# mRNA Cancer Vaccines

Katja Fiedler, Sandra Lazzaro, Johannes Lutz, Susanne Rauch and Regina Heidenreich

**Abstract**

mRNA cancer vaccines are a relatively new class of vaccines, which combine the potential of mRNA to encode for almost any protein with an excellent safety profile and a flexible production process. The most straightforward use of mRNA vaccines in oncologic settings is the immunization of patients with mRNA vaccines encoding tumor-associated antigens (TAAs). This is exemplified by the RNActive® technology, which induces balanced humoral and cellular immune responses in animal models and is currently evaluated in several clinical trials for oncologic indications. A second application of mRNA vaccines is the production of personalized vaccines. This is possible because mRNA vaccines are produced by a generic process, which can be used to quickly produce mRNA vaccines targeting patient-specific neoantigens that are identified by analyzing the tumor exome. Apart from being used directly to vaccinate patients, mRNAs can also be used in cellular therapies to transfect patient-derived cells in vitro and infuse the manipulated cells back into the patient. One such application is the transfection of patient-derived dendritic cells (DCs) with mRNAs encoding TAAs, which leads to the presentation of TAA-derived peptides on the DCs and an activation of antigen-specific T cells in vivo. A second application is the transfection of patient-derived T cells with mRNAs encoding chimeric antigen receptors, which allows the T cells to directly recognize a specific antigen expressed on the tumor. In this chapter, we will review preclinical and clinical data for the different approaches.

**Keywords**

RNActive® vaccines · Personalized mRNA vaccines · mRNA-pulsed dendritic cells · Chimeric antigen receptor (CAR) T cell immunotherapy

K. Fiedler (✉) · S. Lazzaro · J. Lutz · S. Rauch · R. Heidenreich
CureVac AG, Paul-Ehrlich-Str. 15, 72076 Tübingen, Germany
e-mail: katja.fiedler@curevac.com



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**Abbreviations**

| | |
|---|---|
| A | Adenine |
| APC | Antigen-presenting cell |
| CAR | Chimeric antigen receptor |
| CTL | Cytotoxic T lymphocyte |
| CTLA4 | Cytotoxic T lymphocyte antigen 4 |
| DC | Dendritic cell |
| DNA | Deoxyribonucleic acid |
| EGFR | Endothelial growth factor receptor |
| ELISA | Enzyme-linked immunosorbent assay |
| GC | Guanine and cytosine |
| GITR | Glucocorticoid-induced tumor necrosis factor receptor |
| HLA | Human leukocyte antigen |
| HMGB1 | High-mobility group box 1 |
| IDH1 | Isocitrate dehydrogenase 1 |
| IFN | Interferon |
| Ig | Immunoglobulin |
| LLC | Lewis lung cancer |
| MAGEC1 | Melanoma antigen family C1 |
| MAGEC2 | Melanoma antigen family C2 |
| MHC | Major histocompatibility complex |
| mRNA | Messenger ribonucleic acid |
| MUC1 | Mucin 1 |
| NK | Natural killer |
| NSCLC | Non-small-cell lung cancer |
| NY-ESO-1 | New York esophageal squamous cell carcinoma-1 |
| ORF | Open reading frame |
| OVA | Ovalbumin |
| PAP | Prostatic acid phosphatase |
| PD1 | Programmed cell death 1 |
| PDL1 | Programmed cell death 1 ligand 1 |
| PDL2 | Programmed cell death 1 ligand 2 |
| PFS | Progression-free survival |
| PSA | Prostate-specific antigen |
| PSCA | Prostate stem cell antigen |
| PSMA | Prostate-specific membrane antigen |
| RNA | Ribonucleic acid |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| STEAP1 | Six-transmembrane epithelial antigen of the prostate 1 |
| TAA | Tumor-associated antigen |
| TERT | Telomerase reverse transcriptase |
| Th | T helper |
| TLR | Toll-like receptor |

| | |
|---|---|
| Tmeso | CAR T cells specifically targeting mesothelin |
| TPBG | Trophoblast glycoprotein |
| UTR | Untranslated region |

# 1 mRNA Cancer Vaccines

## 1.1 Introduction

The concept of mRNA vaccines is founded on the observation that injection of messenger RNA (mRNA) leads to local protein expression (Wolff et al. 1990) and immune responses against the encoded antigen (Martinon et al. 1993). This was rather unexpected given the abundant presence of RNases in body fluids and on the skin, which leads to the rapid degradation of RNA, for example, in serum-containing media (Rammensee 2006), and most work on nucleic acid-based vaccine development continued to focus on DNA rather than on RNA vaccines. This view has changed with the advent of several technological platforms, which have demonstrated the potential of mRNA vaccines to express antigen and to induce humoral and cellular immune responses.

While both nucleic acid-based platforms share many characteristics, mRNA vaccines have several advantages over DNA-based platforms (Table 1). Firstly, RNA only needs to pass the plasma membrane in order to induce protein synthesis, facilitating vaccine delivery in comparison with DNA that needs to additionally cross the nuclear membrane. Secondly, mRNA is unable to integrate in the genome and therefore has no oncogenic potential. Finally, expression of mRNA-encoded proteins is intrinsically transient, defined by the short half-life of mRNA. Hence, mRNA vaccines feature a significantly increased safety profile over DNA.

In addition, mRNA vaccines provide important advantages over the more widely used protein-based vaccine platforms: Endogenous production of proteins in the cells of the vaccinee upon injection of mRNA supports correct protein modifications, such as glycosylation patterns, and abolishes the need for elaborate protein or particle purification steps. From a technical perspective, mRNA, unlike protein-based vaccines, can be produced in a fully synthetic production process and allows the production of mRNA encoding any protein or combination of proteins of choice using the same biologic compounds and production steps, greatly facilitating vaccine manufacturing processes. Furthermore, the ability to support quick sequence adjustments makes RNA vaccines highly versatile, which is of particular importance in pandemic settings or for individualized therapies (see below).

