Methods in Molecular Biology 1499

Springer Protocols

Thomas Kramps · Knut Elbers *Editors*

RNA Vaccines

Methods and Protocols

EXTRAS ONLINE

Humana Press

METHODS IN MOLECULAR BIOLOGY

Series Editor
John M. Walker
School of Life and Medical Sciences
University of Hertfordshire
Hatfield, Hertfordshire, AL10 9AB, UK

For further volumes:
http://www.springer.com/series/7651

RNA Vaccines

Methods and Protocols

Edited by

Thomas Kramps

Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim am Rhein, Germany

Knut Elbers

Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany

Humana Press

Editors
Thomas Kramps
Boehringer Ingelheim Pharma GmbH & Co. KG
Ingelheim am Rhein, Germany

Knut Elbers
Boehringer Ingelheim GmbH
Ingelheim am Rhein, Germany

ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-6479-6 ISBN 978-1-4939-6481-9 (eBook)
DOI 10.1007/978-1-4939-6481-9

Library of Congress Control Number: 2016958318

Printed on acid-free paper

This Humana Press imprint is published by Springer Nature
The registered company is Springer Science+Business Media LLC
The registered company address is: 233 Spring Street, New York, NY 10013, U.S.A.

Preface

Vaccines are currently regaining attention from members of the medical and scientific communities but even the broader public, including heads of state. This level of public awareness of the fundamental relevance of vaccines for global human well-being has been rekindled by dramatic threats of rapidly emerging infectious diseases (predominantly caused by viruses) and increasingly widespread multidrug-resistant bacterial infections. Insect-borne Zika virus and Ebola fever are only the most recent examples demonstrating a persistent vulnerability of human society to such primordial threats. In another area, cancer immunotherapy, vaccines are a promising, innovative treatment modality, too. In future, integrated treatment regimens that include cancer vaccines may enable patients to better regain immunological control over the tumor, superseding or complementing today's immune checkpoint inhibitors.

RNA vaccines, the subject of this volume, span a spectrum from recombinant viruses to self-amplifying mRNA and nonreplicating mRNA vectors. Given this breadth, we firmly believe that RNA technology will eventually spawn vector platforms of enormous medical and commercial potential. All RNA vaccines share distinct features, which will likely contribute to their continuing relevance:

- Like viruses, they provide integrated stimuli to adaptive and innate immunity, i.e., antigen expression in situ and danger signaling, e.g., via toll-like receptor pathways.
- Like live vectors, they induce "balanced" immune responses that comprise humoral and cellular effectors as well as immunological memory.
- Synthetic RNA vaccines allow for a combination of different antigens without increasing the complexity of vaccine formulation, thus facilitating speedy and flexible production.
- Due to "vector neutrality" they generally allow for highly repetitive vaccination schedules with consistent boost potential and no or little immune response directed against the vector.
- Thermostable RNA vaccines could simplify transport and stockpiling even in the absence of a cold chain, a frequently underestimated hurdle for global disease control.

In any case, unlocking this potential will require continued optimization as well as informed choice of applications.

Thus, the aim of this volume is to facilitate both efforts by assembling an overview of the field and practical hints for vaccinologists in academia and industry. Different RNA vaccines exhibit diverse sets of trade-offs with respect to efficacy, reactogenicity, and handling that reflect the great versatility of this class of vaccines. To choose the best way ahead, a basic understanding of the regulatory framework, including aspects of nonclinical safety testing and good manufacturing practice, is essential. The scope of protocols included in this book is laid out and discussed in more detail (together with some scientific context and additional references) in the introductory, first chapter. The protocols include relevant pointers to current "best practice" with concrete tips and tricks in the notes section of each chapter.

Finally, we are well aware that the relevant body of knowledge is rapidly developing and cannot realistically be captured in a single volume. We, therefore, sincerely hope that this compendium may engender increased collaboration on RNA vaccines between basic and applied scientists in academia, government, and industry to develop future solutions for today's challenges. In any technological field, we need reliable maps that are drawn from facts and open discourse to safely navigate both hyperbole and pessimism. We hope that this book will offer helpful orientation.

Ingelheim am Rhein, Germany *Thomas Kramps*
Knut Elbers

Contents

Contributors

Lukasz Bialkowski • *Laboratory of Molecular and Cellular Therapy, Vrije Universiteit Brussel, Brussels, Belgium*

Karine Breckpot • *Laboratory of Molecular and Cellular Therapy, Vrije Universiteit Brussel, Brussels, Belgium*

Cedrik M. Britten • *R&D Oncology, Glaxo Smith Kline, Stevenage, UK*

Kate E. Broderick • *Inovio Pharmaceuticals, Plymouth Meeting, PA, USA*

Thomas Démoulins • *Institute of Virology and Immunology (IVI), Mittelhäusern, Switzerland*

Mustafa Diken • *TRON – Translational Oncology at the University Medical Center, Johannes Gutenberg University, Mainz, Germany; Biopharmaceutical New Technologies (BioNTech) Corporation, Mainz, Germany*

Jan Dörrie • *Department of Dermatology, Universitätsklinikum Erlangen, Erlangen, Germany*

Thomas Ebensen • *Department of Vaccinology and Applied Microbiology, Helmholtz Centre for Infection Research, Braunschweig, Germany*

Knut Elbers • *Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany*

Pavlos C. Englezou • *Institute of Virology and Immunology (IVI), Mittelhäusern, Switzerland*

Bruno Flamion • *URPhyM, NARILIS, University of Namur, Namur, Belgium*

Kerstin F. Gerer • *Department of Dermatology, Universitätsklinikum Erlangen, Erlangen, Germany*

Ulrich Granzer • *Granzer, Regulatory Consulting & Services, Munich, Germany*

Christian Grunwitz • *Biopharmaceutical New Technologies (BioNTech) Corporation, Mainz, Germany; Research Center for Immunotherapy (FZI), Mainz, Germany*

Carlos A. Guzmán • *Department of Vaccinology and Applied Microbiology, Helmholtz Centre for Infection Research, Braunschweig, Germany*

Gundel Hager • *Aurigon GmbH, Munich, Germany*

Regina Heidenreich • *CureVac AG, Tübingen, Germany*

Carlo Heirman • *Laboratory of Molecular and Cellular Therapy, Vrije Universiteit Brussel, Brussels, Belgium*

Thomas Hinz • *Section for Therapeutic Vaccines, Division for Immunology, Paul-Ehrlich-Institut, Langen, Germany*

Axel Hoos • *Glaxo Smith Kline, Collegeville, PA, USA*

Stefanie Hoyer • *Department of Dermatology, Universitätsklinikum Erlangen, Erlangen, Germany*

Christoph Huber • *Association for Cancer Immunotherapy, Mainz, Germany*

Laurent M. Humeau • *Inovio Pharmaceuticals, Plymouth Meeting, PA, USA*

Ulrich Kalinke • *Twincore, Hannover, Germany*

Kajo Kallen • *Kallen Consulting, Köln/Frechen, Germany*

Christopher M. Kearney • *Institute of Biomedical Studies, Baylor University, Waco, TX, USA; Department of Biology, Baylor University, Waco, TX, USA*

Marleen Keyaerts • *In Vivo Cellular and Molecular Imaging Laboratory, Vrije Universiteit Brussel, Brussels, Belgium*
Samir Khleif • *GHSU Cancer Center, Augusta, GA, USA*
Aleksandra Kowalczyk • *CureVac AG, Tübingen, Germany*
Thomas Kramps • *Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim am Rhein, Germany*
Sebastian Kreiter • *Association for Cancer Immunotherapy, Mainz, Germany; TRON – Translational Oncology at the University Medical Center, Johannes Gutenberg University, Mainz, Germany; Biopharmaceutical New Technologies (BioNTech) Corporation, Mainz, Germany*
Mirko Kummer • *Experimentelle Immuntherapie, Hautklinik, Universitätsklinikum Erlangen, Erlangen, Germany*
Johannes Lutz • *CureVac AG, Tübingen, Germany*
Alison A. McCormick • *College of Pharmacy, Touro University California, Vallejo, CA, USA*
Kenneth C. McCullough • *Institute of Virology and Immunology (IVI), Mittelhäusern, Switzerland*
Gregor Meyers • *Institut für Immunologie, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany*
Panagiota Milona • *Institute of Virology and Immunology (IVI), Mittelhäusern, Switzerland*
Norbert Pardi • *Department of Medicine, University of Pennsylvania, Philadelphia, PA, USA*
Steve Pascolo • *Department of Dermatology, University Hospital of Zurich, Zurich, Switzerland*
Chantal Pichon • *Centre de Biophysique Moléculaire, CNRS UPR4301, Orléans, France*
Hans-Georg Rammensee • *Department of Immunology, Institute for Cell Biology, University of Tübingen, Tübingen, Germany; German Cancer Consortium, DKFZ Partner Site, Tübingen, Germany*
Susanne Rauch • *CureVac AG, Tübingen, Germany*
Dries Renmans • *Laboratory of Molecular and Cellular Therapy, Vrije Universiteit Brussel, Brussels, Belgium*
Nicolas Ruggli • *Institute of Virology and Immunology (IVI), Mittelhäusern, Switzerland*
Ugur Sahin • *TRON – Translational Oncology at the University Medical Center, Johannes Gutenberg University, Mainz, Germany; Biopharmaceutical New Technologies (BioNTech) Corporation, Mainz, Germany; Research Center for Immunotherapy (FZI), Mainz, Germany*
Cédric Sapet • *OzBiosciences, Parc scientifique de Luminy, Marseille, France*
Niels Schaft • *Department of Dermatology, Universitätsklinikum Erlangen, Erlangen, Germany*
Sandra Scheiblhofer • *Division of Allergy and Immunology, Department of Molecular Biology, University of Salzburg, Salzburg, Austria*
Thomas Schlake • *CureVac AG, Tübingen, Germany*
Andreas Schmid • *University of Applied Sciences Albstadt-Sigmaringen, Sigmaringen, Germany*
Beatrice Schuler-Thurner • *Experimentelle Immuntherapie, Hautklinik, Universitätsklinikum Erlangen, Erlangen, Germany*
Harpreet Singh-Jasuja • *Immatics Biotechnologies GmbH, Tübingen, Germany*

Birke Andrea Tews • *Institut für molekulare Virologie und Zellbiologie, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany*
Josef Thalhamer • *Division of Allergy and Immunology, Department of Molecular Biology, University of Salzburg, Salzburg, Austria*
Kris Thielemans • *Laboratory of Molecular and Cellular Therapy, Vrije Universiteit Brussel, Brussels, Belgium*
Nicola Tirelli • *Centre of Regenerative Medicine, University of Manchester, Manchester, UK*
Özlem Türeci • *Cluster for individualized Immune Intervention (CI3), Mainz, Germany; TRON – Translational Oncology at the University Medical Center, Johannes Gutenberg University, Mainz, Germany*
Marina Tusup • *Department of Dermatology, University Hospital of Zurich, Zurich, Switzerland*
Kevin Van der Jeught • *Laboratory of Molecular and Cellular Therapy, Vrije Universiteit Brussel, Brussels, Belgium*
Alexia van Weijnen • *Laboratory of Molecular and Cellular Therapy, Vrije Universiteit Brussel, Brussels, Belgium*
Mathias Vormehr • *Biopharmaceutical New Technologies (BioNTech) Corporation, Mainz, Germany; Research Center for Immunotherapy (FZI), Mainz, Germany*
Richard Weiss • *Division of Allergy and Immunology, Department of Molecular Biology, University of Salzburg, Salzburg, Austria*
Drew Weissman • *Department of Medicine, University of Pennsylvania, Philadelphia, PA, USA*
Yiyang Zhou • *Institute of Biomedical Studies, Baylor University, Waco, TX, USA*

Chapter 1

Introduction to RNA Vaccines

Thomas Kramps and Knut Elbers

Abstract

RNA vaccines are attractive, because they exhibit characteristics of subunit vaccines and live-attenuated vectors, including flexible production and induction of both humoral and cellular immunity. While human proof-of-concept for RNA vaccines is still pending, the nascent field of RNA therapeutics has already attracted substantial industry and government funding as well as record investments of private venture capital. Most recently, the WHO acknowledged messenger RNA (mRNA) as a new therapeutic class. In this chapter, we briefly review key developments in RNA vaccines and outline the contents of this volume of Methods in Molecular Biology.

Key words RNA vaccine, Messenger RNA, Self-amplifying RNA, Replicon, RNA virus vector

1 Introduction

Vaccination remains a key medical innovation. In essence, vaccines stimulate the immune system to form a prophylactic or curative response against a given disease and could offer a powerful treatment modality for a wide range of conditions with unmet medical needs [1]. However, realizing this conceptual potential faces considerable challenges [2]. In many instances, insufficient understanding of immune correlates and mechanisms of protection are major impediments [3]. Also, induction of potent effectors and long-lasting memory can be difficult, e.g., against pathogens localized at mucosal or immune privileged sites. The induction of effective T cell responses or of broadly neutralizing antibodies in particular remains a key challenge in addressing mutable microbial pathogens [4, 5]. Finally, an additional layer of complexity exists in individualized approaches, for example in the tailored immunotherapy of cancer [6].

However, the recent integration of vaccinology and "omics" technology offers exciting prospects of addressing such challenges [3]. For example, they may allow researchers to systematically unravel correlates of protection [7] or better understand dynamic

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_1,

host–pathogen interactions [8, 9]. On the other hand, we still lack validated vaccine platforms that complement such analytic capabilities and facilitate effective vaccine development [10]. Suitable vaccine technologies would enable high-throughput screening for protective antigens as well as rapid synthesis and testing of selected lead compounds [11]. Due to their simplicity and versatility, synthetic RNA vectors offer particular promise as tools for rapid screening and development of vaccine products than traditional approaches (including lower cost) [12–17].

2 Messenger RNA and Self-Amplifying RNA (Replicon) Vaccines

2.1 Historical Background

The concept of synthetic RNA vaccines is not new, but ingenious: In a seminal paper published a quarter century ago, Wolff et al. first showed that injection of uncomplexed messenger RNA (mRNA) led to protein expression in mice [18]. Instead of applying the protein antigen, RNA vaccines carry genetic information for endogenous protein expression in the vaccinee, similar to infection with a virus. In short order of this initial discovery, the immunogenicity of the format was shown in different test systems (reviewed in ref. 12), but overall the impression prevailed that producing and handling synthetic RNA vectors were prohibitive in terms of cost and complexity. By and large, attention focused on plasmid DNA technology or recombinant viral vectors instead [19].

Initial efforts by groups that pioneered mRNA vaccines mostly addressed cancer immunotherapy with no validated benchmarks to compare and optimize the format [20–23]. While some researchers favored direct injection of naked mRNA [20, 24], others used in vitro transfection of dendritic cells (DC) with mRNA to boost immunogenicity [25, 26]. For both approaches, academic and start-up initiatives established clinical grade (GMP)-conform production and provided important basic data on the safety and immunogenicity in humans [13]. The first successful preclinical proof-of-concept studies of prophylactic RNA vaccines in small and large animals, which also included head-to-head comparison with licensed comparators, have been reported only relatively recently [27–29]. These studies indicated principal feasibility and encouraged extended testing of an mRNA-based prophylactic vaccine in a first human clinical trial (NCT02241135). These activities involved increasing industry and government funding and led to record investments of venture capital [30]. Most recently, the WHO acknowledged mRNA as a new therapeutic class with its own international nonproprietary nomenclature (the suffix "-meran" as first used for "nadorameran," a rabies-specific vaccine) [31, 32].

2.2 Vector Design

RNAs are composed of strings of alternating nucleotides (generally uridylate, adenylate, guanylate, and cytidylate) which can also be subject to chemical modification [33]. Synthetic RNA vaccine vectors contain an open reading frame that encodes the antigen of interest and optimized, *cis*-acting flanking structures: the 5′ and 3′ untranslated regions (UTRs) flanking the open reading-frame (ORF), terminal 5′ 7-methyl guanosine cap structure (cap), and 3′ polyadenylated tail (polyA). Ultimately, all these elements serve to increase antigen yield by maximizing the rate of translation and/or vector persistence within transformed cells through interactions with regulatory proteins, other RNAs, and metabolites. As such, the 5′ cap, 5′ UTR, ORF, 3′ UTR, and polyA offer relevant targets for optimization of mRNA vectors [22, 34]. In the sequence of events leading to protein synthesis, translational initiation is rate-limiting and tightly regulated by the orchestrated recruitment of *trans*-acting factors to specific RNA sequences. Thus, improving translational initiation by sequence optimization is also important for the design of better mRNA vectors. We believe that continuing optimization will result in greater carrying capacity, further increasing potency, reducing cost, and facilitating the formulation of multivalent products.

RNA replicon vaccines present a complementary approach and very interesting alternative to non-replicating mRNA vectors [35]. This alternative setup makes use of accessory viral elements that lead to self-amplification of the messenger RNA [36]. A major strength of this approach is that, due to self-amplification of the vector in vivo, high-level and long-lasting protein expression is readily feasible with available technology. A persistent challenge, however, remains in the lower yield and specificity of production of these much larger molecules and—arguably—interference by anti-vector immunity [35, 37].

2.3 Production

The typical product profile of synthetic RNA vaccines differs substantially from that of traditional protein- or pathogen-based vaccines:

- For synthesis of the RNA vector, only information about the nucleic acid sequence is required. Thereby, handling of infectious agents, environmental risks, or restrictions of global vaccine distribution can be eliminated [15].
- While it can take years and hundreds of millions of dollars for a new manufacturing facility for traditional vaccine products to become productive, RNA vaccines are produced by a highly standardized process with relatively minor adaptations to account for variations in sequence length or composition. This generally reduces lead-time and cost [15].

- RNA represents a relatively stable drug-substance, as long as exposure to RNase is prevented [38]. RNA can be lyophilized for prolonged storage at ambient temperature, greatly facilitating distribution and storage [27].

The manufacture of bulk RNA by enzymatic in vitro transcription is well established [38]. Alternative protocols to generate template DNA, e.g., by polymerase chain reaction, currently limit design, fidelity, and yield. They have been employed for antigen screening [11], but remain much less common and are not discussed in this book. In the context of cancer vaccines flexible antigen selection is key to match the most relevant antigens with a given cancer type or for the design of patient-specific vaccines [9]. Such personalized therapeutics recently received much stronger attention and several academic groups and biotech companies initiated efforts to validate RNA vaccines encoding patient specific neoantigens in the clinic [39].

2.4 Adjuvantation

RNA exerts direct immune-stimulating effects [33, 40]. This RNA-mediated adjuvanticity may be modulated by composition and formulation: In the case of synthetic RNA vaccines, factors such as stabilization against RNase-mediated degradation, particle size, and charge influence the localization of RNA in cells or lymphatic organs and their resulting adjuvant activity [41–43].

The signaling pathways involved in RNA-mediated stimulation of the immune system are understood in some detail [44–46]. Innate responses to RNA are induced by dedicated pattern recognition receptors (PRR) upon detection of aberrant localization or unusual structural features of the RNA adjuvant [47]. RNA-specific PRR include endosomal toll-like receptors (TLR) 3, 7, and 8, the cytoplasmic receptors retinoic acid inducible gene I (RIG-I), melanoma differentiation antigen 5 (MDA5), protein kinase R (PKR), and others. They are differentially expressed in various cells and tissues, ranging from narrow expression in specific immune cells like plasmacytoid dendritic cells (pDC) and B cells for TLR7, to virtually ubiquitous expression, e.g., for RIG-I and PKR [48]. The differential stimulation of these molecules and cell types will thus shape the immune response to a given RNA vaccine. In designing preclinical test strategies, it is important to keep in mind that expression patterns and specificities of RNA-specific PRR may vary between humans and an animal test species of choice [48].

Apart from deriving adjuvant effects from their chemical composition, protein-coding RNA vectors can serve as “genetic adjuvant”. Here, co-expression of antigen with immune modulatory factors, such as cytokines, would enhance interactions of antigen presenting cells with immune effectors [49]. Genetic adjuvants extend design space vastly, but also raise additional complexities regarding delivery and—possibly—safety.

Another option that is currently explored is intra-tumoral application of vaccines with systemic check-point blockade to more strongly influence effector T-cell activity at the site of immune-driven tumor destruction [6, 50, 51]. RNA vector vaccines have been shown to be highly immunogenic, which likely is due to their transient amplification in the host and ability to spread to secondary lymphoid tissues (e.g., various lymph nodes) [52]. Most recently, RNA vector vaccine biology is being extended to newly emerging concepts of oncolytic RNA viruses [53].

2.5 Delivery

Apart from the administered antigen, vaccines contain a characteristic adjuvant, while the mode of delivery—generally needle-mediated injection into skeletal muscle—modulates efficacy and safety. The dose, pharmacokinetics, and spatial distribution of antigen and adjuvant shape the immune response against a given vaccine like, for example, TH1/TH2-bias [54]. A key advantage of genetic vaccine vectors is their ability to generate effective $CD8^+$ T cell responses by synthesizing vaccine antigen directly into the major histocompatibility complex class I (MHCI) processing pathway [1].

In principle, exogenous RNA only needs to cross a single lipid bilayer to initiate cytoplasmic protein expression, even in post-mitotic cells. Studies have shown that naked mRNA is spontaneously taken up by a large range of cell types and expressed within minutes (reviewed in ref. 16), even upon direct injection. Nevertheless, it is clear that upon injection, only minute fractions of naked mRNA remain intact and are taken up into the cytoplasm. Therefore, yields of protein antigen remain low (probably in the nanogram range) and it appears likely that improving directed delivery to, e.g., secondary lymphatic organs or specific, highly expressing cell types remains a central area for future improvement [55]. To address these challenges, researchers have used nonviral transfection of DC [26] in vitro or intranodal injection in vivo to increase delivery to professional antigen-presenting cells and, thereby, vaccine potency [56]. While such approaches are technically demanding, potentially simpler approaches include synthetic formulations that increase RNA uptake, e.g., with lipids or polymers or physical delivery techniques as described in this book. In general, enabling less invasive routes of needle-less administration might gain importance in mass vaccinations due to their simplicity of use [57, 58].

2.6 Immuno-monitoring

The active principle of vaccines is antigen-specific immunity, i.e., it is not the antigen that exerts the prophylactic or therapeutic effects, but antigen-specific immunity. Therefore, measuring B and T cell activity against defined antigen is important in identifying protective vaccine antigens and in assessing immunogenicity in vaccinees. In principle, an antigen-encoding RNA can be used as a specific

immunomonitoring agent and corresponding protocols have been developed to eliminate limitations of traditional approaches [59–61].

2.7 Regulatory Aspects

The ultimate goal in researching and developing RNA vaccines is the licensure and provision of effective and safe products for subjects with unmet medical needs [62, 63]. For all parties involved, it is important to understand the basic rules and regulatory mechanisms in place to control risk in developing novel medicinal products. Preclinical results will provide key clues in designing an appropriate clinical study program, e.g., by guiding choice of first-in-human dosing [64]. Before initiating phase I clinical testing, preclinical safety is generally studied in cellular in vitro systems and/or in vivo animal models to identify any potential dose-limiting toxicities and target organs. Here, the choice of suitable test systems must be guided by mechanistic understanding to better judge capabilities and limitations. For example, mice exhibit structural and biological differences in TLR7 and TLR8 when compared to humans that may need to be taken into account [48].

3 Contents of This Book

3.1 General Overview

We believe that this book provides vaccinologists in academia and industry with a relevant overview of the field of RNA vaccines as well as practical points-of-entry: After reviewing a few fundamental aspects of RNA vaccines in this introductory chapter, the following chapters provide concrete examples, protocols, and tips on synthesizing and testing different types of RNA vaccines. The book is divided into four sections that contain details on self-replicating RNA vectors (*part I*, including Chapters 2–4), non-replicating mRNA vectors (*part II*, Chapters 5–7), aspects of adjuvantation and delivery (*part III*, Chapters 8–12), and preclinical and clinical development (*part IV*, Chapters 13–17). While most protocols could, in principle, be grouped into different sections, we hope our approach facilitates a structured overview. The interested reader may complement protocols in this volume of Methods in Molecular Biology by an earlier monograph on Synthetic Messenger RNA Therapeutics [65].

3.2 Part I: Self-Replicating RNA Vectors

In Chapter 2, Tews and Meyers review the molecular biology of self-replicating RNA vectors and the variety of approaches that can be used to derive synthetic replicon vectors from various positive strand RNA viruses. This introductory overview is extended in Chapter 3 by Démoulins et al. with the example of a classical swine fever virus (CSFV)-derived replicon vaccine. The authors describe relevant in vitro production and test systems as well as nanoparticle

formulations. Chapter 4 by Zhou et al. complements these protocols by another, elegant approach to produce and package animal replicons with a viral coat protein in plants. Such approaches may offer significant benefits for cost-effective, large-scale vaccine production and side-step challenges associated with the in vitro synthesis of very large RNA molecules that are fraught with notoriously low yields of specific product. Furthermore, the resulting vaccine format should be very resilient to environmental stress.

3.3 Part II: Non-replicating mRNA Vectors

The second section on non-replicating mRNA vaccine vectors starts with Chapter 5 by Rauch et al. that presents a protamine-complexed, non-replicating mRNA vector format. Similar vaccines are currently undergoing clinical testing and are injected by the intradermal route with a syringe and needle. The chapter includes details on testing protein-expression in vivo by encoding luciferase, a useful technique for vector optimization. Chapter 6 by Pardi et al. offers a variation on the mRNA vaccine format that includes nucleoside modified mRNA packaged in lipid nanoparticles for enhanced delivery. Certain nucleoside modifications have been shown to enhance translation and may result in increased antigen-expression, especially in a proinflammatory environment that inherently suppresses translation. Finally, in Chapter 7 Weiss et al. lay out protocols of conventional as well as self-replicating mRNAs from commercially available vectors as a prophylactic strategy against allergy. This chapter presents an example for benchmarking replicating and non-replicating RNA vaccines and exemplifies innovative applications for these novel technologies that could be extended even further to the production of therapeutic proteins in vivo.

3.4 Part III: Adjuvantation and Delivery

In the third section on adjuvantation and delivery, Chapter 8 by Pardi and Weissman contains fundamental protocols to study the adjuvanticity of RNA-based vaccines. Potential applications are twofold: On the one hand it allows for testing adjuvant effects in vitro and improving vaccine immunogenicity. On the other hand, it might serve to assess the reproducible formulation of RNA vaccine nanoparticles and could thus be used as a potency assay in batch release for clinical use. Chapter 9 by Tusup and Pascolo nicely extends aspects discussed in Chapter 8 by presenting physical determinants of RNA vaccine adjuvanticity, namely particle size and charge. This approach opens ways to rationally optimize RNA adjuvanticity and better control vaccine quality.

Gerer et al. in Chapter 10 present protocols for electroporation of primary cells with mRNA. This could serve for vaccination upon adaptive transfer or to enhance delivery of RNA vaccines or for testing antigen expression, e.g., in potency assays or for immunomonitoring purposes. In Chapter 11 Bialkowski et al. outline various

approaches to improving RNA vaccine immunogenicity in vivo, particularly intranodal and intratumoral injection. The authors previously also developed a genetic adjuvant by co-administering mRNA encoding CD40L and a constitutively active variant of Toll-like receptor 4 (caTLR4) together with the co-stimulatory molecule CD70, highlighting the variability of RNA vaccine adjuvanticity [49]. Chapter 12 by Broderick and Humeau outlines methods to enhance RNA vaccine delivery and immunogenicity by in vivo electroporation. This physical method was initially developed to enhance plasmid DNA delivery and has been successfully applied to RNA vaccines as well [66–68].

3.5 Part IV: Preclinical and Clinical Development

The fourth and last section addresses preparations for clinical testing of RNA vaccines. It starts with a comprehensive review of the European regulatory framework applying to RNA vaccines by Hinz et al. in Chapter 13. While no dedicated guidance on mRNA vaccines exists today, it is important to understand that regulatory requirements depend on both, the RNA vaccine type and the application. The adaptability of RNA vaccines offers the promise of patient-specific therapeutic development; this is especially relevant for cancer immunotherapy that targets a highly mutable and heterogeneous population of tumor cells [69]. Diken et al. describe a strategy to identify tumor-specific neoantigens as a basis for individualized therapy in Chapter 14. The reproducible production of test product is central to safe and productive clinical testing. Andreas Schmid lays out basic considerations for the production of clinical-grade in vitro transcribed RNA in Chapter 15. On this basis of reproducible manufacture and leading up to clinical studies, preclinical safety testing may follow. Toxicology follows an established framework of assessing a test product's toxicological potential and proven profile and Gundel Hager lays out a structured approach to preclinical safety testing of RNA vaccines in Chapter 16, including selection of appropriate animal models and test strategies. While these are early days in a rapidly developing field, Chapter 17 by Kummer and Schuler-Thurner shows that specific RNA vaccine approaches are progressing and have already entered advanced clinical testing. The authors describe the example of a therapeutic vaccine approach with mRNA-transfected DCs in uveal melanoma.

4 Conclusion

Recent technological advances promise increasing progress toward the rational design of vaccines against diverse targets. Important challenges that remain include better efficacy against highly variable or persistent pathogens, cheaper and more resilient technology for worldwide use, and adaptability and scalability for individualized or

emergency production. To demonstrate superior efficacy and solve relevant medical problems, future vaccines will induce balanced responses that include humoral and cellular effectors.

RNA vaccines may become an important enabling technology: They can mimic desirable immunogenic characteristics of acute infections, including coordinated exposure to antigen and adjuvant stimuli. Importantly, they also offer a modular format to add or omit such stimuli in a highly specific and defined manner, enabling continued, stepwise optimization. We hope that this collection of protocols and perspectives will foster innovation and contribute to tapping the full potential of RNA vaccines.

Acknowledgments

We thank colleagues in the field for continuing discussions. We also thank Dr. Michel Pairet and Dr. Holger Gellermann of Boehringer Ingelheim for support.

References

1. Levine MM, Dougan G, Good MF, Liu MA, Nabel GJ, Nataro JP, Rappuoli R (eds) (2009) New generation vaccines, 4th edn. CRC Press, New York
2. Hilleman MR (1998) A simplified vaccinologists' vaccinology and the pursuit of a vaccine against AIDS. Vaccine 16(8):778–793
3. Nakaya HI, Pulendran B (2015) Vaccinology in the era of high-throughput biology. Phil Trans R Soc Lond B Biol Sci 370. pii:20140146. doi:10.1098/rstb.2014.0146
4. Sadanand S, Suscovich TJ, Alter G (2016) Broadly neutralizing antibodies against HIV: new insights to inform vaccine design. Annu Rev Med 67:185–200. doi:10.1146/annurev-med-091014-090749
5. Walker CM, Grakoui A (2015) Hepatitis C virus: why do we need a vaccine to prevent a curable persistent infection? Curr Opin Immunol 35:137–143. doi:10.1016/j.coi.2015.06.010
6. van der Burg SH, Arens R, Ossendorp F et al (2016) Vaccines for established cancer: overcoming the challenges posed by immune evasion. Nat Rev Cancer 16:219–233. doi:10.1038/nrc.2016.16
7. Nakaya HI, Hagan T, Duraisingham SS et al (2015) Systems analysis of immunity to influenza vaccination across multiple years and in diverse populations reveals shared molecular signatures. Immunity 43:1186–1198. doi:10.1016/j.immuni.2015.11.012
8. Stern-Ginossar N (2015) Decoding viral infection by ribosome profiling. J Virol 89:6164–6166. doi:10.1128/JVI.02528-14
9. Schumacher TN, Schreiber RD (2015) Neoantigens in cancer immunotherapy. Science 348:69–74. doi:10.1126/science.aaa4971
10. Ulmer JB, Valley U, Rappuoli R (2006) Vaccine manufacturing: challenges and solutions. Nat Biotechnol 24(11):1377–1383
11. Hondowicz BD, Schwedhelm KV, Kas A et al (2012) Discovery of T cell antigens by high-throughput screening of synthetic minigene libraries. PLoS One 7(1):e29949
12. Pascolo S (2004) Messenger RNA-based vaccines. Expert Opin Biol Ther 4:1285–1294
13. Pascolo S (2015) The messenger's great message for vaccination. Expert Rev Vaccines 14:153–156. doi:10.1586/14760584.2015.1000871
14. Sahin U, Karikó K, Türeci Ö (2014) mRNA-based therapeutics--developing a new class of drugs. Nat Rev Drug Discov 13:759–780. doi:10.1038/nrd4278
15. Ulmer JB, Mansoura MK, Geall AJ (2015) Vaccines "on demand": science fiction or a future reality. Expert Opin Drug Discov 10:101–106. doi:10.1517/17460441.2015.996128
16. Kramps T, Probst J (2013) Messenger RNA-based vaccines: progress, challenges, applications. Wiley Interdiscip Rev RNA 4:737–749. doi:10.1002/wrna.1189

17. Youn H, Chung J-K (2015) Modified mRNA as an alternative to plasmid DNA (pDNA) for transcript replacement and vaccination therapy. Expert Opin Biol Ther 15:1337–1348. doi:10.1517/14712598.2015.1057563
18. Wolff JA, Malone RW, Williams P et al (1990) Direct gene transfer into mouse muscle in vivo. Science 247:1465–1468
19. Leitner WW, Ying H, Restifo NP (1999) DNA and RNA-based vaccines: principles, progress and prospects. Vaccine 18:765–777. doi:10.1016/S0264-410X(99)00271-6
20. Weide B, Carralot J-P, Reese A et al (2008) Results of the first phase I/II clinical vaccination trial with direct injection of mRNA. J Immunother 31(2):180–188
21. Rittig SM, Haentschel M, Weimer KJ et al (2011) Intradermal vaccinations with RNA coding for TAA generate CD8+ and CD4+ immune responses and induce clinical benefit in vaccinated patients. Mol Ther 19(5):990–999
22. Vallazza B, Petri S, Poleganov MA et al (2015) Recombinant messenger RNA technology and its application in cancer immunotherapy, transcript replacement therapies, pluripotent stem cell induction, and beyond: Recombinant mRNA technology and its application. Wiley Interdiscip Rev RNA. doi:10.1002/wrna.1288
23. Van Lint S, Heirman C, Thielemans K, Breckpot K (2013) mRNA: from a chemical blueprint for protein production to an off-the-shelf therapeutic. Hum Vaccin Immunother 9(2):265–274
24. Granstein RD, Ding W, Ozawa H (2000) Induction of anti-tumor immunity with epidermal cells pulsed with tumor-derived RNA or intradermal administration of RNA. J Invest Dermatol 114(4):632–636
25. Boczkowski D, Nair SK, Snyder D, Gilboa E (1996) Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. J Exp Med 184(2):465–472
26. Boczkowski D, Nair S (2010) RNA as performance-enhancers for dendritic cells. Expert Opin Biol Ther 10:563–574. doi:10.1517/14712591003614749
27. Petsch B, Schnee M, Vogel AB et al (2012) Protective efficacy of in vitro synthesized, specific mRNA vaccines against influenza A virus infection. Nat Biotechnol 30(12):1210–1216
28. Geall AJ, Verma A, Otten GR et al (2012) Nonviral delivery of self-amplifying RNA vaccines. Proc Natl Acad Sci U S A 109(36): 14604–14609
29. Brito LA, Chan M, Shaw CA et al (2014) A cationic nanoemulsion for the delivery of next-generation RNA vaccines. Mol Ther. doi:10.1038/mt.2014.133
30. Dolgin E (2015) Business: The billion-dollar biotech. Nature 522:26–28. doi:10.1038/522026a
31. Chambers M ChemIDplus - 1613055-09-6 - Nadorameran - Searchable synonyms, formulas, resource links, and other chemical information. http://chem.sis.nlm.nih.gov/chemidplus/rn/1613055-09-6. Accessed 29 Mar 2016
32. WHO Drug Information Vol. 29, No. 2, 2015 - Proposed International Nonproprietary Names, List 113. http://apps.who.int/medicinedocs/en/m/abstract/Js22000en/. Accessed 29 Mar 2016
33. Karikó K, Weissman D (2007) Naturally occurring nucleoside modifications suppress the immunostimulatory activity of RNA: implication for therapeutic RNA development. Curr Opin Drug Discov Devel 10(5):523–532
34. Schlake T, Thess A, Fotin-Mleczek M, Kallen K-J (2012) Developing mRNA-vaccine technologies. RNA Biol 9:1319–1330. doi:10.4161/rna.22269
35. Brito LA, Kommareddy S, Maione D et al (2014) Self-amplifying mRNA vaccines. Adv Genet 89:179–233
36. Ulmer JB, Mason PW, Geall A, Mandl CW (2012) RNA-based vaccines. Vaccine 30(30):4414–4418
37. Uematsu Y, Vajdy M, Lian Y et al (2012) Lack of interference with immunogenicity of a chimeric alphavirus replicon particle-based influenza vaccine by preexisting antivector immunity. Clin Vaccine Immunol 19:991–998. doi:10.1128/CVI.00031-12
38. Nielsen H (2011) RNA. Humana Press, Totowa, NJ
39. Boisguérin V, Castle JC, Loewer M et al (2014) Translation of genomics-guided RNA-based personalised cancer vaccines: towards the bedside. Br J Cancer 111:1469–1475. doi:10.1038/bjc.2013.820
40. Desmet CJ, Ishii KJ (2012) Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination. Nat Rev Immunol 12(7):479–491
41. Scheel B, Teufel R, Probst J et al (2005) Toll-like receptor-dependent activation of several human blood cell types by protamine-condensed mRNA. Eur J Immunol 35:1557–1566. doi:10.1002/eji.200425656
42. Rettig L, Haen SP, Bittermann AG et al (2010) Particle size and activation threshold: a new dimension of danger signaling. Blood 115(22):4533–4541
43. Fotin-Mleczek M, Duchardt KM, Lorenz C et al (2011) Messenger RNA-based vaccines with dual activity induce balanced TLR-7 dependent adaptive immune responses and provide antitumor activity. J Immunother 34(1):1–15

44. Barbalat R, Ewald SE, Mouchess ML, Barton GM (2011) Nucleic acid recognition by the innate immune system. Annu Rev Immunol 29:185–214
45. Wu J, Chen ZJ (2014) Innate immune sensing and signaling of cytosolic nucleic acids. Annu Rev Immunol 32:461–488. doi:10.1146/annurev-immunol-032713-120156
46. Kagan JC, Barton GM (2015) Emerging principles governing signal transduction by pattern-recognition receptors. Cold Spring Harb Perspect Biol 7:a016253. doi:10.1101/cshperspect.a016253
47. Brencicova E, Diebold SS (2013) Nucleic acids and endosomal pattern recognition: how to tell friend from foe? Front Cell Infect Microbiol 3:37. doi:10.3389/fcimb.2013.00037
48. Barchet W, Wimmenauer V, Schlee M, Hartmann G (2008) Accessing the therapeutic potential of immunostimulatory nucleic acids. Curr Opin Immunol 20(4):389–395
49. Van Lint S, Goyvaerts C, Maenhout S et al (2012) Preclinical evaluation of TriMix and antigen mRNA-based antitumor therapy. Cancer Res 72(7):1661–1671
50. Hammerich L, Binder A, Brody JD (2015) In situ vaccination: cancer immunotherapy both personalized and off-the-shelf. Mol Oncol 9:1966–1981. doi:10.1016/j.molonc.2015.10.016
51. Schlom J, Hodge JW, Palena C et al (2014) Chapter two - therapeutic cancer vaccines. In: Tew KD, Fisher PB (eds) Advances in cancer research. Academic, New York, pp 67–124
52. Mogler MA, Kamrud KI (2015) RNA-based viral vectors. Expert Rev Vaccines 14:283–312. doi:10.1586/14760584.2015.979798
53. Chiocca EA, Rabkin SD (2014) Oncolytic viruses and their application to cancer immunotherapy. Cancer Immunol Res 2:295–300. doi:10.1158/2326-6066.CIR-14-0015
54. Bachmann MF, Jennings GT (2010) Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. Nat Rev Immunol 10(11):787–796
55. Phua KKL, Nair SK, Leong KW (2014) Messenger RNA (mRNA) nanoparticle tumour vaccination. Nanoscale 6:7715–7729. doi:10.1039/c4nr01346h
56. Kreiter S, Selmi A, Diken M et al (2010) Intranodal vaccination with naked antigen-encoding RNA elicits potent prophylactic and therapeutic antitumoral immunity. Cancer Res 70(22):9031–9040
57. Ravi AD, Sadhna D, Nagpaal D, Chawla L (2015) Needle free injection technology: a complete insight. Int J Pharm Investig 5:192–199. doi:10.4103/2230-973X.167662
58. Foged C (2016) Thermostable subunit vaccines for pulmonary delivery: how close are we? Curr Pharm Des 22(17):2561–2576
59. Etschel JK, Hückelhoven AG, Hofmann C et al (2012) HIV-1 mRNA electroporation of PBMC: a simple and efficient method to monitor T-cell responses against autologous HIV-1 in HIV-1-infected patients. J Immunol Methods 380:40–55. doi:10.1016/j.jim.2012.03.005
60. Van Camp K, Cools N, Stein B et al (2010) Efficient mRNA electroporation of peripheral blood mononuclear cells to detect memory T cell responses for immunomonitoring purposes. J Immunol Methods 354:1–10. doi:10.1016/j.jim.2010.01.009
61. Cools N, Van Camp K, Van Tendeloo V, Berneman Z (2013) mRNA electroporation as a tool for immunomonitoring. Methods Mol Biol 969:293–303
62. Scully IL, Swanson K, Green L et al (2015) Anti-infective vaccination in the 21st century — new horizons for personal and public health. Curr Opin Microbiol 27:96–102. doi:10.1016/j.mib.2015.07.006
63. Lopalco PL, DeStefano F (2015) The complementary roles of Phase 3 trials and post-licensure surveillance in the evaluation of new vaccines. Vaccine 33:1541–1548. doi:10.1016/j.vaccine.2014.10.047
64. Goetz KB, Pfleiderer M, Schneider CK (2010) First-in-human clinical trials with vaccines—what regulators want. Nat Biotechnol 28:910–916. doi:10.1038/nbt0910-910
65. Rabinovich PM (2013) Synthetic messenger RNA and cell metabolism modulation: methods and protocols, 2013th edn. Humana Press, New York
66. Johansson DX, Ljungberg K, Kakoulidou M, Liljeström P (2012) Intradermal electroporation of naked replicon RNA elicits strong immune responses. PLoS One 7:e29732. doi:10.1371/journal.pone.0029732
67. Cu Y, Broderick KE, Banerjee K et al (2013) Enhanced delivery and potency of self-amplifying mRNA vaccines by electroporation in situ. Vaccines 1:367–383. doi:10.3390/vaccines1030367
68. Broderick KE, Humeau LM (2015) Electroporation-enhanced delivery of nucleic acid vaccines. Expert Rev Vaccines 14(2):195–204. doi:10.1586/14760584.2015.990890
69. Britten CM, Singh-Jasuja H, Flamion B et al (2013) The regulatory landscape for actively personalized cancer immunotherapies. Nat Biotechnol 31:880–882. doi:10.1038/nbt.2708

Part I

Self-Replicating RNA Vectors

Chapter 2

Self-Replicating RNA

Birke Andrea Tews and Gregor Meyers

Abstract

Self-replicating RNA derived from the genomes of positive strand RNA viruses represents a powerful tool for both molecular studies on virus biology and approaches to novel safe and effective vaccines. The following chapter summarizes the principles how such RNAs can be established and used for design of vaccines. Due to the large variety of strategies needed to circumvent specific pitfalls in the design of such constructs the technical details of the experiments are not described here but can be found in the cited literature.

Key words Self-replicating RNA, Positive strand RNA virus, Alphavirus, Flavivirus, Pestivirus, Classical swine fever virus

1 Introduction

The story of self-replicating RNA started with the recognition of the infectious nature of some viral RNA genomes in the 1950s and 1960s [1–7]. The evidence that naked RNA upon introduction into cells is able to promote a full replication cycle including release of infectious virus particles represented the starting point for a new era of research on RNA virus molecular biology and its application. Due to the technical difficulties, RNA is not amenable to site specific manipulation so that reverse genetics systems for RNA viruses always rely on a cDNA intermediate [8, 9]. First successful approaches towards recovery of replicating viruses from cloned cDNA were published for positive-strand RNA viruses relying on transfection of plasmid DNA containing a virus derived cDNA insert [10]. Soon afterwards, in vitro transcription and transfection of viral genome-like RNA was described leading to recovery of infectious progeny virus [11, 12].

Positive-strand RNA viruses were the first RNA viruses amenable to direct genetic manipulation due to their simple strategy of gene expression and replication [13]. The genomic RNA (vRNA) represents an mRNA able to govern the production of all viral proteins necessary for the initiation of virus replication. Products of the

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_2,

first round of translation of the viral genomic RNA assemble into a replicase complex that polymerizes a minus strand complementary to the genome (cRNA) as a template for the synthesis of additional mRNA molecules. Thus, for all positive-strand RNA viruses the components of the replicase complex have to be translated directly from the genomic RNA. Viral polypeptides not required for RNA replication, which mainly constitute structural proteins, can either also be translated from the genomic RNA or from one or more subgenomic mRNAs transcribed from the negative sense cRNA template, depending on the specific type of virus. Genomes of members of the group using the former expression strategy contain one long open reading frame (ORF). Translation of this RNA leads to a polyprotein that is co-translationally and posttranslationally processed by viral and host cellular proteases. The members of the families *Picornaviridae* and *Flaviviridae* belong to this first group (Fig. 1). The second group comprises the families *Togaviridae*, *Coronaviridae*, *Arteriviridae*, and *Caliciviridae*. These viruses are characterized by the subgenomic RNAs used for expression of part of their genes (Fig. 1). In contrast to the first group, the replicase genes of these viruses are located in the 5′ part of the genome upstream of the structural genes. For all of these viruses the subgenomic RNAs are 3′ co-terminal with the genomic RNA.

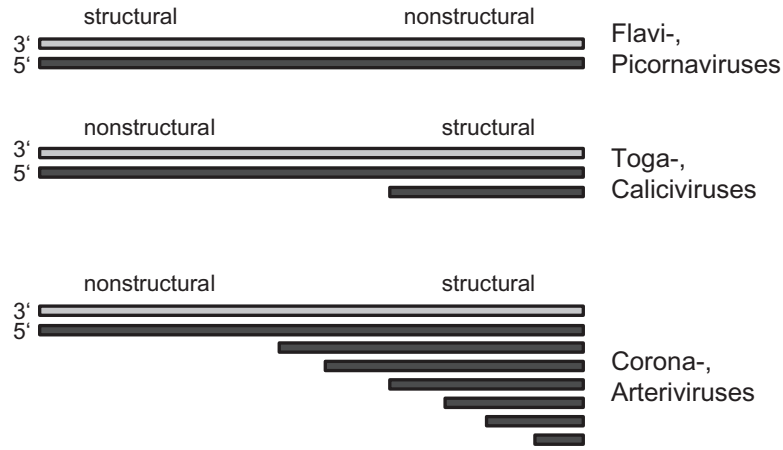

Fig. 1 Genome structures and gene expression strategies of different positive strand RNA viruses. Schematic representation of the RNA species found in cells infected with the indicated viruses. For flaviviruses and picornaviruses, only RNA of genome size is generated. The RNA with positive polarity (genome orientation) is translated into one polyprotein that is subsequently processed into the viral proteins. Togaviruses and caliciviruses transcribe one RNA of subgenomic length encoding the structural proteins. Coronaviruses and arteriviruses use multiple subgenomic mRNAs for expression of structural and accessory proteins. RNA in coding orientation (mRNA sense) is represented by *black bars* whereas *grey bars* symbolize negative strand intermediates of viral genome replication. The location of structural and nonstructural genes in the viral genomes is indicated

The possibility to recover self-replicating viral RNA from cloned cDNA sequences opened a window to sophisticated studies on the mechanisms of RNA virus replication. Moreover, this knowledge was crucial for establishment of rationally attenuated viruses as well as development of strategies for use of self-replicating RNA expressing foreign genes for vaccine purposes and other applications. In this chapter, we present the technical principles used for establishment of self-replicating RNA and selected examples for its application in the context of vaccine development.

2 Methods for Establishment of Self-Replicating RNAs

2.1 Basic Strategies: A Historical Review

Due to the greater instability of (single stranded) RNA versus DNA and the wealth of techniques for DNA manipulation in contrast to the difficulties of direct RNA manipulation recombinant virus systems are based on DNA constructs, even in the case of RNA viruses where these systems rely on cDNA of the viral RNA. Due to the infectious nature of the positive strand RNA virus genome reverse genetics systems for positive strand RNA viruses need not be much more complicated than to be a way to deliver genome-like RNA into cells for successful replication of said RNA and for virus recovery. The history of reverse genetic systems for positive strand RNA viruses highlights the pitfalls that may be encountered in the design of a reverse genetic system and show solutions how to circumvent these difficulties. Some of these difficulties are covalently linked to the genome structures found in different positive strand RNA virus families. The genome can be capped or linked to a so-called VPg-protein or contain a naked 5′ end. The 3′ end can form loop structures or be a poly-A tail as would be expected for mRNAs. Depending on the virus the correct 5′ and 3′ end is very important as they can be crucial for replication and/or translation, or the production of subgenomic RNA (Fig. 1).

The first infectious cDNA clone of a eukaryotic virus was a cDNA clone for poliovirus [10]. This construct had the complete cDNA-sequence including a 37 residue poly (A) tail in the plasmid pBR322 and yielded infectious virus particles upon transfection in mammalian cells. This first construct did not contain a dedicated promoter to ensure the transcription of viral RNA, but nevertheless led to enough RNA expression for virus recovery. Three years later, the performance of poliovirus cDNA clones could be significantly ameliorated through the introduction of SV40 transcription and replication signals and transfection of the resulting construct into cells expressing the SV40 large T antigen [14], thus ensuring replication of the DNA-plasmid in eukaryotic cells leading to a higher yield of viral RNA and recovered virus (Fig. 2, left part). For other picornaviruses, cloning the cDNA into a bacterial

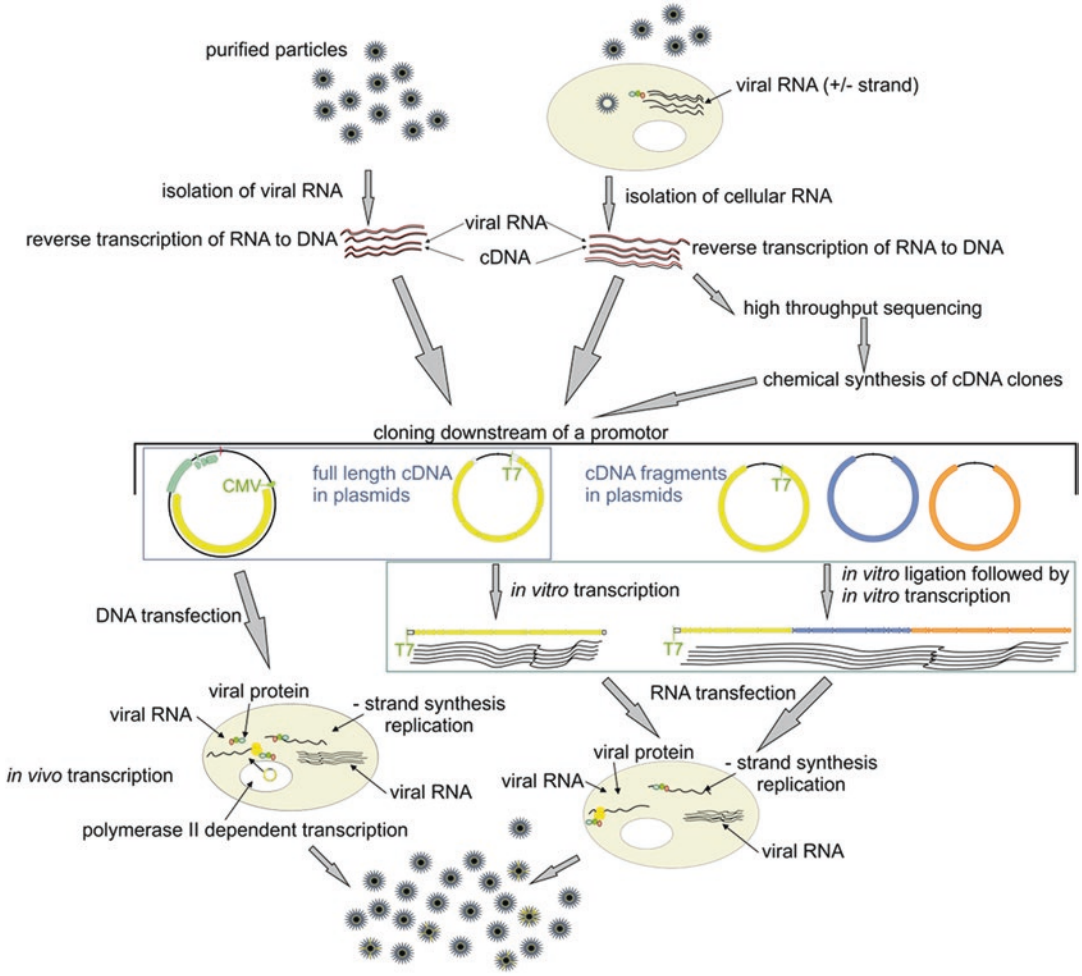

Fig. 2 Different strategies to generate reverse genetic systems for positive strand RNA viruses. *Upper part*: Viral RNA can either be obtained from purified virus particles or from infected cells trough total RNA extraction. cDNA of the viral genome can be generated using a specific primer complementary to the 3′ end of the viral genome if the sequence is known, oligo-d(T) primers for polyadenylated genomes or random priming in case of unknown sequences. RNA can also be used in high throughput sequencing approaches to determine the viral genomic sequence including the genomic ends. *Middle part*: To obtain efficient reverse genetics systems the cDNA needs to be cloned downstream of promoter sequences. This can either be a RNA polymerase II promoter if the vRNA shall be transcribed in the nucleus of transfected cells, or bacteriophage promoters like T7 for in vitro transcription. When possible, the cDNA is assembled in one full length construct (*left*). Alternatively, the cDNA can be cloned in fragments into different plasmids to avoid instability or to break down large genomes to sizes that are more amenable to manipulation (*right*). *Lower part*: From full length plasmids containing a eukaryotic promoter vRNA will be transcribed by the cellular machinery upon transfection of the cDNA construct. After export of the RNA into the cytoplasm its translation will provide the viral proteins necessary for replication (*left*). Full length plasmids with bacteriophage promoters are linearized before RNA synthesis via run-off in vitro transcription (*middle*). When the viral cDNA is cloned in several fragments, the complete cDNA needs first to be assembled into a full length cDNA template by in vitro ligation to obtain a template for in vitro transcription (*right*). The resulting RNA is transfected into cells where it is translated. In all cases translation of the RNA within transfected cells generates the viral replicase proteins that are necessary and sufficient to initiate virus replication and production of viral particles

plasmid was not enough to establish constructs leading to the production of infectious virus progeny. Indeed, the first cDNA clone for rhinovirus type 14 failed to produce infectious viral particles, but addition of an SP6 bacteriophage promoter upstream of the cDNA combined with in vitro transcription of the cDNA, produced RNA that led to infectious progeny upon transfection into cells [12] (Fig. 2, middle). An equivalent approach was also used in the reverse genetics system for brome mosaic virus, which consists of three plasmids containing the cDNAs to the three viral genomic RNAs immediately downstream of a λ-phage promoter to drive in vitro transcription. Combined transfection of the three in vitro-transcribed RNAs led to virus infection in plants [11].

Another problem encountered in the generation of reverse genetic systems was the fact that some plasmids containing viral cDNA were unstable in *E. coli* and/or induced cytotoxicity. In many cases, cytotoxicity or instability of the viral cDNA could be countered successfully by use of low copy plasmids with for example P15A origins of replication restricting the plasmid copy number to 1 or very few per cell. This approach was successful in all of the first infectious clones for pestiviruses (ncpBVDV, cpBVDV, and CSFV) [15–18] but failed in case of yellow fever virus (YFV). This problem led to the development of a strategy using two or more plasmids, each of which contained a different part of the virus-derived cDNA. The first YFV infectious cDNA clone (17D vaccine strain, first flavivirus infectious clone at all) consisted of two separated fragments corresponding to the 5′ and 3′ half of the genome, respectively. Infectious RNA was generated through in vitro ligation of the two fragments followed by in vitro transcription [19] (Fig. 2 right part).

Correct 5′ end 3′ ends of the viral genome are often very important for the success of a reverse genetics system, as many viruses rely on special structures at their termini for replication and/or translation. All systems described above used restriction enzyme sites introduced directly downstream of the viral cDNA to linearize the plasmids before run-off in vitro transcription to obtain RNA 3′ ends identical or as similar as possible to those of the viral genome. With regard to the 5′ end of the RNA the use of bacteriophage promoters (mostly T7 or SP6) allowed to transcribe RNA with a marginally modified or even the desired start since only a 5′ G residue is necessary for efficient transcription by these enzymes. All infectious cDNA clones for members of the *Flaviviridae* were established with a T7 promoter directly upstream of the genomic cDNA and a blunt cutting restriction enzyme with a recognition site that directly overlaps the 3′ end of the genome to allow run-off in vitro transcription resulting in RNA identical to viral genomic RNA [15–19].

As an alternative to in vitro ligation of cDNA fragments an interesting approach based on reconstitution of full length viral

genomic RNA via intracellular RNA recombination has been developed. RNA recombination is a naturally occurring process and very widespread in RNA viruses. It gives rise to new virus variants such as the cytopathic biotype of pestiviruses [20–24]. Recombination of RNA of positive strand RNA viruses that replicate in the cytoplasm of infected cells, is different from DNA recombination or cellular RNA splicing, in which dedicated cellular machinery joins the ends of the respective nucleic acids. Recombination of cytoplasmic RNA is thought to occur either through template switching by the RNA-dependent polymerase during genome replication or through breakage of the RNA and joining with other RNA ends [22]. Several experiments with pestivirus and poliovirus mutants have shown that RNA recombination can happen independently of active RNA replication [25, 26]. In these experiments RNA fragments that each encoded only part of the RNA-dependent RNA polymerase were co-transfected into cells and were sufficient to lead to the recovery of infectious virus. The fact that intracellular recombination of viral RNA occurs rather frequently has been used as a tool to manipulate viral genomes not (yet) accessible to reverse genetics systems by cDNA clones or similar approaches via recombination of (mutated) genome fragments [27–30].

The above mentioned instability of viral full length cDNA clones is in part dependent on the size of the viral genome. The first cDNA clones were established for members of the *Picornaviridae* with genome sizes of about 7.5 kb [10, 12, 14]. Members of the *Flaviviridae* have genome sizes of 9.5–14 kb. Coronaviruses have the largest RNA genomes and therefore remained inaccessible to reverse genetic systems based on cDNA clones for a long time. Instead, targeted mutagenesis was achieved through recombination of transfected in vitro-transcribed RNA representing only a part of the viral genome and full length viral RNA in infected cells [27–29]. It took 19 years from the first infectious full length cDNA clone of a picornavirus to a full length infectious cDNA clone of a member of the *Coronaviridae* [31]. The latter construct was for the transmissible gastroenteritis virus (TGEV) and used a bacterial artificial chromosome (BAC) to propagate the large virus derived cDNA with low copy number, as parts of the genome were toxic to the bacteria. Furthermore, this cDNA clone contained the TGEV sequence downstream of a cytomegalovirus immediate early promoter and upon transfection of the DNA into cells, viral RNA was produced by the cellular RNA polymerase II, which then led to the production of infectious viral particles. The same year, a second cDNA system for TGEV was published using five separate plasmids which together contained the full length genome and needed to be assembled through in vitro ligation before RNA synthesis [32]. Yet another approach followed a year later for the avian coronavirus infectious bronchitis virus in which the genomic cDNA was inserted into the

genome of vaccinia virus, a large DNA virus from the family *Poxviridae* [33]. However, also strategies based on the use of RNA recombination are still employed for establishment of recombinant coronaviruses [30].

Methods to generate the long viral cDNA have changed in the last 35 years. The first approaches were based on cDNA libraries made from purified virion RNA or RNA of infected cells [10, 15–19, 34–36]. Later, full length PCR amplification of viral genomes became feasible through the availability of proofreading polymerases that allowed generation of an infectious clone after a single round of reverse transcription, followed by long-range PCR [37, 38]. With the rapid development of nucleic acid synthesis and high throughput sequencing it is now possible to generate cDNA clones through synthesis of the corresponding DNA sequences simply with the knowledge of the sequence. This was first demonstrated once again with poliovirus, but recently a cDNA clone system based on synthetic plasmids was published for the coronavirus porcine epidemic diarrhea virus [39, 40].

2.2 Road Map to Recovery of Self-Replicating RNA

Development of a strategy for establishment of a reverse genetics system for a new virus, which allows generation of self-replicating RNA and recovery of recombinant virus, requires knowledge on the molecular biology of this virus and a variety of considerations with regard to the final aim of the approach. The first step will usually be the determination of the sequence of the viral genome including the correct 5′ and 3′ ends. The latter information can be obtained by so-called RACE technology (rapid amplification of cDNA ends), PCR based systems that nowadays are provided by different commercial suppliers. The knowledge of the sequence will provide the necessary information on the genome organization which helps to understand the gene expression strategy of the virus. An important question in this context concerns the mechanism promoting initiation of translation and replication of the viral genomic RNA. As described above, translation of the genome is necessary to provide the components of the replicase that starts genome replication and thereby initiates the viral life cycle. Positive strand RNA viruses have developed a variety of strategies to ensure initiation of translation of their RNA [41–43]. In most systems, the infectious cDNA construct can be designed in a way that cis-acting structures important for translation and replication of the genome-like RNA derived from the construct will be equivalent to what is found in the viral genome. There are, however, special cases providing problems. Caliciviruses have a protein called VPg covalently bound to 5′ end of the viral RNA, which functions as a substitute for the cap structure driving translation initiation in eukaryotic mRNAs. This protein is most likely also crucial for the RNA to be accepted as a substrate for RNA replication but cannot be easily linked to in vitro-transcribed viral RNA. Replacing VPg by a

standard cap structure was found to be sufficient for translation and initiation of replication of the in vitro-transcribed RNA, but with quite low efficiency [36, 44, 45].

Similarly, the 3′ end of the viral RNA is important for successful recovery of self-replicating RNA. Many viruses contain a poly(A) tail at the 3′ end and thereby mimic the structure of a standard eukaryotic mRNA ensuring efficient translation. The poly(A) tail should also be important for replication of the viral RNA since it is the sequence at which transcription has to start during minus strand synthesis. Other viral genomes contain no poly(A) but for example specific secondary structures representing important cis-acting elements for both translation and RNA replication. As a general rule, any virus with a genome containing a poly(A) tail should also have such a sequence in its infectious cDNA construct, whereas viruses without a poly(A) tail can be expected to be very sensitive to changes in the sequence at their genomic 3′ end, so that steps should be undertaken to ensure generation of the correct genomic end during transcription.

When the necessary information on the viral genome and strategy of gene expression are available the next point to be decided is where and how transcription of the cDNA construct should occur. For the majority of reverse genetics systems for positive strand RNA viruses the genome-like RNA is generated in vitro and subsequently introduced into cells via transfection. This strategy is characterized by some methodical advantages, especially the simple generation of correct end sequences through use of bacteriophage RNA polymerases and "run-off" transcription. The transcription procedure was improved over the years so that highly efficient kits yielding large amounts of RNA became commercially available. The most common promoters used in in vitro transcription systems are the phage promoters T7 and SP6. These can be placed immediately upstream of the cDNA sequence ensuring a correct 5′ end of the resulting RNA. To obtain capped transcripts either a cap analog (like m7G(5′)ppp(5′)G) has to be included in the in vitro transcription reaction or the RNA needs to be capped after the in vitro transcription (using vaccinia virus derived capping systems). If the genomic RNA should contain a poly-A tail this needs to be either included in the template construct or added after transcription using a poly(A) polymerase. The second choice adds yet another step to the generation of the RNA and thus might reduce yield.

The above mentioned ways to introduction of a cap structure into in vitro-transcribed RNA work with only rather low efficiency. Thus, the alternative strategy relying on transfection of the plasmid DNA followed by in vivo transcription of the genome-like RNA can be advantageous when the production of capped transcripts is necessary, since RNA produced in transfected cells is 5′ capped and 3′ polyadenylated by cellular enzymes. A problem with this approach is the relatively high chance of further post-transcriptional

modification of the RNA like splicing, which could abrogate any infectivity. To obtain correct genomic ends for non-polyadenylated viruses with this approach ribozyme sequences such as the hepatitis delta ribozyme can be added at the ends, which will cleave themselves off and leave the correct terminus [46]. In fact, reverse genetics systems for positive strand RNA viruses using direct transfection of DNA into cells are much rarer than in vitro transcription based approaches.

An interesting alternative combining features of the in vitro transcription system with the advantages of DNA transfection is based on helper viruses like the vaccinia virus MVA-T7. Cells infected with the latter virus contain bacteriophage T7 RNA polymerase expressed by MVA-T7 which upon introduction of plasmid constructs with T7 promoters will transcribe the desired RNA in the cytoplasm of the cell which avoids nuclear location and the danger of unwanted splicing of the RNA product. The defined start site of T7 based transcription allows for an easy production of the correct 5′ end just as during in vitro transcription. Insertion of ribozyme sequences into the plasmid can ensure the formation of the desired defined 3′ end of the transcript. Since vaccinia virus replicates in the cytoplasm it expresses enzymes that cap and polyadenylate its own transcripts efficiently, which is also true for the T7 transcripts. The final result is the efficient production of a capped and polyadenylated transcript with correct ends within the cell which can lead to superior performance compared to in vitro transcription/transfection of RNA [45].

As mentioned before instability of viral sequences in E. coli while propagating the cDNA plasmids can be countered by different measures. It is preferable to use low copy plasmids or BACs to minimize the amount of plasmids with toxic sequences in the bacteria. Moreover, BACs can carry large inserts and thus are suitable for every positive strand RNA virus genome including those of coronaviruses. Sequences that seem to be deleterious for the propagation in E. coli can be disrupted by strategically placed intron sequences, if virus recovery is achieved via plasmid transfection into cells and intracellular RNA synthesis through RNA polymerase II. The intron will be spliced out of the produced RNA regenerating the viral sequence within the cells. This approach was employed in the production of a TGEV infectious clone [47].

Taken together, the establishment of systems for generation of self-replicating RNAs and recovery of infectious recombinant positive strand RNA viruses are in principle straight forward today but have to pay attention to the individual features of the respective system and the aims to be achieved. Depending on the system, more rarely used strategies like RNA recombination based generation of mutants might show up as the feasible solution. In fact, system specific problems had to be solved during development of almost any reverse genetics system that is routinely used now but

the available repertoire of possible solutions for such problems will facilitate such approaches in the future.

3 Use of Self-Replicating RNA for Vaccine Purposes

The development of techniques allowing the recovery of self-replicating viral RNA from cDNA was not only a milestone for basic research on RNA virus biology but also opened a door to new approaches towards modified live vaccines against viral diseases. In contrast to the traditional ways relying for attenuation on elaborate passaging of viruses in tissue culture cells or unusual host animals, reverse genetics systems allow for defined mutagenesis and rational attenuation.

3.1 Vaccines Based on Full Length Viral RNA

Pestiviruses represent a good example for different approaches towards live attenuated viral vaccines. Members of the genus *Pestivirus* are economically important pathogens of farm animals that are grouped in the family *Flaviviridae* together with their closest relatives, the hepaciviruses. Most important pestiviruses are the classical swine fever virus (CSFV) and the bovine viral diarrhea virus (BVDV) [48]. All members of the *Flaviviridae* are enveloped viruses with positive strand RNA genomes containing one long open reading frame. The economic impact of pestiviruses results at least in part from causing a wide range of pregnancy disorders and persistent infection due to their ability to cross the placenta in pregnant animals [48]. Persistently infected animals represent an important reservoir for virus spread. Vaccination represents a feasible means to interrupt the cycle of transmission as long as the vaccines do not only prevent disease but also fetal transmission of the pathogens. To fulfill the latter demand pestivirus vaccines have to be very potent.

The so-called CSFV C-strain represents an example of a successful pestivirus vaccine. It is a traditional modified live vaccine that was attenuated via serial passages in rabbit cells resulting in a very safe and efficient vaccine virus with so far undefined basis of attenuation. The latter is also true for different live BVDV strains used for vaccination in various countries worldwide. As an important disadvantage of these vaccines the attenuated viruses can still cross the placenta and infect the fetus in pregnant animals which in case of the conventional live BVDV vaccines can even lead to abortion. Using a reverse genetics approach we were able to establish a BVDV mutant with defined genomic deletions of nonessential sequences that knocked out two viral factors interfering with the host's type 1 interferon response without significantly impairing viral replication [49]. As a consequence of these changes affecting viral mechanisms blocking the innate immune response to BVDV infection not only complete attenuation of the mutant virus was observed but also the inability to

infect the fetus in pregnant animals, the prerequisite for pregnancy disorders and persistent infection.

Another approach based on deletion of nonessential sequences was described for coronaviruses. Members of the family *Coronaviridae* represent important pathogens of man and animals among which SARS and MERS coronavirus (SARS-CoV and MERS-CoV) are best known [50, 51]. As outlined above, coronaviruses have by far the largest known RNA genomes which encode not only essential but also some nonessential accessory proteins. Deletion of five of the eight group-specific ORFs (ORF3a, OF3b, ORF6, ORF7a, and ORF7b), either alone or in various combinations, from the SARS-CoV genomic RNA did not result in clear indications for attenuation in a mouse model. In contrast, a viable SARS-CoV mutant lacking the sequence coding for the E protein (ORF4) was recovered that exhibited reduced virulence in two animal models probably by enhanced response of the immune response to the infection [52–56]. E represents one of the membrane bound structural proteins of the virus and is involved in virion assembly and release. Such deletion mutants are still being characterized and improved but might provide a basis for the development of coronavirus vaccines in the future.

Not only deletion of sequences but also exchange of genomic fragments between related viruses is easily done via reverse genetics and can lead to attenuation and other desired features. As an example, a chimeric pestivirus was established as a vaccine against classical swine fever (Fig. 3). This concept was based on the replacement of the region coding for the major envelope protein E2 of a BVDV genome by the corresponding sequence of CSFV. The resulting virus CP7_E2alf was only able to infect pigs and thus displayed the

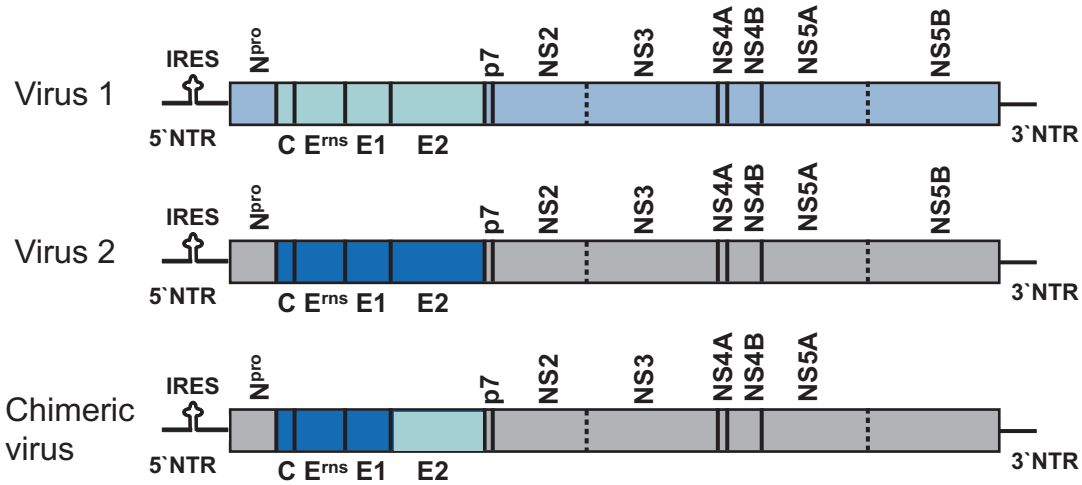

Fig. 3 Generation of a chimeric viral genome from two parental RNAs. On the level of a cDNA construct, one protein-coding sequence is replaced by the corresponding gene of the other virus (principle used for the pestivirus vaccine CP7_E2alf [58]). *IRES* internal ribosome entry site

tropism of CSFV. The chimeric virus was fully attenuated but nevertheless induced strong protective immunity [57–59]. As an important further advantage, the configuration of this chimera allows for serologic differentiation between vaccinated animals and those having been infected by a CSFV field virus, a feature of major importance for control and eradication programs in veterinary medicine.

Similar approaches as for CP7_E2alf were also used for members of the genus *Flavivirus* in the family *Flaviviridae*. The first approach employed the yellow fever virus (YFV) vaccine strain 17D, a virus developed in 1936 by empirical passage. YFV 17D is a very effective and safe vaccine that was found to highly trigger the innate immune response which helps driving the adaptive immune response to long lasting protective immunity [60–63]. Therefore, YFV 17D was chosen as backbone for a chimeric Japanese encephalitis/yellow fever virus vaccine (ChimeriVax™-JE) in which the surface protein prM/E coding region of YFV was replaced by the corresponding but modified JEV sequence resulting in a safe and effective vaccine launched by the end of 2012 (trade name IMOJEV™) [64]. Similar constructs in the 17D background were established with prM-E sequences from West Nile virus or the four dengue virus (DENV) genotypes and tested as vaccine candidates [65–69]. Also chimeric DENV composed of sequences from two different DENV genotypes and encompassing attenuating mutations were established and tested, as well as chimeras of DENV with tick-borne encephalitis virus sequences [70, 71].

The chimeric approach has also been followed in vaccine trials in alphaviruses, another group of positive strand RNA viruses belonging to the family *Togaviridae*. Low virulent Sindbis virus provided the backbone for these approaches that used exchange of the complete structural protein coding regions with sequences from highly virulent alphaviruses like Eastern or Western or Venezuelan equine encephalitis virus (EEE or WEE or VEE). The chimerization process itself led to significant attenuation of the resulting viruses that were found to be highly immunogenic (for review, *see* ref. 72). Nevertheless, the safety issue is a major concern in such approaches since biomarkers for the attenuation of Sindbis virus are not known and small animal models for testing virulence in most cases not adequate to evaluate attenuation in humans. Further research is needed to fully evaluate these vaccine candidates.

It has to be stressed that all the approaches described above employ self-replicating RNAs that represent either full length viral genomes or such RNA with deletion of nonessential sequences. Accordingly, these constructs allow the recovery of infectious virus particles. As presented above, introduction of the in vitro-transcribed recombinant RNA into a cell via transfection starts its autonomous replication leading to release of infectious viruses that after amplification in tissue culture serve as vaccine. Upon

administration, the immune response is triggered because the vaccine virus mimics all steps of a field virus infection but without induction of significant symptoms.

3.2 Replicons as Vaccines

The use of fully replication competent recombinant viruses bears a certain risk of reversion to virulence. Depending on the type of attenuating mutations this risk can be significant or only theoretically relevant as for viruses containing more than one deletion. Introduction of deletions into RNA virus genomes can lead to recovery of attenuated viruses in some special cases but will in most cases result in RNAs that are no longer able to promote the generation of infectious progeny. As long as the deletions do not concern the sequences responsible for replication of the RNA, such mutant RNAs will behave as replicons that amplify autonomously when introduced into a cell and lead to translation of significant amounts of the encoded proteins. A typical replicon approach is based on deletion of sequences coding for one or more structural components of the virus (Fig. 4). Such replicons were important tools for research on RNA replication of for example pestiviruses and hepaciviruses [73, 74]. For pestiviruses, replicons have also been tested in vaccination approaches [75]. In all cases, essential sequences were deleted from the genomes so that the vaccine candidates need complementation in trans by stably transfected cells providing the missing factors. Infection of a host organism with the virus particles secreted from these complementing cells represents a dead-end since no infectious virus can be released from non-complementing cells. Thus, these vaccines cross the border between live attenuated viruses and killed vaccines exhibiting safety features at least very close to killed vaccines. The big advantage over killed vaccines is the ability to express viral proteins within cells leading to MHC presentation of viral peptides and activation of a T-cell response in addition to the humoral response.

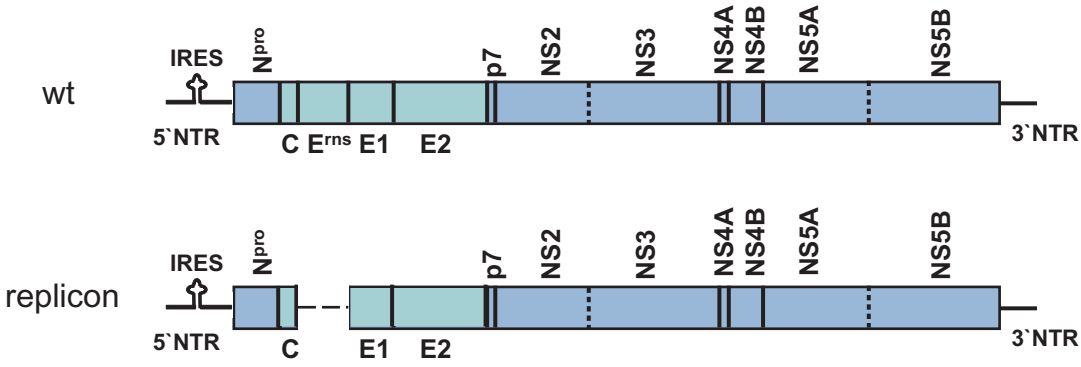

Fig. 4 Genome structure of a replicon. Deletion of a structural protein-coding region from the viral genome (represented by a *dotted line*) without interrupting the reading frame results in an RNA able to replicate autonomously within cells but deficient in production of infectious progeny

An interesting example of a further developed replicon approach is found in flaviviruses with the so-called pseudo-infectious vaccines. Integration of deletions of different length into the capsid protein coding region of the viral genomes results in autonomously replicating RNAs that are no longer able to promote the generation of infectious virus particles [72, 76, 77]. However, cells harboring these replicons will secrete large amounts of immunogenic prM/E particles. Propagation of such replicons in stably transfected cells providing the missing C protein in trans leads to virus-like particles able to conduct a single round of infection with highly efficient establishment of cells producing the prM/E particles. As a further approach, a DNA based vaccination has been developed that relies on two separate plasmids, one containing a cDNA representing the capsid-deleted viral genome and another expressing the missing capsid protein [72]. Cells that have taken up both plasmids will not only translate and present viral sequences but will also release infectious virus particles that can infect further cells leading to an enhanced stimulus of the immune system. Again, chimeric approaches with replicon backbones derived from one flavivirus species and prM/E coding sequences from another species have been tested successfully [72].

3.3 Self-Replicating RNAs as Vectors for Expression of Foreign Genes

The chimeric systems described above represent a special case of a more general approach towards vaccines based on self-replicating RNA that contain foreign sequences. Similar to the chimeric constructs mentioned before the replacement of viral protein-coding sequences by foreign genes can be used to express the desired proteins for immunization. In contrast to the chimeric viruses with a structural protein exchange between closely related viruses, such constructs will usually not yield autonomously propagating infectious viruses but replicons harboring non related sequences instead of the original structural proteins. Alternatively, the foreign sequences can be inserted into the viral genome as additional information without loss of essential viral functions so that fully replication-competent recombinant viruses can be produced. A prerequisite for the successful establishment of such self-replicating RNAs expressing foreign genes is the development of a strategy for integration and expression of the latter sequences without disturbing the autonomous replication of the RNA. Due to the fact that positive strand RNA viruses use expression strategies based on translation of polyproteins and subsequent proteolytic processing, the integration of foreign sequences into a viral open reading frame has to be combined with a specific processing step. A common approach avoiding fusion of significant numbers of unwanted residues to the proteins of interest is to place the foreign sequence at the 5′ end of the viral ORF and insert the foot and mouth disease virus (FMDV) 2A-coding region between the foreign sequence and the viral polyprotein (Fig. 5). FMDV 2A is a short peptide of

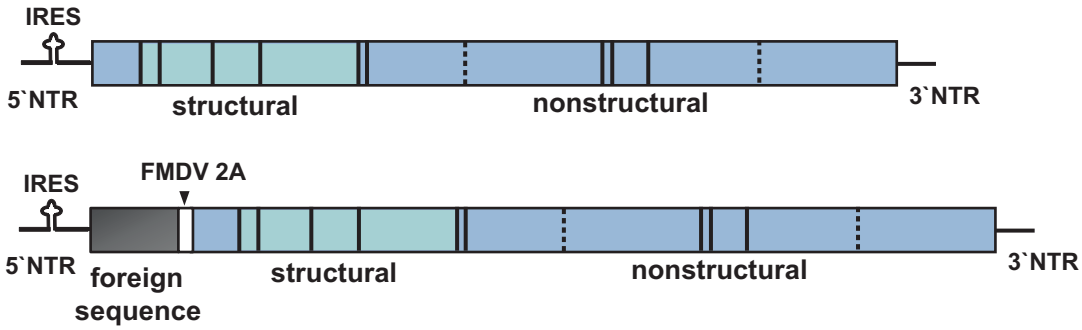

Fig. 5 Expression of a foreign protein from a viral genome via the viral polyprotein. The foreign sequence is inserted at the 5′ end of the viral ORF followed by a sequence coding for the FMDV 2A that promotes the separation of the foreign protein from the viral polyprotein during translation of the recombinant RNA

18 amino acids that is able to induce an irregular stop and restart of translation [78, 79]. In fact, 2A provokes an interruption of the polyprotein translation at its own carboxy terminus leading to release of an upstream protein fragment with 2A at its C-terminus and restart of translation with the proline following 2A in the polyprotein, so that the viral proteins following downstream are free of any added residues. An elegant approach avoiding any fusion of unwanted residues relies on the establishment of bicistronic RNAs. In such constructs the foreign sequence is usually also placed at the 5′ end of the ORF with a stop codon at the desired end of the translated region. Instead of a protein coding region ensuring processing of proteins an internal ribosome entry site (IRES) is integrated between foreign sequence and the viral ORF (Fig. 6) [74, 80]. The foreign sequence is expressed using the strategy that initiates translation of the viral proteins in the wt virus. Its translation terminates at a stop codon at the 3′ end. The IRES recruits ribosomes to the start site at the 5′ end of the viral ORF and thereby promotes translation of the proteins necessary for replication of the recombinant RNA. An alternative arrangement has been published for BVDV, in which IRES and foreign sequence are placed in the 3′ NTR (Fig. 6) [81].

