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mRNA modification and delivery strategies towards the establishment of a platform for safe and effective gene therapy

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Here's to the crazy ones. The rebels. The troublemakers. The ones who see things differently. While some may see them as the crazy ones, we see genius. Because the people who are crazy enough to think they can change the world, are the ones who do.

- Apple Inc. 1997

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LIST OF ABBREVIATIONS

2A	ribosome skipping peptide 2A
3′UTR	3′ untranslated regions
4-OHT	4-hydroxytamoxifen
AAV	adeno-associated virus
ACTB	actin bèta
AFM	atomic force microscopy
aIRES	anti-IRES
APC	antigen-presenting cell
ARCA	anti-reverse-cap-analogue
ARE	adenylate uridylate rich element
BLI	bioluminescence imaging
BSA	bovine serum albumin
CBP	cap-binding protein
cDNA	complementary DNA
CP	coat protein
CPE	cytoplasmic poly-adenylation element
CpG	cytosine-phosphate-guanine
Cq	quantification cycle
CTL	cytotoxic T lymphocyte
DAMPs	damage-associated molecular patterns
dATP	deoxyadenosine triphosphate
DCs	dendritic cells
dCTP	deoxycytosine triphosphate
DD	destabilizing domain
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMPE	dimyristoylphosphatidylethanolamine
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DOPE	1,2 dioleoyl-sn-glycero-3-phosphoethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOTMA	N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammoniumchloride
dsRNA	double-stranded RNA
eIF	eukaryotic initiation factor

e.p.	electroporation
FBF	fem-binding factor
FBS	foetal bovine serum
FDA	food and drug administration
GFP	green fluorescent protein
GL67	Genzyme Lipid 67; GL67:DOPE:DOTAP-PEG5000 (1:2:0,05)
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCRs	G protein-coupled receptors
GPR	global pattern recognition
HD	homology domain
hEGF	human epidermal growth factor
HIV	human immunodeficiency virus
Hsp	heat shock protein
i.d.	intradermal
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
IFN	interferon
IL	interleukin
iPS	inducible pluripotent stem
IRE	iron responsive elements
IRF3	interferon regulatory factor 3
IRP	iron regulatory protein
IVT	*in vitro* transcription
KTR	kinase translocation reporters
K-turn	kink-turn
LGP2	Laboratory of Genetics and Physiology 2
LID	ligand-induced degradation
m5C	5-methylcytidine
m^7G	7-methyl-guanosine
MDA-5	Melanoma Differentiation-Associated protein 5
MFI	mean fluorescence intensity
MOPS	3-(N-morpholino) propansulfonic acid
mRNA	messenger RNA
m1Ψ	N1-methyl-pseudouridine
NES	nuclear export signal
NK	natural killer cells

NLR	NOD-like receptors = nucleotide-binding oligomerization domain receptors
NMD	nonsense-mediated decay
NMR	nucleic magnetic resonance
nsP	nonstructural protein
nt	nucleotide
OAS	2'-5'-oligoadenylate synthetase
ORF	open-reading frame
PABP	poly(A)-binding protein
PAMPs	pathogen-associated molecular patterns
pDNA	plasmid DNA
PKR	protein kinase R
PSA	prostate specific antigen
PTC	premature termination codons
PUF	Pumilio and FBF homology proteins
PUM	Pumilio
RANTES	Regulated on Activation, Normal T-cell Expressed and Secreted = CCL5
RBP	RNA-binding protein
RIG-I	retinoic acid-inducible gene 1
RISC	RNA induced silencing complex
RLR	RIG-I-like receptors
RLU	relative light units
RNase L	ribonuclease L
RNP	ribonucleoprotein
s.c.	subcutaneous
s^2U	2-thiouridine
SafeR	Synthetic amplified RNA
SELEX	Systematic evolution of ligands by exponential enrichment
SGP	subgenomic promoter
shRNA	short hairpin RNA
siRNA	short interfering RNA
TAA	tumor-associated antigen
TetR	Tet repressor
Th	T helper cells
TLR	toll-like receptor
TMP	trimethoprim

tRNA	transfer RNA
tTA	tetracycline-controlled transactivator
VEE	Venezuelan Equine Encephalitis
Ψ	pseudouridine
ISG	interferon-stimulated genes

CHAPTER 1

GENERAL INTRODUCTION and AIMS OF THE STUDY

GENERAL INTRODUCTION

mRNA as a therapeutic modality is becoming increasingly popular in the fields of gene therapy and vaccination. mRNA has various advantages over pDNA-based therapeutics, for instance, 1) it can immediately express a protein of interest even in non-dividing cells, 2) it carries virtually no risk of genomic integration and oncogenic mutagenesis, and 3) due to its transient nature, there is no risk of potential side effects from permanent production of the therapeutic protein. mRNA has been shown to be useful for various applications including vaccination against infectious diseases [1-3], cancer immunotherapy [4,5], protein-replacement therapy [6,7], generation of induced pluripotent stem (iPS) cells (genetic reprogramming) [8], desensitization of allergies [9,10], and genome engineering [11,12].

However, unmodified mRNA produced by in vitro transcription (IVT) can stimulate innate immune receptors upon transfection into cells and cause substantial cell death [13-16]. For instance, toll-like receptors (TLRs) 3 and 7 are stimulated by double- and single-stranded RNA (ssRNA and dsRNA), respectively, inside the endosomes. Another group of innate immune sensors, the RIG-I-like receptors (RLRs), detect exogenous RNA in the cytoplasm. Members of the RLR group include: retinoic acid-inducible gene I (RIG-I) which recognizes short ssRNA, dsRNA, or uncapped RNA and melanoma differentiation-associated protein 5 (MDA-5) which recognizes longer dsRNAs or mRNAs without 2'-O-methylation of the penultimate nucleoside. Other pattern recognition receptors (PRRs) include the NOD-like receptors (NLRs), which bind nucleic acids or peptidoglycans of pathogens and cause the activation of inflammasomes leading to caspase-dependent programmed cell death. Stimulation of PRRs by exogenous RNA triggers overexpression of type I interferons (IFN-α and IFN-β) as well as type III IFN (IL-28A and IL-28B) resulting in the activation of interferon-stimulated genes (ISGs), such as Protein Kinase R (PKR) or RNase L, which play a role in the anti-viral response [17]. Upon stimulation of PRRs by exogenous RNA, mammalian cells use several mechanisms at different phases of the viral lifecycle to inhibit the replication of the pathogen. One of the most widely recognized defense mechanisms is the phosphorylation of eukaryotic translation initiation factor 2, α subunit, (eIF2α); at the serine 51 by PKR and cessation of cellular translation [18]. As a

consequence, viral replication is arrested due to deficiencies in essential viral proteins. However, many viruses have discovered ways to subvert this response by utilizing cap-independent initiation of translation using internal ribosome entry sites (IRESs) typically located within the 5' untranslated region (UTR) of the viral RNA. Additionally, activation of OAS2 by dsRNA and subsequent RNase L dimerization/activation causes degradation of all viral and cellular RNA, often resulting in cell death [19].