## 1.2 RNActive® Vaccines

The first section focuses on the RNActive® technology (CureVac AG), as an example for an mRNA vaccine platform currently evaluated in several clinical trials for oncologic indications.

RNActive® vaccines consist of formulated mRNA which encodes for the antigenic protein of choice and features modifications to enhance translation efficiency, delay mRNA decay, and improve immune stimulation.

In 2000, Hoerr et al. described that the in vivo application of mRNA encoding the model antigen β-galactosidase led to the induction of specific cytotoxic T lymphocytes (CTLs) and antibodies (Hoerr et al. 2000). The RNA used for these experiments had the basic design of a mRNA and consisted of a cap, an open reading frame (ORF) encoding for β-galactosidase that was flanked 5′ and 3′ by the untranslated regions (UTRs) of β-globin and a poly(A) tail. For the current RNActive® technology, several aspects of the mRNAs have been modified to increase the extent and duration of the antigen expression. The β-globin UTRs have been replaced by UTRs selected for higher translation efficiency and stability of the mRNAs, and the ORF was optimized for enhanced protein expression by enriching the guanine and cytosine (GC) content according to a proprietary algorithm. These changes, as well as the use of a template-encoded poly(A) tail of defined length, optimized buffers, and purification led to an increase in protein expression by four to five orders of magnitude in various test systems (Kallen and Theß 2014). Importantly, this technology exclusively employs unmodified nucleotides. Studies by Karikó (Karikó et al. 2008) and Anderson (Anderson et al. 2010) have demonstrated that modified nucleotides can lead to increased protein expression. However, they also reduced immunogenicity, making the use of modified nucleotides unfavorable in the context of vaccines.

In addition to antigen availability, adjuvanticity is essential for inducing strong immune responses. In RNActive® vaccines, immunostimulatory capacity is achieved via suitable formulation, e.g., by employing protamine, a cationic peptide that forms stable complexes with nucleic acids. Protamine binds to the mRNA and leads to the formation of larger particles that activate the immune system in a process involving the endosome-resident TLR7 (Toll-like receptor 7) (Fotin-Mleczek et al. 2011; Kallen et al. 2013; Scheel et al. 2005; Kowalczyk et al. 2016) (Table 1).

Hence, RNActive® vaccines are containing two components that serve complementary functions: “naked” mRNA, which serves as a translation template responsible for strong expression of the encoded antigen, and mRNA–protamine complexes, which enhance the immune stimulatory capacity of the vaccine. Indeed, vaccination with RNActive® vaccines has been shown to induce strong and balanced immune responses, i.e., Th1 and Th2, humoral and cellular, and effector and memory responses. Encouraging results have been gained in various animal model systems, among others mice, ferrets, and pigs, both in the field of cancer

**Table 1** Advantages of RNA over DNA vaccines

| | DNA | RNA |
|---|---|---|
| Delivery | DNA needs to cross both cell and nuclear membranes and be first transcribed in the nucleus before protein expression occurs | RNA only needs to gain entry into the cytoplasm, where translation and thus protein expression directly occur |
| Integration | DNA vaccines are able to integrate into the host genome, which might result in insertional mutagenesis and chromosomal instability | RNA cannot integrate into the genome and therefore has no oncogenic potential |
| Expression | Long-term expression possible (months to years), depending on vector | Transient expression |

immunotherapy (Fotin-Mleczek et al. 2011, 2012) and prophylactic vaccines (Petsch et al. 2012; Kowalczyk et al. 2016; Schnee et al. 2016).

### 1.2.1 Induction of Antitumor Responses with RNActive® Vaccines

The antitumor efficacy of RNActive® vaccines was initially studied in the E.G7-OVA tumor model. This system uses C57BL/6 mice, which are inoculated subcutaneously with the syngeneic E.G7-OVA cell line, a clone of the mouse thymoma EL4 cell line that has been stably transfected to express ovalbumin (Moore et al. 1988). The vaccine was first studied in a prophylactic setting. Mice were immunized twice intradermally with the mRNA vaccine encoding ovalbumin and challenged one week later with E.G7-OVA cells. Compared to control mice that had received an mRNA vaccine encoding for an irrelevant antigen, the OVA-RNActive®-treated group displayed a significant delay in tumor growth (Fotin-Mleczek et al. 2011). Additionally, vaccination with OVA-RNActive® induced superior tumor protection in comparison with vaccination with ovalbumin protein or OVA-peptide SIINFEKL (Fotin-Mleczek et al. 2012).

A detailed analysis revealed that the antigen-specific vaccination had induced a balanced humoral and cellular immune responses with high titers of ovalbumin-specific IgG1 and IgG2a antibodies and cytotoxic T cells, which secreted IFN-γ upon stimulation with an OVA-derived peptide in vitro and killed OVA-peptide-loaded cells in vivo. Further experiments employing the antigen PSMA showed that the cytotoxic response was boostable and led to the formation of T memory cells. Increasing the number of biweekly vaccinations with the PSMA-RNActive® vaccine from 2 to 4 or 6 vaccinations induced a significant increase in both number of IFN-γ-secreting $CD8^+$ T cells and in vivo cytotoxicity during the acute phase. When analyzing these mice in the memory phase 8 weeks after the last immunization, a similar increase in the number of IFN-γ-secreting $CD8^+$ T cells and in vivo cytotoxicity was observed by increasing the number of vaccinations. Importantly, these IFN-γ-secreting $CD8^+$ T cells displayed the memory T cell marker CD44 and predominantly exhibited an effector memory

phenotype ($CD44^+$, $CCR7^-$, $CD62L^-$). Moreover, repeated vaccinations did not induce detectable frequencies of regulatory T cells in mice in comparison with controls (Fotin-Mleczek et al. 2011).

To test the efficacy of this technology in a therapeutic setting, the E.G7-OVA model was again employed. Mice were challenged subcutaneously with the tumor cells and received the OVA-RNActive® vaccine twice a week once the tumor was palpable. The treatment led to a significant delay in tumor growth but could not eradicate the tumor. A subsequent quantitative RT-PCR analysis showed that the ovalbumin expression in the outgrown tumors of all mice treated with the ovalbumin-encoding mRNA vaccine was reduced or even absent, while this was only the case in 1/5 of the untreated mice. This indicates that the tumors in RNActive® vaccine-treated mice had escaped immunotherapy due to downregulation of ovalbumin (Fotin-Mleczek et al. 2011).