Similarly, viruses like alphaviruses that use subgenomic RNAs for expression of their structural proteins can be adapted to expression of foreign sequences with an approach relying on the standard genome organization and expression strategy of the viruses. The viral RNA contains promoter sequences that recruit the viral RNA polymerase to internal sites of the minus strand RNA replication intermediate and start transcription of a mRNA of subgenomic length [82, 83]. Replacing the viral structural protein coding sequence downstream of this internal promoter with the desired foreign sequence will lead to a replicon that transcribes an mRNA coding for the foreign protein (Fig. 7). Alternatively, the subgenomic RNA promoter can be duplicated and inserted together

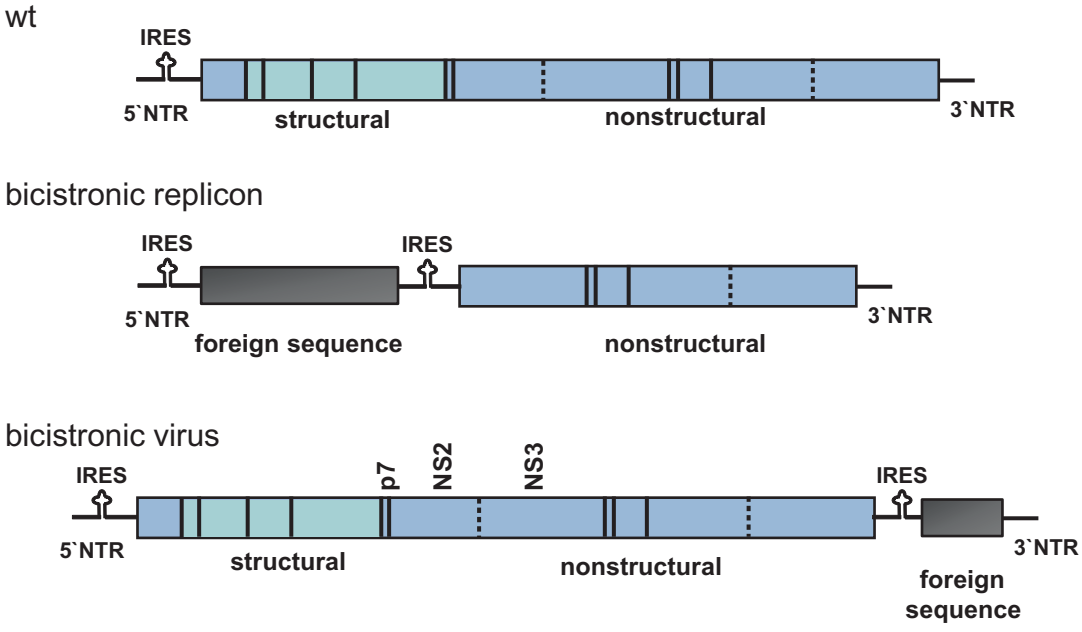

Fig. 6 Schematic representation of bicistronic self-replicating RNAs. On *top*, a standard positive strand virus genome with a single long open reading frame is shown (similar to RNA of picornaviruses or pestiviruses). Foreign sequences can be inserted together with a second IRES (internal ribosome entry site) instead of structural protein-coding sequences (bicistronic replicon—*middle*) or as insertion (bicistronic virus, *bottom panel*)

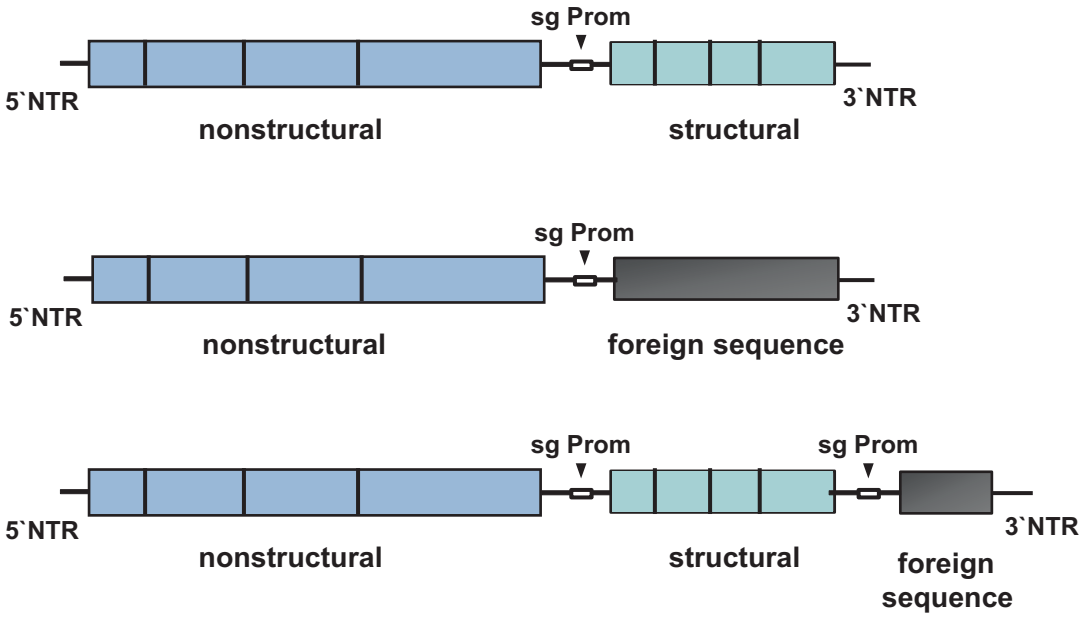

Fig. 7 Strategies to express foreign sequences in alphaviruses. On *top*, a standard alphavirus genome is shown. It contains two open reading frames, the second of which is expressed from as subgenomic mRNA transcribed under control of the subgenomic promoter (sg Prom). Replacement of the structural protein coding second ORF by a foreign sequence will lead to a replicon expressing the desired protein (*middle*), whereas insertion of the foreign sequence downstream of a duplicated sg promoter generates a recombinant virus expressing the desired sequence via a second sg mRNA (*bottom*). *See* also Fig. 1

with the desired foreign sequence as an additional cistron into the viral genomic RNA thereby preserving its ability to drive the generation of infectious replication competent virus particles. Based on these principles, a variety of vaccine strategies has been developed [71].

As a matter of fact, basically all approaches using self-replicating RNA for vaccination employ packaging of the RNA into virions or virus-like particles. The reason for this preference over naked or stabilized RNA is based on the extraordinary performance of the viral infection machinery resulting in highly efficient delivery of the RNA into cells. Since self-replicating RNAs derived from viral genomes exhibit the intrinsic property for specific packaging into viral particles, the use of this strategy is easy and straight forward. It has, however, to be mentioned that many virus particles display quite limited stability so that approaches relying on stabilized RNA could well be advantageous in certain situations especially when a cold chain during transport and delivery cannot be provided. An interesting opportunity for the future could be a vaccine formulation containing the RNA genome of a fully replication competent attenuated virus in stabilized form so that infectious virus is generated in the vaccinee upon administration. This approach would combine the superior resistance of stabilized RNA with the efficacy of a modified live viral vaccine.

References

1. Colter JS, Bird HH, Brown RA (1957) Infectivity of ribonucleic acid from Ehrlich ascites tumour cells infected with Mengo encephalitis. Nature 179(4565):859–860
2. Colter JS, Bird HH, Moyer AW, Brown RA (1957) Infectivity of ribonucleic acid isolated from virus-infected tissues. Virology 4(3):522–532
3. Sprunt K, Redman WM, Alexander HE (1959) Infectious ribonucleic acid derived from enteroviruses. Proc Soc Exp Biol Med Soc Exp Biol Med 101:604–608
4. Holland JJ, Hoyer BH, Mc LL, Syverton JT (1960) Enteroviral ribonucleic acid. I. Recovery from virus and assimilation by cells. J Exp Med 112:821–839
5. Holland JJ, Mc LL, Hoyer BH, Syverton JT (1960) Enteroviral ribonucleic acid. II. Biological, physical, and chemical studies. J Exp Med 112:841–864
6. Ketler A, Hamparian VV, Hilleman MR (1962) Characterization and classification of ECHO 28-rhinovirus-coryzavirus agents. Proc Soc Exp Biol Med Soc Exp Biol Med 110:821–831
7. Dimmock NJ (1966) Biophysical studies of a rhinovirus. Extraction and assay of infectious ribonucleic acid. Nature 209(5025):792–794
8. Mogler MA, Kamrud KI (2015) RNA-based viral vectors. Expert Rev Vaccines 14(2):283–312. doi:10.1586/14760584.2015.979798
9. Conzelmann KK, Meyers G (1996) Genetic engineering of animal RNA viruses. Trends Microbiol 4(10):386–393. doi:10.1016/0966-842X(96)10062-7
10. Racaniello VR, Baltimore D (1981) Cloned poliovirus complementary DNA is infectious in mammalian cells. Science 214(4523):916–919
11. Ahlquist P, French R, Janda M, Loesch-Fries LS (1984) Multicomponent RNA plant virus infection derived from cloned viral cDNA. Proc Natl Acad Sci U S A 81(22):7066–7070
12. Mizutani S, Colonno RJ (1985) In vitro synthesis of an infectious RNA from cDNA clones of human rhinovirus type 14. J Virol 56(2):628–632
13. Baltimore D (1971) Expression of animal virus genomes. Bacteriol Rev 35(3):235–241
14. Semler BL, Dorner AJ, Wimmer E (1984) Production of infectious poliovirus from cloned cDNA is dramatically increased by SV40 transcription and replication signals. Nucleic Acids Res 12(12):5123–5141
15. Moormann RJ, van Gennip HG, Miedema GK, Hulst MM, van Rijn PA (1996) Infectious

RNA transcribed from an engineered full-length cDNA template of the genome of a pestivirus. J Virol 70(2):763–770
16. Meyers G, Tautz N, Becher P, Thiel HJ, Kümmerer BM (1996) Recovery of cytopathogenic and noncytopathogenic bovine viral diarrhea viruses from cDNA constructs. J Virol 70(12):8606–8613
17. Meyers G, Thiel HJ, Rümenapf T (1996) Classical swine fever virus: recovery of infectious viruses from cDNA constructs and generation of recombinant cytopathogenic defective interfering particles. J Virol 70(3):1588–1595
18. Ruggli N, Tratschin JD, Mittelholzer C, Hofmann MA (1996) Nucleotide sequence of classical swine fever virus strain Alfort/187 and transcription of infectious RNA from stably cloned full-length cDNA. J Virol 70(6): 3478–3487
19. Rice CM, Grakoui A, Galler R, Chambers TJ (1989) Transcription of infectious yellow fever RNA from full-length cDNA templates produced by in vitro ligation. New Biol 1(3):285–296
20. Becher P, Orlich M, König M, Thiel HJ (1999) Nonhomologous RNA recombination in bovine viral diarrhea virus: molecular characterization of a variety of subgenomic RNAs isolated during an outbreak of fatal mucosal disease. J Virol 73(7):5646–5653
21. Becher P, Orlich M, Thiel HJ (2001) RNA recombination between persisting pestivirus and a vaccine strain: generation of cytopathogenic virus and induction of lethal disease. J Virol 75(14):6256–6264. doi:10.1128/JVI.75.14.6256-6264.2001
22. Becher P, Tautz N (2011) RNA recombination in pestiviruses: cellular RNA sequences in viral genomes highlight the role of host factors for viral persistence and lethal disease. RNA Biol 8(2):216–224
23. Meyers G, Rümenapf T, Thiel HJ (1989) Ubiquitin in a togavirus. Nature 341:491
24. Meyers G, Thiel HJ (1996) Molecular characterization of pestiviruses. Adv Virus Res 47:53–117
25. Gallei A, Pankraz A, Thiel HJ, Becher P (2004) RNA recombination in vivo in the absence of viral replication. J Virol 78(12):6271–6281. doi:10.1128/JVI.78.12.6271-6281.2004
26. Gmyl AP (2003) Nonreplicative homologous RNA recombination: promiscuous joining of RNA pieces? RNA 9(10):1221–1231. doi:10.1261/rna.5111803
27. Koetzner CA, Parker MM, Ricard CS, Sturman LS, Masters PS (1992) Repair and mutagenesis of the genome of a deletion mutant of the coronavirus mouse hepatitis virus by targeted RNA recombination. J Virol 66(4):1841–1848
28. Liao CL, Lai MMC (1992) RNA recombination in a coronavirus - recombination between viral genomic RNA and transfected RNA fragments. J Virol 66(10):6117–6124
29. van der Most RG, Heijnen L, Spaan WJ, de Groot RJ (1992) Homologous RNA recombination allows efficient introduction of site-specific mutations into the genome of coronavirus MHV-A59 via synthetic co-replicating RNAs. Nucleic Acids Res 20(13):3375–3381
30. Masters PS, Rottier PJ (2005) Coronavirus reverse genetics by targeted RNA recombination. Curr Top Microbiol Immunol 287:133–159
31. Almazan F, Gonzalez JM, Penzes Z, Izeta A, Calvo E, Plana-Duran J, Enjuanes L (2000) Engineering the largest RNA virus genome as an infectious bacterial artificial chromosome. Proc Natl Acad Sci U S A 97(10):5516–5521
32. Yount B, Curtis KM, Baric RS (2000) Strategy for systematic assembly of large RNA and DNA genomes: transmissible gastroenteritis virus model. J Virol 74(22):10600–10611
33. Casais R, Thiel V, Siddell SG, Cavanagh D, Britton P (2001) Reverse genetics system for the avian coronavirus infectious bronchitis virus. J Virol 75(24):12359–12369. doi:10.1128/JVI.75.24.12359-12369.2001
34. Racaniello VR, Baltimore D (1981) Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proc Natl Acad Sci U S A 78(8):4887–4891
35. Rice CM, Levis R, Strauss JH, Huang HV (1987) Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of a temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. J Virol 61(12):3809–3819
36. Sosnovtsev S, Green KY (1995) RNA transcripts derived from a cloned full-length copy of the feline calicivirus genome do not require VpG for infectivity. Virology 210(2):383–390. doi:10.1006/viro.1995.1354
37. Chumakov KM (1996) PCR engineering of viral quasispecies: a new method to preserve and manipulate genetic diversity of RNA virus populations. J Virol 70(10):7331–7334
38. Rasmussen TB, Reimann I, Hoffmann B, Depner K, Uttenthal A, Beer M (2008) Direct recovery of infectious pestivirus from a full-length RT-PCR amplicon. J Virol Methods 149(2):330–333. doi:10.1016/j.jviromet.2008.01.029

39. Cello J, Paul AV, Wimmer E (2002) Chemical synthesis of poliovirus cDNA: generation of infectious virus in the absence of natural template. Science 297(5583):1016–1018. doi:10.1126/science.1072266
40. Beall A, Yount B, Lin CM, Hou Y, Wang Q, Saif L, Baric R (2016) Characterization of a pathogenic full-length cDNA clone and transmission model for porcine epidemic diarrhea virus strain PC22A. MBio 7(1):e01451–15. doi:10.1128/mBio.01451-15
41. Bushell M, Sarnow P (2002) Hijacking the translation apparatus by RNA viruses. J Cell Biol 158(3):395–399
42. Firth AE, Brierley I (2012) Non-canonical translation in RNA viruses. J Gen Virol 93(Pt 7):1385–1409. doi:10.1099/vir.0.042499-0
43. Lopez-Lastra M, Ramdohr P, Letelier A, Vallejos M, Vera-Otarola J, Valiente-Echeverria F (2010) Translation initiation of viral mRNAs. Rev Med Virol 20(3):177–195. doi:10.1002/rmv.649
44. Sosnovtsev SV, Belliot G, Chang KO, Onwudiwe O, Green KY (2005) Feline calicivirus VP2 is essential for the production of infectious virions. J Virol 79(7):4012–4024
45. Thumfart JO, Meyers G (2002) Feline calicivirus: recovery of wild-type and recombinant viruses after transfection of cRNA or cDNA constructs. J Virol 76(12):6398–6407
46. Schnell MJ, Mebatsion T, Conzelmann KK (1994) Infectious rabies viruses from cloned cDNA. EMBO J 13(18):4195–4203
47. Gonzalez JM, Penzes Z, Almazan F, Calvo E, Enjuanes L (2002) Stabilization of a full-length infectious cDNA clone of transmissible gastroenteritis coronavirus by insertion of an intron. J Virol 76(9):4655–4661. doi:10.1128/jvi.76.9.4655-4661.2002
48. Tautz N, Tews BA, Meyers G (2015) The molecular biology of pestiviruses. Adv Virus Res 93:47–160. doi:10.1016/bs.aivir.2015.03.002
49. Meyers G, Ege A, Fetzer C, von Freyburg M, Elbers K, Carr V, Prentice H, Charleston B, Schürmann EM (2007) Bovine viral diarrhoea virus: prevention of persistent foetal infection by a combination of two mutations affecting the Erns RNase and the Npro protease. J Virol 81(7):3327–3338
50. Masters PS (2006) The molecular biology of coronaviruses. Adv Virus Res 66:193–292. doi:10.1016/S0065-3527(06)66005-3
51. Ziebuhr J (2004) Molecular biology of severe acute respiratory syndrome coronavirus. Curr Opin Microbiol 7(4):412–419. doi:10.1016/j.mib.2004.06.007
52. Yount B, Roberts RS, Sims AC, Deming D, Frieman MB, Sparks J, Denison MR, Davis N, Baric RS (2005) Severe acute respiratory syndrome coronavirus group-specific open reading frames encode nonessential functions for replication in cell cultures and mice. J Virol 79(23):14909–14922. doi:10.1128/JVI.79.23.14909-14922.2005
53. DeDiego ML, Alvarez E, Almazan F, Rejas MT, Lamirande E, Roberts A, Shieh WJ, Zaki SR, Subbarao K, Enjuanes L (2007) A severe acute respiratory syndrome coronavirus that lacks the E gene is attenuated in vitro and in vivo. J Virol 81(4):1701–1713. doi:10.1128/JVI.01467-06
54. Dediego ML, Pewe L, Alvarez E, Rejas MT, Perlman S, Enjuanes L (2008) Pathogenicity of severe acute respiratory coronavirus deletion mutants in hACE-2 transgenic mice. Virology 376(2):379–389. doi:10.1016/j.virol.2008.03.005
55. Lamirande EW, DeDiego ML, Roberts A, Jackson JP, Alvarez E, Sheahan T, Shieh WJ, Zaki SR, Baric R, Enjuanes L, Subbarao K (2008) A live attenuated severe acute respiratory syndrome coronavirus is immunogenic and efficacious in golden Syrian hamsters. J Virol 82(15):7721–7724. doi:10.1128/JVI.00304-08
56. Netland J, DeDiego ML, Zhao J, Fett C, Alvarez E, Nieto-Torres JL, Enjuanes L, Perlman S (2010) Immunization with an attenuated severe acute respiratory syndrome coronavirus deleted in E protein protects against lethal respiratory disease. Virology 399(1):120–128. doi:10.1016/j.virol.2010.01.004
57. Beer M, Reimann I, Hoffmann B, Depner K (2007) Novel marker vaccines against classical swine fever. Vaccine 25(30):5665–5670
58. Koenig P, Lange E, Reimann I, Beer M (2007) CP7_E2alf: a safe and efficient marker vaccine strain for oral immunisation of wild boar against Classical swine fever virus (CSFV). Vaccine 25(17):3391–3399
59. Leifer I, Lange E, Reimann I, Blome S, Juanola S, Duran JP, Beer M (2009) Modified live marker vaccine candidate CP7_E2alf provides early onset of protection against lethal challenge infection with classical swine fever virus after both intramuscular and oral immunization. Vaccine 27(47):6522–6529
60. Gaucher D, Therrien R, Kettaf N, Angermann BR, Boucher G, Filali-Mouhim A, Moser JM, Mehta RS, Drake DR 3rd, Castro E, Akondy R, Rinfret A, Yassine-Diab B, Said EA, Chouikh Y, Cameron MJ, Clum R, Kelvin D, Somogyi R, Greller LD, Balderas RS, Wilkinson P, Pantaleo G, Tartaglia J, Haddad EK, Sekaly RP

(2008) Yellow fever vaccine induces integrated multilineage and polyfunctional immune responses. J Exp Med 205(13):3119–3131. doi:10.1084/jem.20082292

61. Querec T, Bennouna S, Alkan S, Laouar Y, Gorden K, Flavell R, Akira S, Ahmed R, Pulendran B (2006) Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. J Exp Med 203(2):413–424. doi:10.1084/jem.20051720
62. Querec TD, Akondy RS, Lee EK, Cao W, Nakaya HI, Teuwen D, Pirani A, Gernert K, Deng J, Marzolf B, Kennedy K, Wu H, Bennouna S, Oluoch H, Miller J, Vencio RZ, Mulligan M, Aderem A, Ahmed R, Pulendran B (2009) Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans. Nat Immunol 10(1):116–125. doi:10.1038/ni.1688
63. Querec TD, Pulendran B (2007) Understanding the role of innate immunity in the mechanism of action of the live attenuated Yellow Fever Vaccine 17D. Adv Exp Med Biol 590:43–53. doi:10.1007/978-0-387-34814-8_3
64. Monath TP, Guirakhoo F, Nichols R, Yoksan S, Schrader R, Murphy C, Blum P, Woodward S, McCarthy K, Mathis D, Johnson C, Bedford P (2003) Chimeric live, attenuated vaccine against Japanese encephalitis (ChimeriVax-JE): phase 2 clinical trials for safety and immunogenicity, effect of vaccine dose and schedule, and memory response to challenge with inactivated Japanese encephalitis antigen. J Infect Dis 188(8):1213–1230. doi:10.1086/378356
65. Guirakhoo F, Kitchener S, Morrison D, Forrat R, McCarthy K, Nichols R, Yoksan S, Duan X, Ermak TH, Kanesa-Thasan N, Bedford P, Lang J, Quentin-Millet MJ, Monath TP (2006) Live attenuated chimeric yellow fever dengue type 2 (ChimeriVax-DEN2) vaccine: phase I clinical trial for safety and immunogenicity: effect of yellow fever pre-immunity in induction of cross neutralizing antibody responses to all 4 dengue serotypes. Hum Vaccines 2(2):60–67
66. Guirakhoo F, Weltzin R, Chambers TJ, Zhang ZX, Soike K, Ratterree M, Arroyo J, Georgakopoulos K, Catalan J, Monath TP (2000) Recombinant chimeric yellow fever-dengue type 2 virus is immunogenic and protective in nonhuman primates. J Virol 74(12):5477–5485
67. Monath TP, Arroyo J, Miller C, Guirakhoo F (2001) West Nile virus vaccine. Curr Drug Targets Infect Disord 1(1):37–50
68. Monath TP, Liu J, Kanesa-Thasan N, Myers GA, Nichols R, Deary A, McCarthy K, Johnson C, Ermak T, Shin S, Arroyo J, Guirakhoo F, Kennedy JS, Ennis FA, Green S, Bedford P (2006) A live, attenuated recombinant West Nile virus vaccine. Proc Natl Acad Sci U S A 103(17):6694–6699. doi:10.1073/pnas.0601932103
69. Pugachev KV, Guirakhoo F, Trent DW, Monath TP (2003) Traditional and novel approaches to flavivirus vaccines. Int J Parasitol 33(5-6):567–582
70. Pletnev AG, Bray M, Lai CJ (1993) Chimeric tick-borne encephalitis and dengue type 4 viruses: effects of mutations on neurovirulence in mice. J Virol 67(8):4956–4963
71. Monath TP (2011) Recombinant, chimeric, live, attenuated vaccines against Flaviviruses and Alphaviruses. In: Dormitzer PR, Mandel CW, Rappuoli R (eds) Replicating vaccines: a new generation. Springer, Basel, pp 349–438
72. Roby JA, Hall RA, Khromykh AA (2011) Nucleic acid-based infections and pseudo-infectious flavivirus vaccines. In: Dormitzer PR, Mandel CW, Rappuoli R (eds) Replicating vaccines: a new generation. Springer, Basel, pp 299–320
73. Behrens SE, Grassmann CW, Thiel HJ, Meyers G, Tautz N (1998) Characterization of an autonomous subgenomic pestivirus RNA replicon. J Virol 72(3):2364–2372
74. Lohmann V, Körner F, Koch JO, Herian U, Theilmann L, Bartenschlager R (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285:110–113
75. Reimann I, Semmler I, Beer M (2007) Packaged replicons of bovine viral diarrhea virus are capable of inducing a protective immune response. Virology 366(2):377–386
76. Kofler RM, Aberle JH, Aberle SW, Allison SL, Heinz FX, Mandl CW (2004) Mimicking live flavivirus immunization with a noninfectious RNA vaccine. Proc Natl Acad Sci U S A 101(7):1951–1956. doi:10.1073/pnas.0307145101
77. Mandl CW (2004) Flavivirus immunization with capsid-deletion mutants: basics, benefits, and barriers. Viral Immunol 17(4):461–472. doi:10.1089/vim.2004.17.461
78. Donnelly ML, Hughes LE, Luke G, Mendoza H, ten Dam E, Gani D, Ryan MD (2001) The 'cleavage' activities of foot-and-mouth disease virus 2A site-directed mutants and naturally occurring '2A-like' sequences. J Gen Virol 82(Pt 5):1027–1041. doi:10.1099/0022-1317-82-5-1027
79. Donnelly ML, Luke G, Mehrotra A, Li X, Hughes LE, Gani D, Ryan MD (2001) Analysis of the aphthovirus 2A/2B polyprotein 'cleavage'

mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'skip'. J Gen Virol 82(Pt 5):1013–1025. doi:10.1099/0022-1317-82-5-1013

80. Tautz N, Harada T, Kaiser A, Rinck G, Behrens SE, Thiel HJ (1999) Establishment and characterization of cytopathogenic and noncytopathogenic pestivirus replicons. J Virol 73(11):9422–9432
81. Baroth M, Peters Y, Schönbrunner ER, Behrens SE (2010) Stable recombinants of bovine viral diarrhea virus containing a hepatitis C virus insert. J Gen Virol 91(Pt 5):1213–1217. doi:10.1099/vir.0.016998-0
82. Agapov EV, Frolov I, Lindenbach BD, Pragai BM, Schlesinger S, Rice CM (1998) Noncytopathic Sindbis virus RNA vectors for heterologous gene expression. Proc Natl Acad Sci U S A 95:12989–12994
83. Strauss JH, Strauss EG (1994) The alphaviruses: gene expression, replication, and evolution. Microbiol Rev 58:491–562

Chapter 3

Self-Replicating RNA Vaccine Delivery to Dendritic Cells

Thomas Démoulins, Pavlos C. Englezou, Panagiota Milona, Nicolas Ruggli, Nicola Tirelli, Chantal Pichon, Cédric Sapet, Thomas Ebensen, Carlos A. Guzmán, and Kenneth C. McCullough

Abstract

Most current vaccines are either inactivated pathogen-derived or protein/peptide-based, although attenuated and vector vaccines have also been developed. The former induce at best moderate protection, even as multimeric antigen, due to limitations in antigen loads and therefore capacity for inducing robust immune defense. While attenuated and vector vaccines offer advantages through their replicative nature, drawbacks and risks remain with potential reversion to virulence and interference from preexisting immunity. New advances averting these problems are combining self-amplifying replicon RNA (RepRNA) technology with nanotechnology. RepRNA are large self-replicating RNA molecules (12–15 kb) derived from viral genomes defective in at least one structural protein gene. They provide sustained antigen production, effectively increasing vaccine antigen payloads over time, without the risk of producing infectious progeny. The major limitation with RepRNA is RNase-sensitivity and inefficient uptake by dendritic cells (DCs)—absolute requirements for efficacious vaccine design. We employed biodegradable delivery vehicles to protect the RepRNA and promote DC delivery. Encapsulating RepRNA into chitosan nanoparticles, as well as condensing RepRNA with polyethylenimine (PEI), cationic lipids, or chitosans, has proven effective for delivery to DCs and induction of immune responses in vivo.

Key words Replicon-RNA, Self-replicating vaccine, Universal influenza vaccine, Dendritic cell delivery, Chitosan nanoparticles, Polyplexes, Cationic lipids

1 Introduction

Vaccination is the cornerstone of prophylaxis. However, current vaccines suffer from several drawbacks. Most employed vaccines are inactivated virus or protein/peptide-based. The low antigen loads of such vaccines provide limited capacity for inducing robust immune defense, and tend to favor induction of humoral immunity rather than combined humoral plus cell mediated immune (CMI) responses. Consequently, robust and durable immunity are not guaranteed. Although live, attenuated, or vector vaccines offer advantages in terms of antigen load due to their replicative nature,

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_3,

they often remain reliant on cell culture or egg production. Importantly, these vaccines can pose risks from potential reversion to virulence and/or interference from preexisting immunity.

The disadvantages of the above vaccines can be overcome by application of the self-amplifying replicon RNA (RepRNA) technology [1–7]. RepRNA is derived from defective virus genomes, allowing translation of encoded antigens without progeny virus production. Their replicative nature provides several rounds of antigen production, thus enhancing the antigen dosage available for activating humoral and CMI responses, as well as the duration and therefore robustness of that response [5]. Although RepRNA application in vaccinology has gained momentum over the past two decades, their delivery has required application as virus-like replicon particles (VRPs) [1–4, 6–11]. This necessitates employment of expensive complementing cell cultures to provide the missing gene products *in trans* for encapsidation of the RepRNA in VRPs. Their application can still encounter problems—potential neutralization by preexisting immunity against VRP epitopes; species/individual restrictions in terms of VRP interaction with host cells; and production difficulties due to the prerequisite for the complementing cell lines. Moreover, application of VRPs cannot ensure targeting to dendritic cells (see below). Recent advances have applied nanotechnology to replace VRPs by biocompatible and biodegradable delivery vehicles to enhance the applicability of RepRNA vaccines [5, 12–14]. Synthetic biology approaches allow for production of both the RepRNA and delivery vehicles, thus avoiding any need for cell lines, serum, or other animal products.

Here we propose synthetic vaccines employing RepRNA technology for delivery to dendritic cells (DCs). Thereby, the RepRNA can auto-amplify within the cell for sustained antigen production without infectious progeny assembly, increasing the potential for humoral and CMI response induction [5, 12–16]. Being in vitro-transcribed and purified, the RepRNA is free from animal-, plant- or microbe-related impurities, and can be manufactured cost-effectively on a large scale under good manufacturing practice (GMP) conditions.

Delivery of the RepRNA to DCs is key event for efficient vaccination, due to the presence of different DC subsets in most tissues and organs of the body, thus determining their central role in immune surveillance and therefore as a target for vaccines [5, 17–22]. Moreover, DCs are the principal players in delivering and presenting antigen to the adaptive immune system, as well as maintaining and regulating homeostasis. With respect to the adaptive immune system, DCs are referred to as the "professional antigen presenting cells," due to their essential roles in activating naïve T-lymphocyte responses and cross-presenting antigen to promote cytotoxic T-lymphocyte responses.

Our work advancing the replacement of VRP-mediated delivery by synthetic, nonviral vectors employs RepRNA encapsulation or complexing with delivery vehicles to promote interaction with DCs. Concomitant with facilitating delivery to these centrally important cells of the immune system, the RepRNA is protected from nuclease degradation, while the delivery vehicle remains nonimmunogenic. Three distinct delivery vehicles have been successfully applied: (1) chitosan nanoparticles (encapsulation of RepRNA within alginate-coated or hyaluronic-coated chitosan nanoparticles); (2) polyethylenimine (PEI)-based polyplexes (complexing RepRNA with cationic biodegradable polymers); and (3) lipoplexes (complexing RepRNA with cationic biodegradable lipids). This innovative approach is currently developing the first synthetic influenza vaccine of its kind, with potential as a universal vaccine application.

2 Cell Preparations and Cultures

2.1 Chemicals and Solutions

All reagents used for the procedures are listed in Table 1.

2.2 Main Equipment and Other Materials

Main equipment and other materials are listed in Table 2.

2.3 DCs and Monocytes for RepRNA Delivery Assessment

Current efforts on synthetic, biodegradable delivery vehicles for RepRNA have been focussing on replicons derived from the classical swine fever virus (CSFV), due to the biosafety for humans engendered by the virus being nonpathogenic for humans. As such, a major advantage lies with the cells employed for assessment of RepRNA integrity and functionality, particularly when employed with the delivery vehicles. Thus, cells of porcine origin are utilized, due to the known efficacy of the RepRNA for translation and replication in these cells. The porcine cells are always employed as a reference control when assessing the translation and replication of the RepRNA in other cells, including humans DCs.

2.3.1 Porcine Peripheral Blood Mononuclear Cell Preparation

Experiments employing porcine blood were approved by the Animal Welfare Committee of the Canton of Bern, Switzerland, under license number BE26/11 and BE88/14, and conducted in compliance with the Swiss animal protection law. The use of porcine blood offers a reliable source of cells, through the regular availability of large quantities of blood cells from donor animals. Moreover, the porcine immune system has been proposed as a model for human immunology, including studies on influenza vaccines. For assessing RepRNA delivery, one isolates the DCs as described below. These cells are employed to determine the

efficacy of RepRNA delivery, as well as the efficiency of RNA translation. Thereafter, DCs expressing the antigens encoded by the RepRNA can be employed in restimulation assays with lymphocytes isolated from the blood of the same donor animals that provided the blood for the DC isolation.

1. Prepare a sterile 1 l bottle with rubber cap for needle puncture, containing 200 ml (1/3 final volume with blood) Alsever's solution (is actually a 2× solution, to allow for mixing with twice the volume of blood rather than an equal volume—*see* Table 1); this will collect up to 400 ml blood (final volume must not exceed 600 ml). Draw vacuum in the bottle. Blood is collected from the *vena cava cranialis* (preferred) or the *vena jugularis* of a blood donor pig (≥100 kg bodyweight) by a veterinarian or a trained animal caretaker, using sterile, heparinized silicon tubing (carrying sterile Luer needle attachments at either end) and appropriate needles (see below). The tubings are prepared in advance by rinsing 5 times with 20 ml heparin solution (100 U/ml), removing the excess heparin, sealing the Luer ends with aluminum foil and autoclaving. Before blood drawing, a sterile 15G needle (1.8 × 40 mm) is attached aseptically to one end of the sterile silicon tubing (this will serve for puncturing the rubber lid of the bottle when starting the blood drawing—see below). A syringe with an appropriate sterile needle adapted to the size of the pig and the vein to be punctured (for example 15G, 1.8 × 80–100 mm for the *vena cava cranialis*) is used to puncture the vein and blood is drawn into the syringe. The syringe is then removed and the free Luer end of the tubing attached aseptically to the needle still in the *vena cava cranialis* or *vena jugularis*. The other end of the tubing carrying the short 1.8 × 40 mm needle is then inserted through the rubber lid of the bottle containing the Alsever's solution. The vacuum within the bottle then exerts its effect by drawing blood into the bottle. As this flows into the bottle, gently and continually mix the blood and Alsever's solution in a swirling motion until the blood collection has reached the maximum volume. Keep the blood at room temperature (RT) until further processing.
2. Split the blood into 50 ml sterile centrifuge tubes (NOT polystyrene); centrifuge for 20 min at 1000 × *g* at RT, no brake, to separate the buffy coat from the erythrocytes and plasma.
3. In the meantime, fill eight 50 ml LeucoSep™ tubes with 17 ml Ficoll-Paque and spin for 1 min at 1000 × *g*; remove any Ficoll-Paque from above the filter.
4. Carefully remove the buffy coat (white interface between plasma and erythrocytes) by pipetting from each tube, and combine in a sterile bottle; dilute 1:1 with PBS no calcium, no magnesium ($PBS^{-/-}$)/EDTA (0.8 mM) to a final volume of 200 ml at RT.

Table 1
List of general compounds

Reagents	Manufacturer	Catalog number
Chloroform	Sigma-Aldrich	C2432-25ml
Isoamyl alcohol	Sigma-Aldrich	19392-25ml
Phenol–chloroform–isoamyl alcohol 25:24:1	Sigma-Aldrich	77617
Tuerk solution	Sigma-Aldrich	93770
NaAC (sodium acetate)	Sigma-Aldrich	S2889
3-amino-9-ethyl-carbazole–*N*,*N*-dimethyl-formamide (AEC)	Sigma-Aldrich	A6926
Nuclease-free H_2O	Ambion®/ThermoFisher Scientific	AM9930
MEGAscript® T7 Transcription Kit	Ambion®/ThermoFisher Scientific	AM1334
RNase*Zap*® RNase Decontamination Solution	Ambion®/ThermoFisher Scientific	AM9780
Sodium Acetate (3 M), pH 5.5	Ambion®/ThermoFisher Scientific	AM9740
SfiI	BioConcept/New England BioLabs	R0123S
SrfI	BioConcept/New England BioLabs	R0629S
NucleoBond® Xtra Midi	Macherey-Nagel	740410.10
NEBuffer 2.1	BioConcept/New England BioLabs	B7202S
MACS buffer: $PBS^{-/-}$ EDTA (2 mM) BSA 0.1 %	Home made	–
Heparin sodium	Serva	24590
Ficoll® Paque Plus	GE Healthcare	17-1440-03
Illustra MicroSpin S-400 HR Columns	GE Healthcare	27-5140-01
EDTA	Eurobio	GAUEDT0065
Label IT® Fluorescein Nucleic Acid Labeling Kit	Mirus/LabForce AG	MIR 3200
Label IT® CX-Rhodamine Labeling Kit	Mirus/LabForce AG	MIR 3100
Dy490-UTP	Dyomics GMBH	490-34

(continued)

Table 1
(continued)

Reagents	Manufacturer	Catalog number
Alsever's solution: $C_6H_{12}O_6 \cdot H_2O$ (1.55 mol/L) $Na_3C_6H_5O_7 \cdot 2H_2O$ (408 mmol/L) NaCl (1.078 mol/L) $C_6H_8O_7$ (43 mmol/L)	Home made	–
Recombinant porcine GM-CSF	Home made	[23]
Recombinant porcine IL-4	Home made	[24]
Corning® Fibronectin, Human, 1 mg	Corning Incorporated	354008
DPBS, calcium, magnesium (1×) ($PBS^{+/+}$) *Calcium and magnesium important for cell adhesion*	Gibco Thermo Fisher Scientific	14040-174
DPBS (10×), no calcium, no magnesium ($PBS^{-/-}$) *Absence of cations avoid aggregation of cells during the procedure of isolation*	Gibco Thermo Fisher Scientific	14200-067
MEM Earle's *Red color, for SK6 culture (medium A)*	Gibco Thermo Fisher Scientific	11095080
MEM Hank's *Red color, for SK6 culture (medium B)*	Gibco Thermo Fisher Scientific	21575-022
DMEM, high glucose, HEPES, no phenol red *White color, for bDC and MoDC culture*	Gibco Thermo Fisher Scientific	21063-029
Penicillin–streptomycin (10,000 U/ml)	Gibco Thermo Fisher Scientific	15140-122
Trypsin–EDTA (0.05%), phenol red	Gibco Thermo Fisher Scientific	25300-054
MACS Blood Dendritic Cell Isolation Kit II	Miltenyi Biotec	130-091-379
CD14 MicroBeads Human	Miltenyi Biotec	130-050-201
Porcine serum	Biochrom Merck Millipore	S0163
Fetal bovine serum	Biochrom Merck Millipore	S0115
Horse serum	SVA Hatunaholm Bro Sweden	HS 9/02

5. Dispense the diluted buffy coats by overlaying the Ficoll-Paque now under the filters of the LeucoSep™ tubes with no more than 25 ml of the diluted buffy coats, and centrifuge for 25 min at 800 × *g*, RT, no brake.

6. Take the interface on top of the Ficoll-Paque, which has been displaced at least in part above the filter (the interface is visible as a "cloudy" band), and transfer each interface into a 50 ml tube (1 per LeucoSep™ tube); fill up to 50 ml with cold (held in an ice bath) $PBS^{-/-}$/EDTA (0.8 mM) and centrifuge for 10 min at 350×*g*, 4 °C.
7. Remove the supernatants from the cell pellets, and gently resuspend the pellets in a small amount (≤1 ml) of cold $PBS^{-/-}$/EDTA (0.8 mM), then top up to 10 ml with cold $PBS^{-/-}$/EDTA (0.8 mM) and transfer to 15 ml centrifuge tubes (1 for each 50 ml tube resuspended pellet); centrifuge for 10 min at 350×*g*, 4 °C.
8. Remove the supernatants from the cell pellets, and gently resuspend the pellets in a small amount (≤1 ml) of cold $PBS^{-/-}$/EDTA (0.8 mM). If no aggregation of the cells is observable, two cell pellets can now be combined. After resuspending in the ≤1 ml of cold $PBS^{-/-}$/EDTA (0.8 mM), 10 ml cold $PBS^{-/-}$/EDTA (0.8 mM) is added to each pellet for two resuspended pellets to be combined in one tube; centrifuge for 10 min at 250×*g*, 4 °C (*see* **Note 1**).
9. Repeat step 8.
10. At this stage, after resuspending each pellet again, all the PBMCs are pooled into a single 15 ml tube, and the volume made up to a final volume of 10 ml with cold $PBS^{-/-}$/EDTA (0.8 mM). A small volume is taken aseptically to make a 1/100 dilution using Tuerk solution, for counting the cell number. While counting, the PBMCs are centrifuged for 10 min at 350×*g*, 4 °C.

2.3.2 Isolation of Porcine DCs and Monocytes

The procedure starts with steps 1–10 from Subheading 2.3.1.

1. Prepare MACS buffer ($PBS^{-/-}$/EDTA (2 mM)/BSA 0.1%) and keep on ice.
2. Dilute the anti-CD172a antibody (Ab) (can be purchased at 1 mg/ml; *see* Table 5) in MACS buffer to have a 1 µg/ml stock solution.
3. Resuspend the pellet from step 10 under Subheading 2.3.1 in ≤1 ml MACS buffer, then make up to 500 µl with MACS buffer; add the diluted anti-CD172a Ab to have 100 µL/10^8 PBMC; incubate for 20 min on ice.
4. Add 50 ml of MACS buffer to the cells/Ab mix, and centrifuge for 10 min at 350×*g*, 4 °C.
5. Prepare 20 µl goat anti-mouse IgG microbeads with 80 µl MACS buffer per 10^8 PBMC.
6. Resuspend the pellet of PBMCs with the anti-mouse IgG microbeads, using the appropriate volume for the number of cells counted; make up to 1000 µl with MACS buffer; incubate for 15 min on ice.

7. Add 50 ml of MACS buffer and centrifuge for 10 min at 350 × g, 4 °C.
8. During this centrifugation time, attach a Miltenyi Biotec LD column (Table 2) to the magnet of the MACs holder (Table 2), then equilibrate by adding 2 ml MACS buffer and letting flow through by gravity.
9. Resuspend the cell pellet in 2 ml MACS buffer; apply the cell suspension to the equilibrated LD column in volumes of 500 µl aliquots, allowing volume to enter the column before adding next.
10. Wash the column 3 times with 2 ml of MACS buffer per wash; remove the LD column from the MACS holder and place on top of a 15 ml tube.
11. Add 2 ml of MACS buffer into the LD column and flush out the cells with the provided plunger. Count this positive fraction which has been eluted from the column and thus contains the blood DCs + monocytes.

Table 2
List of equipment and materials

Equipment and materials	Manufacturer	Catalog number
LD Columns	Miltenyi Biotec	130-042-901
LS Columns	Miltenyi Biotec	130-042-401
MS Columns	Miltenyi Biotec	130-042-201
MACS MultiStand	Miltenyi Biotec	130-042-303
QuadroMACS Separator	Miltenyi Biotec	130-090-976
Leucosep™	Greiner Bio-One	227 290
Screw cap micro tubes	Sarstedt	72.692.005
GelDoc-It® TS Imaging System	UVP	TS 310
NanoDrop 2000c	Thermo Scientific	–
Lab-Tek® II Chamber Slide™ System	Nalge Nunc International	154534
FACS Canto™ II	BD Biosciences	–
Inverted Research Microscope Eclipse ti	Nikon	–
ECM 830 Square Wave Electroporation System	BTX Harvard Apparatus	–
PowerPac™ Basic Power Supply	Bio-Rad	–
Wide Mini-Sub Cell GT Cell	Bio-Rad	–

2.3.3 Culture of Monocytes to Differentiate Monocyte-Derived DCs

The procedure employs the steps 1–7 from Subheading 2.3.2. Then, the following, using a MACS LS column in place of the LD column used to isolate DCs.

1. During this centrifugation time, attach a Miltenyi Biotec LS column (Table 2) to the magnet of the MACs holder (Table 2), then equilibrate by adding 2 ml MACS buffer and letting flow through by gravity.
2. Resuspend the cell pellet in 2 ml MACS buffer; apply the cell suspension to the equilibrated LS column in volumes of 500 µl aliquots, allowing volume to enter the column before adding next.
3. Wash the column 3 times with 2 ml of MACS buffer per wash; remove the LS column from the MACS holder and place on top of a 15 ml tube.
4. Add 2 ml of MACS buffer into the LS column and flush out the cells with a provided plunger. Count this positive fraction which has been eluted from the column and thus contains the blood monocytes.
5. Centrifuge the cells at 300 × *g* for 10 min at 4 °C.
6. Resuspend the cell pellet in ≤1 ml phenol red-free Dulbecco's modified Eagle's Medium (DMEM), then add phenol red-free DMEM containing 10% serum, 150 ng/ml GM-CSF, and 100 U/ml IL-4 to have 50×10^6 cells in 30 ml; seed into a 150 cm^2 tissue culture flask, and incubate at 39 °C (for porcine cells, the serum must be porcine serum, preferably from specific pathogen-free (SPF)-animals).
7. At day 1 and 3, replace 5 ml of the medium with fresh DMEM containing 10% serum, 150 ng/ml GM-CSF and 100 U/ml IL-4.
8. At day 4, use a pipette to wash the medium in the flask over the cells on the flask surface—this will dislodge the DCs, but leave any macrophages attached; then harvest the cells by removing the medium and centrifuging for 5 min at 350 × *g*, RT; discard the media.
9. Resuspend the cell pellet in ≤1 ml $PBS^{-/-}$/EDTA (4 mM).
10. At the same time, remove the slightly more semi-adherent cells by shaking the flask with 10 ml cold $PBS^{-/-}$/EDTA (4 mM) for 5 min on ice.
11. Combine the cells from step 10 with the cell pellet from step 9, and centrifuge again for 5 min at 350 × *g*, RT; resuspend in ≤1 ml $PBS^{-/-}$/EDTA (4 mM), make up to 10 ml and count the cells.
12. Centrifuge again for 5 min at 350 × *g*, RT; resuspend in ≤1 ml DMEM containing 10% serum for use as monocyte-derived DCs (MoDCs).

For long term culture beyond 3 days, the additional presence of 150 ng/ml GM-CSF is beneficial.

2.3.4 Human Peripheral Blood Mononuclear Cell Preparation

Again, appropriate licenses, permissions to use the blood and trained and certified staff for taking human blood are required. Blood samples from donors are usually collected into 20–50 ml syringes containing either heparin (20 U/ml) or EDTA (1.5–2 mg/ml) as anticoagulant. Split the blood into 15 ml sterile centrifuge tubes (NOT polystyrene); centrifuge for 20 min at 1000×*g* at RT, no brake, to separate the buffy coat from the erythrocytes and plasma.

It is also possible to use preprepared buffy coats, which may be obtained from, for example, the local blood bank, with the appropriate licenses and permissions in place. Such sources of buffy coats often provide larger volumes and therefore larger quantities of cells for preparing the PBMCs.

This preparation of human PBMCs from buffy coats follows the same procedure as described under Subheading 2.3.1.

2.3.5 Isolation of Human DCs and Monocytes

The procedure starts with the preparation of the human PBMCs (Subheading 2.3.4). Thereafter, the procedure follows that for porcine DCs (Subheading 2.3.2) wherein step 2 using anti-CD172a antibody is replaced by the use of the MACS Blood Dendritic Cell Isolation Kit II (Miltenyi Biotec). This isolation kit allows the concurrent isolation of pDCs, cDC1s, and cDC2s. Firstly, the non-DCs are depleted with a cocktail of monoclonal biotin-conjugated antibodies against human CD1c (BDCA-1) (clone: AD5-8E7; isotype: mouse IgG2a), and MicroBeads conjugated to monoclonal antibodies against human CD14 (isotype: mouse IgG2a) and human CD19 (isotype: mouse IgG1). Monocytes and B cells are thus depleted prior to positive selection of plasmacytoid and myeloid dendritic cells by magnetic labeling for CD304 (BDCA-4), CD1c (BDCA-1), and CD141 (BDCA-3). Three markers are used for immunomagnetic labeling of all DC subsets: CD304 (BDCA-4/Neuropilin-1), CD1c (BDCA-1), and CD141 (BDCA-3). The incubations are performed at 2–8 °C.

1. Determine the cell concentration for the PBMCs prepared under Subheading 2.3.4.
2. Centrifuge the PBMC suspension at 300×*g* for 10 min.
3. Remove the supernatant and resuspend the cell pellet in 300 µl of MACs buffer per 10^8 total cells.
4. Add 100 µl of the FcR Blocking Reagent supplied in the Blood Dendritic Cell Isolation Kit II, and 100 µl of the Non-DC Depletion Cocktail supplied in the Blood Dendritic Cell Isolation Kit II per 10^8 total cells.
5. Mix well and incubate for 15 min at 2–8 °C.
6. Wash the cells by adding 5–10 ml of MACS buffer per 10^8 cells and centrifuge at 300×*g* for 10 min.

7. Remove the supernatant and resuspend the cell pellet in MACS buffer: 500 µl for up to 10^8 cells
8. Place a MACS LD Column into the magnetic holder of the MACS Separator.
9. Rinse the column with 2 ml MACS buffer.
10. Apply the cell suspension on to the column.
11. Collect the unlabeled cells that flow through the column and also in the washings coming through with two washes each of 1 ml MACS buffer.
12. Wash the column a further two times, and collect the total effluent together with that from step 11—this is the unlabeled, pre-enriched DC fraction.
13. Centrifuge the cell suspension derived from steps 11 and 12, at 300 × *g* for 10 min.
14. Remove the supernatant and resuspend the cell pellet in 400 µl MACS buffer.
15. Add 100 µl of the DC Enrichment Cocktail supplied in the Blood Dendritic Cell Isolation Kit II.
16. Mix well and incubate for 15 min at 2–8 °C.
17. Wash the cells by adding 5–10 ml of MACS buffer and centrifuge at 300 × *g* for 10 min.
18. Remove the supernatant and resuspend the cell pellet in MACS buffer: 500 µl for up to 10^8 cells.
19. Place an MS Column into the magnetic holder of the MACS Separator.
20. Rinse the column with 500 µl MACS buffer.
21. Apply the cell suspension on to the column.
22. Collect the unlabeled cells that flow through the column.
23. Wash column with 3 times 500 µl of MACS buffer.
24. Collect the unlabeled cells that flow through the column, and combine with the flow-through from step 22.
25. Remove column from the MACS Separator, and place it on a 15 ml collection tube.
26. Pipette 500 µl of buffer on to the column, and immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column; these are the DCs (*see* **Note 2**).

2.3.6 Culture of Monocytes to Differentiate Human Monocyte-Derived DCs

The procedure starts with isolation of human monocytes, which employs a similar procedure to that in Subheading 2.3.5, but using the CD14 MicroBeads Human kit (Miltenyi Biotec).

1. Once the monocytes are isolated, count and centrifuge the cells at 300 × *g* for 10 min at 4 °C.

2. Resuspend the cell pellet in ≤1 ml phenol red-free DMEM, then add phenol red-free DMEM containing 10% serum, 150 ng/ml GM-CSF, and 100 U/ml IL-4 to have 50×10^6 cells in 30 ml; seed into a 150 cm^2 tissue culture flask, and incubate at 39 °C (for human cells, pooled human serum from normal donors must be employed.)
3. At day 1 and 3, replace 5 ml of the medium with fresh DMEM containing 10% serum, 150 ng/ml GM-CSF and 100 U/ml IL-4.
4. At day 4, use a pipette to wash the medium in the flask over the cells on the flask surface—this will dislodge the DCs, but leave any macrophages attached; then harvest the cells by removing the medium and centrifuging for 5 min at 350 × *g*, RT; discard the media.
5. Resuspend the cell pellet in ≤1 ml $PBS^{-/-}$/EDTA (4 mM).
6. At the same time, remove the slightly more semi-adherent cells by shaking the flask with 10 ml cold $PBS^{-/-}$/EDTA (4 mM) for 5 min on ice.
7. Combine the cells from step 6 with the cell pellet from step 5, and centrifuge again for 5 min at 350 × *g*, RT; resuspend in ≤1 ml $PBS^{-/-}$/EDTA (4 mM), make up to 10 ml and count the cells.
8. Centrifuge again for 5 min at 350 × *g*, RT; resuspend in ≤1 ml DMEM containing 10% serum for use as monocyte-derived DCs (MoDCs).

For long term culture beyond 3 days, the additional presence of 150 ng/ml GM-CSF is beneficial.

2.4 Other Cells

As mentioned above, with the RepRNA being derived from a porcine virus, assessment in porcine cells provides a reliable reference. As a reproducible positive control, alongside analyses with dendritic cells and monocytes, the swine kidney epithelial cell line SK-6 (kindly provided by Prof. M. Pensaert, Faculty of Veterinary, Medicine, University of Gent, Belgium) is employed due to its efficiency in propagating virus and supporting replication of the RepRNA (Fig. 1). These SK-6 cells are particularly sensitive to CSFV [25].

For seeding in cell culture flasks, the cells are kept in Medium A (MEM Hank's—consisting of Eagle's Minimal Essential Medium (MEM) supplemented with Hank's salts, 2 mM l-glutamine, and 7% (v/v) pestivirus- and *Mycoplasma*-free horse serum) (*see* **Note 3**) to facilitate cell adherence to the plastic for 2–3 h at 37 °C. Once the cells have adhered to the plastic, Medium A is replaced with Medium B to facilitate cell growth (MEM Earle's, consisting of MEM supplemented with Earle's salts, 2 mM l-glutamine, and 7% (v/v) pestivirus- and *Mycoplasma*-free horse serum). For further details, *see* **Notes 4** and **5**.

The cell passage procedure for T150 flasks is as follows:

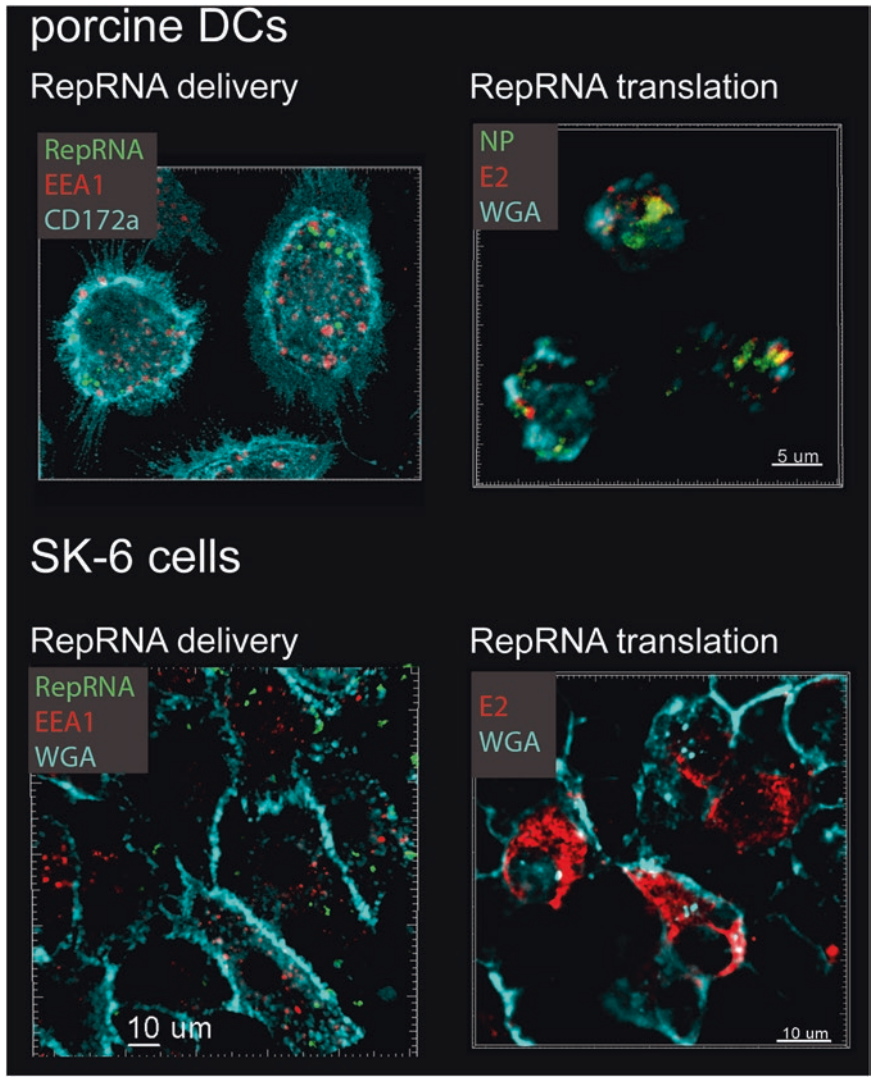

Fig. 1 RepRNA delivery to and translation in DCs and SK-6 cells. *Left panel*: Intracellular delivery of RepRNA to porcine DCs (*upper image*) and SK-6 cells (*lower image*). Cells were incubated for 2–3 h at 39 °C (DCs)/37 °C (SK-6) with 1 μg FITC-labeled RepRNA (*green*) complexed to lPEI (polyplexes). Samples were fixed (*p*-formaldehyde), permeabilized (saponin), and labeled with antibody against EEA-1 (*red*); cell surfaces were stained with antibody against CD172a (DCs) or WGA-Alexa$_{633}$ (SK-6) (*blue*). *Right panel*: translation of delivered RepRNA in porcine DCs and SK-6 cells. Cells were exposed 2 h to 1 μg RepRNA complexed to lPEI (polyplexes). After incubation for 48 h at 39 °C (DCs)/37 °C (SK-6), the cells were washed, fixed, and permeabilized as above, then labeled with antibody against the RepRNA encoded influenza virus NP antigen (*green*; DCs only) and E2 antigen (*red*); cell surfaces were stained with WGA-Alexa$_{633}$ (*blue*)

1. Remove medium from the SK-6 cell monolayer (*see* **Note 6**), rinse twice with 5 ml of fresh, pre-warmed (37 °C) trypsin–EDTA (0.05 %), then add 7 ml of this trypsin–EDTA solution per 150 cm^2 flask.

2. Distribute the trypsin solution evenly and allow the treated culture to sit at 37 °C until the cell monolayer is disrupted, typically for 5–10 min.
3. Tap the flask firmly to detach the cells and add 3 ml of Medium A (the horse serum will neutralize the trypsin). Pipette the cells up and down towards the bottom of the flask avoiding generation of foam, and transfer the cells to a sterile 50 ml tube.
4. Take a sample of cells for counting, and centrifuge the remainder at 250 × *g* for 10 min at RT to pellet the cells (this should be done as soon as possible after collecting the cells to remove the trypsin).
5. Resuspend the cell pellet in ≤1 ml of Medium A (serum-free), then make up to the required volume with Medium A containing serum to have the desired cell concentration—see next step.
6. Resuspend the cells gently and dispense the suspension into new flasks or plates. For routine passaging, a split ratio of 1:6 to 1:10 is most commonly employed. This corresponds to a seeding concentration of approximately $8–15 \times 10^6$ cells per 150 cm^2 flask, which allows for a passage frequency of once per week.
7. After 1.5–3 h at 37 °C, replace MEM Hank's (Medium A) with MEM Earle's (Medium B), and incubate the culture at 37 °C under 5% CO_2 until the required confluence is reached or until next cell passage (typically 7 days). Do NOT passage more frequently than once per week, because the cells will be forced to over-work and thus "burn out" before the 20 passages one normally employs (*see* **Notes 4** and **5**).
8. For seeding plates or multi-well slides (such as Lab-Tek® II Chamber), a seeding concentration of $0.5–1 \times 10^5$ cells per ml is employed (2 ml/well of 6-well plates, 1 ml/well of 24-well plates, or 200 µl/well of Lab-Tek® II Chamber), suing the same routine with Medium A and Medium B as in step 7. These cells can be employed from 24 to 72 h, dependent on the required level of confluence, but the total length of time in culture should not exceed 7 days. In order to prevent excessive growth for long-term cultures (4–7 days), the medium can be replaced with Medium B containing 1% (v/v) instead of 7% (v/v) horse serum.

3 Replicon Generation

3.1 Characteristics of the RepRNA

3.1.1 Overview of CSFV-Derived RepRNA

The RepRNA is derived from the genome of the non-cytopathogenic CSFV strain Alfort/187 (Fig. 2), which is nonpathogenic for humans and therefore a biosafe vaccine vector. The replicon is generated by deleting at least one structural gene from the viral genome [8, 11, 14]. A complementary DNA (cDNA) copy was

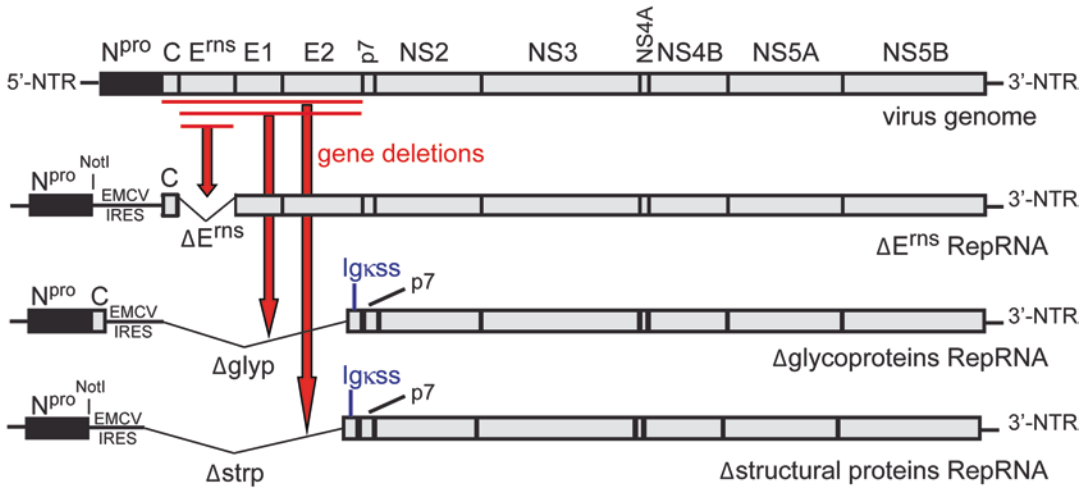

Fig. 2 Replicon derivations. Gene arrangement of the CSFV genome parent (virus genome) employed for generating the RepRNA constructs: E^{rns} gene deletion (ΔE^{rns}) for the ΔE^{rns} RepRNA; glycoprotein gene deletion (E^{rns}, E1, E2; Δglyp) for the Δglycoproteins or Δglyp RepRNA; structural protein gene deletion (C, E^{rns}, E1, E2; Δstrp) for the Δstructural proteins or Δstrp RepRNA. The 5′-NTR carries the ribosomal entry site to initiate translation. With the Δglyp and Δstrp RepRNA constructs, it is necessary to replace the C-terminal part of E2 coding for a transmembrane domain and a signal peptidase cleavage site between E2 and p7 by the signal sequence from the Igk gene (Igkss) to ensure that the polymerase complex (p7 to NS5B) is correctly associated with the ER

inserted in a low-copy number plasmid downstream of a bacteriophage T7 polymerase promoter [26]. Unique SrfI and SfiI restriction endonuclease sites were placed at the precise 3′-end of the viral genome cDNA sequence (Fig. 3a shows an example carrying an inserted luciferase gene) for linearization and run-off RNA transcription of the plasmids. Deletion or mutation of the N^{pro} gene can be applied for attenuating the regulation of type I interferon induction [14, 27, 28].

3.1.2 *Important Genetic Considerations for the RepRNA*

Partial or complete removal of replicon genes encoding viral structural proteins, while retaining genes encoding the polymerase complex results in self-replicating RepRNA incapable of producing progeny virus [10]. An efficacious replicon vaccine against CSFV was generated by removing the viral glycoprotein E^{rns} (ΔE^{rns} RepRNA) and packaging the ΔE^{rns} RepRNA in VRP using a complementary cell line [8, 11] (Fig. 3b shows a schematic of the viral genome and the linearized and transcribed version of the replicon from Fig. 3a; the luciferase gene insertion is also referred to by a more generic term "GOI" representing inserted "gene of interest"). Despite the lack of the structural glycoprotein E^{rns}, the RepRNA could still translate and replicate, but was unable to produce infectious progeny. The non-translated region at the 5′ end of the RNA (5′-NTR) retained the viral ribosomal entry site to initiate translation of the N^{pro} gene and GOI; insertion of the GOI was provided

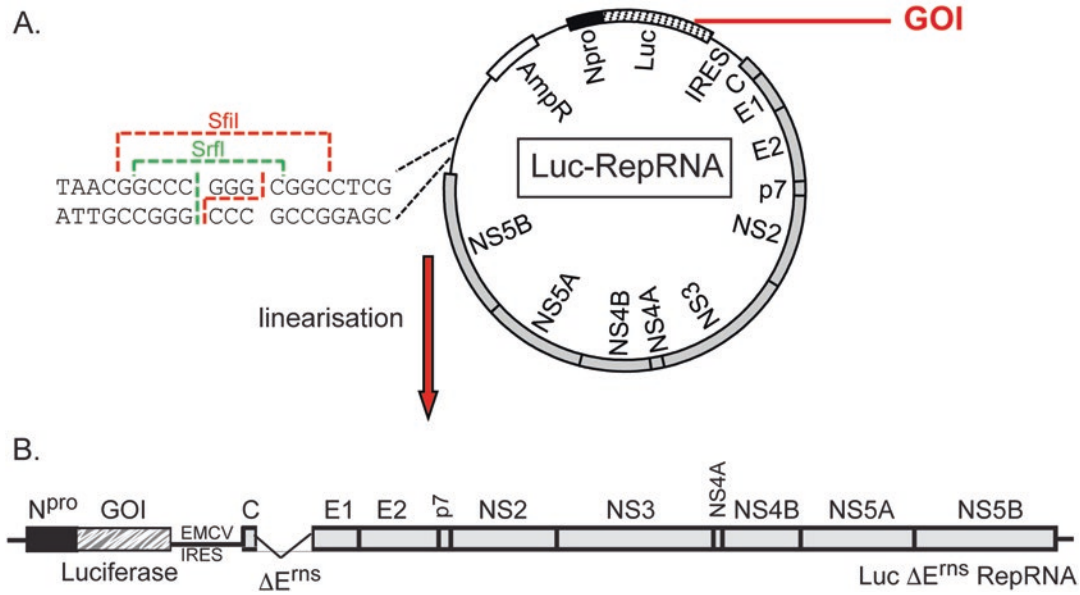

Fig. 3 Baseline replicon constructs. (**a**) The constructs carrying the influenza virus genes are derived from a plasmid carrying the RepRNA encoding luciferase (Luc); Luc-RepRNA. The luciferase gene is inserted via a NotI restriction endonuclease site at the 3′ end of the N^{pro} gene (*see* also Fig. 2), upstream of an internal ribosomal entry site (IRES) from EMC virus (EMCV) which permits reinitiation of the translation for the downstream genes, including those encoding the polymerase complex (p7 to NS5B). Prior to in vitro transcription of RNA, the plasmid is linearized at the 3′ end of the RepRNA cDNA sequence with the SrfI restriction endonuclease. Alternatively, a SfiI restriction site can be employed in case SrfI is present in the gene of interest (GOI) for instance. These run-off sites used to linearize the DNA plasmid to produce RepRNA RNA with precise 3′ ends are expanded on the left, showing the sequences and cleavage sites of SrfI (preferred) and SfiI. (**b**) Representation of the linearized sequence of the ΔE^{rns} RepRNA carrying the luciferase gene in the position of the GOI. This is the template RepRNA sequence for generation of RepRNA encoding other GOI, whereby the luciferase gene if replaced by the GOI

by further modifying the constructs to carry a unique NotI restriction endonuclease site downstream of the N^{pro} autoprotease gene. An encephalomyocarditis virus internal ribosomal entry site (EMCV IRES) was engineered downstream of the GOI insertion site for initiating translation of the second open reading frame, resulting in a bicistronic RepRNA genome. This permitted translation of the downstream genes including those encoding the polymerase complex (p7 to NS5). EMCV IRES, thus providing the means for replicon replication. Overall, by such means, the RepRNA possesses high potential for increasing antigen load within DCs [5, 11–13].

3.1.3 Constructing RepRNA for Influenza Vaccine Delivery

As shown in Fig. 3b, the first cistron of the replicon contains the N^{pro} gene fused in frame to the GOI terminated with a stop codon, and the second cistron mediates translation of the remaining viral polyprotein (C to NS5B) [11]. We have employed this construct for inserting as GOI that encoding influenza virus hemagglutinin [HA, H5N1/Yamaguchi/2004 and H1N1/California/2009],

neuraminidase [NA, H1N1/California/2009], or nucleoprotein [NP, H5N1/Yamaguchi/2004] (Fig. 4) [12–14]. This employs a replacement of the luciferase gene in the Luc-RepRNA with genes encoding the above influenza virus antigens (Fig. 4). An additional exercise was necessary to improve expression of the glycoprotein HA-encoding GOI through optimized translocation into the endoplasmic reticulum (ER). The HA-glycoprotein GOI was placed downstream of a codon-optimized C protein gene following N^{pro}, relating to the natural position of the first glycoprotein gene (the E^{rns} gene that was deleted) of the RepRNA sequence [14]. With this approach, the C-terminal part of the C protein representing the ER translocation signal of the deleted E^{rns} is exploited to drive ER translocation of the HA-glycoprotein GOI (*see* Fig. 4). In addition, codon modification and optimization of the C gene avoids homologous recombination with the C gene duplicate downstream of the EMCV IRES.

3.2 In Vitro Transcription of RepRNA from Plasmid DNA

The plasmid DNA construct shown in Fig. 3a is linearized with the SrfI endonuclease [11, 14] or SfiI endonuclease (Englezou, Démoulins, et al., in preparation). RepRNA is prepared from this cDNA template by run-off in vitro transcription using the MEGAscript T7 kit (Ambion) [11, 14]. The insertion of the alternative SfiI restriction site overlapping with the SrfI restriction site ensures a choice of enzymes for the linearization, which increases the applicability of the construct and its availability in the face of commercial changes with respect to availability of the endonucleases. By this method, in vitro transcription yields typically 10–25 μg RepRNA per μg plasmid template.

3.2.1 Preparation and Linearization of the Plasmid DNA

1. Bacterial *E. coli* XL-1 blue glycerol stocks from each RepRNA construct are stored at −80 °C. Alternatively, purified plasmid can be used to transform competent *E. coli* XL-1 blue cells. Typically, bacterial colonies are recovered from the frozen glycerol stock by streaking on LB agar supplemented with 50 μg/ml ampicillin, together with incubation at 37 °C in a humidified incubator overnight. Bacteria from a single colony are then amplified in 200 ml LB supplemented with 50 μg/ml ampicillin, inoculated with 200 μl of a log-phase LB preculture, by shaking at 37 °C overnight.
2. The plasmid DNA is extracted and purified with the NucleoBond® Xtra Midi Plasmid DNA purification kit (*see* Table 1), according to the standard procedure of the manufacturer's protocol for low copy number plasmids (double volumes of cell resuspension, lysis, and neutralization solutions).
3. Determine the concentration and purity of the plasmid DNA by photometry (NanoDrop 2000c, Table 2; for DNA, the A_{260}/A_{280} ratio should be between 1.8 and 2), and adjust the DNA concentration to 1 μg/μl.

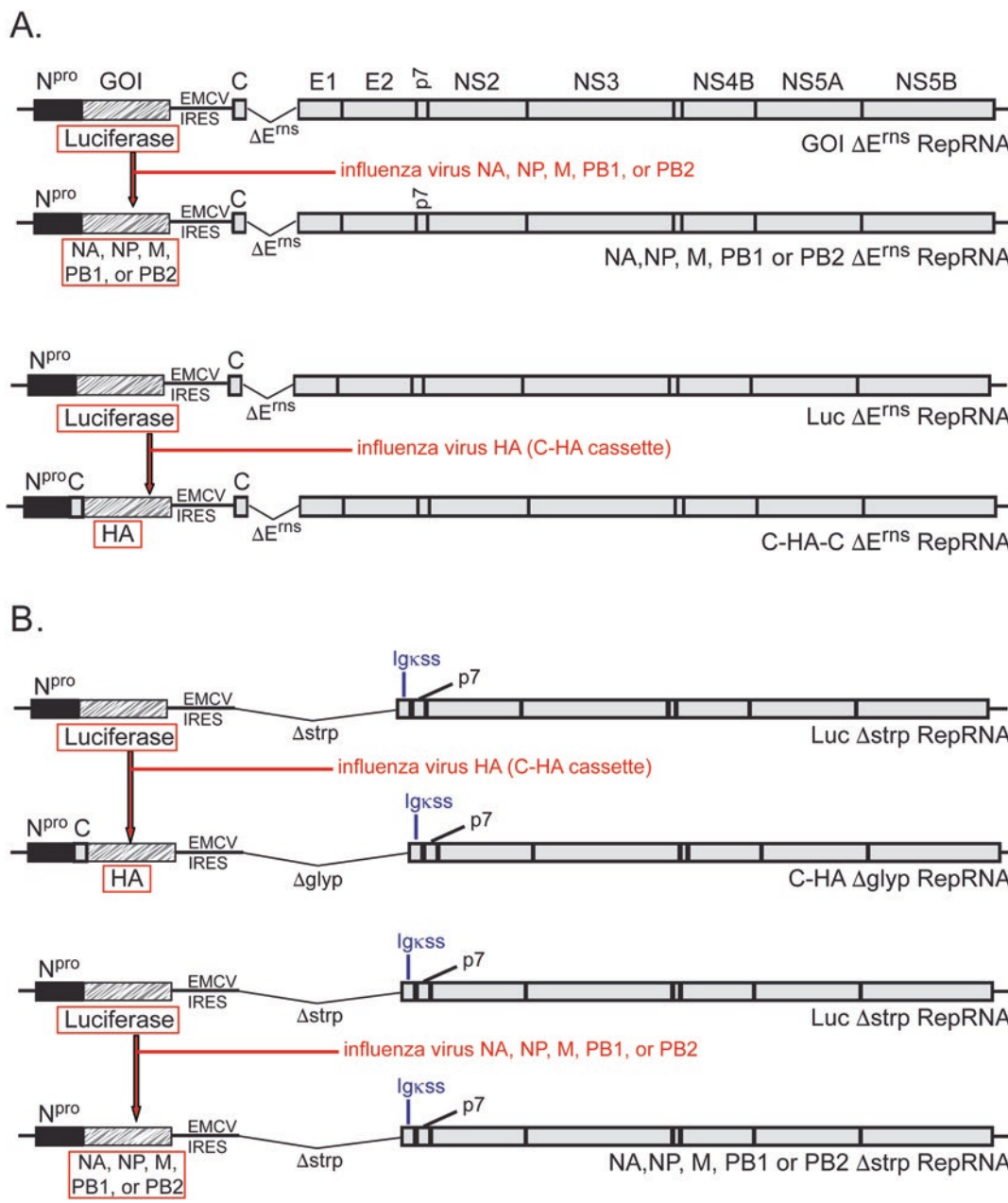

Fig. 4 Replicon constructs encoding influenza virus antigens. The luciferase gene of the construct shown in Fig. 3 is replaced by the gene of interest (GOI), which in these examples encode influenza virus antigens. The derivations shown in Fig. 2 are employed to generate ΔE^{rns} RepRNAs, Δglyp RepRNAs, or Δstrp RepRNAs. (**a**). For ΔE^{rns} RepRNAs, the luciferase gene is replaced by the GOI encoding influenza virus NA, NP, M, PB1, or PB2 at the 3′ end of the N^{pro} gene (NA, NP, M, PB1, or PB2 ΔE^{rns} RepRNA). When the GOI is a glycoprotein gene such as influenza virus HA for instance, this has to be placed downstream of the gene encoding the RepRNA C protein, which is at the 3′ end of the N^{pro} gene (C-HA-C ΔE^{rns} RepRNA). This ensures the correct ER translocation of the glycoprotein (HA). (**b**) For Δglyp RepRNA the glycoptrotein GOI is placed downstream of N^{pro}-C, and the CSFV glycoprotein genes are deleted; the replicon is referred to as a Δglyp RepRNA (C-HA Δglyp RepRNA). For Δstrp RepRNAs lacking all the CSFV structural proteins (C and all glycoprotein genes), the luciferase gene is replaced by the GOI encoding influenza virus NA, NP, M, PB1, or PB2 at the 3′ end of the N^{pro} gene (NA, NP, M, PB1, or PB2 Δstrp RepRNA)

4. The plasmid DNA is then linearized at the run-off site (*see* **Note 7**). For one reaction by way of example, *see* Table 3. Incubate at least 2 h at 37 °C; after 1.5 h, check 1 µl (100 ng) of the reaction mix by gel electrophoresis. Once linearization is complete, proceed to Subheading 3.2.2.

3.2.2 Phenol Extraction of Linearized DNA

1. Add 50 µl of H_2O to the 50 µl linearization reaction and then 100 µl of phenol–chloroform–isoamyl alcohol 25:24:1 (P:C:I); shake by hand for ~20 s and centrifuge for 2 min at 12–14,000 × *g* at RT (or longer if a lower *g* force is used).
2. Transfer the aqueous phase into a new 1.5 ml Screw Cap Micro Tube (*see* **Note 8**) containing 100 µl of P:C:I; shake by hand for ~20 s and centrifuge again for 2 min at 12–14,000 × *g* at RT (or longer if a lower *g* force is used).
3. Transfer the aqueous phase into a new 1.5 ml Screw Cap Micro Tube containing 100 µl of chloroform–isoamyl alcohol 25:1 (C:I); shake by hand for ~20 s and centrifuge for 2 min at 12–14,000 × *g* at RT (or longer if lower *g* force is used).
4. Transfer the aqueous phase (be careful not to transfer any chloroform) into a new 1.5 ml Screw Cap Micro Tube and add 10 µl (1/10 of the volume) of 3 M RNase-free NaOAc pH 5.2, 2.5 volumes of 100% RNase-free EtOH (250 µl); hold for 15–30 min on ice or at −20 °C, then centrifuge for 10 min at 14,000 × *g*, 4 °C.
5. Carefully discard the supernatant, then wash the pellet with 1 ml of 75% RNase-free EtOH; shake by hand for ~20 s and centrifuge for 10 min at 14,000 × *g*, 4 °C.
6. Carefully discard most of the supernatant (approximately 1 ml) avoiding the pellet—perform a short additional centrifuge spin (1 min at 14,000 × *g*, 4 °C) keeping the orientation of the tube as in the first centrifugation step, and remove the remaining ethanol. Allow the pellet to dry for ~10 min at 37 °C with the lid removed (do not dry excessively!).

Table 3
SrfI restriction digestion for one reaction

Components	Volume
NEB 10× CutSmart® Buffer	5 µl
SrfI (NEB) (20,000 U/ml)	1 µl
Plasmid DNA	5 µg (typically 5 µl)
RNase-free H_2O	to 50 µl final

7. When the traces of 75% RNase-free EtOH have evaporated, resuspend the pellet with 10 µl of RNase-free H_2O; let sit for at least 10 min at RT or 37 °C and then vortex and centrifuge briefly. Keep 0.5 µl (250 ng) for comparison to nonlinear plasmid by gel electrophoresis.

3.2.3 In Vitro Transcription

1. Using the MEGAscript® T7 Transcription Kit, set up the following mix per reaction, as shown in Table 4. (It is preferable to pool RNA from several reactions rather than to upscale the reaction volume).
2. Incubate for 2–3 h at 37 °C (longer incubation results in a higher proportion of degraded RNA transcripts).
3. Add 1 µl of turbo DNase provided by the MEGAscript® T7 Transcription Kit and incubate for 15 min at 37 °C (*see* **Note 9**).

3.2.4 RNA Cleaning Step

1. Vortex a MicroSpin S-400 HR gel filtration column with the cap in place, to resuspend the resin; loosen cap and remove the plug; place the column in a collection tube, centrifuge for 1 min at 735 × *g* at RT (time and 735 × *g* are critical for optimal packing of the gel) (*see* also **Note 10**).
2. Place the column in a labeled RNase-free Eppendorf tube; add 40 µl of RNase-free H_2O to the 21 µl of RepRNA sample (in vitro transcription mix + 1 µl of turbo DNase) and load the 61 µl on to the middle of the gel; avoid touching the gel with the pipette tip.
3. Centrifuge for 2 min at 735 × *g* at RT (again, time and 735 × *g* are critical for optimal purification), then transfer aliquots of 3 µl into new 1.5 ml Screw Cap Micro Tubes (samples must then be stored at −80 °C); keep 1 µl of RepRNA for assessment with gel electrophoresis.

3.2.5 Evaluation of the Physical Properties of the RepRNA

1. Determine the RepRNA concentration and assess its purity using NanoDrop measurements (*see* **Note 11**).

Table 4
In vitro transcription mix for one reaction

Components	Volume
Linearized/phenol extracted DNA	4–5 µg (typically 4–5 µl)
NTP mix	8 µl
Buffer 10×	2 µl
T7 polymerase enzyme mix	2 µl
RNase-free H_2O	to 20 µl final

2. Run a 1% (w/v) agarose gel with the in vitro transcripts (RepRNA) to assess the quality of the RNA; gel electrophoresis should be performed first for no more than 10–15 min at 130 V until the first picture is taken, and may then be extended to 30 min (*see* Fig. 5a and **Note 12**). Electrophoresis tank, TBE buffer, casting plates, and any other material used for electrophoresis are kept as close as possible to RNase-free conditions.