These antiviral mechanisms limit the therapeutic potential of IVT mRNA. However, the realization that nucleotide base modifications greatly improve the properties of mRNA as an expression platform by reducing the immunogenicity and increasing the stability of the RNA molecule has been pivotal in overcoming these hurdles [6,7,16,20-25]. Inclusion of specific nucleotide modifications, such as 5-methylcytidine (m5C), pseudouridine (Ψ) or 2-thiouridine (s2U) [21] makes the mRNA molecule less recognizable by pattern recognition receptors (PRRs).

AIMS OF THE STUDY

Gene-based immunotherapy has gathered much attention in the last decade as a promising approach to treat cancer or genetic disorders. Successful clinical trials led to the FDA approval of the first veterinary and human gene- and cell-based immunotherapies (Oncept™ and Provenge®, respectively).

While plasmid DNA (pDNA) is commonly used as the method of choice for vectored immunotherapy, it has many caveats including the necessity of the DNA to overcome the nuclear barrier, a particularly difficult challenge in an *in vivo* setting, where cells are non- or slowly dividing. Furthermore, the presence of an antibiotic resistance gene in pDNA and the possibility of mutagenesis due to integration of the vector into the genome raises safety concerns, which makes such therapies particularly difficult to obtain regulatory approval. Thus, more recently, mRNA-based approaches have become increasingly popular as an alternative to pDNA. In order to improve the stability and enable prolonged expression from mRNA, nucleotide modifications have been incorporated into therapeutic RNA to evade recognition by endosomal Toll-like receptors (TLR3, TLR7 or TLR8) or cytosolic RIG-I-like receptors (RIG-I, MDA-5).

The general goal of this PhD project was to develop a safe yet potent mRNA-based protein expression platform. To this end the following questions are addressed in this dissertation:

1. What are the advantages and disadvantages of mRNA and pDNA as gene therapy platforms?
2. What are the hurdles of mRNA-based gene therapy and how can we overcome them?
3. What are the molecular mechanisms that underlie the cytotoxic effects caused by transfected mRNA?
4. How can we alleviate the toxicity/immunogenicity of *in vitro* transcribed mRNA?
5. Which ribonucleoside modifications enable mRNA to express proteins most robustly *in vitro* and *in vivo*?

I. LITERATURE REVIEW

CHAPTER 2

mRNA as a safe and effective platform for gene-based therapeutics

The chapter is based on the publications:

Geertrui Tavernier[2], Oliwia Andries[1], Jo Demeester[2], Niek N. Sanders[1], Stefaan C. De Smedt[2], Joanna Rejman[2]; mRNA as gene therapeutic: how to control protein expression. *Journal of Controlled Release* 2011, 150(03): 238-247

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Oliwia Andries[†1], Tasuku Kitada[†3], Katie Bodner[3], Niek N. Sanders[§*1] and Ron Weiss[§*3]; Synthetic biology devices and circuits for RNA-based "smart vaccines": a propositional review. *Expert Review of Vaccines (SPECIAL FOCUS | RNA-Based Vaccines),* 2015

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INTRODUCTION

mRNA-based therapeutics have the potential to be used for a myriad of applications including protein replacement therapy and vaccination. The principles behind the two therapies are relatively straightforward: the introduction of wild-type proteins into cells to “correct” for an abnormal gene (protein replacement therapy) or expression of antigens from RNA to trigger an immune response (vaccination). However, in practice, a great amount of optimization is required to transform an mRNA molecule into an effective therapeutic. In this introductory chapter, we provide an overview of some of the currently understood principles behind how to optimize an mRNA molecule for therapy. Furthermore, we discuss various strategies to efficiently deliver RNA into cells and then we describe a few possible applications for mRNA therapy.

Half-life and translatability of IVT mRNA

Since the elucidation of mRNA structure and its chemical synthesis [26], it was generally believed that, compared to DNA, mRNA is a fairly unstable molecule, especially once it reaches the cytoplasm where it is exposed to degrading enzymes. The main reason for its instability is the presence of a hydroxyl group on the second carbon atom of the sugar moiety, which, due to sterical hindrance, prevents mRNA from adopting a stable double β-helix structure and which makes the molecule more prone to hydrolytic degradation. Initial reports of intracellular mRNA delivery were subject to skepticism, mainly because of the belief that mRNA is extremely labile and could not withstand the transfection protocols.

Cap structure

The 5′ ends of mRNA are modified post-transcriptionally in the nucleus with a methylated m7GpppN-cap structure. This modification plays a role in mRNA splicing, stabilization, transport and, most importantly, it facilitates the translation process by recruiting ribosomes. The eukaryotic initiation factor 4G (eIF4G), which is the scaffold molecule of the holo-enzyme complex eIF4F, contains a cap-binding eIF4E, an RNA helicase eIF4A and eIF3, a complex that associates directly with the 40S ribosomal

subunit. The concept that the cap structure is essentially required for recruitment of ribosomes was put to the test with the discovery that internal ribosome entry sites (IRES), present in some viral and cellular mRNAs, are able to attract ribosomes even when the 5′ cap is blocked or missing [27]. Still, the cap structure has proven to be imperative for normal mRNA function [28]. Initially, the mRNA cap binds to the cap-binding protein (CBP) heterodimer CBP80-CBP20. This protein complex regulates transport of the mRNA from the cytoplasm to the nucleus and plays a crucial role in monitoring the quality of the mRNA molecule via nonsense-mediated mRNA decay (NMD), a process by which mRNAs with premature stop codons (e.g. due to errors introduced by RNA polymerase) become degraded. The degradation of mRNAs takes place in the cytoplasm at sites called P-bodies [29]. Until now, up to forty P-body proteins have been described, including Xrn 5′-3′ exonucleases, decapping and de-adenylating enzymes. The cap structure protects against Xrn1 in the cytoplasm and against Xrn2 in the nucleus because of its 5′-5′ linkage [30]. The cap structure is an essential part of the mRNA molecule, especially if one wishes to introduce an exogenous mRNA into the cell [28]. When mRNA is synthesized *in vitro*, the cap structure may be incorporated into the RNA in the reverse orientation, causing only half of the *in vitro* generated mRNAs to be functional. This can be averted by the use of an anti-reverse-cap analogue (ARCA), a modified cap structure in which the 3' OH (closer to the m7G) is methylated. This forces the ARCA to be incorporated in the right orientation, leading to close 100 % yield of translatable mRNA [31].