Analysis of the cellular composition of tumors at several time points after vaccination demonstrated an increased and sustained influx of activated $CD8^+$ T cells into the tumors in vaccinated mice. Depletion of $CD8^+$ T cells, but not of $CD4^+$ T cells, during the effector phase completely abolished the antitumor effect of the vaccine. In contrast, $CD4^+$ T cells are required during the priming phase of the immune response as their depletion during vaccination significantly reduced vaccine efficacy (Fotin-Mleczek et al. 2011).

### 1.2.2 Clinical Studies with RNActive® Vaccines

Based on the encouraging preclinical results, the decision was taken to advance this technology to clinical testing in cancer patients (Table 2). The first-in-class first-in-man clinical trial using mRNA as a therapeutic approach was CV9103 for patients with prostate cancer. CV9103 is an RNActive® vaccine targeting the following 4 tumor-associated antigens: prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), prostate stem cell antigen (PSCA), and six-transmembrane epithelial antigen of the prostate 1 (STEAP1). The vaccine was tested in a phase I/IIa study that enrolled 44 eligible patients with castrate-resistant prostate cancer with rising levels of PSA (all patients) and metastatic disease in 84 % of patients and comprised 12 men in the phase I and 32 in the phase IIa study (Kübler et al. 2015). The study was conducted as an open, uncontrolled, multicenter, international, and prospective trial with safety and tolerability defined as the primary endpoints and the induction of antigen-specific humoral and cellular immune responses as secondary endpoints. The recommended dose was established via a dose escalation in the phase I trial (259, 640, and 1280 μg total RNA tested), while the phase IIa study was designed to confirm safety and address the induction of antigen-specific cellular and humoral immune responses following injection with the highest dose. This study demonstrated that CV9103 exhibits a favorable safety profile with most frequent adverse events being injection site reactions, fatigue, pyrexia, chills, and flulike symptoms. The majority of related adverse events was of mild-to-moderate intensity and resolved upon therapy.

Importantly, immune responses against all mRNA-encoded antigens were detectable independent of the cellular localization of the antigen and the HLA

**Table 2** Overview of clinical trials using RNActive® vaccines

| Biologic | Indication | Phase | Antigens | *n* | Outcome | Reference |
|---|---|---|---|---|---|---|
| CV9103 | Hormone refractory prostate cancer | I/IIa | PSA<br>PSMA<br>PSCA<br>STEAP1 | 44 | – Favorable safety profile<br>– Induction of multiple antigen-specific immune responses<br>– Induction of immune responses against all encoded antigens | Kübler et al. (2015) |
| CV9104 | Metastatic castrate-refractory prostate cancer<br>Asymptomatic, minimally symptomatic, chemonaive | I/IIb | MUC1<br>PAP<br>PSA<br>PSMA<br>PSCA<br>STEAP1 | Ph IIb 197 | Double-blind placebo-controlled study<br>Recruitment completed | |
| CV9104 | Intermediate- or high-risk prostate cancer prior to radical prostatectomy | II | MUC1<br>PAP<br>PSA<br>PSMA<br>PSCA<br>STEAP1 | 48 | Open-label randomized trial of presurgical CV9104 vs no treatment<br>Recruitment completed | |
| CV9201 | Advanced-stage non-small-cell lung cancer | I/IIa | MAGEC1<br>MAGEC2<br>NY-ESO-1<br>Survivin<br>5T4 | 46 | – Favorable safety profile<br>– Induction of multiple antigen-specific immune responses<br>– Induction of immune responses against all encoded antigens | Sebastian et al., manuscript in preparation |
| CV9202+ local radiation | Advanced-stage non-small-cell lung cancer | Ib | MAGEC1<br>MAGEC2<br>MUC1<br>NY-ESO-1<br>Survivin<br>5T4 | 26 | Study ongoing | |

subtype of the patient. More specifically, antigen-specific cellular immune responses were detected in 76 % of all patients treated with the highest dose. Of note, 58 % of responding patients and 45 % of all evaluable patients at the highest dose level showed responses to multiple antigens. The assessment of humoral immune responses was restricted to PSA and PSCA, since no proteins suitable for ELISA were available for the other vaccination antigens at that time. An increase of PSA-specific antibodies could be detected in 12 % of patients, but no increase of anti-PSCA antibodies was observed.

Clinical efficacy of CV9103 was assessed mainly by progression of PSA serum levels, since radiographic progression-free survival (PFS) was not assessed in this clinical trial. Similar to other cancer vaccine trials, a median time to PSA-related PFS is 1.8 months and an objective PSA response in only one patient was observed.

Additionally, the study estimated a median survival of 31.4 months for a subgroup of 36 patients with metastatic castrate-resistant prostate cancer. In this group, a non-significant correlation between survival time and multivalent immune response was observed. Interestingly, outcome further improved in patients with responses to multiple vaccination antigens. Yet a correlation between immune responses against more than one antigen and improved survival time does not necessarily imply a therapeutic effect of vaccination. The ability to mount an immune response after vaccination might be instead a surrogate of an improved prognosis (Kübler et al. 2015).

Nevertheless, these encouraging results justified the investigation of RNActive® vaccines in a controlled clinical trial. Hence, 197 patients with castrate-resistant asymptomatic to minimally symptomatic castration-resistant prostate cancer were enrolled and randomized in an ongoing placebo-controlled phase IIb study with CV9104. This updated vaccine is based on CV9103 and encodes two additional tumor-associated antigens, namely prostatic acid phosphatase (PAP) and mucin 1 (MUC1), a glycoprotein that is overexpressed and aberrantly glycosylated in various cancers including prostate cancer. The clinical efficacy and safety of PAP as vaccine antigen have previously been demonstrated in the clinical trial of Sipuleucel-T (Kantoff et al. 2010), while MUC1 has mainly been used in immunotherapy approaches against non-small-cell lung cancer with a favorable safety profile (Palmer et al. 2001). Primary endpoint of the study is overall survival from time of randomization (up to 3.5–4 years). Secondary endpoints include PFS from date of randomization and from start of first subsequent systemic therapy, respectively, immune response against the CV9104 antigens, time to symptom progression, and change in quality of life.

