 2000). Around the same time, Granstein et al. demonstrated that intradermal (i.d.) injection of total tumor mRNA delayed tumor growth in a prophylactic methylcholanthrene (MCA)-induced fibrosarcoma model (Granstein et al., 2000). Studies by Scheel et al. revealed that immune responses elicited by

Table 1. Clinical trials using direct mRNA vaccination

Sponsor	Indication	Administration route	Status	Reference/ clinical trial number
University Hospital Tübingen, CureVac	Metastatic melanoma	Intradermal (mRNA + GM-CSF)	Completed	(Weide et al., 2008b)
University Hospital Tübingen, CureVac	Metastatic melanoma	Intradermal (protamine-complexed mRNA + GM-CSF, +/- KLH)	Completed	(Weide et al., 2009) NCT00204607
University Hospital Tübingen, CureVac	Renal cell carcinoma	Intradermal (mRNA + GM-CSF)	Completed	(Rittig et al., 2011, 2016)
CureVac	Non-small cell lung cancer	Intradermal (self-adjuvanted mRNA)	Completed	(Sebastian et al., 2011) NCT00923312
BioNTech RNA Pharmaceuticals	Metastatic melanoma	Intranodal (mRNA)	Completed	NCT01684241
CureVac	Castration-resistant prostate cancer	Intradermal (self-adjuvanted mRNA)	Completed	NCT00906243
CureVac	Castration-resistant prostate cancer	Intradermal (self-adjuvanted mRNA)	Completed	(Kübler et al., 2015) NCT00831467
CureVac	Castration-resistant prostate cancer	Intradermal (self-adjuvanted mRNA)	Ongoing	NCT01817738
BioNTech RNA Pharmaceuticals	Metastatic melanoma	Intranodal (poly-neo-epitopic mRNA)	Ongoing	NCT02035956
BioNTech RNA Pharmaceuticals	Metastatic melanoma	Intravenous (liposome-formulated mRNA)	Ongoing	(Kranz et al., 2016) NCT02410733

β-galactosidase mRNA complexed with protamine injected i.d. could be shifted towards Th1 immunity by combination with GM-CSF (Carralot et al., 2004). Protamine-stabilized mRNA activated human and mouse immune cells most likely through TLR7 (Scheel et al., 2004, 2005), inducing therapeutic antitumor immunity after i.d. injection at distant sites in a tumor model of SMA-560 glioblastoma (Scheel et al., 2006). The adjuvant effect of protamine-stabilized mRNA, however, came at the cost of very weak antigen expression (Fotin-Mleczek et al., 2011; Schlake et al., 2012) due to the tight complexing of mRNA. The search for the optimal ratio of naked antigen-expressing mRNA with preformed, immunostimulatory mRNA/protamine complexes led to the development of a two-component, self-adjuvanted 'RNActive' vaccine platform for i.d. delivery by the biotechnology company CureVac (Tübingen, Germany) (Fotin-Mleczek et al., 2011). Preclinical studies demonstrated delay of tumor growth in prophylactic and therapeutic settings (Fotin-Mleczek et al., 2011, 2012), and combination with chemotherapy or αCTLA-4 (Fotin-Mleczek et al., 2012) as well as radiotherapy revealed strong synergistic effects in the improvement of survival (Fotin-Mleczek et al., 2014). Clinical trials of RNActive in patients with advanced castration-resistant prostate carcinoma (Kübler et al., 2015) and stage IIIB/IV non-small cell lung cancer (Sebastian et al., 2011) revealed substantial immunity against multiple antigens in the majority of immune responders, which may be indicative of an improved overall survival in vaccinated patients (Table 1).