Poly(A) tail

Also the 3′ ends of mRNAs are post-transcriptionally tailored by an enzyme which adds a series of adenine nucleotides. The length of this poly(A) tail is crucial. It has been shown that all actively translated mRNAs in mammalian cells contain 100 to 250 A residues [32]. To be translated efficiently, the poly(A) tail of exogenously delivered mRNAs should consist of at least 20 A residues [33,34]. Moreover, it has been described that mRNA expression positively correlates with poly(A) tail length [33-35]. Several groups have reported that mRNAs containing a cytoplasmic polyadenylation element (CPE, a specific nucleotide sequence at the 3′ UTR), can initiate a process, which elongates the poly(A) tail in the cytoplasm, so that mRNAs can be turned from a repressed into an active molecule [36,37]. However, up until now, this process has only been shown in

cells in early development. Interestingly, a synergistic effect of the cap structure and the poly(A) tail on translation efficiency has been demonstrated by several research groups [28,38-41]. This synergism has been explained by the formation of a cap-eIF4E-eIF4G-PABP-poly(A) closed loop structure that could facilitate the recycling of ribosomes [40] and/or protect the mRNA against exonucleolytic nucleases [42]. On the other hand it has been reported that disruption of eIF4G–PABP interaction, still leads to a synergistic effect, albeit of smaller magnitude [41]. The fact that synergy is only seen in cells and not in cell-free translation systems, has been proven to be a result of the presence of competitor mRNAs in cells, which enforces the combined use of both cap and poly(A) [35]. This is also supported by the notion that co-delivery of exogenous free poly(A) tails results in a 2 to 9-fold higher transfection efficiency [41,43].

3' and 5' UTRs

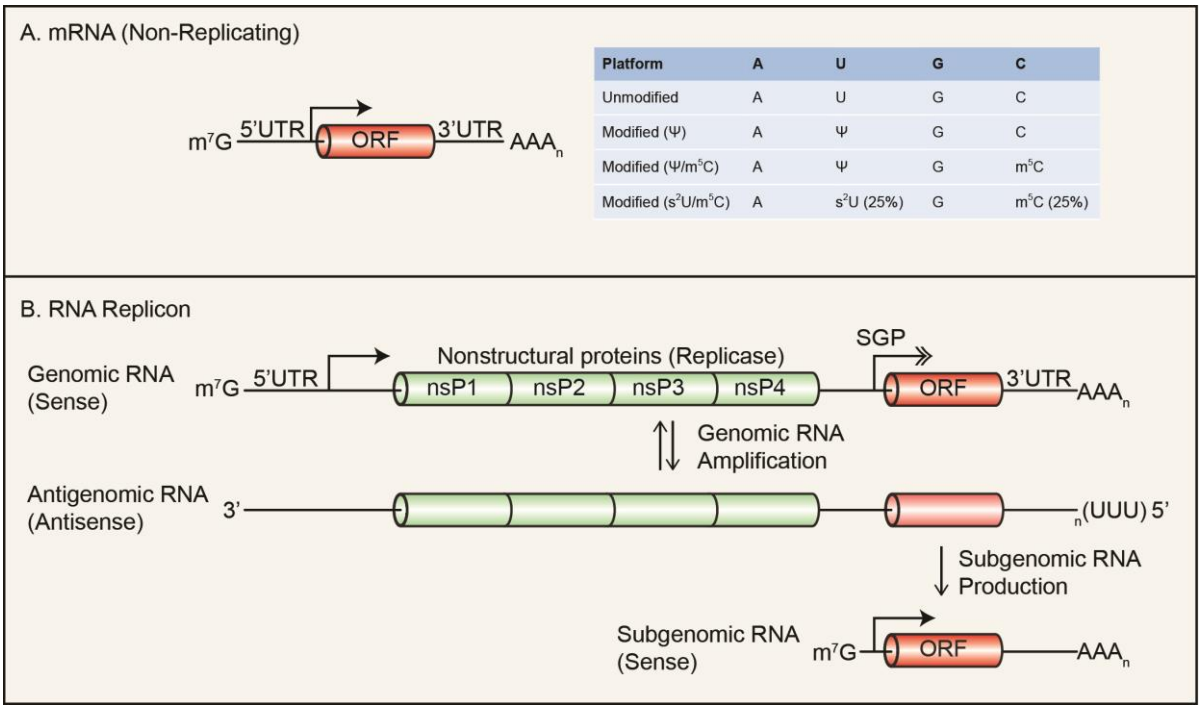
Most eukaryotic mRNAs contain mRNA decay signals in their 3′ untranslated regions (3′ UTRs). The most extensively studied are the Adenylate Uridylate Rich Elements (AREs). Many AU-rich mRNA sequences exist. They affect mRNA stability to different extent. It has been demonstrated that mRNAs that contain ARE are unstable (mostly because of rapid removal of the poly(A) tail) [44] and that their half-life increases when ARE is replaced by the 3′ UTR of a stable mRNA (e.g. β-globin or Venezuelan Equine Encephalitis virus - VEEV)[45,46]. The mechanism of the destabilizing power of ARE is not very well understood. It appears, however, that specific AU sequences destabilize mRNA in their own manner, which depends on the mRNA itself, as well as on the cell type and growth conditions. Indeed, the destabilizing activity of ARE can be decreased or increased due to interactions with other particular mRNA sequences (e.g. U-rich region) or with ARE binding proteins. Interestingly, ARE can destabilize constitutively or they can work as regulatory elements [47]. Another form of 3′ UTRs are the Iron Responsive Elements (IREs), present in mRNAs encoding proteins that affect iron homeostasis (e.g. transferrin and ferritin). They respond to intracellular iron concentration by binding of the IRP (Iron Regulatory Protein). The effect of IREs depends on their precise location. They regulate mRNA half-life when present at the 3′ UTR and will affect translation when located at the 5′ UTR [48]. Several other destabilizing 3′ UTR, and also 5′ UTR, have been discovered (e.g. stem-loop of insulin-like growth factor II) [49].