In a second study, CV9104 is tested in patients with high- and intermediate-risk non-metastatic prostate cancer. For these patients, radical prostatectomy is a standard treatment option. After surgery, the estimated risk of relapse is considerable, even more in case of positive surgical margins. Hence, new adjuvant or neoadjuvant treatments that can prevent relapses after primary therapy are highly needed.

The study is aimed to assess the immune responses in the peripheral blood as well as immunological parameters in the tumor tissue such as cellular immune infiltrates, cytokines, and gene expression upon application of CV9104.

In addition to its application in the treatment of prostate cancer, RNActive® vaccines were also tested as an immunotherapeutic against non-small-cell lung carcinoma (NSCLC) in a clinical phase I/IIa trial (Sebastian et al., manuscript in preparation).

In this study, the immunotherapeutic CV9201 was intradermally administered at different dose levels to evaluate the safety and tolerability in patients with advanced NSCLC. CV9201 encodes five NSCLC-associated cancer antigens selected for their role in NSCLC oncogenesis, differential expression between malignant and normal tissue and to induce cellular or humoral immune responses: New York esophageal squamous cell carcinoma-1 (NY-ESO-1), melanoma antigen family C1 (MAGEC1), melanoma antigen family C2 (MAGEC2), survivin, and trophoblast glycoprotein (TPBG; synonym 5T4). Eligible patients had stage IIIB/IV NSCLC and at least stable disease after first-line treatment. During the open, uncontrolled, multicenter, and prospective trial, 9 patients received CV9201 in the phase I and 37 patients in the phase IIa part of the study. Overall, 45/46 patients received at least two treatments and 33/46 patients received all five planned doses of CV9201. This study demonstrated that similar to CV9103, also CV9201 exhibits a favorable safety profile with most frequent adverse events being injection site reactions, including injection site erythema, pruritus, discoloration, and pain. Other treatment-related adverse events were pyrexia and fatigue, chills, and flulike symptoms. Furthermore, immune responses against all encoded antigens were detectable. In detail, 65 % of all patients exhibited antigen-specific cellular or humoral immune responses and 65 % of responders reacted against multiple antigens. The strength of the induced T cell response varied, but most patients displayed a more-than-twofold increase in frequency and number of antigen-specific T cells detected ex vivo, with some patients showing very strong responses. Moreover, 48 % of the patients treated in phase IIa had antigen-specific humoral responses, with more than twice as many patients exhibiting IgM than IgG responses.

Tumor response was evaluable in 29 patients of which nine patients had stable disease and twenty patients experienced progressive disease as best overall response. The median PFS in the total study population was 2.7 months. Importantly, the study revealed an encouraging clinical course in five patients with stage IV NSCLC being without further systemic cancer therapy after 1 year.

Overall, these results demonstrate that RNActive® vaccines represent a highly promising new vaccine platform that is safe, highly specific, versatile, and able to induce a balance immune response in the context of clinical studies.

### 1.2.3 Combination of RNActive® Vaccines with Chemotherapy or Radiation Therapy

From a clinical perspective, it is important to assess whether RNActive® vaccines can be combined with the current standard of care. Therefore, the combination of these vaccines with chemotherapy or radiation therapy was investigated.

The combination with chemotherapy was tested in the E.G7-OVA tumor model using docetaxel or cisplatin and the mRNA vaccine against ovalbumin.

Having received a subcutaneous tumor challenge followed by RNActive® vaccinations, mice were subsequently treated with an intraperitoneal application of docetaxel followed by further vaccinations. This treatment schedule led to a significant delay in tumor growth compared to docetaxel or RNActive® vaccine alone. Similar results were observed when using the same treatment schedule but cisplatin as a chemotherapeutic drug. In agreement with published results using viral vector vaccines (Garnett et al. 2008), a delay of tumor growth was not detectable when chemotherapy preceded vaccination, indicating a negative effect of docetaxel during the induction phase of the immune response (Fotin-Mleczek et al. 2012).

An interesting option is the combination of vaccination with radiation therapy because of the complementary features of the two approaches, which can synergize to restrict tumor growth. Radiation is a well-established therapeutic method, especially for the treatment of single solid tumors. It acts on the tumor tissue locally and, for a very short period of time, is effective on tumors with a local immunosuppressive environment due to increased release of proinflammatory cytokines (Formenti and Demaria 2013) and induces local MHC expression (Reits et al. 2006). Moreover, radiotherapy-induced cell death seems to represent a form of immunogenic cell death characterized by cell surface translocation of calreticulin and extracellular release of ATP and the high-mobility group box 1 (HMGB1) protein (Golden et al. 2014). These factors are able to activate dendritic cells (DCs) via pattern recognition receptors and promote the cross-presentation of tumor antigens between DCs and T cells (Demaria and Formenti 2012).

Vaccination, on the other hand, is able to induce systemic immune responses against non-symptomatic metastatic disease that are long-lasting and boostable.

Hence, the combination of radiotherapy and RNActive® vaccines was tested in an E.G7-OVA tumor model. After the establishment of large subcutaneous tumors, the mice received three local radiations on consecutive days. Concomitantly, several vaccinations with OVA-RNActive® vaccine were administered. These experiments demonstrated a pronounced tumor regression in the group treated with combination therapy and complete eradication of large established E.G7-OVA tumors in 3 out of 7 mice, while single therapies remained significantly less effective (Fotin-Mleczek et al. 2014). Additional experiments were performed in the Lewis lung cancer (LLC) model as a second tumor system, which features low immunogenicity and is resistant to different kinds of therapeutic regimens (Savai et al. 2007; Shojaei et al. 2007; Knapp et al. 2003). After subcutaneous tumor challenge, the mice were treated with three local radiations on consecutive days in combination with several vaccinations with RNActive® vaccines encoding the described tumor-associated antigens EGFR and connexin (Mandelboim et al. 1995). The combined treatment showed a synergistic antitumor effect demonstrating the efficacy of radioimmunotherapy even in a low immunogenic carcinoma model system (Fotin-Mleczek et al. 2014).