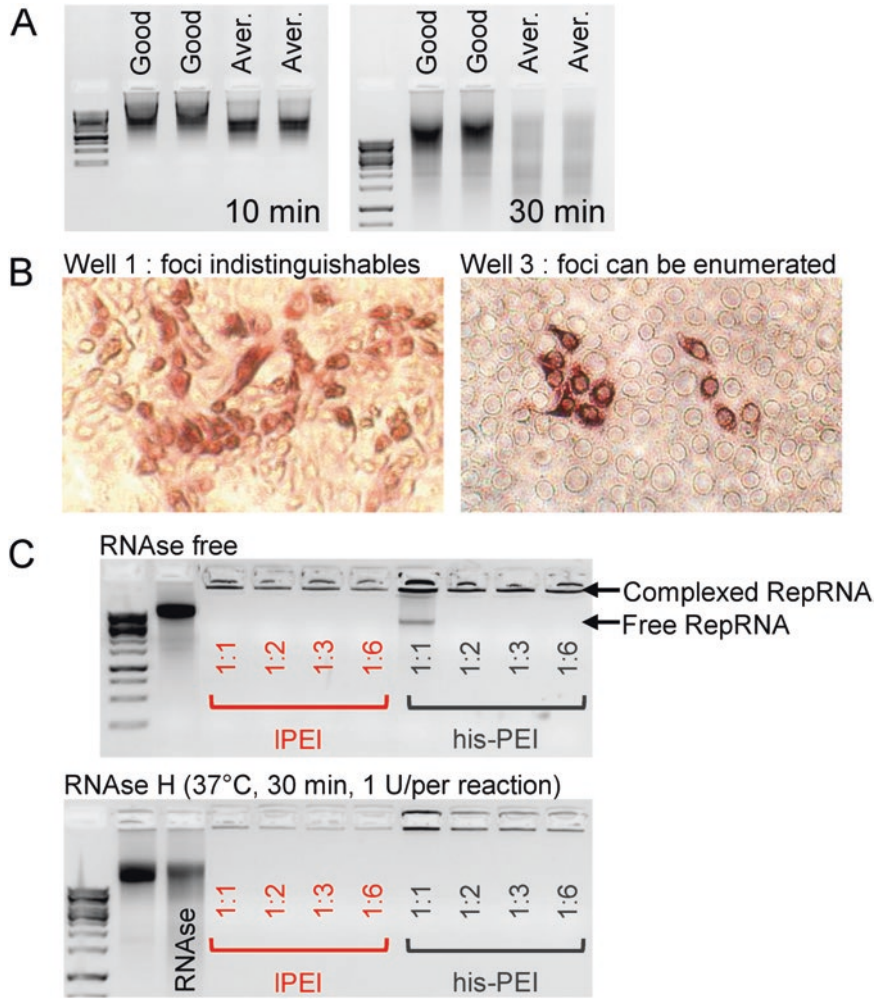

Fig. 5 (**a**) Evaluation of the physical properties of the in vitro transcripts of RepRNA on a 1 % (w/v) agarose gel after 10 min (*left panel*) and 30 min (*right panel*) of migration at 130 V. Two RepRNA productions were of good quality (Good) and displayed a band profile after both 10 and 30 min of electrophoresis. In contrast, two RepRNA productions were of an average quality (Aver.) and were not detectable anymore after 30 min, suggesting that the corresponding RepRNA had been degraded. (**b**) Immune-peroxidase staining (E2) of SK-6 cells electroporated with RepRNA and analyzed in the infectious center assay; typically, foci of 2, 4, or 8 cells can be observed, originating from 1 to 3 cycles of SK-6 cell mitosis. (**c**) Gel retardation assay and RNAse protection. When treated with RNAse H, naked RepRNA displayed a characteristic smear of degradation, whereas the complexed RNA protected from the RNase remained associated with the delivery vehicles at the top of the gels

3.3 Functional Assay of the Freshly Produced RepRNA

Good physical properties of RepRNA cannot solely guarantee its functional activity. Accordingly, the so-called "*RepRNA infection center assay (ICA)*" is routinely employed to quantify the specific infectivity of the transcripts [29]. The assay employs medium instead of the semisolid overlay traditionally used with ICA for virus-infected cells, because the RepRNA cannot produce any progeny virus and will not spread to cells other than those initially transfected.

3.3.1 Electroporation of RepRNA

1. Split confluent SK-6 cells 1:4 into 150 cm^2 flasks typically 48 h prior to electroporation (one 150 cm^2 flask yields cells for 2–3 electroporation events); seed cells in complete medium A and replace with medium B after 2–3 h (*see* Subheading 2.4).
2. Once the SK-6 cell monolayer is confluent, remove the medium, rinse twice with 5 ml of fresh, pre-warmed (37 °C) trypsin–EDTA (0.05%), then add 7 ml of this trypsin–EDTA solution and allow cells to detach for 5–10 min at 37 °C.
3. Dislodge the cells by tapping the flask firmly, and resuspend the cells by adding an additional 3 ml of complete medium A (which inactivates the trypsin). Pipette the cells up and down towards the bottom of the flask avoiding generation of foam, and transfer into a 50 ml sterile centrifuge tube; pellet the cells by centrifugation for 5 min at 250 × *g*; take a sample and count the cells.
4. Use one 6-well plate per electroporation. Add complete medium A alone in *well 1* and seed 7×10^5 SK-6 cells into each well of *wells 2–6* (2 ml per well, 3.5×10^5 cells per ml of medium A); let the cells adhere 2–3 h at 37 °C before electroporation.
5. In the meantime, resuspend the remaining cells with 50 ml ice-cold RNase-free PBS with calcium/magnesium (1×) ($PBS^{+/+}$); collect the cells by centrifugation for 5 min at 250 × *g*; repeat this step twice (for a total of 3 wash steps).
6. Thaw 1 μg RepRNA and add to 10 μl RNase-free H_2O in a 1.5 ml Screw Cap Micro Tube. Keep on ice.
7. Resuspend the cells in ice-cold RNase-free $PBS^{+/+}$ and adjust the cell concentration to 2×10^7 cells/ml (keep cells on ice at all times).
8. Precool the electroporation cuvettes (2 mm gap, long electrodes) on ice; prepare the ECM 830 Square Wave Electroporation System as follows: Volts set to 980 V, 2 pulses of 100 μs, interval of 1 s between the two pulses (if an alternative electroporation device is used, or other cells, the electroporation conditions must be optimized beforehand).
9. Add 400 μl cells (8×10^6 cells) to the tube containing the 1 μg RepRNA (in 10 μl), mix by pipetting up and down 5–6 times, and transfer immediately into the precooled electroporation cuvette.

10. Electroporate immediately and allow the cells to recover for 5–10 min at RT. The time between mixing the cells with RNA and electroporation must be kept to a minimum.
11. For each electroporation, prepare an empty sterile 5 ml tube (tube 1 for well 1) and 5 tubes (tubes 2–6 for wells 2–6) containing 900 µl dilution buffer ($PBS^{+/+}$ + 1% heat-inactivated horse serum).
12. Resuspend the electroporated cells with 600 µl dilution buffer and transfer into tube 1; perform a tenfold serial dilution by pipetting 100 µl cells into the prepared tubes 2–6 (change pipette tip every time and mix well between each dilution).
13. Transfer 100 µl from each dilution 1–6 into the corresponding wells containing 2 ml of complete medium (well 1 should not have pre-seeded cells); distribute cells evenly (no rotational motion, no tapping!) and allow them to adhere for 4–6 h in the 37 °C incubator.
14. Replace complete medium A by fresh and pre-warmed 2 ml/well of complete medium B; 48–72 h later, proceed to Subheading 3.3.2.

3.3.2 Quantifying the Specific Infectivity of RepRNA by Immunoperoxidase Staining

1. Carefully aspirate the supernatant from the wells and wash twice with $PBS^{+/+}$.
2. Fix and permeabilize the cells with 80% EtOH (stored at −20 °C) for 15 min on ice; wash twice with $PBS^{+/+}$.
3. Incubate the fixed cells with the primary antibody (anti-E2 or anti-NS3—Table 5) diluted in $PBS^{+/+}$ for 30 min at RT; wash twice with $PBS^{+/+}$.
4. Incubate the cells for 30 min at RT with the secondary antibody (anti-mouse Ig/HRP) diluted in $PBS^{+/+}$; wash twice with $PBS^{+/+}$.
5. Prepare the substrate solution (this solution is carcinogenic; wear appropriate protective device, and discard properly). For two plates, mix:
   (a) 12 ml of 50 mM NaOAc pH 5
   (b) 0.5 ml of H_2O_2 (1%)
   (c) 0.5 ml of 4 mg/ml 3-amino-9-ethyl-carbazole–N,N-dimethyl-formamide (AEC), (add directly before use).
   Add 1 ml/well of substrate and incubate for 10–30 min at RT
6. Stop the reaction by removing the substrate (discard properly) when the signal-to-noise ratio is optimal (10–30 min, follow under the microscope; do not stain too long to avoid excessive background; *see* Fig. 5b for an example of how the foci of RepRNA translation/replication appear). Wash twice with $PBS^{+/+}$.
7. Add 1 ml per well of $PBS^{+/+}$; store at 4 °C; the staining is stable for a few weeks.

Table 5

List of antibodies

Antibodies Employed	Dilution	Host	Clone	Application	Reference	Source
Anti-E2	1:15–1:40, varies with batches	Mouse, IgG2b	HC/TC 26	FC, ICA RepRNA translation	–	Dr. Greiser-Wilke, Hannover, Germany
Anti-NS3	1:80	Mouse, IgG1	C16	FC, ICA RepRNA translation	–	Dr. Greiser-Wilke, Hannover, Germany
Anti-Mouse Ig/HRP (1.3 mg/ml)	1:200	Rabbit,	Polyclonal	ICA	P0260	Dako
Anti-CD172a (1 mg/ml)	1:1000	Mouse, IgG1	74-22-15 (PG2049)	FC, CM, ICA	–	Washington State University Monoclonal Antibody Center
Anti-CD172a (1 mg/ml)	1:1000	Mouse, IgG2b	74-22-15A (PG2031)	FC, CM, ICA	–	Washington State University Monoclonal Antibody Center
Wheat Germ Agglutinin, Alexa Fluor® 633 (1 mg/ml)	1:200	–	–	CM	W21404	Molecular Probes/Invitrogen
Anti-HA (H5N1/Yamaguchi)	from a hybridoma		Fus. 82, mAK AIV H5 82_3C1_1G10_3E11	FC, CM, ICA RepRNA translation		
Anti-HA (H1N1/California) (1 mg/ml)	1:200	Mouse, IgG1	clone 26-D11	FC, CM, ICA RepRNA translation	IT-003-001 M4	Immuno-Tech
Anti-NP (H5N1/Yamaguchi)	Hybridoma: B lymphocyte	Mouse, IgG2a	HB-65™	FC, CM, ICA RepRNA translation	H16-L10-4R5	ATCC®

APC Annexin V Apoptosis Detection Kit with 7-AAD	1:40 1:40			FC Cell viability	640930	BioLegend, Lucerna, Switzerland
Goat anti-mouse IgG microbeads		Goat		Cell sorting	130-048-401	Miltenyi Biotec
Anti-EEA1 (0.25 mg/ml)	1:200	Mouse, IgG1	14/EEA1	CM RepRNA internalization	610456	BD Transduction Laboratories™
Anti-Clathrin (0.25 mg/ml)	1:50	Mouse, IgG1	23/Clathrin HC	CM RepRNA internalization	610499	BD Transduction Laboratories™
Anti-Caveolin-1 (0.25 mg/ml)	1:200	Mouse, IgG2b	7C8	CM RepRNA internalization	MA3-600	Thermo Fisher Scientific
Anti-CD9 (1 mg/ml)	1:200	Mouse, IgG2b	MM2/57	CM RepRNA internalization	AHS0902	Thermo Fisher Scientific
Anti-Calnexin (1 mg/ml)	1:100	Rabbit, IgG	pAB; SPA-860	CM RepRNA internalization	ADI-SPA-860-D	Enzo

FC flow cytometry, *CM* confocal microscopy, *ICA* infectious center assay

Analyze cells for positive staining with microscopy enumerating the number of small foci per well. Calculate the specific infectivity of the RepRNA (infectious units [IU]/μg RNA) using the formula of Lorenz and Bogel [30].

An example is provided in Fig. 5b. The number of foci was too large and overlapping to be enumerated in well 1 and 2 (foci indistinguishable); in well 6, there were no detectable foci. Accordingly, the number of foci and titre were calculated from wells 3–5 as follows:

Well 3: 10^{-3} μg RepRNA = 69 foci

Well 4: 10^{-4} μg RepRNA = 9 foci

Well 5: 10^{-5} μg RepRNA = 1 focus

$\rightarrow N = 69 + 9 + 1 = 79$

$\text{Titre} = 79/111 \times 10^5 = 0.71 \times 10^5 = 7 \times 10^4$ IU/μg RNA

3.4 RepRNA Association with Synthetic Delivery Vehicles

RepRNA are large molecules (12–15 kb) with a high RNase-sensitivity that are poorly internalized by DCs. Efficient internalization by DCs leading to cytosolic release of the RepRNA for translation and replication are absolute requirements for an efficient vaccine. This has been achieved by i) RepRNA condensation with PEI; ii) RepRNA encapsulation into chitosan nanoparticles; iii) RepRNA condensation with cationic lipids. The list of the various compounds used for the different formulations is provided in Table 6.

3.4.1 PEI-Based Polyplexes

Polyplex Formulation

Perform all steps under an RNase-free laminar flow hood using RNase*Zap*®. Solutions, pipettes, tips, tubes and any other consumables used for the formulation must be RNase-free. The following protocol is given for 1 μg RepRNA; volumes have to be increased accordingly for higher RepRNA amounts. This protocol can also be employed for different RepRNA/polymer weight ratios, which may be required for different sources, different forms and different molecular weights of the polymer; examples are given below for 1:1, 1:2, 1:3, and 1:6 ratios.

1. Dilute 1 μg RepRNA in 14 μl 10 mM RNase-free HEPES buffer pH 7.4 ("RepRNA solution").
2. Proceed to a twofold dilution of the linear PEI (lPEI) and/or his-PEI in 10 mM RNase-free HEPES buffer pH 7.4 ("PEI solution") (calculate the volume according to the number of samples and the RepRNA/polymer weight ratios) (*see* **Note 13**).
3. Add dropwise 2, 4, 6, or 12 μl of PEI solution (for 1:1, 1:2, 1:3, or 1:6 ratios, respectively) to the 14 μl RepRNA solution while vortexing, and incubate for 30 min, RT.
4. Volumes are adjusted with serum-free Opti-MEM® (200–500 μl for FACS; 200 μl for confocal microscopy).

Table 6
List of the compounds used for the various formulations

Compound	Stock concentration	Source
Polyethylenimine-based polyplex		
lPEI (MW 22 kDa)	1 mg/ml, store at −20 °C	Pr. P. Guégan, Ivry-sur-Seine, France
his-PEI (PEI modified with 16% histidine residues *per* molecule; MW = 34,5 kDa)	1 mg/ml, store at −20 °C	Polytheragene, Evry, France [31]
OptiMEM I + Glutamax		Gibco—Life Technologies, 51985
RNase-free HEPES solution	10 mM pH7.4, store at 4 °C	Sigma-Aldrich, H3537-100ML
Chitosan nanoparticles		
Low viscosity chitosan from crustacean cell with deacetylation degree of approximately 95% (MW 100 kDa)	1% (w/v) in RNase-free H2O, store at −20 °C	Primex, Siglufjordur, Iceland
Sodium triphosphate pentabasic (TPP), purum p.a., ≥98.0% (T)	Powder store at RT	Sigma-Aldrich, 72061
Sodium alginate	Powder store at RT	Medipol SA, Lausanne, Switzerland (raw material: Keltone LVCR, ISP, San Diego, CA)
Lipofectamine® 2000 Reagent	Store at 4 °C	11668-019
Lipoplexes with cationic lipids		
NL10	1 mM, stored at 4 °C	OZ Biosciences, Marseille, France
NL21	1 mM, stored at 4 °C	OZ Biosciences, Marseille, France
NL42	1 mM, stored at 4 °C	OZ Biosciences, Marseille, France
DreamFect™	1 mM, stored at −20 °C	OZ Biosciences, Marseille, France DF40500
Lullaby	1 mM, stored at 4 °C	OZ Biosciences, Marseille, France LL70500
Dogtor	1 mM, stored at −20 °C	OZ Biosciences, Marseille, France
EcoTransfect	1 mM, stored at 4 °C	OZ Biosciences, Marseille, France ET10500
NL124	1 mM, stored at 4 °C	OZ Biosciences, Marseille, France
Dog-CNE	1 mM, stored at 4 °C	OZ Biosciences, Marseille, France
NL10-CNE	1 mM, stored at 4 °C	OZ Biosciences, Marseille, France

5. Size, polydispersity index (PDI) and ζ-potential can be measured in 10 mM RNase-free HEPES buffer pH 7.4 and serum-free Opti-MEM®, employing Zetasizer Nano ZS (Malvern Instruments, UK) or qNano (Izon, UK) [12].

RepRNA Protection from RNases Offered by Polyplexes

Protecting RepRNA from degradation by RNAse is an essential function that any delivery system has to achieve. Interaction of RepRNA with the delivery vehicles and RNAse protection are investigated by electrophoretic gel mobility shift assay.

1. The PEI/RepRNA polyplexes are prepared and loaded on to a 1% (w/v) agarose gel containing ethidium bromide, and electrophoresis run with Tris-acetate buffer at 130 V for 30 min. RepRNA retardation is visualized and photographed by a GelDoc-It® TS Imaging System.
2. For the RNA gel retardation assay, 1 U of RNAse H is added to the preformed polyplexes and incubated for 30 min at 37 °C, and then loaded as above. RepRNA retardation is visualized and photographed by a GelDoc-It® TS Imaging System, as shown in Fig. 5c.

Polyplex Internalization by Porcine and Human Cells

Visualization of RepRNA interaction with DCs, endocytic trafficking and cytosolic release requires labeled RepRNA. This employs fluorescein or rhodamine Mirus labeling kits at 1:2 reagent/RepRNA weight ratio, or RepRNA labeled by incorporating Dy490-UTP during synthesis [12, 13].

1. Lab-Tek® Chamber Slides II are coated with 200 µl per well of human fibronectin diluted in $PBS^{-/-}$ at 15 µg/ml, allowing the fibronectin to adhere for 1 h, at RT.
2. Lab-Tek® chambers are washed with H_2O for three consecutive cycles to remove excess fibronectin before 200 µl of cell suspension—200,000 blood DCs (bDCs) or monocyte-derived DCs (MoDCs) diluted in DMEM/10% serum/IL4/GM-CSF (*see* Subheading 2)—is dispensed per well; cells are adhered at least overnight.
3. The Lab-Tek® Chambers are centrifuged for 10 min at 350 × *g*, at 4 °C, to collect ensure no loss of any loosely adherent cells.
4. The Lab-Tek® Chambers receive three consecutive wash cycles with DMEM to remove residual serum that could interfere with endocytic processes.
5. DCs are prechilled 30 min on ice, then exposed to the polyplexes for 30 min at 37/39 °C (from Subheading "Polyplex Formulation", 1 µg of complexed RepRNA in 200 µl of Opti-MEM® per well); then the temperature is switched to 37/39 °C for 1 h.
6. Wash with $PBS^{+/+}$, and then proceed to the classical two-step intracellular staining, as described previously [12, 13, 32]. The various antibodies that can be used for confocal microscopy are listed in Table 5.

RepRNA Translation/ Replication Offered by Polyplexes in Transfected Cells

We found that the above labeling of RepRNA (Subheading "Polyplex Internalization by Porcine and Human Cells") abrogates its ability to translate and replicate (data not shown). Therefore studies on the translation of RepRNA after delivery employ unlabeled RepRNA.

The procedure starts with **steps 1–4** from Subheading "Polyplex Internalization by Porcine and Human Cells".

1. Cells are exposed to polyplexes for 2 h at 37/39 °C; wash twice with DMEM/10% serum/IL-4/GM-CSF (*see* Subheading 2); culture the cells for 48–72 h in DMEM/10% serum/IL-4/GM-CSF.
2. Wash with $PBS^{+/+}$, and then proceed to the classical two-step intracellular staining, as described previously [12, 13, 32]. The various antibodies that can be used for confocal microscopy (CM) are listed in Table 5.

Polyplex Formulation to Be Injected in Mice

The immunization trials in mice were approved by the Animal Welfare Committee of the Canton of Berne under license number BE72/12, and conducted in compliance with the Swiss animal protection law. Balb/c mice were vaccinated subcutaneously at days 0, 28, and 56, as described previously [12, 13]. Each vaccine dose contains 0.4 μg of RepRNA-HA and 0.4 μg of RepRNA-NP. The protocol below is given for six mice (volumes correspond to seven mice, given the dead volume within the syringe); volumes should be adjusted according to the different number of mice. Also, similar applications can be employed for lPEI (present case) or his-PEI.

1. Dilute 2.8 μg of RepRNA-HA (7 mice × 0.4 μg) in [10 mM HEPES buffer pH 7.4 + glucose 5%], final volume = 39.2 μl ("RepRNA-HA solution").
2. Depending on the RepRNA–lPEI ratio, dilute 2.8/5.6/8.4 or 16.8 μl lPEI (1 mg/ml) in 2.8/5.6/8.4 or 16.8 μl 10 mM HEPES buffer pH 7.4, respectively ("lPEI solution").
3. Dropwise add the lPEI solution to the RepRNA-HA solution, vortex at the same time, and incubate for 30 min, at RT ("lPEI/RepRNA-HA").
4. As soon after, repeat **steps 1–3** for RepRNA-NP ("lPEI/RepRNA-NP").
5. When the 30 min incubation time is over, mix up the two formulations lPEI/RepRNA-HA and lPEI/RepRNA-NP (total volume = 78.4 μl).
6. Make up to 1400 μl with 1321.6 μl of [HEPES buffer pH 7.4 + Glucose 5%] (1089.1 μl).
7. Proceed to the injection subcutaneously; use 200 μl per mouse (*see* **Note 14**).

3.4.2 Chitosan Nanoparticles

Chitosan Nanoparticle Formulation

Perform all steps under an RNase-free laminar flow hood using RNase*Zap*®. Solutions, pipettes, tips, tubes and any other consumables used for the formulation must be RNase-free. The following Protocol is given for 1 ml final volume chitosan nanoparticles containing 8 μg RepRNA; volumes have to be increased accordingly for higher RepRNA concentrations.

1. Prepare a 1 % (w/v) chitosan stock solution in RNase-free H_2O by adding slowly 1 M HCl until all chitosan is dissolved (HCl must be filtered using 0.2 μm filter); the pH of the solution should be between 3.5 and 4.0. This is a slow process and must be performed under stirring conditions. As chitosan dissolves it increases the solution pH, therefore HCl has to be added several times to keep pH below 4.0. Chitosan is completely dissolved when the pH of the solution doesn't increase above 4. This solution can be stored at –20 °C in aliquots for 1 year. Avoid freeze-thawing the aliquots more than twice.
2. Prepare 0.1 % (w/v) chitosan by diluting the 1 % chitosan stock solution 1:10 in RNase-free H_2O; filter using 0.2 μm filters.
3. Prepare 0.1 % (w/v) alginate solution in RNase-free H_2O. Dissolve the alginate by vortexing for 2 min at maximum speed; filter using 0.2 μm filters.
4. Prepare 0.1 % (w/v) TPP in RNase-free H_2O. Briefly vortex the solution to ensure that TPP is completely dissolved; filter using 0.2 μm filters (*see* **Note 15**).
5. Admix by pipetting 25 μl of 0.1 % TPP with 8 μg RepRNA (bring the RepRNA concentration to 1–2 μg/μl before the addition to TPP); incubate for 10 min at RT (solution [TPP/RepRNA]).
6. Place 250 μl of 0.1 % chitosan solution in a 5 ml glass container with a magnetic stirrer; employ maximum speed avoiding the presence of bubbles in the solution (*see* **Note 16**); add solution [TPP/RepRNA] dropwise while stirring, and stir for 2 h, at RT (solution [chitosan/TPP/RepRNA]).
7. Dilute the [chitosan/TPP/RepRNA] solution with RNase-free H_2O to 500 μl; stir for another 10 min.
8. For the chitosan nanoparticle coating step, place 500 μl of 0.1 % alginate solution in a 5 ml glass container with a magnetic stirrer; employ maximum speed avoiding the presence of bubbles in the solution; bring the solution up to pH of 8.5–9.0.
9. Place the 500 μl [chitosan/TPP/RepRNA] solution in a 1 ml syringe with a 21-22G hypodermic needle. Add slowly this solution into the 0.1 % alginate solution while stirring; monitor the pH at all times and adjust when is needed with 0.1 M NaOH solution (filtered with 0.2 μm filter and RNase-free); avoid having pH less than 6.5 [chitosan/TPP/RepRNA/alginate]; stir for 2 h, at RT; use the preparation on the same day.

10. Size, polydispersity index (PDI) and ζ-potential can be measured in 10 mM RNase-free HEPES buffer pH 7.4 and serum-free Opti-MEM®, employing Zetasizer Nano ZS (Malvern Instruments, UK) or qNano (Izon, UK) [12] (*see* **Note 17**).

RepRNA Protection from RNases Offered by Chitosan Nanoparticles

For the assay of RNase protection offered by chitosan nanoparticles, the procedure described in Subheading "RepRNA protection from RNases offered by polyplexes" was not applicable because of the small volume that can be loaded on an agarose gel. We therefore use the procedure described previously—10% (w/v) polyacrylamide and 35% (w/v) urea gel in 133 mmol/l Tris–HCl, 45.5 mmol/l boric acid, and 3.2 mmol/l EDTA [13, 33].

Chitosan Nanoparticle Internalization by Cells

The procedure starts with **steps 1–4** from Subheading "Polyplex Internalization by Cells".

1. DCs are prechilled 30 min on ice; then exposed to chitosan nanoparticles at 37 °C, during 2 or 24 h.
2. Wash with $PBS^{+/+}$ and then proceed to the classical two-step intracellular staining, as described previously [12, 13, 32]. The various antibodies that can be used for confocal microscopy are listed in Table 5.

RepRNA Translation/ Replication Offered by Chitosan Nanoparticles in Transfected Cells

The procedure starts with **steps 1–4** from Subheading "Polyplex Internalization by Cells".

1. Cells are exposed to chitosan nanoparticles for 2 h at 37 °C in [DMEM + 10% serum]; wash 2 times with [DMEM + 10% serum + IL4 + GM-CSF]; culture the cells for 48–72 h in [DMEM + 10% serum + IL4 + GM-CSF].
2. Wash with $PBS^{+/+}$, and then proceed to the classical two-step intracellular staining, as described previously [12, 13, 32]. The various antibodies that can be used for confocal microscopy are listed in Table 5.

Chitosan Nanoparticle Formulation to Be Injected in Mice

The immunization trials in mice were approved by the Animal Welfare Committee of the Canton of Berne under license number BE72/12, and conducted in compliance with the Swiss animal protection law. Balb/c mice are vaccinated by subcutaneous injection at days 0, 28, and 56, as described previously [12, 13]. 0.4 μg of RepRNA-HA and 0.4 μg of RepRNA-NP are injected per vaccination. The protocol is given for six mice (volumes correspond to seven mice, given the dead volume with the syringe); calculate the volumes accordingly for a different number of mice.

1. The protocol to formulate [chitosan/TPP/RepRNA-NP/alginate] and [chitosan/TPP/RepRNA-HA/alginate] is the same with what is described in Subheading "Chitosan nanoparticle Formulation".

2. Admix 350 µl of the two formulations (total volume = 700 µl).
3. Add sequentially 35 µl of BPPcysMPEG (2 µg/µl), 31 µl of sterile RNase-free H_2O and 350 µl of 4× sterile $PBS^{-/-}$.
4. Proceed to injection subcutaneously; use 200 µl per mouse.

3.4.3 Lipid-Based Lipoplexes

Lipoplex Formulation

All steps should be performed under RNase-free and sterile conditions using RNase-free equipment and reagents. The following protocol describes the formulation of 1 µg of RepRNA with cationic lipids obtained from OzBiosciences®, namely NL10, NL21, NL42, Dogtor (DOG), DreamFect™ (DREAM), Lullaby (LUL), Ecotransfect, NL124, Dogtor-Cationic nano emulsion (DOG-CNE), and NL10-cationic nano emulsion (NL10-CNE). Classically, a RepRNA–lipid ratio of 3:1 (w:v) is employed to formulate cationic lipoplexes with lipids 1–7 and a ratio of 1:1 (v:v) using lipids 8–10, however, this ratio can be varied accordingly.

1. Dilute 1 µg RepRNA in a total volume of 50 µl of serum-free Opti-MEM® (RepRNA solution).
2. Allow the lipid of interest to equilibrate to RT before diluting 3 µl in 47 µl of serum-free Opti-MEM® (Lipid solution) (*see* **Note 18**).
3. Slowly admix the lipid solution with the RepRNA solution or 50 µl of NL124/DOG-CNE/NL10-CNE in a dropwise manner.
4. Shortly vortex the mix and allow the lipid to complex the RepRNA molecules for 20 min at RT (or 1 h at 4 °C for DOG-CNE and NL10-CNE based complexes).
5. Size, polydispersity index (PDI) and ζ-potential of the lipoplexes, diluted in RNase-free H_2O are assessed using a Zetasizer Nano ZS (Malvern Instruments, UK) or qNano (Izon, UK) [12].

RepRNA Protection from RNases Offered by Lipoplexes

Protecting RepRNA from degradation by RNases is an essential property for any successful delivery vehicle. Interaction of RepRNA with its delivery vehicle and RNase protection are investigated by an electrophoretic gel mobility shift assay.

1. The Lipid-RepRNA complexes are prepared and loaded on to a 1% agarose gel matrix containing ethidium bromide and run with Tris-acetate buffer at 130 V for 30 min. RepRNA retardation is visualized and photographed by a GelDoc-It® TS Imaging System.
2. In the RNAse assay, 1 U of RNAse H is added to the preformed lipoplexes incubated for 30 min at 37 °C, and subsequently loaded as in Subheading "RepRNA Protection from RNases Offered by Polyplexes". RepRNA retardation is visualized and photographed by a GelDoc-It® TS Imaging System, as shown in Fig. 5c for polyplexes.

Lipoplex Internalization by Cells

In order to visualize the interaction of RepRNA molecules with DCs as well as their transit through the various endocytic routes and cytosolic release, RepRNA was labeled with fluorescein molecules using the Mirus® labeling kits at 1:1 reagent/RepRNA weight ratio [13].

The procedure starts with **steps 1–4** from Subheading "Polyplex Internalization by Porcine and Human Cells".

1. DCs are pulsed with the lipoplexes for either 30 min or 1 h at 37 °C (*see* Subheading "Lipoplex Formulation", 1 μg of complexed RepRNA in 200 μl of Opti-MEM® per well).
2. Subsequently, cells receive 1 wash cycle with DMEM to remove excess lipoplexes, and are stained for the antigens of interest as described previously [12, 13, 32]. The various antibodies that can be employed for confocal microscopy are listed in Table 5.

RepRNA Translation/ Replication Offered by Lipoplexes in Transfected Cells

1. To investigate lipid-mediated translation of the RepRNA, cells are pulsed with non-labeled RepRNA as described in Subheading "Lipoplex Internalization by Cells", and the cells are subsequently cultured (DMEM + 10 % PS + IL-4 + GM-CSF) for 48 h or 72 h to facilitate RepRNA translation.
2. The cells receive at least one wash cycle with $PBS^{+/+}$, and the classical two-step intracellular staining protocol described previously [12, 13, 32] is followed to stain for the antigens of interest. Table 5 lists the various antibodies that can be used for confocal microscopy.

Lipoplex Formulation to Be Injected in Mice

The use of animals for experimentation was approved both by the authorities of Canton Bern, as well as the authorities of the Federal government of Switzerland (License BE 72/12). Balb/c mice were vaccinated subcutaneously as previously described [12, 13] on day 0, 28, and 56. The RepRNA-HA and the RepRNA-NP constructs were used at 0.4 μg per RNA construct per animal per vaccination wave. The protocol below describes the formulation of the vaccination cocktail prepared for 6 animals.

1. Dilute 12 μg of RepRNA-HA and 12 μg of RepRNA-NP (6 mice × 2 μg for each RepRNA) in $PBS^{+/+}$ in a final volume of 300 μl (RepRNA solution).
2. Allow all cationic lipids of interest to equilibrate to RT before use.
3. Depending of the lipid used: dilute 96 μl of Dogtor or Lullaby in 204 μl of $PBS^{+/+}$; or dilute 72 μl of Econtransfect in 228 μl of $PBS^{+/+}$; or use DOG-CNE and NL124 undiluted.
4. In a dropwise manner, add 300 μl of Dogtor, Lullaby, or Ecotransfect solution into a tube containing 300 μl of RepRNA solution and vortex briefly. Allow the lipids to complex the RepRNA molecules for 20 min, at RT. Similarly, add 300 μl of undiluted DOG-CNE or NL124 to separate tubes containing 300 μl of RepRNA solution and allow DOG-CNE to complex the RepRNA at 4 °C for 1 h, and the NL124 for 20 min at RT.

3.5 Experimental Readouts

3.5.1 In Vitro Readouts

For flow cytometry and confocal microscopy, the list of the Abs used is provided in Table 5. Confocal microscopy employed either a Leica TCS-SL or a Nikon Eclipse Ti microscope. Both techniques have been described previously in detail [12, 13, 32, 34–36].

For the luciferase reporter assay, standard procedures are used as described previously [11, 37].

3.5.2 In Vivo Readouts

For humoral response evaluation, serum anti-HA and anti-NP antibody titres were assessed by indirect ELISA [12, 13].

For cellular response evaluation, either standard T-cell restimulation assays [12, 13] or cytokine profiling were used [12, 38–40].

4 Application of Synthetic RepRNA Delivery

4.1 Biodegradable Formulations for Delivery of RepRNA to DCs

Following the first proposed delivery of self-amplifying RepRNA vaccines by synthetic, biodegradable particles in 2008 [14], complexing the RNA for delivery to DCs has shown applicability for polysaccharide, polyplex and lipoplex. Chitosan-based nanoparticles (termed nanogels due to their gel-like matrix structure) and polyplex formulations have already proven efficiency for RepRNA delivery to DCs. The RepRNA associates physically with the delivery vehicles, which is important due to the incapacity of the RNA to enter DCs—or inducing immune responses in vivo—in the absence of a delivery vehicle [12–14]. This delivery of RepRNA to DCs facilitated translation of the GOI carried by the RepRNA for vaccine purposes, as well as RNA replication in the DCs. In turn, this was related to induction of humoral and cell-mediated immune responses in vivo against the antigen encoded by the GOI, namely influenza virus HA and NP.

4.2 Future for RepRNA Delivery to DCs

Due to their capacity for accommodating GOI encoding vaccine antigens of choice, and their self-replicating or self-amplifying nature, RepRNA-based vaccines lend themselves readily for delivery of many vaccine antigens particularly weakly immunogenic antigens including tumor antigens. These RepRNA molecules can be produced under cell-free conditions, as can the delivery vehicles and formulation into the final vaccine, which is important considering the avoidance of complex and expensive infrastructures for cell cultures or egg production, as well as avoiding potential contamination by animal, plant or microbial products.

With the biodegradable delivery vehicles both protecting the RepRNA and promoting delivery, induction of efficient immune defense development can be tackled. If the RepRNA is non-cytopathic, as with the RepRNA derived from CSFV, targeting to DCs ensures survival of the cells to maintain antigen synthesis, thus furthering robust immune defense development. Translation of the

RepRNA in critically important antigen-presenting cells such as DCs promotes antigen production at the heart of the immune system involved in the induction of humoral and CMI defenses.

The process of associating self-amplifying RepRNA with biodegradable and biosafe delivery vehicles promotes novel synthetic vaccine development, currently being assessed in terms of robust and broadly protective influenza vaccines. The rapidity with which the RepRNA can be modified and formulated with the delivery vehicle facilitates tackling emergency situations, such as during epidemics and pandemics. The cationic nature of the delivery vehicle is advantageous for the RNA delivery, but also provides potential for association of synthetic adjuvants. Indeed, the adjuvants mentioned above with the in vivo assessment of RepRNA delivery have been proving particularly effective.

5 Conclusions

RepRNA vaccines are entirely synthetic and biodegradable. As such, they are not encumbered by delays and risks associated with current, more traditional vaccine production methods. They have high potential for prophylactic and therapeutic application. However, the large and complex nature of RepRNA requires particular investigation to overcome their inability to survive in biological environments (protection) and cross the cell membrane barrier (DC targeting). In the present report, we provide in depth description of our current PEI-, chitosan-, or cationic lipid-based formulations for delivery of RepRNA encoding influenza virus antigens to DCs. Importantly, DC targeting by our synthetic delivery vehicles can be modulated by the inclusion in the formulation of cell penetrating peptides (CPPs) or ligands targeting cell surface receptors, such as pathogen-associated molecular patterns. This successively impacts on the endocytic route employed for uptake by DCs, RepRNA cytosolic translocation, translation/self-replication, and ultimately the likelihood of success for RepRNA vaccines.

6 Notes

1. At this stage, it is important to reduce the centrifugation speed from $350 \times g$ to $250 \times g$ to remove the platelets.
2. To increase the purity of DCs, the eluted fraction can be enriched over a second MS or LS Column. Repeat the magnetic separation procedure as described in steps 19–26 by using a new column.
3. Use only horse serum, never bovine serum—bovine serum may contain pestiviruses (bovine viral diarrhea virus) or

pestiviral RNA, which interferes with RepRNA in SK-6 cells, resulting in contamination and false positive results.

4. This protocol is designed to subculture the cells typically every 5–7 days. Exceptionally, it is possible to passage the cells with shorter intervals. However, this should be avoided whenever possible, because it forces to cells to metabolize more rapidly. They will eventually grow more slowly, look more granular and may become less susceptible to virus or replicon infection.
5. One tends to keep the number of passages to a certain limit. We tend to passage up to 20 times, and then take a new ampoule of cells from the liquid nitrogen stocks to start afresh. This ensures that the cells remain most sensitive to CSFV infection, and therefore supportive of the replicon. Nevertheless, the cells can be passaged beyond passage 20, until there are signs of increased granularity, slowing of cell growth, or reduced capacity to support replicon translation and replication. At this point, new cells must be thawed from liquid nitrogen stocks, or cloned to isolate cells showing the highest capacity for supporting replicon translation/replication. Starting fresh cultures with a new ampoule from liquid nitrogen stocks is preferred.
6. SK-6 cells may tend to grow in "islets" of cells after passage, before starting to create a monolayer. Due to this, one often remarks that a complete monolayer does not form, but there are gaps. This is not a problem and the cells can still be passaged once per week.
7. The endonuclease restriction site was designed to contain both SrfI and SfiI run-off restriction endonuclease sites; digestion with SrfI is preferred because it generates a blunt end (Fig. 3a). SfiI can be used if a GOI contains SrfI.
8. At this stage it is essential to ensure that all tubes, tips, and solutions are RNase-free.
9. Nucleoside-modified RepRNA can also be prepared with the same methodology. Partial or total replacement of UTP or CTP with 5-Methyl-CTP, pseudo-UTP, or 2-Thio-UTP can lead to enhanced RepRNA translation by increasing its stability protecting it from nucleases.
10. Work with gloves and avoid touching the tip of the column when removing the plug. Clean the centrifuge lid and rotor with RNase*Zap*® RNase Decontamination Solution prior to use limits exposure of the transcripts to RNases when spinning the open columns.
11. For RNA, the A_{260}/A_{280} and A_{260}/A_{230} ratios must be in the range of 1.8–2.1 and larger than 2, respectively; if not, it is recommend to start a new RepRNA production.

12. From our expertise, the thin shape of the RepRNA band after 10 min of gel electrophoresis is an indicative criterion for RepRNA quality. However, average quality RepRNA may also display a thin band after short run. Therefore, electrophoresis must be extended to 30 min: unstable RepRNA will progressively degrade and cannot be considered for experimental work (Fig. 5a).
13. A pre-step can be performed with CPPs. Dropwise addition of 2, 4, 6, or 12 μl of PEI solution (for 1:1, 1:2, 1:3, or 1:6 ratios, respectively) to 0.5 μM of Arg_9 (BAP-301), HIV-1 TAT (47–57) (BAP-303), *Penetratin* (BAP-306), or CyLoP-1 (BAP-307), all from EMC microcollections GmbH, Tübingen, Germany; vortex at the same time and incubate for 30 min, at RT.
14. After **step 5**, 70 μg of adjuvants such as Pam3Cys-SK4 and *S*-[2,3-bispalmitoyiloxy-(2*R*)-propyl]-*R*-cysteinyl-amido-monomethoxy polyethylene glycol (BPPcysMPEG) can be added in the reconstitution to 1400 μl (then each mouse receives 10 μg of adjuvant).
15. All 0.1% (w/v) chitosan, alginate and TPP solutions must be prepared on the same day.
16. At this step of the formulation, Lipofectamine® 2000 Reagent can be added dropwise into chitosan while stirring for 10 min. The 8 μl/ml of Lipofectamine® 2000 was found to be the most efficient concentration. Then add solution [TPP/RepRNA] dropwise to [chitosan/Lipofectamine® 2000] /while stirring, and stir for 2 h, at RT (solution [chitosan/Lipofectamine® 2000/TPP/RepRNA]). The rest of the procedure is unchanged.
17. As an alternative to alginate, hyaluronic acid can be employed for decorating the chitosan nanoparticle surface. As such, it is possible to omit the TPP. It is also possible to employ glycoconjugates to decorate the nanoparticle surface for targeting particular cell surface or intracellular receptors on DCs.
18. NL124, DOG-CNE, and NL10-CNE do not require to be diluted in medium prior use.

Acknowledgement

We thank Markus Gerber and Samira Locher for their help and input with the RepRNA technology, and Brigitte Herrmann for helping with the DC studies. We are also grateful to Patrick Midoux and Laure Magrangeas-Janot for help with the polyplex technology, Olivier Zelphati and Florent Poulhes for help with the lipoplex technology, and Kai Schulze for the adaptive immune response profiling. The work was funded by the Marie Curie IAPP Project Replixcel (251420) and the EU FP7 Project UniVax (HEALTH-F3-2013-60173).

References

1. Atkins GJ, Fleeton MN, Sheahan BJ (2008) Therapeutic and prophylactic applications of alphavirus vectors. Expert Rev Mol Med 10:e33
2. Khromykh AA (2000) Replicon-based vectors of positive strand RNA viruses. Curr Opin Mol Ther 2:555–569
3. Ljungberg K, Liljestrom P (2015) Self-replicating alphavirus RNA vaccines. Expert Rev Vaccines 14:177–194
4. Lundstrom K (2002) Alphavirus-based vaccines. Curr Opin Mol Ther 4:28–34
5. McCullough KC, Bassi I, Démoulins T, Thomann-Harwood LJ, Ruggli N (2012) Functional RNA delivery targeted to dendritic cells by synthetic nanoparticles. Ther Deliv 3:1077–1099
6. Pijlman GP, Suhrbier A, Khromykh AA (2006) Kunjin virus replicons: an RNA-based, non-cytopathic viral vector system for protein production, vaccine and gene therapy applications. Expert Opin Biol Ther 6:135–145
7. Rayner JO, Dryga SA, Kamrud KI (2002) Alphavirus vectors and vaccination. Rev Med Virol 12:279–296
8. Frey CF, Bauhofer O, Ruggli N, Summerfield A, Hofmann MA, Tratschin JD (2006) Classical swine fever virus replicon particles lacking the Erns gene: a potential marker vaccine for intradermal application. Vet Res 37:655–670
9. Maurer R, Stettler P, Ruggli N, Hofmann MA, Tratschin JD (2005) Oronasal vaccination with classical swine fever virus (CSFV) replicon particles with either partial or complete deletion of the E2 gene induces partial protection against lethal challenge with highly virulent CSFV. Vaccine 23:3318–3328
10. Moser C, Stettler P, Tratschin JD, Hofmann MA (1999) Cytopathogenic and noncytopathogenic RNA replicons of classical swine fever virus. J Virol 73:7787–7794
11. Suter R, Summerfield A, Thomann-Harwood LJ, McCullough KC, Tratschin JD, Ruggli N (2011) Immunogenic and replicative properties of classical swine fever virus replicon particles modified to induce IFN-alpha/beta and carry foreign genes. Vaccine 29:1491–1503
12. Démoulins T, Milona P, Englezou PC, Ebensen T, Schulze K, Suter R, Pichon C, Midoux P, Guzman CA, Ruggli N, McCullough KC (2016) Polyethylenimine-based polyplex delivery of self-replicating RNA vaccines. Nanomedicine 12(3):711–722
13. McCullough KC, Bassi I, Milona P, Suter R, Thomann-Harwood L, Englezou P, Démoulins T, Ruggli N (2014) Self-replicating replicon-RNA delivery to dendritic cells by chitosan-nanoparticles for translation in vitro and in vivo. Mol Ther Nucleic Acids 3:e173
14. Tratschin JD, Ruggli N, McCullough KC (2008) Pestivirus replicons providing an RNA-based viral vector system. PCT/EP2009/003892 WO 2009146867
15. McCullough KC, Milona P, Démoulins T, Englezou P, Ruggli N (2015) Dendritic cell targets for self-replicating RNA vaccines. J Blood Lymph 5:132. doi:10.4172/2165-7831.1000132
16. McCullough KC, Milona P, Thomann-Harwood L, Démoulins T, Englezou P, Suter R, Ruggli N (2014) Self-amplifying replicon RNA vaccine delivery to dendritic cells by synthetic nanoparticles. Vaccines 2:735–754
17. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K (2000) Immunobiology of dendritic cells. Annu Rev Immunol 18:767–811
18. Medzhitov R, Janeway C Jr (2000) Innate immune recognition: mechanisms and pathways. Immunol Rev 173:89–97
19. Mellman I, Steinman RM (2001) Dendritic cells: specialized and regulated antigen processing machines. Cell 106:255–258
20. Steinman RM (1991) The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 9:271–296
21. Steinman RM (2012) Decisions about dendritic cells: past, present, and future. Annu Rev Immunol 30:1–22
22. Steinman RM, Hemmi H (2006) Dendritic cells: translating innate to adaptive immunity. Curr Top Microbiol Immunol 311:17–58
23. Summerfield A, Horn MP, Lozano G, Carrasco CP, Atze K, McCullough K (2003) C-kit positive porcine bone marrow progenitor cells identified and enriched using recombinant stem cell factor. J Immunol Methods 280:113–123
24. Carrasco CP, Rigden RC, Schaffner R, Gerber H, Neuhaus V, Inumaru S, Takamatsu H, Bertoni G, McCullough KC, Summerfield A (2001) Porcine dendritic cells generated in vitro: morphological, phenotypic and functional properties. Immunology 104:175–184
25. Kasza L, Shadduck JA, Christofinis GJ (1972) Establishment, viral susceptibility and biological characteristics of a swine kidney cell line SK-6. Res Vet Sci 13:46–51
26. Ruggli N, Tratschin JD, Mittelholzer C, Hofmann MA (1996) Nucleotide sequence of classical swine fever virus strain Alfort/187 and transcription of infectious RNA from stably cloned full-length cDNA. J Virol 70:3478–3487

27. Ruggli N, Summerfield A, Fiebach AR, Guzylack-Piriou L, Bauhofer O, Lamm CG, Waltersperger S, Matsuno K, Liu L, Gerber M, Choi KH, Hofmann MA, Sakoda Y, Tratschin JD (2009) Classical swine fever virus can remain virulent after specific elimination of the interferon regulatory factor 3-degrading function of Npro. J Virol 83:817–829
28. Ruggli N, Tratschin JD, Schweizer M, McCullough KC, Hofmann MA, Summerfield A (2003) Classical swine fever virus interferes with cellular antiviral defense: evidence for a novel function of N(pro). J Virol 77:7645–7654
29. Mayer D, Hofmann MA, Tratschin JD (2004) Attenuation of classical swine fever virus by deletion of the viral N(pro) gene. Vaccine 22:317–328
30. Lorenz RJ, Bogel K (1973) Laboratory techniques in rabies: methods of calculation. Monograph series. World Health Organization 23(23):321–335
31. Bertrand E, Goncalves C, Billiet L, Gomez JP, Pichon C, Cheradame H, Midoux P, Guegan P (2011) Histidinylated linear PEI: a new efficient non-toxic polymer for gene transfer. Chem Commun (Camb) 47:12547–12549
32. Sharma R, Ghasparian A, Robinson JA, McCullough KC (2012) Synthetic virus-like particles target dendritic cell lipid rafts for rapid endocytosis primarily but not exclusively by macropinocytosis. PLoS One 7:e43248
33. Python S, Gerber M, Suter R, Ruggli N, Summerfield A (2013) Efficient sensing of infected cells in absence of virus particles by plasmacytoid dendritic cells is blocked by the viral ribonuclease E(rns.). PLoS Pathog 9:e1003412
34. Démoulins T, Bassi I, Thomann-Harwood L, Jandus C, Kaeuper P, Simon HU, von Gunten S, McCullough KC (2013) Alginate-coated chitosan nanogel capacity to modulate the effect of TLR ligands on blood dendritic cells. Nanomedicine 9:806–817
35. Démoulins T, Milona P, McCullough KC (2014) Alginate-coated chitosan nanogels differentially modulate class-A and class-B CpG-ODN targeting of dendritic cells and intracellular delivery. Nanomedicine 10:1739–1749
36. Thomann-Harwood LJ, Kaeuper P, Rossi N, Milona P, Herrmann B, McCullough KC (2013) Nanogel vaccines targeting dendritic cells: contributions of the surface decoration and vaccine cargo on cell targeting and activation. J Control Release 166:95–105
37. Liniger M, Summerfield A, Zimmer G, McCullough KC, Ruggli N (2012) Chicken cells sense influenza A virus infection through MDA5 and CARDIF signaling involving LGP2. J Virol 86:705–717
38. Mittal A, Schulze K, Ebensen T, Weissmann S, Hansen S, Guzman CA, Lehr CM (2015) Inverse micellar sugar glass (IMSG) nanoparticles for transfollicular vaccination. J Control Release 206:140–152
39. Mittal A, Schulze K, Ebensen T, Weissmann S, Hansen S, Lehr CM, Guzman CA (2015) Efficient nanoparticle-mediated needle-free transcutaneous vaccination via hair follicles requires adjuvantation. Nanomedicine 11:147–154
40. Rharbaoui F, Drabner B, Borsutzky S, Winckler U, Morr M, Ensoli B, Muhlradt PF, Guzman CA (2002) The Mycoplasma-derived lipopeptide MALP-2 is a potent mucosal adjuvant. Eur J Immunol 32:2857–2865

Chapter 4

Plant Expression of Trans-Encapsidated Viral Nanoparticle Vaccines with Animal RNA Replicons

Yiyang Zhou, Alison A. McCormick, and Christopher M. Kearney

Abstract

In this protocol, we outline how to produce a live viral nanoparticle vaccine in a biosafety level 1 (BSL1) environment. An animal viral vector RNA encapsidated with tobacco mosaic virus (TMV) coat protein can be fully assembled *in planta*. *Agrobacterium* cultures containing each component are inoculated together into tobacco leaves and the self-assembled hybrid nanoparticle vaccine is harvested 4 days later and purified with a simple PEG precipitation. The viral RNA delivery vector is derived from the BSL1 insect virus, Flock House virus (FHV), and replicates in human and animal cells but does not spread systemically. A polyethylene glycol purification protocol is also provided to collect and purify these vaccines for immunological tests.

Key words Trans-encapsidation, Viral vaccine, Agroinoculation, Polyethylene glycol purification

1 Introduction

Our system comprises an RNA-based, replicating viral vaccine. This has advantages over single-protein vaccines or virus-like particles (VLPs) in that viral RNA replication triggers strong immune activation [1–3]. To avoid the use of an intact infectious virus, viral coat proteins coded on a separate genetic unit can be used to trans-encapsidate the vector RNA. In this way, a replicating viral RNA can be protected on its journey to the target cell and can be absorbed as a nanoparticle. Tobacco mosaic virus (TMV) is an excellent candidate for providing the coat protein to create stable nanoparticles [4]. A unique packaging sequence (origin of assembly, Oa, [5]) allows trans-encapsidation occur by TMV coat protein of any RNA containing the Oa RNA sequence [6–8]. Experimentally, TMV virions are highly stable [4] and trans-encapsidated vaccines demonstrate better antibody response and dendritic cell activation [6, 9].

In our system, expression in tobacco (*Nicotiana benthamiana*) is used to assemble hybrid nanoparticles. An in vivo plant-based

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_4,

expression system very well addresses the expense and biosafety concerns of animal cell lines and cell cultures. Furthermore, by using the BSL1 insect virus, Flock House virus, this system can produce encapsidated vaccines completely in a BSL1 environment, without introducing any endotoxins or extraneous human viruses [6, 10]. Flock House virus RNA 1 is used as the vaccine viral vector, and RNA 1, in the absence of RNA 2, has been shown to replicate in plants and mammalian cells but not to spread systemically [11]. An authentic in vivo replication of viral RNA in plant cells also overcomes the challenge of low efficiency of in vitro RNA 5′-capping when using in vitro RNA synthesis [8]. Finally, an unpurified form of raw plant materials could potentially be used for veterinary vaccination use.

Our protocol utilizes agroinoculation, which provides a rapid and convenient way to express heterogeneous genes in plant tissues. By introducing the gene of interest into a T-DNA cassette in a binary shuttle vector in *E. coli* and then transferring the shuttle vector to *Agrobacterium tumefaciens,* the agrobacterium will readily incorporate the T-DNA segment randomly into the plant chromosome [12]. In contrast to using plant protoplasts [13] or leaf disks [14] to regenerate whole plants from tissue culture, agroinoculation uses leaves on whole, non-sterile plants [15], and, since proteins are harvested within a week from inoculation, this is considered a transient expression system rather than a long-term expression system. This allows for higher yields, quicker set-up and lesser maintenance than obtainable with long-term transgenic plants.

We previously described a successful application of this system to produce hybrid Flock House virus RNA trans-encapsidated nanoparticle vaccines [8]. Briefly (Fig. 1), a BSL1, nonpathogenic, multi-host Flock House virus [11, 16–18] RNA was engineered to contain tobacco mosaic virus (TMV) virion packaging signal (origin of assembly (Oa)). The plant virus, Foxtail mosaic virus, was engineered to express TMV coat protein at high levels while not itself being encapsidated by the coat protein, since it lacks the TMV Oa. Both viral components were introduced to separate agrobacterium inocula, and co-delivered to plant leaves, along with the aid of a gene silencing suppressor gene, p19, driven by a 35S promoter in a third co-delivered agrobacterium inoculum [19]. A simple polyethylene glycol precipitation method was used to collect highly purified rod-shaped nanoparticle vaccines in leaf tissue, indicating successful trans-encapsidation of Flock House virus RNA by TMV coat protein.

Here we describe the full details of conducting agroinoculation and vaccine purification. Besides the demonstrated production of plant-produced encapsidated FHV vaccine, we envision that this method could also be applied to the production of other *in planta* TMV coat protein trans-encapsidated viral vaccines.

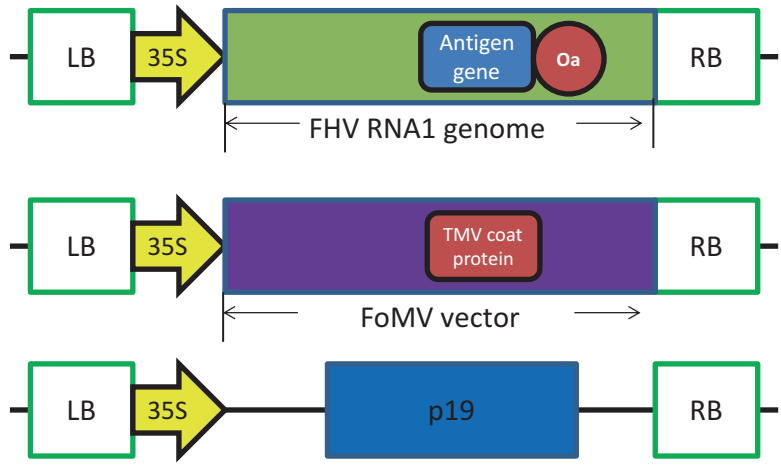

Fig. 1 Scheme of agro-coinoculation to produce trans-encapsidated vaccines. Cauliflower mosaic virus 35S promoter (35S) and terminator (not shown) flank the viral vector genome or (*bottom*) the p19 sequence. Left border (LB) and right border (RB) sequences are for T-DNA insertion into plant chromosome. On Flock House virus vector, the TMV origin of assembly (Oa) packaging sequence was inserted to allow trans-encapsidation by TMV coat protein produced by the FoMV plant viral vector

2 Materials

2.1 Agro-inoculation

1. *Agrobacterium tumefaciens* strain: GV3101 (*see* **Note 1**).
2. LB-agar plate: 5 g NaCl, 5 g yeast extract, 10 g Bacto tryptone, 15 g agar per L, with appropriate antibiotics (*see* **Note 2**).
3. LB media: 5 g NaCl, 5 g yeast extract, 10 g Bacto tryptone per L, with appropriate antibiotics (*see* **Note 2**).
4. L-MESA media (LB-MES-Acetosyringone): 20 mM MES (pH 7.5) and 200 μM acetosyringone final concentration in LB media, with appropriate antibiotics. A 0.5 M stock MES solution is prepared by adding 5.33 g MES monohydrate into 50 ml distilled water and adjusting the pH to 5.7 with KOH. MES media can be autoclaved or sterile filtered. A 0.1 M acetosyringone stock solution is prepared with DMSO solvent and separated into aliquots. Acetosyringone needs to be used fresh.
5. Induction media: 10 mM $MgCl_2$, 10 mM MES (pH 5.7), and 200 μM acetosyringone final concentration in distilled water (*see* **Note 3**).
6. Needleless syringes (1 or 3 ml volume).
7. Plant host: *Nicotiana benthamiana* (*see* **Note 4**).

2.2 Nanoparticle collection and purification

1. Oak Ridge centrifuge tubes
2. Fine grade blender or pestle and mortar

3. Virion extraction buffer (1×): 50 mM sodium acetate, 0.86 M NaCl (5% w/v), 0.04% sodium metabisulfite (w/v), adjust pH to 5.0 with acetic acid
4. 50 mM Tris–HCl, pH 7.2
5. Polyethylene glycol (PEG) solutions: 20% (w/v) PEG 8000 solution and 20% (w/v) PEG/NaCl solutions. Both 20% PEG and 20% PEG/NaCl solutions are prepared with PEG 8000. For a 100 ml solution, weigh 20 g of PEG 8000 (or 20 g PEG 8000 and 20 g NaCl), and add 80 ml distilled water. Place in a 60 °C water bath to help dissolving and stir constantly. Autoclaving is optional. Wait until PEG is completely dissolved, then add distilled water to 100 ml. The 20% PEG/NaCl solution will separate into phases when hot. The separation will disappear when the solution cools to room temperature; swirl to ensure complete mixing. Both solutions can be kept in 4 °C for few weeks. However, using fresh solution is recommended.
6. Phosphate buffer (10×): Make 0.1 M Na_2HPO_4 (pH 9.0) and 0.1 M KH_2PO_4 (pH 4.4) respectively, add 100 ml Na_2HPO_4 buffer in a beaker, adjust to pH 7.2 with KH_2PO_4 buffer (*see* **Note 5**).

3 Methods

3.1 Agro-coinoculation

Steps 2–4 have to be conducted under a sterile hood. Involved buffer containers, forceps, pipettes, and other tools must be wiped with 70% ethanol before using. Pipet tips and Eppendorf tubes are sterilized by autoclaving.

1. Plasmids containing FHV replicon sequence and CP-embracing vector sequence were constructed in *E. coli*. Agrobacterium competent cells were transformed with plasmid preparations from *E. coli* by electroporation, and plated on agar plate with antibiotics (*see* **Note 6**).
2. Agrobacterium colonies with FHV vector containing the TMV origin of assembly signal (Oa), and colonies of Foxtail mosaic virus (FoMV) vector containing TMV coat protein, are picked by toothpick swipes from agar plates, and transferred into 3 ml LB broth with appropriate antibiotics. An agrobacterium culture of 35S/p19 should also be prepared at the same time (*see* **Note 2**)
3. Incubate the cultures by shaking in 28 °C until late log or stationary phase (*see* **Note 7**)
4. After incubation, a 10% glycerol stock can be prepared with agrobacterium culture and stored in −80 °C for future use. Otherwise, 250 μl of LB culture is added into 5 ml L-MESA

culture, with appropriate antibiotics. O.D. 600 is measured after 8–16 h incubation (*see* **Note 8**)

5. Pellet cells by centrifugation at 4000 × *g* for 10 min. Drain supernatant from cell pellet. Thoroughly resuspend pellet with 5 ml induction media (*see* **Note 9**)
6. Resuspended cells need to be kept still (without shaking) in room temperature from 3 h to overnight (*see* **Note 10**).
7. Equal parts of inocula of Oa-containing FHV, CP-containing FECT, and 35S/p19 are mixed gently. Agro-inoculation is done by infiltrating culture from underside of leaf using 1 or 3 ml syringe with no needle. At the opposite side of infiltration, block the hole with gloved finger (Fig. 2) (*see* **Note 4**).
8. If a reporter gene (e.g., eGFP) is used as the antigen sequence in FHV vector (Fig. 1), a faint fluorescence will be detected as early as 48 h post inoculation, in contrast to the brighter fluorescence when inoculating "coat protein-less" control (with only FHV vector and without CP-containing vector) (Fig. 3). This indicates successful coinoculation of both Oa-containing vector and CP containing vector (*see* **Note 11**). Plants are allowed to grow for 7 days, then proceed to vaccine collection.

3.2 Viral Nanoparticle Vaccine Extraction and Purification

All buffers should be prechilled unless otherwise mentioned. All procedures should be performed on ice where feasible.

1. Harvest inoculated leaves. Cut off the midribs of leaves, which contain less cytoplasm and are harder for grinding. Record fresh weight of leaf tissue. Mix leaf tissue with 2 volumes (2 ml per gram of fresh weight) of virion extraction buffer. Thoroughly homogenize leaves with a blender.

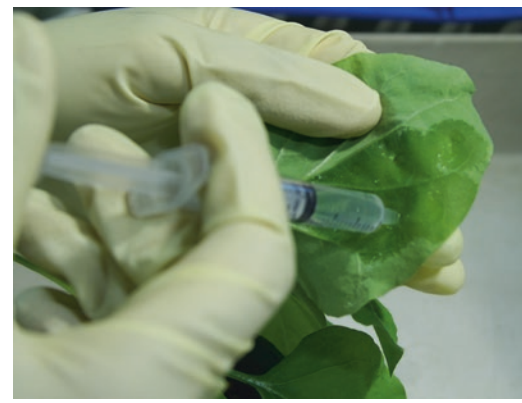

Fig. 2 Agro-inoculation technique. A needleless syringe is used to inoculate *N. benthamiana* leaves from the underside. A slow push allows agrobacterial solution to infiltrate leaf tissue. Gloved fingers gently block and support the opposite side of the leaf

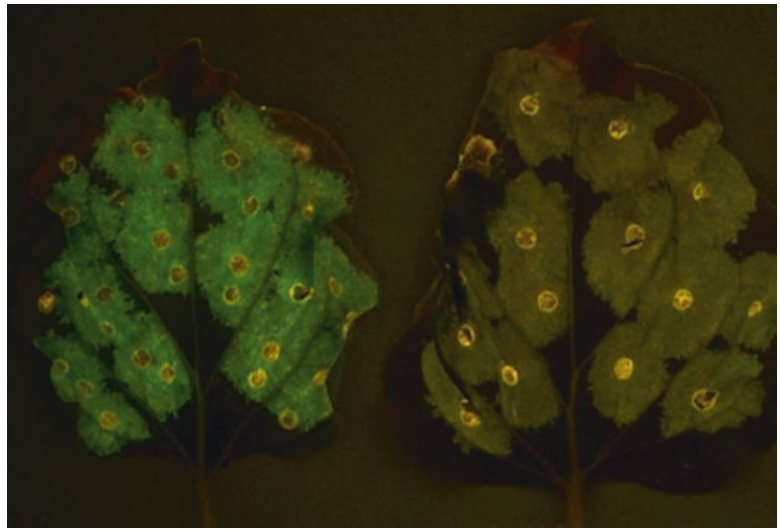

Fig. 3 Reduced fluorescence indicates trans-encapsidation. *Left*: Strong GFP fluorescence is seen with agrobacterium culture carrying Flock House viral vaccine vector with the TMV packaging signal (Oa) inoculated into leaf along with agrobacterium carrying the 35S/p19 silencing suppressor construct. *Right*: When CP-containing vector is coinoculated with this inoculum, a weaker fluorescence is observed, indicating successful trans-encapsidation of the RNA vector template

2. Pour the homogenized materials through four layers of cheese cloth into a proper sized beaker. Squeeze out the remaining liquid from cheese cloth as much as possible. Measure the pH. A small amount of liquid can be set aside for SDS-PAGE analysis (*see* **Note 12**).
3. Cover the beaker with foil and a rubber band. Insert a thermometer into the beaker. Place the beaker in 60 °C water bath for 15 min. Swirl beaker occasionally (*see* **Note 13**)
4. Agitate the beaker in an ice water bath to lower the temperature to 15 °C. Transfer the homogenate to Oak Ridge centrifuge tubes. Record the volume in each tube. Leave on ice for 15 min (*see* **Note 14**).
5. Centrifuge at 6000 × *g* for 10 min, 4 °C. Supernatant should be almost clear. Decant supernatant into new centrifuge tubes. Record volume. Another SDS-PAGE sample can be collected (*see* **Note 15**)
6. Precipitate nanoparticles by adding 20% (w/v) freshly made PEG 8000 to a final concentration of 4% (w/v). Mix well and place on ice for at least 1 h (*see* **Note 16**)
7. Centrifuge at 10,000 × *g* for 10 min at 4 °C. Remove all remaining supernatant carefully without disrupting the pellet. Resuspend pellet in half volume 50 mM Tris–HCl (pH 7.5) (*see* **Note 17**).

8. Centrifuge the resuspended solution at 10,000 × *g* for 10 min. Collect the supernatant into new tubes, record volume (*see* **Note 18**).
9. Precipitate nanoparticles again with 20% PEG/20% NaCl to a final concentration of 4% PEG/4% NaCl. Mix well and place on ice for at least 1 h.
10. Centrifuge at 10,000 × *g* for 10 min, at 4 °C. Drain supernatant as stated above.
11. Resuspend the virus pellets in appropriate volume of 10 mM phosphate buffer. Clarify nanoparticle solution by centrifuging at 10,000 × *g* for 10 min, discarding the pellet. A final SDS-PAGE sample can be collected (*see* **Note 19**).
12. Optionally, a third round of PEG precipitation can be performed in order to obtain nanoparticles of greater purity or higher concentration. This is done by repeating **steps 9–11**.
13. Bicinchoninic acid protein assay and SDS-PAGE analysis are recommended to assess the concentration and purity of nanoparticle vaccines, prior to animal tests.

4 Notes

1. Other *Agrobacterium tumefaciens* strains can also be used. However, GV3101 was found to yield the best results in our hands
2. In previous research [8], the agrobacterium strain GV3101 contains both rifampicin-resistant gene and gentamycin-resistant gene. An additional kanamycin-resistant gene, which is encoded in the plasmid backbone of FHV and FECT binary vectors, is introduced into GV3101 as well. A triple antibiotic screening is recommended for both liquid and solid culture, with final concentrations of gentamycin to 25 μg/ml, kanamycin to 50 μg/ml, and rifampicin to 10 μg/ml.
3. A 1 M $MgCl_2$ stock solution can be prepared and sterilized.
4. A "coat protein-less" control is recommended at this point, by only mixing FHV and 35S/p19 inocula and inoculating separate plant or leaf. Plant to plant and leaf to leaf variations were found. Best results are obtained by inoculating 20-day-old plants post-seeding, with 4–6 fully opened leaves. A plant older than this may have significantly reduced expression for FHV vector. However, FECT vector's expression seems less affected by plant age, by our observations. Plants should be maintained in a high humidity, 28 °C growth room, under sufficient lighting, proper fertilizing and watering conditions. It has been noticed that, over or under fertilized, and over-watered plants will result in significantly lower expression.

5. This buffer is made to 10×. A working buffer will be diluted to 1× to resuspend virus pellet.
6. Plasmids should be constructed with the gene of interest flanked by plant promoter and terminator sequences (e.g., Cauliflower mosaic virus 35S promoter and terminator [20]). In our previous application [8], viral vectors were constructed from the CB301-based plasmid, JL22 [21], which is a binary vector reduced in size to allow for the cloning of large inserts like a viral genome.
7. Shaking is normally carried out at 180–250 rpm, 28 °C, for 16–24 h. When a 28 °C shaker is not available, a room temperature incubation can also be used with a longer time and faster speed. An even and dense culture is optimal for agroinoculation. A moderate clumping pattern may occur, which can result in difficulties in resuspension, accurate O.D. measuring, and leaf inoculation. However, the final yield of protein is rarely affected by clumping.
8. For FECT- and 35S/p19-containing agrobacteria, an O.D. 600 value of 0.6–0.8 is optimal. Lower O.D. 600 values will result in reduced expression. However, the O.D. value for FHV containing agrobacterium is less important. By our observations, the protein of interest expression level of FHV vectors remains unchanged from 0.2 to 0.8 (O.D. 600), while a much higher O.D. may cause a greater necrotic effect on plant cells.
9. Draining of the supernatant has to be as thorough as possible. Alternatively, an additional washing step can be used after draining, by resuspending in 1 M $MgCl_2$, recentrifugation and draining.
10. The acetosyringone in induction media activates agrobacterium's DNA transfer activity [22, 23]. Thus a non-shaking incubation before inoculation is necessary. A 4–6 h incubation has proved to give good result in our lab. However, longer time (up to overnight) can also be applied if needed.
11. The hypothesized reason for reduced fluorescence is because of the premature binding of coat protein to Oa sequence on FHV RNA. This prevents the further expression of reporter gene on the FHV vector [8].
12. The pH of homogenized materials should be about 5.0. However, depending on the amount of leaves and expressed proteins, pH may vary between 5.0 and 6.0. Under this circumstance, pH should be adjust to 5.0 with concentrated H_3PO_4. Due to possible high concentration of protein in the homogenate, the pH meter may respond slowly. Dropwise application of acid and thorough stirring are recommended.
13. The 60 °C water bath is to aid in removal of rubisco.

14. Homogenate can be transferred into Oak Ridge tubes by using a graduated pipet, with the volume recorded. If Oak Ridge tubes are not available, other centrifuge tubes compatible with the rotor and speed can be used. The amount of homogenate in each tube should not be too low, otherwise centrifugation may not yield a solid pellet.
15. If the supernatant remains "cloudy" and large particles are visible in supernatant, it can be collected by filtering through two layers of cheese cloth into centrifuge tubes.
16. 1–2 h on ice will result in thorough precipitation. However, time can be extended to overnight if necessary.
17. When Oak Ridge tubes are not available and low speed tubes are used, centrifugation can also be conducted at 6000 × *g* for 45 min to 1 h. After draining the supernatant, tubes can be centrifuged again and the remaining supernatant can be taken out by pipetting. Alternatively, remaining supernatant can be wiped off by using cotton swabs. The resuspended solution should have a pH higher than 7.2; if not, adjust with NaOH.
18. The centrifugation in this step is preferably done at room temperature.
19. The amount of final buffer is typically 3–5 % the volume of original homogenate. However, the amount can be adjusted according to need. If the clarified solution still appears slightly green, a more thorough clarification can be performed by overnight chilling and centrifugation at maximum speed.

References

1. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C (2004) Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 303:1529–1531
2. Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, Iwasaki A, Flavell RA (2004) Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci U S A 101:5598–5603
3. Schwarz K, Storni T, Manolova V, Didierlaurent A, Sirard JC, Rothlisberger P, Bachmann MF (2003) Role of Toll-like receptors in costimulating cytotoxic T cell responses. Eur J Immunol 33:1465–1470
4. Fraile A, Escriu F, Aranda MA, Malpica JM, Gibbs AJ, Garcia-Arenal F (1997) A century of tobamovirus evolution in an Australian population of Nicotiana glauca. J Virol 71:8316–8320
5. Turner DR, Butler PJ (1986) Essential features of the assembly origin of tobacco mosaic virus RNA as studied by directed mutagenesis. Nucleic Acids Res 14:9229–9242
6. Smith ML, Corbo T, Bernales J, Lindbo JA, Pogue GP, Palmer KE, McCormick AA (2007) Assembly of trans-encapsidated recombinant viral vectors engineered from tobacco mosaic virus and Semliki Forest virus and their evaluation as immunogens. Virology 358:321–333
7. Sacher R, French R, Ahlquist P (1988) Hybrid brome mosaic virus RNAs express and are packaged in tobacco mosaic virus coat protein in vivo. Virology 167:15–24
8. Zhou Y, Maharaj PD, Mallajosyula JK, McCormick AA, Kearney CM (2015) In planta production of flock house virus transencapsidated RNA and its potential use as a vaccine. Mol Biotechnol 57:325–336
9. Kemnade JO, Seethammagari M, Collinson-Pautz M, Kaur H, Spencer DM, McCormick AA (2014) Tobacco mosaic virus efficiently targets DC uptake, activation and antigen-specific T cell responses in vivo. Vaccine 32:4228–4233

10. Lundstrom K (2003) Semliki Forest virus vectors for gene therapy. Expert Opin Biol Ther 3:771–777
11. Johnson KL, Ball LA (1999) Induction and maintenance of autonomous flock house virus RNA1 replication. J Virol 73:7933–7942
12. Francis KE, Spiker S (2005) Identification of Arabidopsis thaliana transformants without selection reveals a high occurrence of silenced T-DNA integrations. Plant J 41:464–477
13. De Buck S, Jacobs A, Van Montagu M, Depicker A (1998) Agrobacterium tumefaciens transformation and cotransformation frequencies of Arabidopsis thaliana root explants and tobacco protoplasts. Mol Plant-Microbe Interact 11:449–457
14. van der Meer IM (2006) Agrobacterium-mediated transformation of Petunia leaf discs. Methods Mol Biol 318:265–272
15. Liu Z, Kearney CM (2010) An efficient Foxtail mosaic virus vector system with reduced environmental risk. BMC Biotechnol 10:88
16. Selling BH, Allison RF, Kaesberg P (1990) Genomic RNA of an insect virus directs synthesis of infectious virions in plants. Proc Natl Acad Sci U S A 87:434–438
17. Dasgupta R, Cheng LL, Bartholomay LC, Christensen BM (2003) Flock house virus replicates and expresses green fluorescent protein in mosquitoes. J Gen Virol 84:1789–1797
18. Maharaj PD, Mallajosyula JK, Lee G, Thi P, Zhou Y, Kearney CM, McCormick AA (2014) Nanoparticle encapsidation of flock house virus by auto assembly of tobacco mosaic virus coat protein. Int J Mol Sci 15:18540–18556
19. Scholthof HB (2006) The Tombusvirus-encoded P19: from irrelevance to elegance. Nat Rev Microbiol 4:405–411
20. Odell JT, Nagy F, Chua NH (1985) Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313:810–812
21. Lindbo JA (2007) High-efficiency protein expression in plants from agroinfection-compatible tobacco mosaic virus expression vectors. BMC Biotechnol 7:52
22. Schrammeijer B, Beijersbergen A, Idler KB, Melchers LS, Thompson DV, Hooykaas PJ (2000) Sequence analysis of the vir-region from Agrobacterium tumefaciens octopine Ti plasmid pTi15955. J Exp Bot 51:1167–1169
23. Sheikholeslam SN, Weeks DP (1987) Acetosyringone promotes high efficiency transformation of Arabidopsis thaliana explants by Agrobacterium tumefaciens. Plant Mol Biol 8:291–298

Part II

Non-replicating mRNA Vectors

Chapter 5

RNActive® Technology: Generation and Testing of Stable and Immunogenic mRNA Vaccines

Susanne Rauch, Johannes Lutz, Aleksandra Kowalczyk, Thomas Schlake, and Regina Heidenreich

Abstract

Developing effective mRNA vaccines poses certain challenges concerning mRNA stability and ability to induce sufficient immune stimulation and requires a specific panel of techniques for production and testing. Here, we describe the production of stabilized mRNA with enhanced immunogenicity, generated using conventional nucleotides only, by introducing changes to the mRNA sequence and by complexation with the nucleotide-binding peptide protamine (RNActive® technology). Methods described here include the synthesis, purification, and protamine complexation of mRNA vaccines as well as a comprehensive panel of in vitro and in vivo methods for evaluation of vaccine quality and immunogenicity.

Key words mRNA vaccines, RNActive®, Protamine complexation, GC enrichment, Adjuvanticity, Stabilized mRNA

1 Introduction

Using mRNA as a basis for vaccine development provides several advantages over more conventional vaccination strategies: any protein or combination of proteins of choice can be delivered on a minimal genetic construct whose expression is intrinsically self-limiting and safe. Antigen expression in the cells of the vaccinee supports correct protein modifications and abolishes the need for elaborate protein or particle purification steps. However, the use of mRNA as a vaccine has long been hampered by the instability of the molecule, low expression levels upon mRNA delivery and insufficient immune stimulation. Several solutions have been found to overcome these problems, one of which will be introduced in the following chapter. In the described technology, termed RNActive®, mRNA is modified by enriching the guanine and cytosine (GC) content of the open reading frame (ORF) and by introducing regulatory elements (untranslated regions (UTRs) and poly(A) tail) that enhance translation efficiency and delay mRNA decay. Importantly,

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_5,

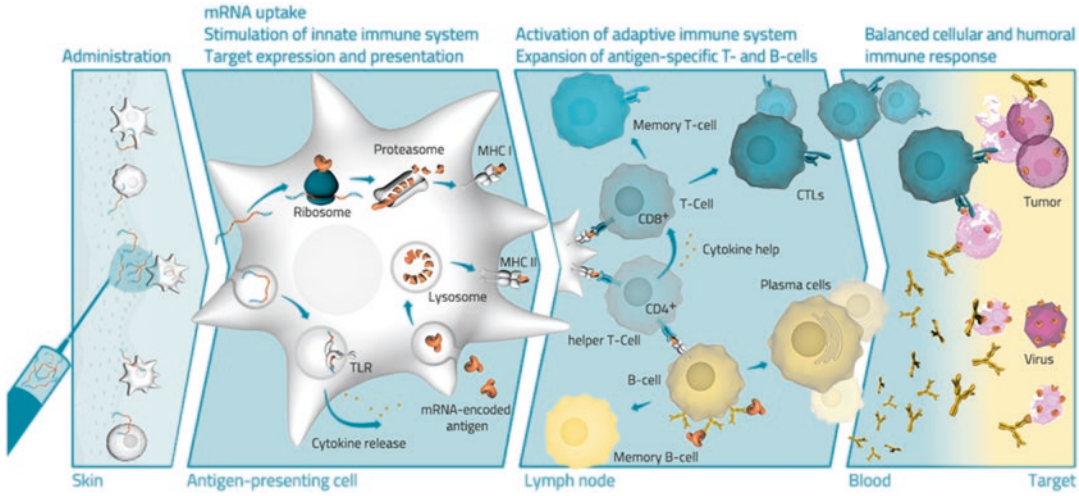

Fig. 1 Vaccination with RNActive® vaccines leads to balanced cellular and humoral responses. After intradermal application of an RNActive® vaccine, the naked antigen-coding mRNA is taken up by various cells and expressed as a protein. The protamine-complexed mRNA is recognized by innate receptors, e.g., Toll-like receptors (TLRs), triggering an activation of the innate immune system including antigen-presenting cells (APCs). The activated APCs then present peptides derived from endogenously expressed or phagocytosed antigens on MHC-I or MHC-II, respectively, which leads to an efficient priming of the adaptive immune system, the expansion of antigen-specific T and B cells, and a balanced cellular and humoral immune response

the mRNA is composed of conventional nucleotides and does not require the incorporation of chemically modified nucleotides. Introducing these mRNA sequence modifications has been shown to lead to enhanced mRNA stability and protein expression both in vitro and in vivo [1]. In order to enhance its immune-stimulatory capacity, the mRNA in this approach is partly formulated with protamine, a cationic peptide that forms stable complexes with nucleic acids. The final vaccine therefore comprises two components: naked mRNA and mRNA–protamine complexes. Upon vaccine injection, the naked mRNA serves as a translation template, while the protamine–mRNA complex triggers enhanced TLR (Toll like receptor) activation [2, 3]. A schematic drawing of the mode of action of mRNA vaccines is shown in Fig. 1. Using this technology, encouraging results have been generated both in the fields of cancer immunotherapy [4, 5] and vaccines against infectious diseases [6].

The following chapter describes the production and protamine formulation of RNActive® vaccines as well as their application and a panel of possible functional tests.