In summary, when aiming at transfecting cells with exogenous mRNA, the *in vitro* transcribed mRNA molecule should at least be provided with a cap structure and a poly(A) tail containing at least 20 A residues to ensure an acceptable half-life [50]. Further optimization of the mRNA structure can be done by replacing unstable non-coding sequences with non-coding sequences of mRNAs known as stable (e.g. β-globin). Also coding mRNA regions can accelerate mRNA decay. To tackle this problem, one could change nucleotides so that a different codon triplet is formed, still matching with a tRNA carrying the same amino acid (codon optimization) [51].

mRNA platforms: modified and replicating

Modified mRNA and replicating mRNA are two of the most promising platforms on which therapeutic genes may be encoded. One of the challenges that must be overcome when using such mRNAs for gene expression in mammalian cells is the antiviral innate immune response (i.e. activation of the interferon (IFN) and NF-κB pathways). mRNAs transfected into mammalian cells are subject to detection by PRRs such as the endosomal toll-like receptors (TLRs) TLR3, TLR7, and TLR8 and the cytosolic RIG-I-like receptors (RLRs) RIG-I, MDA-5, and LGP2 [52]. These sensors are involved in the recognition of RNA species that are “non-self” (e.g. viral RNA). Stimulation of these receptors leads to activation of the IFN and NF-κB signaling pathways and subsequent translation inhibition by protein kinase R (PKR), mRNA degradation by ribonuclease L (RNase L), inflammatory cytokine expression and programmed cell death. The innate immune response is particularly problematic when carriers such as cationic liposomes or polymers are used for the delivery of mRNAs into cells (for a recent review on nucleic acid delivery methods see [53]). Carrier-mRNA complexes, which often have a net positive charge, bind the negatively charged cell membrane through electrostatic interactions and are subsequently taken up into endosomes via endocytosis, where the mRNAs are sensed by TLRs. Depending on the efficiency of the carrier, this may result in a very strong innate immune response. In contrast, when physical mRNA delivery methods such as electroporation or the gene gun approach are used, the mRNA does not encounter endosomal TLRs, and thus, the innate immune response may be less severe compared to when chemical carriers are used. However, the induction of an innate immune response is still a major concern

in cells that are known to possess high levels of PRRs such as epithelial cells as shown by us and others [14,54]. This problem has now been largely solved by the pioneering research of Kariko and colleagues which demonstrated that the immunogenicity of mRNA molecules could be greatly reduced by the incorporation of base modifications such as pseudouridine (Ψ) into the mRNA [16]. Kariko and colleagues showed that mRNAs with Ψ can evade PRRs, reduce PKR activation, and are more resistant to RNase L [16,20,21,23]. Subsequently, others followed suit and identified other combinations of base modifications that provide similar types of effects [6,55] as depicted in Figure 2.1A. For the purpose of mRNA vaccination, however, some level of innate immune activation may be beneficial to induce a potent adaptive immune response.



Platform	A	U	G	C
Unmodified	A	U	G	C
Modified (Ψ)	A	Ψ	G	C
Modified (Ψ/m^{5}C)	A	Ψ	G	m^{5}C
Modified (s^{2}U/m^{5}C)	A	s^{2}U (25%)	G	m^{5}C (25%)

Figure 2.1. Structures of RNA platforms for vaccination. (A) Unmodified and modified (non-replicating) RNA structures. (B) Alphaviral RNA replicon structure.
m^{7}G: 7-methyl-guanosine; UTR: untranslated region; ORF: open reading frame; AAA$_n$: poly(A) tail; Ψ: pseudouridine; m^{5}C: 5-methyl-cytosine; s^{2}U: 2-thiouridine; nsP: nonstructural protein; SGP: subgenomic promoter.

While cellular antiviral pathways have evolved into very complex innate immune signaling networks [52], viruses have also developed a myriad of sophisticated counter-strategies to dampen the IFN response or to avoid being recognized by the host cell

[56]. Thus, RNAs derived from viruses provide another attractive option for a therapeutic platform. In particular, the RNA "replicon" approach in which non-essential structural proteins (but not RNA replicase proteins) are deleted from the genome of the virus and replaced with a gene of interest has gained popularity as a safe and robust mean of exogenous protein expression [57]. Major advantages of the RNA replicon approach include its strong expression level and long duration of expression due to its "self-replicating" properties. As an example, the mechanism of replication of an alphaviral RNA replicon has been depicted in Figure 2.1 B (for a review see [58]). Geall and colleagues recently showed that gene expression from alphaviral RNA replicons can last for at least seven weeks *in vivo* when replicon RNA was packaged in lipid nanoparticles and injected into the muscle of mice for vaccination [59]. Other groups have successfully used alphaviral replicons for the purpose of induced pluripotent stem cells (iPS) reprogramming [60] or even *in vivo* artificial miRNA delivery [61] demonstrating their potential as a broad-purpose gene expression vector. More recently, to facilitate the use of alphaviral replicons as a platform for synthetic gene circuit engineering, our collaborating group created a mathematical model for *Alphavirus* gene expression kinetics using high-density time course data [62].

In Table 2.1, we summarize the differences in the properties of the non-replicating and replicating mRNA platforms discussed above.

Platform	Size	Expression level	Duration of expression *in vivo* (i.m. injection)	Innate immune Stimula-tion	Amplifica-tion in cells	Ref.
Unmodi-fied mRNA	Typically > ~500 nt	Low	~1 week*	High	No	Reviewed in [63-65]
Modified mRNA	Typically > ~500 nt	Medium	~4 weeks*	Low	No	[6,23,55]
RNA replicon	> ~8000 nt	High	~7 weeks	High	Yes	Reviewed in [65-67]

*Authors' results presented in Chapter 5.
i.m.: intramuscular.

Table 2.1: Comparison of RNA platforms for vaccination.

It should be emphasized that one mRNA platform is not generally better than the other, and the specific application of interest will ultimately determine which platform to choose to bring out the maximum potential of mRNA-based therapy.