### 1.2.4 Clinical Study with RNActive® Vaccines in Combination with Radiotherapy

Based on the promising preclinical results, a phase Ib study is ongoing, evaluating the clinical potential of the RNActive® vaccine CV9202 in combination with local radiation of individual metastases as consolidation and maintenance treatment for patients with stage IV non-small-cell lung cancer. CV9202 is an updated version of CV9201 that encodes in addition to NY-ESO-1, MAGEC1, MAGEC2, survivin, and 5T4, the antigen MUC1. As described earlier, the glycoprotein MUC1—overexpressed and aberrantly glycosylated in various cancers—has previously been used in immunotherapy approaches against non-small-cell lung cancer (Palmer et al. 2001). The exploratory, open-label multicenter trial assesses the safety and tolerability of CV9202 vaccination combined with local radiation in patients with stage IV NSCLC of different subtypes (squamous and non-squamous cell with or without EGFR mutations) who achieved a response or stable disease after first-line therapy (chemotherapy or EGFR tyrosine kinase inhibitors). All patients receive two initial vaccinations with CV9202 prior to local radiotherapy followed by further vaccinations until disease progression. The primary endpoint of the study is the number of patients experiencing treatment-related adverse events above grade 3. Secondary endpoints include evaluation of cellular and humoral immune responses to antigens encoded by CV9202 and antigens not covered by the vaccine allowing the investigation of potential antigen spreading in response to the treatment. In addition, assessment of PFS, time to start of second-line cancer treatment, response to second-line treatment, and overall survival are secondary efficacy endpoints of the study (Sebastian et al. 2014).

### 1.2.5 Combination of RNActive® Vaccines with Immune Checkpoint Inhibitors

The immune response to cancer evolves over many years and ultimately fails due to immunoediting (downregulation of MHC I and immunogenic antigens) and immunoevasion (immunosuppressive microenvironment) (Vesely and Schreiber 2013). Moreover, the antitumor immune response has many characteristics of a chronic immune response, with T cell exhaustion mediated by immune checkpoint receptors such as programmed cell death 1 (PD1). PD1 is also expressed by B cells, NK cells, and regulatory T cells in addition to activated T cells and is engaged by two different ligands PDL1 (B7-H1) and PDL2 (B7-DC). In contrast, the immune checkpoint receptor cytotoxic T lymphocyte antigen 4 (CTLA4) is only expressed by activated T cells or regulatory T cells, and binding of its ligands CD80 (B7-1) or CD86 (B7-2) leads to inhibition of T cells by antagonizing the costimulatory signals delivered by CD28 during T cell activation (Melero et al. 2015).

Various antibodies targeting these immune checkpoints are currently in clinical testing and show promising results for some of the patients. But there are still a substantial proportion of patients not reacting to immune checkpoint blockade. Newly published studies imply a strong correlation between the occurrence of antigen-specific T cells and response to immune checkpoint blockade (reviewed in Schumacher and Schreiber 2015). Hence, the induction of antigen-specific cellular

response against tumor-associated antigens by vaccination would be beneficial during immune checkpoint blockade therapy. To test whether checkpoint inhibitors can also be combined with mRNA vaccines, an RNActive® vaccine was combined with an anti-CTLA-4 antibody. Mice were challenged with E.G7-OVA tumors and treated alternatingly with an ovalbumin-encoding mRNA vaccine and an anti-CTLA-4 antibody. The combination led to significantly reduced tumor growth, while anti-CTLA-4 treatment alone did not delay tumor growth. In addition, mice in which treatment had led to complete rejection were rechallenged with the parental tumor cell line, which lacks OVA expression. These experiments showed that the mice were nonetheless completely protected, strongly indicative of the induction of epitope spreading (Fotin-Mleczek et al. 2012).

In summary, the superior antitumor response of RNActive® vaccine treatment in combination with immune checkpoint blockade could be demonstrated in a preclinical animal model.

## 1.3 Personalized mRNA Vaccines

In the past, development of cancer vaccines was mainly dedicated toward the so-called tumor-associated proteins as antigenic source. Mostly, these tumor-associated antigens can be divided into two classes: Antigens derived from fetal genes overexpressed in cancer cells (van der Bruggen et al. 1991; Van den Eynde et al. 1991) or antigens derived from tissue-specific proteins also expressed in cancer cells (Boon et al. 1994). To offer broadly applicable immune therapies against cancer, the latest developmental activity in the field of cancer vaccines was focused on shared tumor-associated antigens that are expressed not only in abundance in different cancer types but also in a certain amount of cancer patients.

But the use of such shared tumor-associated antigens is accompanied with certain drawbacks: Firstly, T cells easily recognize foreign antigens but in general are unable to recognize self-antigens due to the negative selection process in the thymus preventing induction of autoimmunity. Unfortunately, most tumor-associated antigens fall into the group of self-antigens hampering the initiation of a proper immune response toward these antigens. Secondly, tumor-associated antigens are not solitarily expressed in tumor tissue leading to possible on-target effects in healthy tissue through the induced immune response. Finally, the expression of tumor-associated antigens in different tumor tissues or different cancer patients can be highly variable due to normal biologic heterogeneity. Recent research has shown that ninety-five percent of the mutations in a given patient seem to be unique to that tumor (Stratton 2011) and that even on a subclonal level a high variability can be found in a certain tumor (Gerlinger et al. 2015; Martincorena et al. 2015). Therefore, it can be assumed that expression of shared tumor-associated antigens is subjected to a similar variability. Moreover, tumor tissues often exploit different escape mechanisms to evade the antitumoral immune response like downregulation of tumor-associated antigens or preferential outgrowth of non-expressing clones (Matsushita et al. 2012).