2 Materials

2.1 Media

Opti-MEM® Medium (Gibco); X-Vivo 15 (Lonza)

alpha-MEM complete: alpha-MEM medium with 10% FCS, 1% Pen-Strep, 1% L-glutamine, 10 mM HEPES, 50 μM beta-mercaptoethanol

2.2 Reagents

RNase-ExitusPlus™

Ringer's lactate solution

Lipofectamine® 2000 (Invitrogen)

100 mg/ml ketamine hydrochloride

2% xylazine

D-luciferin sodium salt: Dissolve 1 g in 50 ml DPBS, sterile-filter (0.2 μm Filter) and prepare 1 ml aliquots. Store at −80 °C in the dark.

mRNA encoding *Photinus pyralis* luciferase (Pp Luc)

Beetle-Juice BIG KIT

QuantiLum® Recombinant Luciferase

Basic Lysis Buffer: Tris–HCl (25 mM), EDTA (2 mM), glycerol (10% (w/v), Triton X-100 (1% (w/v))

PpLuc Dilution Buffer: prepare in Basic Lysis Buffer: acetylated BSA (1 g/l), DTT (2 mM)

Coating Buffer: 15 mM Na_2CO_3, 15 mM $NaHCO_3$, 0.02% NaN_3, pH 9.6

Blocking Buffer: PBS (pH 7.4) with 0.05% Tween 20, 1% BSA, 0.02% NaN_3

Blocking Buffer without sodium azide (NaN_3): PBS (pH 7.4) with 0.05% Tween 20, 1% BSA; sterile-filtered

Wash Buffer: PBS (pH 7.4) with 0.05% Tween 20

Tetramethylbenzidine (TMB)

20% sulfuric acid (H_2SO_4)

Permwash: 1× PBS with 0.5% BSA, 0.1% saponin, 0.02% NaN_3

PFEA: 1× PBS with 2% FCS, 2 mM EDT, 0.01% NaN_3

PMA (phorbol 12-myristate 13-acetate)/ionomycin

Stimuli (exemplary for HA): Influenza A (H1N1) peptide mix (0.5 μg/ml), influenza HA peptide 1 (461–469) LYEKVKSQL (5 μg/ml), influenza HA peptide 2 (518–526) IYSTVASSL (5 μg/ml), recombinant protein of A/California/07/09 (2.5 μg/ml)

Visine Intensiv

2.3 Equipment

U-100 Insulin 0.5 ml syringe, BD Micro Fine™+, 0.30 mm (30 G) × 8 mm

1 ml Sub-Q syringe, BD Plastipak™, 26G × ½″ (0.45 mm × 12.7 mm)

Animal clipper ISIS GT 420

Heated Operating Pad with Control Unit

IVIS Lumina II System

Living Image® software

Plate Reader Synergy™ HT

Tissue Lyser

LIA plates white 96-well flat bottom

96-well Maxisorp ELISA plates (colorless flat bottom)

Tecan Sunrise ELISA plate reader

3 Methods

3.1 mRNA Synthesis

A detailed protocol for the synthesis of mRNA has recently been published [7]. While the published protocol uses modified nucleotides, it can also be applied to the generation of unmodified mRNA. Moreover, most steps utilize commercially available kits, the leaflets of which provide detailed information on experimental execution. Thus, the following outline focuses on the explanation of the critical steps and is aimed at providing helpful notes.

1. Minimal requirements for the sequence of the DNA template for in vitro transcription are a T7, T3 or Sp6 promoter and the gene of interest. For efficient translation, the translational start site should be as close to the Kozak consensus sequence (A/GCC**ATG**G; start codon in bold) as possible. The use of 5′- and/or 3′-UTR elements can further improve protein expression. Here, the widely used globin sequences are a good start [8]. Alternatively, the natural mRNA sequence of the gene of interest can be used. In addition, the template may code for a poly(A) tail as an alternative to enzymatic polyadenylation (*see* **step 7**). Importantly, if plasmid DNA is used as a template, the mRNA sequence should be followed by a unique restriction site (*see* **step 3**).
2. The DNA template for in vitro transcription can be generated by standard methods of choice (*see* **Note 1**). Emphasis should be put on using high quality DNA preparations, i.e., if plasmid DNA is used as a template, the method of choice should provide material free of endotoxins.
3. To avoid undesired sequences contaminating the mRNA, circular DNA has to be linearized with a restriction enzyme that cuts directly downstream of the putative mRNA sequence (*see* **Note 2**). A linear template should also be cut in a similar manner, if its downstream and does not coincide with the end of the putative mRNA.
4. Linearized DNA is purified by phenol–chloroform extraction, followed by precipitation.
5. For in vitro transcription, RNA transcription kits from suppliers such as CellScript or ThermoFisher can be used. If co-transcriptional instead of enzymatic (*see* **step 7**) capping is applied, rGTP has to be partially replaced by a cap analog in the transcription mix (*see* **Note 3**). It is important to remove the template DNA by digestion with RNase-free DNase after the completion of RNA synthesis.
6. Small amounts of RNA can be purified for further enzymatic treatments (*see* **step 7**), most easily by spin column chromatography (Qiagen, Macherey-Nagel). Amounts that vastly exceed the capacity of such spin columns can be purified by LiCl precipitation.

7. If the RNA has not been capped during in vitro transcription, the Capping System and 2′-O-Methyltransferase kit from CellScript can be used to enzymatically generate a cap1 structure. If a poly(A) tail is to be added enzymatically, kits applying either yeast (USB) or bacterial (e.g., CellScript) poly(A) polymerase can be deployed. If both enzymatic modifications of the RNA are applied, the capping product should be purified according to **step 6** before polyadenylation.

3.2 mRNA Purification

After the last step of mRNA synthesis and before formulation of the mRNA vaccine, impurities have to be removed from the preparation. Contaminants may reduce the activity of the mRNA vaccine or elicit undesired biological effects. Moreover, they may cause differences among independent preparations leading to unreliable results.

LiCl precipitation, possibly complemented by phenol/chloroform extraction, usually provides good depletion of most contaminants such as proteins, DNA, and non-incorporated nucleotides. The addition of spin column chromatography removes small abortive transcripts but can only be employed for small quantities of mRNA. Overall, purification by HPLC is highly recommended as the method of choice. This technique does not only remove all aforementioned contaminants but has the potential to even deplete larger abortive transcripts that can occur in in vitro transcription reactions. A detailed protocol for HPLC purification of mRNA has recently been published [9].

3.3 Protamine Formulation

Good vaccines provide both antigen and stimulation of the innate immune system, i.e., adjuvanticity. Although mRNA is able to interact with various cellular sensors leading to cytokine secretion and an activation of the innate immune system, mRNA alone might not be sufficiently immune-stimulatory in the context of a vaccine [10]. Hence, RNActive® vaccines contain mRNA complexed with protamine as an immunostimulatory component [11].

1. Dissolve the protamine in Ringer lactate solution.
2. While stirring, slowly add the protamine-Ringer lactate solution to half of the mRNA until you reach a weight ratio of mRNA and protamine of 2:1.
3. Stir the solution for another 10 min to ensure the formation of stable complexes.
4. Add the remaining half of free mRNA and stir briefly.
5. Adjust the final concentration of the vaccine with Ringer lactate solution.

3.4 Transfection

Expression of mRNA-encoded antigens can be tested after transfection by flow cytometry (if antigen is membrane-bound or cytoplasmic), Western blotting or ELISA (if mRNA-encodes a secretory

protein). Here, a detailed protocol of a transfection with mRNA is presented. All numbers specified below are for transfection in a 6 well format. If other formats are used, adjust all material employed accordingly.

1. On day 0, HEK 293T cells are seeded at a concentration of 5×10^5 cells/well in a 6-well plate to ascertain 70–90% of confluency on the day of transfection (day 1).
2. For transfection, a ratio of 2 μl of Lipofectamine® reagent to 1 μg of RNA is used. Cells are transfected with 1 and 2 μg of mRNA. Transfection is performed in duplicates.
3. On the day of transfection, an appropriate volume of Lipofectamine® reagent is prepared in Opti-MEM® medium and incubated at room temperature for 5 min.
4. 1 and 2 μg of mRNA are diluted in Opti-MEM® medium. If required, pre-dilute mRNA to a concentration of 0.5 μg/μl. Mix Lipofectamine® reagent and Opti-MEM®-diluted mRNA gently in a total volume of 500 μl at a 1:1 ratio (v/v) and incubated at room temperature for 20 min.
5. Wash cells twice with 500 μl DMEM medium containing glutamine but without FBS. Add 2 ml of DMEM to the cells.
6. Add 500 μl of mRNA–lipid complexes to each well. Mix gently.
7. Incubate cells at 37 °C in a CO_2-incubator for 4–6 h.
8. After the incubation, replace the medium with 2 ml of fresh DMEM medium containing 10% FBS.
9. Incubate cells at 37 °C in a CO_2-incubator for 24–48 h. For establishing expression levels of a protein, it may be useful to analyze both time points.
10. Depending on the cellular localization of the mRNA-encoded protein, cells (for membrane-bound and intracellular proteins) or supernatants (for secretory proteins) are collected and analyzed by flow cytometry, Western blotting, or ELISA (*see* **Note 4**). Figure 2 depicts a representative flow cytometry histogram showing the expression of HA protein of H1N1 influenza virus in 293T cells after transfection with an mRNA construct.

3.5 In Vitro Stimulation

RNA can be recognized by endosomal (e.g., TLR7, TLR3) and cytoplasmic (RIG-I, MDA-5) receptors within a cell which results in the induction of proinflammatory cytokines. Human peripheral blood mononuclear cells (PBMCs) express both types of receptors and can therefore be used to test the stimulatory capacity of mRNA vaccines in vitro.

1. Isolate PBMCs under sterile conditions from whole blood or use frozen PBMCs (*see* **Note 5**). Count the cells.

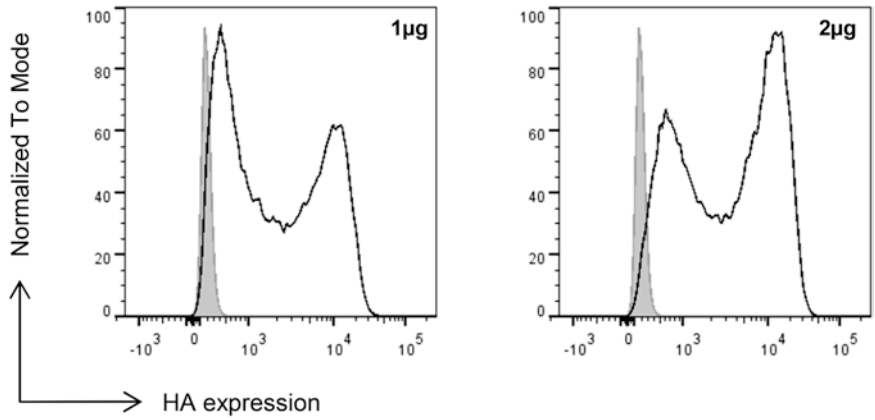

Fig. 2 Expression of HA protein of H1N1 Influenza virus in HEK 293T cells after mRNA transfection. HEK 293T cells were transfected with 1 µg (*left panel*, *solid black line*) or 2 µg (*right panel*, *solid black line*) of mRNA coding for HA protein of H1N1 influenza virus. Cells were collected 24 h after transfection and HA expression was evaluated by flow cytometry. Mock-transfected cells were used as a negative control (*filled histogram*)

2. Dilute PBMCs in warm X-Vivo 15 medium (with a final concentration of 1% penicillin–streptomycin) to a concentration of 2×10^6 cells/ml. Invert the tube three times to mix.
3. Pour the cell suspension into a sterile tissue culture dish. Using a multichannel pipette, pipette 100 µl of the cell suspension (2×10^5 cells) into each well of a 96-well flat bottom plate.
4. Close the lid and place the plate in a 37 °C incubator for 1–4 h.
5. After the incubation, add 100 µl of the stimulants at an appropriate concentration. Dilute mRNA to a final concentration of 40 µg/ml and add mRNA and positive controls (*see* **Note 6**) to each well containing 100 µl cell suspension (final volume is 200 µl). Use PBMCs incubated in medium without stimulants to determine the background cytokine production (negative control). Test each sample in triplicates.
6. Pipette up and down to mix. Place the lid on the culture plate and incubate at 37 °C (in a humidified incubator/5% CO_2) for 16–24 h.
7. Transfer 180 µl of cell free supernatant to a 96-well round bottomed plate using a multichannel pipette. Supernatants can be used directly or may be frozen at –20 °C until required.
8. Use the supernatants to test cytokine/chemokine production (e.g., TNF, IFN-α, CXCL10) by ELISA or CBA (cytometric bead array). Figure 3 shows the TNF concentration in supernatants of PBMCs stimulated with various mRNA vaccine formulations.

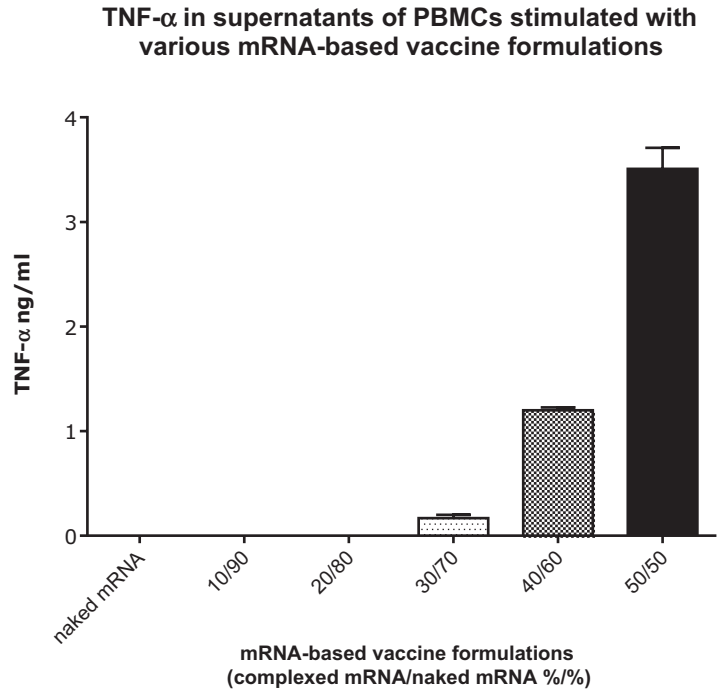

Fig. 3 In vitro stimulation of PBMCs with various mRNA-based vaccine formulations. RNActive® vaccine is a two-component vaccine consisting of protamine-complexed mRNA (component 1) and naked mRNA (component 2). PBMCs were stimulated with 40 μg of various formulations (different ratios of component 1 to component 2; in %) of mRNA-based vaccine. 24 h after stimulation, supernatant was collected and tested by ELISA for TNF expression

3.6 Intradermal and Intramuscular Injections

The intradermal and intramuscular routes have successfully been used to apply mRNA vaccines and induce strong immune responses [6, 12]. The intradermal route offers the benefit that the antigen is expressed in close proximity to the antigen presenting cells of the epidermis (e.g., Langerhans cells) and the dermis (e.g., dermal dendritic cells, macrophages), while the advantage of the intramuscular route is the simplicity of the application. Further routes of administration for mRNA vaccines include intranodal [13] or systemic application [14].

3.6.1 Intraperitoneal Anesthesia

1. Calculate the volume of ketamine hydrochloride (1 μl ketamine hydrochloride [100 mg/ml]/g bodyweight of the mouse) and xylazine (0.5 μl xylazine [2%]/g bodyweight of the mouse) required. The anesthetic is prepared to a total volume of 5 μl/g bodyweight of the mouse with sterile 1× PBS.
2. Mix components in a Falcon tube, invert several times and fill 1 ml Sub-Q syringes with the required volumes.
3. Pick the mouse up by the base of the tail with one hand and place the mouse on the cage lid.

4. Grip the mouse by the fold of skin over the neck and back using the thumb and index finger of your other hand. Hold the base of the tail between your palm and ring finger. The hind left leg can be secured between the ring and little finger.
5. Tilt the head of the restrained mouse slightly lower than the body so that the posterior end is elevated.
6. Insert the needle of a 1 ml Sub-Q syringe in at a 10°–30° angle between the needle and the abdominal surface in the lower left quadrant of the mouse's abdomen.
7. Inject 5 µl of anesthetic/g bodyweight of the mouse intraperitoneally. Perform treatment on anesthetized mice. Afterwards apply Visine Intensiv to both eyes to prevent them from drying out. Place cages without lid on a heated operating pad set to 39 °C for 1 h.

3.6.2 Intradermal Injection in the Back

1. Prepare 0.5 ml insulin syringes with 100 µl of mRNA solution/mouse (*see* **Note 7**). Cap syringes. The syringes with the mRNA solution can be stored at room temperature until use.
2. Anesthetize the mouse as described above.
3. Shave the area to be injected (Fig. 4a).
4. Place the mouse on a table with the area to be injected, usually the back, facing upwards.
5. Using straight tweezers, pull up a fold of skin so that 0.5 mm of skin protrude above the upper edge of the tweezers as shown in Fig. 4b.
6. Using a 0.5 ml insulin syringe, pierce the fold of skin very closely to the surface.
7. Keep the needle parallel to the tweezers and the bevel of the needle facing upwards and insert the needle so that half of it is inserted into the skin (Fig. 4c). Mind that the needle does not pierce the skin on the other side of the fold. If this happens, reposition the tweezers and insert the needle at a different position.
8. Remove the tweezers and keep the fold of skin pulled up with the needle as shown in Fig. 4d.
9. Slowly inject the solution (maximum 25 µl/ injection site) so that a blister forms as depicted in Fig. 4e. Resistance must be felt while injecting the solution. If no resistance is perceivable, remove the needle and start again (*see* **Note 8**).
10. Several injections can be administered. However, injection blisters should at least be 1 cm apart.
11. Place the mouse back in its cage, apply Visine Intensiv to both eyes and place the cage without lid on a heated operating pad for 1 h.

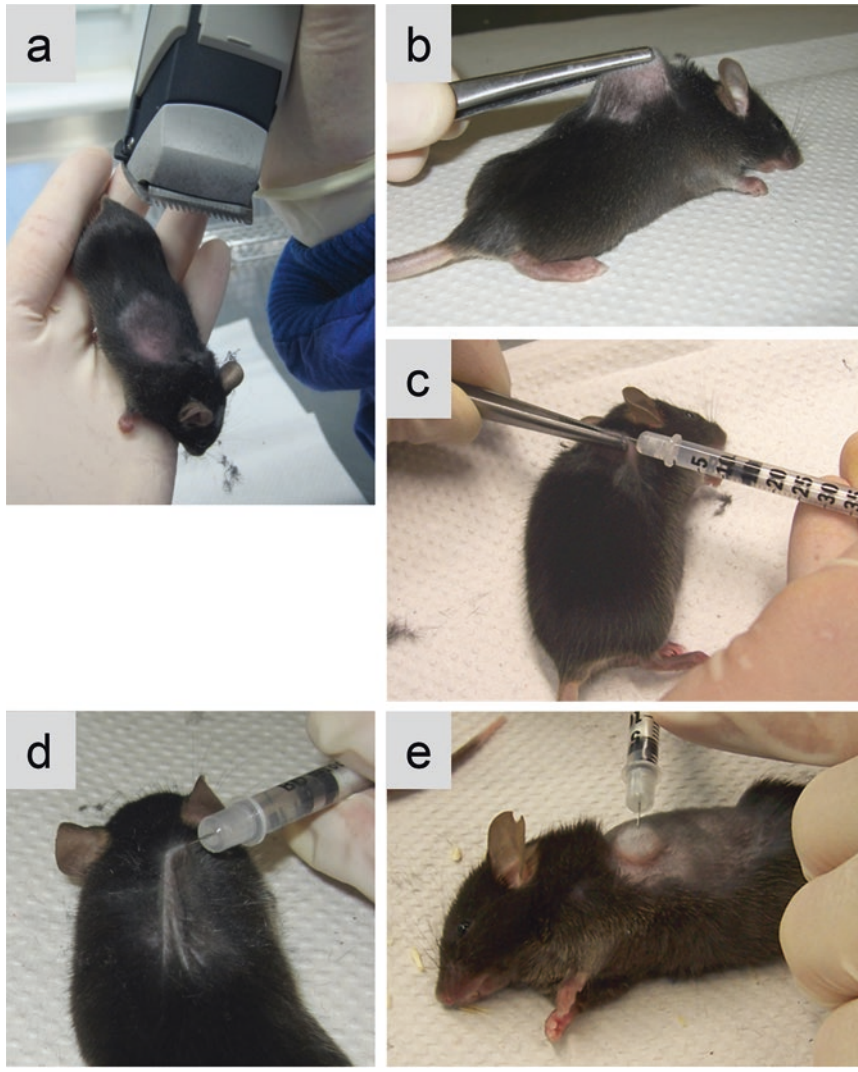

Fig. 4 Intradermal injection. See text for details

3.6.3 Intramuscular Injection

1. Anesthetize the mouse as described above and place the mouse on its back.
2. Using a cotton swab, wipe the leg with 70% ethanol.
3. Lightly pull the hind leg to a straight position with the thumb and forefinger.
4. Keep the mouse steady using the middle finger.
5. Insert the needle in the middle of the posterior tibialis muscle.
6. Inject the RNA solution. Use 25 µl/injection site for the tibialis muscle.

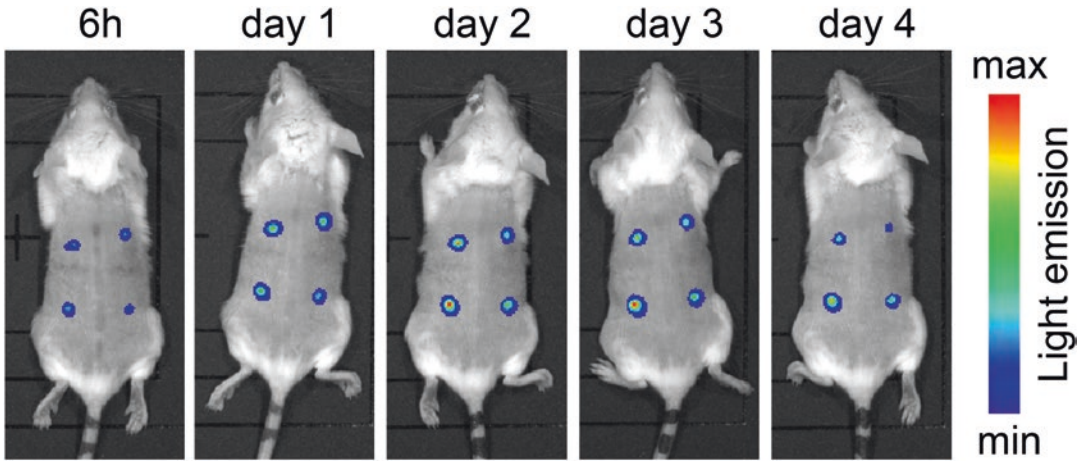

Fig. 5 Luciferase expression in vivo after intradermal injection. Balb/c mice were injected intradermally at four sites with 10 μg of firefly luciferase-encoding mRNA per site. Luciferase expression was detected by in vivo imaging after injection of luciferin at different time points

3.7 Detection of Luciferase Expression After Intradermal Injection of Luciferase mRNA by In Vivo Imaging

Luciferase-encoding mRNA is a useful tool to measure mRNA expression in vivo and can for example be used to compare transfection efficiencies of different mRNA formulations or to visualize targeting of mRNAs. It is especially useful to measure expression kinetics after intradermal injection (*see* Fig. 5).

1. Use Balb/c mice for intradermal injection. Anesthetize and shave mice as described above.
2. Inject 5 μg of naked PpLuc-encoding mRNA in Ringer's lactate buffer per site either intradermally or intramuscularly. If intradermal injection is performed, you can inject at four sites on the back as shown in Fig. 5.
3. Measure luciferase expression at 24 h after injection or at multiple time points as shown in Fig. 5.
4. Anesthetize mice by intraperitoneal injection of a ketamine hydrochloride and xylazine mixture as described above.
5. Inject 150 μl of Luciferin solution (20 g/l) intraperitoneally (i.p. 3 mg/150 μl).
6. Perform optical imaging on the IVIS Lumina II System 10 min after Luciferin injection.

3.8 Detection of Luciferase Expression in Tissue Lysates

In addition to in vivo imaging, expression of Luciferase-encoding mRNA can also be measured in tissues lysates.

*3.8.1 Preparation of PpLuc Measuring Buffer and PpLuc Standard (See **Note 9**)*

1. Reconstitute Beetle-Juice with the included D-luciferin and ATP as described in the manual. Store aliquots at −20/−80 °C.
2. Dilute PpLuc in PpLuc dilution buffer to a final concentration of 100 ng/ml (=1.64 nM). Freeze 25 μl aliquots and store at −80 °C.

3.8.2 Preparation of Tissue Samples

1. Euthanize mouse and shave injection site.
2. Prepare the skin or muscle tissue and place in 2 ml Eppendorf tube containing metal beads (*see* **Note 10**).
3. Freeze Eppendorf tube in liquid nitrogen and store at –80 °C.

3.8.3 Preparation of Tissue Lysates

1. Store shaker boxes of Tissue Lyser at –20 °C for 30 min.
2. Supplement 10 ml Basic lysis buffer (at room temperature) with 20 µl DTT and 100 µl PMSF.
3. Place frozen tissue samples with metal beads into Tissue Lyser and homogenize skin or muscle tissues at 30 Hz for 3 or 2 min, respectively.
4. Add 600 µl of lysis buffer for skin or 800 µl for muscle sample and homogenize again at 30 Hz for 4 min.
5. Spin down samples at 15,000 rcf and 4 °C for 10 min.
6. Transfer supernatant to new Eppendorf tube and freeze until use.

3.8.4 Preparation of LIA Plate with Samples and PpLuc Standard

1. Thaw PpLuc Measuring Buffer and wrap the tube in aluminum foil. Make sure the buffer is at room temperature (20–25 °C) during the complete measurement.
2. Place white LIA plate with flat bottom on ice and keep on ice until measurement.
3. Thaw tissue lysates and vortex. Store samples on ice.
4. Transfer 50 µl of each sample to the LIA-plate. Leave the first 8 wells of the first plate empty. They will be used for the PpLuc standards and blanks.
5. Titrate PpLuc standard. Pipette 50 µl of acetylated BSA [20 g/l] into an Eppendorf tube and add 2 µl of DTT [1 M] and 948 µl of Basic Lysis Buffer. Vortex the tube. Prepare three Eppendorf tubes with 180 µl of PpLuc Dilution Buffer. Pipette 20 µl of the 100 ng/ml PpLuc Standard into the first Eppendorf tube, mix and transfer 20 µl to the next Eppendorf tube. Repeat to prepare Eppendorf tubes with PpLuc concentrations of 10, 1 and 0.1 ng/ml.
6. Transfer 50 µl of each standard and the PpLuc dilution buffer in duplicates to the LIA-plate.
7. Immediately proceed to the measurement using a suitable device, e.g., the Plate Reader Synergy™ HT.

3.9 Prophylactic Vaccination of Mice

1. Prepare antigen-encoding mRNA by T7 polymerase-based in vitro run-off transcription, e.g., mRNA encoding influenza hemagglutinin as published in Petsch et al. [6].
2. Select the mouse strain depending on the requirements of the planned analyses.

3. Anesthetize and shave mice as described above for intradermal injection.
4. Dilute 80 μg of antigen-encoding mRNA in 100 μl Ringer's lactate buffer (*see* **Note 11**)
5. Perform intradermal injections at 4 sites on the back as described above. Use 20 μl/injection site.
6. Perform booster immunization 21 days after prime immunization with the same amounts of RNA. If required, add a second boost immunization another 21 days later.
7. T cell responses are typically analyzed from splenocytes 7 days after boost immunization by intracellular cytokine staining and flow cytometry as described below. Splenocytes can also be prepared at a later time point. However, this will yield lower frequencies of antigen-specific T cell since the T cell response will already be in the contraction phase.
8. Antibody responses can be determined from mouse sera as described below. For this, blood samples are typically taken 21 days after prime and 14 days after boost vaccinations.

3.10 Assessing Immunogenicity of mRNA Vaccinations

Immunogenicity of mRNA vaccination can be assessed by determining antibody titers induced upon vaccination. For this, an ELISA (enzyme-linked immunosorbent assay) is performed, which determines the titers of antibodies able to bind to a specific antigen. Importantly, an ELISA will detect all binding antibodies, which includes both antibodies flagging the antigen and antibodies able to neutralize the targeted virus.

Protection against many pathogenic viruses can be mediated via virus neutralizing antibodies, which are functional antibodies able to directly inhibit viral infectivity. In order to analyze this antibody subset, virus neutralizing titers (VNTs) need to be determined. Depending on the virus, this is typically done via plaque assays, which asses the ability of a serum to inhibit the cytopathic effect (CPE) of a virus or, if the virus is not lytic, by immunofluorescence staining or cell-based reporter assays. A special case to determine functional titers in sera are HI (hemagglutination inhibition) assays, which detect the ability of a serum to block the binding between receptors on red blood cells to the hemagglutinin glycoprotein on the surface of certain viruses (e.g., influenza virus). This assay is not directly a test for virus neutralization but monitors the ability of a serum to block viral receptor binding.

3.10.1 ELISA

The ELISA described below is specific for serum IgG titers but can be adapted to detect all antibody serotypes isolated from serum or mucus by choosing different detection antibodies. To assess the immunogenicity of an mRNA vaccine, different time points after vaccination should be analyzed. Typically, the highest antibody titers can be expected at approximately 2 weeks after the last vaccination.

1. Choose an appropriate coating reagent. Ideally, coat with the protein encoded by the mRNA used for vaccination. If the antigen is encoding for a viral surface antigen, inactivated virus may alternatively be employed for coating.
2. Prepare 11 ml of coating buffer with the desired concentration of coating reagent per plate. If the optimal concentration of the coating reagent is unknown, perform test experiment to establish a suitable concentration.
3. Using a multichannel pipette, pipette 100 µl of coating solution into each well of a 96 well plate.
4. Cover the plate with a lid, wrap in Parafilm, and incubate at 4 °C overnight.
5. Discard the solution and remove all residual liquid by patting the plate on tissue paper several times.
6. Wash the plate three times with 200 µl of wash buffer. Remove all residual liquid after every washing step.
7. Pipette 200 µl of blocking buffer into each well using a multichannel pipette.
8. Cover the plate with a lid, wrap in Parafilm, and incubate at 37 °C for 2 h.
9. Discard the solution and remove all residual liquid.
10. For testing 1:50 serum dilutions: For each serum to be tested, add 122.5 µl of blocking buffer in a well in row A and pipette 100 µl of blocking buffer in the rows underneath (row B-H). Pipette 2.5 µl of each serum in a well in row A and mix by pipetting up and down. Transfer 25 µl from row A to row B. Mix by pipetting up and down. Repeat for residual rows. In row H, mix the wells carefully and discard 25 µl of the solution in this last dilution step.

    If necessary, adjust starting dilution and dilution steps.

    Always include wells filled with blocking buffer as a background control on every plate.
11. Cover the plate with a lid and incubate at room temperature for 2–4 h.
12. Discard the solution and remove residual liquid.
13. Wash the plate three times with 200 µl of wash buffer. Remove all residual liquid after every washing step.
14. Prepare 11 ml of blocking buffer without NaN_3 with the appropriate detection antibody per plate.
15. Using a multichannel pipette, pipette 100 µl of the diluted detection antibody into each well.
16. Cover the plate with a lid and incubate at room temperature for 1–1.5 h.

If detection is performed with a biotin coupled detection antibody followed by an incubation step with HRP-streptavidin, wash three times in 200 µl wash buffer and incubate with a suitable dilution of HRP-streptavidin blocking buffer without NaN_3 at room temperature for 30 min.

17. Discard the solution and remove all residual liquid.
18. Wash the plate three times with 200 µl of wash buffer. Remove all residual liquid after every washing step.
19. Add 100 µl of TMB substrate into each well and incubate at room temperature. Stop the reaction with 100 µl of 20% H_2SO_4 into each well when the serum wells start turning blue or according to the manufacturer's instructions.
20. Measure the absorbance at a wave length of 450 nm (*see* **Note 12**).

3.10.2 Intracellular Cytokine Staining (ICS)

An important readout for determining vaccine efficacy is the analysis of antigen specific T cells which can be accessed via intracellular cytokine staining (ICS). In the context of mRNA vaccines, both $CD4^+$ and $CD8^+$ responses are generally detectable. For this, effector $CD4^+$ and $CD8^+$ T cells are analyzed for the presence of intracellular cytokine production, typically IFNγ, TNF, and IL-2, upon antigen specific stimulation. $CD8^+$ T-cells can be stimulated with peptides of an optimal length of 8–10 amino acids, while $CD4^+$ T cell stimulation can be performed with either proteins or peptides of approximately 15 amino acids in length. For simultaneous stimulation of both $CD4^+$ and $CD8^+$ T cells, peptide pools featuring overlapping 15mer peptides may be used.

1. Count splenocytes (either freshly isolated or from frozen sample) (*see* **Note 13**).
2. Pipette 2×10^6 cells/well for each sample into a 96-well round bottom plate and note plate layout. For frozen cells, optionally use 3 wells with 2×10^6cells each.

   Prepare one additional well for PMA/Ionomycin positive control and one additional well for unstained cells.
3. Prepare stimulants by diluting to a suitable final concentration together with α-CD28 (final concentration of 2.5 µg/ml) in alpha-MEM complete.
4. For a positive control, prepare PMA/ionomycin (final concentration PMA 5 ng/ml, Ionomycin 500 ng/ml) in alpha-MEM complete.
5. Centrifuge the plate (500 rcf, 4 °C, 3 min), discard supernatant and resuspend the cells by pipetting up and down with a multichannel pipette or by vortexing carefully.
6. Pipette 200 µl of stimulant plus α-CD28 antibody or PMA/Ionomycin positive control into the relevant wells.

7. Mix each well by pipetting up and down with a multichannel pipette and incubate the plate at 37 °C. The incubation time is dependent on the stimulant employed. In general, use 24 h or 1 h for stimulation with proteins or peptides, respectively.
8. Block protein secretion with Brefeldin A and/or Monensin. For detection of IFNγ, TNF, and IL-2, the following protocol has been employed with good results: Dilute BD GolgiPlug™ 1:200 and BD GolgiStop™ 1:300 in alpha-MEM complete and pipette 50 µl/well to all stimulated wells (BD Golgi Plug™ final 1:1000 and BD GolgiStop™ 0.67:1000). Mix each well and incubate at 37 °C for additional 5–6 h (*see* **Note 14**).
9. Centrifuge the plate (500 rcf, 4 °C, 3 min), discard supernatant and resuspend the cells. Add 200 µl of alpha-MEM complete medium to each well.
10. Store the plate at 4 °C overnight.
11. Centrifuge the plate (500 rcf, 4 °C, 3 min), discard supernatant and resuspend the cells. Wash cells twice in 200 µl PBS.
12. Perform life/dead staining. This protocol has been established using dye for 405 nm excitation (LIVE/DEAD™ Fixable Aqua Stain (Molecular Probes)). Pipette 200 µl of Aqua stain solution at a dilution of 1:1000 in PBS into each well for life/dead staining (without unstained control). Incubate the cells in the dark at 4 °C for 30 min.
13. Centrifuge the plate (500 rcf, 4 °C, 3 min), discard supernatant and resuspend the cells. Wash cells twice with 200 µl PBS^+ 0.5 % BSA
14. Prepare a mix of the following antibodies/FcγR-block for cell surface staining: CD4-V450 (1:200); CD8 PE-Cy7 (1:200); Thy1.2-FITC (1:300); FcγR-block (1:100) (in PBS + 0.5 % BSA for 100 µl/well).
15. Centrifuge the plate (500 rcf, 4 °C, 3 min), discard supernatant and resuspend the cells.
16. Pipette 100 µl of the antibody-mix into each well (excluding the unstained control) and incubate the cells at 4 °C for 30–45 min.
17. Centrifuge the plate (500 rcf, 4 °C, 3 min), discard supernatant and resuspend the cells. Wash cells twice with 200 µl PBS + 0.5 % BSA per well.
18. Pipette 200 µl of cell fixing and permeabilization solution (Cytofix/Cytoperm) into each well and incubate in the dark at room temperature for 20 min. Optional: Incubate at 4 °C overnight.
19. Centrifuge the plate (500 rcf, 4 °C, 3 min), discard supernatant and resuspend the cells. Wash the cells twice with 200 µl of washing buffer (Permwash).

20. Optional: block the cells with 100 µl of Permwash + 2% rat-serum. Incubate the cells in the dark at room temperature for 15 min. Centrifuge the plate (500 rcf, 4 °C, 3 min), discard supernatant and resuspend the cells.

    This step is recommended to reduce background for intracellular staining with cytokines produced at low levels or unknown antibodies.

21. Prepare a mix of the following antibodies (1:100) for intracellular staining: IFNγ-APC; TNF-PE; IL-2-PerCpCy5.5 or IL-17-PerCPCy5.5 (in Permwash for 100 µl/well).
22. Pipette 100 µl of the antibody-mix into each well (excluding the unstained control) and incubate in the dark at 4 °C for 30 min.
23. Centrifuge the plate (500 rcf, 4 °C, 3 min), discard supernatant and resuspend cells. Wash cells twice with 200 µl of Permwash.
24. Centrifuge the plate (500 rcf, 4 °C, 3 min) discard supernatant and resuspend cells in 200 µl of PFEA per well.
25. If necessary, store the plate in the dark at 4 °C for a maximum of 2 days until FACS analysis.

3.10.3 Challenge Experiments

In order to proof the efficacy of a given vaccination, a challenge experiment might be required. This is especially necessary if a clear correlate of protection for a given pathogen is not known. This can sometimes be performed in the context of a human challenge model (e.g., for Malaria or RSV (respiratory syncytial virus)) but mostly requires the use of a suitable animal model system.

4 Notes

1. Usually, plasmid preparations are used as template for in vitro transcription. Kits for the isolation of high quality plasmid DNA can be obtained from various suppliers such as Qiagen and Macherey-Nagel. Alternatively, templates could be directly generated by polymerase chain reaction [15]. While the latter approach may overcome cloning difficulties and save time, template fidelity will be usually lower compared to plasmids.
2. For linearization, 5′-overhangs are preferred over blunt ends over 3′-overhangs, since the latter can cause undesired mRNA synthesis independent of a T7, T3 of Sp6 promoter that may compromise your vaccine.
3. If not part of the RNA transcription kit, cap analogs can be purchased for instance from TriLink. While different analogs are available, the anti-reverse cap analog (ARCA) should give the

best expression results. The most widely used ratio of cap analog and rGTP is 4:1 which appears to be the best compromise for getting good capping efficiency (about 80%) and high RNA yield. Increasing the ratio will improve capping efficiency at the expense of RNA yield, whereas reducing the ratio will provide more RNA with a lower portion of molecules being capped.

4. For detection of membrane-bound proteins, either Western blotting or FACS analysis can be used. For FACS analysis, use detach buffer (40 mM Tris–HCl pH 7.5/150 mM NaCl/1 mM EDTA) to remove cells from the plates to prevent cleavage of membrane-bound proteins by trypsin. The best methods to detect cytoplasmic proteins are Western blotting or FACS analysis for which intracellular staining must be performed. Expression of soluble proteins can be shown by ELISA or Western blot analysis.
5. Due to donor to donor variability, use PBMCs isolated from 2 to 3 different donors.
6. The following reagents can be included in the assay as positive controls: R848 (TLR7/8 agonist; InvivoGen; 10 ng/ml to 10 μg/ml) and poly(I:C) HMW (TLR3 agonist; InvivoGen; 30 ng/ml to 10 μg/ml).
7. Handle RNA in a flow cabinet and use RNase-free reagents and filter tips to avoid RNase contaminations. Clean working surfaces with an RNase decontamination solution before use, e.g., with RNase-ExitusPlus™.
8. In contrast to intradermal injections, no resistance is felt during inadvertent subcutaneous injections. The blister is less clearly defined and more oval.
9. Addition of acetylated BSA is required to stabilize the Pp Luc standard. This is not required for cell or tissue samples.
10. Skin samples should be free of residual hair or fat, which can interfere with the measurements.
11. 80 μg are generally a good starting point for assessing immunogenicity. Lower amounts of RNA may be tested once the immunogenicity of the vaccine has been established.
12. If you experience a high level of background try using 1% milk instead of BSA or a higher percentage of BSA in the blocking buffer. Make sure the ELISA plate does not dry out at any point during the experiment and carefully follow instructions concerning the washing steps. If background appears only in single wells, this might be due to air bubbles in the wells which have to be removed before measurement.
13. Freshly isolated splenocytes can be stored at 4 °C overnight. If frozen splenocytes are employed, the sample will contain a higher percentage of dead cells.

14. Using a different panel of cytokines will require adjustments in the choice of inhibitors of protein secretion and their incubation times.

Acknowledgements

This work was partly funded by the German Federal Ministry of Education and Research (BMBF, grant number: 031A061A).

References

1. Schlake T, Thess A, Fotin-Mleczek M, Kallen K-J (2012) Developing mRNA-vaccine technologies. RNA Biol 9:1319–1330. doi:10.4161/rna.22269
2. Kallen K-J, Heidenreich R, Schnee M et al (2013) A novel, disruptive vaccination technology: self-adjuvanted RNActive(®) vaccines. Hum Vaccin Immunother 9:2263–2276. doi:10.4161/hv.25181
3. Kallen K-J, Theß A (2014) A development that may evolve into a revolution in medicine: mRNA as the basis for novel, nucleotide-based vaccines and drugs. Ther Adv Vaccines 2:10–31. doi:10.1177/2051013613508729
4. Kübler H, Scheel B, Gnad-Vogt U et al (2015) Self-adjuvanted mRNA vaccination in advanced prostate cancer patients: a first-in-man phase I/IIa study. J Immunother Cancer 3:26. doi:10.1186/s40425-015-0068-y
5. Sebastian M, Papachristofilou A, Weiss C et al (2014) Phase Ib study evaluating a self-adjuvanted mRNA cancer vaccine (RNActive®) combined with local radiation as consolidation and maintenance treatment for patients with stage IV non-small cell lung cancer. BMC Cancer 14:748. doi:10.1186/1471-2407-14-748
6. Petsch B, Schnee M, Vogel AB et al (2012) Protective efficacy of in vitro synthesized, specific mRNA vaccines against influenza A virus infection. Nat Biotechnol 30:1210–1216. doi:10.1038/nbt.2436
7. Pardi N, Muramatsu H, Weissman D, Karikó K (2013) In vitro transcription of long RNA containing modified nucleosides. Methods Mol Biol 969:29–42. doi:10.1007/978-1-62703-260-5_2
8. Strong TV, Hampton TA, Louro I et al (1997) Incorporation of beta-globin untranslated regions into a Sindbis virus vector for augmentation of heterologous mRNA expression. Gene Ther 4:624–627. doi:10.1038/sj.gt.3300423
9. Weissman D, Pardi N, Muramatsu H, Karikó K (2013) HPLC purification of in vitro transcribed long RNA. Methods Mol Biol Clifton NJ 969:43–54. doi:10.1007/978-1-62703-260-5_3
10. Thess A, Grund S, Mui BL et al (2015) Sequence-engineered mRNA without chemical nucleoside modifications enables an effective protein therapy in large animals. Mol Ther 23:1456–1464. doi:10.1038/mt.2015.103
11. Fotin-Mleczek M, Duchardt KM, Lorenz C et al (2011) Messenger RNA-based vaccines with dual activity induce balanced TLR-7 dependent adaptive immune responses and provide antitumor activity. J Immunother 34:1–15. doi:10.1097/CJI.0b013e3181f7dbe8
12. Geall AJ, Verma A, Otten GR et al (2012) Nonviral delivery of self-amplifying RNA vaccines. Proc Natl Acad Sci U S A 109:14604–14609. doi:10.1073/pnas.1209367109
13. Kreiter S, Selmi A, Diken M et al (2010) Intranodal vaccination with naked antigen-encoding RNA elicits potent prophylactic and therapeutic antitumoral immunity. Cancer Res 70:9031–9040. doi:10.1158/0008-5472.CAN-10-0699
14. Midoux P, Pichon C (2015) Lipid-based mRNA vaccine delivery systems. Expert Rev Vaccines 14:221–234. doi:10.1586/14760584.2015.986104
15. Warren L, Manos PD, Ahfeldt T et al (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell 7:618–630. doi:10.1016/j.stem.2010.08.012

Chapter 6

Nucleoside Modified mRNA Vaccines for Infectious Diseases

Norbert Pardi and Drew Weissman

Abstract

In recent years, numerous studies have demonstrated the outstanding abilities of mRNA to elicit potent immune responses against pathogens, making it a viable new platform for vaccine development (reviewed in Weissman, Expert Rev Vaccines 14:265–281, 2015; Sahin et al., Nat Rev Drug Discov 13:759–780, 2014). The incorporation of modified nucleosides in mRNA has many advantages and is currently undergoing a renaissance in the field of therapeutic protein delivery. Its use in a vaccine against infectious diseases has only begun to be described, but offers advantages for the generation of potent and long-lived antibody responses. FPLC purification and substitution of modified nucleosides in the mRNA make it non-inflammatory and highly translatable (Kariko et al., Immunity 23:165–175, 2005; Kariko et al., Mol Ther 16:1833–1840, 2008; Kariko et al., Nucleic Acids Research 39:e142, 2011) that are crucial features for therapeutic relevance. Formulation of the mRNA in lipid nanoparticles (LNPs) protects it from degradation enabling high levels of protein production for extended periods of time (Pardi et al., J Control Release, 2015). Here, we describe a simple vaccination method using LNP-encapsulated 1-methylpseudouridine-containing FPLC purified mRNA in mice. Furthermore, we describe the evaluation of antigen-specific T and B cell responses elicited by this vaccine format.

Key words Messenger RNA, Modified nucleoside, Pseudouridine, Lipid nanoparticles, Infectious diseases, Vaccine

1 Introduction

Vaccine studies with nucleic acids have been pursued since the 1990s targeting cancer and infectious diseases [1, 2]. Interestingly, most studies focused on the DNA-based systems and mRNA vaccination has lagged far behind. However, vaccination with mRNA has some advantages over other nucleic acid-based approaches, such as direct uptake into the cytosol resulting in rapid protein production and the lack of chromosomal integration that makes mRNA a safe and fully controllable delivery platform.

Several mRNA/self-replicating RNA vaccines against infectious diseases have been developed, but none of them contain modified nucleosides with their identified enhancements to safety

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_6,

and translation. A protamine-complexed mRNA vaccine against influenza that elicited protective immune responses in mice, ferrets and pigs has been described [3]. A nanoparticle or nanoemulsion-complexed self-replicating RNA vaccine demonstrated that low dose administration could elicit strong T cell and B cell immune responses against a series of infectious pathogens [4–7]. An interesting approach is the reinjection of autologous dendritic cells after ex vivo electroporation with in vitro generated mRNAs encoding HIV-1 antigens to HIV-1 infected individuals on antiretroviral therapy [8, 9]. This vaccine format induced strong $CD4^+$ and $CD8^+$ T cell immune responses and has entered phase I/II clinical trials [10], but is not currently applicable to large-scale immunization programs.

While in vitro transcribed unmodified mRNA has become an attractive new therapeutic tool, issues related to mRNA instability, insufficient translatability and in vivo delivery need to be resolved. We previously demonstrated that codon optimization of the coding sequence, introduction of stabilizing 5′ and 3′ UTR sequences, replacement of uridine with modified nucleosides, including pseudouridine, 1-methylpseudouridine, and 5-methylcytosine, addition of cap1 and poly(A) tail modifications combined with FPLC purification make mRNA non-inflammatory and highly translatable [11–13]. Moreover, we have recently shown that ionizable amino lipid-containing lipid nanoparticles (LNPs) are excellent mRNA carriers in vivo [14]. Numerous, heterogeneous populations of immune cells are found in the skin [15] making it an optimal anatomical site for vaccination. This method describes the administration of nucleoside-modified mRNA-LNPs encoding immunogens by the intradermal route. Single or multiple immunizations can be delivered depending on the immunogen and the required level of response. This new vaccine format induced strong antigen-specific T and B cell immune responses demonstrating that nucleoside modified mRNA in a LNP format is superior for vaccination against infectious pathogens and potentially other vaccine targets.

2 Materials

2.1 mRNA Production and Purification

1. Plasmid linearization with appropriate restriction enzymes that cleave at the 3′ end of desired mRNA sequence.
2. Phenol–chloroform–isoamyl alcohol (25:24:1), pH 8.0.
3. Chloroform.
4. Ultrapure water (UP).
5. 7.5 M LiCl, 3.0 M NaOAc, pH 5.5.
6. Isopropanol.

7. 75 % ethanol.
8. Siliconized microcentrifuge tube (Research Products International Corp, Mount Prospect, IL).
9. In vitro transcription kit (Megascript) (Ambion, Grand Island, NY) that includes the unmodified nucleoside triphosphate solutions, reaction buffer, DNase, and RNA polymerase enzyme.
10. INCOGNITO kits from CellScript (Madison, WI) (T7 ± ARCA) with Ψ and m5C, T7 with Ψ, SP6 with Ψ and kits without modified nucleosides; SP6-Scribe, T7 mScript, and T7-Scribe.
11. Modified nucleoside triphosphates (NTPs) can be obtained separately for transcription kits: Ψ, m1Ψ, m5C (TriLink, San Diego, CA), or purchased with the transcription kit (CellScript).
12. ÄKTApurifier 10 FPLC with a Frac-920 fraction collector (GE Healthcare Biosciences, Piscataway, NJ) and a TL105 column heater (Timberline Instruments, Boulder, CO) or similarly configured system for FPLC purification of in vitro transcribed mRNA.
13. ScriptCap m7G Capping System and 2′-O-Methyltransferase kits (CellScript) to introduce cap1 modification into the FPLC-purified mRNA.
14. Yeast poly(A) polymerase polyadenylation kit (600 U/μl) (USB, Cleveland, OH) to add poly(A) tail to the mRNA, if desired.

2.2 LNP Complexing of mRNA

1. Ionizable cationic lipid, varies with LNP formulation.
2. Phosphatidylcholine, varies with LNP formulation.
3. Cholesterol, varies with LNP formulation.
4. PEG-lipid, varies with LNP formulation.
5. Ethanol.
6. Dynamic light scattering instrument, such as a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, Worchestershire, UK).

2.3 Intradermal mRNA-LNP Administration to Mice

1. Mice (inbred or outbred strains, including BALB/c, C57bl/6, and others, as indicated by experimental system).
2. Isoflurane (Piramal Healthcare Limited, Coldstream, KY) and regulated nebulizer (Forane model 100F regulated nebulizer, Ohio Medical Products, Madison, WI) to anesthetize mice.
3. Electric shaver to remove hair from the back of the animals.
4. 3/10 cc 29½ G insulin syringe (BD Biosciences, Franklin Lakes, NJ) to intradermally administer mRNA-LNPs to mice.
5. Dulbecco's Phosphate Buffered Saline (PBS) to dilute mRNA-LNPs.

2.4 Retro-orbital Blood Collection from Mice

1. Microcentrifuge tubes.
2. Microhematocrit capillary tubes (Fisher Scientific, Pittsburg, PA).
3. Paper towels and Kimwipes.
4. Isoflurane and regulated nebulizer to anesthetize mice.
5. Anticoagulant: 0.3 M EDTA, pH 7.4.

2.5 Spleen and Lymph Node Isolation

1. Scissors and forceps.
2. UP water and 75% ethanol.
3. RPMI 1640 medium containing 2 mM L-glutamine (Life Technologies) and 10% fetal calf serum (FCS) (HyClone) or as indicated by experimental system.
4. 15 ml conical tubes (BioExpress, Kaysville, UT).
5. Isoflurane and regulated nebulizer to anesthetize mice.

2.6 Stimulation and Staining of Mouse Splenocytes and Lymph Node Cells

1. RPMI 1640 medium containing 2 mM L-glutamine and 10% FCS.
2. FACS buffer: 1% FCS in PBS, FACS tubes 12 × 75 mm (Fisher Scientific), 15 and 50 ml conical tubes, Eppendorf tubes.
3. 10 ml syringes (BD Biosciences), nylon mesh, 100 μm cell strainer (BioExpress), 100 × 15 mm petri dishes (Crystalgen, Commack, NY), hemocytometer (Reichert Technologies, Buffalo, NY).
4. Peptide pools or identified specific peptides corresponding to immunogen, such as HIV-1 Subtype B (MN) Env Peptide Set (NIH AIDS Reagent Program) or influenza hemagglutinin (HA) peptide pools (BEI Resources, NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH), or purified immunogen proteins or mRNA encoded immunogen
5. GolgiPlug (brefeldin A, BD Biosciences) and GolgiStop (monensin, BD Biosciences).
6. PMA and ionomycin (Sigma).
7. Antibodies: anti-CD4 PerCP/Cy5.5 (Clone GK1.5, Biolegend), anti-CD8a PB (Clone 53-6.7, Biolegend), anti-CXCR5 BV605 (Clone L138D7, Biolegend), anti-PD-1 BV785 (Clone 29F.1A12, Biolegend), anti-Bcl6 PE (Clone K112-91, BD Biosciences), anti-ICOS BV421 (Clone 7E.17G9, BD Biosciences), anti-CD27 PE (Clone LG.3A10, BD Biosciences), anti-CD107a FITC (Clone 1D4B, BD Biosciences), anti-CD3 APC-Cy7 (Clone 145-2C11, BD Biosciences), anti-TNF-α PE-Cy7 (Clone MP6-XT22, BD Biosciences), anti-IFN-γ AF700 (Clone XMG1.2, BD Biosciences) and anti-IL-2 APC (Clone JES6-5H4, BD Biosciences). Additional antibodies can be used depending on the experiment.

8. LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies).
9. Cytofix/Cytoperm kit (BD Biosciences).
10. FoxP3 transcription factor buffer kit (eBioscience).
11. Fixing buffer: 1% paraformaldehyde in PBS, made fresh.

2.7 Preparation of Compensation Controls

1. ArC Amine Reactive Compensation Bead Kit (Life Technologies) and anti-rat and anti-hamster compensation beads (BD Biosciences) or appropriate species to match staining antibodies.
2. Aqua Blue and all antibodies used for the staining of splenocytes and lymph node resident cells.
3. FACS tubes, FACS buffer.
4. Fixing buffer: 1% paraformaldehyde in PBS.

2.8 Flow Cytometry and T Cell Data Analysis

1. LSR II (BD Biosciences) or similarly enabled flow cytometer.
2. FlowJo 9.8.5, Excel (Microsoft), Prism 5.0f (GraphPad), PESTLE 1.7, and SPICE 5.35 software for data analysis.

2.9 Enzyme-Linked Immunosorbent Assays (ELISA)

1. Detection antibody: goat anti-mouse IgG HRP conjugate (Sigma).
2. Coating antigen: purified immunogen or related protein, such as HIV-1 gp120 for HIV envelope responses or HA for influenza HA responses.
3. Blocking buffer: 2% bovine serum albumin (BSA) (Sigma) in PBS.
4. Wash buffer: 0.05% Tween-20 in PBS.
5. Substrate solution (KPL Inc, Gaithersburg, MD): TMB peroxidase substrate and Peroxidase Substrate Solution B mixed in 1:1 ratio.
6. Stop solution: 2 N sulfuric acid.
7. Immulon 4 HBX high-binding plates (Thermo Scientific, Rochester).
8. Positive control mAb or affinity purified polyclonal antibody to use as a positive control.
9. Dynex MRX Revelation (or similar) microplate reader (Dynex Technologies, Chantilly, VA).

2.10 Virus Neutralization, Bacterial Protection, and Cancer Killing Assays

As described for virus, bacteria, or cancer being used.

3 Methods

mRNA is in vitro transcribed from a linearized plasmid and FPLC purified to remove double stranded RNA contamination. Cap1 is added enzymatically by the vaccinia virus capping enzyme and 2′-O-methyltransferase. mRNA is then formulated in ionizable amino lipid containing lipid nanoparticles (LNPs).

Mice are immunized intradermally using increasing amounts of mRNA-LNPs. The optimal dose of mRNA-LNPs and the number of immunizations depend on the immunogenicity of the antigen. Blood is collected prior to each immunization and plasma is used for ELISA and functional assays to measure the quantitative and qualitative properties of the B cell responses. Two weeks after the last immunization, for the measurement of T cell responses, animals are sacrificed and spleen, lymph nodes and blood is collected to analyze antigen-specific T cell and B cell responses. If only B cell responses will be analyzed, increased time post immunization allows further maturation of the antibody response. Splenocytes and/or lymph node cells are stimulated with appropriate peptide pools, whole protein or transfected with encoding mRNA and stained with antibodies against cell surface markers and intracellular cytokines. Samples are analyzed by a multicolor flow cytometer and data is evaluated using appropriate computer software.

3.1 mRNA Production and Purification

mRNA is synthesized from a linearized plasmid using appropriate in vitro transcription kit(s) (*see* ref. [16] for detailed information and tips on plasmid linearization and mRNA production). mRNA is then FPLC-purified and cap1 is added (*see* ref. [17] for detailed information on FPLC purification). Enzymatic addition of poly(A) tail is optional, as poly(A) tail can be encoded in the plasmid DNA.

1. 100 μg plasmid is linearized by incubation with 50 U of restriction enzyme(s) in a 100 μl reaction volume for 1 h to overnight, depending on the optimal conditions for the restriction enzyme(s).
2. Analyze DNA by agarose gel electrophoresis with EtBr staining to confirm complete cleavage of the plasmid DNA
3. Isolate the linearized plasmid DNA by extracting with 50 μl of phenol–chloroform-isoamyl alcohol following standard techniques. Dissolve linearized plasmid DNA in UP water.
4. For a 10 μl in vitro transcription reaction volume add 1 μg linearized plasmid, reaction buffer, appropriate nucleoside-triphosphate solutions, and RNA polymerase in a siliconized microcentrifuge tube.
5. Incubate the in vitro transcription reaction at 37 °C for 2–4 h.

6. Add 1 μl DNase per 10 μl reaction and incubate at 37 °C for 15 min.
7. Precipitate mRNA with cold LiCl, incubate overnight at −20 °C.
8. Centrifuge the precipitated mRNA at 13,000 × *g* for 5 min, wash 3 times with cold 75% ethanol and dissolve in UP water. Analyze mRNA by agarose gel electrophoresis.
9. FPLC purify the mRNA following the protocol in ref. [17] and precipitate mRNA by adding 3 μl glycogen, 1/10th volume NaOAc and 1 volume of isopropanol.
10. After overnight precipitation, centrifuge the mRNA at 13,000 × *g* for 5 min, wash 3 times with cold 75% ethanol and dissolve in UP water. Analyze mRNA by agarose gel electrophoresis.
11. To enzymatically cap and 2-O-methylate the cap use kits available from CellScript. Add 60 μg of in vitro transcribed FPLC-purified mRNA and UP water to a final volume of 67.5 μl in a siliconized microcentrifuge tube and incubate at 65 °C for 5 min.
12. Put the reaction tubes on ice for 20 s and centrifuge for 5 s. Add 10 μl 10× reaction buffer, 10 μl GTP, 5 μl SAM, 2.5 μl ScriptGuard RNase inhibitor, 5 μl ScriptCap m^7G capping enzyme, and 5 μl 2-O-methyltransferase in a 100 μl final volume.
13. Incubate at 37 °C for 1 h.
14. Precipitate mRNA with 50 μl of cold LiCl solution per 100 μl reaction volume and incubate at −20 °C overnight.
15. After overnight incubation, centrifuge mRNA at 13,000 × *g* for 5 min, wash 3 times with cold 75% ethanol and dissolve in UP water. Analyze mRNA by agarose gel electrophoresis.
16. Polyadenylation of mRNA is optional. Add 33 pmol of mRNA, which correspond to ~10 μg of a 1 kb-long RNA to a final volume of 16.2 μl in a siliconized microcentrifuge tube.
17. Add 1.3 μl ATP stock, 5 μl 5× reaction buffer, and 2.5 μl poly(A) polymerase enzyme. Incubate at 37 °C for 1 h.
18. Precipitate mRNA with 12.5 μl of cold LiCl solution per 25 μl reaction volume and incubate at −20 °C overnight.
19. Centrifuge mRNA at 13,000 × *g* for 5 min, wash 3 times with cold 75% ethanol and dissolve in UP water. Analyze mRNA by agarose gel electrophoresis.

3.2 LNP Complexing of mRNA

Lipid nanoparticle-mRNA complexes are typically made by companies (Arcturus, Acuitas, and others) due to the combined requirements of lipid synthesis and microfluidic mixing. mRNA is encapsulated in LNPs using a self-assembly process in which an aqueous solution of mRNA at pH 4.0 is rapidly mixed with a

solution of lipids dissolved in ethanol. LNPs contain ionizable cationic lipid/phosphatidylcholine/cholesterol/PEG-lipid at different mol/mol ratios and are typically encapsulated at an RNA-to-total lipid ratio of ~0.05 (wt/wt). LNPs have a diameter of ~60–100 nm that is measured by dynamic light scattering using a Zetasizer Nano ZS or similar instrument. Certain mRNA-LNP formulations are stored at −80 °C, while others require storage at 4 °C. For detailed information *see* ref. [18].

3.3 Intradermal mRNA-LNP Administration to Mice

1. Dilute mRNA-LNPs in PBS in a microcentrifuge tube or 15 ml conical tube and place on ice.
2. Remove hair from the back of mice with an electric shaver.
3. Anesthetize animals in a chamber with 3% isoflurane before administration of mRNA-LNPs.
4. Inject 30 μl of fluid intradermally using a 3/10 cc 29½ G insulin syringe. Inject each animal at 4 points on the back (4 × 30 μl) (*see* **Note 1**).
5. Place the animal back to the cage and confirm that it has completely recovered from anesthesia.

3.4 Retro-orbital Blood Collection from Mice

1. Add 1/10th volume of EDTA to the microcentrifuge tube (for example 10 μl EDTA to 100 μl blood).
2. Anesthetize animals in a chamber with 3% isoflurane.
3. Use a microhematocrit capillary tube to collect blood from the peri-orbital sinus of the mouse eye (*see* **Note 2**).
4. Transfer blood from the capillary into the microcentrifuge tube with EDTA.
5. After the required amount of blood is obtained gently push a Kimwipe to the peri-orbital sinus to stop further bleeding and remove extra blood from the eyeball.
6. Apply ophthalmologic ointment to the eye.
7. Place the animal back to the cage and confirm that it has completely recovered from anesthesia.
8. Centrifuge blood at 1000 × *g* for 10 min and pipette the plasma into a clean microcentrifuge tube. Store at −80 °C until analysis.

3.5 Spleen and Lymph Node Isolation

1. Anesthetize animals in a chamber with 3% isoflurane.
2. After complete anesthesia sacrifice the mice (one at the time) by performing cervical dislocation.
3. Open the chest and the abdomen of the animal with scissors and remove the spleen and lymph nodes.
4. Place the organs in 5 ml medium in a 15 ml conical tube on ice.

3.6 Stimulation and Staining of Mouse Splenocytes and Lymph Node Cells

1. Place the spleen/lymph nodes in a petri dish with 5 ml complete medium and grind the spleen on a cell strainer with the barrel of a 10 ml syringe.
2. Filter the suspension through a nylon mesh and place it into a 15 ml conical tube.
3. Centrifuge the conical tubes at 350 × *g* for 7 min.
4. Remove supernatant and resuspend the pellet in 10 ml PBS.
5. Centrifuge the conical tubes at 350 × *g* for 7 min.
6. Remove supernatant and resuspend the pellet in 2 ml medium.
7. Red blood cells can be lysed with AKC lysis buffer, but we find this impairs viability.
8. Count the nucleated cells using a hemocytometer (*see* **Note 3**).
9. Make a cell suspension with a concentration of 10^7 nucleated cells/ml. Dilute the peptides or protein in medium or transfect immunogen encoding mRNA using transfection reagent and protocol of choice (*see* **Note 4**).
10. Calculate the number of samples per animal and add 100 µl cell suspension to a FACS tube for each staining. Add 50 µl diluted peptide to each tube. Always include an unstimulated control for each animal. Use a PMA (10 ng/ml) and ionomycin (250 ng/ml) stimulated sample as a positive control.
11. Incubate samples at 37 °C for 1 h.
12. Add a total volume of 50 µl diluted GolgiPlug (1:100 in medium) and GolgiStop (1:143 in medium) solution to each FACS tube. If you include CD107a staining, add the antibody to the cells in this step.
13. Cover samples with aluminum foil and incubate them at 37 °C for 5 h.
14. Add 2 ml PBS to samples.
15. Centrifuge the FACS tubes at 350 × *g* for 7 min.
16. Decant supernatant and remove any drops from the FACS tube with a paper towel.
17. Dilute Aqua Blue stock 1:60 in PBS and resuspend the cells in 5 µl diluted solution.
18. Incubate at room temperature (RT) for 10 min in the dark. In the meantime, prepare the surface stain master mix in FACS buffer including appropriate antibodies: anti-CD4, anti-CD8, and anti-CD27 for Th1 panel and anti-CD4, anti-CXCR5, anti-ICOS, anti-PD-1 for Tfh panel, or any panel you develop.
19. Resuspend the cells in 50 µl surface stain master mix.
20. Incubate at room temperature (RT) for 30 min in the dark.
21. Add 3 ml FACS buffer to samples.

22. Centrifuge the FACS tubes at 350 × *g* for 7 min.
23. Decant supernatant and remove any drops from the FACS tube with a paper towel.
24. Resuspend the cells in 250 μl Fix/Perm solution for the Th1 panel and 1 ml diluted (1 part concentrate and 3 parts diluent) FoxP3 Fix/Perm solution for the Tfh panel.
25. Incubate at room temperature (RT) for 20 min (Th1) or 1 h (Tfh) in the dark. In the meantime, prepare the intracellular stain master mix in diluted Perm/Wash (Th1) or FoxP3 Perm buffer (Tfh) including appropriate antibodies: anti-CD3a, anti-IFN-γ , anti-IL-2 for Th1 panel and anti-CD3, anti-Bcl6, anti-TNF-α and anti-IL-2 for Tfh panel, or any panel you develop.
26. Resuspend the cells in 50 μl intracellular stain master mix.
27. Incubate at RT for 1 h in the dark.
28. Add 2 ml diluted Perm/Wash (Th1) or FoxP3 Perm buffer (Tfh) to the samples. Both are diluted in 1:10 with distilled water.
29. Centrifuge the FACS tubes at 350 × *g* (Th1) or at 700 × *g* (Tfh) for 7 min (*see* **Note 5**).
30. Decant supernatant and remove any drops from the FACS tube with a paper towel.
31. Resuspend the cells in 200 μl fixing buffer
32. Store the cells at 4 °C in the dark until flow cytometry analysis.

3.7 Preparation of Compensation Controls

1. Prepare compensation controls before analyzing the samples. Use the ArC Amine Reactive Compensation Bead Kit for aqua blue compensation and anti-rat and anti-hamster compensation beads (or whatever species your labeled mAbs are derived from) as the antibody compensation controls.
2. Prepare aqua blue compensation control: add 30 μl of beads to a FACS tube. Add 0.25 μl aqua blue (undiluted from the stock) to the beads. Incubate at RT for 30 min in the dark. Add 1 ml of FACS buffer and centrifuge the beads at 700 × *g* for 5 min. Remove the supernatant and add 300 μl fixing buffer to the beads.
3. For antibody compensation controls: add 40 μl of beads to a FACS tube. Add the same amount of antibody to each tube that is used to stain each sample. Incubate at RT for 30 min in the dark. Add 300 μl fixing buffer to the beads.
4. Store the samples at 4 °C in the dark until flow cytometry analysis.
5. Optional: instead of beads, splenocytes or lymph node resident cells can be used to obtain compensation controls.

3.8 Flow Cytometry and T Cell Data Analysis

Use a multicolor flow cytometer to collect data. First, set up the compensation matrix using the compensation beads. Confirm that flow cytometer settings are appropriate, each fluorophores can be detected without interference from others and the desired cell populations can be identified. Next, run all your samples and collect at least 100,000 events per specimen. Analyze the data using your desired software: FlowJo 9.8.5, Excel, Prism 5.0f, PESTLE 1.7, or SPICE 5.35.

3.9 Enzyme-Linked Immunosorbent Assays (ELISA)

1. Coat the ELISA plate with an appropriate antigen. Let it sit at RT for 1 h or at 4 °C overnight (depending on the antigen).
2. Remove the coating antigen completely by flicking the plate over a sink and then patting it on a paper towel.
3. Add 100 μl blocking buffer and incubate at RT for 1 h.
4. Wash the plate 3 times with 300 μl wash buffer and remove the fluid completely after each wash.
5. Dilutions of plasma samples and standard are made in blocking buffer and incubated on the plate (100 μl/well) at RT for 1 h.
6. Wash the plate 3 times with 300 μl wash buffer and remove the fluid completely after each wash.
7. Detection antibody is diluted in blocking buffer and incubated (100 μl/well) at RT for 1 h.
8. Wash the plate 3 times with 300 μl wash buffer and remove the fluid completely after each wash.
9. Add TMB substrate mixture at 1:1 ratio (100 μl/well) and incubate the plate in the dark at RT for 20 min or until appropriate level of color develops.
10. Add 2 N sulfuric acid (50 μl/well) to stop the reaction.
11. Read the optical density at 450 nm with a microplate reader (*see* **Note 6**).

3.10 Virus Neutralization, Bacterial Protection and Cancer Killing Assays

Perform as directed for pathogen or antigenic target of interest.

4 Notes

1. For successful intradermal delivery insert the needle at a shallow angle, just under the superficial layer of epidermis. A hard bleb will be seen upon successful intradermal injection of even a small quantity (30 μl) of fluid.
2. The microhematocrit tube is inserted into the orbital sinus by quickly rotating the tube. The eye will not be damaged as the

tube passes under the eye. The blood flow can be increased by slightly changing the angle of the tube.

3. 40–100 million cells can be obtained from a mouse spleen. Dilute a small amount of cell suspension 1:1000 to be able to count using a hemocytometer.
4. Most peptide pool stocks have a concentration of 1 mg/ml. Peptides are diluted in medium 1:200 and 50 µl diluted peptide is used for each sample.
5. It is essential to centrifuge cells at 700 × *g* after treatment with FoxP3 Fix/Perm solution, as most cells will be lost at a lower centrifugation speed.
6. Read the optical density within 15 min after addition of the stop solution.

References

1. Yang B, Jeang J, Yang A et al (2014) DNA vaccine for cancer immunotherapy. Hum Vaccin Immunother 10:3153–3164
2. Villarreal DO, Talbott KT, Choo DK et al (2013) Synthetic DNA vaccine strategies against persistent viral infections. Expert Rev Vaccines 12:537–554
3. Petsch B, Schnee M, Vogel AB et al (2012) Protective efficacy of in vitro synthesized, specific mRNA vaccines against influenza A virus infection. Nat Biotechnol 30:1210–1216
4. Geall AJ, Verma A, Otten GR et al (2012) Nonviral delivery of self-amplifying RNA vaccines. Proc Natl Acad Sci U S A 109:14604–14609
5. Hekele A, Bertholet S, Archer J et al (2013) Rapidly produced SAM((R)) vaccine against H7N9 influenza is immunogenic in mice. Emerg Microbes Infect 2:e52
6. Brito LA, Chan M, Shaw CA et al (2014) A cationic nanoemulsion for the delivery of next-generation RNA vaccines. Mol Ther 22:2118–2129
7. Bogers WM, Oostermeijer H, Mooij P et al (2015) Potent immune responses in rhesus macaques induced by nonviral delivery of a self-amplifying RNA vaccine expressing HIV type 1 envelope with a cationic nanoemulsion. J Infect Dis 211:947–955
8. Routy JP, Boulassel MR, Yassine-Diab B et al (2010) Immunologic activity and safety of autologous HIV RNA-electroporated dendritic cells in HIV-1 infected patients receiving antiretroviral therapy. Clin Immunol 134:140–147
9. Van Gulck E, Vlieghe E, Vekemans M et al (2012) mRNA-based dendritic cell vaccination induces potent antiviral T-cell responses in HIV-1-infected patients. AIDS 26:F1–F12
10. Allard SD, De Keersmaecker B, de Goede AL et al (2012) A phase I/IIa immunotherapy trial of HIV-1-infected patients with Tat, Rev and Nef expressing dendritic cells followed by treatment interruption. Clin Immunol 142:252–268
11. Kariko K, Buckstein M, Ni H et al (2005) Suppression of RNA recognition by toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunity 23:165–175
12. Kariko K, Muramatsu H, Welsh FA et al (2008) Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. Mol Ther 16:1833–1840
13. Kariko K, Muramatsu H, Ludwig J et al (2011) Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. Nucleic Acids Res 39:e142
14. Pardi N, Tuyishime S, Muramatsu H et al (2015) Expression kinetics of nucleoside-modified mRNA delivered in lipid nanoparticles to mice by various routes. J Control Release. doi:10.1016/j.jconrel.2015.08.007
15. Klechevsky E (2013) Human dendritic cells - stars in the skin. Eur J Immunol 43:3147–3155

16. Pardi N, Muramatsu H, Weissman D et al (2013) In vitro transcription of long RNA containing modified nucleosides. Methods Mol Biol 969:29–42
17. Weissman D, Pardi N, Muramatsu H et al (2013) HPLC purification of in vitro transcribed long RNA. Methods Mol Biol 969:43–54
18. Semple SC, Akinc A, Chen J et al (2010) Rational design of cationic lipids for siRNA delivery. Nat Biotechnol 28:172–176

Chapter 7

Generation and Evaluation of Prophylactic mRNA Vaccines Against Allergy

Richard Weiss, Sandra Scheiblhofer, and Josef Thalhamer

Abstract

Due to the worldwide increase in allergies and a limited efficacy of therapeutic interventions, the need for prophylactic vaccination against allergies has been recognized. mRNA and DNA vaccines have demonstrated their high potential for preventing allergic sensitization by inducing an immunological bias that prevents TH2 sensitization. However, only mRNA vaccines fulfill the stringent safety requirements for vaccination of healthy children. In this chapter, we describe the generation of conventional as well as self-replicating mRNA vaccines and methods to test their prophylactic efficacy in animal models.

Key words mRNA vaccine, Self-replicating mRNA, Genetic immunization, Prophylactic, TH1/TH2 responses, Allergy, BALB/c

1 Introduction

Although therapeutic DNA vaccines against cedar and peanut allergy have recently entered clinical trials [1], it is unlikely that this type of vaccines will fulfill the stringent safety demands necessary for prophylactic vaccination in healthy individuals. mRNA vaccines have reemerged as a promising alternative that avoid risk factors associated with DNA vaccines. We have previously demonstrated that mRNA vaccines have the potential to prevent from allergic sensitization, i.e., the generation of TH2 biased immune responses characterized by high levels of allergen specific IgE and the hallmark cytokines IL-4, IL-5, and IL-13 [2, 3]. Notably, the requirements for prophylactic vaccination against allergies markedly differ from traditional vaccines. While vaccines against infectious diseases aim at inducing high titers of protective antibodies and/or cellular immunity, a prophylactic vaccine against allergy introduces an immune bias, which is subsequently boosted upon natural contact with the respective allergen. It has been shown, that even a barely detectable primary immune response induced by the vaccine, is sufficient to prevent from allergic sensitization [4]. Moreover, we

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_7,

could show that memory responses after vaccination are long lasting and are maintained by repeated allergen challenges via the airways, without inducing detrimental side effects [5]. Thus, mRNA vaccines against allergic diseases meet all requirements for safe and effective prophylactic vaccines against allergies.

In this book chapter we describe the generation of conventional as well as self-replicating mRNAs from commercially available vectors. While conventional mRNA vaccines are limited to the minimal elements required for efficient translation of an antigen, self-replicating mRNA vaccines make use of a replicase, which can amplify the mRNA vaccine and trigger additional immunostimulatory pathways. A more detailed description of this vaccine type can be found in another book chapter of this series [6]. Additionally, we provide methods for quality control of the vaccines and suggestions which parameters can be optimized. Finally, we address in vivo vaccine delivery and describe methods for assessment of vaccine efficacy. Further methods to assess cellular immune responses and lung function in sensitized mice have been described in detail elsewhere [7].

2 Materials

2.1 Construction of Plasmids

1. pTNT (Promega).
2. pSinRep5 (Thermo Fisher).
3. Restriction enzymes, calf intestine alkaline phosphatase (CIAP), T4 DNA Ligase, corresponding buffers.
4. Agarose gel DNA electrophoresis equipment.
5. Gel extraction kit.

2.2 Plasmid Purification and Linearization

1. Escherichia coli XL1-blue competent cells (Thermo Fisher).
2. Standard Luria broth (LB) Medium.
3. Ampicillin.
4. Plasmid DNA preparation kit.
5. BamHI and NotI restriction enzymes and appropriate buffers.
6. MaXtract™ High Density, 2 mL (Qiagen).
7. Phenol–chloroform–isoamyl alcohol (PCI) 25:24:1.
8. Chloroform–isoamyl alcohol (CI) 24:1.
9. 3 M sodium acetate, pH 5.2, RNase free.
10. H_2O, RNase free.
11. 100% ethanol, RNase free.
12. 70% ethanol, RNase free.
13. Filter tips, RNase free.

2.3 In Vitro Transcription of RNA

All reagents need to be RNase free.

1. 5× T7 reaction buffer: 400 mM HEPES-KOH pH 7.5, 120 mM $MgCl_2$, 10 mM spermidine, 200 mM DTT.
2. 5× SP6 reaction buffer: 400 mM HEPES-KOH pH 7.5, 160 mM $MgCl_2$, 10 mM spermidine, 200 mM DTT.
3. rNTP mix: 25 mM rATP, 25 mM rCTP, 25 mM rGTP, 25 mM rTTP.
4. T7 and SP6 RNA polymerase, high concentration (200 U/μL) (Thermo Fisher).
5. Inorganic pyrophosphatase (NEB).
6. RNase-free DNase (Thermo Fisher).
7. 5 M ammonium acetate, pH 6.2–7.5.
8. H_2O, RNase free.
9. 70% ethanol, RNase free.
10. Filter tips, RNase free.

2.4 RNA Capping

2.4.1 All Reagents Need to Be RNase Free

1. ScriptCap m^7G Capping Kit (CELLSCRIPT).
2. 5 M ammonium acetate, pH 6.2–7.5.
3. H_2O, RNase free.
4. 70% ethanol, RNase free.
5. Filter tips, RNase free.

2.5 RNA Quality Control

2.5.1 All Reagents Need to Be RNase Free

1. Agarose.
2. 20× RNA buffer: 500 mM BES or MOPS, 300 mM sodium acetate, 10 mM Na_2EDTA, pH 6.8.
3. RNA loading buffer (FOFAL): To make 1 mL add 50 μL 20× RNA buffer, 500 μL formamide (>99%), 184 μL formaldehyde (37%), 134 μL glycerol (87%), 5 μL ethidium bromide (10 mg/mL), 117 μL H_2O, RNase free.
4. RNA-marker for agarose gel electrophoresis.
5. BHK-21 cell line (ATCC number CCL-10).
6. BHK-21 growth medium: DMEM, 10% FBS, 4.5 g/L glucose, 4 mM L-glutamine, 2.2 g/L $NaHCO_3$.
7. TurboFect transfection reagent (Thermo Fisher).
8. DPBS without Ca^{++} and Mg^{++}.
9. Lysis solution: 100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100.
10. Trichloroacetic acid (TCA), 30% w/v.
11. 2× SDS-sample-buffer: 200 mM β-mercaptoethanol, 4% (w/v) SDS, 20% (v/v) glycerol, 0.2% (w/v) bromophenol blue, 100 mM Tris–HCl, pH 6.8.

12. Equipment for SDS-PAGE and western blotting.
13. Immune serum against the protein encoded by the RNA (*see* **Note 1**).
14. HRP-labeled anti-mouse-IgG1 secondary antibody.

2.6 Immunization and Allergic Sensitization

1. BALB/c mice (female, 6–10 weeks of age).
2. 27-gauge needles.
3. 1-mL syringes.
4. Alu-Gel-S (Serva).
5. Purified allergen-encoding mRNA.
6. 5× Ringer's Solution with optimized $CaCl_2$ concentration: 513.5 mM NaCl, 26.5 mM KCl, 3.75 mM $CaCl_2$ (*see* **Note 2**).
7. Recombinant allergen.
8. 10× endotoxin-free phosphate buffered saline (PBS), pH 7.5 (Sigma).
9. Endotoxin-free H_2O.
10. Curved forceps.
11. Isoflurane anesthesia machine suitable for rodents.

2.7 IgG1 and IgG2a Subclass ELISA

1. White 96-well high-bind flat bottom ELISA plates (Greiner).
2. Standard PBS, pH 7.5.
3. Recombinant allergen.
4. Wash buffer: PBS, 0.1% (v/v) Tween-20.
5. Blocking buffer: PBS, 0.1% (v/v) Tween-20, 2% (w/v) blotting-grade skim milk.
6. Detection antibodies: anti-mouse IgG1 and IgG2a, HRP-conjugated.
7. BM Chemiluminescence ELISA substrate (POD) (Roche).
8. Luminescence microplate reader.

2.8 Basophil Release Assay

1. Rat basophil leukemia (RBL)-2H3 cell line (ATCC no. CRL-2256; DSMZ no. ACC 312).
2. Culture medium: 70% MEM (with Earle's salts) and 20% RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin/streptomycin, 2 mM sodium pyruvate.
3. Cell culture flasks with 25 cm^2, 75 cm^2, and 175 cm^2 area.
4. DPBS (Sigma).
5. Trypsin–ethylene-diamine tetraacetic acid (EDTA): 0.05% trypsin, 0.02% EDTA in DPBS (Sigma).
6. 96-well flat-bottom cell culture plates.

7. Tyrode's buffer: 9.6 g Tyrode's salts (Sigma), 1 g $NaHCO_3$/L; adjust pH to 7.2 with NaOH or HCl; add 0.1% BSA freshly before use.
8. Citrate buffer: 0.1 M citric acid or sodium citrate in H_2O; adjust pH to 4.5 with NaOH.
9. Glycine buffer: 15 g glycine, 11.7 g NaCl/L; adjust pH to 10.7 with NaOH.
10. 4-MUG (4-methyl umbelliferyl-N-acetyl-β-D-glucosaminide) (Sigma): 10 mM solution in DMSO; store aliquots of 80 µL or multiples thereof at −70 °C.
11. 10% Triton X-100.
12. Non-sterile 96-well flat-bottom microtiter plates.
13. Fluorescence microplate reader.

3 Methods

3.1 Construction of Plasmids for RNA Transcription

Plasmid vectors for transcription of mRNA vaccines employ a promoter specific for a viral RNA polymerase (typically T7 or SP6), which drives downstream transcription of the mRNA of interest. Elements to stabilize the transcribed RNA are usually incorporated into the vector. These include untranslated sequences before (5′ UTR), and/or after (3′ UTR) the cDNA sequence encoding the gene of interest [8]. The 3′ UTR also contains a synthetic poly(A) tail, which is important for efficient translation of the protein. Longer poly(A) tails usually result in increased mRNA half-life and more efficient protein translation [9]. A commercially available vector employing these features is pTNT (Promega). This vector contains tandem SP6 and T7 promoters, a 5′ UTR from rabbit β-globin [10], and a synthetic poly$(A)_{30}$ tail (*see* Fig. 1).