Dermal delivery based on the gene gun technology represents a needle-free approach where mRNA is precipitated on gold particles which, upon firing the gun, penetrate the stratum corneum and reach skin-resident DCs for antigen expression and triggering of an immune response. Qiu et al. were the first to demonstrate the generation of antibodies against human α-1 antitrypsin in response to mRNA-loaded gold particle bombardment (Qiu et al., 1996). Gene gun-based immunization with tyrosinase-related protein 2 (TRP2) mRNA induced antigen-specific cellular as well as humoral immunity and protected against B16 melanoma growth in a preclinical mouse model (Steitz et al., 2006). Several advantages of this technique would allow for prophylactic vaccination of large populations (e.g. reliable and safe, low amounts of mRNA required, easy storage), ideally to be

used against pandemic viruses. However, the cost of gold particles and the inconvenience of gene-gun bombardment presumably hampered the exploitation of ballistic delivery for mRNA vaccination, and consequently clinical translation has so far not been reported.

Acknowledging the pivotal role of reaching adequate numbers of DCs in priming of immune responses, Sahin and colleagues explored the effect of different delivery routes of naked mRNA on *in vivo* transfection of DCs and T cell priming (Kreiter et al., 2010; Selmi et al., 2016). In parallel, mRNA structure as well as sequence optimization were investigated for improved stability, translation and MHC presentation (Holtkamp et al., 2006; Kreiter et al., 2007, 2008; Kuhn et al., 2010). Intranodal (i.n.) injection was found to be superior to the subcutaneous (s.c.), near-nodal or i.d. route in terms of expansion of antigen-specific Th1 polarized CD4 and CD8 T cells as well as prophylactic and therapeutic antitumor immunity (Kreiter et al., 2010), most likely due to the direct bioavailability of the mRNA molecule to a high number of APCs and efficient uptake before elimination by extracellular RNases. mRNA administered i.n. is selectively taken up by resident DCs by macropinocytosis (Diken et al., 2011), resulting in a TLR7-dependent T cell stimulatory environment (Kreiter et al., 2010). The potential of i.n. mRNA delivery for cancer immunotherapy is further highlighted by the finding that i.n. tumor vaccination was superior over s.c. immunization with bone marrow-derived DCs electroporated with antigen-encoding mRNA (Kreiter et al., 2010).

mRNA vaccines can be further optimized by combined administration of recombinant Fms-like tyrosine kinase 3 ligand (FLT3L) which provoked the expansion of pDCs in the lymph node, T cell homing into melanoma tumors and remarkably improved survival of tumor-bearing mice (Kreiter et al., 2011). Rapamycin, an immunomodulator inhibiting mechanistic target of rapamycin (mTOR), was discovered to enhance the quantity and quality of the memory pool, leading to improved recall abilities (Diken et al., 2013). In addition, co-administration of the mRNA-encoded immunomodulator cocktail TriMix (constitutively active TLR4 variant, CD40L and CD70) as employed by Thielemans and colleagues further improved antitumoral immunity (Van Lint et al., 2012). A clinical phase I first-in-human dose escalation study evaluating the safety and tolerability of ultrasound-guided, i.n. administration of an mRNA-based cancer vaccine (MERIT) in patients with advanced melanoma was initiated by BioNTech RNA Pharmaceuticals (Mainz, Germany) and is currently in progress (NCT01684241).

Quite recently, lipid carrier systems such as liposomes and lipid nanoparticles have been revisited as an attractive delivery option also for mRNA (reviewed in Phua, 2015; Phua et al., 2014a). Profiting from the extensive research on liposomal DNA-based gene transfer, the possibility to transfer this approach to mRNA-based vaccines was demonstrated early by Martinon et al. by the induction of influenza nucleoprotein (NP)-specific cytotoxic T cells after s.c. injection of liposomal NP mRNA (Martinon et al., 1993). Moreover, intrasplenic delivery of gp100 mRNA complexed with liposomes improved survival upon challenge with B16 melanoma (Zhou et al., 1999), and HIV GAG-specific T cell immunity was elicited when GAG mRNA complexed with liposomes was injected s.c. (Pollard et al., 2013). Regarding cancer immunotherapy, liposome-complexed chicken ovalbumin (OVA)-encoding mRNA generated antigen-specific CTLs and delayed the growth of established OVA-expressing tumors after i.d. administration (Hess et al., 2006) or intranasal injection (Phua et al., 2014b). Encapsulation of RNA replicons in PEGylated liposomes or cationic nanoemulsions elicited high titers of antibodies as well as IFNγ-producing CD4 and CD8 T cells against respiratory syncytial virus (RSV), HIV and human cytomegalovirus (CMV) antigens after i.m. application in mice and Rhesus macaques (Brito et al., 2014; Geall et al., 2012). Synthetic nanoparticles were also employed to deliver RNA replicon vaccines to DCs (McCullough et al., 2014).