The use of really tumor-specific antigens or neoantigens instead of tumor-associated antigens would be advantageous for the development of cancer vaccines for obvious reasons: Owed to the tumor-specific mutations of these neoantigens, the specific T cell repertoire is not affected by negative selection in the thymus. In addition, neoantigen-specific T cells would not cause on-target effects on healthy tissue as mutated antigens are only expressed in cancer cells. Already in the 1970s and 1980s, the tumor-rejecting abilities of neoantigen-induced immune responses were described in transplantable tumor models (Boon and Kellermann 1977; Lurquin et al. 1989). Moreover, different studies could show that single mutations in defined genes are able to induce antigen-specific cytolytic T cell responses (Wölfel et al. 1995; Coulie et al. 1995; Echchakir et al. 2001).

The advent of next-generation sequencing technologies over the last years allows nowadays the definition of such tumor-specific antigens or neoantigens by comparing genome, exome, or transcriptome data of tumor tissue to healthy tissue for an individual patient. This approach allows the description of non-synonymous mutations solely expressed in tumor cells. Moreover, exome or transcriptome analysis permits the definition of protein abundance for defined non-synonymous mutations in the tumor tissue. This conglomerate of tumor-specific mutations, also named "mutanome," displayed by an individual tumor is a valuable source for highly tumor-specific antigens.

Two studies in mice provided the first direct evidence that neoantigens identified by mutanome analysis can be recognized by T cells (Castle et al. 2012; Matsushita et al. 2012). Briefly, potential MHC-binding peptides were predicted for all tumor-specific mutations that result in the formation of novel protein sequences and the most promising mutated peptides were used to query T cell reactivity in vivo. Castle and colleagues could show that 16 out of 50 mutation-coding peptides elicit a measurable immune response in immunized mice. Moreover, the induced immune response conferred a significant antitumor effect in a B16F10 tumor model. In the study of Matsushita et al., it was demonstrated that one in silico-predicted highly immunogenic neoantigen derived from a mutant spectrin-β2 is sufficient to induce tumor cell elimination in an unedited tumor.

Subsequent studies in a clinical setting have added further evidences for the ability of neoantigens to induce significant antitumoral immune responses and the predictability of such neoantigens by mutanome mining. Robbins and coworkers could identify neoantigens recognized by adoptively transferred tumor-reactive T cells mining exome sequencing data (Robbins et al. 2013), whereas van Rooji and colleagues discovered neoantigen-specific T cell reactivity in an ipilimumab-responsive melanoma by tumor exome analysis (van Rooij et al. 2013). Particularly, the correlation between successful immune checkpoint blockade and the occurrence of tumor-specific neoantigens has been demonstrated in several publications over the last years. Gubin and colleagues identified neoantigens following anti-PD1 or anti-CTLA-4 therapy of mice bearing progressively growing sarcoma. Additionally, they could show that therapeutic synthetic long-peptide vaccines incorporating these mutant epitopes are able to induce tumor rejection comparably to immune checkpoint blockade (Gubin et al. 2014). Even more

interesting are the results of Snyder et al. and Rizvi et al. demonstrating a close correlation between the abundance of clonal neoantigens and sensitivity to immune checkpoint blockade by anti-PD1 or anti-CTLA-4 treatment in patients suffering from non-small-cell lung cancer or melanoma. Moreover, the predicted neoantigens elicited specific $CD8^+$ T cell responses in both studies (Snyder et al. 2014; Rizvi et al. 2015). Also for therapeutic approaches with adoptive tumor-infiltrating lymphocyte transfer in melanoma patients, a similar correlation between neoantigen load and therapy efficacy was revealed (Lu et al. 2014; Linnemann et al. 2015). Additionally, Tran and colleagues could provide evidence that immunotherapy based on mutation-specific $CD4^+$ T cells in a patient with epithelial cancer is possible (Tran et al. 2014).

The above-mentioned studies and several further publications demonstrated the feasibility of tumor exome mining plus immunogenicity prediction based on HLA allotypes and peptide-binding probability to identify patient-specific highly immunogenic neoantigens in different types of cancer with a variety of approaches (Rajasagi et al. 2014; Duan et al. 2014; Yadav et al. 2014; Tran et al. 2015). Moreover, the potential value of such neoantigens for personalized immunotherapy approaches has been frequently implied (Gubin et al. 2014; Tran et al. 2014). One possibility to target specifically neoantigens in cancer patients is to engineer neoantigen-specific T cell receptors and adoptively transfer these T cells (Leisegang et al. 2016) using methods also described here. A further option would be the immunization with vaccines incorporating the mutated sequences of neoantigens. The efficacy of such an immunization approach using peptides is currently under evaluation in a phase I clinical trial in IDH1R123H-mutated grade III–IV gliomas (NCT02454634). This clinical study is based on the results published by Schumacher and colleagues showing the antitumor potential of mutant IDH1 peptide vaccination in tumor-bearing MHC-humanized mice (Schumacher et al. 2014). However, mutant IDH1 seems to be a unique exception, since the mutation can be found frequently in diffuse grade II and grade III gliomas.

This high penetrance of a neoantigen appears to be in contrast to other tumor entities. In most human tumors, a large fraction of the mutations is not shared between patients at a meaningful frequency. Moreover, only a small portion of mutations within expressed genes have antigenic potential leading to T cell reactivity (Lu et al. 2014; Linnemann et al. 2015). Due to these limitations, broadly applicable neoantigens that can be used in huge patient cohorts for vaccinations are highly unlikely. Hence, mRNA vaccines represent a superior approach to satisfy the specific demands of a personalized immunotherapy based on neoantigens. The obvious advantages of mRNA vaccines in comparison with peptide vaccines were already mentioned above. Particularly, the production under well-defined and controlled conditions by in vitro transcription and the possibility to produce vaccines against different neoantigens by a common process and in a relatively short period of time fulfill the requirements for a personalized immunotherapy approach.