Plasmid vectors for transcription of self-replicating mRNA have to fulfill different requirements. These vectors are used for transcription of a so called genomic RNA, which encodes the nonstructural proteins 1–4 of an alphavirus (e.g., Sindbis or Semliki Forest Virus), which form the viral replicase. Downstream of the replicase lies a subgenomic promoter, also called 24-nucleotide (nt) conserved sequence element (CSE) [11], which drives the expression of the gene of interest. This is followed by a viral 3′ UTR including a conserved 19-nt CSE, which forms the core promoter for synthesis of negative strand RNA [12], and a synthetic poly(A) tail. Interaction of the viral 3′ and 5′ UTRs is necessary to initiate replication and to regulate both minus- and plus-strand synthesis [13].

The pSFV and pSinRep5 (Fig. 1) vectors are available from Thermo Scientific and can be used to generate self-replicating RNAs employing replicases from SFV and Sindbis virus, respectively.

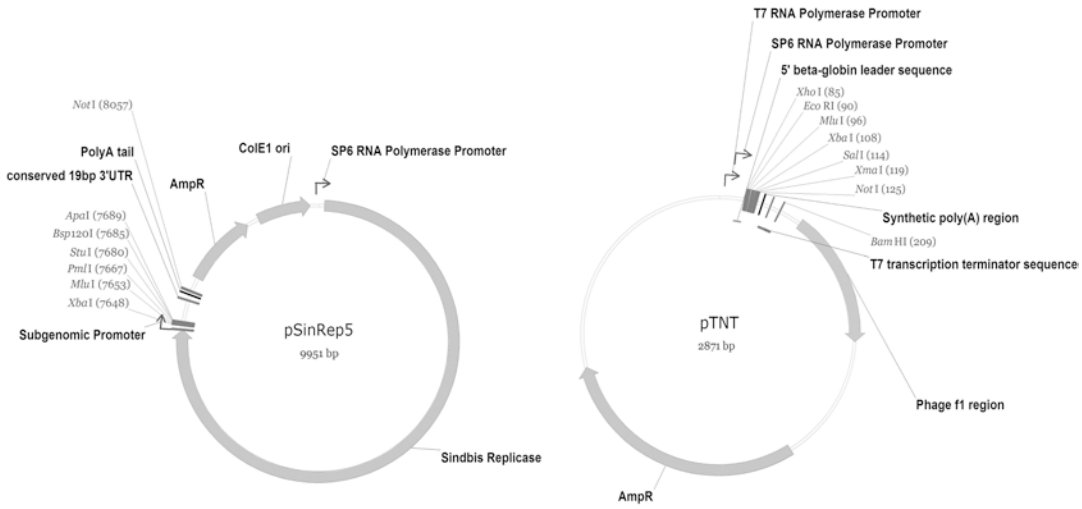

Fig. 1 Commercially available vectors for in vitro transcription of self-replicating (pSinRep5) or conventional (pTNT) mRNA vaccines

Constructions of pTNT-P5 and pSin-P5 vectors can be performed by standard recombinant DNA methods. Vectors pTNT and pSinRep5 are linearized with XbaI and treated with calf intestine alkaline phosphatase (CIAP) to prevent religation. The cDNA encoding Phl p 5 is excised from pCMV-Phl p 5 [14] using NheI/XbaI and ligated into the linearized vectors. The resulting vectors are named pTNT-P5 and pSin-P5, respectively.

3.2 Plasmid Purification and Linearization

Plasmid templates can be prepared with any commercially available plasmid preparation kit that gives endotoxin-free plasmid DNA of good quality. This plasmid DNA has to be linearized after the poly(A) tail before mRNA can be transcribed (*see* **Note 3**).

Complete linearization of plasmid DNA is important to avoid circular transcription, resulting in RNA of incorrect size. It is preferable to generate a larger batch of linearized plasmid DNA which can then be stored at −20 °C.

1. Add 200 µg of plasmid DNA (pTNT-P5 or pSin-P5) dissolved in H_2O to a 1.5 mL reaction tube.
2. Add 50 µL of appropriate 10× reaction buffer.
3. Add 20 µL of BamHI (10 U/µL), or NotI (10 U/µL) for linearization of pTNT-P5 or pSin-P5, respectively (*see* **Note 4**).
4. Fill up to 500 µL with H_2O, vortex, and incubate for 2 h at 37 °C (*see* **Note 5**).
5. From this point on work under RNase-free conditions: wear gloves, use certified RNase-free filter tips, reaction tubes, and reagents.

6. Prepare 2 MaXtract™ High Density columns by centrifugation at 1500 × *g* for 2–3 min.
7. Transfer reaction to a MaXtract™ High Density column (*see* **Note 6**).
8. Add 500 µL PCI (25:24:1) and mix thoroughly by repeated inversion.
9. Centrifuge at 1500 × *g* for 5 min to separate phases.
10. Transfer aqueous phase to the second MaXtract™ High Density column prepared at **step 6**.
11. Add 500 µL CI (24:1) and mix thoroughly by repeated inversion.
12. Centrifuge at 1500 × *g* for 5 min to separate phases.
13. Transfer the aqueous phase to a new reaction tube.
14. Add 1/10 Vol of 3 M sodium acetate, pH 5.2 and vortex.
15. Add 2 Vol of 100% ethanol and vortex.
16. Centrifuge at 16,000 × *g* for 10 min at RT.
17. Wash pellet 3 times with 70% ethanol.
18. Air-dry pellet and dissolve in 100 µL H_2O.
19. Measure concentration and check complete linearization on 1% agarose gel.
20. Store linearized plasmid DNA at −20 °C.

3.3 In Vitro Transcription of RNA

RNA is in vitro-transcribed from the linearized plasmid DNA using T7 or SP6 RNA polymerase. Milligram quantities of RNA can be produced by using optimized buffers and high concentrations of rNTPs [15] and inorganic pyrophosphatase [16], which prevents the inhibitory effects of pyrophosphate released during ribonucleoside triphosphate incorporation. We have successfully employed homemade buffers as described in this chapter; however, all necessary components are available in kits from Promega (RiboMAX™ Large Scale RNA Production Systems).

Conventional mRNA encoding Phl p 5 can be transcribed from linearized pTNT-P5 template DNA using T7 or SP6 polymerase. A 1 mL reaction results in approximately 2–5 mg of RNA. Similarly, self-replicating RNA can be transcribed from linearized pSin-P5 using SP6 polymerase. A 1 mL reaction will yield approximately 1 mg of self-replicating RNA.

1. Add 200 µL of 5× T7 or SP6 reaction buffer to a 1.5 mL reaction tube.
2. Add 200 µL of rNTP mix.
3. Add 50–100 µg of linearized pTNT-P5 or pSin-P5.
4. Add 30 U T7 or SP6 RNA polymerase per µg template DNA.

5. Add 15 U inorganic pyrophosphatase.
6. Fill up to 1 mL with H_2O.
7. Mix by repeated inversion.
8. Incubate at 37 °C for 2–3 h.
9. Add RNase-Free DNase to a concentration of 1 U/µg of template DNA.
10. Mix by repeated inversion.
11. Incubate at 37 °C for 15 min.
12. Add 1 mL of 5 M ammonium acetate, pH 6.2–7.5.
13. Mix by repeated inversion.
14. Incubate on ice for 15 min.
15. Centrifuge at 16,000 × *g* for 15 min at 4 °C.
16. Wash pellet in 70% EtOH.
17. Air-dry pellet and dissolve in 100 µL H_2O (*see* **Note 7**).
18. Measure RNA concentration (*see* **Note 8**).

3.4 RNA Capping

A 7-methyl-guanosine cap structure is essential for mRNA stability and efficient translation [17]. Cap analogons can be incorporated during the transcription process. Alternatively, cap 0 structures (m7G(5′)ppp(5′)NpN) can be added to the 5′ end of RNA using vaccinia virus capping enzyme [18]. The latter method has the advantage that up to 100% of transcripts can be capped. In this chapter, we describe the enzymatic capping approach using the ScriptCap m^7G Capping Kit (CELLSCRIPT). The kit contains the vaccinia virus capping enzyme including all three enzymatic activities needed for cap construction, i.e., mRNA triphosphatase, guanylyltransferase, and guanine-7-methyltransferase.

1. Add 50–60 µg in vitro-transcribed RNA to a 1.5 mL reaction tube and fill up to 68.5 µl with RNase-free water.
2. Heat-denature sample at 65 °C for 5–10 min and then immediately transfer on ice.
3. On ice add in the following order and mix after each step:
   (a) 10 µL 10× ScripCap buffer.
   (b) 10 µL rGTP (10 mM).
   (c) 5 µL S-adenosyl methionine (SAM) (2 mM).
   (d) 4 µL ScriptCap Capping Enzyme (10 U/µl) (*see* **Note 9**).
4. Fill up to 100 µL with RNase-free water and mix.
5. Incubate for 30–60 min at 37 °C.
6. Add 100 µL of 5 M ammonium acetate, pH 6.2–7.5.
7. Mix by repeated inversion.

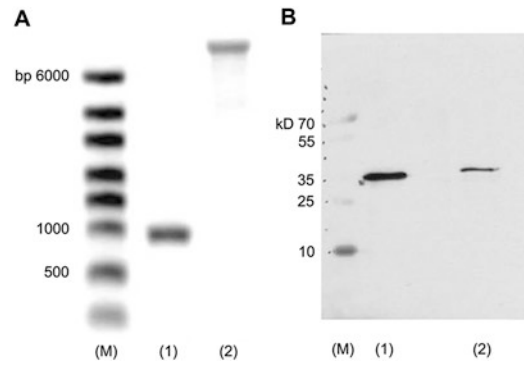

Fig. 2 RNA vaccine quality control. (**a**) Correct length of transcribed RNA is assessed by denaturing agarose gel electrophoresis. (M) marker, (1) Phl p 5 mRNA transcribed from pTNT-P5, (2) self-replicating RNA transcribed from pSin-Rep5-P5. (**b**) Western blot of cell lysates of BHK-21 cells transfected with conventional (1) or self-replicating (2) RNA encoding Phl p 5

8. Incubate on ice for 15 min.
9. Centrifuge at 16,000 × *g* for 15 min at 4 °C.
10. Wash pellet in 70% EtOH.
11. Air-dry pellet and dissolve in 100 µL H_2O.
12. Measure RNA concentration.

3.5 RNA Quality Control

Quality control of capped mRNA is done by means of denaturing agarose gel electrophoresis, in vitro transfection, and western blot analysis.

3.5.1 Denaturing Agarose Gel Electrophoresis

Integrity and correct length of the transcribed RNA is checked by denaturing agarose gel electrophoresis. Ideally, transcripts show as a single band of the expected size (Fig. 2). Longer transcripts point to insufficient linearization of the plasmid template or the presence of cryptic antisense promoters. Smaller transcripts can be the result of RNA secondary structures such as poly-T regions or repeats (*see* **Note 10**).

For 20 mL of a 1% agarose gel:

1. Add 0.2 g agarose to 17.6 mL RNase-free water.
2. Boil up in microwave until agarose is completely dissolved.
3. Cool down to approximately 60 °C.
4. Under a fume hood add 1 mL of 20× RNA buffer and 1.4 mL of 37% formaldehyde solution. Mix by swirling.
5. Cast gel.
6. Mix 1 µg of RNA with 1/10 Vol of RNA loading buffer (FOFAL).

7. Add an appropriate amount of RNA marker (ready to use) to a reaction tube.
8. Incubate RNA and RNA marker at 95 °C for 5 min and put on ice afterwards.
9. Immerse gel in 1× RNA buffer, add RNA and marker and run at 100 V.

3.5.2 In Vitro Transfection and Western Blot

Translation efficacy of the RNA can be tested by in vitro transfection of BHK-21 cells. Effective translation is dependent on the integrity and secondary structure of the RNA, and presence of a cap 0 structure. Also codon usage may affect translation efficacy (*see* **Note 11**).

1. On the day before transfection, seed BHK-21 cells into 24-well tissue culture plate at a density of 50–70% (2.5×10^5 cells per well).
2. For each transfection dilute 1 μg of RNA in 100 μL DMEM without serum and vortex.
3. Add 1 μL of TurboFect.
4. Mix solution by pipetting gently up and down.
5. Incubate for 15–20 min at RT.
6. Add 100 μL of the polymer/RNA mixture dropwise to the well.
7. Incubate for 24 h at 37 °C, 5% CO_2.
8. The next day, remove supernatant and wash wells once with 500 μL DPBS.
9. Add 100 μL of lysis solution.
10. Incubate 1–2 min at 37 °C.
11. Resuspend cells by pipetting up and down and thoroughly rinse the well bottom.
12. Transfer lysate to a fresh tube.
13. Precipitate proteins by adding 100 μL of 30% TCA.
14. Incubate on ice for 10 min.
15. Centrifuge at 16,000 × *g* for 10 min at 4 °C.
16. Discard supernatant.
17. Resuspend pellet in 50 μL SDS-sample-buffer (buffer turns yellow).
18. Add 1 M NaOH until sample becomes blue again.
19. Incubate at 95 °C for 10 min.
20. Add 15 μL of sample to a 12.75% polyacrylamide gel.
21. Run SDS-PAGE.
22. Perform western blot and stain protein with suitable antibody (*see* **Note 12**).

3.6 Immunization and Allergic Sensitization of Mice

Due to the fact that BALB/c mice are prone to develop TH2-biased immune responses, they are frequently employed for allergy models. Following sensitization with recombinant allergen together with adjuvants such as aluminum hydroxide (Alu-Gel-S), high titers of allergen-specific IgE and elevated levels of IL-4, IL-5, and IL-13 can be detected in these animals. Furthermore, the ratio of allergen-specific IgG1:IgG2a subclass antibodies is elevated in sensitized mice, indicative of a TH2-dominated response type. By immunizing mice with allergen-encoding mRNA vaccines prior to sensitization, the formation of IgE and IgG1 as well as the production of allergy-associated cytokines can be prevented.

A typical experimental setup for protective mRNA immunization against allergy requires 2–3 immunizations at weekly intervals followed by 2–3 sensitization rounds. To avoid antigen-independent suppression of TH2 sensitization, an interval of at least 2 weeks between vaccination and sensitization should be maintained. Longer intervals are possible, as vaccine memory lasts for at least 9 months [5]. For control purposes, groups of animals receiving mRNA encoding an irrelevant antigen/allergen and/or only sensitizations have to be included.

3.6.1 Intradermal mRNA Immunization

1. Prepare allergen-encoding mRNA in a volume of 200 μL 1× Ringer's solution per mouse and keep the RNA on ice until injected (*see* **Note 13**).
2. Carefully shave the back of the mice with an electric clipper.
3. Anesthetize the mice by using an isoflurane anesthesia machine and keep them under anesthesia during the whole procedure.
4. Lift a small fold of the skin with the forceps.
5. Inject a small amount of the mRNA superficially into this fold (*see* **Note 14**).
6. Repeat this step 5 times to distribute the solution into several injection sites.

3.6.2 Subcutaneous Sensitization

1. Prepare a mixture of 1–5 μg recombinant allergen (*see* **Note 15**) with 100 μL Alu-Gel-S and 20 μL endotoxin-free 10× PBS and fill up with endotoxin-free water to a volume of 200 μL per mouse.
2. Let protein-adjuvant complex formation take place by shaking the solution in a large enough tube at room temperature for at least 2 h.
3. Distribute the solution between two sites on the shaved back of the mice by lifting up the skin and injecting subcutaneously.
4. Repeat the sensitization after 2 weeks (*see* **Note 16**).

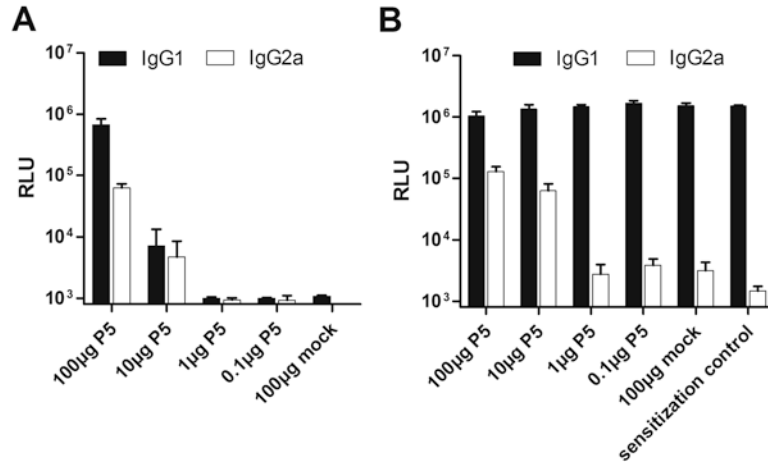

Fig. 3 (**a**) Phl p 5-specific IgG1 and IgG2a responses after vaccination of mice ($n=5$) with 0.1–100 µg of conventional mRNA encoding Phl p 5, or 100 µg of mRNA encoding an irrelevant antigen (mock). (**b**) IgG1 and IgG2a level after sensitization with recombinant Phl p 5/Alu-gel-S. A boost of IgG2a in the 100 µg and 10 µg groups indicates successful induction of TH1 memory and correlates with protection from sensitization. All sera were diluted 1:1000. Data are shown as means ±SEM

3.7 Measurement of Antibody Subclasses

To test whether prophylactic immunization has worked out, the easiest parameter to measure is the ratio of IgG1:IgG2a in sera. Although the humoral responses after immunization might be barely detectable, a boost of IgG2a titers after allergic sensitization is a strong indicator of a TH1 bias set by the RNA vaccine and successful protection (Fig. 3).

1. Coat ELISA plates with 50 µL/well of a 1 µg/mL allergen solution in PBS at 4 °C overnight.
2. Wash wells 3 times with 200 µL PBS, 0.1% Tween-20.
3. Block with 200 µL blocking buffer/well for 1 h at RT.
4. Wash wells 3 times with 200 µL PBS, 0.1% Tween-20.
5. Add 50 µL of serial dilutions of individual sera in blocking buffer to the wells and incubate for 1 h at room temperature.
6. Wash wells 5 times with 200 µL PBS, 0.1% Tween-20. Incubate 1 min between each wash.
7. Add 50 µL of detection antibodies diluted in blocking buffer to the wells and incubate for 1 h at room temperature.
8. Wash wells 5 times with 200 µL PBS, 0.1% Tween-20. Incubate 1 min between each wash.
9. Prepare Chemiluminescence substrate according to the manufacturer's instructions and dilute the substrate 1:2 with H_2O.

10. Add 50 µL of the substrate to the wells and incubate for 3 min at room temperature.
11. Measure chemiluminescence in photon counts per second in a luminometer.

3.8 Basophil Release Assay

To assess the amount of free allergen-specific IgE in sera of sensitized mice, basophils expressing FcεRI, the high affinity receptor for IgE, are passively loaded with IgE. Addition of the respective allergen leads to cross-linking of IgE and subsequently to mediator release. One of these mediators, β-hexosaminidase, cleaves the substrate 4-MUG, leading to formation of cleavage products detectable by fluorescence spectroscopy.

In contrast to measurement of IgE by ELISA, the RBL release assay provides a functional readout for IgE-mediated degranulation. Additionally, the amount of cell-bound IgE in the blood of sensitized animals can be determined by a basophil activation test, which has been described in detail elsewhere [7].

3.8.1 Culture of RBL Cells

The RBL-2H3 cells are adherent, fibroblast-like cells forming monolayers. They are cultured in a mixture of MEM and RPMI (*see* **Note 17**) with supplements in an incubator at 37 °C, 95% RH, 5% CO_2.

1. Culture cells in 25 cm^2, 75 cm^2, or 175 cm^2 cell culture flasks with 5 mL, 20 mL, or 50 mL culture medium, respectively.
2. Split culture before becoming confluent (*see* **Note 18**).
3. Remove culture medium.
4. Wash cells 3 times with DPBS (*see* **Note 19**).
5. Cover the cell monolayer with pre-warmed trypsin–EDTA.
6. Place culture flask in the incubator until cells detach (*see* **Note 20**).
7. Add warm culture medium (*see* **Note 21**).
8. Rinse off the cells (*see* **Note 22**) and transfer them into a fresh tube.
9. Centrifuge for 10 min at 300 × *g*.
10. Discard the supernatant and dissolve the cell pellet in culture medium.
11. Transfer cells into fresh culture flasks at the desired dilution.
12. For storage, freeze cells in 70% medium, 20% FBS, 10% DMSO on liquid nitrogen vapor or at −70 °C.

3.8.2 Mediator Release

1. Harvest cells from a dense culture as described under Subheading 3.8.1 (*see* **Note 23**).
2. Count cells and plate them in culture medium at 7×10^5/mL in 100 µL per well of a 96-well flat-bottom tissue culture plate.

3. Place the plate in the incubator overnight.
4. Add serial dilutions of sera (*see* **Note 24**) and incubate for 2 h in the incubator. Wells for background and maximum release are left untreated at this point.
5. Discard the supernatant and dry the plate by tapping on a paper towel.
6. Carefully wash the cells 3 times by adding 200 µL Tyrode's buffer.
7. Add 100 µL Tyrode's buffer containing dilutions of allergen or allergen extract (*see* **Note 25**). For maximum release, 10 µL of a 10% Triton solution is added to the maximum release wells. Background wells receive Tyrode's buffer only.
8. Place the plate in the incubator for 1 h.
9. Carefully resuspend the solution contained in the maximum release wells.
10. Transfer 50 µL supernatant of each well into a fresh, non-sterile 96-well flat-bottom plate.
11. Thaw 4-MUG and add 80 µL thereof per 5 mL of citrate buffer to prepare assay solution.
12. Add 50 µL assay solution to each supernatant and incubate for 1 h at 37 °C.
13. Add 100 µL glycine buffer per well.
14. Measure fluorescence (in relative fluorescence units, rfu) at λex 360 nm, λem 465 nm in a fluorescence microplate reader.
15. Calculate specific release in percent according to the following equation: $((\text{experimental}_{\text{rfu}} - \text{background}_{\text{rfu}})/(\text{maximum}_{\text{rfu}} - \text{background}_{\text{rfu}})) \times 100$.

4 Notes

1. You can use immune sera from previous experiments of mice sensitized with the recombinant protein adjuvanted with Alu-Gel-S as described in Subheading 3.6.2.
2. The in vivo uptake of naked mRNA strongly depends on the presence of Ca^{++} ions [19]. We optimized the $CaCl_2$ concentration in Ringer's solution using in vivo expression of luciferase mRNA as readout.
3. Although pTNT contains a T7 terminator after the poly(A) tail, this termination is not 100% efficient. Therefore, linearization of the vector is necessary to avoid too long transcripts.
4. If possible, restriction enzymes producing 5′ overhangs should be used as 3′ overhangs may initiate transcription and generated unwanted transcripts.

5. Although the digest should be finished in less time, we found that longer incubation gives better reproducible results.
6. We found that purification of linearized template DNA by organic extraction results in the best transcription rates. It is important to avoid any phenol contamination of the template DNA as this would inhibit downstream enzymatic reactions. Therefore, use of MaXtract gel, which separates the aqueous and organic phase, is highly recommended.
7. Do not overdry the pellet. Depending on the pellet size, solubilization of the RNA can take some time. We usually incubate the pellet in water over night at 4 °C and resuspend on the next day. If the pellet is still not completely dissolved, incubate at 50 °C and vortex every 2–3 min until fully dissolved.
8. Measurement of RNA concentration by UV absorption at 280 nm can lead to an overestimation due to the presence of free rNTPs. Although ammonium acetate precipitation minimizes contamination with free rNTPs we routinely experience an overestimation of ~10% by UV absorption measurement. To determine the exact RNA concentration, the use of RNA intercalating dyes, which are not affected by the presence of free rNTPs are recommended, e.g., using the Quant-iT RNA Assay Kit (Thermo Fisher).
9. To save enzyme, we tested the minimum amount of Capping Enzyme required to achieve 100% incorporation of radioactively labeled SAM. We found that by increasing the incubation time to 2–2.5 h, 0.8 μL of capping enzyme are sufficient to incorporate the same amount of methyl groups as 4 μL.
10. Though in vitro-transcribed RNA is often of high enough purity for animal experiments, large transcripts or GMP requirements can make additional chromatographic purification steps necessary. A protocol to further purify in vitro-transcribed RNA by size exclusion chromatography can be found here [20].
11. RNA vaccines that fail to translate after in vitro transfection may have to be optimized. Several companies offer services for optimizing codon usage and secondary structural elements of the RNA encoding the antigen of interest.
12. We use sera from mice immunized with recombinant Phl p 5 adjuvanted with Alu-Gel-S at a dilution of 1:5000. Detection is performed with a HRP-conjugated goat anti-mouse IgG1 secondary antibody. Use a dilution as recommended by the manufacturer.
13. 10–100 μg of conventional mRNA are required for protective vaccination, depending on the antigen. For self-replicating RNA, five to tenfold lower doses are sufficient [21].
14. Intradermal injection leads to formation of a small blister, which persists for several minutes. If the solution quickly disappears instead, the injection has been delivered into subcutaneous tissue.

15. Depending on the allergenicity of the protein used, the amount necessary for proper sensitization may vary and has to be tested on a case-by-case basis.
16. For weak allergens even a third sensitization round might be necessary. Take blood at regular intervals and check the sensitization status by mediator release assay.
17. Other media formulations are also frequently used, including RPMI 1640 alone with supplements. However, a higher proportion of RPMI may increase the proliferation rate of the cells, but potentially decreases production of mediators.
18. Usually, cells have to be split every 3–4 days at a dilution of 1:10. If cells are growing too dense, they tend to accumulate in suspension instead of adhering to the plastic. It is recommended to check the density/quality of the cells under the microscope before performing an assay.
19. Washing is essential to remove any traces of serum as this contains trypsin inhibitor.
20. This usually takes about 5 min. You can check in an inverted microscope whether the cell layer is already dispersed and if so, quickly proceed to the next step.
21. This has to be done as quickly as possible after the cells have been detached to dilute the trypsin–EDTA. Longer incubation with trypsin leads to reduced numbers of receptors on the cell surface and hence can diminish receptor cross-linking.
22. Avoid tapping against the culture flask as this may lead to clogging of the cells.
23. It has been demonstrated that histamine release capacity may be seriously reduced after too much subculturing [22]. This may also apply for release of β-hexosaminidase. Hence, we recommend starting a new culture after approx. 30 passages.
24. We recommend starting at a dilution of 1:50. If this results in weak or undetectable mediator release, the sensitization protocol might have failed.
25. A standard concentration for recombinant or purified allergens is 0.1 μg/mL. For allergen extracts we recommend to start with 1 μg/mL.

Acknowledgements

This work was supported by the Austrian Science Fund, grant # W1213.

References

1. Weiner L, Mackler B, Hearl B et al. (2013) Poster 2011: a DNA vaccine immunotherapy for japanese red cedar allergy. Paper presented at the WAO Symposium on Immunotherapy and Biologics, Chicago, Feb 2014
2. Weiss R, Scheiblhofer S, Roesler E et al (2012) mRNA vaccination as a safe approach for specific protection from type I allergy. Expert Rev Vaccines 11:55–67
3. Weiss R, Scheiblhofer S, Roesler E et al (2010) Prophylactic mRNA vaccination against allergy. Curr Opin Allergy Clin Immunol 10:567–574
4. Weiss R, Scheiblhofer S, Thalhamer J (2014) Allergens are not pathogens: why immunization against allergy differs from vaccination against infectious diseases. Hum Vaccin Immunother 10:703–707
5. Hattinger E, Scheiblhofer S, Roesler E et al (2015) Prophylactic mRNA vaccination against allergy confers long-term memory responses and persistent protection in mice. J Immunol Res 2015:797421. doi:10.1155/2015/797421
6. Scheiblhofer S, Weiss R, Gabler M et al (2006) Replicase-based DNA vaccines for allergy treatment. Methods Mol Med 127:221–235
7. Isakovic A, Weiss R, Thalhamer J et al (2014) Protective and therapeutic DNA vaccination against allergic diseases. Methods Mol Biol 1143:243–258
8. Schlake T, Thess A, Fotin-Mleczek M et al (2012) Developing mRNA-vaccine technologies. RNA Biol 9:1319–1330
9. Holtkamp S, Kreiter S, Selmi A et al (2006) Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells. Blood 108:4009–4017
10. Annweiler A, Hipskind RA, Wirth T (1991) A strategy for efficient in vitro translation of cDNAs using the rabbit beta-globin leader sequence. Nucleic Acids Res 19:3750
11. Levis R, Schlesinger S, Huang HV (1990) Promoter for Sindbis virus RNA-dependent subgenomic RNA transcription. J Virol 64:1726–1733
12. Ou JH, Strauss EG, Strauss JH (1981) Comparative studies of the 3'-terminal sequences of several alpha virus RNAs. Virology 109:281–289
13. Frolov I, Hardy R, Rice CM (2001) Cis-acting RNA elements at the 5' end of Sindbis virus genome RNA regulate minus- and plus-strand RNA synthesis. RNA 7:1638–1651
14. Gabler M, Scheiblhofer S, Kern K et al (2006) Immunization with a low-dose replicon DNA vaccine encoding Phl p 5 effectively prevents allergic sensitization. J Allergy Clin Immunol 118:734–741
15. Gurevich VV, Pokrovskaya ID, Obukhova TA et al (1991) Preparative in vitro mRNA synthesis using SP6 and T7 RNA polymerases. Anal Biochem 195:207–213
16. Cunningham PR, Ofengand J (1990) Use of inorganic pyrophosphatase to improve the yield of in vitro transcription reactions catalyzed by T7 RNA polymerase. Biotechniques 9:713–714
17. Kuhn AN, Diken M, Kreiter S et al (2010) Phosphorothioate cap analogs increase stability and translational efficiency of RNA vaccines in immature dendritic cells and induce superior immune responses in vivo. Gene Ther 17:961–971
18. Guo PX, Moss B (1990) Interaction and mutual stabilization of the two subunits of vaccinia virus mRNA capping enzyme coexpressed in Escherichia coli. Proc Natl Acad Sci U S A 87:4023–4027
19. Probst J, Weide B, Scheel B et al (2007) Spontaneous cellular uptake of exogenous messenger RNA in vivo is nucleic acid-specific, saturable and ion dependent. Gene Ther 14:1175–1180
20. McKenna SA, Kim I, Puglisi EV et al (2007) Purification and characterization of transcribed RNAs using gel filtration chromatography. Nat Protoc 2:3270–3277
21. Roesler E, Weiss R, Weinberger EE et al (2009) Immunize and disappear-safety-optimized mRNA vaccination with a panel of 29 allergens. J Allergy Clin Immunol 124:1070–1077
22. Barsumian EL, Isersky C, Petrino MG et al (1981) IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and nonreleasing clones. Eur J Immunol 11:317–323

Part III

Adjuvantation and Delivery

Chapter 8

Measuring the Adjuvant Activity of RNA Vaccines

Norbert Pardi and Drew Weissman

Abstract

mRNA has recently arisen as a promising new drug class with the potential to be applied to various therapeutic modalities, including protein replacement and vaccination against cancer and infectious diseases. Numerous approaches have been pursued to develop potent mRNA vaccines, many of them have proved to be successful and have already entered human clinical trials. RNA, especially in vitro transcribed, is extremely immunogenic as it induces innate immune responses through the activation of a variety of pattern recognition receptors. This feature of RNA is potentially beneficial for vaccine development, where antigen-encoding RNA might also function as an adjuvant to elicit potent antigen-specific T and B cell immune responses. Here, we describe the methods that can be used to evaluate the immunogenicity of RNA vaccines in vitro and in vivo.

Key words Messenger RNA, Vaccine, Adjuvant, Cancer, Infectious diseases, Immune system

1 Introduction

Vaccination with in vitro transcribed mRNA was first reported in 1993, when injection of liposome-encapsulated mRNA encoding influenza nucleoprotein was demonstrated to elicit antigen-specific immune responses [1]. Since then, numerous mRNA-based vaccine formats have been evaluated targeting cancer and infectious diseases and some have entered clinical trials. A two-component vaccine in which a protamine-complexed adjuvant RNA was mixed with the antigen-encoding naked mRNA has been developed [2]. This vaccine proved to be efficacious against influenza in mice, ferrets and pigs [3]. An alphavirus-based self-amplifying RNA vaccine platform (SAM) that could be delivered in unformulated, synthetic lipid nanoparticle or cationic nanoemulsion manners has been investigated. Both complexed formulations displayed high immunogenicity and could induce strong immune responses against a series of infectious pathogens [4–8]. A unique method in which naked mRNA is injected directly into lymph nodes inducing the maturation of lymph node-resident dendritic cells that generates

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_8,

potent immune responses has also been developed [9]. This approach has entered human trials to treat melanoma patients with injection of cancer antigen-encoding naked mRNA. An alternative approach is the injection of dendritic cells electroporated ex vivo with melanoma-associated antigen fused to a HLA-class II targeting signal (DC-LAMP) along with mRNAs encoding immune stimulatory molecules, including CD40 ligand, a constitutively active Toll-like receptor 4, and CD70 (TriMix) to melanoma patients [10, 11]. Strong antitumor activity with long-term disease control was observed in treated individuals.

One of the key components of a potent vaccine is the adjuvant that can increase the magnitude and breadth of the immune response and direct the expansion of certain subsets of T helper cells, IgG subclasses, or mucosal antibody responses. In RNA vaccines, the source of the adjuvant activity can be (1) the in vitro-transcribed RNA [12], as it is a highly immunogenic molecule without codon optimization [13] or nucleoside modification and purification [14–16], (2) direct acting adjuvants (TLR, helicase, NOD and inflammasome agonists), (3) mRNA-encoded immune stimulatory molecules (CD40L, constitutively active TLR4, CD70, GM-CSF, and others) [10], and (4) the RNA complexed with specific agents (protamine, lipid-based reagents) ([3] and reviewed in ref. 17). A general rule of thumb for vaccines is that as the adjuvant activity increases so do the adverse events. Thus, evaluating adjuvant activity is a crucial step in the process of vaccine development. In this chapter, we present the methods that can be used to measure RNA vaccine immunogenicity in vitro and in vivo.

2 Materials

2.1 Cell Treatment and Cytokine Measurement

For spleen and lymph node cells, peripheral blood mononuclear cells (PBMC) or whole blood (human and mouse).

1. 24-well plates (Greiner Bio-One), 96-well plates (Greiner Bio-One), hemocytometer (Reichert Technologies, Buffalo, NY) or similar cell counting device.
2. RPMI 1640 medium containing 2 mM L-glutamine (Life Technologies) and 10% fetal calf serum (FCS) (HyClone).
3. Lactobacillus acidophilus M-TriLYS-D-ASN (Invivogen), lipopolysaccharide (LPS) (Sigma) and poly(I:C) (Invivogen) or other positive control innate immune receptor agonists.
4. Appropriate ELISA kits to measure IFN-α, TNF-α proinflammatory cytokines in cell culture supernatant.

2.2 Generation and Culturing of Human and Murine Dendritic Cells (DCs)

1. Six-well plates (Greiner Bio-One), 15 ml conical tubes (BioExpress, Kaysville, UT).
2. Ultrapure (UP) water, 100 × 15 mm petri dishes (Crystalgen, Commack, NY).

3. Hemocytometer or similar cell counting device.
4. Scissors, forceps, 75% ethanol.
5. Mice (inbred or outbred strains, including BALB/c, C57bl/6, and others).
6. 3/10 cc 29½G insulin syringe (BD Biosciences, Franklin Lakes, NJ).
7. RPMI 1640 medium containing 2 mM L-glutamine and 10% FCS.
8. Human IL-4 (100 ng/ml), human GM-CSF (50 ng/ml), mouse GM-CSF (50 ng/ml) (R&D, Minneapolis, MN).

2.3 Transfection of Cells

For in vitro measurement of adjuvant activity in DCs, PBMCs, spleen or lymph node cells.

1. 96-well plates, 50 ml reagent reservoir (USA Scientific, Ocala, FL).
2. Multichannel pipettes, UP water.
3. RPMI 1640 medium with 2 mM L-glutamine (Life Technologies) with and without 10% fetal calf serum (FCS) (HyClone).
4. Transfection reagents: TransIT mRNA (TransIT) (Mirus Bio), Lipofectin (Invitrogen), Lipofectamine RNAiMAX (Invitrogen), mRNA-lipid nanoparticles (mRNA-LNPs) (obtained from Acuitas Therapeutics), and others.
5. Potassium-phosphate buffer, 0.4 M with 10 mg/ml BSA, pH 6.2.
6. Human recombinant ApoE protein (Sigma).

2.4 Cytokine Assays

Assays for proinflammatory cytokines, chemokines, other ligands and interferons (IFNs). Enzyme-linked immunosorbent assays (ELISA), Luminex multiplex, functional assays and other techniques for measurement are available from a variety of companies.

2.5 mRNA Administration

Intradermal, intramuscular, intranodal, subcutaneous injection for in vivo measurement of adjuvant activity in mice.

1. Mice (inbred or outbred strains, including BALB/c, C57bl/6, and others, as indicated by experimental system).
2. Isoflurane (Piramal Healthcare Limited) (Coldstream, KY) and Forane model 100F regulated nebulizer (Ohio Medical Products, Madison, WI) or similar device to anesthetize mice.
3. Electric shaver to remove hair from the animals.
4. 3/10 cc 29½G insulin syringe to administer mRNA to mice.
5. Dulbecco's Phosphate Buffered Saline (PBS) to dilute mRNA.
6. 1% Evans Blue dye (Sigma, St. Louis, MO) in water.

7. BD ULTRA-FINE™ Short Needle Insulin Syringe, 3/10 ml Syringe, 31G × 8 mm, (BD Biosciences).
8. Nair or similar depilatory cream.

2.6 Retro-orbital Blood Collection from Mice

1. Microcentrifuge tubes.
2. Microhematocrit capillary tubes (Fisher Scientific, Pittsburg, PA).
3. Paper towels and Kimwipes.
4. Isoflurane and regulated nebulizer to anesthetize mice.
5. Anticoagulant: 0.3 M EDTA, pH 7.4.

2.7 Dot Blot to Detect dsRNA

1. Super charged Nytran membranes.
2. TBS-T buffer: 50 mM Tris–HCl, 150 mM NaCl, 0.05%, Tween-20, pH 7.4.
3. Blocking buffer: 5% nonfat dried milk in TBS-T buffer.
4. Incubation buffer: 1% nonfat dried milk in TBS-T buffer.
5. dsRNA-specific mAb J2 (English & Scientific Consulting, Szirák, Hungary).
6. HRP-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA).
7. ECL Plus Western blot detection reagent (Amersham/GE Healthcare Biosciences).
8. Fujifilm LAS1000 digital imaging system or similar.

3 Methods

3.1 Spleen and Lymph Node Cell, Peripheral Blood Mononuclear Cells (PBMC), or Whole Blood (Human and Mouse) Treatment and Proinflammatory Cytokine Measurement

3.1.1 Whole Blood

1. Dilute whole blood 1:4 in serum-free medium.
2. Incubate 500 μl diluted blood with positive controls (3 μg/ml *Lactobacillus acidophilus* M-TriLYS-D-ASN, 0.01–1 μg/ml LPS, 1–25 μg/ml poly(I:C), or others) or and negative control (medium) in triplicates in a 24-well plate or transfect with mRNA, as described below.
3. Incubate at 37 °C for 24 h.
4. Remove supernatant and store at −80 °C until analysis.
5. Use appropriate ELISA, Luminex or other multiplexing technology kits for measurement of proinflammatory cytokines, chemokines, ligands, or interferons following the manufacturer's instructions.

3.1.2 Spleen Cells, Lymph Node Cells, or PBMCs

1. Count spleen cells, lymph node cells or PBMCs and seed in a 96-well plate (5.5 × 105 cells in 220 μl) complete medium.
2. Incubate with 1 μg/ml Lactobacillus acidophilus M-TriLYS-D-ASN, 0.01–0.1 μg/ml LPS, 1–25 μg/ml poly(I:C) or medium

in triplicates in a 24-well plate or transfect with mRNA as described below.

3. Incubate at 37 °C for 24 h.
4. Remove supernatant and store at −80 °C until analysis.
5. Use appropriate ELISA, Luminex or other multiplexing technology kits for measurement of proinflammatory cytokines, chemokines, ligands, or interferons following the manufacturer's instructions.

3.2 Generation and Culturing of Human and Murine Dendritic Cells (DCs)

3.2.1 Human Dendritic Cells

1. Human monocyte-derived DCs are generated and cultured in six-well plates.
2. 3 ml medium is placed in a well and 3×10^6 monocytes, obtained by elutriation, adherence, or negative selection, are added to each well.
3. Human IL-4 (100 ng/ml) and human GM-CSF (50 ng/ml) are added to each well.
4. Cells are maintained with 1 ml/well fresh medium containing cytokines every 3 days and used on day 7–10.

3.2.2 Murine Dendritic Cells

1. Sacrifice a mouse by performing CO_2 anoxia and cervical dislocation.
2. Cut off the legs of the animal and remove muscle from the femurs.
3. Put the femurs into ethanol for 3 min.
4. Put the femurs in complete medium for 1 min.
5. Cut off the ends of the femurs and wash out the bone marrow into a petri dish containing complete medium by injecting medium into the bone cavity with a syringe.
6. Count the nucleated cells. RBCs can be lysed with RBC lysis buffer, if desired.
7. Add three million cells in 3 ml complete medium to every well of a six-well plate.
8. Supplement the medium with murine GM-CSF (50 ng/ml).
9. Cells are maintained with 1 ml/well fresh medium containing murine GM-CSF every 3 days and used on day 7–11.

3.3 Transfection of Cells

Transfection of DCs, PBMCs, spleen or lymph node cells with naked mRNA or mRNA complexed with TransIT, Lipofectin, RNAiMAX, or lipid nanoparticles for in vitro measurement of adjuvant activity.

For all methods of transfection, harvest cell culture supernatant after overnight incubation and store at −80 °C until analysis.

3.3.1 Add 5 × 10⁴ DCs, PBMCs, Spleen or Lymph Node Cells per Well of a 96-Well Plate

1. Add 5×10^4 DCs, PBMCs, spleen or lymph node cells per well of a 96-well plate.

3.3.2 Transfection with Naked mRNA

1. Wash DCs in PBS to remove FCS that contains RNases.
2. Add appropriate amount of mRNA to DCs in a small volume, typically 50 μl of PBS.
3. Incubate for 1 h and then add complete medium.

3.3.3 Transfection with TransIT mRNA

1. Remove medium from cells and add 183 μl complete medium prior to transfection.
2. Combine 0.1 μg mRNA with TransIT reagents, TransIT mRNA (0.34 μl), and Boost (0.22 μl) in a final volume of 18 μl serum-free medium in a standard microcentrifuge tube.
3. Mix it well and add 17 μl of complex to cells (*see* **Note 1**).

3.3.4 Transfection with Lipofectin

1. Mix 6.7 μl serum-free medium with 0.37 μl potassium phosphate buffer with BSA in a standard microcentrifuge tube (*see* **Note 2**). Add 0.8 μl Lipofectin and mix again. Incubate at room temperature (RT) for 10 min. In the meantime, mix 0.1–1.0 μg mRNA with serum-free medium in a siliconized microcentrifuge tube in a final volume of 3.3 μl.
2. After incubation add mRNA to the Lipofectin, mix and incubate at RT for 10 min to generate complexes.
3. Add 38.8 μl serum-free medium and mix well (total volume is 50 μl).
4. Remove medium from cells, wash with serum-free medium, and add 47 μl of complex to cells.
5. After 1 h incubation, remove mRNA and add 200 μl complete medium to cells (*see* **Note 3**).

3.3.5 Transfection with Lipofectamine RNAiMAX

1. Dilute 0.5–1.5 μg of mRNA into serum-free media, 5 μl total volume in a standard microcentrifuge tube.
2. Dilute 0.5 μl of RNAimax into serum-free media, 5 μl total volume.
3. Add diluted mRNA to diluted Lipofectamine RNAiMAX reagent (1:1 ratio).
4. Incubate at RT for 5–15 min.
5. Add the complex to the cells in 190 μl complete medium in a 96-well plate.

3.3.6 Transfection with Lipid Nanoparticles (LNPs)

1. mRNA is formulated to lipid nanoparticles by a self-assembly process in which an aqueous solution of mRNA at pH 4.0 is rapidly mixed with a solution of lipids dissolved in ethanol.

2. mRNA-LNPs are preincubated with 0.1 μg human recombinant ApoE3 protein in 6 μl serum-free medium at 37 °C for 5 min (human DCs express ApoE receptors but do not produce ApoE).
3. After preincubation, mRNA-LNPs are added to DCs cultured in 194 μl complete medium.

3.4 Cytokine Assays

Assays for proinflammatory cytokines, chemokines, associated ligands, and interferons (IFNs). Enzyme-linked immunosorbent assays (ELISA), Luminex multiplex, functional assays, and other techniques for measurement are available from a variety of companies.

1. Use kits, as directed by manufacturer.
2. Other methods to measure innate immune responses can be utilized, including Northern blotting, real-time PCR, and other molecular methods to quantitate specific mRNA.

3.5 mRNA Administration

Intradermal, intramuscular, intranodal, subcutaneous injection to mice for in vivo measurement of adjuvant activity.

3.5.1 Animals

1. Anesthetize animal with isoflurane for intradermal and intranodal delivery.

3.5.2 mRNA-LNP Prep

1. Dilute mRNA-LNPs in PBS in a microcentrifuge tube or 15 ml conical tube and place on ice.

3.5.3 Intradermal Injection

1. For intradermal delivery remove hair from the back of the mice with an electric shaver.
2. Anesthetize animals in a chamber with 3% isoflurane before administration of mRNA.
3. Intradermal delivery: inject 30 μl of fluid using a 3/10 cc 29½G insulin syringe. Inject each animal at four points on the back (4 × 30 μl) (*see* **Note 4**).

3.5.4 Intramuscular Delivery

Inject 30 μl of mRNA using a 3/10 cc 29½G insulin syringe (*see* **Note 5**).

3.5.5 Intranodal Delivery [18]

1. Anesthetize mouse using isoflurane and a regulated nebulizer.
2. Shave hair at base of tail and hindquarter.
3. Inject Evans Blue tracer dye to identify lymph nodes without dissection: For each dye injection, one per side, transfer 10 μl of dye solution into a microcentrifuge tube with a micropipette and aspirate the entire 10 μl with a 31G needle attached to a 0.3 ml syringe. Inject 10 μl of dye subcutaneously, on each side of the base of the tail.

4. Remove remaining hair from the ventral side of the animal and laterally around to the dorsal side just above the hip joint of the hind leg with depilatory cream. Be sure to treat in between the abdomen and hind thigh.
5. Allow depilatory cream to incubate on skin for 3 min. Use a wet gloved hand with warm water and gently rub depilatory cream into skin.
6. Immediately remove depilatory cream with warm water and rubbing treated areas. Repeat until excess cream is removed.
7. Remove any residual depilatory cream with a wet soft cloth or paper towel and in a single motion, wiping lower portion of mouse. Avoid rubbing to prevent abrasion to the mouse.
8. On the next day, anesthetize mouse using isoflurane and a regulated nebulizer.
9. Examine the mouse to confirm drainage of tracer dye into each inguinal lymph node. The lymph node should be visible through the hairless skin as a dark spot near the hind thigh and abdomen.
10. Transfer 10 μl of mRNA solution into a microcentrifuge tube and aspirate the entire 10 μl with a 31G needle attached to a 0.3 ml syringe.
11. Tighten skin around LN using thumb and index and middle fingers by pulling skin taut to allow for accurate and controlled placement into the lymph node.
12. Inject perpendicular to the skin over the dyed LN to a depth of 1 mm.
13. Slowly inject the 10 μl volume, observing the LN size through the skin to confirm injection by visible enlargement (*see* **Note 6**).

3.5.6 Subcutaneous Delivery

Inject 200 μl of mRNA using a 3/10 cc 29½ G insulin syringe (*see* **Note 7**).

3.5.7 Completion

Place animals back in cages and confirm they completely recover from anesthesia.

3.6 Retro-Orbital Blood Collection from Mice

1. Add EDTA (1/10th the volume of blood to be collected) to a microcentrifuge tube (e.g., 10 μl EDTA for 100 μl blood).
2. Anesthetize animals in a chamber with 3% isoflurane.
3. Use a microhematocrit capillary tube to collect blood from the peri-orbital sinus of the mouse eye (*see* **Note 8**).
4. Transfer blood from the capillary into the microcentrifuge tube with EDTA.
5. After the required amount of blood is obtained gently push a Kimwipes to the peri-orbital sinus to stop further bleeding and remove extra blood from the eyeball.

6. Place the animal back in the cage and confirm that it completely recovers from anesthesia.
7. Centrifuge blood at 1000 × *g* for 10 min in a microcentrifuge and pipette the plasma into a clean microcentrifuge tube. Store at −80 °C until analysis.
8. Other forms of blood collection can be used, including cheek lancet and tail cut down.

3.7 Dot Blot to Detect dsRNA

dsRNA is a highly immunogenic molecule and is often present in IVT mRNA. It is recognized by a wide range of cellular immune sensors resulting in proinflammatory cytokine production and inhibition of translation. Thus, detection (and removal) of dsRNA contamination from RNA vaccines is essential.

1. Blot 200 ng of mRNA onto super charged Nytran membranes and dry for a minimum of 30 min.
2. Incubate membrane with blocking buffer for 1 h.
3. Rinse membrane with TBS-T buffer twice.
4. Incubate membrane with J2 mAb (1:5000 dilution) in incubation buffer at RT for 1 h.
5. Rinse membrane four times and wash six times (5 min each wash) with TBS-T buffer.
6. Incubate membrane with HRP-conjugated donkey anti-mouse IgG (1:5000 dilution) in incubation buffer at RT for 1 h.
7. Rinse membrane four times and wash six times (5 min each wash) with TBS-T buffer.
8. Detect membrane with ECL Plus Western blot detection reagent.
9. Capture images with an appropriate digital imaging system.

4 Notes

1. Transfection with TransIT-mRNA is time sensitive. Once the complexes are formed add them to the cells within 5 min.
2. Gently mix the components by tapping the sides of the tube four to six times. Do not use a vortex machine.
3. Lipofectin–mRNA complexes are made in serum-free medium and Lipofectin is toxic to cells, so it is essential to remove the complexes after 1 h incubation and add complete medium to cells.
4. For successful intradermal delivery the needle is inserted at a shallow angle, just under the superficial layer of epidermis. A hard bleb will be seen upon successful intradermal injection of even a small quantity (30 μl) of fluid.

5. Insert the needle at a shallow angle in the thigh muscle and slowly inject 30–50 μl of fluid.
6. A visual presentation of lymph node injection is available in ref. [19].
7. Lift the skin over the back to form a tent. Insert the needle at the tent base and inject the volume at a moderate rate. Your fingers should be at the top of the tent to avoid accidents.
8. The microhematocrit tube is inserted into the orbital sinus by quickly rotating the tube. The eye will not be damaged as the tube passes under the eye. The blood flow can be increased by slightly changing the angle of the tube.

References

1. Martinon F, Krishnan S, Lenzen G et al (1993) Induction of virus-specific cytotoxic T lymphocytes in vivo by liposome-entrapped mRNA. Eur J Immunol 23:1719–1722
2. Fotin-Mleczek M, Duchardt KM, Lorenz C et al (2011) Messenger RNA-based vaccines with dual activity induce balanced TLR-7 dependent adaptive immune responses and provide antitumor activity. J Immunother 34:1–15
3. Petsch B, Schnee M, Vogel AB et al (2012) Protective efficacy of in vitro synthesized, specific mRNA vaccines against influenza A virus infection. Nat Biotechnol 30:1210–1216
4. Geall AJ, Verma A, Otten GR et al (2012) Nonviral delivery of self-amplifying RNA vaccines. Proc Natl Acad Sci U S A 109:14604–14609
5. Hekele A, Bertholet S, Archer J et al (2013) Rapidly produced SAM® vaccine against H7N9 influenza is immunogenic in mice. Emerg Microbes Infect 2:e52
6. Brito LA, Chan M, Shaw CA et al (2014) A cationic nanoemulsion for the delivery of next-generation RNA vaccines. Mol Ther 22:2118–2129
7. Bogers WM, Oostermeijer H, Mooij P et al (2015) Potent immune responses in rhesus macaques induced by nonviral delivery of a self-amplifying RNA vaccine expressing HIV type 1 envelope with a cationic nanoemulsion. J Infect Dis 211:947–955
8. Brazzoli M, Magini D, Bonci A et al (2015) Induction of broad-based immunity and protective efficacy by self-amplifying mRNA vaccines encoding influenza virus hemagglutinin. J Virol. doi:10.1128/JVI.01786-15
9. Kreiter S, Selmi A, Diken M et al (2010) Intranodal vaccination with naked antigen-encoding RNA elicits potent prophylactic and therapeutic antitumoral immunity. Cancer Res 70:9031–9040
10. Van Lint S, Wilgenhof S, Heirman C et al (2014) Optimized dendritic cell-based immunotherapy for melanoma: the TriMix-formula. Cancer Immunol Immunother 63:959–967
11. Wilgenhof S, Van Nuffel AM, Benteyn D et al (2013) A phase IB study on intravenous synthetic mRNA electroporated dendritic cell immunotherapy in pretreated advanced melanoma patients. Ann Oncol 24:2686–2693
12. Weissman D, Ni H, Scales D et al (2000) HIV gag mRNA transfection of dendritic cells (DC) delivers encoded antigen to MHC class I and II molecules, causes DC maturation, and induces a potent human in vitro primary immune response. J Immunol 165:4710–4717
13. Thess A, Grund S, Mui BL et al (2015) Sequence-engineered mRNA without chemical nucleoside modifications enables an effective protein therapy in large animals. Mol Ther 23:1456–1464
14. Kariko K, Buckstein M, Ni H et al (2005) Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunity 23:165–175

15. Kariko K, Muramatsu H, Welsh FA et al (2008) Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. Mol Ther 16:1833–1840
16. Kariko K, Muramatsu H, Ludwig J et al (2011) Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. Nucleic Acids Res 39:e142
17. Midoux P, Pichon C (2015) Lipid-based mRNA vaccine delivery systems. Expert Rev Vaccines 14:221–234
18. Harrell MI, Iritani BM, Ruddell A (2008) Lymph node mapping in the mouse. J Immunol Methods 332:170–174
19. Andorko JI, Tostanoski LH, Solano E et al (2014) Intra-lymph node injection of biodegradable polymer particles. J Vis Exp 83: e50984

Chapter 9

Generation of Immunostimulating 130 nm Protamine–RNA nanoparticles

Marina Tusup and Steve Pascolo

Abstract

Nanoparticles of defined size can be easily obtained by simply mixing Protamine, a pharmaceutical drug that is used to neutralize heparin after surgery, and RNA in the form of oligonucleotides or messenger RNA. Depending on the concentrations of the two reagents and their salt contents, homogenous nanoparticles with a mean diameter of 50 to more than 1000 nm can spontaneously be generated. RNA is a danger signal because it is an agonist of for example TLR-3, -7, and -8; therefore, Protamine–RNA nanoparticles are immunostimulating. We and others have shown in vitro that nanoparticle size and interferon-alpha production by human peripheral blood mononuclear cells (PBMCs) are inversely correlated. Conversely, nanoparticle size and TNF-alpha production by PBMCs are positively correlated (Rettig et al., Blood 115:4533–4541, 2010). Particles of less than 450 nm are most frequently used for research and clinical applications because they are very stable, remain polydispersed and induce interferon-alpha proteins, which are a natural antiviral and anticancer protein family with 12 members in humans. Herein, we describe a method to generate 130 nm nanoparticles as well as some of their physical and biological characteristics.

Key words RNA, Protamine, Nanoparticles, Toll like receptor, Interferon-alpha, TNF-alpha

1 Introduction

Danger signals are immunostimulatory molecules that can stimulate surface, intravesicular, and intra-cytosolic receptors. Danger signals can be used as vaccine adjuvants as well as monotherapies to induce/boost innate and adaptive immunity against pathogens and tumor cells [1]. Toll-like receptors (TLRs) are specialized in the recognition of danger signals [2] and are expressed in different immune cells and different subcellular locations. When triggered, TLRs induce specific intracellular activation pathways that can result in the expression of different types of innate immune response molecules, such as alpha interferons (all 12 human alpha interferon

Electronic supplementary material: The online version of this chapter (doi:10.1007/978-1-4939-6481-9_9) contains supplementary material, which is available to authorized users.

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_9,

protein family members) and/or TNF-alpha. Unmodified single-stranded RNA (ssRNA) is recognized by human TLR-7 (expressed, for example, in plasmacytoid dendritic cells, the main producers of interferon-alpha) and human TLR-8 (expressed, for example, in monocytes, which are capable of producing large amounts of TNF-alpha) [3]. Thereby, ssRNA can trigger a broad range of immune cells and activation pathways [4, 5]. To protect it from RNase activity and successfully deliver it to endosomes in which TLR-7 and TLR-8 are located, ssRNA must be formulated for example in lipoplexes or polyplexes (or lipopolyplexes). Several teams including us have developed RNA polyplex formulations based on Protamine, a natural cationic peptide that is used as a drug to inhibit heparin and that also spontaneously associates with nucleic acids [4–9]. Thus, immunostimulating polyplexes can be produced for injections into humans, and we used these polyplexes as vaccines [10] or peptide vaccine adjuvants [11, 12] as experimental immunotherapy in cancer patients. Remarkably, the size of the Protamine–RNA polyplexes can be specifically and simply determined by regulating the salt concentration in the Protamine and RNA components before mixing to promote the spontaneous formation of specifically sized particles [13]. As the salt concentration decreases, the particle size decreases. In this manner, particles from 50 nm to above 1000 nm can be easily produced. Surprisingly, the size of the particles dictates their immunostimulating features. Only particles less than 450 nm efficiently stimulate plasmacytoid dendritic cells and thereby induced high levels of interferon-alpha. Larger particles activate monocytes but not plasmacytoid dendritic cells and thereby trigger the production of TNF-alpha but not alpha interferons [13]. Thus, Protamine–RNA particles of different sizes are ideal, versatile tools to activate immune cells of interest, such as in dendritic cell-based vaccination [14]. Meanwhile, because alpha-interferons are of great interest for anticancer and antivirus therapies, we have focused our research on Protamine–RNA particles that are less than 450 nm in size. These particles are further being developed as immunomodulatory drugs (Tusup and Pascolo, ongoing studies) and are also used as adjuvants for mRNA vaccines [15]. This article presents detailed methods for generating immunostimulating Protamine–RNA particles approximately 130 nm in size.

2 Materials

For dilutions, resuspension, and analysis, we use ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18.2 M Ω cm at 25 °C, 4 ppb TO—Milli-Q® Advantage A10 ultrapure water).

2.1 Particle Components

1. Protamine. Pharmaceutical Protamine is available at two concentrations: 1000 (1 ml neutralizes 1000 IU of heparin) or 5000 (1 ml neutralizes 5000 IU of heparin). To generate particles of 130 nm, we use Protamine Ipex 5000 from MEDA Pharmaceuticals (Protamine hydrochloride 5000 IU/ml, *see* **Note 1**). The Protamine is stored at 4 °C (*see* **Note 2**).
2. Messenger RNA. Highly purified mRNA coding for firefly Luciferase was produced by in vitro transcription and provided by BioNTech Ag (Prof. Ugur Sahin, Mainz, Germany). The transcript is approximately 1800 bases and contains canonic A, C, G and U residues with a 5′ cap and 3′ poly-A tail (*see* **Note 3**). The mRNA is stored at −20 °C (*see* **Note 4**).

2.2 Particle Analyzer

1. Zetasizer (Malvern) 3000HSA Particle Analyzer equipped with PCS software.
2. Transparent cuvettes (Disposable cuvettes, 1.5 ml, semi-micro, Brand + CO Gmbh, Germany).

2.3 Stimulation of Human Blood Cells

1. Heparin tubes (BD vacutainer LH 17 IU/ml, cat no. 367526).
2. Ficoll solution (Ficoll-Paque™ Plus, GE Healthcare Life Sciences, 17-1440-02).
3. Pasteur pipets.
4. Centrifuge with controllable brake (Eppendorf™ Model 5810 Centrifuge).
5. Phosphate Buffered Saline (PBS) (without Ca and Mg, pH 7.2, sterile, not for infusion, Kantonsapotheke Zurich, Switzerland).
6. 15 ml Falcon tubes.
7. Complete medium: RPMI medium 1640 (Sigma cat no. R0883) containing 10% heat-inactivated fetal bovine serum (Gibco, Life Technologies, Thermo Fischer Scientific), 1:100 antibiotics (Penicillin-Streptomycin Thermo Fischer, cat no. 15140-122) and L-Glutamine (200 mM, Merck Milipore cat no. K 0282).
8. 96-well U bottom plate (Falcon).
9. Humidified 37 °C CO_2 incubator.

2.4 Detection of Alpha Interferons

1. Pan interferon-alpha kit (MABTECH, cat no. 3425-1A-6).
2. ELISA reader (Biotek, ELx808 Absorbance Reader, software Gen 5, 2.07 version).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Production of the 130 nm Protamine–RNA Nanoparticles

This step is performed under laminar flow using sterile equipment.

1. Prepare a 0.5 mg/ml Protamine solution (*see* **Note 5**) by diluting 10 µl of Protamine 5000 in 280 µl of water.
2. Prepare a 0.5 mg/ml RNA solution (*see* **Note 5**) by diluting the RNA stock with the appropriate amount of water.
3. Add an equal amount of Protamine solution to the RNA solution (*see* **Note 6**), and pipet quickly up and down at least ten times (*see* **Note 7**).
4. Leave the solution at room temperature for 10 min (*see* **Note 8**).

3.2 Particle Size Measurement

1. Dilute 40 µl of the Protamine–RNA formulation (containing 10 µg of RNA and 10 µg of Protamine) with 1 ml of water (*see* **Note 9**).
2. Set the viscosity of the analyzer to 0.89 (select within the software setting either measurement in water or a viscosity of 0.89). As shown in Fig. 1, particles made in the conditions described above are approximately 130 nm in size with a polydispersity index (PDI) of less than 0.3 (*see* **Note 10**).

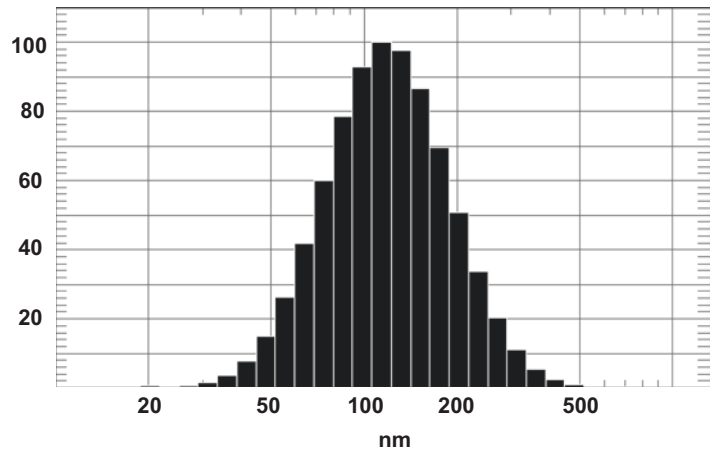

Fig. 1 Measurement of particle size displaying "Intensity-Weighted Gaussian Distribution Analysis" for solid particles. Average size is 131 nm and PDI is 0.214

3.3 Evaluation of Interferon-Alpha-Inducing Capacities in Human Blood Cells

This step is performed under laminar flow using sterile equipment.

1. Place 5 ml of fresh venous blood (collected from a healthy donor in a heparin tube, *see* **Note 11**) in a 15 ml Falcon tube.
2. Add a Pasteur pipet to the tube, and make sure that the tip of the Pasteur pipet is at the bottom of the tube.
3. Using a 5-ml pipet attached to a pipet aid, pipet 5 ml of Ficoll inside the Pasteur pipette. This fluid will underlay the blood. Elevate the Pasteur pipet slightly if the Ficoll does not flow easily into the bottom of the tube. When the maximum of Ficoll has gone in the 15 ml tube (when the level of Ficoll in the Pasteur pipet is at the level of the blood in the tube), close the Pasteur pipet with an index finger and lift it out of the Falcon tube. Discard the Pasteur pipet.
4. Carefully place the tube in a centrifuge with a balance tube on the opposite side of the rotor and centrifuge the tubes at 805 × *g*, 20 °C for 20 min without braking (*see* **Note 12**).
5. Prepare a 15 ml Falcon tube with 10 ml PBS.
6. Carefully place the tube containing blood and Ficoll under laminar flow, aspirate the upper phase (plasma) up to approximately 5 mm above the interface, and collect up to 3 ml of the interface liquid (it will contain some cells in plasma and some Ficoll) using a 5-ml pipet attached to a pipet aid. Perform small rotations with the pipette tip to collect cells that may be close to the tube wall.
7. Dilute the 3 ml collected in 10 ml PBS (tube prepared in **step 5**).
8. Mix by inverting the tube three times and place it in a centrifuge (equilibrated with a balance tube on the opposite of the rotor), and centrifuge the tubes at 453 × *g*, 20 °C for 10 min.
9. Discard the supernatant, loosen the pellet by tapping the bottom of the Falcon tube several times, and repeat the PBS wash (add 10 ml of PBS on the cells and centrifuge as in **step 7**). Loosen the pellet and add 1 ml of complete medium.
10. Count the cells (Peripheral Blood Mononuclear Cells [PBMCs]), which should total approximately 5 million.
11. Adjust the cell concentration to 5 million per ml (if needed, spin the cells again at 453 × *g* for 10 min and after having loosened the pellet, resuspend in an adequate amount of complete medium).
12. Place 4 µl of 130 nm Protamine–RNA particles (containing 1 µg of RNA and 1 µg of Protamine, obtained in **step 4** of Subheading 3.1) at the bottom of a well in a 96-well U bottom plate.
13. Add 200 µl of cells (one million) on top of the 4 µl of particles. As controls, prepare one well with 200 µl of cells alone, one well with 200 µl of cells and 1 µg of Protamine and one well with 200 µl of cells and 1 µg of RNA.

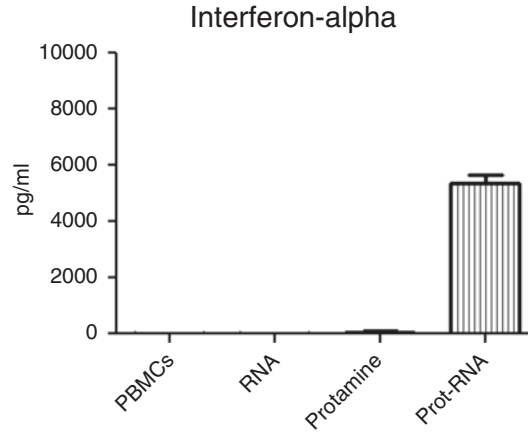

Fig. 2 Quantification of alpha interferons. The graph reports the calculated values of alpha-interferon content in supernatants from PBMCs alone ("PBMCs") or incubated with RNA ("RNA"), Protamine ("Protamine"), or Protamine–RNA nanoparticles approximately 130 nm in size (Prot-RNA). Each bar represents the average value of three data points (three culture wells) and the standard deviation

14. Incubate overnight at 37 °C in a humidified CO_2 incubator.
15. Transfer cell culture supernatants to a fresh 96-well plate. Proceed to **step 15** or store at −80 °C (*see* **Note 13**).
16. Use 20 µl of cell culture supernatant (add 80 µl per well of assay diluent: 1% BSA in PBS) in the pan-interferon-alpha ELISA kit strictly following the manufacturer's instructions (*see* **Note 14**).
17. Calculate experimental values. As shown in Fig. 2, Protamine–RNA nanoparticles of approximately 130 nm efficiently induce interferon-alpha expression in PBMCs in vitro (*see* **Notes 15** and **16**).

4 Notes

1. Protamine sulfate, such as Valeant Protamine, gave identical results.
2. We have observed that freezing Protamine solutions may jeopardize its functionality as far as production of immunostimulating RNA polyplex is concerned. Pharmaceutical Protamine should be stored at 4 °C as indicated on the package.
3. Any RNA (oligonucleotide, long uncapped and/or non-polyadenylated mRNA, or capped mRNA) can be used to generate the 130 nm particles.

4. Highly pure sterile RNA in water is very stable at room temperature. However, because contaminating RNases could theoretically be present, we store RNA at 4 °C (day storage) or −20 °C (long term storage) and bring it to room temperature when doing the experiments before immediately placing it back at 4 °C or −20 °C.
5. The more RNA and Protamine are diluted, the smaller the resulting particles will be (Supplementary Fig. 1a). Particles of 50–250 nm in size induce interferon-alpha in human blood cells in vitro to similar extents (Supplementary Fig. 1b).
6. Mixing equal mass amounts of Protamine and RNA will generate nearly neutral or slightly positive particles (zeta potential). Using a twofold mass excess of Protamine or a twofold mass excess of RNA will generate particles with positive or negative zeta potentials, respectively, as previously described [13]. Size (Supplementary Fig. 2a) and in vitro immunostimulation characteristics (Supplementary Fig. 2b) of the particles are similar when using particles with a positive, neutral, or negative surface charge within this range (twofold excess of one of the two components).
7. We always add Protamine to RNA, not RNA to Protamine.
8. This formulation is very stable at room temperature (as long as it is made using sterile RNA, Protamine, and water and in sterile conditions, i.e., under a laminar flow) or in the fridge. We have tested storage of this material up to 1 week and found that the storage period did not affect particle size or immunostimulatory capacity (Supplementary Fig. 3). By contrast, freezing the particles at −20 °C destroys their immunostimulatory capacity. Thus, it is of utmost importance to store the 130 nm Protamine–RNA nanoparticles in liquid solutions, and we recommend storage of undiluted particles at 4 °C.
9. We observed that the particles aggregate over time when diluted in salt-containing solutions, such as PBS or Ringer lactate, and thereby will appear larger than their original size if analyzed in those solutions. The original particle size is preserved when the particles are undiluted or diluted in water or other low-salt solution (e.g., isotonic 5% glucose). Thus, for particle size measurement, Protamine–RNA nanoparticles must be preserved by dilution in water or other low-salt solution.
10. A PDI value of 1 indicates that the sample has a very broad size distribution and may contain large particles or aggregates that could be slowly sedimenting. A PDI value closer to zero denotes a monodispersed system (one unique particle size in the sample). For biological particles, it is usually accepted that a PDI value below 0.5 represents a relatively homogenous formulation.

11. Blood stored up to 24 h at 4 °C can be used. However, frozen blood cells did not respond as well as did fresh or stored (up to 24 h) blood. We suspect that plasmacytoid dendritic cells, an important producer of interferon-alpha, do not survive freeze–thaw cycles well when cells are frozen in physiologic solutions containing 10% DMSO.
12. The centrifuge should accelerate and decelerate as slowly as possible. Should acceleration or deceleration be too fast, the interface between blood and Ficoll will be perturbated, and the collection of peripheral blood mononuclear cells will be jeopardized. Should this happen (for example, by mistake the brake is not disabled during centrifugation), the blood and Ficoll can be manually further mixed together (inverting the tube few times) and 5 ml Ficoll can again be underlay with 5 ml of Ficoll as in **step 3** of Subheading 3.3 before centrifugation under the appropriate conditions.
13. We observed that supernatants stored at −20 °C were not well preserved as far as interferon-alpha is concerned.
14. Using 20 μl of cell culture supernatants usually allows experimental ELISA optical densities (ODs) that are in the range of the standard titration (from 0 to 1000 pg/ml).
15. The total amount of alpha interferon detected in supernatants varies from donor to donor and can be in the range of 1000–10,000 pg/ml.
16. For each condition, we performed three PBMC cultures (triplicates) and presented the data as the means plus standard deviation.

Acknowledgement

The research leading to these results has received funding from the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 601939 (MERIT).

References

1. Parvanova I, Rettig L, Knuth A et al (2011) The form of NY-ESO-1 antigen has an impact on the clinical efficacy of anti-tumor vaccination. Vaccine 29:3832–3836
2. Kawai T, Akira S (2011) Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 34:637–650
3. Jarrossay D, Napolitani G, Colonna M et al (2001) Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. Eur J Immunol 31:3388–3393
4. Scheel B, Braedel S, Probst J et al (2004) Immunostimulating capacities of stabilized RNA molecules. Eur J Immunol 34:537–547
5. Scheel B, Teufel R, Probst J et al (2005) Toll-like receptor-dependent activation of several human blood cell types by protamine-condensed mRNA. Eur J Immunol 35:1557–1566
6. Hoerr I, Obst R, Rammensee HG et al (2000) In vivo application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies. Eur J Immunol 30:1–7

7. Scheel B, Aulwurm S, Probst J et al (2006) Therapeutic anti-tumor immunity triggered by injections of immunostimulating single-stranded RNA. Eur J Immunol 36:2807–2816
8. Amos H, Kearns KE (1963) Influence of bacterial ribonucleic acid on animal cells in culture. II. Protamine enhancement of RNA uptake. Exp Cell Res 32:14–25
9. Billiau A, Buckler CE, Dianzani F et al (1969) Induction of the interferon mechanism by single-stranded RNA: potentiation by polybasic substances. Proc Soc Exp Biol Med 132:790–796
10. Weide B, Pascolo S, Scheel B et al (2009) Direct injection of protamine-protected mRNA: results of a phase 1/2 vaccination trial in metastatic melanoma patients. J Immunother 32:498–507
11. Feyerabend S, Stevanovic S, Gouttefangeas C et al (2009) Novel multi-peptide vaccination in Hla-A2+ hormone sensitive patients with biochemical relapse of prostate cancer. Prostate 69:917–927
12. Widenmeyer M, Griesemann H, Stevanovic S et al (2011) Promiscuous survivin peptide induces robust CD4(+) T-cell responses in the majority of vaccinated cancer patients. Int J Cancer 131:140–149
13. Rettig L, Haen SP, Bittermann AG et al (2010) Particle size and activation threshold: a new dimension of danger signaling. Blood 115:4533–4541
14. Skold AE, van Beek JJ, Sittig SP et al (2015) Protamine-stabilized RNA as an ex vivo stimulant of primary human dendritic cell subsets. Cancer Immunol Immunother 64:1461–1473
15. Fotin-Mleczek M, Duchardt KM, Lorenz C et al (2011) Messenger RNA-based vaccines with dual activity induce balanced TLR-7 dependent adaptive immune responses and provide antitumor activity. J Immunother 34:1–15

Chapter 10

Electroporation of mRNA as Universal Technology Platform to Transfect a Variety of Primary Cells with Antigens and Functional Proteins

Kerstin F. Gerer*, Stefanie Hoyer*, Jan Dörrie*, and Niels Schaft*

Abstract

Electroporation (EP) of mRNA into human cells is a broadly applicable method to transiently express proteins of choice in a variety of different cell types. We have spent more than a decade to optimize and adapt this method, first for antigen-loading of dendritic cells (DCs), and subsequently for T cells, B cells, bulk PBMCs, and several cell lines. In this regard, antigens were introduced, processed, and presented in context of MHC class I and II. Next to that, functional proteins like adhesion receptors, T-cell receptors (TCRs), chimeric antigen receptors (CARs), constitutively active signal transducers, and others were successfully expressed. We have also established this protocol under full GMP compliance as part of a manufacturing license to produce mRNA-electroporated DCs for therapeutic vaccination in clinical trials. Therefore, we here want to share our universal mRNA electroporation protocol and the experience we have gathered with this method. The advantages of the transfection method presented here are: (1) easy adaptation to different cell types, (2) scalability from 10^6 to approximately 10^8 cells per shot, (3) high transfection efficiency (80–99%), (4) homogenous protein expression, (5) GMP compliance if the EP is performed in a class A clean room, and (6) no transgene integration into the genome. The provided protocol involves: Opti-MEM® as EP medium, a square-wave pulse with 500 V, and 4 mm cuvettes. To adapt the protocol to differently sized cells, simply the pulse time is altered. Next to the basic protocol, we also provide an extensive list of hints and tricks, which in our opinion are of great value for everyone who intends to use this transfection technique.

Key words mRNA electroporation, Monocyte-derived DC (moDC), T cell, CAR, TCR, B cell, Antigen expression, Protein expression, GMP, Immunotherapy

1 Introduction

Electroporation (EP) is a standard method to transfer nucleic acid into eukaryotic cells. The exact mechanism how the membrane is passed is still unknown, but according to current scientific knowledge the nucleic acid moves, due to its electric charge, in the

*Kerstin F. Gerer, Stefanie Hoyer, Jan Dörrie, and Niels Schaft contributed equally to this manuscript.

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_10,

electric field, which is applied between the electrodes of the cuvette. Thereby the nucleic acid crosses the cell membranes of cells present in the cuvette [1]. Besides DNA, mRNA is increasingly used in this context. Although the transfer of RNA into cells is defined as genetic engineering, mRNA has the large advantage over DNA that there is no risk of integration into the host genome. Further benefits of mRNA electroporation are: (1) high transfection efficiency (*see* Fig. 1) [2–7], (2) rapid expression of protein, (3) transiency of the expression, (4) high reproducibility [8], (5) absence of an influence on cell phenotype [9, 10], (6) ability to introduce several proteins at the same time [2, 3, 11–13], (7) scalability of the method, and (8) GMP compatibility [4, 14]. Thus, this method creates a highly reproducible and validatable product that can be used for cellular immunotherapies.

mRNA electroporation of dendritic cells (DCs) has emerged as a means to load the DCs with antigen and to introduce functional proteins to mature and activate these DCs [11, 15–20]. This strategy also found its way from the bench to the bedside, since in the last 5 years alone, over 20 publications about clinical application of mRNA-electroporated DCs appeared. These studies were performed with cancer patients, suffering from malignant melanoma [11, 15–19, 21–23], but also from kidney cancer [15, 20], pancreatic cancer [15], glioblastoma [15, 24], multiple myeloma [15], acute myeloid

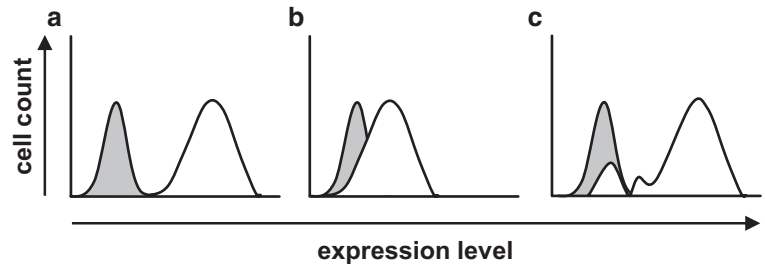

Fig. 1 Schematic examples of different transfection efficiencies. The expression level of your protein of interest and the transfection efficiency of your cells can vary between different mRNAs and cells types. A schematic illustration of different transfection efficiencies is depicted. The *gray histograms* represent cells transfected without mRNA, whereas the *white histograms* show the expected expression of the protein of interest. (**a**) The histogram is representative for a successful transfection with high protein expression. Almost 100 % of the cells are transfected. (**b**) Cells depicted in this plot were also electroporated with high efficiency, but the introduced protein is expressed at a lower level. Thus, you may use more mRNA, or, if your cells can stand it, increase the pulse time for gaining better results. (**c**) Here, not all cells are transfected with your protein hence you have a mixture of non-transfected and transfected cells, which can in addition display a diverse expression of the protein. This can happen if you start with a mixture of cells (e.g., bulk primary cells like PBMCs), or if some of the cells are damaged. You may want to reduce the pulse time to improve your results

leukemia [25], colorectal cancer [26], uterine cancer [27], and others [15, 28]. Next to cancer, also HIV was treated [29, 30]. All of these trials used monocyte-derived DCs. DC vaccination was very well tolerated, and seemed to induce clinical benefits, but unfortunately only in an unsatisfyingly small percentage of the patients [11]. Therefore, the optimization of DC vaccines is still ongoing.

Adoptive T-cell transfer was proven to be a powerful immunotherapeutic tool during the last decades, in particular to specifically target tumors with cytolytic T cells [31]. Engineering T cells that express chimeric antigen receptors (CARs) or normal T-cell receptors (TCRs) has emerged as a strategy to redirect them to effectively recognize and lyse tumor cells [32–34] (and reviewed in refs. 35–39). Retroviral and lentiviral transduction are currently the preferred procedures to equip T cells with antigen-specific receptors [35–43]. Safety concerns, however, have been raised regarding permanent and high level receptor expression resulting in autoimmunity by the modified T cells [44–46]. Therefore, the transient receptor transfer is considered a safer approach, with mRNA electroporation providing a robust and easy-to-perform method [47–51], which recently yielded first clinical data [52, 53].

Besides the clinical application, mRNA electroporation of various cell types is widely used in preclinical and basic research. By transfection of antigen-presenting cells (APCs), they are forced to process and present the exogenous protein, generating all naturally processed epitopes of the full length antigen. Hence, targets for immunological assays can be generated, which are suitable to assess the immunogenic potential of tumor-associated [54] or viral antigens. The mRNA-electroporated APCs can be used to prime and expand T cells [55], to monitor the quantity and quality of T cells, which are specific for the respective antigen, or to examine the intracellular pathways of antigen-processing [56]. Normal B cells or Epstein-Barr virus (EBV)-transformed B-cell lines were transfected in this fashion and used as APCs [57–59]. DCs have also been used as targets in functional readout assays [60]. As any MHC class I-positive cell can serve as APCs, bulk peripheral blood mononuclear cells (PBMCs) [61, 62], and even the $CD8^+$ T cells themselves were electroporated with the antigen and then successfully used to reciprocally stimulate each other in an IFNγ-enzyme linked immuno spot (ELISPOT) assay [54]. Taken together, this shows the broad applicability of mRNA electroporation, which is described in this chapter.

2 Materials

2.1 Equipment

1. Cell culture equipment.
2. Liquid handling equipment.
3. Electroporation device capable to deliver a square-wave pulse (e.g., the Bio-Rad Gene Pulser Xcell or the BTX ECM 830).