The nuclear barrier: challenging for pDNA but irrelevant for mRNA

Multiple extracellular and intracellular barriers pose serious limitations to non-viral gene delivery. Newly designed lipid and polymer formulations have significantly improved the uptake and the endosomal escape of pDNA, leaving the nuclear envelope the main obstacle for non-viral pDNA transfer. Indeed, several groups have demonstrated that microinjections of plasmid DNA into the cytoplasm of non-dividing cells result in very low levels of gene expression. In contrast, intra-nuclear injection of the same number of pDNA copies leads to 100 % transfection of the injected cells [68-70]. One possibility for pDNA to enter the nucleus is during cell division, when the integrity of the nuclear envelope is temporarily lost. In fact, it has been shown that dividing cells are more easily transfected than cell-cycle arrested cells [71-75]. However, the advantage of the temporary absence of the nuclear envelope during mitosis will not be generally applicable in gene therapy because in most cases the target cells will divide slowly or not at all.

The easiest approach to overcome the obstacle presented by the nuclear envelope would be to develop a cytoplasmic expression system. mRNA, being translated in the cytosol, would seem to serve that purpose perfectly. mRNA does not need to enter the nucleus to perform its function and thus avoids a major limiting factor for efficient gene transfer. In this way, mRNA allows transfection of different cell types in the human body, including quiescent or slowly proliferating cells, such as vascular endothelia, muscle cells, hepatocytes or brain cells.

Methods for mRNA delivery

The spontaneous uptake of naked nucleic acids by cells is a very inefficient process. In principal two methods of nucleic acid delivery can be distinguished: the viral and the non-viral delivery systems. The viral vectors have been studied extensively for pDNA

delivery, although reports exist also where mRNA is packaged into RNA viruses [76-78]. However, gene expression after viral transfection is difficult to control and certain viral vectors integrate their genome into that of the host cells. Moreover, the immune system is also an important barrier for viral vectors. Finally, the production of clinical grade viral vectors is expensive and time consuming. Therefore in this chapter, we will focus on non-viral delivery methods (illustrated in Figure 2.2), which can be classified in two subgroups; those that physically disturb the barrier function of the cell membrane and thus provide a passage for mRNA (electroporation, ultrasound or gene gun) and those that employ cationic carriers (lipo- and polyplexes), which are taken up by endocytosis and thus facilitate the entry of the mRNA.

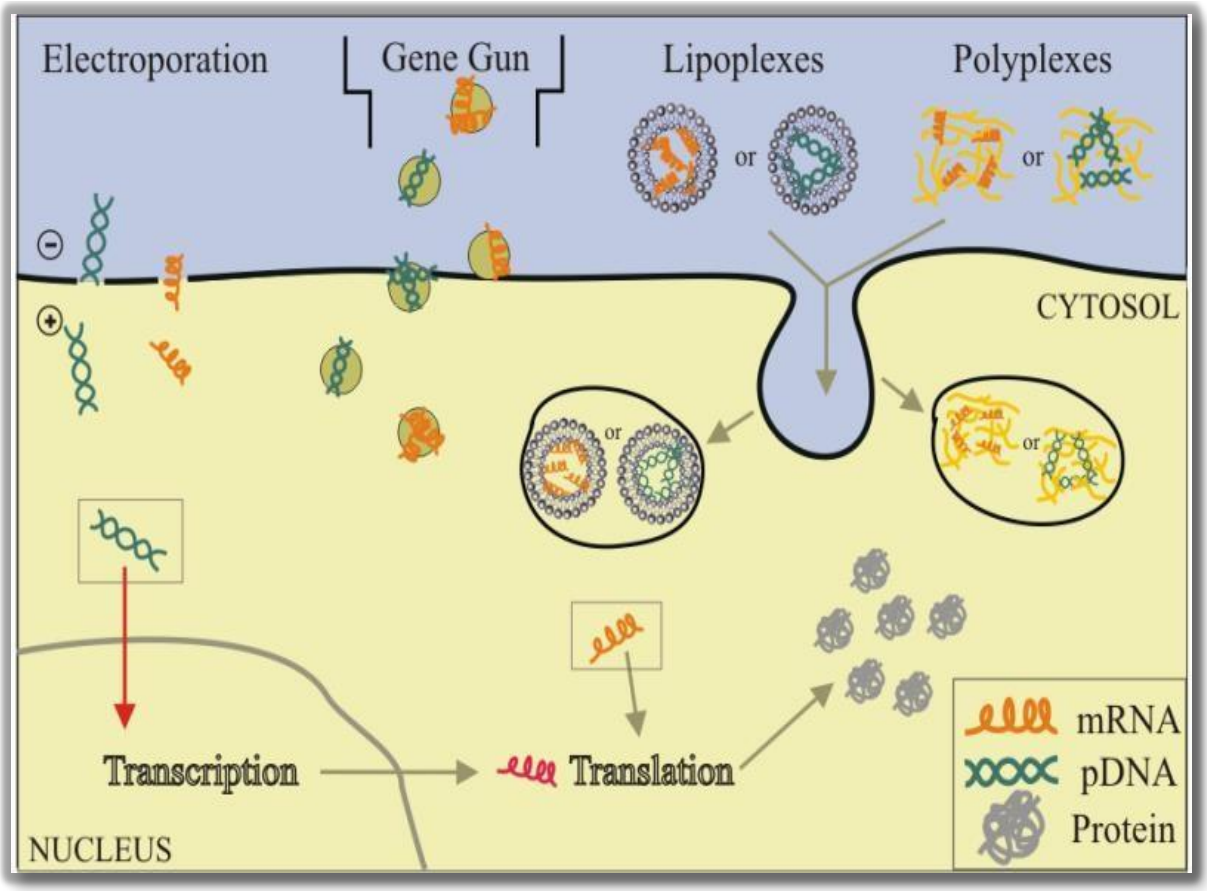


Figure 2.2. Non-viral delivery methods of mRNA. Electroporation is a physical delivery method based on applying of an electrical current to cellular membrane, increasing its permeability for gene-based therapeutics. Gene gun (biolistic technique) delivers gold particles precoated with nucleic acids straight to the cytoplasm. Lipoplexes and polyplexes are non-viral delivery methods in which negatively charged pDNA or mRNA is complexed with positively charged lipids or polymers, respectively.