In a recent study of Kreiter and colleagues, the tumor-rejecting properties of neoantigen-based RNA vaccines were demonstrated in murine tumor models. In principle, mutanome analysis and MHC-binding prediction lead to selection of

neoepitopes with proven in vivo immunogenicity for several of the selected neoepitopes. Additionally, the immunization of tumor-bearing mice with an mRNA vaccine coding for one B16-specific neoantigen resulted in a significantly increased survival of immunized mice in comparison with untreated mice. For the clinical setting, a combination of several neoantigens in one vaccine would be preferable to address tumor heterogeneity and immune editing which could mediate clinical failure of vaccines in humans. It was recently demonstrated that immunotherapy of CT26 lung metastasis after i.v. injection of tumor cells employing mRNA-encoded pentatope resulted in a drastic reduction of the tumor burden in the lung of vaccinated mice (Kreiter et al. 2015).

Based on these convincing results, a first phase I clinical trial was started in 2014 with melanoma patients, where a poly-neoepitope coding RNA vaccine is administered that targets the unique mutation signature of an individual patient (NCT02035956). In addition, a second clinical trial was set up in 2015 using a slightly different approach for the treatment of triple-negative breast cancer (NCT02316457).

In summary, induction of neoantigen-specific T cell reactivity via mRNA vaccines seems to be a promising strategy for successful cancer immunotherapies. Because of the tumor-restricted expression of the antigens that are being targeted, these personalized antitumor therapies offer the promise of high specificity and safety. Additionally, T cell reactivity that can be achieved with such personalized immunotherapies will further increase the spectrum of human malignancies that respond to cancer immunotherapy. Particularly, the correlation between immune checkpoint blockade and neoantigen burden implies synergistic effects by combination of personalized cancer immunotherapy with checkpoint blockade.

## 1.4 Cellular Vaccines: mRNA-Pulsed Dendritic Cells

mRNA-transfected dendritic cell (DC) vaccines represent a distinct type of vaccine strategy involving RNA. Among different types of antigen-presenting cells (APCs), DCs are considered to be the most potent ones because they can efficiently prime naïve T cells, thereby stimulating an adaptive immune response. Because of their ability to induce potent antitumor CTLs both in vitro and in vivo, DCs have been employed as cancer vaccination platform. The basis of this therapeutic strategy is to use cancer patient's DCs expressing tumor-associated antigens (TAAs) in order to activate antigen-specific T cells which after differentiation in cytotoxic effector T cells will be able to eradicate tumor cells, irrespective of their location. Moreover, these T cells can form an immunological memory providing in this way a defense against recurring cancer cells.

For the delivery of tumor antigens to DCs, mRNA is considered as an attractive vector as it overcomes some limitations associated with the use of antigen-derived peptides, the most commonly used strategy of antigen loading (Cerundolo et al. 2004; Schuler et al. 2003; Jager et al. 2002). Synthetic peptides represent only defined epitopes from known TAAs and are dependent on prior identification and

on HLA restriction of the patients, meaning that the induced immune response by the vaccine is limited to the peptides used and that only patients with specific HLA allotypes can be treated (Van Nuffel et al. 2012). Instead, mRNA molecules can encode the entire tumor antigen. Therefore, multiple immunogenic epitopes within the same protein can be presented. In addition, since the protein is endogenously expressed and presented after mRNA transfection of DCs, multiple peptide–MHC complexes (pMHC) are generated; thereby, vaccine development is independent on the patient's genetic background.

Boczkowski and collaborators were the first ones to describe in the late 1990s that DCs pulsed with mRNA encoding for tumor antigens are potent antigen-presenting cells (Boczkowski et al. 1996). Only few years later, an early clinical trial using DCs loaded with RNA encoding the PSA proved the feasibility and safety of this approach. CTL responses against metastatic prostate tumors expressing PSA were indeed induced in some patients (Heiser et al. 2002). This approach was based on passive pulsing of DCs, which relies on the ability of these cells to take up mRNA through micropinocytosis (Diken et al. 2011). But this mechanism, involving transport into the endosomes, entails the risk that only a fraction of the mRNA can reach the cytosol and can then be translated into the protein. Therefore, several approaches have been established in order to deliver mRNA directly to the cytoplasm such as electroporation, nucleofection, lipofection, and more recently sonoporation (Benteyn et al. 2015). Above all, electroporation has been shown to be a powerful technique to introduce tumor antigens into DCs (Van Tendeloo et al. 2001).

mRNA encoding specific TAAs or total tumor RNA can be used to transfect DCs. Vaccine strategies employing DCs transfected with defined TAA mRNA avoid the need for growth of patient-specific tumor cells and reduce the risk of autoimmunity, which can be induced in patients by the presence of normally expressed self-proteins (Nair et al. 1999; Ponsaerts et al. 2003). However, there are some limitations due to the fact that for many cancers, the TAAs are unknown. An attractive alternative is to utilize DCs transfected with patient-derived total tumor RNA. Through this approach, the entire spectrum of tumor-specific antigens is displayed, thereby eliminating the need for identification of TAAs and allowing the immune system to use the most effective antigens while reducing the risk of escape mutants. Another advantage of using tumor-derived RNA as a source of whole-tumor antigen is that it can be quickly and easily amplified by RT-PCR from even a small amount of tumor (Heiser et al. 2002).

To date, several clinical trials have been reported using mRNA-transfected DCs. The majority of these studies have shown that tumor-specific T cell responses can be induced by mRNA-transfected DCs in several tumor entities such as brain cancer, melanoma, lung adenocarcinoma, renal cell carcinoma, and ovarian cancer (Bonehill et al. 2009; Caruso et al. 2004, 2005; Kyte et al. 2007; Nair et al. 2002; Su et al. 2003; Morse et al. 2003; Dannull et al. 2005; Van Nuffel et al. 2012). In particular, in the renal cell carcinoma study by Su et al., patients displayed no evidence of dose-limiting toxicity or induction of autoimmunity (Su et al. 2003).

Similarly, brain tumor and neuroblastoma studies conducted in nine and seven patients, respectively, showed a clinical response in a total of three of the patients.