Despite strong efficacy in preclinical studies and promising results in early clinical trials, local administration restricts the target population to a limited number of tissue-resident DCs, constraining the strength of the induced T cell response. Broad-scale antigen availability to the maximum number of DCs should be the aim of any vaccine, since the extent of presentation is expected to determine the extent of the immune response. In order to unleash the full potential of mRNA vaccination, lymphoid-resident DCs must consequently be reached at multiple priming

sites on a systemic scale, which can most easily be achieved by i.v. application. Systemic targeting requires a suitable carrier system that is able to assure extracellular mRNA stability, and selectively targets the tissue of interest to maximize pharmacological dosing while minimizing potential side effects. Splenocytes from mice injected i.v. with Unifectin-encapsulated, protamine-condensed β-galactosidase mRNA exhibited antigen-specific cytotoxicity upon *in vitro* restimulation (Hoerr et al., 2000). Phua et al. used Stemfect to demonstrate antigen expression in the spleen, lungs and liver (Phua et al., 2013), and mRNA encoding melanoma antigen recognized by T cells 1 (MART1) complexed with histidylated and mannosylated lipopolyplexes delayed B16 melanoma tumor progression in a prophylactic setting in mice after i.v. injection (Mockey et al., 2007; Perche et al., 2011).

Very recently, Kranz et al. utilized the central role of DCs and the favorable physicochemical properties of liposomes by directing mRNA translation precisely to DCs residing in secondary lymphoid compartments including the spleen, lymph nodes and bone marrow, which provide the ideal microenvironment for efficient priming and recall expansion of T cell responses (Kranz et al., 2016). During this systematic approach, solely the RNA-to-lipid ratio of lipid-formulated, tumor antigen-encoding mRNA nanoparticles [RNA-lipoplexes (RNA-LPX)] was discovered to determine the biodistribution of RNA-LPX, irrespective of the types of lipids used, and a slightly negative particle net charge was able to specifically transfect lymphoid-resident APCs, completely omitting the need for ligand conjugation or functionalization. RNA recognition via TLR7 serving signaling cascades that prepare for antiviral defense established a type I IFN (IFNα and β)-dependent inflammatory milieu reminiscent of that initiated during the early systemic phase of viral infection. IFNα receptor (IFNAR)-dependent immune mechanisms stimulated maturation of DCs, and presentation on MHC class I and II in the context of upregulated CD40, CD69 and CD86 elicited strong effector and memory CD8 and CD4 T cell immunity against viral, mutant neo-antigens or self-antigens, which was able to reject progressive tumors in therapeutic mouse models of melanoma, colon carcinoma and human papilloma virus (HPV)-associated cancer. In an ongoing phase I dose escalation study initiated by BioNTech RNA Pharmaceuticals (Mainz, Germany), patients with advanced melanoma received RNA-LPX encoding four shared tumor antigens starting with an extremely low dose, lower than the total amount of RNA-LPX used in preclinical studies (Lipo-MERIT, NCT02410733). All patients showed a dose-dependent IFNα- and IP-10-dominated cytokine response, developed *de novo* CD8 and CD4 T cell responses or enhanced pre-existing immunity against the encoded self-antigens NY-ESO-I, Tyrosinase and MAGE-A3. By mimicking infectious non-self and thus mobilizing both adaptive and innate immune mechanisms including a strong type I IFN-driven immuno-stimulatory program, this systemic mRNA vaccine connects effective cancer immuno-therapy with host pathogen-defense mechanisms. The important role of type I IFN for full functionality of antigen-specific T cells induced by i.v. delivery of lipid-formulated mRNA has also been demonstrated by Broos et al. *(*2016). Further clinical trials will soon be opened for recruitment for the treatment of triple receptor-negative breast cancer as well as the treatment of HPV-induced head and neck cancer.