Electroporation and gene gun delivery

Electroporation is a gene delivery method which was originally developed for *in vitro* transfection. An external electrical field is applied to a cell in the presence of a nucleic acid containing solution, leading to enhanced electrical conductivity and permeability of the cell membrane. When the voltage over the plasma membrane becomes higher than its dielectric strength, pores are formed resulting in the transmembrane passage of the nucleic acids. Both strength and duration of the applied electrical field should be chosen carefully, so that pores can close again when extracellular material has been introduced into the cell. If not, cells can be severely damaged or even die [79]. The *in vivo* applicability of electroporation was first demonstrated by Mir et al. [80], who used this technique to deliver a drug (bleomycin) in several types of tumors. Since then, the technique has been shown to introduce naked pDNA *in vivo* into several types of tissue; however the limited accessibility of less superficially localized organs remains an issue. mRNA electroporation has several advantages over pDNA electroporation. First of all, it is less toxic because less stringent electrical settings are required as the mRNA has to cross only the cell membrane to perform its function as opposed to both the cell and nuclear membrane in the case of pDNA [81]. Electroporation with mRNA has been explored elaborately in dendritic cells (DCs) because of their possible use in vaccination strategies [82]. Electroporation of DCs with mRNA is a safe and relatively easy method and it has already been tested in clinical trials (e.g. transfection of mRNA encoding prostate specific antigen (PSA)) [83]. In addition to DCs, also other cell types have been successfully electroporated with mRNA and used in adoptive cell therapy [84,85].

Another method, which can intracellularly deliver genetic material by breaking the existing barriers, is the gene gun, a biolistic delivery system. This transfection device, originally designed for plant transformation [86], uses high velocity heavy metal (often gold) particles coated with nucleic acids, which are released once they reach the aqueous intracellular environment. Since the initial work was performed, the technique has been refined: a hand-held device facilitates its use; both transfection efficiency and cell viability have been improved. Moreover, the applicability on most tissues, including several mammalian, has been demonstrated [87-89]. Initial reports about biolistic delivery

of mRNA were aimed at the evaluation of mRNA decay rates. Rajagopalan et al. [90] used a gene gun to deliver exogenous mRNA (encoding granulocyte-macrophage colony stimulating factor or β-globin) into peripheral blood mononuclear cells and found mRNA half-lives varying between 9 and 80 min, depending on whether or not destabilizing factors were present. Gene gun bombardment for successful mRNA transfection has been shown both *in vitro* as *in vivo* in several cell types and tissues. When mRNA encoding alpha-1 antitrypsin was delivered in mice, a strong antibody response was seen, indicating the possibility of using this technique as a vaccination strategy [91]. Sohn et al. used the technique to deliver mRNA encoding human epidermal growth factor (hEGF) and observed increased wound healing [92].

Lipo- and polyplexes

The complexation of nucleic acids (negatively charged) with cationic lipids or polymers occurs spontaneously through charge–charge interaction, forming lipo- or poly-plexes, respectively. The complexes thus formed are usually slightly positive, facilitating interaction with the negatively charged cell membrane, after which they can be taken up in the cell by endocytosis [93-96]. The advantage of net positive charge of complexes *in vitro* is, however, overshadowed *in vivo* by possible interactions with negatively charged serum proteins, which results in the rapid clearance of such formed aggregates [97]. This hurdle can be partially overcome by shielding the cationic complexes with charge-neutralizing polyethylene glycol (PEG). Cationic carriers not only serve to condense nucleic acids into small particles (several hundred nm) but also to protect them against degradation [98]. A wide variety of cationic lipids and polymers has been elaborately tested for their potential to complex and deliver pDNA into cells, both *in vitro* and *in vivo*. It is only since the beginning of the millennium that the technique has been implemented for mRNA delivery, although a first report where a polymer (DEAE-dextran) is used to complex *in vitro* synthesized mRNA to transduce cells already dates back to 1973 [99]. The first mRNA transfection by means of lipofection was performed by Malone et al. [100]. They were able to deliver mRNA encoding luciferase to different cell lines by condensing it with DOTMA/DOPE (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl-ammoniumchloride/1,2dioleo-yl-sn-glycero-3-phosphoethanolamine). The authors observed a linear relationship between activity of luciferase and the quantity of introduced mRNA. An overall conclusion when

considering all studies on mRNA transfection by means of non-viral cationic carriers is that the delivery of mRNA by means of cationic lipids resulted in a significantly better outcome than when cationic polymers were used. Bettinger et al. [50] transfected different cell types with a variety of cationic carriers that were already tested for pDNA delivery. They tested linear and branched polyethylene imine (PEI), poly-L-lysine and polyamidoamine dendrimer and demonstrated a very low potency for mRNA translation. However, if shorter polymers were used, the electrostatic interaction with mRNA was weaker, resulting in a slightly better expression. DOTAP (1,2-dioleoyl-3-trimethylam-monium-propane) is one of the most extensively studied lipid carriers for cellular delivery of mRNA [50,101-104] and it proved to possess superior efficiency in several comparative studies.

Applications for mRNA as a drug molecule

mRNA-loaded dendritic cells vaccine

Dendritic cells (DCs) are the most potent cells in presenting antigens through major histocompatibility complex (MHC) class I and II proteins and are thus capable of eliciting both cellular and humoral immune responses. The pioneered by Gilboa group [105] principle of classical vaccination is based on pulsing DCs with previously defined antigenic peptides. Although this method has proven its relevance in the past, the main drawback is the restriction of the immune response to a limited number of human leukocyte antigen (HLA) type-restricted leukocytes. This problem can be overcome by loading DCs with proteins, cDNAs or mRNAs to induce immune responses to a host of immunogenic epitopes. However, in case of cancer vaccination one has to take into account that many patients have microscopic amounts of tumor, limiting the practicability of loading DCs with whole cell protein extracts. Moreover, whole cell protein extracts contain many irrelevant antigens, which can cause autoimmune responses or present immunodominance problem. For that reason, nucleic acid vaccinations represent an interesting alternative. Moreover, when considering vaccination against infectious diseases, mRNA vaccinations eliminate the risk of mutation and uncontrollable proliferation of inactivated pathogens [106].

Cancer is no longer considered a single disease but instead a complex interaction of many pathologies that actively change the tumor microenvironment. Tumors are heterologous compositions of many cell types in abnormal states. This feature of cancer pathology renders cancer vaccines that target just one tumor antigen, less effective. On the other hand, vaccination with several antigens may introduce a new problem, namely immunodominance, in which CD4+ and/or CD8+ T cells preferentially respond to certain epitopes leaving others unattended [107,108]. Another hurdle in cancer vaccination, especially in tumors diagnosed in an advanced stage, is the immunosuppressive network of immune cells, cytokines and other proteins that subvert tumor surveillance. Monoclonal antibodies that block T cell inhibitory signaling are very effective in immunomodulation of the cancer environment. For example, the anti-CTLA-4 antibody (Ipilimumab) enhances activation of antitumor effector T cells and has been approved by the FDA as a cancer drug. Immunomodulation has proven to boost anti-cancer vaccination and thus should be treated as an essential component of immunotherapy.