Despite many progresses in mRNA-DC immunotherapies, clinical responses remain modest and new strategies on how to enhance the efficacy of mRNA-DC vaccines are being explored. Besides the delivery of tumor antigens, mRNA can be used to deliver also proteins that can modulate the function of DCs. Cotransfection of DCs with mRNA encoding TAAs and costimulatory molecules such as CD83 (Aerts-Toegaerr et al. 2007), OX40 (Dannull et al. 2005), and 4-1BBL (Grünebach et al. 2005) has shown to improve mRNA-DC vaccine efficacy in preclinical models. Moreover, DCs activated through electroporation with mRNAs encoding four tumor antigens as well as mRNAs encoding CD40 ligand and constitutively active TLR4 and CD70 (TriMix-DCs) resulted in a broad T cell response and durable tumor response in chemorefractory advanced melanoma patients (Van Nuffel et al. 2012). DCs have been also modified with mRNAs encoding immunomodulating cytokines. For example, DCs transfected with mRNAs encoding IL-12 and TAAs were shown to induce high-avidity cytotoxic T cells and enhance their effector function. In addition, the migratory capacity of DCs has been modulated using mRNA encoding a chimeric E/L-selectin, a strategy that led to increased migration of the DCs to lymph nodes upon intravenous administration (Dörrie et al. 2008).

Finally, further preclinical strategies have focused on increased DC function by coadministration of DCs transfected with TAA-encoding mRNAs or mRNAs encoding for antagonistic anti-CTLA-4 or agonistic anti-GITR antibodies (Pruitt et al. 2011). Thus, the engineered DCs have the ability not only to present the tumor antigen of interest but also to locally modulate immune checkpoints and the tumor microenvironment. Because of promising results in preclinical studies, this approach is currently under investigation in a phase I clinical trial for the treatment of melanoma patients (NCT01216436).

Taken together, these strategies have the potential to improve cancer immunotherapy.

## 1.5 CAR T Cells

Chimeric antigen receptor (CAR) T cell immunotherapy has recently emerged as a promising strategy for treating tumors (Cheadle et al. 2012; Restifo et al. 2012; Kershaw et al. 2013).

CAR T cells are a form of personalized cell therapy using patient-derived T cells. After the collection of T cells, they are genetically engineered to express receptors that allow them to recognize a specific antigen expressed by the patient's cancer cells and to attack the tumor, once the cells are infused back into the patient. Chimeric receptors are usually composed of an extracellular TAA-specific antibody-binding domain fused to intracellular T cell-signaling domains. CAR T cells can be engineered to target virtually any tumor-associated antigen (Jensen and Riddell 2015).

Since CARs are based on TAA-specific antibody-binding domains, recognition of the tumor antigen is HLA-independent, thereby extending its applicability to many patients and overcoming some tumor escape mechanisms (Ramos and Dotti 2011).

The adoptive transfer of genetically modified T cells engineered to express a CAR has shown early promising results in the treatment of hematologic malignancies (Kochenderfer et al. 2012; Brentjens et al. 2013; Porter et al. 2011; Grupp et al. 2013). However, by now, the use of CAR T cells was less successful for the therapy of solid cancers, one of the major issues being toxicity. Indeed, clinical trials have revealed the potential of engineered T cells to recognize and attack normal cells that share the expression of the tumor CAR-specific antigen, causing off-target toxicity (Lamers et al. 2006, 2013; Maus et al. 2013).

The main approach to modify the T cells uses viral vectors such as γ-retroviruses and lentiviruses that are expensive to produce and involve safety concerns associated with their integration into the genome (Kershaw et al. 2013).

Recently, mRNA electroporation has been used to engineer T cells with transient CAR expression. Prof. June and his colleagues at the University of Pennsylvania's Perelman School of Medicine were the first to prove the feasibility of this approach and the potential of mRNA-engineered T cells to induce robust antitumor effects in preclinical tumor models (Zhao et al. 2010; Barett et al. 2011, 2013).

Consequently, they conducted the first clinical trial to evaluate the feasibility and safety of repetitive infusions of mRNA CAR T cells in patients (Beatty et al. 2014). Two patients, one with advanced mesothelioma and the other one having metastatic pancreatic cancer, were recruited into a phase I clinical trial. Researchers used mRNA electroporation to engineer patient-derived T cells with a CAR specifically targeting mesothelin (Tmeso cells), a TAA overexpressed in several cancers. After ensuring their viability and specificity, engineered T cells were repetitively infused into the patients. Surprisingly, clinical and laboratory evidence of antitumor activity without explicit evidence of off-tumor toxicity against normal tissues was shown in both patients. In addition to the antitumor activity, CAR Tmeso cells were able to trigger a broad antitumor immune response consistent with epitope spreading. Moreover, mRNA CAR Tmeso cells were shown to persist transiently within the peripheral blood after intravenous administration and migrate to tumor tissue.

More recently, mRNA has been used not only to engineer T cells to recognize specific TAAs but also to transiently deliver a modified telomerase reverse transcriptase (TERT) to CD19 CAR T cells. This study provided an effective and safe method to extend the T cell replicative life span improving in this way the persistence and antitumor effects of CAR T cell in mouse xenograft tumor models of B cell malignancies compared with conventional CAR T cells (Bai et al. 2015).

All these findings support the development of mRNA-engineered T cells as a novel approach for adoptive cell transfer, providing a flexible platform for the treatment of cancer that may complement the use of retroviral and lentiviral engineered T cells.

## 2 Conclusion

The versatility of mRNA in encoding TAAs, patient-derived tumor neoantigens, or even chimeric antigen receptors makes mRNA vaccines a promising candidate for use in cancer immunotherapy. The encouraging results of the first clinical studies have prompted intensive research into strategies to increase the immunogenicity of the vaccines, which include modifications to the mRNA and the application or the combination with other treatments like checkpoint inhibitors or radiation therapy. These efforts will hopefully result in novel treatment options in cancer therapy, which will make full use of the unique properties of mRNA vaccines.

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