Accompanied by the advances in next-generation sequencing systems, use of tumor specimens for identification of patient-specific mutations and vaccination with neoantigens holds the promise for new generation cancer immunotherapy (Castle et al., 2012; Kreiter et al., 2012). mRNA can also serve as a powerful format for this type of vaccines such that several identified immunogenic epitopes which possess patient-specific mutations can be incorporated into a personalized mRNA vaccine for induction of immune responses. Kreiter et al. showed the feasibility of this approach in different preclinical tumor models with such a poly-epitope mRNA (Kreiter et al., 2015), providing the proof of feasibility for mRNA vaccines. This approach is currently being tested in a first of its kind clinical trial in advanced melanoma by BioNTech RNA Pharmaceuticals (IVAC MUTANOME, NCT 02035956).

Challenges and prospects

Thanks to accumulating preclinical and clinical data, mRNA vaccines have been proven to be a potent platform against cancer and infectious diseases. Compared to production of recombinant proteins, mRNA production includes remarkably lower risks due to the cell and animal

component-free mRNA production process. Nevertheless, several challenges have to be addressed for optimal therapy, such as purification, scale-up and the source as well as the processing of the reagents used for *in vitro* transcription. Process automation may also be needed in cases where mRNA is used to deliver patient-specific neoantigens in the context of individualized vaccination. Rapid assembly of the synthetic genes required for the DNA template would decrease the production time of mRNA. In terms of stability, use of special buffers can increase mRNA stability under alkaline environment. Moreover, the effect of the mRNA sequence length and the sequence of coding as well as regulatory regions on the yield and stability of mRNA have to be assessed in detail.

While mRNA can be injected locally in a naked form with a suitable buffer, the systemic administration of mRNA requires a proper formulation which not only renders it resistant to RNases but also targets the mRNA to professional APCs for efficient processing and presentation of the encoded antigen in the context of MHC class I or II molecules. The formulation can be of different nature (lipid, polymer, protein/peptide), but should exhibit favorable pharmacokinetics and biodistribution without accumulation in vital organs, and a safety profile devoid of formulation-based toxicities.

As mRNA is a potent activator of the immune system, its secondary pharmacodynamics should be followed carefully in both preclinical and clinical studies. Both should include monitoring of proinflammatory cytokines (such as IFNα, IFNβ, IL-6, IP-10) during mRNA vaccination, and *in vitro* systems should be established to predict the outcome of mRNA-mediated immune activation. These systems together with *in vivo* toxicology studies in different species can foster the understanding of mRNA-mediated immune activation, as the affinity and cell/tissue distribution of mRNA-sensing receptors can vary between species. Fine-tuning the mRNA molecule itself can also alter the activatory potential. mRNA immunogenicity, stability and translational efficiency can be modified through the introduction of modified nucleosides (Karikó and Weissman, 2007; Karikó et al., 2012) and sophisticated purification methods (Karikó et al., 2011) to eliminate residual double-stranded fragments, or sequence-engineered mRNA (Thess et al., 2015). However, the potential toxicity of these analogs and sequence alterations should be carefully addressed.

Conclusion

Despite challenges, the available data on mRNA vaccines suggest great versatility and a favorable safety profile, and make mRNA a potent vaccination platform. Clinical studies in various cancer types are moving towards late-stage clinical development and promising results with favorable clinical outcome are awaited [reviewed in (Sahin et al., 2014)]. Further understanding of the complex mRNA pharmacology combined with carefully designed clinical studies using tailored mRNA molecules will pave the way for regulatory approval of mRNA-based vaccines in the near future.

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