2.2 Consumables

1. Electroporation cuvettes 4 mm electrode gap (e.g., Cell Projects; Mat.No. EP-104; *see* **Note 6** and Fig. 2).
2. RNase-free pipette filter tips.
3. Wet ice.
4. mRNA can be generated by in vitro transcription (IVT) from any plasmid with suitable promoter (e.g., T7). A plasmid-encoded polyA stretch is advisable, but not absolutely necessary, since enzymatic polyadenylation is also possible. Several companies offer suitable IVT-kits. Proper capping is required and a minimum polyA of at least 50 A. Enzymatic polyadenylation can be beneficial (*see* **Note 2**). The concentration of the mRNA should be sufficient (*see* **Note 10**).
5. Cells should be healthy and vital. DCs should be prepared and can be matured according to Pfeiffer et al. [55]. T cells can be purified by magnetic cell sorting or expanded from PBMCs according to Krug et al. [4]. Transformed cells lines should be split 1 day prior to electroporation.
6. RPMI 1640 without L-glutamine.
7. Opti-MEM® without phenol red.
8. Cell culture media: Use the respective cell culture media for your cell type or cell line, which is usually used for their cultivation. Typical media compositions for T-cell, DC, and B-cell cul-

Fig. 2 No plastic ridges should cover the inside of the electrodes of the cuvette. An electroporation cuvette was cut in half. The image shows the inside surface of the electrodes. There must not be any plastic ridges covering the metal, as depicted on the *left-hand side*. On the *right-hand side*, a plastic ridge covering the lower edge of the electrode is present (see *arrow*). Especially if only a small volume of cell suspension is used, this will result in improper contact of the suspension to the electrode, thus negatively affecting the electroporation. This may even result in arc formation

tivation are listed below (concentrations of stock solutions are mentioned in brackets; for the choice of medium *see* Table 1). The mentioned media consist of the indicated ingredients. Medium components are added to RPMI 1640 and the mixture is subsequently filtered sterile. All steps are performed in a laminar flow cabinet.

(a) DC medium: 500 ml RPMI 1640, 5 ml human serum (heat-inactivated for 30 min at 56 °C), 5 ml L-glutamine (200 mM), 200 µl gentamicin (20 µg/ml).

(b) MLPC medium: 500 ml RPMI 1640, 50 ml human serum (heat-inactivated for 30 min at 56 °C), 5 ml L-glutamine (200 mM), 5 ml hepes buffer (1 M), 5 ml sodium pyruvate (100 mM), 5 ml nonessential amino acids (100×), 200 µl gentamicin (20 µg/ml).

(c) R10 medium: 500 ml RPMI 1640, 50 ml FCS or FBS (heat-inactivated for 30 min at 56 °C), 5 ml L-glutamine (200 mM), 5 ml penicillin–streptomycin (10,000 UI/ml each), 1 ml hepes buffer (1 M), 200 µl β-mercaptoethanol (50 mM).

9. Supplements: Certain supplements, e.g., cytokines, might be needed after electroporation. Whether you apply any supplements or not depends on the kind of assay you will perform afterwards. Consecutively, examples of cytokine additions (final concentration) are listed: DCs 800 U/ml GM-CSF and 275 IU/ml IL-4, T cells 10 ng/ml IL-7.

Table 1
Electroporation conditions for different types of cells

Cell type		DCs	B cells	PBMCs	T cells	Cell lines
EP[a] settings	Voltage [V]	500	500	500	500	500
	Electrode gap [mm]	4	4	4	4	4
	Pulse duration [ms]	1	3	3	5	1–3[d]
Cell concentration	Maximum cell number per 100 µl Opti-MEM®	6×10^6	10×10^6	8×10^6	12×10^6	$8–10\times10^{6e}$
Cultivation after EP	Medium[b]	DC medium	R10	MLPC	MLPC	R10[f]
	Supplement[c]	800 U/ml GM-CSF + 275 IU/ml IL-4	–	–	10 ng/ml IL-7	–

[a] *EP:* electroporation
[b] Distinct ingredients of the respective media are described in the materials section
[c] Final concentration is displayed
[d] Larger cells require shorter pulse durations
[e] Larger cells require lower cell concentrations
[f] Or medium required for the respective cell line

3 Methods

3.1 General Remarks

1. Prepare your experimental setup carefully (*see* **Note 1**).
2. Comply with the general rules of working with RNA, i.e., use only RNase-free materials, wear gloves, use filter-tips and disposable materials whenever handling the mRNA (*see* **Note 2**).
3. Carry out all working steps at room temperature (18 to 22 °C) unless mentioned otherwise.
4. Perform all centrifugation steps for 10 min at 140 × *g* for dendritic cells, or 215 × *g* for all other cell types with maximal acceleration and deceleration.
5. Perform all individual working steps with open cell products under a laminar flow.
6. Set your incubator conditions to standard 37 °C, 5 % CO_2, and 95 % relative humidity.

3.2 Preparatory Work

1. Remove the RPMI 1640, Opti-MEM®, and the appropriate cell culture medium (*see* Table 1) from the refrigerator to allow for warming to room temperature.
2. Thaw your mRNAs gently, resuspend them, and keep them on ice (*see* **Notes 2–4**).
3. Prepare and label the 4 mm gap cuvettes and tissue culture plates (*see* **Notes 5** and **6**).
4. Adjust the settings of your electroporation device to square-wave protocol and use the appropriate settings suitable for your cell type (*see* Table 1).
5. Perform a test pulse without inserting a cuvette.

3.3 Protocol

1. Harvest your cells by rinsing them with RPMI 1640 (*see* **Notes 7–9**).
2. Count your cells to determine the cell number.
3. During the centrifugation steps (**step 4** and following), calculate your cell numbers, electroporation volumes, and mRNA volumes (*see* **Notes 10–12**). You should at least electroporate 1×10^6 cells per pulse. The maximum cell concentrations for each cell type are described in Table 1. At least 100 µl Opti-MEM® are necessary per pulse. You can increase the volume up to 600 µl, but be aware that you have to augment the quantity of mRNA according to the increase of the used quantity of Opti-MEM®.
4. Centrifuge your cells with the appropriate settings (*see* Table 1).
5. Aspirate and discard the supernatant.
6. Resuspend the cells (and pool in one tube if needed) in RPMI.

7. Centrifuge the cells again and discard the supernatant.
8. While centrifuging the cells, prepare the plates, on which you will put the cells after electroporation. After electroporation you should seed the cells with a final concentration of roughly 1×10^6 cells/ml. Use the appropriate medium and supplements for the respective cell type (*see* Table 1 and **Note 13**). Resuspend the cells in 5–10 ml Opti-MEM® to wash away any residual RPMI.
9. Centrifuge the cells with the appropriate centrifugation settings according to the cell type used (*see* Table 1).
10. During this last centrifugation step transfer the calculated quantities of mRNA onto the bottom of your cuvette (*see* **Note 14**). Use filter tips for mRNA pipetting.
11. Discard the supernatant and subsequently resuspend the cells in the calculated quantity of Opti-MEM®.
12. Add the respective quantity of cell suspension to the mRNA into the cuvette. No additional mixing is necessary (*see* **Note 14**). Ensure that the fluid is at the bottom of the cuvette and forms a concave meniscus (*see* Fig. 3 and **Troubleshooting 2**). Try to avoid air bubbles by knocking the cuvette gently on your laminar flow (*see* **Note 15**).
13. Place the closed cuvette into the shock pod of the electroporation device.
14. Pulse the cells (*see* Table 1 for electroporation settings).
15. Remove the cuvette from the shock pod and transfer the cells immediately into the prepared medium. Rinsing of the cuvettes is not necessary (*see* **Note 16**).

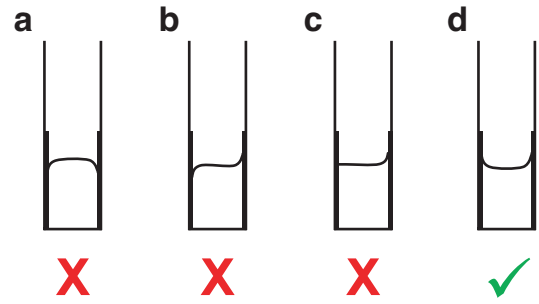

Fig. 3 The cell suspension has to form a concave meniscus in the cuvette. It should be assured that the current can flow easily through the cell suspension from one to the other electrode inside the cuvette. The contact surface between the electrodes and the fluid should be as large as possible. Hence, fluid levels as depicted in (**a**), (**b**), and (**c**) should be avoided, since the fluid makes only very little contact to the electrodes. It is very important that the cell suspension forms a concave meniscus (**d**) inside the cuvette

16. Incubate your cells (as described above at standard incubator conditions) for at least 1 h before proceeding with further experiments (*see* **Notes 17** and **18**).

4 Notes

1. Electroporation (EP) is a stressful procedure for the cells, but also the EP medium (Opti-MEM®) can have some detrimental effect on the cell, therefore plan your experiments carefully. Prepare your experimental setup in detail and have everything ready before you start to work with the cells to keep the time in which the cells are resuspended in Opti-MEM® as short as possible. Transfer the cells immediately to the culture medium after electroporation.
2. mRNA can be generated with commercially available in vitro transcription kits. It must be capped and should have a polyA tail of at least 50 A. Longer polyA tails generated for example by enzymatic polyadenylation may improve your mRNA stability inside the transfected cell, hence resulting in better and longer protein expression. The pure mRNA should be dissolved in water (free of endotoxins and other noxious substances) or Opti-MEM®.
3. RNA is always endangered by the omnipresent RNases, so when handling the mRNA for electroporation, work scrupulously cleanly. Be also aware that even without RNases, RNA is not very stable at room temperature. As soon as you start working with the mRNA, ensure that the electroporation process is carried out as rapidly as possible.
4. Due to the low mRNA stability, keep the time during which your mRNA is thawed as short as possible and always keep it on ice. Aliquot your mRNA batches in small quantities to keep thawing cycles at a minimum. mRNAs can be stored at –20 °C until usage (for longer storage times, preserve them at –80 °C).
5. You can also use 2 mm gap cuvettes instead of 4 mm gap cuvettes, but then you have to use half the voltage, i.e., 250 V. You may also have trouble pipetting the cells out of the smaller gap. Hence, try in advance if you can reach the bottom of the cuvette with your pipette tips.
6. An immaculate quality of your cuvettes is essential, especially when transfecting small volumes. Some vendors offer cuvettes that have small plastic ridges that cover the lower end of the electrodes (*see* Fig. 2). This will negatively affect the electroporation process and often results in arc formation.
7. You can not only use freshly isolated or generated cells, but you can also use thawed cells. If you use thawed cells, let these cells rest for at least 1 h after thawing before you start the electro-

poration process. Since the freeze and thaw process means additional stress for the cells, they will most likely survive the electroporation process inferior to fresh cells.

8. If you use cells isolated by magnetic beads, let them rest at least for 4 h. For better results let them rest over night. During this time, the beads can drop off, and thus the electroporation process will not be disturbed by the magnetic beads.
9. Harvesting of adherent cells is a critical step, especially if you have sensitive cells (e.g., DCs). The cells should not be stressed already in advance of the electroporation process. Do not tap the plates or cell culture flasks for harvesting! Do not harvest the cells by incubating at low temperature, but work at room temperature. Rinsing the cells off with a sharp jet of medium from the 10 ml pipet is, however, well tolerated.
10. The quantity of water, in which the mRNAs are dissolved, must not be too high, otherwise the resulting osmotic stress will damage the cells. If the concentration of your mRNA is too low, precipitate your mRNA and dissolve it in a smaller quantity of water. The volume of water (containing mRNA) during the electroporation should never exceed 25% of the volume of Opti-MEM® used. Alternatively, you may also dissolve your mRNA directly in Opti-MEM®.
11. It is possible to electroporate different mRNAs simultaneously. The different mRNAs can be applied concurrently into the electroporation cuvette. Subsequently, the electroporation procedure can be performed as described, and the different mRNAs are co-electroporated.
12. It is also possible to co-electroporate DNA together with the mRNA into the cells. The DNA has to enter the nucleus and therefore it is more difficult to achieve sufficient expression in DNA-transfected cells compared to mRNA-transfected cells. Yet luciferase reporter plasmids were already successfully transfected into Jurkat T cells [63]. In case that you use DNA for electroporation, the DNA (like the mRNA) has to be dissolved in nuclease-free water or Opti-MEM®.
13. Consider for which experiment you want to use the cells after electroporation – for some experiments the addition of cytokines could impact your results. Thus, you might seed the cells without any additional supplements. The supplements for dendritic cells, however, are quite essential. GM-CSF and IL-4 prevent dendritic cells from sticking to the cell culture plates. Without these cytokines, the cell harvesting might be difficult.
14. Always apply the mRNA first and add the cells into the cuvette afterwards. Thereby the mRNA is automatically mixed with your cell suspension and you avoid additional mixing steps, which might only cause the formation of bubbles.

15. In order to avoid inhomogeneities in the electric field inside your cuvette, be sure that no bubbles are present. They could disturb the electrical flow and may even result in arc formation.
16. It is not necessary to rinse your cuvettes after transferring the cells to the prepared plates. By rinsing the cuvettes you transfer mainly dead cells and cell debris from the electroporation cuvette onto the cell culture plate.
17. Each mRNA behaves slightly different. Consider that you have to perform an expression kinetics assay to assess at which time-point your mRNA leads to the highest expression and to decide how to perform your individual experimental setup.
18. Since each mRNA has its own individual expression kinetics, you should titrate the amount of mRNA needed to reach a sufficient expression of your protein of interest.

5 Troubleshooting

1. *Cells die*: Typically, 30–90 % of the cells survive the electroporation process if you follow the instructions provided. If fewer cells survive the electroporation you should improve your procedure. One reason might be that the cells were already stressed before, e.g., by incorrect, i.e., too harsh, harvesting. Moreover, the used media might not have been warmed to room temperature and the cells therefore suffered from a thermal shock. If you electroporate cell lines be sure they are cultured properly in advance. Therefore, best split them the day before electroporation. Additionally, make sure that they are mycoplasma-free. Some maturation stimuli for DCs also result in poor survival. Hence, you can also reduce the pulse time, but this will also result in lower protein expression levels.
2. *Arc formation*: It is possible that you see (and hear) an arc formation inside the cuvette during pulsing of the cells. This may involve a flash of light and a loud bang, which usually produces disastrous results. It may even damage your electroporation device. This can occur if the current cannot flow properly and homogenously from one to the other electrode inside the cuvette. To avoid this, be sure to always check the fluid level in your cuvette (*see* Fig. 3). If the fluid level does not form a concave meniscus (*see* Fig. 3, **a**-**c**), hold the top of the cuvette between index finger and thumb, and shake it gently from the wrist until the fluid level adjusts to a concave meniscus (*see* Fig. 3d). Another reason for an arc formation might be the usage of low quality cuvettes (*see* Fig. 2, right cuvette).
3. *Low or no protein expression*: Although you have followed all instructions, you may not see any or a sufficient expression of your protein. There can be several reasons for this issue. On the

one hand, your mRNA might be of low quality (e.g., due to a too short polyA tail) or be degraded. Whether your mRNA is degraded can be easily checked by performing an agarose gel electrophoresis. On the other hand, the quantity of your used mRNA might be not sufficient. In this case you have to increase the quantity of mRNA. The required quantities vary from RNA to RNA. As a reference value, between 5 and 30 μg per 100 μl of Opti-MEM® of polyadenylated mRNA are commonly used. If you have good survival rates, you can sacrifice some cells by increasing the pulse time. This reduces the yield, but usually increases the expression level of the introduced protein. Furthermore, each protein is expressed with different kinetics, so you have to determine the time-point of highest expression.

4. *Unexpected behavior of cells*: Cells usually cope with the electroporation very well and are not influenced by the electroporation process *per se*. However, in some cases the cells might change their activation or maturation status due to the electroporation (e.g., immature DCs might be slightly activated by the whole process, or calcium might enter the cytoplasm of T cells through permeabilized membranes). Always remember that the electroporation is a stressful procedure for the cells.

Acknowledgements

We thank Gerold Schuler and Beatrice Schuler-Thurner for their support during the establishment and improvement of mRNA electroporation. Furthermore, we thank the former and the current members of the RNA-group and our collaborators, who participated in the establishment of the mRNA electroporation protocol, or its adaption and improvement: Peter Thumann, Verena Wellner, Ina Müller, Stefanie Baumann, Tanja Moritz, Manuel Wiesinger, Michael Erdmann, Katrin Birkholz, Christian Hofmann, Thomas Harrer, Christian Wohn, Isabell Pfeiffer, Christian Krug, Sabrina Prommersberger, and Sandra Höfflin.

References

1. Gehl J (2003) Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research. Acta Physiol Scand 177:437–447
2. Schaft N, Dorrie J, Thumann P et al (2005) Generation of an optimized polyvalent monocyte-derived dendritic cell vaccine by transfecting defined RNAs after rather than before maturation. J Immunol 174:3087–3097
3. Dorrie J, Schaft N, Muller I et al (2008) Introduction of functional chimeric E/L-selectin by RNA electroporation to target dendritic cells from blood to lymph nodes. Cancer Immunol Immunother 57:467–477
4. Krug C, Wiesinger M, Abken H et al (2014) A GMP-compliant protocol to expand and transfect cancer patient T cells with mRNA encoding a tumor-specific chimeric antigen receptor. Cancer Immunol Immunother 63:999–1008
5. Strobel I, Berchtold S, Gotze A et al (2000) Human dendritic cells transfected with either RNA or DNA encoding influenza matrix protein M1 differ in their ability to stimulate cytotoxic T lymphocytes. Gene Ther 7:2028–2035

6. Van Tendeloo VF, Ponsaerts P, Lardon F et al (2001) 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 98:49–56
7. Saeboe-Larssen S, Fossberg E, Gaudernack G (2002) mRNA-based electrotransfection of human dendritic cells and induction of cytotoxic T lymphocyte responses against the telomerase catalytic subunit (hTERT). J Immunol Methods 259:191–203
8. Schaft N, Wellner V, Wohn C et al (2013) CD8(+) T-cell priming and boosting: more antigen-presenting DC, or more antigen per DC? Cancer Immunol Immunother 62:1769–1780
9. Hoyer S, Gerer KF, Pfeiffer IA et al (2015) Electroporated antigen-encoding mRNA is not a danger signal to human mature monocyte-derived dendritic cells. J Immunol Res. ID 952184
10. Lundqvist A, Noffz G, Pavlenko M et al (2002) Nonviral and viral gene transfer into different subsets of human dendritic cells yield comparable efficiency of transfection. J Immunother 25:445–454
11. Van Lint S, Wilgenhof S, Heirman C et al (2014) Optimized dendritic cell-based immunotherapy for melanoma: the TriMix-formula. Cancer Immunol Immunother 63:959–967
12. Hofflin S, Prommersberger S, Uslu U et al (2015) Generation of CD8(+) T cells expressing two additional T-cell receptors (TETARs) for personalised melanoma therapy. Cancer Biol Ther 16:1323–1331
13. Hofmann C, Hofflin S, Huckelhoven A et al (2011) Human T cells expressing two additional receptors (TETARs) specific for HIV-1 recognize both epitopes. Blood 118:5174–5177
14. Erdmann M, Dorrie J, Schaft N et al (2007) Effective clinical-scale production of dendritic cell vaccines by monocyte elutriation directly in medium, subsequent culture in bags and final antigen loading using peptides or RNA transfection. J Immunother 30:663–674
15. Bloy N, Pol J, Aranda F et al (2014) Trial watch: dendritic cell-based anticancer therapy. Oncoimmunology 3:e963424
16. Van Nuffel AM, Benteyn D, Wilgenhof S et al (2012) Dendritic cells loaded with mRNA encoding full-length tumor antigens prime CD4+ and CD8+ T cells in melanoma patients. Mol Ther 20:1063–1074
17. Van Nuffel AM, Benteyn D, Wilgenhof S et al (2012) Intravenous and intradermal TriMix-dendritic cell therapy results in a broad T-cell response and durable tumor response in a chemorefractory stage IV-M1c melanoma patient. Cancer Immunol Immunother 61:1033–1043
18. Wilgenhof S, Van Nuffel AM, Corthals J et al (2011) Therapeutic vaccination with an autologous mRNA electroporated dendritic cell vaccine in patients with advanced melanoma. J Immunother 34:448–456
19. Wilgenhof S, Corthals J, Van Nuffel AM et al (2015) Long-term clinical outcome of melanoma patients treated with messenger RNA-electroporated dendritic cell therapy following complete resection of metastases. Cancer Immunol Immunother 64:381–388
20. Amin A, Dudek AZ, Logan TF et al (2015) Survival with AGS-003, an autologous dendritic cell-based immunotherapy, in combination with sunitinib in unfavorable risk patients with advanced renal cell carcinoma (RCC): phase 2 study results. J Immunother Cancer 3:14
21. Aarntzen EH, Schreibelt G, Bol K et al (2012) Vaccination with mRNA-electroporated dendritic cells induces robust tumor antigen-specific CD4+ and CD8+ T cells responses in stage III and IV melanoma patients. Clin Cancer Res 18:5460–5470
22. Bol KF, Mensink HW, Aarntzen EH et al (2014) Long overall survival after dendritic cell vaccination in metastatic uveal melanoma patients. Am J Ophthalmol 158:939–947
23. Bol KF, Figdor CG, Aarntzen EH et al (2015) Intranodal vaccination with mRNA-optimized dendritic cells in metastatic melanoma patients. Oncoimmunology 4:e1019197
24. Mitchell DA, Batich KA, Gunn MD et al (2015) Tetanus toxoid and CCL3 improve dendritic cell vaccines in mice and glioblastoma patients. Nature 519:366–369
25. Van Tendeloo VF, Van de Velde A, Van Driessche A et al (2010) Induction of complete and molecular remissions in acute myeloid leukemia by Wilms' tumor 1 antigen-targeted dendritic cell vaccination. Proc Natl Acad Sci U S A 107:13824–13829
26. Lesterhuis WJ, de Vries IJ, Schreibelt G et al (2010) Immunogenicity of dendritic cells pulsed with CEA peptide or transfected with CEA mRNA for vaccination of colorectal cancer patients. Anticancer Res 30:5091–5097
27. Coosemans A, Vanderstraeten A, Tuyaerts S et al (2013) Wilms' Tumor Gene 1 (WT1)-loaded dendritic cell immunotherapy in patients with uterine tumors: a phase I/II clinical trial. Anticancer Res 33:5495–5500
28. Bigalke I, Honnashagen K, Lundby M et al (2015) A new generation of dendritic cells to

improve cancer therapy shows prolonged progression-free survival in patients with solid tumors. [abstract 2516]. In: Proceedings of the 106th Annual Meeting of the American Association for Cancer Research; 2015 Apr 18-22; Philadelphia (PA): AACR. Cancer Res 75:SY26-02-5568

29. Allard SD, De KB, de Goede AL et al (2012) A phase I/IIa immunotherapy trial of HIV-1-infected patients with Tat, Rev and Nef expressing dendritic cells followed by treatment interruption. Clin Immunol 142:252–268
30. Van Gulck E, Vlieghe E, Vekemans M et al (2012) mRNA-based dendritic cell vaccination induces potent antiviral T-cell responses in HIV-1-infected patients. AIDS 26:F1–F12
31. Gattinoni L, Powell DJ Jr, Rosenberg SA, Restifo NP (2006) Adoptive immunotherapy for cancer: building on success. Nat Rev Immunol 6:383–393
32. Biagi E, Marin V, Giordano Attianese GM et al (2007) Chimeric T-cell receptors: new challenges for targeted immunotherapy in hematologic malignancies. Haematologica 92:381–388
33. Abken H, Hombach A, Heuser C et al (2002) Tuning tumor-specific T-cell activation: a matter of costimulation? Trends Immunol 23:240–245
34. Eshhar Z (2010) Adoptive cancer immunotherapy using genetically engineered designer T-cells: first steps into the clinic. Curr Opin Mol Ther 12:55–63
35. Anurathapan U, Leen AM, Brenner MK, Vera JF (2013) Engineered T cells for cancer treatment. Cytotherapy 16(6):713–733
36. Bonini C, Brenner MK, Heslop HE, Morgan RA (2011) Genetic modification of T cells. Biol Blood Marrow Transplant 17:S15–S20
37. Gill S, Kalos M (2013) T cell-based gene therapy of cancer. Transl Res 161:365–379
38. Wieczorek A, Uharek L (2013) Genetically modified T cells for the treatment of malignant disease. Transfus Med Hemother 40:388–402
39. Park TS, Rosenberg SA, Morgan RA (2011) Treating cancer with genetically engineered T cells. Trends Biotechnol 29:550–557
40. Hombach A, Wieczarkowiecz A, Marquardt T et al (2001) Tumor-specific T cell activation by recombinant immunoreceptors: CD3 zeta signaling and CD28 costimulation are simultaneously required for efficient IL-2 secretion and can be integrated into one combined CD28/CD3 zeta signaling receptor molecule. J Immunol 167:6123–6131
41. Kershaw MH, Westwood JA, Parker LL et al (2006) A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. Clin Cancer Res 12:6106–6115
42. Xue S, Gillmore R, Downs A et al (2005) Exploiting T cell receptor genes for cancer immunotherapy. Clin Exp Immunol 139:167–172
43. Cheadle EJ, Sheard V, Hombach AA et al (2012) Chimeric antigen receptors for T-cell based therapy. Methods Mol Biol 907:645–666
44. Lamers CH, Willemsen R, van Elzakker P et al (2011) Immune responses to transgene and retroviral vector in patients treated with ex vivo-engineered T cells. Blood 117:72–82
45. Lamers CH, Sleijfer S, Vulto AG et al (2006) Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. J Clin Oncol 24:e20–e22
46. Morgan RA, Yang JC, Kitano M et al (2010) Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Mol Ther 18:843–851
47. Birkholz K, Hombach A, Krug C et al (2009) Transfer of mRNA encoding recombinant immunoreceptors reprograms CD4+ and CD8+ T cells for use in the adoptive immunotherapy of cancer. Gene Ther 16:596–604
48. Zhao Y, Moon E, Carpenito C et al (2010) Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor. Cancer Res 70:9053–9061
49. Almasbak H, Rian E, Hoel HJ et al (2011) Transiently redirected T cells for adoptive transfer. Cytotherapy 13:629–640
50. Barrett DM, Zhao Y, Liu X et al (2011) Treatment of advanced leukemia in mice with mRNA engineered T cells. Hum Gene Ther 22:1575–1586
51. Riet T, Holzinger A, Dorrie J et al (2013) Nonviral RNA transfection to transiently modify T cells with chimeric antigen receptors for adoptive therapy. Methods Mol Biol 969:187–201
52. Beatty GL, Haas AR, Maus MV et al (2014) Mesothelin-specific chimeric antigen receptor mRNA-engineered T cells induce anti-tumor activity in solid malignancies. Cancer Immunol Res 2:112–120
53. Maus MV, Haas AR, Beatty GL et al (2013) T cells expressing chimeric antigen receptors can cause anaphylaxis in humans. Cancer Immunol Res 1:26–31
54. Prommersberger S, Hofflin S, Schuler-Thurner B et al (2015) A new method to monitor antigen-specific CD8 T cells, avoiding additional target cells and the restriction to human leukocyte antigen haplotype. Gene Ther 22(6):516–520

55. Pfeiffer IA, Hoyer S, Gerer KF et al (2014) Triggering of NF-kappaB in cytokine-matured human DCs generates superior DCs for T-cell priming in cancer immunotherapy. Eur J Immunol 44:3413–3428
56. Setz C, Friedrich M, Hahn S et al (2013) Just one position-independent lysine residue can direct MelanA into proteasomal degradation following N-terminal fusion of ubiquitin. PLoS One 8, e55567
57. Hofmann C, Harrer T, Kubesch V et al (2008) Generation of HIV-1-specific T cells by electroporation of T-cell receptor RNA. AIDS 22:1577–1582
58. Coughlin CM, Vance BA, Grupp SA, Vonderheide RH (2004) RNA-transfected CD40-activated B cells induce functional T-cell responses against viral and tumor antigen targets: implications for pediatric immunotherapy. Blood 103:2046–2054
59. Van den Bosch GA, Van Gulck E, Ponsaerts P et al (2006) Simultaneous activation of viral antigen-specific memory CD4+ and CD8+ T-cells using mRNA-electroporated CD40-activated autologous B-cells. J Immunother 29:512–523
60. Holtkamp S, Kreiter S, Selmi A et al (2006) Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells. Blood 108:4009–4017
61. Etschel JK, Huckelhoven AG, Hofmann C et al (2012) HIV-1 mRNA electroporation of PBMC: A simple and efficient method to monitor T-cell responses against autologous HIV-1 in HIV-1-infected patients. J Immunol Methods 380(1-2):40–55
62. Van Camp K, Cools N, Stein B et al (2010) Efficient mRNA electroporation of peripheral blood mononuclear cells to detect memory T cell responses for immunomonitoring purposes. J Immunol Methods 354:1–10
63. Birkholz K, Hofmann C, Hoyer S et al (2009) A fast and robust method to clone and functionally validate T-cell receptors. J Immunol Methods 346:45–54

Chapter 11

Adjuvant-Enhanced mRNA Vaccines

Lukasz Bialkowski, Kevin Van der Jeught, Dries Renmans, Alexia van Weijnen, Carlo Heirman, Marleen Keyaerts, Karine Breckpot, and Kris Thielemans

Abstract

Recent advances in molecular biology have led to dramatic enhancement of the stability of in vitro transcribed (IVT) messenger RNA (mRNA) and improvement in its translational efficacy. Nowadays, mRNA-based vaccines represent a promising approach in the field of anticancer immunotherapy, gaining attention over the earlier-established bacteria-, virus-, or cell-based vaccination approaches. Here, we present the experimental procedures employed in our laboratory to induce anticancer immune responses in different murine tumor models using IVT mRNA encoding for immune activation signals and antigens of interest.

Key words mRNA, Electroporation, Intranodal, Intralymphatic, Intratumoral, Bioluminescence imaging, Therapeutic vaccine, Cancer immunotherapy, Dendritic cells

1 Introduction

Dendritic cells (DCs) are the central paradigm of cancer immunotherapy. Their ability to capture, process, and present antigens have been deeply explored in recent years, resulting in better understanding of DC biology and consequently leading to multiple clinical trials [1, 2]. DCs can be exploited in different manners for immunization purposes. For instance, they can be cultured from $CD14^+$ patients' autologous monocytes and loaded with tumor-associated antigens (TAAs), matured and readministered to the patients. The maturation process is crucial for the induction of potent anticancer immune responses. Many different approaches were described to achieve an optimal DC maturation status [2]. Our group has developed an mRNA-based platform to activate the autologous DCs by electroporation with three mRNA molecules, collectively called TriMix. This mRNA mixture encodes for two DC activation stimuli: CD40L and a constitutively active variant of Toll-like receptor 4 (caTLR4) together with the co-stimulatory

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_11,

molecule CD70, and can be supplemented with TAA mRNA molecules [3].

Lately, we have expanded our expertise and proposed a novel in situ vaccination strategy that allows circumventing the costly and time-consuming patient-specific process of ex vivo DC generation and further manipulations. We proposed to inject the mRNA directly into lymph nodes, where priming of naïve T cells takes place. This approach was shown to be at least as efficient as DC-based vaccines in inducing potent T-cell immune responses in mice [4]. A variant of this in situ mRNA administration is the intratumoral injection of mRNA that grants the possibility to act directly on tumor-infiltrating T cells (via DC-T cell interaction) and the suppressive tumor microenvironment (by exploiting tumor-resident DCs as "factories" for the secretion of mRNA-encoded immunomodulatory factors) [5–7]. Importantly, intratumoral mRNA-injection offers a TAA-independent vaccination system by direct activation of tumor-resident DCs that are already loaded with antigens derived from dying tumor cells.

The techniques presented in this chapter were used to induce antitumor immune responses in different mouse models and are described according to the approach tested: immunization with mRNA-electroporated DCs, immunization by intranodal mRNA delivery, and immunization and immunomodulation by intratumoral mRNA delivery. The description of the intranodal and intratumoral mRNA injection techniques is preceded by a detailed procedure of post-synthesis mRNA preparation. Moreover, we briefly describe an in vivo bioluminescence imaging technique that could be used to evaluate the correctness of performed mRNA injection.

2 Material

2.1 DC Electroporation

1. Genepulser X cell system (Bio-Rad, Belgium).
2. Electroporation cuvettes 4 mm (Cell Projects, UK).
3. Micropipette (10, 20, 200 μl).
4. Neptune Pipette barrier tips (Biotix Inc., USA).
5. Class II vertical laminar flow cabinet.
6. Centrifuge.
7. Culture medium: RPMI 1640 medium (Sigma-Aldrich, Belgium) supplemented with 5% fetal calf serum (Harla, the Netherlands), 100 IU penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids and 50 μM beta-mercaptoethanol (supplements from Sigma-Aldrich, Belgium).
8. Opti-MEM buffer (Life Technologies, Belgium).

9. Sterile PBS.
10. Sterile 50 ml Falcon tubes, conical base.
11. Sterile red blood cell lysis buffer.
12. 100 × 20 mm sterile tissue culture dish.
13. Recombinant mouse GM-CSF (prepared in-house).
14. Scissors and forceps.
15. Falcon Cell Strainer 40 μm Nylon (Corning, the Netherlands).
16. 1 ml 26 G syringe.

2.2 Post-synthesis mRNA Preparation for Intranodal and Intratumoral Injection

1. Messenger RNA (eTheRNA, Belgium).
2. LiCl (Sigma-Aldrich, Belgium).
3. 70% EtOH.
4. Sterile water for injection.
5. Hartmann Solution (Baxter, Belgium).
6. Microcentrifuge.
7. Freezer –20 °C.
8. Refrigerator +4 °C.
9. Class II vertical laminar flow cabinet.
10. RNase wipes (Ambion—Life Technologies, Lithuania).
11. DNA LoBind tubes 1.5 ml (Eppendorf, Germany).
12. Micropipette (10 μl, 20 μl, 200 μl, 1000 μl)
13. Neptune Pipette barrier tips (Biotix Inc., USA)
14. Agilent 2100 Bioanalyzer (Agilent Technologies, Germany)

2.3 Intranodal Injection

1. 100 mg/ml ketamine (Ceva, Belgium).
2. 20 mg/ml xylazine (Bayer SA-NV, Belgium).
3. Syringe BD Micro-Fine + Demi 0.3 (30G), 8 mm (BD Medical, France).
4. Scissors and forceps.
5. Michel clips (Fine Science Tools, Germany) or 6-0 coated VICRYL suture Ethicon (Johnson & Johnson Medical, Belgium).
6. 60 × 60 cm diapers.
7. Double-edged razor blades.
8. Magnifying glass or microscope with light source.
9. Soap water.
10. 10 × 10 cm sterile gauze swabs.
11. 0.9% NaCl.
12. 10% Iso-Betadine Dermicum (Meda Pharma SA, Belgium).
13. Infrared lamp or heating blanket.

2.4 Intratumoral Injection

1. Isoflurane (Forene, AbbeVie SA, Belgium) and delivery apparatus (Minerve, Module Anesth Compact, France).
2. Syringe BD Micro-Fine + 0.3 (30G), 5 mm (BD Medical, France).
3. Electric shaver appropriate for mice.

2.5 In Vivo Bioluminescence Imaging (BLI)

1. D-luciferin (Promega, Belgium).
2. Syringe BD Micro-Fine + Demi 0.3 (30G), 8 mm (BD Medical, France).
3. Isoflurane (Forene, AbbeVie SA, Belgium) and delivery apparatus (Minerve, Module Anesth Compact, France).
4. BLI apparatus (Photoimager Optima, Biospacelab, France).
5. Acquisition Software—Photo Acquisition Version 3.4 (Biospacelab, France).
6. Analysis Software—M3 Vision Version 1.0.7.1178 (Biospacelab, France).

3 Methods

3.1 Immunization with mRNA-Electroporated DCs

It has been demonstrated that DC-based vaccines are feasible and safe for cancer patients, leading to the induction of clinical responses in some cases [2]. Previous experiments have shown that large numbers of functional DCs can be generated in humans for instance from circulating blood precursors or in mice from bone marrow cells. In order to induce potent T-cell immune responses, DCs have to be loaded with TAAs and properly activated. Various methods have been designed and described so far. One possibility explored by our group is DC electroporation with mRNA encoding a mix of TAAs and immune-activating molecules, including DC activation stimuli, co-stimulatory molecules and immune checkpoint blocking molecules [8–12]. Of these, the aforementioned TriMix was shown to be a potent mRNA mixture to activate TAA-specific antitumor T-cell responses. The DC electroporation approach resulted in several clinical trials [13–15], some of which are still ongoing (e.g., n° NCT01676779).

3.1.1 DC Generation

1. Sacrifice a 6-week-old female C57BL/6 mouse and excise femora and tibiae (*see* **Note 1**). Remove all musculature. Wash the isolated bones briefly in 70% EtOH and keep for further processing in culture medium at room temperature.
2. Carefully cut off both ends of each bone and use medium and a sterile 26 G-syringe to flush out the bone marrow into a sterile tissue culture dish.

3. Resuspend the bone marrow thoroughly to obtain a single cell suspension and filter it through a pre-wetted 40 μm nylon filter placed on a sterile 50 ml Falcon tube.
4. Pellet the cells by centrifuging for 10 min at 435 × *g*.
5. Resuspend the pellet in 2 ml of red blood cell lysis buffer and incubate for 2 min.
6. Add an excess of fresh culture medium (20 ml) and centrifuge for 5 min at 435 × *g*.
7. Resuspend the cells in culture medium supplemented with 200 IU/ml rmoGM-CSF. Seed 2×10^6 cells in 10 ml culture medium into a sterile tissue culture dish and incubate for 3 days at 37 °C, 5 % CO_2 and 95 % humidity.
8. On day 3, add additional 10 ml of culture medium to each tissue culture dish. Supplement the medium with double amount of rmoGM-CSF (i.e., 10 ml of new medium containing 400 IU/ml rmoGM-CSF).
9. On day 5, refresh 50% of the culture medium. To this aim, aspirate 10 ml of cell suspension from each tissue culture dish, transfer to a sterile 50 ml tube and centrifuge for 5 min at 435 × *g*. Resuspend the cell pellet in 10 ml of fresh culture medium supplemented with 400 IU/ml rmoGM-CSF and put the cells back in culture.
10. On day 7, harvest the cells by gently rinsing the tissue culture dishes with pre-warmed PBS. Transfer the cells to a sterile 50 ml tube and centrifuge for 5 min at 435 × *g*. We strongly advise to perform a flow-cytometric analysis in order to evaluate the purity of CD11c⁺ DCs (Fig. 1a).

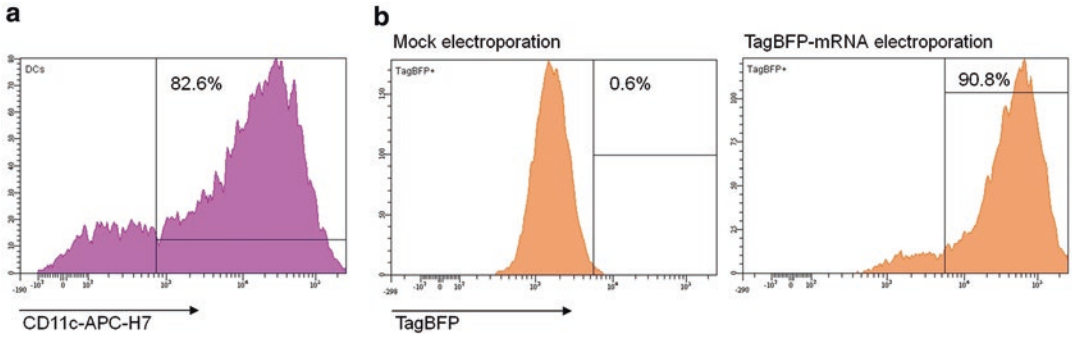

Fig. 1 DC culture purity and electroporation efficacy. (**a**) After a 7-day culture, DC purity was evaluated using flow cytometry. Cells were harvested by gently rinsing the tissue culture plates with PBS, washed once with PBS/BSA/Azide and stained with anti-CD11c-APC-H7 antibodies. CD11c-expression within living single cells is shown. (**b**) DCs were electroporated with 10 μg TagBFP-mRNA and expression of TagBFP protein was assessed 24 h later using flow cytometry. TagBFP-expression within CD11c⁺ living single cells is shown

3.1.2 DC Electroporation

1. Immediately prior to the transfection, wash the cells twice with warm PBS and repeat this step twice with warm Opti-MEM buffer. After centrifugation (5 min at 435 × *g*), resuspend the cells in Opti-MEM so the final concentration is 40×10^6 cells per ml. Prepare mRNA-mixes by adding a desired amount of mRNA to Opti-MEM in nuclease-free Eppendorf tubes (the total volume: 100 μl). Transfer 100 μl of the cell suspension (4×10^6 cells) to the tube containing the mRNA-mix, resuspend gently and transfer the mix to a 4 mm gap sterile disposable electroporation cuvette.
2. Electroporate the cells using the following parameters: voltage pulse 300 V, capacitance 150 μF, resistance ∞ Ω.
3. After electroporation, transfer the cells immediately into warm culture medium and incubate for 1 h at 37 °C, 5% CO_2, and 95% humidity in a sterile tissue culture dish.
4. Wash the cells twice with PBS and resuspend them in PBS. If necessary, use PBS supplemented with 2 mM EDTA to detach the cells that strongly adhere to the plastic. Inject intravenously 5×10^5 cells per mouse.
5. We advise to check the efficiency of the electroporation by including a reporter gene, for instance Blue Fluorescence Protein (TagBFP), into the mixture of mRNA molecules used (Fig. 1b).

3.2 Post-synthesis mRNA Preparation for Intranodal and Intratumoral Injection

The IVT mRNA is dissolved in RNase-free water and stored at −20 °C. Different mRNAs are mixed according to the amount of mRNA needed for injection, resulting in different volumes of final solution (*see* **Note 2**). In order to adjust the volume of injected solution (to for example 10 μl per injection), the mixture of mRNAs has to be precipitated with use of LiCl and purified as described below.

1. Mix the mRNA solution with LiCl at ratio 2:1 and store overnight or longer at −20 °C.
2. Once thawed, place the eppendorf in a microcentrifuge. Centrifuge the solution for 15 min at 18,600 × *g*.
3. Remove the supernatant carefully. The mRNA will be visible as a white pellet. Avoid touching the pellet.
4. Add 500 μl of 70% EtOH and centrifuge for 5 min at 18,600 × *g*. Remove the supernatant. Be careful not to aspirate the pellet since it can detach from the bottom of the tube in this step.
5. Spin down briefly and remove the residual EtOH. Dissolve the pellet firstly in RNase-free water and leave it for 30 min at room temperature. Vortex for 30 s and briefly spin down. Repeat this step three times. Add Hartmann Solution in desired

volume (water–Hartmann solution ratio is 1:4), vortex and spin down three times. Leave the solution for 15 min at room temperature, vortex and spin down once. Fill the syringes. The solution is ready to be injected (*see* **Note 3**).

6. We advise to test the quality of injected mRNA *post factum* using capillary gel electrophoresis (Agilent).

3.3 Immunization by Intranodal mRNA Delivery

Intralymphatic immunization with different vaccine types has been shown to exert more potent immune responses when compared to other administration routes [16, 17]. In order to circumvent the laborious procedures and costs of ex vivo DC manipulation, we and others proposed that mRNA can be injected directly into lymph nodes [4]. In this context, the primary objective is to target the tumor-draining lymph nodes. However, it seems plausible that the intranodally injected vaccine could spread through lymphatic vessels connecting different lymph nodes (Lukasz Bialkowski, unpublished observations). The intranodal mRNA delivery platform is now being investigated in a series of clinical trials including hepatocellular carcinoma (n°EudraCT 2012-005572-34), melanoma (n°NCT01684241, n°NCT02035956) and anti-HIV vaccine (n°NCT02413645).

1. Anesthetize mice with ketamine–xylazine solution by intraperitoneal injection (*see* **Note 4**).
2. Shave the fur using a razorblade in order to uncover the subiliac lymph nodes (i.e., tumor-draining lymph nodes for subcutaneously implanted tumors on the back and flank).
3. Place the animal under a microscope, adjust the lenses and the light intensity.
4. Make a small incision along the longitudinal body axis (Fig. 2a).
5. Broaden the incision in order to uncover the entire lymph node. Do not cut neighboring lymphatic vessels and blood vessels. Should blood appear in the field of surgical manipulation, wash the incision with 0.9 % NaCl and dry carefully with sterile gauze. This will reduce the risk of mRNA degradation due to RNases that are present in blood.
6. Grab the lymph node with tweezers and pull it up slightly.
7. Once the lymph node is stabilized using the tweezers, the needle can be inserted. Try to inject the mRNA exactly in the center of the lymph node (*see* **Note 5**).
8. Slowly remove the needle. Do not manipulate the injected lymph node anymore. Let it find its correct position. Close the incision with Michel clips or a surgical suture (*see* **Note 6**).
9. Let the animal recover. Remember that the anesthesia induces depression of the cardiovascular and the respiratory systems. The mice can become hypothermic. If necessary, place an infrared

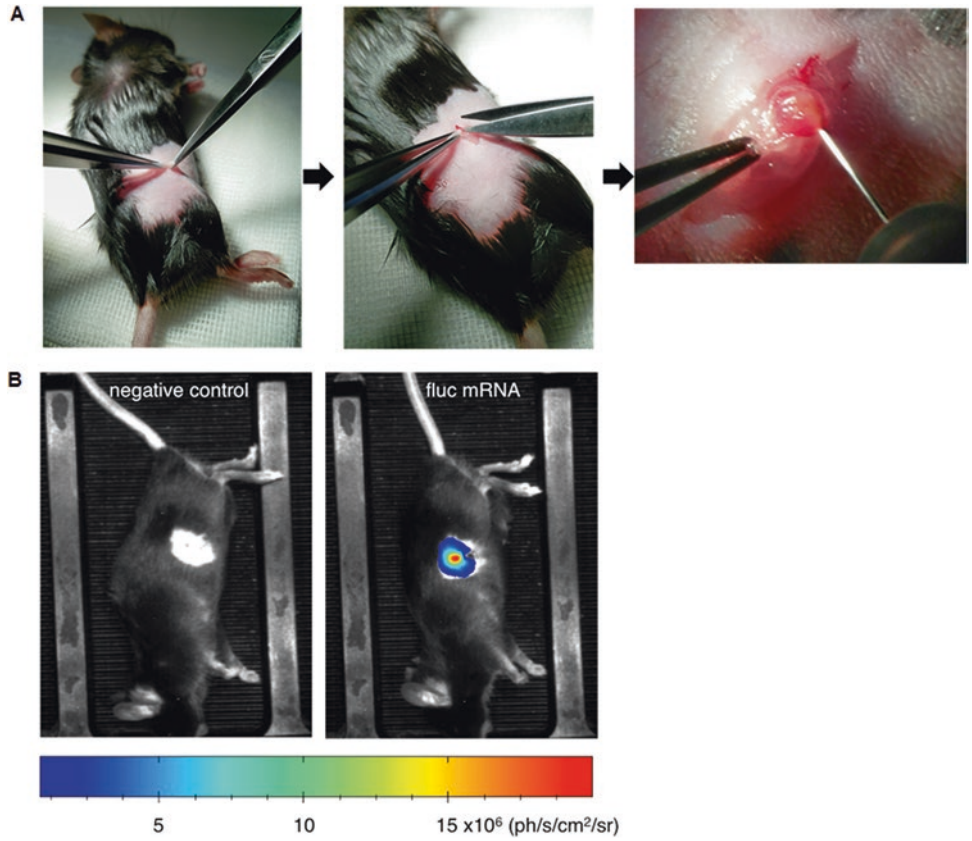

Fig. 2 The procedure of intranodal mRNA administration. (**a**) Make an incision along the longitudinal body axis and uncover the lymph node (*left* and *central panel*). Grab the lymph node, insert the needle in the center of the lymph node and carefully inject the mRNA (*right panel*). (**b**) Six-week-old C57BL/6 female mouse was injected with 1 μg fluc mRNA and an in vivo BLI was performed 24 h later in order to evaluate the correctness of the injection. A non-injected mouse was used as a negative control

lamp in front of the cage with recovering mice. Do not place the lamp too close to the animals to avoid overheating or burn wounds. Optionally, heating blankets with a rectal probe to adjust the heat to the real body weight of the mouse can be used.

10. The correctness of the injection can be verified by means of in vivo bioluminescence imaging (Fig. 2b). To that aim, a reporter mRNA encoding firefly luciferase has to be incorporated into the injected mixture of mRNAs.

3.4 Immunization and Immunomodulation by Intratumoral mRNA Delivery

Although significant progress has been made in the field of anticancer vaccines, a growing body of evidence indicates that as long as the hostile tumor environment is not reversed, the clinical benefits of immunotherapy will stay limited [5, 18]. It has therefore been proposed to target tumor lesions directly. This approach allows boosting the tumor-infiltrating T cells, while simultaneously acting specifically on the tumor microenvironment.

3.4.1 Tumor Cell Inoculation

1. Anesthetize the mice with 2.5% isoflurane.
2. Shave the hair on the right flank of the mouse in order to uncover the area of tumor inoculation. Disinfect the skin using 70% EtOH.
3. Fill the syringe with the tumor cell suspension. For the subcutaneous tumors, the cells are resuspended in a final volume of 50 µl of PBS per mouse (*see* **Note 7**).
4. Insert the needle right under the skin holding it parallel to the skin surface and slowly inject the cells. If necessary, use tweezers to hold the skin in order to correctly insert the needle.

3.4.2 Tumor Growth Monitoring

The tumor growth should be carefully monitored two to three times per week using a caliper (*see* **Note 8**).

1. Anesthetize the mice with 2.5% isoflurane.
2. The tumor volume is calculated using the formula for a prolate ellipsoid: $(\text{width}^2 \times \text{length})/2$.

3.4.3 The mRNA Injection Procedure

1. The intratumoral injection is performed when the tumors reach a desired volume (*see* **Note 9**).
2. Anesthesize the mice with 2.5% isoflurane.
3. Shave the hair overlying the tumor if needed in order to uncover the area for injection. Disinfect the skin using 70% EtOH.
4. Fill the syringe with an appropriate volume of the mRNA solution. For tumor volumes above 100 mm^3 a total injection volume of 50 µl is advised. For volumes between 50 and 100 mm^3, a volume of 30 µl is appropriate. For volumes below 50 mm^3, minimum 10 µl is advised (*see* **Note 10**).
5. Insert the needle into the tumor at the desired depth and slowly inject the mRNA solution (Fig. 3a) (*see* **Note 11**).
6. Due to the presence of many factors that can potentially degrade the injected mRNA, we propose to always incorporate a reporter system such as firefly luciferase mRNA in order to verify the correctness of the performed injection using the in vivo bioluminescence imaging system (Fig. 3b) (*see* **Note 12**).
7. Note any observations with regard to the quality of the injection e.g.: blood (*see* **Note 13**) or efflux of the injected solution in order to optimize the intratumoral injection procedure for each tumor model independently. Very often these observations can be correlated to the in vivo bioluminescence data.

3.5 In Vivo Bioluminescence Imaging After the mRNA Injection

Although structural modifications potently increase stability and translational efficacy of in vitro transcribed mRNA, it can still be degraded in the presence of ubiquitous RNases. We therefore strongly advise to verify the correctness of the mRNA injection using in vivo bioluminescence imaging. To this aim, firefly luciferase (fluc) mRNA has to be incorporated in the mixture of

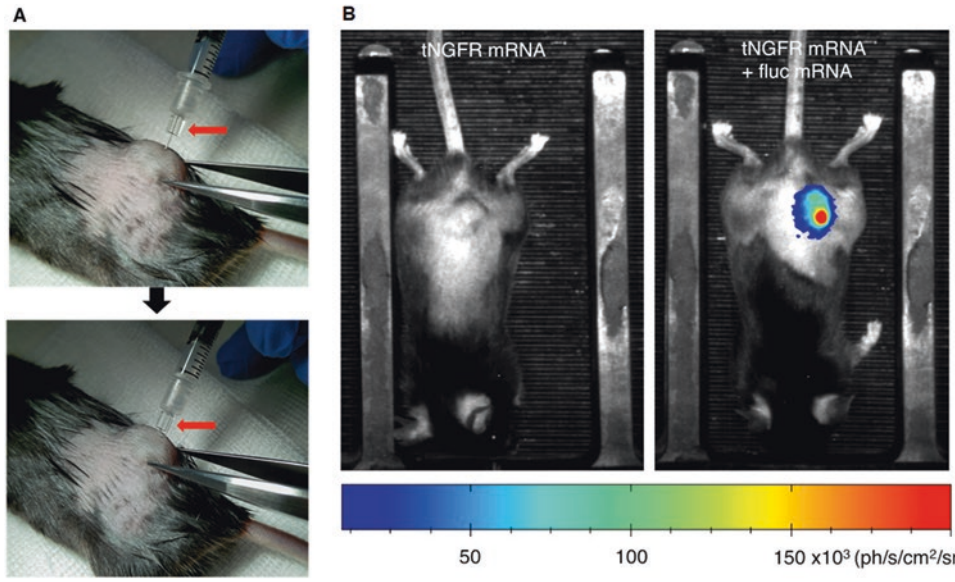

Fig. 3 The procedure of intratumoral mRNA administration. (**a**) Stabilize the tumor nodule with tweezers and place a ring—indicated by the *red arrow*—on the needle to ensure the desired depth of injection (*upper panel*). Insert the needle carefully into the tumor tissue and inject the mRNA solution (*lower panel*). (**b**) Six-week-old C57BL/6 female mice were injected with 10 μg of control mRNA (truncated nerve growth factor receptor, tNGFR) or 10 μg of tNGFR mRNA together with 1 μg of fluc mRNA. An in vivo BLI was performed 24 h later to evaluate the correctness of the injection

administered mRNAs. The fluc mRNA will be translated into fluc enzyme that will mediate the redox reaction of D-luciferin (substrate). The photons generated during this redox reaction are detected by a dedicated camera and translated into electric signal. The software allows to quantitatively evaluate the signal's strength that is proportional to the amount of engulfed fluc mRNA. The procedure for in vivo bioluminescence imaging is adapted from the procedure described in Keyaerts et al. [19].

1. When preparing the mRNA, add 1 μg of fluc mRNA into the mixture.
2. The optimal signal can be measured between 4 and 24 h after the injection, depending on the experimental objectives.
3. Anesthetize the mice using gas anesthesia (2.5% isoflurane).
4. Ensure that the skin above the injected lymph node is shaved. If necessary, remove the Michel clips or excess of the suture.
5. Inject D-luciferin at a concentration of 30 mg/ml (*see* **Note 14**).
6. Position the animal in a black box equipped with cooled charge-coupled device (CCD) detectors to quantify the light photons produced by the animal. The obtained color-scaled image is typically overlayed with a grey-scale photo of the mouse to allow the 2D localization of the bioluminescent sig-

nal in the mouse body. An acquisition time of 4–5 min is enough to determine whether the mRNA-injection was successful or not. It can however be adjusted to the individual needs, depending for instance on the quality of injected mRNA and used equipment.

4 Notes

1. From one mouse up to 100×10^6 bone marrow cells can be obtained.
2. The amount of mRNA used depends on the experimental setup and objectives. It has to be noted that mRNA from different manufacturers can differ in their capacity to induce immune responses. In our approach, 10 μg of each of the TriMix components and 10 μg of an antigen mRNA are used for intranodal vaccination purposes.
3. Depending on the duration of the procedure, it might be considered to store the ready-to-inject solution at 4 °C before filling the syringes. It is also recommended to keep the syringes on ice during the procedure.
4. In order to prepare the stock solution for injection, add 1 ml ketamine and 0.5 ml xylazine to 8.5 ml of NaCl 0.9% and store at 4 °C. Inject 0.1 ml of stock solution per 10 g of body mass (i.e., 100 mg/kg of ketamine and 10 mg/kg of xylazine).
5. For the optimal performance, the final volume of injected solution should be equal to 10 μl per lymph node.
6. We advise to use coated suture instead of nylon suture to limit the risk of infectionc. Disinfect the closed wound with sterile gauze dipped in iso-Betadine. Wrap the mouse in sterile gauze; avoid placing the mouse directly on sawdust after the surgical manipulations. Always place the animal in prone position.
7. Culture the tumor cells according to the SOPs available at your laboratory. It is recommended that the cells are in the logarithmic growth phase (80% confluence) at the day of inoculation. Before injection it is advised to perform a Mycoplasma test. The number of injected tumor cells strictly depends on the kinetics of the tumor model used and the experimental objectives and should therefore be optimized empirically for each model. Depending of the tumor model, Matrigel may be needed to allow tumor development.
8. It often happens that hair starts regrowing where the tumor is inoculated. Therefore, in order to properly determine the size of the tumor, it is recommended to reshave the mice carefully.
9. The desired tumor volume is determined based on the experimental needs and the institutional Ethical Committee requirements.

10. Please refer to the section *Post-synthesis mRNA preparation* for further details. Injection of large volumes of mRNA into small tumors can result in the efflux of the injected solution.
11. When injecting the tumors, it is important to take into account that the needle might be inserted at different depths. In order to limit the interindividual variability we advise the use of rings that can be put around the needle (Fig. 3a). This will result in a constant depth of the intratumoral injections. Depending on the tumor model, bigger tumors can develop necrotic regions. These areas have a scarce number of cells capable of engulfing the injected mRNA and possibly abound in the RNA-degrading factors. Immunohistochemical analysis of the tumor nodule (areas of necrosis, regions rich in dendritic cells) could help to define the optimal spot for injection. When working with large tumors (>300 mm^3) a common problem is the efflux of the injected mRNA due to high interstitial pressure. The discharged volume can be reabsorbed and slowly injected on the opposite side of the tumor.
12. It is important to note that dark substances in tumors (such as the melanin produced by some types of melanoma cell lines) and dark skin or hair of the mice can attenuate the emitted light and reduce the sensitivity of this technique. Upon repetitive shaving, the skin irritation can result in increased pigmentation of the skin and further reduce the sensitivity.
13. The appearance of blood depends on the vascularity of the used tumor model.
14. Two routes of substrate administration can be chosen. The intraperitoneal injection is technically easier. If you opt for this route, inject 100 μl of D-luciferin per 20 g body mass and wait 10 min before starting data acquisition. The intravenous injection demands more technical skills but the data acquisition can be started immediately after the injection. For the intravenous route, inject 100 μl of D-luciferin per 20 g body mass and immediately start data acquisition. Please mind that intraperitoneal injection gives much more variation of the signal than the intravenous injection [19]. Therefore, it is worth to consider that a weak BLI signal may not always be ascribed to the degradation of mRNA but can also be associated with unsuccessful substrate administration.

Acknowledgements

This work was supported by the Agency for Innovation by Science and Technology (IWT-Vlaanderen), Interuniversity Attraction Poles Program, the National Cancer Plan, the Stichting Tegen Kanker, the

Kom op tegen Kanker, the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO-Vlaanderen), the Hercules Foundation Flanders (Middelzware onderzoeksinfrastructuur), and the EU FP7-cancer immunotherapy program. Marleen Keyaerts is a Senior Clinical Investigator of FWO-Vlaanderen.

References

1. Palucka K, Banchereau J (2013) Dendritic-cell-based therapeutic cancer vaccines. Immunity 39:38–48
2. Constantino J, Gomes C, Falcão A et al (2015) Antitumor dendritic cell-based vaccines: lessons from 20 years of clinical trials and future perspectives. Transl Res. doi:10.1016/j.trsl.2015.07.008
3. Van Lint S, Wilgenhof S, Heirman C et al (2014) Optimized dendritic cell-based immunotherapy for melanoma: the TriMix-formula. Cancer Immunol Immunother 63:959–967
4. Van Lint S, Goyvaerts C, Maenhout S et al (2012) Preclinical evaluation of TriMix and antigen mRNA-based antitumor therapy. Cancer Res 72:1661–1671
5. Van der Jeught K, Bialkowski L, Daszkiewicz L et al (2015) Targeting the tumor microenvironment to enhance antitumor immune responses. Oncotarget 6:1359–1381
6. Van der Jeught K, Van Lint S, Thielemans K, Breckpot K (2015) Intratumoral delivery of mRNA: overcoming obstacles for effective immunotherapy. Oncoimmunology 4:e1005504
7. Van Lint S, Renmans D, Broos K et al (2015) Intratumoral delivery of TriMix mRNA results in T-cell activation by cross-presenting dendritic cells. Cancer Immunol Res 4:146–156. doi:10.1158/2326-6066.CIR-15-0163
8. Tuyaerts S, Van Meirvenne S, Bonehill A et al (2007) Expression of human GITRL on myeloid dendritic cells enhances their immunostimulatory function but does not abrogate the suppressive effect of CD4+CD25+ regulatory T cells. J Leukoc Biol 82:93–105
9. Pen JJ, Keersmaecker BD, Heirman C et al (2014) Interference with PD-L1/PD-1 co-stimulation during antigen presentation enhances the multifunctionality of antigen-specific T cells. Gene Ther 21:1–10
10. De Keersmaecker B, Heirman C, Corthals J et al (2011) The combination of 4-1BBL and CD40L strongly enhances the capacity of dendritic cells to stimulate HIV-specific T cell responses. J Leukoc Biol 89:989–999
11. Bonehill A, Van Nuffel AMT, Corthals J et al (2009) Single-step antigen loading and activation of dendritic cells by mRNA electroporation for the purpose of therapeutic vaccination in melanoma patients. Clin Cancer Res 15:3366–3375
12. Aerts-Toegaert C, Heirman C, Tuyaerts S et al (2007) CD83 expression on dendritic cells and T cells: correlation with effective immune responses. Eur J Immunol 37:686–695
13. Van Nuffel AMT, Benteyn D, Wilgenhof S et al (2012) Intravenous and intradermal TriMix-dendritic cell therapy results in a broad T-cell response and durable tumor response in a chemorefractory stage IV-M1c melanoma patient. Cancer Immunol Immunother 61:1033–1043
14. Wilgenhof S, Corthals J, Van Nuffel AMT et al (2015) Long-term clinical outcome of melanoma patients treated with messenger RNA-electroporated dendritic cell therapy following complete resection of metastases. Cancer Immunol Immunother 64:381–388
15. Wilgenhof S, Van Nuffel AMT, Benteyn D et al (2013) A phase IB study on intravenous synthetic mRNA electroporated dendritic cell immunotherapy in pretreated advanced melanoma patients. Ann Oncol 24:2686–2693
16. Johansen P, Häffner AC, Koch F et al (2005) Direct intralymphatic injection of peptide vaccines enhances immunogenicity. Eur J Immunol 35:568–574
17. Van Lint S, Heirman C, Thielemans K, Breckpot K (2013) mRNA: from a chemical blueprint for protein production to an off-the-shelf therapeutic. Hum Vaccin Immunother 9:265–274
18. Joyce JA, Fearon DT (2015) T cell exclusion, immune privilege, and the tumor microenvironment. Science 348:74–80
19. Keyaerts M, Verschueren J, Bos TJ et al (2008) Dynamic bioluminescence imaging for quantitative tumour burden assessment using IV or IP administration of D: luciferin: effect on intensity, time kinetics and repeatability of photon emission. Eur J Nucl Med Mol Imaging 35:999–1007

Chapter 12

Enhanced Delivery of DNA or RNA Vaccines by Electroporation

Kate E. Broderick and Laurent M. Humeau

Abstract

Nucleic acid vaccines are a next-generation branch of vaccines which offer major benefits over their conventional protein, bacteria, or viral-based counterparts. However, to be effective in large mammals and humans, an enhancing delivery technology is required. Electroporation is a physical technique which results in improved delivery of large molecules through the cell membrane. In the case of plasmid DNA and mRNA, electroporation enhances both the uptake and expression of the delivered nucleic acids. The muscle is an attractive tissue for nucleic acid vaccination in a clinical setting due to the accessibility and abundance of the target tissue. Historical clinical studies of electroporation in the muscle have demonstrated the procedure to be generally well tolerated in patients. Previous studies have determined that optimized electroporation parameters (such as electrical field intensity, pulse length, pulse width and drug product formulation) majorly impact the efficiency of nucleic acid delivery. We provide an overview of DNA/RNA vaccination in the muscle of mice. Our results suggest that the technique is safe and effective and is highly applicable to a research setting as well as scalable to larger animals and humans.

Key words Electroporation, Muscle, Plasmid DNA, Mouse, DNA vaccine, RNA vaccine

1 Introduction

Electroporation (EP) involves the application of brief electrical pulses that result in the creation of aqueous pathways within the lipid bilayer membranes of mammalian cells. This allows the passage of large molecules, including DNA and other macromolecules, through the cell membrane which would otherwise unable to cross. As such, EP increases both the uptake and the extent to which drugs and DNA are delivered to the target tissue of interest [1–4]. Historically, EP has been primarily targeted to muscle tissue and currently multiple clinical trials are being conducted using this route of delivery [5–7].

EP as a mode of enhanced delivery provides a platform for the in vivo manufacture of the desired gene product which, in the case of both DNA and RNA vaccines, can lead to both antibody and cellular immune responses [7, 8]. Optimized vectors through

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_12,

Table 1
Nucleic acid vaccination in human muscle

Site of injection	Intramuscular
Method of *i*njection	Needle and syringe, jet injection
Injection depth	10–50 mm
Typical *i*njection *v*olume	1–2 ml
Targeted *t*issue *r*egion	Skeletal muscle
Targeted *c*ell *t*ype	Myocytes
Max. *d*ose (10 mg/ml formulation)	10–20 mg

codon optimization, RNA optimization, the addition of leader sequences and optimized consensus sequences, are vital to elicit robust responses [9–13]. The potency of both plasmid DNA and RNA vaccines can be enhanced by EP, suggesting that EP facilitates nucleic acid delivery across both the plasma and nuclear membranes [14, 15].

Both DNA and RNA vaccine technologies have been previously shown to be effective in animal models at generating immune responses and in the case of DNA vaccines, have shown clinical efficacy in human trials [16–24]. Intramuscular (IM) EP has also been evaluated extensively in the clinic and been shown to be an efficient and acceptable mode of delivery for DNA vaccine [22, 25]. Table 1 summarizes aspects of nucleic acid vaccination related to delivery in muscle.

While EP clearly improves the delivery of DNA and RNA in vivo, the electrical parameters must be adjusted to ensure optimal delivery while limiting destructive tissue damage. In this protocol, we describe administration of a DNA plasmid encoding green fluorescent protein (GFP) and a large, self-amplifying mRNA vector encoding GFP. GFP expression is determined over a 2 day period since this was previously determined to be the peak of expression in the mouse muscle. We provide strategies to optimize the intramuscular injections in mice as well as improve the reproducibility of the EP procedure.

2 Materials

2.1 Plasmid/ Replicon Preparation

1. Plas\mid DNA, pgWIZ-GFP (Aldevron, ND). Plasmid DNA is formulated into a volume of 30 μl per injection site.
2. GFP expressing RNA replicons (Novartis, Cambridge, MS). Replicon RNA is formulated into a volume of 30 μl per injection site.
3. Sterile 1× PBS for formulation.

2.2 Animals

1. Female Balb/c mice at 3 weeks old (Charles River Laboratories, Worcester, MA, USA). Approved animal experimental protocol (in accordance with the NIH, Animal Welfare Act and USDA).
2. Electric clippers (for hair removal).
3. Oxygen and isoflurane.

2.3 Intramuscular Injection

1. 29-gauge insulin needle and syringe.
2. Plastic depth spacer.

2.4 Electroporation Procedure

1. ELGEN pulse generator (Inovio Pharmaceuticals, Plymouth Meeting, PA, USA) (*see* Fig. 1a).
2. Mouse-IM EP device (Inovio Pharmaceuticals) (*see* Fig. 1b).
3. 27-gauge needles.

2.5 Tissue Harvest and Analysis

1. Oxygen and isoflurane.
2. Scalpel.
3. Scissors.
4. Microscope slides.
5. Fluorescent microscope.

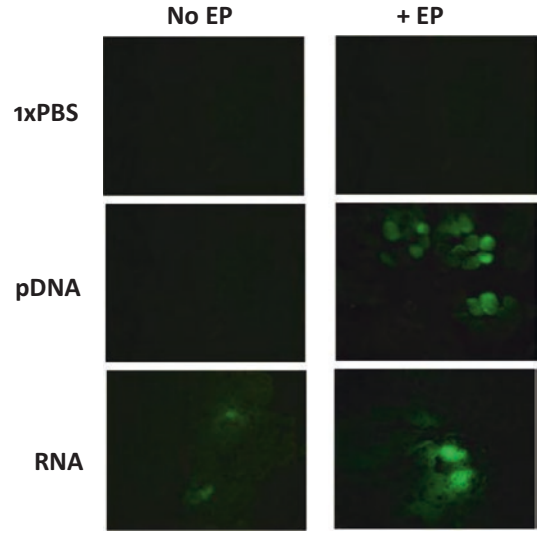

Fig. 1 Electroporation Procedure. (**a**) Muscle EP device (Inovio Pharmaceuticals). Photo shows electrode configuration. (**b**) ELGEN pulse generator (Inovio Pharmaceuticals) which is tethered to the EP device and delivers the electrical pulses. (**c**) Positioning of the EP device in a mouse muscle

3 Methods

3.1 Plasmid Preparation

1. Dilute plasmid preparations to desired dose (5 μg per treatment site) in 1 ml injectable 1× PBS.
2. Prepare injection syringes with the volume of plasmid to be injected (30 μl per injection site).

3.2 Replicon Preparation

1. Dilute GFP-expressing self-replicating RNA vector preparations (5 μg per treatment site) in 1 ml injectable 1× PBS.
2. Prepare syringes with the volume of replicon to be injected (30 μl per injection site).

3.3 Intramuscular Injection and Electroporation Procedure

1. House animals in groups or individually based on intuitional cage size with ad lib access to food and water. Randomly assign mice to treatment groups. Allow acclimatization for 5 days.
2. Anesthetize animals with inhaled isoflurane (5%) and maintain light anesthesia with isoflurane (3%) through appropriate tank while waiting to treat.
3. Identify the quadriceps muscles of the hindleg (*see* **Note 1**) and note which side will be treated (right or left).
4. Shave the leg of the animals to be treated. Clean the area with ethanol to ensure full removal of oil, dust and dander.
5. Initiation of electroporation and DNA/RNA injection (*see* Fig. 1). Administer intramuscular injection using standard IM technique (*see* **Note 2**). Lengthening the limb and positioning the leg between the operators thumb and forefinger can assist with positioning (*see* **Note 3**). Inject the full volume of solution into the quadriceps muscle perpendicularly to the skin. A successful intramuscular injection should result in a visible inflation of the muscle. Following the injection, the needle and syringe should be disposed of in a Sharps container.
6. Immediately (not more than 2 min; *see* **Note 4**), following administering the injection, the mouse EP device electrodes should be inserted into the region of the muscle where the drug was injected (*see* Fig. 1c). Care must be taken to ensure full penetration of the electrodes into the muscle (*see* **Note 5**). Electroporation should be initiated through activation of the foot switch. The mouse EP device and the mouse leg must be held firmly thought the procedure since the electrical pulses will cause involuntary muscle contractions. Two distinct contractions should be observed with a successful treatment. The pulse generator should sound an audible series of beeps to mark a successful treatment. The device can now be removed from the mouse muscle.

7. If multiple treatments are planned, the electrodes in the device should be replaced every five treatments (*see* **Note 6**). Once completed the electrodes should be disposed of in a Sharps container.
8. Animals should be monitored for 2 h to ensure a full recovery from anesthesia and assessed for any issues related to motor function in the treated limb.
9. Peak GFP reporter gene expression in the mouse muscle is detected between 24 and 72 h (*see* **Note 7**).
10. Animals should be euthanized using standard institutional procedures at the desired time point (*see* **Note 8**).
11. Using a scalpel blade and scissors, the treated muscle is excised from the animal through an initial incision of the skin. Care is taken to remove the whole quadriceps muscle group. The excised muscle can then be kept flat on a microscope slide and stored in a Ziplock bag on ice. If the muscle will not be processed immediately, it can be frozen at −20 °C.
12. To view gross GFP expression, the excised muscle can be dissected lengthwise using a scalpel blade, mounted on a flat surface such as a microscope slide (*see* **Note 9**) and viewed under low magnification fluorescent microscopy. To assess the number of transfected myocytes, the muscle can be sliced as a cross section. The entire treated muscle can be captured in its entirety or each cross section addressed individually. Images can be saved as TIFF or BITMAP files (*see* Fig. 2a).

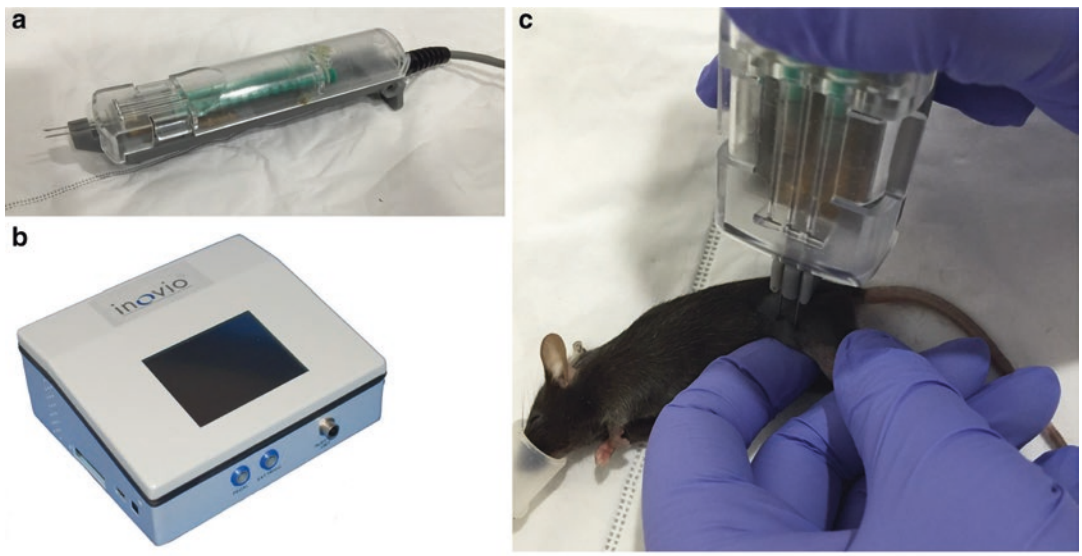

Fig. 2 Expression of reporter protein (GFP) as delivered by intramuscular injection without EP (No EP) or with EP (+EP). Mice were injected intramuscularly with 50 μl of 1× PBS, pDNA or self-amplifying RNA vectors (pDNA or RNA, 5 μg/site). Images were obtained 2 days following treatment and are shown as representative of six to eight slides taken per area, $n = 4$ muscles per group

13. Using commercially available pixel counting software (i.e., Photoshop), each treated muscle can be analyzed for expression (*see* Fig. 2b) or the number of GFP positive myocytes calculated.
14. Statistical analysis between treatments can be performed using the Student's *t*-test program in the Microsoft Excel statistics package.

4 Notes

1. Due to the small nature of the mouse leg, it is easy to confuse the quadriceps muscle with the tibialis muscle. Although either muscle will work, the quadriceps is larger than the tibialis and as such a larger injection volume can be used. It is vital that the treated muscle is correctly identified so that during the excision process, the wrong muscle is not removed resulting in a false negative.
2. The mouse intramuscular injection method involves the insertion of a thin gauge needle at a prescribed depth into the mouse muscle. Since the mouse muscle is small, care must be taken not to inject too deep which may result in the fluid passing out of the muscle and into the subcutaneous space. To assist with this depth assessment, the plastic shield from the Insulin syringe can be removed and cut to the desired length (generally 2–3 mm). This results in the penetration of the needle only to that depth past the shield.
3. To assist with the needle positioning when injecting intramuscularly, extending the mouse leg and using the thumb and forefinger to gently push the muscle group upwards can assist with the process.
4. The intramuscularly delivered drug will begin to dissipate at the treatment site approximately 2 min following injection. It is imperative to EP before this occurs. It is best practice to inject and immediately EP.
5. For the EP procedure to work effectively, the full depth of the muscle must be in contact with the electrodes. The strongest electric field (and the region where most transfection will occur) is between the electrodes. Therefore, more transfection will result from full penetration of the electrodes in the muscle.
6. Although the electrodes can be used multiple times between treatments, it is good practice to replace the electrodes after every five treatments. Multiple insertions of the electrode tips into mouse muscle will result in a dulling of the tips and less efficient entry of the electrodes into the tissue. Additionally, repeated EPs will result in a build-up of material on the electrodes, resulting in reduced EP efficiency. If a multiple vaccine regimen is planned, we recommend alternating limbs.

7. GFP expression can be detected in the muscle as early as 1 h post treatment (microscopically) and persist out for over a month.
8. Here we choose CO_2 asphyxia. The animals are monitored for 10 min to ensure no life signs.
9. A glass microscope slide or square of Perspex plastic is ideal to seat the muscle for imaging.

Acknowledgments

The authors would like to thank Janess Mendoza, Rachel Elward, and Lauren Gites for help with compiling the manuscript and the animal procedures. This work was supported by Inovio Pharmaceuticals (Plymouth Meeting, PA).

References

1. Widera G, Austin M, Rabussay D et al (2000) Increased DNA vaccine delivery and immunogenicity by electroporation in vivo. J Immunol 164:4635–4640
2. Prud'homme GJ, Draghia-Akli R, Wang Q (2007) Plasmid-based gene therapy of diabetes mellitus. Gene Ther 14:553–564
3. Otten G, Schaefer M, Doe B et al (2004) Enhancement of DNA vaccine potency in rhesus macaques by electroporation. Vaccine 22:2489–2493
4. Mathiesen I (1999) Electropermeabilization of skeletal muscle enhances gene transfer in vivo. Gene Ther 6:508–514
5. Bagarazzi ML, Yan J, Morrow MP et al (2012) Immunotherapy against HPV16/18 generates potent TH1 and cytotoxic cellular immune responses. Sci Transl Med 4:155ra138
6. El-Kamary SS, Billington M, Deitz S et al (2012) Safety and tolerability of the Easy Vax clinical epidermal electroporation system in healthy adults. Mol Ther 20:214–220
7. Trimble CL, Morrow MP, Kraynyak KA et al (2015) Safety, efficacy, and immunogenicity of VGX-3100, a therapeutic synthetic DNA vaccine targeting human papillomavirus 16 and 18 E6 and E7 proteins for cervical intraepithelial neoplasia 2/3: a randomised, double-blind, placebo-controlled phase 2b trial. Lancet. doi:10.1016/S0140-6736(15)00239-1
8. Cu Y, Broderick KE, Banerjee K et al (2013) Enhanced delivery and potency of self-amplifying mRNA vaccines by electroporation in situ. Vaccines (Basel) 1:367–383
9. Andre S, Seed B, Eberle J et al (1998) Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. J Virol 72:1497–1503
10. Deml L, Bojak A, Steck S et al (2001) Multiple effects of codon usage optimization on expression and immunogenicity of DNA candidate vaccines encoding the human immunodeficiency virus type 1 Gag protein. J Virol 75:10991–11001
11. Muthumani K, Zhang D, Dayes NS et al (2003) Novel engineered HIV-1 East African Clade-A gp160 plasmid construct induces strong humoral and cell-mediated immune responses in vivo. Virology 314:134–146
12. Schneider R, Campbell M, Nasioulas G et al (1997) Inactivation of the human immunodeficiency virus type 1 inhibitory elements allows Rev-independent expression of Gag and Gag/protease and particle formation. J Virol 71:4892–4903
13. Yang JS, Kim JJ, Hwang D et al (2001) Induction of potent Th1-type immune responses from a novel DNA vaccine for West Nile virus New York isolate (WNV-NY1999). J Infect Dis 184:809–816
14. Miyazaki S, Miyazaki J (2008) In vivo DNA electrotransfer into muscle. Dev Growth Differ 50(6):479–483
15. Draghia-Akli R, Khan AS, Cummings KK et al (2002) Electrical enhancement of formulated plasmid delivery in animals. Technol Cancer Res Treat 1:365–372
16. Atkins GJ, Fleeton MN, Sheahan BJ (2008) Therapeutic and prophylactic applications of alphavirus vectors. Expert Rev Mol Med 10:e33
17. Barnett SW, Burke B, Sun Y et al (2010) Antibody-mediated protection against mucosal

simian-human immunodeficiency virus challenge of macaques immunized with alphavirus replicon particles and boosted with trimeric envelope glycoprotein in MF59 adjuvant. J Virol 84:5975–5985

18. Robert-Guroff M (2007) Replicating and non-replicating viral vectors for vaccine development. Curr Opin Biotechnol 18:546–556
19. Smerdou C, Liljestrom P (1999) Non-viral amplification systems for gene transfer: vectors based on alphaviruses. Curr Opin Mol Ther 1:244–251
20. Zimmer G (2010) RNA replicons - a new approach for influenza virus immunoprophylaxis. Viruses 2:413–434
21. Rayner JO, Dryga SA, Kamrud KI (2002) Alphavirus vectors and vaccination. Rev Med Virol 12:279–296
22. Gronevik E, von Steyern FV, Kalhovde JM et al (2005) Gene expression and immune response kinetics using electroporation-mediated DNA delivery to muscle. J Gene Med 7:218–227
23. Lin F, Shen X, McCoy JR et al (2011) A novel prototype device for electroporation-enhanced DNA vaccine delivery simultaneously to both skin and muscle. Vaccine 29:6771–6780
24. Sardesai NY, Weiner DB (2011) Electroporation delivery of DNA vaccines: prospects for success. Curr Opin Immunol 23:421–429
25. Diehl MC, Lee JC, Daniels SE et al (2013) Tolerability of intramuscular and intradermal delivery by CELLECTRA® adaptive constant current electroporation device in healthy volunteers. Hum Vaccin Immunother 9:2246–2252

Part IV

Preclinical and Clinical Development

Chapter 13

The European Regulatory Environment of RNA-Based Vaccines

Thomas Hinz*, Kajo Kallen*, Cedrik M. Britten, Bruno Flamion, Ulrich Granzer, Axel Hoos, Christoph Huber, Samir Khleif, Sebastian Kreiter, Hans-Georg Rammensee, Ugur Sahin, Harpreet Singh-Jasuja, Özlem Türeci, and Ulrich Kalinke

Abstract

A variety of different mRNA-based drugs are currently in development. This became possible, since major breakthroughs in RNA research during the last decades allowed impressive improvements of translation, stability and delivery of mRNA. This article focuses on antigen-encoding RNA-based vaccines that are either directed against tumors or pathogens. mRNA-encoded vaccines are developed both for preventive or therapeutic purposes. Most mRNA-based vaccines are directly administered to patients. Alternatively, primary autologous cells from cancer patients are modified ex vivo by the use of mRNA and then are adoptively transferred to patients. In the EU no regulatory guidelines presently exist that specifically address mRNA-based vaccines. The existing regulatory framework, however, clearly defines that mRNA-based vaccines in most cases have to be centrally approved. Interestingly, depending on whether RNA-based vaccines are directed against tumors or infectious disease, they are formally considered gene therapy products or not, respectively. Besides an overview on the current clinical use of mRNA vaccines in various therapeutic areas a detailed discussion of the current regulatory situation is provided and regulatory perspectives are discussed.