Most groups using the mRNA vaccination strategy described earlier, tested its application in the immunotherapeutic treatment of different cancers. The precedent was set by Conry et al. [109], who measured the immune response in mice after injection of a liposome/mRNA vaccine encoding human CEA (carcinoembryonic antigen). Other groups showed specific immune responses against OVA (chicken ovalbumin) [110], hTERT (human telomerase catalytic subunit) [111], AFP (α-fetoprotein, a protein specifically expressed by hepatocellular carcinoma cells) [112], tTERT (truncated TERT which can serve as a universal tumor-associated antigen) [113], RHAMM (the receptor for hyaluronan-mediated motility, frequently overexpressed in brain tumors) [114] and IL-13ra2 (often overexpressed in brain tumors) [115] when DCs were loaded with the respective mRNA.

Transfection of DCs with patient's total tumor RNA and their subsequent re-administration is not only feasible but is also beneficial because of the broad array of epitopes that can be presented. The success of this method was demonstrated by tumor-specific responses both *in vitro* [116,117] and in phase I and II clinical trials [118-120]. Although the patient-specific antigens cannot be presented when non-autologous tumor-mRNAs are used, Mu et al. [117] demonstrated an improved clinical outcome of

patients treated with DCs electroporated with mRNA from allogeneic prostate cancer cell lines. It is worth mentioning that traditional clinical trial designs, aimed at assessing the safety of chemotherapeutic or biological agents, are not suited for cell-based therapies such as DC vaccines. The reason is that in the classical design the maximally tolerated dose is defined in phase I and this concentration is then further used in phase II and phase III clinical trials. Because DC vaccines seem to be inherently safe, it is always possible to increase the dose, however a higher dose will not necessarily render the optimal immunological or clinical response. Nonetheless, (pre)clinical trials show that DC vaccines are well tolerated and only minimal toxicities (such as grade I skin reactions and/or flu-like symptoms) were observed [83,121].

Therapeutic cancer vaccination with mRNAs that encode tumor-associated antigens (TAAs) *in vivo* has in the last decade gathered much attention as a promising alternative for dendritic cell (DC)-based vaccines. Although, clinical trials with the latter vaccines have resulted in promising outcomes, they do not allow mass production due to their laborious manufacturing process. Recently, the potential of mRNA cancer vaccines has been confirmed in several finished and ongoing clinical trials [120,122]. These trials demonstrated that mRNA cancer vaccines are at least as effective as DC-based vaccines.

mRNA vaccine against infectious diseases

Different groups have shown that mRNA is at least equally potent as proteins in eliciting CD8+ and CD4+ T-cell responses [123,124]. Nucleic acid vaccines are easy to manufacture and relatively inexpensive. Although pDNA can be taken up and expressed by cells *in vitro and in vivo*, its use as nucleic acid vaccine has some disadvantages as compared to the use of mRNA. As mentioned before, DNA can integrate into the host genome, causing inactivation of cellular genes or oncogenesis. Another disadvantage is the fact that DNA provides a long duration of expression of immunizing antigens, while it has been demonstrated that the capacity of mRNA to cause a boost in antigen expression is desired when aiming for optimal vaccination [121,125].

The mRNA vaccination strategy can be of interest to induce protective anti-viral immunity. In 1993, Martinon et al. demonstrated the potential of a liposome-entrapped mRNA vaccine against influenza in a mouse model [126]. Since then, murine DCs have

been electroporated with several viral antigens in the form of their corresponding mRNAs (lymphocytic choriomeningitis virus glycoprotein by Zarei et al. [127]), HCV–NS3/4A (Hepatitis C virus type NS3/4A by Yu et al. [128]) and HPV16 E7 (human papillomavirus type 16 oncoprotein E7 by Dell et al. [129]). They all showed a specific CTL response. Moreover, Dell et al. [129] demonstrated an enhanced DC migration due to higher cytokine production. Very recently, the german RNA vaccine company CureVac opened, aside their mRNA cancer vaccination activities, a new Phase I clinical trial with an anti-rabies vaccine that is based on their RNActive® platform [130]. Additionally, different injection sites have been examined (intravenous, intradermal, intramuscular, intranodal, intra-pinnal) demonstrating that the administration route of the mRNA vaccine is critically important. Hoerr et al. [131] showed in their study a huge difference in specific CTL response after intravenous (i.v.), subcutaneous (s.c.), intramuscular (i.m.) or intradermal (i.d.) injection of protamine-condensed mRNA into the ear pinna. Only the latter administration route showed a significant CTL response. Interestingly, mRNA can serve not only as a molecule encoding the antigen but also as an adjuvant by enhancing immunological responses and antigen presentation [15]. Indeed, as mentioned previously, mRNAs can be recognized by TLRs which can initiate an innate immune response. Therefore, the use of mRNA to express antigen has gained more and more attention in the battle against viral infections and cancer.

Anti-allergy immunotherapy

mRNA-based immunomodulation finds also its application in anti-allergy therapies. In 2009, Roesler et al. [132] showed a proof-of-concept that vaccination with mRNAs encoding 29 different pollens was a preventive measure against type I allergies [10].

Passive immunoprophylaxis

Immunoprophylaxis through vector-based expression of broadly neutralizing antibodies is a promising approach for preventing and combating viral infections or cancer[133-138]. Using a viral vector based on an adeno-associated virus (AAV), Balazs et al. demonstrated that expression of neutralizing antibodies can provide long-lasting protection against influenza challenges in mice[139]. His and others' approaches were also shown to be successful in fighting HIV and other pathogens[140-143]. However,

possibly due to viral DNA integration into the host genome, AAV injection leads to life-long protein expression, which is not ideal for immunization against frequently mutating viruses. Hence, we believe that RNA-based expression of antibodies in patients will soon become a safer alternative. Indeed, during this doctoral research, I was able to confirm the feasibility of RNA-based production of antibodies against infectious diseases (influenza, HIV) and cancer cells (CD20-positive non-Hodgkin's lymphoma, Rituximab), as presented in Figure 2.3.