Key words mRNA, Vaccines, Anticancer vaccination, Vaccination against infectious disease, Preventive and therapeutic approaches, Regulatory framework in the EU, Advanced therapy medicinal products (ATMP), Genetically modified medicinal products

1 Medical Application of mRNA

1.1 The History of RNA

The discovery of messenger RNA (mRNA) can be pinpointed to 1961, the year in which Marshall Nirenberg, a young NIH group leader, and Heinrich Matthaei, a German postdoctoral fellow in the Nirenberg lab, published a paper in which they described the

*Thomas Hinz and Kajo Kallen contributed equally to this manuscript.

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_13,

synthesis of repetitive sequences of ribonucleic acids which they in vitro translated to peptides [1, 2]. By correlating the peptide sequences with the original RNA sequences, Nirenberg and Matthaei were able to deduce the genetic code, a fundamental breakthrough that earned Nirenberg the Nobel prize in 1968. Since then, extensive research has been performed to understand how cells transcribe DNA to mRNA and how this mRNA is translated to proteins. Soon it became evident that unlike DNA, mRNA does not have to enter the nucleus in order to become active. Right after reaching the cytoplasm it is translated into proteins, irrespective of whether the mRNA was exported from the nucleus or whether it was brought directly into the cell by some transfection method. Unlike DNA, mRNA delivered to the cell lumen is not integrated into the genome. Hence, there is not even a theoretical risk for insertional mutagenesis upon transfection of mRNA as is the case for certain DNA-based gene transfer approaches. Furthermore, once reaching the cytoplasm mRNA is only transiently active because a plethora of physiological mechanisms exist within the cytoplasm that constantly degrade RNA.

1.2 The Therapeutic Potential of mRNA

The production of proteins under GMP conditions is a considerable resource-, cost-, and time-consuming effort. The use of DNA or mRNA for the expression of virtually any protein in vivo thus appears as an attractive alternative to the ex vivo production of proteins, thereby essentially turning an organism into its own production unit. mRNA therefore appears as a favorable vector for the production of widely needed proteins such as antigens or proteins required for replacement of genetically damaged hypo- or hyperfunctional proteins. 25 years ago, Wolff et al. demonstrated that comparable protein expression was obtained in vivo from mRNA and DNA injected into mouse skeletal muscle [3]. Later it was shown that DNA and mRNA-based vaccines had similar efficacies in the induction of humoral [4–6] and cellular responses [7].

However, before these initial successes lead to a more widespread interest in mRNA as the carrier of the protein code, mRNA technologies had to be significantly improved in particular with regard to translation, intracellular stability, delivery, and not the least costs [8]. Many researchers have long held the erroneous view that mRNA is a rather unstable molecule, possibly due to the ubiquitous presence of RNA-degrading nucleases. Notwithstanding, mRNA is chemically very stable and can easily be produced in cell free systems [8–10]. For this purpose a linearized DNA template is generated that is transcribed in vitro to mRNA using T7 or SP6 RNA polymerase. Coding mRNAs are single stranded, endowed with a 5′ cap structure, and the coding sequence of interest is embraced by untranslated regions and a 3′ poly(A) tail thus making them look like naturally occurring mature and processed mRNA as they are detected in the cytoplasm of eukaryotic cells.

The translation efficacy from such an in vitro-transcribed mRNA can be dramatically improved by the use of modified sequences of naturally occurring nucleotides, modifications of the 5′- and 3′-untranslated regions, the use of chemically modified CAP structures, as well as the introduction of chemically modified nucleosides [11–19]. Unlike naturally transcribed mRNA that is transported from the nucleus to the cytoplasm, in vitro synthesized mRNA is typically brought into the cytoplasm by sophisticated transfection methods. The mechanism operating after injection of synthetic mRNA in vivo is still unclear, but may involve active transport via receptor-mediated endocytosis or micropinocytosis [20, 21].

Engineered mRNA molecules can also be used for in vitro transfection of cells. Since spontaneous mRNA uptake is very low, depending on the cell type, different transfection methods are required. Interestingly, direct injection of mRNA into the cell also leads to substantial protein expression [17]. Lately, intravenous administration of mRNA packed into liposomes has also been applied successfully. The liposomal nanoparticles protect the mRNA from immediate degradation by serum RNases and are also engineered to target mRNA to specific organs [19, 22].

1.3 Current Strategies for RNA-Based Tumor Vaccines

A number of different mRNA-based approaches are now under investigation to induce T-cells directed against tumors [22]. Anti-tumor vaccination with mRNA-based vaccines originated from the observation that injection of antigens encoded as naked (unprotected) or protamine protected mRNA into the ear pinna of mice resulted in substantial antigen-specific humoral and cellular immune responses that were able to lyse antigen expressing cells [7], and T-cells induced by the injected mRNA were able to lyse cells expressing the antigen. A refinement of the initial vaccine by making use of translationally enhanced mRNAs and a formulation imparting adjuvant activity has successfully passed phase I clinical trials in prostate and non-small-cell lung cancer (NSCLC) [23–27]. The flexibility of the mRNA was exploited to encode four (prostate) or five (NSCLC) tumor-associated antigens. The intradermal administration of the non-encapsulated vaccine appeared to be safe and immunogenic with antigen-specific T-cells induced in approximately 80 % of vaccinated individuals [27]. Later stage clinical studies with prostate and NSCLC vaccines with six tumor antigens each are ongoing (ClinicalTrials.gov Identifier: NCT02140138, NCT01817738, NCT01915524).

The versatility of mRNA-based vaccines is also highlighted by the approach to use cellular targeting of encoded tumor antigens of a translationally enhanced mRNA vaccine [12, 28]. This approach also entered clinical phase I testing in melanoma (ClinicalTrials.gov Identifier: NCT01684241). Importantly, novel insights into the immunological relevance of the tumor mutanome, which is unique for each individual patient, led to the development

of the so-called actively personalized immunotherapy [29]. In brief, a cancer mutation-encoding mRNA is tailored for each and every patient based on NGS-informed profiling of individual tumors [30–32]. Thus, an individual drug product is newly manufactured for each patient based on the preceding mutanome analysis. Another type of actively personalized immunotherapy is the so-called warehouse concept that relies on the manufacture of patient-specific combinations of non-mutated tumor antigens [29]. These tumor antigens are selected from an off-the-shelf warehouse after an individual patient has been analyzed for the expression of each of the warehouse antigens. Both mRNA-based actively personalized concepts, the mutanome and the warehouse approach, are currently evaluated in melanoma and triple negative breast cancer, respectively (ClinicalTrials.gov Identifier: NCT02035956, NCT02316457).

Another type of mRNA-based vaccines are dendritic cell vaccines transfected with mRNA-encoded antigens linked to a class II sorting signal antigen together with mRNAs of three different immunostimulatory molecules (a constitutively active TLR4 variant, CD40L and CD70) [33]. Vaccination of melanoma patients in stage III/IV with such electroporated dendritic cells administered intravenously and/or intradermally resulted in robust antigen-specific $CD4^+$ and $CD8^+$ T-cell responses, some clinical responses, and favorable clinical courses [34–36].

The very different approaches summarized above exemplify the broad variability of well tolerated mRNA-based vaccination approaches. Furthermore, they illustrate the opportunity to implement new medical and scientific insights during clinical development.

1.4 Current Strategies for RNA-Based Vaccines Against Infectious Diseases

Current cancer vaccines are administered repeatedly and in situations where presumably high antigen loads are present. Infectious disease vaccines by contrast, are given prophylactically to healthy individuals to elicit a protective immune response with as few administrations as possible. This poses new challenges for safety and the induction of protective immune responses. Different mRNA-based vaccine technologies have now revealed promising results also in this arena. The self-adjuvanted mRNA approach described above was protective against several lethal influenza virus strains in mice and against swine flu in pigs and does not require the expensive and logistically challenging maintenance of a cold chain during transport of protein-based vaccines [18, 37]. An mRNA vaccine constructed this way is currently in phase I testing (ClinicalTrials.gov Identifier: NCT02241135). Substantial humoral and cellular immune responses against several different pathogens have also been achieved using rapidly self-amplifying mRNA vaccines that are based on engineered alphavirus replicons delivered non-virally to

mice and macaques [38–41]. Another important feature of mRNA based vaccinology is that the technology allows rapid development of novel vaccines within a very short time span of weeks rather than months [18, 42]. Hence "on-demand vaccines" might become feasible in the future to combat novel pathogenic challenges [43]. A project to develop a therapeutic mRNA-based vaccine against HIV by encoding conserved T-cell epitopes identified in HIV patients (http://ihivarna.org/partners/) as well as efforts to produce highly individualized vaccines against the mutanome of a cancer patients exemplify therapeutic efforts opened by the possible speed of production [32, 44]. While numerous molecular approaches have been investigated in the last decades, many of them had difficulties to pass the hurdle of scalability to tens of millions, if not hundreds of millions of doses within a limited period of time. A real-world test is still unavailable, but sensitivity analyses suggest that large-scale production at affordable costs will be possible for mRNA-based vaccines against infectious pathogens. Thus, vaccine development could well be revolutionized by mRNA-based vaccines [45].

1.5 Emerging Therapeutic Options for mRNA: Vaccines Against Allergy and In Vivo Production of Proteins by mRNA

Allergy is characterized by an immune response with Th2 bias that causes secretion of cytokines inducing an alteration in the antibody isotype switch occurring in B-cells and the induction of allergen-specific IgE antibodies. Recent experiments with a variety of allergens encoded by mRNA vaccines suggested that these might prevent Th2 skewing [46, 47]. This could offer the possibility to interfere with an epidemiologically very burdensome group of diseases.

Recent research provided the preclinical proof of principle that proteins encoded by mRNA using modified or naturally occurring nucleosides can be expressed in vivo in large animals at levels and durations that are functionally relevant [15, 16, 19, 48]. Research calls have been made to study mRNA technology for the expression of very large proteins such as antibodies. One question arising from these studies is whether highly purified mRNA is really immunogenic per se [19].

Despite the high interest of these very recent developments and the huge therapeutic possibilities offered, the regulatory challenges of these emerging options might be different from those for cancer or infectious disease vaccines. Therefore, regulatory requirements related to mRNA-based allergy and protein replacement/substitution medicinal products are not discussed here. In contrast, the regulatory implications of mRNA or synthetic peptides used in actively personalized immunotherapies has been discussed before by authors also representing the Regulatory Research Group [29] (for more information about this group see acknowledgements below).

2 The European Regulatory Framework

2.1 EMA and National Regulatory Authorities

As any other medicinal product, mRNA-based medicines are regulated in the EU on both the national and the EU level, depending on their developmental stage. While the conduct of clinical trials including the manufacture of investigational medicinal products is regulated on the national level, several products are shifted to the EU level as soon as a marketing authorization is sought. Depending on the class of product an mRNA medicine can obtain a marketing authorization in all EU member states by proceeding through the so-called centralized procedure. Marketing authorization applications are evaluated by the Committee for Human Medicinal Products (CHMP) located at the European Medicines Agency (EMA). CHMP's opinion concerning the approvability of a medicinal product is forwarded to the European Commission. The European Commission thus is the ultimate institution that approves or rejects a marketing authorization application.

Applicants like biotechnology or pharmaceutical companies need to send a dossier to EMA that comprehensively describes the manufacturing and quality control as well as the preclinical and clinical studies. Once started, the centralized EMA procedure is organized along a defined schedule consisting of an in-depth assessment of the submitted data resulting in a list of questions which is sent to the applicant no later than 120 days after the starting day. The assessment is done separately by both a Rapporteur and a Co-Rapporteur who are nominated by the CHMP. In the CHMP, representatives from all EU Member State regulatory authorities are present, thereby forming a large network of European regulatory agencies. The day 120 list of questions is adopted by the CHMP after discussing the separate assessments of Rapporteur and Co-Rapporteur, receiving comments and contributions from CHMP members/national agencies, and assessment of the pharmacovigilance and risk management system by the EMA's Pharmacovigilance Risk Assessment Committee (PRAC).

After sending the day 120 list of question to the applicant a clock stop (from EMA's point of view) of about 90 days follows. During this time the applicant is given the opportunity to prepare a response document. Upon receiving the applicant's responses to each question that has been raised the re-start of the procedure at EMA on day 121 is initiated. Evaluation of the responses by Rapporteur, Co-Rapporteur, CHMP members, and PRAC is finalized at day 180 by adopting a list of outstanding issues which is again forwarded to the applicant. Another clock stop (1–3 months) follows allowing the applicant to send responses to EMA resulting in the re-start of the procedure at day 181. The final opinion on the granting of a marketing authorization is taken by the CHMP on day 210. This CHMP opinion is forwarded to the European

Commission who will ultimately decide on day 277. Applicants should be aware that marketing authorization applications also need to include the results of studies as described in the pediatric investigation plan (PIP), unless the medicine has been exempted from this requirement. EMA's Pediatric Committee (PDCO) is responsible for agreeing or refusing the PIP.

Apart from the 210 days EMA procedure, applicants can request an accelerated procedure which takes 150 days only. The accelerated procedure is applicable should the medicinal product be of major interest from both the public health and the therapeutic innovation point of view.

Besides the above outlined "normal" way of EMA centralized marketing authorization based on comprehensive data, it is also possible to approve products on the basis of incomplete data. Such a conditional marketing authorization may be granted in case only preliminary clinical safety and efficacy data are available. Though the clinical data may be incomplete, the overall benefit/risk ratio as determined by EMA during the assessment procedure needs to be positive. Another prerequisite to this alternative authorization pathway is the ability of the applicant to provide the missing data after conditional approval has been granted. Moreover, the medicinal product is intended for patients with unmet medical needs, and the benefit to public health needs to outweigh the risks that might be associated with the incomplete clinical data. Upon completion of the data the conditional authorization can become a regular marketing authorization. Conditional marketing authorizations are valid for 1 year, on a renewable basis. Yet another scenario is that for a given medicinal product the applicant may be unable to provide comprehensive safety and efficacy data under normal conditions of use, because the disease to be treated is rare or because collection of full information is not possible or is unethical. In this case marketing authorization under exceptional circumstances is possible. In contrast to a conditional authorization, it is not expected that missing data can be provided. Thus, affected products are reviewed annually to reassess their benefit/risk balance. A helpful questions and answers document addressing the possibilities to obtain marketing authorization can be found on the EMA homepage (www.ema.europa.eu: Human regulatory/Pre-authorisation/Presubmission guidance: questions and answers). To obtain more detailed information related, e.g., to the usual clinical development paradigm, authorization based on one pivotal clinical trial only, clinical trial design, etc., it is recommended to consult dedicated guidance instructions [49, 50].

As outlined above the regulatory authorities of individual EU member states can act as Rapporteurs or Co-Rapporteurs in EMA centralized procedures. Delegates of the national authorities can be members of EMA committees, like the CHMP, Pharmacovigilance Risk Assessment Committee (PRAC), Committee for Orphan

Medicinal Products (COMP), Committee for Advanced Therapies (CAT) and others. National delegates can also be members of EMA working parties like the Biologics Working Party (BWP) which is the central EMA platform for discussing quality aspects of biological medicinal products for example those arising in centralized marketing authorization procedures or in scientific advice procedures. Besides their EMA involvement the national regulatory agencies are also involved in activities which are the sole responsibility of the EU member states. One important example is the authorization of clinical trial applications. At the time of writing this manuscript, clinical trial applications need to be submitted to those individual member state authorities where the clinical trial is to be conducted. This procedure will change in the near future upon coming into application of the new clinical trials regulation [49]. Regulations are legally binding for all EU member states as soon as coming into force and do not require implementation into the member state legislation as in case of directives. The new GCP regulation is intended to not only accelerate but also to harmonize the review of clinical trial applications in the EU. In fact, the new clinical trials regulation is already in force since 16 June 2014. Its entry into application, however, is dependent on the functionality of the EU clinical trial portal which will be located at EMA. The portal will be the future entry point for all clinical trial applications in the EU. Clinical trial applications will be forwarded via the portal to the member states concerned (where the trial is being conducted). Sponsors will have the opportunity to suggest a reporting member state performing the primary assessment. After coordinated review by all concerned member states sponsors will be informed via the portal by each member state whether the clinical trial is authorized, authorized with conditions, or if authorization is refused.

In summary, if an mRNA-based medicine is intended to be marketed on the whole EU market via a single authorization, the above-described EMA-centralized procedure applies. Depending on the disease to be treated and the data presented either a "normal" marketing authorization, a conditional one, or an authorization under exceptional circumstances can be granted. On the other hand, if an mRNA-based medicine is intended to be used in a clinical trial a respective application needs to be sent to the concerned member state regulatory agencies. In the future (but no earlier than 28 May 2016) clinical trials have to be sent to concerned member states via the EMA portal.

2.2 Mandatory Versus Optional Centralized EMA Marketing Authorization Procedure

For the marketing of certain medicinal products in the EU the centralized marketing authorization is mandatory. The products affected are listed in Annex of Regulation (EC) No 726/2004 [50]. These are medicines developed by one of the following biotechnological processes: recombinant DNA technology; controlled expression of genes coding for biologically active proteins in prokaryotes and eukaryotes

including transformed mammalian cells; and hybridoma and monoclonal antibody methods. For Advanced Therapy Medicinal Products (ATMPs, see below) the centralized procedure is also mandatory. Similarly affected are medicinal products for human use containing a new active substance which has not been authorized before 20 November 2005 and which are intended for the treatment of the following diseases: acquired immune deficiency syndrome, cancer, neurodegenerative disorders, diabetes, auto-immune diseases and other auto-immune dysfunctions, viral diseases. Medicines for the treatment of rare diseases (prevalence in the EU less than 5/10.000), so-called orphan medicines also need to go through the centralized procedure.

Medicines for which the centralized procedure is optional are those containing a new active substance which has not been authorized in the EU before 20 November 2005. Optional are also medicines that constitute a significant therapeutic, scientific or technical innovation or if the marketing authorization is in the interest of patients. EMA might consider products that provide a new alternative to patients in treating, preventing or diagnosing a disease. Alternatively, the medicinal product development is based on significant new scientific knowledge or on the application of a new scientific knowledge, or, a new technology or a new application of technology is used.

In conclusion, for RNA-based medicines which are gene therapy medicinal products (definition see below) the centralized EMA procedure is mandatory. Should, however, mRNA be used for the purpose of vaccination toward infectious disease it is per definition in the law no more an ATMP. Nevertheless, should recombinant DNA technology be applied such mRNA-based vaccines for infectious disease also need to be authorized via the centralized procedure. Should the criteria for the centralized procedure not be fulfilled mRNA medicines for infectious disease might go via the national route though access to the centralized procedure upon request by the applicant still is possible.

2.3 Role of EMA's Committee for Advanced Therapies for the Approval of mRNA-Based Medicines

The CAT is EMA's Committee for Advanced Therapies. The CAT is responsible for the scientific evaluation of marketing authorization applications for Advanced Therapy Medicinal Products (ATMP). The schedule of the centralized procedure as described above also applies to ATMPs. According to Article 2 of Regulation (EC) No 1394/2007 [51] gene therapy medicinal products, somatic cell therapy products, and the tissue engineered products constitute the ATMPs. While a legally binding definition for the tissue engineered products is provided in Article 2 of Regulation (EC) No 1394/2007, the definitions for somatic cells and gene therapy products can be found in Part IV of Annex I to Directive 2001/83/EC [52]. The latter directive is the codex regulating medicinal products in the EU. As such, its implementation into the legislation of each EU member state is required. The definition of a gene therapy medicinal product as outlined in Annex 1 to Directive 2001/83/EC is as follows:

> Gene therapy medicinal product means a biological medicinal product which has the following characteristics:
>
> (a) it contains an active substance which contains or consists of a recombinant nucleic acid used in or administered to human beings with a view to regulating, repairing, replacing, adding or deleting a genetic sequence;
>
> (b) its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence.
>
> Gene therapy medicinal products shall not include vaccines against infectious diseases.

The following important conclusions can be drawn from this definition: Taking into account that the usual manufacturing process of mRNAs is based on in vitro transcription done with plasmid templates derived from bacteria, such mRNAs presumably have to be considered as a biological medicinal product. Also, mRNAs are usually recombinant due to the introduction of several modifications such as codon optimization, modified CAP structures, introduction of suitable 5′ and 3′ noncoding regions, defined poly(A) tails, etc. Taken together, mRNAs fulfilling the criteria of being a recombinant biological product that is used to add or replace a genetic sequence, and whose therapeutic, prophylactic or diagnostic effect is directly mediated by the nucleic acid it contains are defined as gene therapy medicinal products. Should an RNA molecule be manufactured by pure chemistry as are many RNAi molecules, it would no longer be a biological product, and thus could not be classified as a gene therapy product.

It is also important to notice that in case of treating or preventing infectious disease, an mRNA per law is not a gene therapy product, even though if all the other requirements are fulfilled (recombinant, biological). As a consequence, an mRNA molecule used for the prophylactic vaccination against, e.g., influenza, is not a gene therapy product, while this is the case when for example used for the treatment of cancer. The consequence with respect to the EMA marketing authorization is that mRNA for vaccination against infectious diseases is evaluated by the CHMP, while mRNAs fulfilling the criteria of an ATMP are assessed by the CAT.

2.4 Classification of Medicinal Products by the CAT

Another important task of the CAT is to provide classifications for medicinal products upon request. For developers of medicinal products it might be of considerable interest to know whether or not their medicinal product is an ATMP. A reflection paper has been published by CAT that describes CAT's view on when a medicine is classified as either gene therapy, somatic cell or tissue-engineered product [53]. Short descriptions of the CAT classifications can be found on the EMA homepage (summaries of scientific recommendations on classification of advanced-therapy

medicinal products). In case of transfecting mRNA into, e.g., DCs rather than being directly administered to patients, the resulting genetically modified cells are normally also classified as gene therapy products [52]. One of the classifications described in the above mentioned CAT reflection paper deals with autologous DCs that have been electroporated with in vitro-transcribed RNA. The CAT came to the conclusion, however, that this product is not classified as gene therapy because not all requirements of the legal definition were fulfilled. The mRNA was not administered "with a view to adding a genetic sequence." It was rather intended to mediate the translation of tumor antigens for a short period of time (as long as the mRNA is not degraded in the cells) which are then presented on the surface of the DCs to induce an anti-tumoral immune response in patients.

The meaning of "… *its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence*" as outlined by indent (a) of the legal definition is illuminated by the CAT classifications of T cells genetically modified to express an exogenous thymidine kinase (TK) gene. The T cells have not been classified as gene therapy medicinal product, since they were intended for immune reconstitution after hematopoietic stem cell transplantation. The purpose of the introduced TK gene was to treat the emergence of graft versus host disease should it occur in certain patients. Thus, the genetic sequence introduced into the patient (the TK gene) did not have a direct relationship to the intended therapeutic effect, i.e., immune reconstitution. The cells were therefore classified as somatic cell therapy product. On the other hand, for T cells transfected with mRNA encoding, e.g., a novel T-cell receptor (TCR), the newly introduced TCR clearly has a direct relationship with the intended therapeutic effect, i.e., killing of cancer cells expressing the target antigen recognized by such a TCR. In this scenario, the medicinal product would probably be classified as an ATMP. Such approaches may be attractive for development of adoptive cellular therapies against novel targets for which safety data is not well established but still offer an acceptable risk/benefit profile due to the transient nature of the genetic modification.

As outlined above gene therapy medicinal products do not include vaccines against infectious diseases. The CAT reflection paper outlines, however, that a gene therapy-based vaccine can nevertheless be classified as gene therapy if indicated for the treatment or prevention of pathologies induced by the infection (e.g., malignancies). As an example, an mRNA-based vaccine for the treatment or prevention of HPV16-induced malignancies is a gene therapy product (if the criteria for gene therapy are fulfilled). Using the identical mRNA for vaccination towards HPV16 will result in its classification as a vaccine.

At this time no CAT classification for in vitro-transcribed mRNA to be directly administered to patients exists. Developers of mRNA medicines are thus encouraged to clarify the issue by asking for CAT classification. The details of mRNA manufacture such as the use of chemically synthesized templates versus plasmid templates isolated from bacteria might be important to decide whether or not an mRNA is a biological medicinal product. A definition for biological medicinal products can be found in point 3.2.1.1 of Part I of Annex I to Directive 2001/83/EC. As outlined above both criteria, i.e., biological and recombinant need to be fulfilled for mRNA to be classified as a gene therapy medicinal product.

2.5 National Authorization of ATMPs via the Hospital Exemption

An exemption from the mandatory centralized marketing authorization requirement for ATMPs is laid down in Article 28 (2) of the ATMP regulation [51]. It amends Article 3 Nr. 7 of Directive 2001/83/EC to include the so-called hospital exemption which is applicable to ATMPs fulfilling certain criteria. The hospital exemption is an authorization procedure that is purely applicable at the national level. Applicants thus have to contact their respective national regulatory authorities. Due to implementing the hospital exemption into Directive 2001/83/EC and not into the ATMP regulation itself, the individual member states were obliged to integrate the hospital exemption into their national legislations. As a result, differences exist in the way member states apply the hospital exemption. The prerequisites for an ATMP to be authorized via the hospital exemption are as follows. ATMPs need to be (1) prepared on a non-routine basis according to specific quality standards, (2) used within the same member state in a hospital under the exclusive professional responsibility of a medical practitioner, and (3) need to comply with an individual medical prescription for a custom-made product for an individual patient.

In summary, should mRNA-based medicinal products be intended for vaccination against infectious diseases (prophylactic and therapeutic) they are per law not ATMPs and therefore cannot be authorized nationally via the hospital exemption. On the other hand, should mRNAs be intended for the therapy of other diseases and should the above mentioned criteria be fulfilled (non-routine manufacture, etc.), mRNA medicines in principle can be authorized by individual member states by the hospital exemption. Some EU regulatory authorities envisage this procedure as an opportunity especially for small and medium-sized biotech companies or hospital research groups to apply their medicinal products outside of clinical trials to obtain first safety and efficacy results. Moreover, patients might get early access to innovative medicines, the safety and efficacy of which still needs to be proven in controlled clinical trials.

3 Implications for mRNA Vaccines

3.1 General Considerations Towards Regulatory Guidance Applicable for RNA-Based Vaccines

The information to be provided to regulatory authorities for clinical trial authorization is harmonized in the EU. A detailed form including guidance on the specific information to be provided such as on the manufacturer, description of the manufacturing process, control of materials, control of drug substance/drug product, etc. can be found on the homepage of the European Commission (http://ec.europa.eu/health/documents/eudralex/vol-10/index_en.htm). Additional information can be retrieved from that homepage for example on how to apply for a substantial amendment for an ongoing clinical trial. A detailed guidance document addressing the quality documentation of biological investigational medicinal products is available at EMA [54]. Since the quality data base of investigational medicinal products normally is limited, especially in early developmental stages, it is not expected that investigational products are validated to the extent of a routinely manufactured/marketed product. Nevertheless, premises and equipment are expected to be qualified and sterilizing processes should be validated. When required, virus inactivation/removal and that of other impurities of biological origin should be demonstrated [55]. In general, clinical trial needs to be conducted according to the principles of good clinical practice [56].

For centralized marketing authorization applications the information to be provided is defined in the Common Technical Document (CTD) comprising administrative information (Module 1), summaries of quality, preclinical and clinical data (Module 2); quality data (Module 3), preclinical and clinical information in Modules 4 and 5, respectively [57]. It is strongly recommended that applicants liaise with EMA via pre-submission meetings ahead of actually submitting a marketing authorization application. It is noteworthy that for ATMPs the risk-based-approach can be applied to adapt the content of applicant's marketing authorization applications depending on the individual product [58]. Though the principle technical requirements, i.e., the data to be submitted for ATMPs are defined in Part IV of the Annex to Directive 2001/83/EC [52] deviations are acceptable when justified by the risk-based approach. The methodology of the risk-based approach relies on the establishment of a risk profile by identifying risks and associated risk factors. This allows applicants to justify the extent of data presented in the various sections of the marketing authorization application.

3.2 Quality Regulatory Requirements for mRNA

No specific guidelines have as yet been published by EMA for the development of mRNA-based medicines. The general principles as outlined in overarching guidance documents therefore have to be followed. Though mRNA-based vaccines for the prevention or treatment of infectious disease are not gene therapy products the principles as outlined in EMA guideline for gene therapy medicinal

products should be considered that addresses quality, nonclinical and clinical aspects [59]. At the time of writing this manuscript the latter guideline is only available as a draft version that might be modified after including comments derived from public consultation. Should mRNA be transfected into somatic cells to obtain a cell-based product the EMA guidelines on "human cell-based medicinal products" and the guidance on genetically modified cells should be consulted [60, 61]. As in case for all medicines a suitable manufacturing process needs to be established yielding a medicinal product of consistent quality. For this purpose specifications have to be defined for critical process steps, intermediates, drug substance, and final drug product. Also, the quality of raw and starting materials has to be defined and controlled. The general chapter 5.2.12 on "Raw materials of biological origin for the production of cell-based and gene therapy medicinal products" will soon be published in the European Pharmacopoeia (Ph. Eur.). It covers biological materials like sera, media, proteins of recombinant origin, and proteins extracted from biological materials such as enzymes.

In general, quality attributes to be controlled are appearance, identity (and in case of nucleic acids the integrity), content, potency, product- and process-related impurities, sterility, endotoxin, and physicochemical tests like pH and osmolality. Should mRNA molecules be complexed with, e.g., poly-cationic molecules or liposomes, assays and specifications for particle size distribution should be established. The consistent amount of the complexing materials should either be part of drug product release or it needs to be established in validation studies. The latter option is required in case the complexing materials and drug product are admixed immediately ahead of the administration to patients. In some specific circumstances, especially during early clinical development stages some regulatory authorities might envisage the final preparation of a liposomal formulation or an emulsion as manufacturing (rather than reconstitution) that needs to be controlled appropriately, i.e., by suitable release testing. Irrespective of defining final formulation as manufacturing or reconstitution it appears reasonable to leave this step to qualified pharmacies, whenever possible. Besides the release testing, additional characterization studies should be performed with complexed nucleic acids during product development addressing attributes like form, surface charge, and stability.

While there is a clear requirement in the EU to manufacture investigational drug substances and drug products according to GMP, it is less clear from which manufacturing step onwards GMP has to be applied in case of mRNA manufacture. Examples for other drug substances are provided in Part II of the GMP guidelines (Basic requirements for active substances used as starting materials). Drug substance manufacture in general starts with the drug substance starting materials. For plasmids or non-viral vectors (like mRNA) the starting materials are defined to be the plasmid, the

host bacteria, and the master cell bank of the recombinant microbial cells [52]. No definition, however, of the point from which GMP is required for plasmids and non-viral vectors is provided in the latter reference. In contrast, for viral vectors used to manufacture genetically modified cells, GMP has to be applied from the cell bank system onwards used to produce the vector. For other types of drug substances such as those derived from fermentation/cell culture GMP applies from the maintenance of the working cell bank [62]. Some mRNA drug substances are manufactured using a bacterial cell bank as a starting material. These microbial banks, however, are rather used to manufacture another starting material (the plasmid template) than the mRNA drug substance itself. While it might be beneficial to include microbial cell banks into the GMP system there is no clear requirement at the present time. On the other hand the guidance available is not demanding GMP for the recombinant technology used for initial plasmid/template construction. There is no doubt, however, that the in vitro transcription is drug substance manufacture that needs to be done under GMP. Further detailed information that might be applicable for mRNA manufacture like vector design, development genetics, characterization studies, excipients like complexing materials, and analytical method validation can be found in [59]. Though in Ph. Eur. general chapter 5.14 the production and testing of several types of viral and non-viral vectors is described, mRNA is not yet included. This might hopefully change in future Ph. Eur. editions.

3.3 Preclinical Regulatory Requirements for mRNA Medicinal Products

No dedicated guideline exists specifically addressing the preclinical evaluation of mRNA vaccines. The preclinical pharmacological and toxicological evaluation of mRNA largely depends on the disease to be treated and on the route of administration. The following example might illustrate this fact. Vaccines are usually administered locally via the intradermal or intramuscular route. Pharmacokinetic studies are therefore normally not needed [63]. On the other hand, mRNAs intended for therapeutic cancer vaccination are sometimes administered systemically by the intravenous route. Upon single or repeated systemic administration the evaluation of pharmacokinetic parameters appears to be important from both a safety and an efficacy point of view. Relevant plasma PK parameters in case of systemic administration are exposure, clearance, accumulation. Due to increased stability obtained by complexing with suitable substances mRNA might accumulate or persist in plasma for a certain period of time upon repeated administration. This in turn might be of concern since mRNA per se is immunostimulatory and can induce the secretion of pro-inflammatory cytokines.

Due to the ability of several gene therapy medicinal products to integrate into the recipient's genomes, biodistribution studies are normally considered as being important [59]. Since genome integration is not a concern for mRNA medicines such studies

appear to be less relevant as, e.g., in case of retroviral vector systems used for therapeutic vaccination. Metabolism studies are normally not considered relevant, since it is generally assumed that mRNA-medicines are metabolized in the same way as endogenous mRNA molecules. This might be different when chemically modified nucleosides are used.

Preclinical testing of vaccines (not ATMPs) is outlined in [63]. This EMA guidance also discusses additives like adjuvants, excipients, and preservatives. Potential safety concerns of new investigational adjuvants are injection site reactions, fever, and possibly immune-mediated effects like anaphylaxis that should be addressed in preclinical studies. Besides testing the adjuvant alone the vaccine/adjuvant combination should be tested. This scenario is relevant when noncoding mRNA is used as an adjuvant in vaccines against infectious disease. The duration of preclinical toxicity testing in general depends on the duration of the clinical trial to be conducted. Suitable information in this respect can be obtained from [64].

Preclinical regulatory guidance for therapeutic cancer vaccines is provided in EMA's anticancer guideline [65] in chapter 6.3.2. It is acknowledged that a suitable animal model for therapeutic cancer vaccines is often not available due to the human-specific nature conferred by the presentation of antigens on human HLA molecules. In some cases HLA transgenic animals might be available, although these do not carry the human components of the complex antigen processing machinery and thus may be of limited relevance as well. Should no suitable animal model exist in vitro assays can be employed to show proof-of-principle. Therapeutic cancer vaccines thus might be tested in an in vitro assay demonstrating that specific human T cells can be generated or activated upon repeated stimulation with the respective antigen(s). Preclinical pharmacological studies are aimed to show proof of concept, to determine the starting dose and the schedule.

3.4 Clinical Regulatory Requirements for mRNA Medicines

Clinical requirements for the authorization of vaccines are laid down in a dedicated EMA document that also covers DNA vaccines expressing foreign antigens [66]. Though mRNA is not included in its scope the principles can probably be applied to mRNA vaccines as well. Interestingly, pharmacokinetic studies are not requested by the guideline though nucleic acids are included. On the contrary, pharmacokinetic studies are expected for gene therapy medicinal products [59]. This would apply for a recombinant mRNA (fulfilling the criteria of a gene therapy medicinal product) intended for the treatment of cancer, i.e. a therapeutic cancer vaccine. Detailed guidance for the clinical development of vaccines such as on the characterization of the humoral and cellular immune responses, the populations to be considered (including infants), clinically relevant end points, dose finding, clinical trial design, and analyses of possible immune interference is also

provided in [66]. No such detailed and dedicated EMA guidance exists for therapeutic cancer vaccines. Some information can be found in EMA's anticancer guideline in chapter 6.3.2 [65]. Its content is largely overlapping with available FDA guidance [67].

According to the anticancer guideline [65], early clinical trials are intended to determine the (1) safety as well as (2) dose and (3) schedule to induce the desired immune response. The importance of immune monitoring to determine dose and schedule is also highlighted in the guideline. Whenever possible, tumor biopsies taken before and after vaccination should be analyzed for infiltrating lymphocytes. Multiple monitoring assays might be necessary that should be carefully explored. The relevance of the stage of the disease is highlighted, since it might be difficult to treat a high tumor burden by vaccination for which reason inclusion of patients with low tumor burden might be favorable. On the other hand the realization of clinical studies in early disease stages might be difficult due to the availability of already approved medicinal products with proven clinical efficacy. Since induction of an effective immune response and a clinical response may need time to develop as compared to cytotoxic compounds it might not be advisable to take patients off treatment upon disease progression. Keeping slowly progressing patients in the study might be possible if respective criteria have prospectively been defined in the study protocol, and proper measures of closely monitoring such patients in the study are implemented. Revised criteria for defining progression are acceptable if properly justified.

4 Conclusion and Perspectives

From a regulatory point of view mRNA-based medicines are well embedded in the available EU regulatory system. Depending on the classification, marketing authorization applications are either evaluated by EMA's CAT (gene therapy) or by the CHMP. Since for ATMPs/gene therapy products and for medicines developed by means of recombinant DNA technology the centralized EMA authorization procedure is mandatory and most, if not all, mRNA-based medicinal products have to be authorized by the EMA-centralized procedure. Thereby, access to the whole EU market is granted. National authorization of ATMPs/gene therapy products is possible provided that certain criteria such as production on a non-routine basis are fulfilled. The regulation of the clinical development on the other hand is currently the sole responsibility of the concerned EU member states' regulatory agencies. In principle this will also be the case after the new clinical trials regulation has come into application, though submission of clinical trial applications will have then to be pursued via the new EMA portal. It is nevertheless recommended that developers of mRNA-based

medicines ask EMA for scientific advice ideally ahead of starting pivotal clinical trials. This will result in clarity about the quality, preclinical, or clinical requirements to obtain a marketing authorization from EMA.

Acknowledgements

This chapter was issued as a joint effort by the Regulatory Research Group (RRG). The RRG was founded within the Association for Cancer Immunotherapy (CIMT) in 2008 as an independent group of experts focusing on regulatory aspects of drug development. CIMT was founded in 2002 by physicians and researchers from different fields of clinical and theoretical medicine as an independent nonprofit organization (www.cimt.eu).

References

1. Matthaei H, Nirenberg MW (1961) The dependence of cell-free protein synthesis in E. coli upon RNA prepared from ribosomes. Biochem Biophys Res Commun 4:404–408
2. Matthaei JH, Nirenberg MW (1961) Characteristics and stabilization of DNAase-sensitive protein synthesis in E.coli extracts. Proc Natl Acad Sci U S A 47:1580–1588
3. Wolff JA, Malone RW, Williams P et al (1990) Direct gene transfer into mouse muscle in vivo. Science 247:1465–1468
4. Tang DC, DeVit M, Johnston SA (1992) Genetic immunization is a simple method for eliciting an immune response. Nature 356:152–154
5. Martinon F, Krishnan S, Lenzen G et al (1993) Induction of virus-specific cytotoxic T lymphocytes in vivo by liposome-entrapped mRNA. Eur J Immunol 23:1719–1722
6. Conry RM, LoBuglio AF, Wright M et al (1995) Characterization of a messenger RNA polynucleotide vaccine vector. Cancer Res 55:1397–1400
7. Hoerr I, Obst R, Rammensee HG, Jung G (2000) In vivo application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies. Eur J Immunol 30:1–7
8. Pascolo S (2004) Messenger RNA-based vaccines. Expert Opin Biol Ther 4:1285–1294
9. Ketterer T, von der Mülbe F, Reidel L et al (2008) Method for purifying RNA on a preparative scale by means of hplc. Patent WO2008077592
10. Pardi N, Muramatsu H, Weissman D et al (2013) In vitro transcription of long RNA containing modified nucleosides. Methods Mol Biol 969:29–42
11. Kariko K, Buckstein M, Ni H, Weissman D (2005) Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunity 23:165–175
12. Holtkamp S, Kreiter S, Selmi A et al (2006) Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells. Blood 108:4009–4017
13. Kariko K, Muramatsu H, Welsh FA et al (2008) Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. Mol Ther 16:1833–1840
14. Kuhn AN, Diken M, Kreiter S et al (2010) Phosphorothioate cap analogs increase stability and translational efficiency of RNA vaccines in immature dendritic cells and induce superior immune responses in vivo. Gene Ther 17:961–971
15. Kormann MS, Hasenpusch G, Aneja MK et al (2011) Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. Nat Biotechnol 29:154–157
16. Kariko K, Muramatsu H, Keller JM et al (2012) Increased erythropoiesis in mice injected with submicrogram quantities of pseudouridine-containing mRNA encoding erythropoietin. Mol Ther 20:948–953
17. Schlake T, Thess A, Fotin-Mleczek M et al (2012) Developing mRNA-vaccine technologies. RNA Biol 9:1319–1330

18. Kallen K-J, Thess A (2014) A development that may evolve into a revolution in medicine: mRNA as the basis for novel, nucleotide-based vaccines and drugs. Ther Adv Vaccines 2:10–31
19. Thess A, Grund S, Mui BL et al (2015) Sequence-engineered mRNA without chemical nucleoside modifications enables an effective protein therapy in large animals. Mol Ther 23:1456–1464
20. Lorenz C, Fotin-Mleczek M, Roth G et al (2011) Protein expression from exogenous mRNA: uptake by receptor-mediated endocytosis and trafficking via the lysosomal pathway. RNA Biol 8:627–636
21. Diken M, Kreiter S, Selmi A et al (2011) Selective uptake of naked vaccine RNA by dendritic cells is driven by macropinocytosis and abrogated upon DC maturation. Gene Ther 18:702–708
22. Sahin U, Kariko K, Tureci O (2014) mRNA-based therapeutics--developing a new class of drugs. Nat Rev Drug Discov 13:759–780
23. Fotin-Mleczek M, Duchardt KM, Lorenz C et al (2011) Messenger RNA-based vaccines with dual activity induce balanced TLR-7 dependent adaptive immune responses and provide antitumor activity. J Immunother 34:1–15
24. Sebastian M, von Boehmer L, Zippelius A et al (2011) Messenger RNA vaccination in NSCLC: findings from a phase I/IIa clinical trial. J Clin Oncol 29:(suppl; abstr 2584)
25. Sebastian M, von Boehmer L, Zippelius A et al (2012) Messenger RNA vaccination and B-cell responses in NSCLC patients. J Clin Oncol 30:(suppl; abstr 2573)
26. Sebastian M, Papachristofilou A, Weiss C et al (2014) Phase Ib study evaluating a self-adjuvanted mRNA cancer vaccine (RNActive(R)) combined with local radiation as consolidation and maintenance treatment for patients with stage IV non-small cell lung cancer. BMC Cancer 14:748
27. Kubler H, Scheel B, Gnad-Vogt U et al (2015) Self-adjuvanted mRNA vaccination in advanced prostate cancer patients: a first-in-man phase I/IIa study. J Immunother Cancer 3:26
28. Kreiter S, Selmi A, Diken M et al (2008) Increased antigen presentation efficiency by coupling antigens to MHC class I trafficking signals. J Immunol 180:309–318
29. Britten CM, Singh-Jasuja H, Flamion B et al (2013) The regulatory landscape for actively personalized cancer immunotherapies. Nat Biotechnol 31:880–882
30. Castle JC, Kreiter S, Diekmann J et al (2012) Exploiting the mutanome for tumor vaccination. Cancer Res 72:1081–1091
31. Castle JC, Loewer M, Boegel S et al (2014) Mutated tumor alleles are expressed according to their DNA frequency. Sci Rep 4:4743
32. Kreiter S, Vormehr M, van de Roemer N et al (2015) Mutant MHC class II epitopes drive therapeutic immune responses to cancer. Nature 520:692–696
33. Bonehill A, Tuyaerts S, Van Nuffel AM et al (2008) Enhancing the T-cell stimulatory capacity of human dendritic cells by co-electroporation with CD40L, CD70 and constitutively active TLR4 encoding mRNA. Mol Ther 16:1170–1180
34. Aarntzen EH, Schreibelt G, Bol K, Lesterhuis WJ et al (2012) Vaccination with mRNA-electroporated dendritic cells induces robust tumor antigen-specific CD4+ and CD8+ T cells responses in stage III and IV melanoma patients. Clin Cancer Res 18:5460–5470
35. Wilgenhof S, Corthals J, Van Nuffel AM et al (2015) Long-term clinical outcome of melanoma patients treated with messenger RNA-electroporated dendritic cell therapy following complete resection of metastases. Cancer Immunol Immunother 64:381–388
36. Wilgenhof S, Van Nuffel AMT, Benteyn D et al (2013) Phase IB study on intravenous synthetic mRNA electroporated dendritic cell immunotherapy in pretreated advanced melanoma patients. Ann Oncol 24:2686–2693
37. Petsch B, Schnee M, Vogel AB et al (2012) Protective efficacy of in vitro synthesized, specific mRNA vaccines against influenza A virus infection. Nat Biotechnol 30:1210–1216
38. Geall AJ, Verma A, Otten GR et al (2012) Nonviral delivery of self-amplifying RNA vaccines. Proc Natl Acad Sci U S A 109:14604–14609
39. Lazzaro S, Giovani C, Mangiavacchi S et al (2015) CD8 T-cell priming upon mRNA vaccination is restricted to bone-marrow-derived antigen-presenting cells and may involve antigen transfer from myocytes. Immunology 146:312–326
40. Brazzoli M, Magini D, Bonci A et al (2015) Induction of broad-based immunity and protective efficacy by self-amplifying mRNA vaccines encoding influenza virus hemagglutinin. J Virol. doi:10.1128/JVI.01786-15
41. Bogers WM, Oostermeijer H, Mooij P et al (2015) Potent immune responses in rhesus macaques induced by nonviral delivery of a self-amplifying RNA vaccine expressing HIV

type 1 envelope with a cationic nanoemulsion. J Infect Dis 211:947–955
42. Hekele A, Bertholet S, Archer J et al (2013) Rapidly produced SAMH vaccine against H7N9 influenza is immunogenic in mice. Emerg Microbes Infect 2:e52
43. Ulmer JB, Mansoura MK, Geall AJ (2015) Vaccines 'on demand': science fiction or a future reality. Expert Opin Drug Discov 10(2):101–106
44. Boisguerin V, Castle JC, Loewer M et al (2014) Translation of genomics-guided RNA-based personalised cancer vaccines: towards the bedside. Br J Cancer 111:1469–1475
45. Geall AJ, Mandl CW, Ulmer JB (2013) RNA: the new revolution in nucleic acid vaccines. Semin Immunol 25:152–159
46. Roesler E, Weiss R, Weinberger EE et al (2009) Immunize and disappear-safety-optimized mRNA vaccination with a panel of 29 allergens. J Allergy Clin Immunol 124:1070–1077
47. Weiss R, Scheiblhofer S, Thalhamer J (2014) Allergens are not pathogens: why immunization against allergy differs from vaccination against infectious diseases. Hum Vaccin Immunother 10:703–707
48. Zangi L, Lui KO, von Gise A et al (2013) Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. Nat Biotechnol 31:898–907
49. EU, Regulation (EU) No 536/2014 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 April 2014 on clinical trials on medicinal products for human use, and repealing Directive 2001/20/EC
50. EC, Regulation (EC) No 726/2004 of the European Parliament and of the Council of 31 March 2004 laying down Community procedures for the authorisation and supervision of medicinal products for human and veterinary use and establishing a European Medicines Agency
51. EC, Regulation (EC) No 1394/2007 of the European Parliament and of the Council of 13 November 2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004
52. EU, COMMISSION DIRECTIVE 2009/120/EC of 14 September 2009 amending Directive 2001/83/EC of the European Parliament and of the Council on the Community code relating to medicinal products for human use as regards advanced therapy medicinal products
53. EMA, Reflection paper on classification of advanced therapy medicinal products. 20 June 2014. EMA/CAT/600280/2010 Rev.1. Committee for Advanced Therapies (CAT)
54. EMA, Guideline on the requirements for quality documentation concerning biological investigational medicinal products in clinical trials. EMA/CHMP/534898/2008
55. EU, Guidelines to Good Manufacturing Practice. Medicinal Products for Human and Veterinary Use. Annex 13. Investigational Medicinal Products
56. ICH, Harmonised tripartite guideline for good clinical practice E6(R1)
57. EU, Volume 2B. Notice to applicants. Medicinal products for human use
58. EMA, Guideline on the risk-based approach according to annex I, part IV of Directive 2001/83/EC applied to advanced therapy medicinal products
59. EMA, Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products. EMA/CAT/80183/2014
60. EMA, Guideline on human cell-based medicinal products. EMEA/CHMP/410869/2006
61. EMA, Guideline on quality, non-clinical and clinical aspects of medicinal products containing genetically modified cells. EMA/CAT/GTWP/671639/2008
62. EU, Good manufacturing practice. Medicinal products for human and veterinary use. Part II: basic requirements for active substances used as starting materials
63. EMA, Note for guidance on preclinical pharmacological and toxicological testing of vaccines. CPMP/SWP/465/95
64. EMA, Non-clinical safety studies for the conduct of human clinical trials for pharmaceuticals. ICH M(3)
65. EMA, Guideline on the evaluation of anticancer medicinal products in man. EMA/CHMP/205/95/Rev.4
66. EMA, Guideline on clinical evaluation of new vaccines. EMEA/CHMP/VWP/164653/2005
67. FDA, Guidance for Industry. Clinical Considerations for Therapeutic Cancer Vaccines

Chapter 14

Discovery and Subtyping of Neo-Epitope Specific T-Cell Responses for Cancer Immunotherapy: Addressing the Mutanome

Mustafa Diken, Mathias Vormehr, Christian Grunwitz, Sebastian Kreiter, Özlem Türeci, and Ugur Sahin

Abstract

Cancer accumulates 10s to 1000s of genomic mutations of which a fraction is immunogenic and may serve as an Achilles' heel of tumor cells. Mutation-specific T cells can recognize these antigens and destroy malignant cells. Strategies to immunotherapeutically address individual tumor mutations employing peptide or mRNA based vaccines are now actively investigated in mice and humans. An important step of determining the therapeutic potential of a mutanome vaccine is the detection of mutation reactive T-cell responses. In this chapter we provide protocols to identify and subtype mutation specific T cells in mice based on IFN-γ ELISpot and flow cytometry.

Key words mRNA, Neo-epitopes, Detection and subtyping of $CD4^+$ and $CD8^+$ T-cell responses

1 Introduction

Cancer is a group of diseases characterized by cells with limitless uncontrolled and invasive growth. During carcinogenesis, tumor cells acquire genetic and epigenetic modifications responsible for their malignant behavior. Already seven decades ago it was shown that after prior exposure the immune system is able to specifically recognize and reject tumor cells [1]. Since then, several classes of tumor antigens have been identified (reviewed in ref. 2).

Until recently, most research has focused on the identification of tumor-associated antigens (TAA) such as differentiation and overexpressed antigens as well as cancer-germ line antigens for cancer immunotherapy which can serve as targets for a broad spectrum of tumor patients. At least some of these shared antigens are expressed in the thymus resulting in deletion of high avidity T-cell clones by the

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_14,

central tolerance mechanisms. This tolerance mechanism might be one explanation for the so far largely disappointing outcome of clinical vaccination studies targeting TAA [3].

In contrast to TAA, tumor specific antigens (TSA) such as viral or mutated antigens are solely expressed by malignant cells and thus T-cell responses are not affected by central tolerance. We have recently found that 20–30% of individual amino acid changing point mutations are immunogenic providing a large novel target repertoire for cancer immunotherapy. Besides mutations driving tumor development, neo-epitopes generated by passenger mutations are suitable targets for tumor-specific cytotoxic and helper T cells of the immune system. Thus, genetic instability and neo-epitopes generated by cancer mutations might constitute their Achilles' heel (ref. Castle et al.) [4, 5]. Indeed, several studies demonstrated the importance of mutation specific T cells in cancer. It was shown that the number of neo-epitope candidates in patients correlated with T-cell infiltration and prolonged survival across several cancer types [6]. In patients demonstrating clinical benefit after adoptive transfer of tumor-infiltrating lymphocytes (TILs), T cells were shown to target mutated antigens [7–10]. Furthermore, the clinical success of checkpoint blockade was shown to positively correlate with the mutational load [11–13].

The direct exploitation of mutated antigens for immunotherapy so far was restricted to shared tumor mutations like mutant Ras or p53 [14] since every patient requires a personalized approach to target specific mutations present in the patient's tumor. With the advances in nucleic acid sequencing techniques, analysis, identification and targeting of individual mutation has now become feasible [15, 16].

In this process, somatic nonsynonymous mutations are identified by comparison of exome sequencing data of healthy tissue and tumor DNA. Transcriptome sequencing of tumor RNA then provides information on the expression levels of identified mutations. Those neo-antigen candidates which are likely to induce a T-cell response are selected using in silico tools such as MHC class I or class II binding prediction. Finally, a vaccine encoding the targets of interest is manufactured and delivered to professional antigen-presenting cells such as dendritic cells (DCs) in combination with an adequate adjuvant.

Effective individualized tumor vaccination requires an appropriate vaccine format that bundles features like safety, stability, cost-efficiency and a reliable induction of a proper T-cell response. In this regard, synthetic in vitro transcribed (IVT) mRNA is appealing. IVT mRNA not only encodes the antigen, but acts as a DC-maturating adjuvant as well. mRNA triggers inflammation by activation of several pattern recognition receptors such as toll-like receptors (TLR) 3, 7, 8, retinoic acid-inducible gene 1 (RIG-I), protein kinase R (PKR), and melanoma differentiation-associated protein 5 (MDA5) [17]. Various modifications of IVT mRNA

(e.g., 5′-cap modifications, stabilizing UTR sequences, and modified poly(A) tails) augmented the stability and translational efficacy by several logs leading to the expression of the respective antigen for longer periods of time [18, 19]. In addition, routing the RNA-encoded antigens into the MHC I as well as MHC II presentation pathway via modification of mRNA through signal sequences resulted in superior $CD8^+$ and $CD4^+$ T-cell responses [20]. Importantly for personalized tumor vaccination, mRNA has the ability to encode several neo-epitope candidates at once, thus addressing tumor heterogeneity and increasing the likelihood of inducing a antitumoral T-cell response. As only approx. 25% of point mutations are immunogenic [16], it is important to test for the induction neo-epitope specific T cells.

In this chapter we describe ELISpot- and flow cytometry-based methods to identify and subtype mutation directed T-cell responses in mice. In particular, splenocytes, peripheral blood leukocytes (PBLs), TILs, or isolated T cells as effectors from mice upon immunization with IVT mRNA encoding for mutated epitopes are tested for the recognition of mutated peptides, tumor cells, IVT mRNA electroporated bone marrow-derived dendritic cells (BMDCs), or tumor cells as targets. Moreover, we provide three different protocols for analysis of the mutation directed T-cell subtyping based on MHC blocking antibodies, flow cytometry and magnetic cell separation.

2 Materials

2.1 Identification and Subtyping of Neo-Antigen Specific T-Cell Responses via IFN-γ ELISpot

2.1.1 Culture Medium

1. RPMI 1640 medium with GlutaMAX (Gibco).
2. 100 U/ml penicillin, streptomycin (Gibco).
3. 1 mM sodium pyruvate (Gibco).
4. 1 mM nonessential amino acids (Gibco).
5. 10% heat-inactivated FCS (Gibco).

2.1.2 Preparation of Splenocytes

1. Red blood lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA).
2. Cell strainer (70 μm).
3. 50 ml centrifugation tubes.

2.1.3 Preparation of Peripheral Blood Leukocytes (PBLs)

1. Ficoll-Paque PREMIUM 1,084 (GE Healthcare).
2. Capillary blood collection tubes (1.3 ml, Lithium Heparin, Sarstedt) 15 ml centrifugation tubes.
3. 15 ml centrifugation tubes.
4. 12 × 75 mm polystyrene test tubes.

2.1.4 Preparation of Isolated CD8⁺ or CD4⁺ T Cells from Spleen

Magnetic cell separation system (e.g., MACS Myltenyi).

2.1.5 Preparation of Tumor Infiltrating Lymphocytes (TILs)

1. Collagenase type IV (Gibco).
2. DNAse I (Invitrogen).
3. Hyaluronidase (Sigma-Aldrcich).
4. RPMI 1640 medium with GlutaMAX (Gibco).
5. Ficoll-Paque PREMIUM 1,084 (GE Healthcare).
6. Cell strainer (70 μm).
7. 175 cm^2 cell culture flask (Greiner).
8. 50 ml centrifugation tubes.
9. 15 ml centrifugation tubes.

2.1.6 Preparation of Target Peptides

1. Lyophilized Peptides (*see* **Note 1**).
2. Dimethyl sulfoxide (DMSO, >99% purity, Sigma-Aldrich).
3. Demineralized water.
4. Reaction tubes (500–2000 μl).

2.1.7 Preparation of mRNA Electroporated Cells

1. IVT mRNA encoding for mutated sequence, an irrelevant sequence or enhanced green fluorescent protein.
2. ECM 830 square wave electroporation system (BTX).
3. Electroporation cuvettes (0.4 cm gap, Bio-Rad).
4. X-Vivo 15 serum-free medium (Bio Whittaker).
5. RNase-free pipette tips.
6. RNaseZap (Ambion).
7. Flow cytometry system.
8. 15 ml centrifugation tubes.

2.1.8 Preparation of Tumor Cells

1. Accutase solution (Sigma-Aldrich).
2. Optional: recombinant IFN-γ (Peptrotech).

2.1.9 IFN-γ ELISpot Assay

1. PVDF membrane-based ELISpot plates (Merck Millipore).
2. 35 vol.% aqueous ethanol.
3. Bovine serum albumin (Sigma-Aldrich).
4. Multichannel pipette (10–200 μl range).
5. 1. Antibody (anti-mouse IFN-γ mAb AN18, Mabtech).
6. 2. Antibody (biotinylated anti-mouse IFN-γ mAb R4-6A2, Mabtech).
7. ExtrAvidin-Alkaline Phosphatase (Sigma-Aldrich).

8. BCIP/NBT Liquid Substrate System (Sigma-Aldrich).
9. Optional: MHC class II blocking antibody specific for H-2 I-Ab, I-Ad, I-Aq, I-Ed, I-Ek (clone M5/114, BioXCell).
10. ELISpot reader.

2.2 Identification and Subtyping of Neo-Antigen Specific T-Cell Responses via Flow Cytometry

1. RPMI 1640 medium with GlutaMAX (Gibco).
2. 100 U/ml penicillin, streptomycin (Gibco).
3. 1 mM sodium pyruvate (Gibco).
4. 1 mM nonessential amino acids (Gibco).
5. 10% heat-inactivated FCS (Gibco).

2.2.1 Preparation of Culture Medium

2.2.2 Preparation of Positive Control Cocktail

1. 1 μg/ml PMA.
2. 2 μg/ml ionomycin.
3. 20 μg/ml brefeldin A in culture medium.

2.2.3 Preparation of Negative Control Cocktail

20 μg/ml brefeldin A in culture medium with or without 4 μg/ml irrelevant peptide.

2.2.4 Preparation of Stimulator Cocktail

1. 4 μg/ml peptide.
2. 20 μg/ml brefeldin A in culture medium.

2.2.5 Multicolor Flow Cytometer (6+ Colors)

1. 96-well polystyrene round bottom plate.
2. Brefeldin A (BFA) powder (Sigma-Aldrich).
3. Ionomycin calcium salt (Sigma-Aldrich).
4. Phorbol 12-myristate 13-acetate PMA (Sigma-Aldrich).
5. Fixation/Permeabilization Solution Kit (BD Bioscience).
6. Fixable viability dye, e.g., Fixable Viability Dye eFluor® 506 (eBioscience).
7. Anti-mouse CD4 (clone: GK1.5) for extracellular staining.
8. Anti-mouse CD8 (clone: 53-6.7) for extracellular staining.
9. Anti-mouse IFN-γ (clone: XMG1.2) for intracellular staining.
10. Anti-mouse IL-2 (clone: JES6-5H4) for intracellular staining.
11. Anti-mouse TNF-a (clone: MP6-XT22) for intracellular staining.
12. 12×75 mm polystyrene test tubes.
13. Centrifuge with plate adapter.

3 Methods

3.1 Identification and Subtyping of Neo-Antigen Specific T-Cell Responses via IFN-γ ELISpot

3.1.1 Preparation of Splenocytes

1. Excise the spleen.
2. Grind the spleen through a 70 μm cell strainer into a 50 ml tube with a plunger while rinsing with 20 ml PBS.
3. Centrifuge at 300 × *g* for 6 min at room temperature (RT).
4. Lyse red blood cells by resuspending the cell pellet in 5 ml lysis buffer for 3–5 min.
5. Stop reaction by addition of 20 ml PBS.
6. Centrifuge at 300 × *g* for 6 min at RT.
7. Discard supernatant, resuspend splenocytes in culture medium (*see* **Note 2**).

3.1.2 Preparation of Peripheral Blood Leukocytes (PBLs)

1. Add 2 ml Ficoll-Paque into a 15 ml centrifugation tube.
2. Carefully overlay a 1:1 dilution of blood and PBS (*see* **Note 3**).
3. Centrifuge at 400 × *g* for 30 min at RT without brake.
4. Carefully transfer the interlayer (PBLs) into a new 15 ml tube (*see* **Note 4**).
5. Centrifuge at 300 × *g* for 6 min at RT.
6. Discard supernatant, resuspend cells in culture medium (*see* **Note 5**).

3.1.3 Preparation of Isolated $CD8^+$ or $CD4^+$ T Cells

1. Isolate splenocytes according to Subheading 3.1.1.
2. Separate $CD8^+$ T cells or $CD4^+$ T cells via magnetic cell separation (for example MACS, Miltenyi Biotec) according to the manufacturer's protocol.
3. Discard supernatant, resuspend the cells in culture medium.

3.1.4 Preparation of TILs

1. Mince the isolated tumor into small pieces with a surgical scissor.
2. Incubate for 20 min at 37 °C in 3–4 ml RPMI 1640 medium containing 2 mg/ml Collagenase type IV, 40 μg/ml DNAse I, 500 U/ml Hyaluronidase.
3. Mesh gently through a cell strainer while rinsing with 20 ml PBS.
4. Centrifuge at 300 × *g* for 8 min, at RT.
5. Discard supernatant, resuspend the cells in 30 ml of culture medium.
6. Culture overnight at 37 °C in a 175 cm^2 cell culture flask (*see* **Note 6**).
7. Transfer culture medium including non-adherent cells into a 50 ml tube.
8. Gently rinse culture flask with 10 ml PBS and add to the 50 ml tube.

9. Centrifuge at 300 × *g* for 8 min at RT.
10. Discard supernatant, resuspend cells in 15 ml PBS.
11. Add 10 ml Ficoll-Paque into a 50 ml tube.
12. Gently overlay the Ficoll-Paque with the cell solution.
13. Centrifuge at 400 × *g* for 30 min at RT without brake.
14. Carefully transfer the interlayer (PBLs) into a new 15 ml tube.
15. Centrifuge at 300 × *g* for 8 min at RT.
16. Discard supernatant, resuspend cells in culture medium (*see* **Notes 7** and **8**).

3.1.5 Preparation of Target Peptides

1. Dissolve lyophilized peptide in 10 µl DMSO per 1 mg peptide.
2. Add 490 µl demineralized water per mg peptide to get a 2 mg/ml concentrated solution.
3. Aliquot and store at −80 °C, avoid repetitive freeze and thaw cycles (*see* **Note 9**).

3.1.6 Preparation of mRNA Electroporated Target Cells (BMDC or Tumor Cells)

1. Clean all pipettes as well as working space with RNaseZAP to eliminate RNases.
2. Harvest cells and resuspend cell pellet in 10 ml X-Vivo 15 medium.
3. Centrifuge at 300 × *g* for 6 min at RT.
4. Prepare for each electroporation a 15 ml tube with 750 µl culture medium.
5. Discard supernatant, dilute to a concentration of approximately $1 \times 10^7/240$ µl in X-Vivo 15 medium.
6. Add 10 µg of mRNA (10 µl of a 1 mg/ml solution) to a electroporation cuvette.
7. Add 240 µl of the cell suspension and mix.
8. Electroporate the cells (*see* **Notes 10** and **11**).
9. Immediately transfer cells into the prepared 15 ml tubes.
10. Count cells and dilute to 5×10^5 BMDCs/ml or $1–5 \times 10^5$ tumor cells/ml for ELISpot.
11. Determine the electroporation efficiency and mortality by flow cytometry of eGFP electroporated cells cultured 4–24 h at 37 °C.

3.1.7 Preparation of Tumor Cells

1. Take tumor cells in culture at least 2 days before the read out (*see* **Note 12**).
2. Harvest cells and wash once with PBS (*see* **Note 13**).
3. Resuspend cells in culture medium and dilute to $1–5 \times 10^5$ cells/ml.

3.1.8 IFN-γ ELISpot Assay

1. Add 15 µl of 35 vol.% aqueous ethanol into each well of the PVDF ELISpot plate with a multichannel pipette and discard ethanol immediately.
2. Wash twice with 200 µl PBS.
3. Add 50 µl of 10 µg/ml 1. antibody (anti-mouse IFN-γ mAb AN18) in PBS.
4. Incubate for 4 h at 37 °C or 1–7 days at 2–8 °C.
5. Empty the plate and wash twice with 200 µl PBS.
6. Add effector cells in 100 µl culture medium (5×10^5 splenocytes, $1–5 \times 10^5$ PBLs, $0.5–1 \times 10^5$ isolated $CD8^+$ T cells, $1–2 \times 10^5$ isolated $CD4^+$ T cells, $4–5 \times 10^5$ CD8 or CD4 depleted splenocytes/ml, $0.5–5 \times 10^4$ TILs) (*see* **Notes 14–16**).
7. Add targets in 100 µl culture medium (4–12 µg/ml peptide, 5×10^4 electroporated BMDC, $1–5 \times 10^4$ electroporated tumor cells, $1–5 \times 10^4$ tumor cells) (*see* **Note 17**).
8. Incubate overnight (at least 16 h) at 37 °C.
9. Empty the plate and wash twice with 200 µl PBS.
10. Add 60 µl of 1 µg/ml concentrated 2. antibody (biotinylated anti-mouse IFN-γ mAb R4-6A2) in PBS + 5 g/l BSA (*see* **Note 18**).
11. Incubate for 2 h at 37 °C (*see* **Note 19**).
12. Empty the plate and wash twice with 200 µl PBS.
13. Add 100 µl of ExtrAvidin alkaline phosphatase solution (diluted 1:1000 in PBS + 5 g/l BSA).
14. Incubate for 45 min at RT (*see* **Note 20**).
15. Empty the plate and wash twice with 200 µl PBS.
16. Add 100 µl BCIP/NBT solution.
17. Incubate for 5–10 min in the dark.
18. Empty the plate and rinse wells thoroughly with tap water.
19. Dry the plate for 1 day.
20. Measure spot count with an ELISpot reader (*see* Fig. 1).

3.2 Identification and Subtyping of Neo-Antigen Specific T-Cell Responses via Flow Cytometry

1. Add 100 µl of splenocytes in culture medium ($2–3 \times 10^7$/ml) to 96-well round bottom plate. Include wells for positive, negative and unstained controls (*see* **Note 21**).
2. Add 100 µl of respective cocktails to indicated wells (*see* **Note 22**).
3. Centrifuge at $300 \times g$ for 30 s at RT.
4. Incubate for 5 h at 37 °C.
5. Centrifuge at $300 \times g$ for 5 min at RT.
6. Wash twice with 200 µl PBS.
7. Add 100 µl Fixable Viability Dye solution (e.g., eFluor® 506 1:200 in PBS).
8. Resuspend cells by using a multichannel pipette.

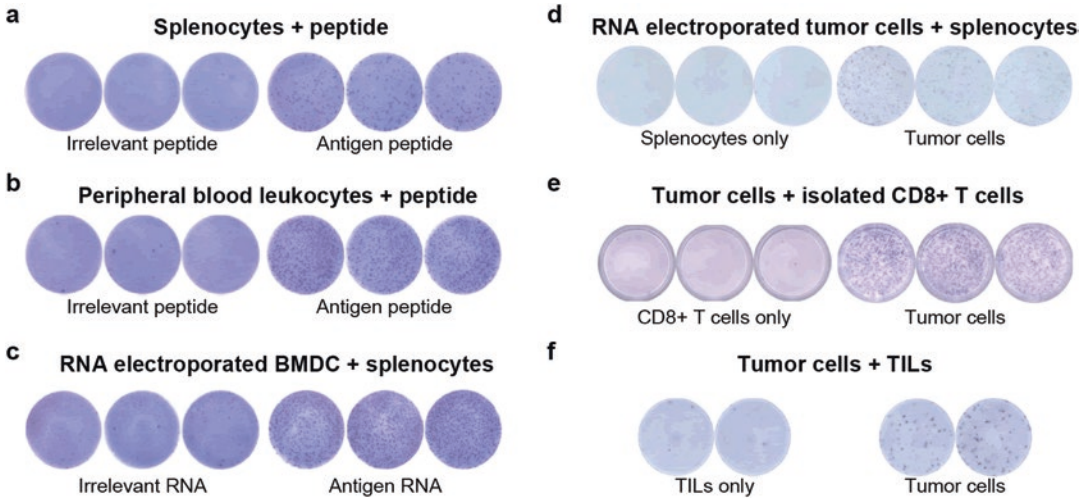

Fig. 1 Identification of mutation specific T cells via IFN-γ ELISpot. In vitro transcribed, mutation encoding mRNA formulated as lipoplex [16] was used to vaccinate C57BL/6 or BALB/c mice. IFN-γ ELISpot was performed according to the protocol described in this chapter. Various effector and target combinations are shown. Peptide antigen can be used to stimulate T cells within splenocytes (**a**) or blood (**b**). In addition, BMDCs (**c**) or antigen negative tumor cells (**d**) electroporated with antigen RNA can serve as T cell targets. Antigen positive tumor cells can be directly recognized by isolated, tumor-specific CD8+ T cells (**e**) or tumor infiltrating T cells (TILs, **f**)

9. Incubate for 25 min at 4 °C in the dark.
10. Wash once with 200 µl FACS buffer (PBS +5 % FCS and 5 mM EDTA).
11. Perform extracellular staining by adding 100 µl antibody mastermix in FACS buffer, resuspend cell by using a multichannel pipette (*see* **Note 23**).
12. Incubate for 30 min at 4 °C in the dark.
13. Centrifuge at 300 × *g* for 5 min at RT.
14. Wash twice with 200 µl FACS buffer.
15. Resuspend cells thoroughly in 100 µl Cytofix/Cytoperm™ (*see* **Note 24**).
16. Incubate for 30 min at 4 °C in the dark.
17. Centrifuge at 300 × *g* for 5 min at RT.
18. Wash once with 200 µl Perm/Wash™.
19. Perform intracellular staining by adding 100 µl antibody mastermix in Perm/Wash™, resuspend cell suspension by using a multichannel pipette (*see* **Note 23**).
20. Incubate for 30 min at 4 °C in the dark.
21. Centrifuge at 450 × *g* for 5 min at RT.
22. Wash twice with 200 µl Perm/Wash™.
23. Wash once with 200 µl FACS buffer.

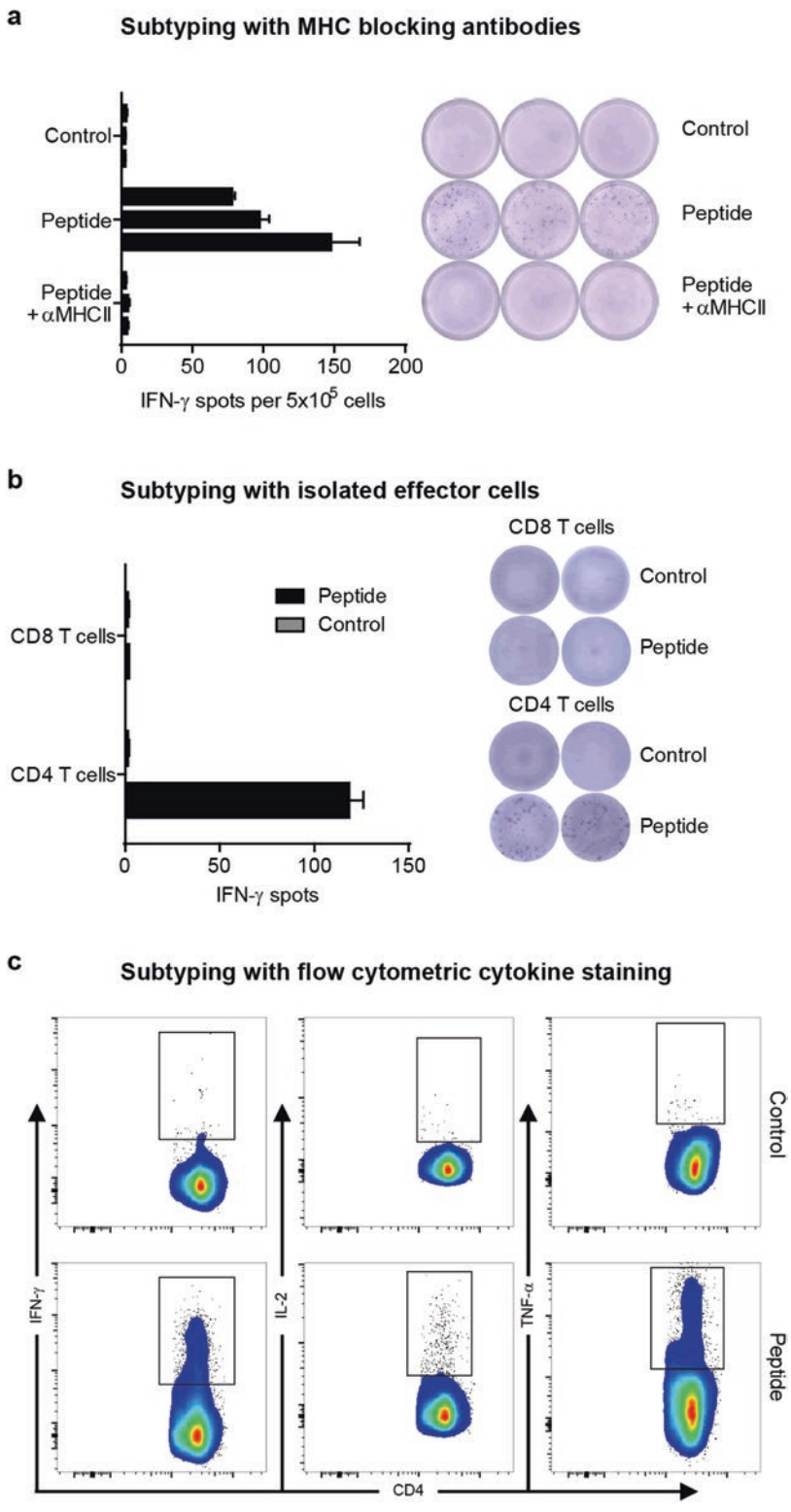

Fig. 2 Subtyping of mutation specific T cells. Exemplary determination of the mutation specific T-cell subtype via an MHC class II blocking antibody (**a**), isolated T-cell populations (**b**) or flow cytometry based cytokine staining (**c**). (**a**) BALB/c mice ($n=3$) were vaccinated with 40 μg IVT mRNA encoding 27 amino acids of a CT26 tumor-derived mutated protein [16]. Spot counts of splenocytes alone (control) or mutated 27-mer peptide

24. Resuspend cells in appropriate volume of FACS buffer and transfer to 12 × 75 mm Polystyrene test tubes.
25. Acquire samples on a flow cytometer (Fig. 2).

4 Notes

1. Peptides should optimally have a purity of higher than 90 %.
2. Alternatively, leukocytes can be isolated from lymph nodes without the need for lysis of erythrocytes.
3. At least 200–300 μl blood for six wells is needed (approximately 1×10^6 cells). A maximum of 6 ml of diluted blood should be added per 15 ml tube.
4. Avoid transferring cells from the upper layer or erythrocytes as this can result in increased background.
5. If 300 μl blood was used, cells should be diluted in approximately 600 μl medium. 10–20 μl of cells is diluted 1:1 with a vital stain and counted on a hemocytometer.
6. Tumor cells will adhere overnight. Alternatively, the cultivation time can be reduced to 4–6 h, depending on the tumor cell line. Large tumors might need to be separated to multiple cell culture flasks. This protocol is not applicable for non-adherent tumor cell lines.
7. The isolated cells should be resuspended in approximately 400 μL of culture medium. 10–20 μl of cells is diluted 1:1 with a vital stain and counted on a hemocytometer.
8. Alternatively, the density gradient centrifugation can be performed prior to the plastic adherence step.
9. Alternatively, peptides can be stored at −20 °C for shorter periods.
10. Murine BMDCs are generated according to published protocols [21]. Bone marrow cells were obtained from femurs and tibias of mice and cultured using 20 ml culture medium supplemented with 1000 U/ml GM-CSF for 7 days with a cell density of 2×10^7 cells per 75 cm^2 cell culture flask. Cells were fed on day 3 with 5 ml culture medium + 1000 U/ml GM-CSF. On day 5, suspension cells were transferred to a new

Fig. 2 (continued) stimulated splenocytes with or without MHC class II blocking antibody are shown (*left*). *Right*, exemplary ELISpot pictures for one mouse. (**b**) BALB/c mice ($n = 5$) were vaccinated with 40 μg IVT mRNA encoding five different 4T1 tumor-derived mutated protein stretches. $CD8^+$ T clls were isolated from pooled splenocytes and along with the CD8 depleted splenocytes ("CD4 T cells") used as effectors cells in an IFN-γ ELISpot together with (Peptide) or without (Control) mutated peptide. Spot counts (*left*) and exemplary ELISpot pictures (*right*) are shown. (**c**) Flow-cytometric analysis of cytokine secretion of splenocytes from a neo-epitope vaccinated C57BL/6 mouse. Signals for IFN-γ, IL-2 and TNF-α among $CD4^+$, living, singlet lymphocytes are shown

75 cm^2 cell culture flask in 20 ml culture medium + 1000 U/ml GM-CSF. BMDCs were harvested on day 7.

11. Optimal electroporation settings should be determined for each cell type. BMDCs are efficiently electroporated at 400 V and one pulse of 5 ms length. 4T1 tumor cells are best treated with 250 V and one pulse of 5 ms length. The A20 B-cell lymphoma can be used as antigen presenting cells after electroporation with 300 V and one pulse of 6 ms length. Note that mRNA electroporation results in upregulation of MHC molecules.
12. To increase the efficacy of tumor antigen presentation, cells might be stimulated with recombinant IFN-γ. Exemplarily, 4T1 tumor cells upregulate MHC class I and MHC class II molecules after coincubation with 1 ng/ml (~1×10^4 U/ml) recombinant IFN-γ for 24 h.
13. We recommend using Accutase solution (Sigma-Aldrich) for detachment of cells. Treatment with Trypsin solutions might negatively affect the cell surface proteins including MHC molecules reducing the efficiency of antigen presentation.
14. The total cell number per well should not exceed 5×10^5 cells. The optimal number of effector cells depends on the frequency of responder T cells.
15. The effector cell number should be increased if frozen cells are used.
16. For subtyping with the MHC class II blocking antibody, effector cells should be added in 50 μl culture medium. The blocking antibody (M5/114, BioXCell) is subsequently added in 50 μl at a concentration of 80 μg/ml (final concentration 20 μg/ml).
17. If isolated T cells are used antigen presenting cells, BMDCs (e.g., 5×10^4) in 50 μl culture medium should be added. This is especially crucial for $CD4^+$ T cells (but not necessarily for CD8 depleted splenocytes). If antigen presenting cells are added, targets should be added in 50 μl (the concentration of the cell and peptide solutions has to be adjusted accordingly).
18. Addition of BSA reduces unspecific binding of the secondary antibody.
19. Longer incubation time for up to 5 h does not affect the assay performance.
20. Incubation time should not exceed 1 h.
21. Number of stimulated and stained splenocytes depends on expected frequencies. For rare event analysis a higher cell number might be applicable.

22. Alternatively to peptide, 2×10^5 electroporated cells or tumor cells can be applied as targets (*see* Subheading 3.1.6 and 3.1.7) in 100 µl culture medium with 20 µg/ml brefeldin A.
23. Reagents should be titrated for optimal performance.
24. Incubation of cells in Cytofix/Cytoperm™ overnight at 4 °C has no negative impact on the subsequent staining.

Acknowledgements

The study was supported by the CI3 excellence cluster program of the Federal Ministry of Education and Research (BMBF).

References

1. Gross L (1943) Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. Cancer Res 3:326–333
2. Coulie PG, Van den Eynde BJ, van der Bruggen P et al (2014) Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. Nat Rev Cancer 14:135–146
3. Melero I, Gaudernack G, Gerritsen W et al (2014) Therapeutic vaccines for cancer: an overview of clinical trials. Nat Rev Clin Oncol 11:509–524
4. Boon T, Kellermann O (1977) Rejection by syngeneic mice of cell variants obtained by mutagenesis of a malignant teratocarcinoma cell line. Proc Natl Acad Sci U S A 74:272–275
5. Wölfel T, Hauer M, Schneider J et al (1995) A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science 269:1281–1284
6. Brown SD, Warren RL, Gibb EA et al (2014) Neo-antigens predicted by tumor genome meta-analysis correlate with increased patient survival. Genome Res 24:743–750
7. Tran E, Turcotte S, Gros A et al (2014) Cancer immunotherapy based on mutation-specific $CD4^+$ T cells in a patient with epithelial cancer. Science 344:641–645
8. Van Rooij N, Van Buuren MM, Philips D et al (2013) Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. J Clin Oncol 31:e439–e442
9. Robbins PF, Lu Y-C, El-Gamil M et al (2013) Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. Nat Med 19:747–752
10. Lu Y-C, Yao X, Li YF et al (2013) Mutated PPP1R3B is recognized by T cells used to treat a melanoma patient who experienced a durable complete tumor regression. J Immunol 190:6034–6042
11. Van Allen EM, Miao D, Schilling B et al (2015) Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. Science 350:207–211
12. Rizvi NA, Hellmann MD, Snyder A et al (2015) Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science 348:124–128
13. Snyder A, Makarov V, Merghoub T et al (2014) Genetic basis for clinical response to CTLA-4 blockade in melanoma. N Engl J Med 371:2189–2199
14. Carbone DP, Ciernik IF, Kelley MJ et al (2005) Immunization with mutant p53- and K-ras-derived peptides in cancer patients: immune response and clinical outcome. J Clin Oncol 23:5099–5107
15. Castle JC, Kreiter S, Diekmann J et al (2012) Exploiting the mutanome for tumor vaccination. Cancer Res 72:1081–1091
16. Kreiter S, Vormehr M, van de Roemer N et al (2015) Mutant MHC class II epitopes drive therapeutic immune responses to cancer. Nature 520:692–696
17. Sahin U, Kariko K, Tureci O (2014) mRNA-based therapeutics - developing a new class of drugs. Nat Rev Drug Discov 13:759–780
18. Holtkamp S, Kreiter S, Selmi A et al (2006) Modification of antigen-encoding RNA increases stability, translational efficacy, and T-cell stimulatory capacity of dendritic cells. Blood 108:4009–4017

19. Kuhn AN, Diken M, Kreiter S et al (2010) Phosphorothioate cap analogs increase stability and translational efficiency of RNA vaccines in immature dendritic cells and induce superior immune responses in vivo. Gene Ther 17:961–971
20. Kreiter S, Selmi A, Diken M et al (2008) Increased antigen presentation efficiency by coupling antigens to MHC class I trafficking signals. J Immunol 180:309–318
21. Lutz MB, Kukutsch N, Ogilvie AL et al (1999) An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods 223:77–92

Chapter 15

Considerations for Producing mRNA Vaccines for Clinical Trials

Andreas Schmid

Abstract

The approval of clinical trials by the competent authorities requires comprehensive quality documentation on the new drug to be used on the clinical trial participant. In the EU quality data is summarized as Investigational Medicinal Product Dossier (IMPD), in the USA as Investigational New Drug (IND) Application. For that, several preconditions concerning production, quality control, and assurance have to be fulfilled. Here, specific requirements related to mRNA vaccines are addressed on the basis of European standards.

Key words Investigational medicinal product, Drug substance, mRNA, IMPD, Specification, Clinical trial

1 Introduction

Holding a manufacturing authorization for investigational medicinal products (IMPs) by the local competent authorities is a prerequisite for a clinical trial application in the EU. The granting of the manufacturing authorization in turn requires that all steps of manufacturing and testing follow the principles of good manufacturing practice (GMP), laid down in Directive 2003/94/EC. This includes inter alia: GMP-compliant premises and equipment, quality assurance, documentation, and appropriate personnel and processes according to the current state of science and technology, specified in Eudralex Volume 4—"Guidelines for good manufacturing practices for medicinal products for human and veterinary use." Annex 13 addresses specific issues and requirements on "Investigational Medicinal Products" and highlights the demand for GMP-conform manufacture of IMPs with high batch-to-batch consistency to ensure the safety of the trial subject and reliable results of the clinical trials unaffected by IMP efficacy or quality variability [1].

Provisions regarding the conduct of clinical trials are defined in Clinical Trials Directive 2001/20/EC (Good Clinical Practice—GCP), translated into concrete guidance in Eudralex Volume

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_15,

10—"Guidelines for clinical trial." Chapter I "Application and Application Form" demands, amongst other documentation, an IMP dossier. Details on the IMPD are given in Chapter III "Quality of the Investigational Medicinal Product" with the two guidelines "Guideline on the requirements to the chemical and pharmaceutical quality documentation concerning investigational medicinal products in clinical trials" [2] and "Guideline on the requirements for quality documentation concerning biological investigational medicinal products in clinical trials". However, the guideline on biological IMPs is limited to proteins and polypeptides and not suited for mRNA-based products [3], means the first-mentioned guideline is relevant for mRNA vaccines.

mRNA vaccines are typically manufactured applying recombinant DNA technology, therefore assigned to the group of biotechnological/biological medicinal products, whereas RNAs synthesized in a purely chemical process would be classified as chemical substances.

Here, the focus is on mRNAs produced by in vitro transcription using recombinant polymerase [4, 5], representing the current large-scale manufacturing technology. Recent in vivo approaches seem to offer new possibilities for the future [6, 7].

2 Investigational Medicinal Product Dossier

As mentioned above, the structure and content of the IMPD (*see* **Note 1**) is outlined in the European Medicines Agency "Guideline on the requirements to the chemical and pharmaceutical quality documentation concerning investigational medicinal products in clinical trials" [2].

Requirements on the quality of investigational medicinal products based on novel active substances like mRNA are given in Chapter 2 of the guideline, discriminating between the drug substance (the active substance) and the drug product (the IMP).

2.1 Drug Substance (2.2.1.S)

The section "General information" (2.1.S.1.1) of the IMPD contains information about the nomenclature of the drug substance (messenger ribonucleic acid/mRNA), means names or laboratory codes or a proposed INN-name (International Nonproprietary Name), if available. Additionally, the structure (Cap, open reading frame, UTRs, Poly A, etc.), sequence, and molecular weight of the mRNA are outlined. Physicochemical properties (potentially affecting pharmacological or toxicological safety) like pKa, osmolality, and solubility complete this section.

In the following detailed information about the "Manufacture" (2.2.1.S.2) of the drug substance is given. Firstly, all manufacturers, contractors and production sites involved in manufacture and testing are listed. Secondly, the manufacturing process and relevant process controls are described, including a flow chart comprising

critical starting materials and intermediates. Here, it is crucial that all relevant steps are laid down in approved standard operating procedures to guarantee reproducible processes. A typical mRNA production process includes generation of the template, i.e., amplification and linearization of plasmid-DNA followed by in vitro transcription, DNase treatment and mRNA purification [5]. A relevant process control would be the verification of plasmid linearity. On the one hand, this could be examined by DNA electrophoresis; on the other hand a small-scale in vitro transcription reaction with subsequent RNA quantification and analysis could be carried out, forecasting that the desired (amounts of) drug substance will be generated on a large scale. Further process controls would focus on mRNA identity and integrity following large-scale in vitro transcription und mRNA purification. Thirdly, information about the quality and control of all raw materials (e.g., RNA polymerase), reagents (e.g., transcription buffer), and solvents used for drug substance manufacture are summarized. If available, certificates of analysis and origin are added. If materials of animal or human origin are used, an adventitious agents safety evaluation has to be performed (7.2.1.A.2), assessing TSE, viral, and microbial safety. Fourthly, in case critical steps in manufacture of the drug substance were identified (by a risk analysis), measures for their control have to be described. If the manufacturing process would have significantly changed from non-clinical to clinical batches, differences should be demonstrated, too.

The section "Characterization" (2.1.2.S.3) focuses on the elucidation of the mRNA structure and possible impurities. The basis for the correctness of the mRNA structure is the identity of the plasmid DNA template, which is verified by fully automated GMP-certified DNA sequencing. As conversion of mRNA to complementary DNA (cDNA) exhibits several shortcomings (e.g., failure rate) and direct RNA sequencing is limited to its read length [8], sequencing of the starting material plasmid DNA is still the best choice to guarantee mRNA sequence accuracy.

Potential impurities of the drug substance include residual DNA, enzymes/proteins and solvents from the manufacturing process as well as degradation products. Plasmid DNA can be degraded and removed by DNase treatment combined with RNA-specific purification (precipitation and chromatography), which also eliminates enzymes and proteins (e.g., RNA polymerase) [5]. Solvents can be removed by freeze-drying of the drug substance, enabling its dissolving in the desired buffer. Additional remarks on the effectiveness of downstream processing steps result in an added value. For that, removal factors of defined impurities, for instance reduction of proteins by RNA-specific precipitation, are stated. Furthermore, potential degradation products should be commented on. These can be traced by RNA integrity analysis (*see* Tables 1 and 2), e.g., RNA electrophoresis.

Subsequently, the following Chapter "Control of the Drug Substance" (2.2.1.S.4) demands a specification of the drug substance, giving information on analytical procedures and their acceptance criteria, addressing mRNA identity, assay and impurities. To this, more details are given in Subheading 3 ("Control of the Drug Substance/IMP") below. All analytical procedures have to be validated. However, for phase I clinical trials it is sufficient to provide the parameters along with acceptance limits for performing validation. Still, this demands extensive experience with the analytical methods. For phase II and III clinical trials a tabulated summary of the validation results is required.

As a key element, data on batch analyses for all batches used in the clinical trial und nonclinical studies have to be listed including the "batch number, batch size, manufacturing site, manufacturing date, control methods, acceptance criteria and the test results" [2]. Complementary to that, justifications of specification of all parameters which may be critical for later drug product performance have to be added. Where available, Ph. Eur. limits [9] should be headed for, e.g., residual solvents (*see* Ph. Eur. 5.4). For analytical procedures not based on Ph. Eur. justifications of specification should be based on thorough characterization of the respective method.

In case reference standards or materials (2.2.1.S.5) are used, parameters of characterization are presented. Reference standards could be used for electrophoresis (RNA ladder), qRT-PCR, or photometry.

In addition, the primary packaging material used for storage of the drug substance (2.2.1.S.6) has to be stated.

Finally, data on parameters which are critical for mRNA stability (2.2.1.S.7) are shown in tabular form. Most important stability indicating parameters for mRNA are RNA integrity, content and potency, supplemented by pH, appearance, and the microbiological status. Stability testing should be performed on representative batches, stored in container closure systems equal to those of clinical batches, in accordance with conditions defined in ICH guideline Q1A(R2)—"Stability testing of new drug substances and products" [10]. Besides studies representing the desired long-term storage temperature, storage under accelerated conditions and stress testing are performed. Both address degradation kinetics and patterns and can give an estimate of worst-case conditions still guaranteeing RNA integrity as well as useful hints for the development of analytical methods. Stress testing could include temperature shifts, pH shifts, photostability, humidity (for freeze-dried RNA), or numerous freeze-thaw cycles (when stored frozen).

2.2 Investigational Medicinal Product Under Test (2.2.1.P)

This part starts with the description and composition of the Investigational Medicinal Product/drug product (2.2.1.P.1), means the sterile mRNA vaccine to be applied (e.g., intradermally) on the clinical trial participant. This should include a designation

of the dosage form, the function of excipients and the quantities/doses per container.

Next, the pharmaceutical development (2.2.1.P.2) of the drug product is pointed out. This is of special interest for clinical phases II and III, whenever changes on the manufacture, composition or dosage form of the IMP with potential clinical relevance have been undertaken. Here, information on the compatibility with solvents (for freeze-dried IMPs), diluents, and admixtures (if applicable) should be presented, including a description of the method of preparation when prepared extemporaneously. In this context, it is also important to examine in-use stability after reconstitution, dilution or addition of admixtures to simulate worst-case conditions occurring during clinical application (e.g., a delay of several hours at room temperature between reconstitution and injection by the physician). The container closure system has to be outlined as well, including a justification for the chosen primary packaging material. It is crucial that the container closure system shows full integrity to protect the sterile mRNA vaccine from microbial contamination throughout the shelf life (for details *see* FDA Guidance for Industry "Container and closure system integrity testing *in lieu* of sterility testing as a component of the stability protocol for sterile products"). For further information on pharmaceutical development see also ICH guideline Q8(R2) [11].

The following section gives details on the manufacture (2.2.1.P.3) of the IMP. As in case of the drug substance, here, all manufacturers, contractors, and sites involved in production and testing have to be listed along with their respective responsibilities, too. The manufacturing process and any relevant process controls should be briefly described, including the disclosure of the batch formula for the clinical batches. As mRNA vaccines cannot be terminally sterilized by moist heat, IMP formulation, fill, and finish (including freeze-drying [5], where applicable) are performed under aseptic conditions (EU GMP clean room class A) [12]. Controls of critical steps do not have to be addressed before phase III, except for measures to ensure sterility of the vaccine. Here, results of media fill, means validation of aseptic processing using a microbiological growth medium in place of the IMP [12], should be presented.

Considering excipients (2.2.1.P.4), it depends whether those reference to pharmacopoeia [9] or not. If not, analytical procedures have to be outlined and an adventitious agents safety evaluation has to be performed. In case of novel excipients more detailed information on their manufacturing process, controls and characteristics has to be supplied.

The "Control of the Investigational Medicinal Product" (2.2.1.P.5) requires IMP specifications adjusted to the current developmental stage. Specifications should be justified and include parameters, test methods, and acceptance criteria. Further particulars are described in Subheading 3 ("Control of the Drug

Substance/IMP") below. Concerning analytical method validation the standards are the same as above for the drug substance. The specifications are accompanied by quality control results of representative IMP batches. Mandatory data include: "batch number, batch size, manufacturing site, manufacturing date, control methods, acceptance criteria and the test results" [2]. In the event of impurities observed in the IMP (but not in the drug substance), details should be given.

Likely, no IMP-specific reference standards or materials (2.2.1.P.6) are needed.

The container closure system (2.2.1.P.7) to be used for the IMP in the clinical trial should be defined. The relevant pharmacopoeia reference for the primary packaging material is stated where applicable. In all other cases specifications and/or material certificates should be provided along with a detailed description.

Finally, information on the stability (2.2.1.P.8) of the IMP and its shelf life is given. For phase I trials, data from development studies can justify a preliminary shelf-life. Stability studies on representative batches are initiated prior to the start of and continued in parallel to the clinical studies, enabling extrapolation and shelf life extension. Data from long-term and accelerated studies are required. Where applicable, it is important not to forget upside down storage. For liquid IMPs, filled in, e.g., vials with rubber stoppers, this is essential to evaluate the influence of the stopper on the RNA vaccine stability. Last but not least, as mentioned before in-use stability simulating the clinical situation after IMP preparation (reconstitution, etc.) should be addressed. For more details on stability studies see ICH guidelines Q1A-1F.

Besides the information related to the quality of the drug substance and the IMP given above, the IMPD contains an overall risk and benefit assessment as well as additional information on nonclinical pharmacology, toxicology and relevant clinical data, which is usually summarized in the Investigator's Brochure (IB)—a guidance for the investigator.

Further aspects to be considered besides the requirements of the IMPD guideline [2] when producing mRNA vaccines for clinical trials are outlined in Subheading 4 (*see* **Notes 2–11**).

3 Control of the Drug Substance/IMP

One of the key elements guaranteeing the quality of the drug substance and IMP is a product specification based on the current state of product development as well as science and technology.

For this, the framework is set by ICH guideline Q6A "Test procedures and acceptance criteria for new drug substances and new drug products: chemical substances" [13]. Additionally, some aspects, above all biological activity, outlined in the ICH guideline

Q6B "Test procedures and acceptance criteria for biotechnological/biological products" [14], which applies to proteins and polypeptides, should be considered, too.

The specification defines a list of tests (test parameters) along with references to analytical procedures and appropriate acceptance criteria for the quality assessment of the respective product regarding identity, assay/quantity, purity/impurities, and potency/biological activity. Additionally, specifications should also contain specific product information, including product name, composition, dosage form, primary packaging, storage conditions, and shelf life (*see* German AMWHV).

An exemplary specification for an mRNA-based drug substance is shown in Table 1 (*see* **Notes 12** and **13**). Please note that no responsibility is accepted for the accuracy of this information. Where available, Ph. Eur. tests have been selected. Two independent identity tests addressing RNA length and accuracy of sequence segments have been chosen (*see* **Notes 16–18**). However, to ensure sequence accuracy of the mRNA transcript sequencing of the plasmid DNA template has to be considered in addition (*see* **Notes 14** and **15**). The section "assay" includes analysis of RNA content and the physicochemical parameters pH and osmolality (*see* **Notes 19** and **20**). Impurity testing comprises analytical procedures for (critical) residual raw material used for up- and downstream processing of the drug substance, RNA integrity and microbiological status (*see* **Notes 21–27**). The potency test addressing translatability of the mRNA may be omitted as biological activity will be analyzed on the IMP anyway.

The specification of the IMP can be derived from the drug substance specification. Here, it is assumed that aseptic formulation of the IMP comprises addition of an injection buffer and an excipient (without any further preservatives), followed by aseptic filling and freeze-drying (all steps performed under EU GMP clean room class A) [12]. Recent developments showed that mRNA-based vaccines can be lyophilized and stored at ambient temperature [5].

Some parameters addressing purity as proteins, plasmid DNA, host DNA, and residual solvents can be omitted as these were (potential) contaminants specific for the drug substance production process. However, according to guideline ICH Q6A, Chapter 3.3.2.3, additional tests have to be performed for parenteral drug product as mRNA-based IMPs. These include sterility and endotoxin testing, osmolality, tests for particulate matter (*see* **Note 28**), water content, reconstitution time, and uniformity of dosage units. Additionally, potency/biological activity should be addressed (*see* **Notes 29** and **30**). An exemplary specification for a lyophilized mRNA-based IMP is shown in Table 2. Please note that no responsibility is accepted for the accuracy of this information.

Table 1
Exemplary specification of a liquid mRNA-based drug substance

Product information		
Product name:	RNA123	
Reference code:	R01-00123	
Application:	Active substance, drug substance	
Condition:	Liquid	
Manufacture reference:	SOP XY	
Concentration:	XX g/l	
Other ingredients:	e.g., water for injection (WFI)	
Primary packaging material:	e.g., polypropylene containers e.g., polypropylene screw cap	
Storage temperature:	–80 °C	
Stability/retest period:	See ongoing stability testing	
RNA sequence:	GG…	
Length in bases:	XX b	
Specification of test parameters		
Description		
Parameter	Analytical procedure	Acceptance criterion
Appearance: clarity and opalescence	Clarity and opalescence of solutions (Ph. Eur. 2.2.1)	e.g., clear liquid
Appearance: coloration	Coloration of solutions (Ph. Eur. 2.2.2)	e.g., colorless to yellowish liquid
Identity		
Parameter	Analytical procedure	Acceptance criterion
DNA sequence plasmid DNA template	Automated DNA sequencing according to SOP XY	Identical to theoretical sequence
RNA length	RNA electrophoresis and determination of run length according to SOP XY	XX ± XX
RNA sequence segment	Reverse transcription, PCR and DNA electrophoresis according to SOP XY	Theoretical band size ± XX
Assay/quantity		
Parameter	Analytical procedure	Acceptance criterion
RNA content	UV absorption, OD_{260} according to SOP XY	XX g/l ± XX %
pH value	pH value (Ph. Eur. 2.2.3)	XX—XX
Osmolality	Osmolality (Ph. Eur. 2.2.35)	≤XX mOsm/kg

(continued)

Table 1
(continued)

Purity/impurities		
Parameter	Analytical procedure	Acceptance criterion
Bacterial count/ Bioburden	Microbial enumeration test (Ph. Eur. 2.6.12)	≤XX cfu/ml
Endotoxins	Bacterial endotoxins (Ph. Eur. 2.6.12)	≤XX EU/ml
RNA integrity	RNA electrophoresis and determination of integrity according to SOP XY	≥XX %
Proteins	Total protein (Ph. Eur. 2.5.33)	≤XX μg/ml
Plasmid DNA	qPCR according to SOP XY	≤XX copies/ml
Host DNA	qPCR according to SOP XY	≤XX copies/ml
Residual solvents	Gas chromatography (Ph. Eur. 2.2.28)	≤XX ppm (*see* Ph. Eur. 5.4)
Potency/biological activity		
Parameter	Analytical procedure	Acceptance criterion
Translatability	In vitro translation according to SOP XY	100 % ± XX %

Table 2
Exemplary specification of a lyophilized mRNA-based IMP

Product information	
Product name:	IMP234
Reference code:	R02-00234
Application:	Investigational medicinal product for intradermal injection
Dosage form:	Freeze-dried powder for reconstitution in, e.g., water for injection (WFI)
Manufacture reference:	SOP XY
Dose per container:	XX mg
API:	RNA123, manufactured according to SOP XY
API concentration after reconstitution:	XX g/l
Other ingredients:	XX % buffer XY after reconstitution in XX ml, e.g., water for injection (WFI) XX g/ml excipient XY after reconstitution in XX ml, e.g., water for injection (WFI)

(continued)

Table 2
(continued)

Primary packaging material:	e.g., glass vial 2R, type 1	
	Bromobutyl rubber stopper, 13 mm, grey	
	Aluminum cap, 20 mm, clear lacquered	
Storage temperature:	XX °C	
Stability/shelf life:	See ongoing stability testing	
RNA sequence:	GG…	
Length in bases:	XX b	
Specification of test parameters		
Description		
Parameter	Analytical procedure	Acceptance criterion
Appearance, coloration	Visual analysis	e.g., colorless to yellowish powder
Appearance after reconstitution: clarity and opalescence	Reconstitution and clarity and opalescence of solutions (Ph. Eur. 2.2.1)	e.g., clear liquid
Appearance after reconstitution: coloration	Reconstitution and coloration of solutions (Ph. Eur. 2.2.2)	e.g., colorless to yellowish liquid
Identity		
Parameter	Analytical procedure	Acceptance criterion
RNA length	Reconstitution, RNA electrophoresis and determination of run length according to SOP XY	XX ± XX
Drug substance identity	Reconstitution, reverse transcription, PCR and DNA electrophoresis according to SOP XY	Theoretical band size ± XX
Assay/quantity		
Parameter	Analytical procedure	Acceptance criterion
RNA content	Reconstitution and UV absorption, OD_{260} according to SOP XY	XX g/l ± XX %
Uniformity of dosage units or mass	Uniformity of dosage units after reconstitution (Ph. Eur. 2.9.40) or uniformity of mass for powder (Ph. Eur. 2.9.5)	Conforms to Ph. Eur.
pH value	Reconstitution and pH value (Ph. Eur. 2.2.3)	XX—XX
Osmolality	Reconstitution and osmolality (Ph. Eur. 2.2.35)	XX—XX mOsm/kg
Reconstitution time	Reconstitution time in WFI	≤XX s
Purity/impurities		
Parameter	Analytical procedure	Acceptance criterion

Table 2
(continued)

Sterility	Sterility test (Ph. Eur. 2.6.1)	Sterile
Endotoxins	Reconstitution and bacterial endotoxins (Ph. Eur. 2.6.12)	≤XX EU/ml
RNA integrity	Reconstitution, RNA electrophoresis and determination of integrity according to SOP XY	≥XX %
Residual moisture	Water content (Ph. Eur. 2.5.12 or Ph. Eur. 2.2.32)	≤XX %
Visible particles	Visible particles (Ph. Eur. 2.9.20)	Visually free of particles
Non-visible particles	Reconstitution and sub-visible particles (Ph. Eur. 2.9.19)	Conforms to Ph. Eur.
Potency/biological activity		
Parameter	Analytical procedure	Acceptance criterion
Translatability	Reconstitution, in vitro translation according to SOP XY	100 % ± XX %
Immunostimulation	Reconstitution, cytokine release according to SOP XY	100 % ± XX %

Moreover, data on extractables from container closure system, which could potentially affect the mRNA vaccine stability, extractable volume (Ph. Eur. 2.9.17), and functionality testing of delivery systems, should be available.

4 Notes

1. Several IMPD templates can be found online, e.g., http://www.mmc.nl/media/portal/mmc_documenten/impd [15].
2. It should be remembered that a pharmaceutical quality system following the "Guidelines for good manufacturing practices for medicinal products for human and veterinary use" has to be established, including GMP manufacture and testing of the mRNA vaccines to guarantee consistent product quality. Besides Annex 13 "Investigational Medicinal Products" another major guideline for parenteral drugs like mRNA vaccines is Annex 1 "Manufacture of Sterile Medicinal Products." Special guidance on biological products is given in Annex 2 "Manufacture of Biological active substances and Medicinal Products for Human Use."
3. Besides validation of analytical procedures equipment qualification, validation of automated systems and cleaning valida-

tion has to be performed. According to the IMPD guideline, process validation data is not required for clinical phases. However, Annex 13 of the EU GMP guideline demands thorough validation of sterilization processes being integral part of the manufacture of sterile products (like mRNA vaccines), including sterilization of material in direct contact with the IMP, as, e.g., vials used for primary packaging [1]. Additionally, media fills have to be performed on a regular basis to assure aseptic manufacturing conditions and product sterility [1, 12].

4. RNA-based IMPs are sterile-filtered as sterilization by moist heat is not applicable. If sterile filtration of the final formulation is not possible, individual components are sterilized separately and combined under aseptic conditions. In both cases, potential product loss during sterile filtration has to be examined. Additionally, filters have to be monitored for their integrity.
5. Analyses for excipient solutions/dilutions (not referencing to pharmacopoeia) have to be established. Stability data on the desired storage should be generated as well. It goes without saying that specifications are required, too.
6. In case an IMP-bulk should be stored (allowing several aseptic fillings from one formulation batch) a bulk specification has to be stated. The development of additional analyses for the bulk might be necessary. Bulk stability should be addressed in any event.
7. Contract manufacturers, external analytical laboratories and suppliers have to be qualified. Measures like audits and questionnaires follow a risk-based approach.
8. Labeling of the primary and secondary IMP packaging are performed based on the requirements of Annex 13 of the EU GMP guideline [1].
9. The IMP packaging and distribution processes have to be established. Actions should be implemented to guarantee the correct storage conditions during transport and on-site.
10. Reference samples of starting and packaging material and the IMP should be stored for analytical purposes. In addition, retention samples of the packaged finished product should be kept safe for each packaging run [1].
11. Annex 13 of the EU GMP guideline requires a product specification file which should be continually updated. It includes, among other things, information or reference to IMP manufacture, testing, packaging, labeling, stability, as well as specifications for raw materials, the drug substance, bulk, and finished products [1].
12. Specifications for clinical phase 1 may have to be adapted throughout later clinical phases. Full traceability of all changes (including rationales) has to be guaranteed.

13. Product specifications for different drug substances or IMPs manufactured along identical processes (except for varying plasmid DNA template or drug substance) might be summarized as sectional specifications. In this way, an elaborate modification of the manufacturing authorization might not be necessary for each newly developed mRNA-based IMP.
14. The above presented analyses for RNA identity (*see* Table 1) do not guarantee full RNA sequence accuracy. Still, GMP sequencing of plasmid DNA ensures correctness of the template. Nonetheless, the outlined RNA identity tests give information on complete in vitro transcription of the template DNA and prevent mixing up of mRNAs of similar length and differing sequence. Sequencing (e.g., of the ORF) following reverse transcription and PCR amplification is possible; however the origin of any sequence deviation could not be directly linked to the drug substance due to the error rate of reverse transcriptase and DNA polymerase (used for PCR).
15. The RNA identity parameter "DNA sequence plasmid DNA template" (*see* Table 1) might not be part of the drug substance specification. As the plasmid DNA template represents the most critical raw material for the identity of the drug substance and product, a supplemental plasmid DNA specification should be available in any case, including the requirement for 100% accuracy of the DNA sequence.
16. The identity test applying RT-PCR followed by DNA electrophoresis could be exchanged for any other suitable sequence-specific method, e.g., northern blot analysis.
17. When determining RNA length by electrophoresis it should be kept in mind that the sequence and GC content of the mRNA can affect the running characteristics. For instance, a length of 1000 b of an mRNA vaccine does not automatically mean a run length equal to a 1000 b size standard.
18. In addition to the identity tests presented in Table 1, the substance class RNA could be verified by RNase treatment. However, no sequence specificity would be given.
19. Acceptance criteria for quantitative assays should take into account the precision of the analytical method, whereas those for limit tests should consider the limit of detection (LOD). For details (on validation characteristics of analytical procedures) *see* ICH guideline Q2(R1).
20. Osmolality testing of the drug substance might be omitted.
21. RNA integrity might also be analyzed by chromatography [5].
22. Limits for RNA integrity depend on sequence complexity, secondary structure, and transcript length. Very long transcripts of several thousand bases and more might not be manufactured with highest purity.

23. In case the drug substance is specified as sterile the microbial enumeration test (Ph. Eur. 2.6.12) has to be replaced by the sterility test (Ph. Eur. 2.6.1).
24. The monocyte activation test (MAT) (Ph. Eur. 2.6.30) might be an alternative for the bacterial endotoxin test (Ph. Eur. 2.6.14). The MAT enables detection of additional pyrogens (sources: gram-positive bacteria, yeast and molds, virus).
25. Plasmid DNA is usually amplified using recombinant *E. coli* cultures. Therefore, limits for residual bacterial RNA should be defined for the starting material plasmid DNA as host RNA would be maintained throughout the manufacture of the mRNA drug substance and IMP. The drug substance has to be analyzed for residual host DNA. Information on bacterial DNA contamination of the plasmid DNA template should be available, too.
26. The definition of the acceptance criterion for residual DNA could be based on WHO Weekly Epidemiological Record, No. 20, 16 May 1997, which gives a recommendation of less or equal 10 ng residual DNA per dose [16].
27. Limits for all residual solvents used in the manufacturing process of the drug substance should be specified. Based on the toxic potential of the solvents limits are defined in Ph. Eur. 5.4 [9].
28. As mRNA vaccines are parenteral products, all filled containers of the IMP have to be inspected individually for defects and extraneous particulate contamination (visible particles) according to Ph. Eur. 2.9.20 [9, 12].
29. To verify the potency/biological activity of the IMP translatability (means antigen production) and immunostimulation should be addressed. Translatability of the mRNA active substance within the IMP can be tested applying cell-free systems for in vitro translation, e.g., rabbit reticulocyte lysate. Immunostimulation of mRNA vaccines can be analyzed using PBMC (peripheral blood mononuclear cells) cytokine release assays [17]. Additionally, antigen-specific humoral and cellular immune response could be analyzed in vivo (animal experiments).
30. Following successful manufacturing and testing, the quality of the IMP is certified (certificate of analysis) and the clinical batch can be released by the qualified person respecting all further provisions laid down in Annex 13 of the EU GMP guideline [1].

References

1. European Commission (2010) EU guidelines to good manufacturing practice - medicinal products for human and veterinary use - Annex 13 Investigational Medicinal Products. http://ec.europa.eu/health/files/eudralex/vol-4/2009_06_annex13.pdf. Accessed 4 Sep 2015
2. European Medicines Agency (2006) Guideline on the requirements to the chemical and pharmaceutical quality documentation concerning investigational medicinal products in clinical trials. http://ec.europa.eu/health/files/eudralex/vol-10/18540104en_en.pdf. Accessed 6 Aug 2015

3. European Medicines Agency (2012) Guideline on the requirements for quality documentation concerning biological investigational medicinal products in clinical trials. http://ec.europa.eu/health/files/eudralex/vol-10/2012-05_quality_for_biological.pdf. Accessed 6 Aug 2015
4. Milligan JF, Groebe DR, Witherell GW et al (1987) Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. Nucleic Acids Res 15:8783–8798
5. Kallen KJ, Theß A (2014) A development that may evolve into a revolution in medicine: mRNA as the basis for novel, nucleotide-based vaccines and drugs. Ther Adv Vaccines 1:10–31
6. Ponchon L, Dardel F (2007) Recombinant RNA technology: the tRNA scaffold. Nat Methods 4:571–576
7. Nelissen FH, Leunissen EH, van de Laar L et al (2012) Fast production of homogeneous recombinant RNA—towards large-scale production of RNA. Nucleic Acids Res 40:e102
8. Ozsolak F, Platt AR, Jones DR et al (2009) Direct RNA sequencing. Nature 461:814–818
9. EDQM (2015) European Pharmacopoeia Online. http://online.pheur.org/EN/entry.htm. Accessed 20 Sep 2015
10. ICH (2003) Stability testing of new drug substances and products Q1A(R2). http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1A_R2/Step4/Q1A_R2__Guideline.pdf. Accessed 7 Aug 2015
11. ICH (2009) Pharmaceutical development Q8(R2). http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q8_R1/Step4/Q8_R2_Guideline.pdf. Accessed 7 Aug 2015
12. European Commission (2008) EU guidelines to good manufacturing practice - medicinal products for human and veterinary use - Annex 1 Manufacture of Sterile Medicinal Products. http://ec.europa.eu/health/files/eudralex/vol-4/2008_11_25_gmp-an1_en.pdf. Accessed 4 Sep 2015
13. ICH (1999) Test procedures and acceptance criteria for new drug substances and new drug products: chemical substances Q6A. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q6A/Step4/Q6Astep4.pdf. Accessed 7 Aug 2015
14. ICH (1999) Test procedures and acceptance criteria for biotechnological/biological products. http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q6B/Step4/Q6B_Guideline.pdf. Accessed 7 Aug 2015
15. Maxima Medical Center (2015) IMPD. http://www.mmc.nl/media/portal/mmc_documenten/impd. Accessed 7 Aug 2015
16. WHO (1997) Weekly Epidemiological Record No. 20. http://www.who.int/docstore/wer/pdf/1997/wer7220.pdf. Accessed 4 Sep 2015
17. Scheel B, Teufel R, Probst J et al (2005) Toll-like receptor-dependent activation of several human blood cell types by protamine-condensed mRNA. Eur J Immunol 35:1557–1566

Chapter 16

Nonclinical Safety Testing of RNA Vaccines

Gundel Hager

Abstract

In this chapter, we first consider the overall goal of nonclinical safety testing during drug development and have a brief overview of its regulatory background. We then discuss some basic requirements of safety/toxicity testing before concentrating on the safety testing of RNA vaccines and developing a sample RNA vaccine safety testing program.

Key words NOAEL, Guidelines, GLP, Study protocol, Study report, Safety and toxicity testing, Safety pharmacology, Immunogenicity, ERA

1 Introduction

1.1 The Goals of Safety and Toxicity Testing in Drug Development

In discussions with people involved in the process of drug development, when the topic of safety respective toxicology comes up, you might often hear the comment: "Our goal is (…) to prove that our drug is not toxic". In order to understand the fatal errors behind this statement please think about the following:

Imagine that you have a potential new drug in your hands. You have information about the substance structure, the mechanism of action, etc. You have some data about efficacy in vitro. You run some promising animal efficacy experiments in which you monitored readouts concerning body weight, survival, and some drug specific efficacy biomarkers. No animals died during treatment and thus you are happy that your substance does not appear to be toxic!

Now imagine that the next step in development would be to use this potential drug for administration in humans for the first time. In preparation for this clinical trial, amongst others, you discuss topics like how to monitor the safety of the respective drug with the physicians involved. Which parameters have to be monitored in humans who, for the first time, have contact with your substance? Is it body weight, survival and the biomarkers from the animal experiments? Is that sufficient? Please consider the extent to which the physiological balance in an organism must be disturbed

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_16,

or damaged before significant body weight reduction or even death occurs! The physicians need reliable, quick, and easy-to-obtain readouts to detect potential toxic effects and targets, and that as early as possible—ideally, when no damage has yet occurred. It is obvious that your answer "in the animal studies no animals died and so my substance was not toxic" is not sufficient. Something like "you have to monitor closely all parameters in clinical findings, hematology, urinalysis, and feces that give you hints on potential liver damage" is what the physicians need.

In order to get that kind of information, a worst-case scenario with respect to the intended clinical use of that specific new drug is simulated in an adequate, most-human-like animal species by, for example:

- Administration of high doses.
- Achievement of high exposure.
- Achievement of on an exaggerated pharmacological effect producing toxicity.

Potential damages can then be visualized/characterized by:

- Reduction or loss of specific functions.
- Biomarkers (e.g., hematology, clinical chemistry, urinalysis, and immune system markers).
- Morphologically ascertained lesions (visualized for example by histopathology).

Because we can identify different types of toxicity, complex models have been designed to accommodate them. Whatever model or scenario is used, you have come to understand that where to draw the line with respect to the toxicity of a drug depends on the circumstances. For instance, the risk related to toxicity increases with treatment duration, the persistence of toxic symptoms, the slope of the dose–effect curve, or the affection of vital organs. As you can see, the picture of safety testing is very complex. Administration of a drug might lead to expected or also unexpected side effects. Risk assessment of a drug has to take into consideration both the pharmacological (wanted) effect and the adverse, toxic (unwanted side) effects.

Getting back to the question of the goal of toxicological testing in drug development, we now understand that it is to characterize the toxicological profile as comprehensively as possible and to assess and evaluate the expected und unexpected risks of the drug under development. Or to put it with the words of one of the International Conference of Harmonisation (ICH) guidelines you should consider studying in our context:

> The development of a pharmaceutical is a stepwise process involving an evaluation of both animal and human efficacy and safety information.

> The goals of the nonclinical safety evaluation generally include a characterisation of toxic effects with respect to target organs, dose dependence, relationship to exposure, and, when appropriate, potential reversibility. This information is used to estimate an initial safe starting dose and dose range for the human trials and to identify parameters for clinical monitoring for potential adverse effects. The nonclinical safety studies, although usually limited at the beginning of clinical development, should be adequate to characterise potential adverse effects that might occur under the conditions of the clinical trial to be supported. [1]

1.2 Regulatory Background

1.2.1 Some Terms and Definitions

Before we enter into further details of nonclinical safety/toxicity testing, it is necessary to learn a few basic terms and definitions, which is why we include a kind of short glossary at this point:

- *ADME*: Absorption, Distribution, Metabolism, Excretion.
- *GLP (Good Laboratory Practice)*: Principles that provide a framework within which laboratory studies are planned, performed, monitored, recorded, reported and archived.
- *LD50 (Median Lethal Dose)*: Statistically derived dose of a chemical or physical agent (radiation) expected to kill 50% of organisms in a given population under a defined set of conditions.
- *LDmin (Minimum Lethal Dose)*: Lowest amount of a substance that, when introduced into the body, might cause death to the individual species of test animals under a defined set of conditions.
- *LED (Lowest Effective Dose)*: Lowest dose of a substance that causes a defined magnitude of response in a given system.
- *LOAEL (Lowest Observed Adverse Effect Level)*: Lowest concentration or amount of a substance, found by experiment or observation, which causes an adverse alteration of morphology, functional capacity, growth, development, or life span of a target organism distinguishable from normal (control) organisms of the same species and strain under defined conditions of exposure.
- *LOEL, LEL (Lowest Observed Effect Level, Lowest Effect Level)*: Lowest concentration or amount of a substance, found by experiment or observation, which causes any alteration of morphology, functional capacity, growth, development, or life span of a target organism distinguishable from normal (control) organisms of the same species and strain under defined conditions of exposure.
- *MABEL (Minimum Anticipated Biological Effect Level)*: Anticipated dose level leading to a minimal biological effect level in humans. When using this approach, potential differences of sensitivity for the mode of action of the investigational medicinal product between humans and animals need to be taken into consideration, for example, derived from in vitro studies.

- *MFD (Maximum Feasible Dose)*: Highest dose that it is feasible to administer.
- *MTD (Maximum Tolerated Dose, Minimal Toxic Dose)*: Dose that is expected to produce limited toxicity when administered for the duration of the test period. It should not induce:
  - Overt toxicity, for example, appreciable death of cells or organ dysfunction.
  - Toxic manifestations that are predicted materially to reduce the life span of the animals except as the result of neoplastic development.
  - 10% or greater retardation of body weight gain as compared with control animals.
- *MTEL (Maximum Tolerable Exposure Level)*: Maximum amount or concentration of a substance to which an organism can be exposed without leading to an adverse effect after prolonged exposure time.
- *NOAEL (No Observed Adverse Effect Level)*: Concentration or amount of a substance, found by experiment or observation, which causes no detectable adverse alteration of morphology, functional capacity, growth, development, or life span of the target organism under defined conditions of exposure.
- *NOEL (No Observed Effect Level)*: Concentration or amount of a substance, found by experiment or observation, that causes no alterations of morphology, functional capacity, growth, development, or life span of target organisms distinguishable from those observed in normal (control) organisms of the same species and strain under the same defined conditions of exposure.
- *NRL (No Response Level)*: Maximum dose of a substance at which no specified response is observed in a defined population and under defined conditions of exposure.
- *Toxic Dose*: Amount of a substance that produces intoxication, not necessarily with lethal outcome.

1.2.2 Regulatory Framework

When we speak about regulations, it is important to understand their relevance for the planning of a testing strategy. On the one hand, we have some requirements that are regulated by laws, like, for example, the Arzneimittelgesetz (Drug Law) and the Chemikaliengesetz (Chemicals Law) in Germany or European directives and regulations. On the other hand, we have guidelines, such as the ICH guidelines or guidelines issued by the Committee for Proprietary Medicinal Products (CPMP) or Committee for Medicinal Products for Human Use (CHMP) in Europe, which are not legally binding. Compliance with these guidelines, however, is a prerequisite for the acceptance of safety testing by the competent authorities. This is understandable, particularly if you

consider that these guidelines are to be regarded as the authorities' interpretation of the various legal requirements. In other words, they are a helping hand for the drug developing industry in how to meet these requirements. Nevertheless, these guidelines cannot be used as nonclinical blueprints that can/have to be followed exactly in every case, but always a tailored, step-by-step approach based on a sound scientific judgment during safety testing of new drugs or therapies should be followed.

ICH Guidelines: The mission of the ICH is to make recommendations towards achieving greater harmonization in the interpretation and application of technical guidelines and requirements for pharmaceutical product registration between its members: the European Union, the USA, and Japan. The ICH guidelines build the international accepted framework for nonclinical safety testing, without giving any details on the concrete design/performance of the tests. The original, actual guidelines are available free of charge on the ICH's Web site (www.ich.org) as well as on the Web sites of the competent authorities of the three ICH members: European Medicines Agency (EMA) for the European Union (www.ema.europa.eu), US Food and Drug Administration (FDA) for the USA (www.fda.gov), and Pharmaceuticals and Medical Devices Agency (PMDA) for Japan (www.pmda.go.jp).

OECD Guidelines: The Guidelines for the Testing of Chemicals of the Organization for Economic Co-operation and Development (OECD) are detailed "cooking recipes" for nonclinical safety studies. They are a collection of the most relevant internationally agreed test methods used by government, industry, and independent laboratories to determine the safety of chemicals and chemical preparations, including pesticides and industrial chemicals. The guidelines cover tests for the physical–chemical properties of chemicals, human health effects, environmental effects, and degradation and accumulation in the environment. However, they have been developed for the testing of chemicals, so you always have to keep in mind, that they do not meet all the requirements for pharmaceutical drug development. The OECD guidelines have fixed the minimal standard for the study design of toxicological testing in more than 40 guidelines, which are valid in all OECD member states. The OECD guidelines are all available, free of charge, in the iLibrary of the OECD (http://www.oecd-ilibrary.org/content/package/chem_guide_pkg-en). Of great importance for the nonclinical toxicology are the OECD Series on Principles of Good Laboratory Practice (GLP) and Compliance Monitoring. The GLP principles have been made mandatory for regulatory toxicological studies by the respective legislative. They set precise requirements on:

- Test facility organization and personnel responsibilities.
- Quality assurance program and responsibilities of the quality assurance personnel.

- Facilities (testing facilities, archiving, waste disposal).
- Apparatus, material, and reagents.
- Test systems.
- Test and reference items (receipt, handling, sampling, storage, characterization).
- Standard Operating Procedures (SOPs).
- Performance and reporting of studies (study plan, conduct of the study, reporting of study results, content of the final report).
- Storage and retention of records and materials.

In addition to the ICH and OECD guidelines, specific regional and national guidelines, regulations, and laws govern the respective region's or country's safety requirements. Current versions of the particular legislation and guidelines can be found on the Web sites of the respective competent authorities.

2 Some Basic Requirements of Safety/Toxicity Testing

As already pointed out, safety/toxicological testing during drug development is quite a regulated process. You have to move under high time and budget pressure within the limits set by the authorities as well as strategic and economic needs, and knowing that at any time the development process could be stopped due to unexpected results. It is obvious that, before starting this complex process, all reasonable efforts should be taken to have all needed information and preceding works in place.

A checklist of some general basic requirements for starting toxicity studies of a *New Chemical Entity* (NCE) would read like the following [2]:

- Are dose and pharmacokinetic (PK) information available?
- Is any test species-related information available?
- Is a sufficient amount of the test item (e.g., drug substance or vehicle) available, and in the needed quality?
- Is an adequate formulation available?
- Is it certain that the test item planned to be used in clinical trials will not display a different impurity pattern than the test item used in toxicity testing?
- Are the handling, storage, and logistics of the test item with respect to drug substance and vehicle planned and tested at the critical points?
- Is information available regarding adhesion to materials used during the preclinical and clinical trials? Are special materials needed?
- Are reasonable package units determined and available?

- Are reasonable concentrations of the drug substance available to secure minimal and maximal application volumes?
- Is an analytical method for formulation and dilution analysis in place and validated?
- Is an analytical method for bioanalytics in different matrices available? What is the validation status of the method?
- Are stability data for the stored test item, stability in formulation and in serum/plasma (including freeze and thaw cycles) available?.

For *New Biologic Entities* (NBE), some additional points have to be considered [3]:

- Ideally, the drug substance is in the (almost) final formulation for use in the clinical trials; the formulation appears neutral concerning modulation of immunogenicity.
- Are activity assays required? If yes, are they established and validated?
- The material should be derived from the production process developed for the clinical testing material. It should come from a GMP or pre-GMP batch (often called the "tox-batch"); both the material and the process have to be fully characterized.
- Are bioactivity assays (batch release tests) established and validated?

Before going into the details of the safety testing strategy and studies in a first step we will learn about the basics of compiling a GLP-compliant study protocol and study report and archiving. Of course the detailed content of the protocols have to be adapted to the specific tests.

2.1 Study Protocol (GLP Compliant)

The GLP-compliant study protocol has to contain all information regarding:

- The item to be tested.
- The regulatory procedures the test is based upon.
- The environment in which the testing is performed (facility, people involved).
- All planned activities.
- The data to be recorded and reported.
- Archiving (what and where).

The following example summarizes main points of a toxicological in vivo study protocol [4]:

General Statements		
Responsibilities		
Protocol Approval		
1.0	Study Objective	
2.0	Regulatory Guidelines and Test Methods	
3.0	Quality Assurance	
	3.1	GLP Compliance
	3.2	Amendment Procedures
	3.3	Deviation Procedures/Interfering Factors
	3.4	Quality Assurance Evaluations
	3.5	Archiving
4.0	Test Item	
	4.1	Test Item Characterization
	4.2	Identification, Storage, and Handling
	4.3	Preparation of Test Item
5.0	Reasons for Choice of Species and Route of Administration	
6.0	Animals and Husbandry	
	6.1	Animal Specification
	6.2	Animal Health
	6.3	Animal Identification
	6.4	Husbandry
	6.5	Food and Feeding
	6.6	Water Supply
	6.7	Water Control
	6.8	Acceptable Levels of Contaminants in Diet and Water
	6.9	Bedding
7.0	Experimental Design	
	7.1	Experimental Groups and Doses
	7.2	Justification for the Dose Selected
	7.3	Administration of the Test Item Solutions and Vehicle
	7.4	Treatment and Recovery (Treatment-Free Observation) Period
	7.5	Mortality
	7.6	Clinical Observations

(continued)

(continued)

	7.7	Body Weight
	7.8	Laboratory Examinations
	7.9	Necropsy and Tissue Preparation
	7.10	Histopathological Examination
	7.11	Statistical Analysis
8.0	Data to Be Recorded	
9.0	Records to Be Maintained	
10.0	Reporting	
11.0	Internal Protocol Distribution	
APPENDIX I	Test Item Characterization and Handling	
APPENDIX II	Acceptable Levels of Contaminants in Diet and Water	
APPENDIX III	Diet composition	

2.2 Study Report (GLP Compliant)

The study report must provide a clear description of all results. However, it is not the place to provide any scientific interpretation nor any statement regarding the implications of the test results with respect to the administration of the drug to humans. Similar to the study protocol, a report contains exhaustive information regarding:

- The item used.
- The regulatory procedures the test was based upon.
- The environment in which the testing was performed (facility, people involved).
- All performed activities.
- All observed findings and recorded endpoints.
- The results of toxicity testing (both summarized and in detailed appendices) and their evaluation.
- Archiving.

The following example summarizes main points of a toxicological animal study report [5]:

Contents of Technical Part
General Statements
Study Schedule
Responsibilities
Project Staff Signatures
Regulatory Guidelines and Test Methods

(continued)

(continued)

	GLP Compliance		
	Archiving		
	Statement of Compliance		
	Statement of the Quality Assurance Unit		
1.0	Summary and Conclusion		
2.0	Study Objective		
3.0	Reasons for Choice of Species and Route of Administration		
4.0	Test Item and Vehicle		
	4.1	Test Item Characterization	
	4.2	Identification, Storage, and Handling of the Test Item	
	4.3	Preparation of the Test Item Solutions	
5.0	Animals and Husbandry		
	5.1	Animal Specification	
	5.2	Groups and Group Size	
	5.3	Animal Health	
	5.4	Randomization	
	5.5	Animal Identification	
	5.6	Husbandry	
	5.7	Food and Feeding	
	5.8	Water Supply	
	5.9	Bedding	
6.0	Experimental Design		
	6.1	Experimental Groups and Doses	
	6.2	Administration of the Test Item Solutions and the Vehicle	
	6.3	Duration of Treatment	
	6.4	Justification for the Doses Selected	
	6.5	Clinical Observations	
		6.5.1	Clinical Observations and Ophthalmoscopy
		6.5.2	Mortality
		6.5.3	Body Weight, Food Consumption, and Food Conversion Ratio
	6.6	Laboratory Examinations	
		6.6.1	Blood Collection and Sampling Schedule
		6.6.2	Hematology

(continued)

		6.6.3	Clinical Biochemistry
		6.6.4	Urine Analysis
	6.7	Toxicokinetics	
	6.8	Terminal Investigations	
		6.8.1	Necropsy Procedure and Macroscopic Examination
		6.8.2	Organ Weights
		6.8.3	Organ Fixation and Tissue Preparation
		6.8.4	Histopathological Examination
	6.9	Statistical Analysis	
	6.10	Environmental Conditions during the Study	
7.0	Results		
	7.1	Clinical Observations	
		7.1.1	General Condition, Behavior, and Mortality
		7.1.2	Ophthalmoscopy
		7.1.3	Body Weight, Food Consumption, and Food Conversion Ratio
	7.2	Laboratory Examinations	
		7.2.1	Hematology
		7.2.2	Clinical Biochemistry
		7.2.3	Urine Analysis
	7.3	Toxicokinetics	
	7.4	Terminal Investigations	
		7.4.1	Organ Weights
		7.4.2	Macroscopic Findings
		7.4.3	Histopathological Findings
8.0	Conclusion		
9.0	Appendices		
APPENDIX 1		Test Item Characterization	
APPENDIX 2		Analytical Report Toxicokinetics	
APPENDIX 3		Special Reports	
		3.1	Clinical Findings
		3.2	Ophthalmology
APPENDIX 4		Figures	
		4.1	Body Weight

(continued)

	4.2	Food Consumption	
APPENDIX 5	Tables		
	5.1	Body Weight	
	5.2	Food Consumption	
		5.2.1	Food Consumption
		5.2.2	Food Conversion Ratio
	5.3	Hematology	
	5.4	Clinical Biochemistry	
	5.5	Urine Analysis	
	5.6	Organ Weights	
		5.6.1	Absolute Organ Weights
		5.6.2	Relative Organ Weights
APPENDIX 6	Individual Data		
	6.1	Body Weight	
	6.2	Food Consumption	
	6.3	Hematology	
	6.4	Clinical Biochemistry	
	6.5	Urine Analysis	
	6.6	Organ Weights	
APPENDIX 7	Pathology: Summary Report		
	7.1	Macroscopical Findings (Incidence Table)	
	7.2	Histological Findings (Incidence Table)	
APPENDIX 8	Individual Macroscopical and Histological Findings		
APPENDIX 9	Study Plan Deviations		
APPENDIX 10	Sample Delivery Formula		
ADDENDUM			
	A Diet Composition		
	B Animal Health Certificate		
	C GLP Certificate		
	D Report: Toxicokinetics		

2.3 Archiving

You will find detailed specifications on what has to be archived and the archiving period in the OECD Series on Principles of Good Laboratory Practice (GLP) and Compliance Monitoring.

3 Methods

In general, the safety evaluation of new medicinal products is highly regulated. Recommendations and guidelines for vaccine safety testing are developed by the World Health Organization (WHO) based on a broad international consulting process; regulatory requirements are produced by the regulatory bodies such as EMA, CBER and in addition there are pharmacopoeial requirements in place too. For newly developed products, such as RNA Vaccines, specific regulatory requirements may not be available. In the absence of a specific guidance, you have to develop the safety testing strategy and the study design on a case-by-case basis. It will be inspired by the more general principles applying to safety testing of medicinal products, of vaccines, of adjuvants, of DNA vaccines and combination vaccines, of gene therapy products and of course on the scientific rationale for the risk assessment.

In a first step you may want to ascertain what are expected risks associated with your new item to be tested:

3.1 What Potential Main Safety Concerns with Vaccines or Adjuvants Are Expected?

One may expect local reactions (e.g., pain, redness, swelling; granuloma formation; abscess; necrosis; and regional lymphadenopathy) and typical systemic reactions like anaphylaxis; pyrogenicity; organ specific toxicity; nausea/diarrhea/malaise; immune-mediated toxicity (e.g., cytokine release, immune suppression, autoimmune disease);teratology and carcinogenicity. To asses these topics and to cover also the question of unexpected toxicity it is recommended to follow the outlined basic toxicity assessment described in the WHO guideline on nonclinical evaluation of vaccines. In certain cases, the additional toxicity assessment may be needed.

3.2 What Potential Main Safety Concerns with Nucleic Acid/RNA-Based Vaccines Are Expected?

They may lead to immunostimulation/inflammatory activation and can have unwanted effects on the host, such as induction of fever or flu-like symptoms and increased expression of autoantigens.

In summary, the nonclinical toxicity testing of RNA vaccines should assess the safety of the vaccine prior to human clinical trials, identify potential toxicities and target organs, ideally determine a safe vaccine dose, and identify an NOAL if needed.

3.3 Prerequisites

Before starting the nonclinical assessment of the RNA vaccine, information on the following of the vaccine formulation should be available.

- Mass.
- Identity.
- Purity.
- Sterility.
- Stability.
- Potency.

The purpose you aim with the nonclinical studies, is to support the intended clinical use of the vaccine and it is thus necessary to know:

- The intended clinical use (clinical indication) and the patient population.
- The intended clinical route/device of administration.
- The formulation.
- The estimated dose level.
- The immunization schedule.

The formulation tested is preferable a GMP lot, a pre-GMP (validation) lot equivalent to the clinical lot is also acceptable to ensure the comparability between nonclinical and clinical data. Control formulations for the treatment of the control groups should be available.

A challenge can be the administration of a full human dose (FHD) since it might be problematic to achieve in smaller animals the full volume used in humans. You can solve this problem partially by using more than one administration site per animal.

3.4 Selection of the Adequate Animal Model

In general, for vaccines, safety testing in a single, relevant species is sufficient. Ideally, the selected species should fulfill a series of criteria:

- The selected species should develop after immunization an immune response to the vaccine and the adjuvants similar to the expected response in humans, in order to be able to identify toxicities related to the pharmacodynamic action of the vaccine.
- The selected species should be susceptible to that pathogen if vaccines are directed against a pathogen.

In addition, previous experience with a specific model may be a good reason for you to use that model for the safety studies too. You will have to consider also the practicability of the model regarding the route and volume to be administered, the volume of biological samples needed for analytical purposes, and the availability of serological kits and reagents for that animal species.

Commonly used species are mice, rats, rabbits, and sometimes minipigs or nonhuman primates. Less common species can be ferrets, hamsters or cotton rats.

3.5 Testing Strategy for the Safety Assessment

To outline it with the words of the WHO guideline on nonclinical evaluation of vaccines, regarding study design you should consider as a minimum necessary:

> The preclinical toxicity study should be adequate to identify and characterize potential toxic effects of a vaccine to allow investigators to conclude that it is reasonably safe to proceed to clinical investigation. The parameters to be considered in designing animal toxicology studies are the relevant animal species and strain, dosing schedule and method of vaccine administration, as well as timing of evaluation of end-points (e.g. sampling for clinical chemistry, antibody evaluation and necropsy). The route of administration should correspond to that intended for use in the clinical trials. When the vaccine is to be administered in human clinical trials using a particular device, the same device should be used in the animal study, where feasible (e.g. measles aerosol vaccine in the monkey model). Potential toxic effects of the product should be evaluated with regard to target organs, dose, route(s) of exposure, duration and frequency of exposure, and potential reversibility. The toxicity assessment of the vaccine formulation can be done either in dedicated-stand alone toxicity studies or in combination with studies of safety and activity that have toxicity endpoints incorporated into the design. The study should also include an assessment of local tolerance. [6]

For RNA vaccines, in addition to the requirements the WHO formulates, the Guideline on the Non-Clinical Studies Required Before First Clinical Use of Gene Therapy Medicinal Products EMEA/CHMP/GTWP/125459/2006 states that studies should be designed and carried out aiming at establishing the following:

- Pharmacodynamic "proof of concept" in nonclinical model(s).
- Bio-distribution of the GTMP.
- Recommendation on initial dose and dose escalation scheme to be used in the proposed clinical trial.
- Identification of potential target organs of toxicity.
- Identification of potential target organs of biological activity.
- Identification of indices to be monitored in the proposed clinical trial.
- Identification of specific patient eligibility criteria [7].

In general, it is highly recommended, and also encouraged by the regulatory bodies, to consult with them, to discuss the adequacy of a proposed testing strategy of the specific RNA vaccine. Based on the topics raised at such a meeting, a testing program can be finalized and started.

A sample nonclinical safety evaluation program for a RNA vaccine might look like this:

- Single dose toxicity.
- Repeated dose toxicity (if feasible including local tolerance, immunological and safety pharmacology endpoints).
- Biodistribution including adsorption by tissues/elimination from the organism.
- Safety Pharmacology (if feasible safety pharmacology evaluations shall be included in single or repeated dose toxicity studies).
- Local tolerance (acute and chronic inflammation).
- Immunogenicity studies (induction of hypersensitivity, anaphylaxis, immune suppression, autoimmunity).

Genotoxicity studies are normally not needed for vaccines. However, if there are components of the vaccine formulation requiring such studies, in vitro tests for mutation and chromosomal damage should be performed prior to first human exposure. Carcinogenicity studies are normally not required for vaccines. However, one has to evaluate whether components of the vaccine formulation may require such studies.

Developmental toxicity studies are usually also not needed for vaccines indicated for immunization during childhood. If women of childbearing age are included in the intended clinical studies/target population developmental and reproductive toxicology (DART) studies should be considered unless sound arguments are in place to show that DART studies are not necessary.

All study designs will have to be adapted to the characteristics of the specific RNA vaccine.

Until up to here, we have taken you through some basics of the regulatory nonclinical safety testing focusing on RNA vaccines. Now we go on with the next, more concrete, step: the nonclinical safety studies. For each study type, you will find a short comment on both the study principle and its structure, some statements on the study goal, the readouts, and their evaluation. Please always remember: Unless stated otherwise, all nonclinical safety studies need to be performed in a GLP-compliant manner.

3.5.1 Single Dose Toxicity

Single dose toxicity studies provide preliminary safety and tolerability data of the acute actions of the vaccine. They have the typically design of rodent acute toxicity studies with administration of the full human dose (FHD) or more. In practice, often when this data are already available from repeated dose toxicity studies, single dose toxicity studies are not performed. In any case, one has to consider that single dose studies might be indispensable when no in vivo data are available previous to starting a repeated dose study or if the immune response induced by the first administration changes the reaction to a repeated administration.

3.5.2 Repeated Dose Toxicity

Repeated dose toxicity testing has a broad scope. Its objectives are:

- To define the toxicological profile and toxicological targets and understand as far as possible the mechanisms of identified toxicities (and, if wisely designed, it is also possible to assess pharmacological parameters and exaggerated pharmacological effects).
- To provide support for the initiation or continuation of clinical trials in humans.

For vaccines, the dose to be tested has to be the full human dose (FHD) and the formulation intended to be used in clinical trials. If the FHD is not feasible, the maximum feasible dose (MFD) shall be administered. In order to have flexibility in setting the clinical dose, it may be desirable to include higher (only small multiples of the FHD to avoid any irrelevant immunological effects) or lower (if unacceptable toxicities are expected and a NOAEL need to be determined) doses. You should also include appropriate control groups (placebo, vehicle, adjuvant-only or antigen-only, etc.) into the study.

The determination of routes and doses (how, how much, and how frequently administered) is based on the intended clinical use of the vaccine. It is possible to compress the time plan, provided that the dosing interval is broadly consistent with the underlying immunological events. In particular, successive administrations in animals should be spaced at sufficient intervals that there is no interference between successive immunological responses. An interval of 2–3 weeks between successive administrations is considered to be sufficient [8]. The number of administrations in the toxicity study should exceed the number planned for human administration to provide safety for the human dosing schedule (at least one more administration should be given than in the proposed clinical scheme).

To get an idea of what safety testing study can be about, in the following a sample case study for a repeated dose study including biodistribution, CNS safety pharmacology and local tolerance evaluation is outlined (Fig. 1).

Please note that for several monitoring purposes (such as for clinical chemistry, blood coagulation, hematology, toxicokinetics), blood collection is required. There are species-specific limitations on how this might be done and how much blood may be collected without compromising the animals. Please note that the tissue list for histopathology is defined in the WHO and EMA guidelines.

Please note, that clinical observations should include, if feasible, ophthalmological evaluations to observe uveitis as possible indication for autoimmune responses.

Please remember, testing, reporting, and archiving has to be performed in compliance with GLP.

3.5.3 Safety Pharmacology

The evaluation, if safety pharmacological studies need to be performed has to be evaluated on product-specific bases. The acute effect that the test substance has on vital organ systems when

Animal species:	Mouse
Duration in-life:	28 days to 3 months
Administration:	Repeated, route as close as possible to the clinical one
Test item:	RNA vaccine clinical formulation, lyophilised as ready to dissolve
Dose level:	1
Groups:	6 groups: 1. saline control group, main 2. saline control group, recovery 3. dose group, main 4. dose group, recovery 5. saline control, satellite 6. dose group, satellite
Group size:	20 (10 m + 10 f)
Total animals:	120

Monitoring:	Mortality Body weight Clinical observation Food consumption Local reaction Body temperature FOB (mod. Irwin) Hematology Clinical chemistry Urinalysis* Bone marrow smear Blood coagulation parameters Cytokines: TNF-α, IFN-γ, IL-6, IL-10, etc...
Post mortem:	Necropsy and weight of selected organs Full histopathological evaluation of all animals including recovery animals RNA biodistribution in satellite animals (administration site, lymph nodes, liver, lung, gonades)
Duration:	Study plan to report: 6 to 8 months

Fig. 1 Repeated Dose Toxicity: Sample case study

administered at doses in the therapeutical range, or higher, is monitored in so-called safety pharmacology studies. A standard set of assays, assessing cardiovascular, central nervous system, and respiratory effects are considered the core battery studies.

An in vivo *cardiovascular safety* study is usually performed in telemetered non-rodents. Animals are dosed successively with a low, medium, and high dose of the test substance, separated by washout periods. After administration cardiovascular parameters (e.g., arterial pressure, heart rate, ECG, and body temperature) are recorded and evaluated. Drugs that prolong the QT interval do so by locking the activity of the human hERG gene channel on the cardiomyocytes. This might lead to a potentially fatal tachyarrhythmia, the so-called torsades des pointes. Therefore, additionally, an in vitro monitoring for QT prolongation (hERG channel test) is performed, for example, by electrophysiological recordings in cells stably transfected with the hERG clone.

Respiratory safety is monitored by recording and evaluating respiratory parameters, in vivo, after administration of test substance.

Central Nervous System (CNS) safety testing (e.g., Irwin test) can either be performed as a stand-alone test, usually in rodents, or it can be integrated in the general toxicity studies (e.g., repeated-dose studies; see case study). It is based on the evaluation of a standard set of behavioral parameters at defined time points after administration of the test substance.

3.5.4 Immunogenicity

The initial screen for immunotoxicity comes from the general toxicity studies where special attention shall be put on hematological changes, alterations in immune system organs (weight and histopathology), changes in serum albumins without plausible explanation, increased infection incidence, increased body temperature, increased tumor occurrence in the absence of plausible genotoxic, immunological biomarker values outside the normal range or compared to controls, liver enzyme induction, or hormonal effect. If immunotoxicity is an issue either due to hints from previous studies and substance properties or due to the intended clinical use (i.e., immunocompromised patients), further immunotoxicity testing has to be performed.

Since for RNA vaccines immune reactions are a main part of the pharmacodynamic activity, testing for unwanted immunological reactions is required. Generally, immunotoxic effects can be:

- Unintended immunosuppression (i.e., reduced immune function leading to infections or malignancies).
- Unintended immunostimulation (rather a general dysregulation of the immune system leading to flu-like reactions, autoimmune diseases, inhibition of drug-metabolizing enzymes, and hypersensitivity reactions to unrelated allergens).
- Immunogenicity (mainly drug allergenicity and the ability of antidrug immune response to alter the pharmacodynamic and kinetic profile of the drug. It is important here to note the limited predictivity of animal testing for the human situation).
- Induction of hypersensitivity resulting in allergic or pseudoallergic reactions (like, for example, a delayed-type hypersensitivity, DTH).
- Induction of autoimmunity (relevant for vaccines or preparations containing immune response modifiers like adjuvants).

Actually, there are no requirements regarding the GLP status of immunogenicity testing. Even so, due to the complex nature of the immune response, ideally a functional, validated immune assay should be used.

3.5.5 Environmental Risk Assessment (ERA)

Other than you might have assumed, toxicity testing is not restricted to research concerning the impact on human health. This becomes clear if you consider that many medicinal products are used, stored, and disposed of. Thus, the request for an Environmental Risk Assessment (ERA) similar to chemicals is plausible. In the EU, the latter is regulated by REACH, the regulation on Registration, Evaluation, Authorisation of Chemicals; an ERA of new drug substances has to be performed according to Article 8(3) (ca) and (g) of Directive 2001/83/EC as amended. The ERA is a mandatory part of all new marketing authorization applications.

The assessment of potential environmental risks is a step-wise, two-phase procedure. The first phase (Phase I) estimates the exposure of the environment to the drug substance, then the environmental fate and effect is analyzed in Phase II. More details are provided in the EMA's Guideline on environmental risk assessment of medicinal products for human use.

Acknowledgements

The author thanks Helga Bleuel for her helpful comments during the preparation of this chapter.

References

1. ICH M3(R2) Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals, p 2. http://www.ich.org/products/guidelines/multidisciplinary/article/multidisciplinary-guidelines.html. Accessed 29 Oct 2015
2. Hager GH (2011) A checklist of some general basic requirements for starting toxicity studies of a New Chemical Entity (NCE). In: Company folder preclinical safety testing. Aurigon Life Science GmbH, Munchen
3. Hager GH (2011) A checklist of some general basic requirements for starting toxicity studies for New Biological Entities (NBE). In: Company folder preclinical safety testing. Aurigon Life Science GmbH, Munchen
4. Aurigon Life Science GmbH (2010) Sample study plan. Aurigon Life Science GmbH, Munchen
5. Aurigon Life Science GmbH (2010) Sample study report. Aurigon Life Science GmbH, Munchen
6. World Health Organization: Annex 1 who guidelines on nonclinical evaluation of vaccines, p 9. http://www.who.int/biologicals/publications/trs/areas/vaccines/nonclinical_evaluation/en/. Accessed 29 Oct 2015
7. European Medicines Agency EMA: guideline on the non-clinical studies required before first clinical use of gene therapy medicinal products (EMEA/CHMP/GTWP/125459/2006). http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000410.jsp&mid=WC0b01ac058002958d. Accessed 29 Oct 2015
8. World Health Organization: Annex 1 WHO guidelines on nonclinical evaluation of vaccines, pp 10–11. http://www.who.int/biologicals/publications/trs/areas/vaccines/nonclinical_evaluation/en/. Accessed 29 Oct 2015

Chapter 17

Immunotherapy of Uveal Melanoma: Vaccination Against Cancer

Mirko Kummer and Beatrice Schuler-Thurner

Abstract

Uveal melanoma is the most frequently occurring primary intraocular tumor in adults, with an incidence of about 5 out of 100,000 per year, the incidence rising with increasing age (Lipski, Klin Monbl Augenheilkd 230:1005–1019, 2013; Metz et al., Klin Monbl Augenheilkd 230:686–691, 2013; Singh and Topham, Ophthalmology 110:956–961, 2003). Often diagnosed late due to a lack of early symptoms, this kind of melanoma is associated with a poor prognosis. Approximately 50% of the patients develop distant metastases (Lipski, Klin Monbl Augenheilkd 230:1005–1019, 2013; Metz et al., Klin Monbl Augenheilkd 230:686–691, 2013; Singh and Topham, Ophthalmology 110:956–961, 2003). In sharp contrast to cutaneous melanoma, uveal melanoma shows a strong liver tropism and spreads exclusively via the hematogenous route (except for tumors with extraocular expansion) (Heindl et al., Arch Ophthalmol 128:1001–1008, 2010). The most likely reason for this observation is the lack of lymphatic vessels in the choroid and alymphatic barrier of the sclera (Schlereth et al., Exp Eye Res 125:203–209, 2014; Schroedl et al., Invest Ophthalmol Vis Sci 49:5222–5229, 2008). Due to its location in the immune-privileged eye, the uveal melanoma is widely protected from the immune system. Therefore, the goal of the approach presented here, of a "personalized vaccination therapy" is to help the immune system recognize and fight the tumor.

Key words Uveal melanoma, Tumor, Immune system

1 Introduction

As prognosis is poor regardless of the therapy applied to the primary tumor (enucleation, endoresection, block excision, brachytherapy, proton therapy, etc.) and cannot be influenced by adjuvant therapies [1–3], new therapeutic approaches are required. In particular, uveal melanoma with monosomy 3 is associated with a dramatically poor prognosis [7]. Uveal melanoma displays a strong liver tropism and spreads exclusively via the hematonegous route, most probably due to the lack of lymphatic vessels in choroid and alymphatic barrier of the sclera [4–6]. In most of these cases, liver metastases leading to death of the patient within several months occur. Only in rare cases can the course of disease be delayed by

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_17,

kinase inhibitors or checkpoint blockade [1–3]. In conclusion, there is currently no adequate approved therapy for patients suffering from uveal melanoma, either in the metastasized or in the adjuvant situation [8–10]. Especially for high risk patients with uveal melanoma and monosomy 3, the approach of a personalized tumor vaccination against the tumor tissue located inside the eye may be a promising concept.

2 Vaccination with Dendritic Cells

Vaccination using dendritic cells is a variant of cancer immunotherapy that has been applied in phase 3 studies for several tumor entities (Table 1), for example, renal cell carcinoma, prostate carcinoma, glioblastoma, cutaneous melanoma, colon carcinoma, and uveal melanoma. One drug (Provenge®) has been already approved for the treatment of metastasized prostate carcinoma in the USA and in Europe. The ongoing phase 3 study for uveal melanoma patients is the only study that is not conducted by a company, but by a university hospital supported by the Deutsche Krebshilfe (Table 2).

Dendritic cells are immune-regulatory cells which can modulate the function of the immune system. They do so by inducing either immunity or tolerance against specific antigens. To be used in therapy, dendritic cells are expanded ex vivo, transferred to the "stimulatory mode" by maturation and finally loaded with specific antigen (e.g., tumor antigen). Once in the patient, these cells can

Table 1
Phase 3 studies/approved products for the immune therapy of tumors using dendritic cells

Tumor	Sponsor	Study-number or approved product
Renal cell carcinoma	Argos Therapeutics, USA	NCT01582672
Prostate carcinoma	Dendreon, USA	Provenge®
	Sotio, Czech Republic	NCT02111577
Glioblastoma	Northwest Biotherapeutics, USA	NCT00045968
	Immunocellular Therapeutics, USA	Planned
	Pharmacell, Netherlands	
Cutaneous melanoma	Caladrius Biosciences, USA	NCT01875653
Colon carcinoma	Dandrit Biotech, Denmark	Planned
Uveal melanoma	University Hospital Erlangen, Dept. of Dermatology, Germany	NCT01983748

Table 2
Participating study centers

Erlangen	Prof. Friedrich Kruse, Dr. Harald Knorr, Dr. Ralph Meiller	+49-9131-85 44141 +49-9131-85 34478	Barbara.Stoll@uk-erlangen.de
Essen	Prof. Norbert Bornfeld, Dr. Claudia Metz	+49-201-72383471 +49-201-72383474	Claudia.Metz@uk-essen.de
Hamburg Eppendorf	PD Lars Wagenknecht, Dr. Bettina Fuisting, Dr. Matthias Keserü	+49-40-741052350	augenklinik@uke.de
Homburg/Saar	PD R. Arne Viestenz, Prof. Berthold Seitz	+49-6841-1622302 +49-6841-1622387	Arne.Viestenz@uks.eu
Cologne	PD Dr. Ludwig M. Heindl, Prof. Claus Cursiefen	+49-221-4784308	Augenklinik-Studien@uk-koeln.de
Lübeck	Prof. Salvatore Grisanti, Dr. Schulz-Wagenbarth	+49-451-5002217	Salvatore.Grisanti@uk-sh.de
Tübingen	PD Dr. Daniela Süsskind, Prof. Dr. Karl-Ulrich Bartz-Schmidt	+49-7071-2983730	Daniela.Suesskind@med.uni-tuebingen.de
Würzburg	Dr. Thomas Ach, Dr. Felix Guggenmoos-Schreyer	+49-931-20120458 +49-931-20120614	AK_Studien@ukw.de

induce an immune response against the loaded tumor antigens through activation of cytotoxic CD8 and CD4-helper T lymphocytes. This can ultimately lead to the elimination of the tumor by the patient's own immune system. The immune response is directed against the vaccinated tumor antigen and is therefore, antigen specific.

For the study, the patient's dendritic cells are loaded with RNA that has been extracted from the patient's tumor tissue and amplified my polymerase chain reaction (PCR). This RNA represents the transcriptome of the tumor cells, including presented antigens. If the vaccination induces immune cells specific for the tumor antigens, remaining tumor cells can be recognized and eliminated by the immune system.

If the promising results obtained from the treatment of cutaneous melanoma, prostate carcinoma, renal cell carcinoma, glioblastoma, and many other tumors can be transferred to uveal melanoma, a prolonged survival due to a delayed metastases can be expected. At best, progression of the disease can be completely stopped by the induction of cytotoxic T lymphocytes against tumor antigens.

3 Phase 3 Vaccination Study of Uveal Melanoma

The opthalmology clinics of the University Hospitals Erlangen, Essen, Hamburg-Eppendorf, Homburg/Saar, Cologne, Lübeck, Tübingen, and Würzburg are conducting a mulitcentric clinical phase 3 trial, offering patients with newly diagnosed, large uveal melanoma (T2-T4, AJCC TNM grading 2009) personalized immune therapy. The goal of the study is the prevention of metastases by the induction of cytotoxic T lymphocytes.

The investigational product, a so-called ATMP (advanced therapy medicinal product), is a personalized vaccine. It consists of autologous dendritic cells loaded with tumor RNA. The RNA has been extracted from the patient's tumor tissue and amplified prior to loading into the dendritic cells. The rationale behind this method is to equip the dendritic cells with the unique antigen repertoire of the tumor cells, to allow them to activate tumor antigen specific killer cells when transferred back into the patient.

To this end, during surgery of the primary tumor (preferentially enucleation), sample of tumor tissue, which must be a least the size of a pea, is dissected. From this sample of tumor tissue, the tumor RNA is prepared in a cleanroom and then transferred into expanded autologous dendritic cells by electroporation. The finished product is frozen and distributed to the participating centers. The vaccine is infused into the patients at the trial center ([11, 12]; Fig. 1).

For inclusion in the study, patients with assumed uveal melanoma must be identified at a participating center, prior to therapy of the primary tumor. The tumor tissue must be obtained in a participating study center under standardized conditions and then sent to the manufacturer of the vaccine (Experimentelle Immuntherapie, Hautklinik, Universitätsklinikum Erlangen). The tumor tissue must be kept in a solution containing RNase-inhibitors to prevent the degradation of tumor RNA, until the RNA is extracted and amplified in a cleanroom facility at the University Hospital Erlangen (Experimentelle Immuntherapie, Hautklinik). The complex procedure of RNA extraction from very small tumor samples was developed by the Dept. of Dermatology, University Hospital Erlangen, in cooperation with the Opthalmology Clinic of the University Hospital Erlangen.

The autologous dendritic cells are generated from precursor cells of the patient (monocytes). After addition of cytokines (GM-CSF and IL-4) and maturation stimuli, the monocytes differentiate into mature dendritic cells within 7 days. To obtain these monocytes, the patient must undergo leukapheresis. For leukapheresis the patient must travel to the University Hospital Erlangen only once. After loading of the dendritic cells with tumor RNA, the cells are aliquoted and frozen (Fig. 1). All production steps are performed in the cleanrooms of the University Hospital Erlangen and are compliant with the current EU GMP-guidelines.

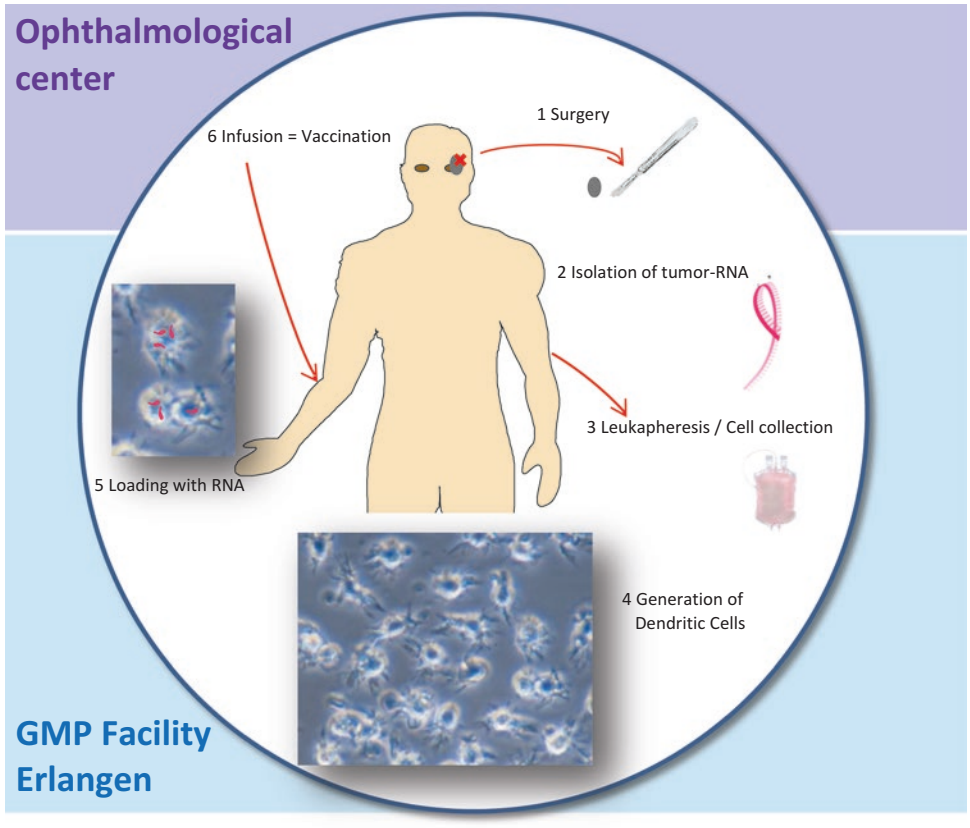

Fig. 1 Schematic representation of vaccination against uveal melanoma

The Department of Dermatology of the University Hospital Erlangen holds a manufacturing license according to §13 AMG. Thus, our production is under surveillance of local authorities. Our production areas meet the most stringent requirements for sterile production. Compliance with the guidelines includes monitoring of the production environment (pressure, particle, microbiological monitoring), qualification of instruments and premises, validation of processes and analytical methods, and adherence to strict manufacturing procedures. Before the vaccine is released and distributed to the centers, the investigational medicinal product undergoes extensive quality control, including sterility testing, endotoxins and mycoplasma testing, cell phenotype identification, cell viability, and cell count evaluations.

The vaccination regimen comprises eight intravenous vaccinations in a period of 2 years, administered in ascending intervals (2, 4, 6 weeks, 3 months, then every 6 months). The quality of life for the treated patients is almost completely unaffected. Side effects are usually limited to elevated temperature and flu-like symptoms shortly after administration of the vaccine. Very rarely, exanthema or vitiligo is observed.

References

1. Lipski AC et al (2013) Diagnosis of and therapy for choroidal melanoma. Klin Monbl Augenheilkd 230:1005–1019
2. Metz CH et al (2013) Uveal melanoma: current insights into clinical relevance of genetic testing. Klin Monbl Augenheilkd 230:686–691
3. Singh AD, Topham A (2003) Incidence of uveal melanoma in the United States: 1973–1997. Ophthalmology 110:956–961
4. Heindl LM et al (2010) Intraocular lymphatics in ciliary body melanomas with extraocular extension: functional for lymphatic spread? Arch Ophthalmol 128:1001–1008
5. Schlereth SL et al (2014) Absence of lymphatic vessels in the developing human sclera. Exp Eye Res 125:203–209
6. Schroedl F et al (2008) The normal human choroid is endowed with a significant number of lymphatic vessel endothelial hyaluronate receptor 1 (LYVE-1)-positive macrophages. Invest Ophthalmol Vis Sci 49:5222–5229
7. Prescher G et al (1996) Prognostic implications of monosomy 3 in uveal melanoma. Lancet 347:1222–1225
8. Moser JC et al (2015) The Mayo Clinic experience with the use of kinase inhibitors, ipilimumab, bevacizumab, and local therapies in the treatment of metastatic uveal melanoma. Melanoma Res 25:59–63
9. Shields JA, Shields CL (2015) Management of posterior uveal melanoma: past, present, and future: the 2014 Charles L. Schepens lecture. Ophthalmology 122:414–428
10. Tarlan B, Kiratli H (2012) Current treatment of choroidal melanoma. Expert Rev Ophthalmol 7:189–195
11. Schuler G, Schuler-Thurner B, Steinman RM (2003) The use of dendritic cells in cancer immunotherapy. Curr Opin Immunol 15:138–147
12. Wilgenhof S et al (2015) Long-term clinical outcome of melanoma patients treated with messenger RNA-electroporated dendritic cell therapy following complete resection of metastases. Cancer Immunol Immunother 64:381–388

INDEX

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9, © Springer Science+Business Media New York 2017

E

F

G

H

I

J

K

L

S

T

U

V

W

X

Y

Z