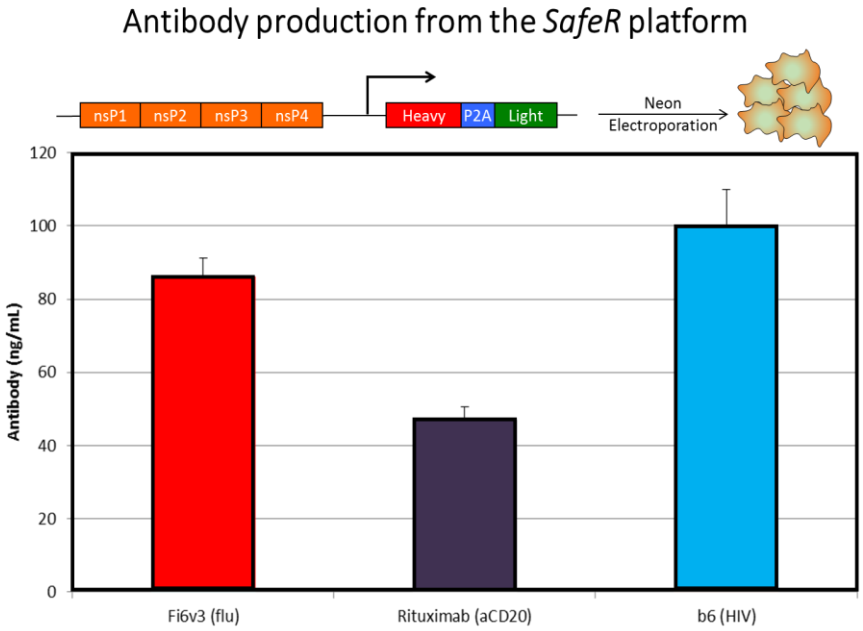


Figure 2.3. Vector-based antibody production in murine muscle cell line (C2C12) following electroporation with Synthetic amplified RNAs (*SafeR*), also called self-replicating RNA (see Chapter 2 - mRNA platforms: modified and replicating).

Tailoring the immune responses for different applications

Numerous modifications were proposed in order to obtain a stronger CTL response after mRNA vaccination. Zhang et al. [144] genetically modified DCs with lymphotactin prior to mRNA loading and they obtained a stronger immune response. Other examples are the incorporation of ubiquitin prior to the TAA sequence in the mRNA construct, species-specific codon optimization of mRNA as well as improvement of stability by addition of UTR sequences from β-globin [145-147]. When developing immunotherapeutic strategies, the main focus has been on inducing potent strong

CD8+ CTL responses but it has become clear that CD4+ T cells also play an important role by providing the tools for the expansion and persistence of these CD8+ T cells [121]. To ensure the concomitant activation of both arms of the immune response, different measures were investigated. A promising technique is co-transfection, in which mRNAs coding for adjuvants improving the stimulation of the CD4+ T-cell response, are delivered in the DCs in addition to the antigen-coding mRNAs. Co-transfection with mRNAs encoding cytokines stimulating signaling pathways showed a clear enhancement in CD4+ T-cell stimulation [5,145,146,148-151].

CONCLUSIONS

mRNA has been considered in the past as too labile to ensure protein expression. However, numerous studies have demonstrated the contrary; not only is mRNA capable of tolerating the impact of transfection protocols and of being translated efficiently, but it also has advantages over the use of pDNA. The high expression in non-dividing cells and the absence of antibiotic resistance genes are two important advantages. Additionally, the higher safety, due to the avoidance of genomic insertion, and no need to provide for a promoter and a terminator decide in favor of further research to advance mRNA's performance in the clinics. We are convinced that mRNA will prove its utility as a therapeutic molecule for many other objectives.

II. EXPERIMENTAL STUDIES

CHAPTER 3

Comparison of the gene transfer efficiency of mRNA/GL67 and pDNA/GL67 complexes in respiratory cells

The chapter is based on the publication:

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INTRODUCTION

The respiratory tract has been the target of many pre-clinical and clinical gene therapy studies. This is due to the fact that the target cells in the respiratory tract are easily accessible as they are only separated from the environment by a thin layer of mucus or liquid [152]. Additionally, a huge variety of lung diseases such as cystic fibrosis (CF), asthma, emphysema, lung cancer, α1-antitrypsin deficiency, and surfactant protein-B (SP-B) can potentially be treated via gene therapy [152]. Promising results have been obtained after the pulmonary administration of certain viral gene vectors [153]. However, the immunogenicity of viral vectors impedes their re-administration, and the risk of insertional mutagenesis and recombination with wild type viruses restrict their clinical use [153]. Therefore, non-viral vectors complexed with pDNAs have been extensively evaluated as safer and less immunogenic alternatives. A major disadvantage of non-viral vectors is their low gene transfer efficacy, which is caused to a large extent by their inability to deliver pDNA into the nucleus of non-dividing cells. It has been shown that after cytoplasmatic microinjection of pDNA less than 0.1 % of the pDNA molecules reached the nucleus [154]. In line with this, Capechhi *et al*. demonstrated that microinjection of pDNA in the nucleus resulted in a gene expression in most of the cells, while no significant expression was detected after microinjection of pDNA in the cytosol [68]. Many strategies have been evaluated to increase the nuclear delivery of pDNA [155,156]. Unfortunately, none of them have resulted in a significant increase of the nuclear localization of pDNA [157,158]. Therefore, we and others consider that the use of mRNA instead of pDNA may overcome this serious obstacle limiting pDNA-mediated gene delivery. In contrast to pDNA, mRNA is translated into proteins in the cytoplasm and hence, it does not have to cross the nuclear membrane to be effective. The idea of using mRNA is not entirely new. In 1985 Mizutani *et al*. already bypassed the nuclear membrane by using mRNA instead of pDNA [159]. Nevertheless, the concept of mRNA delivery has not been picked up by the gene therapy community. Indeed, the use of mRNA to transfect cells is currently only reported in a limited number of papers. The limited interest in mRNA is probably due to the general perception that mRNA is a very labile molecule and hence difficult to handle. However, under RNase-free conditions it is possible to produce and store mRNA without major problems. Moreover, mRNA can

be protected against RNases by complexation with cationic carriers [160]. An overview of the different cationic carriers, that have been evaluated for mRNA delivery, can be found in the recent reviews of Yamamoto *et al*. [161] and Tavernier *et al.* [162]. In general, the intracellular delivery of mRNA seems to be much more efficient with cationic lipids than with cationic polymers [50,163]. Until now, the highest mRNA transfection efficacy has been obtained with Lipofectamine2000, which resulted in transfection of almost 90 % of the cells [163].

In studies that compared delivery of mRNA and pDNA, transfection with mRNA was shown to lead to a faster but shorter lasting expression of a transgene [50,163-165]. Therefore, mRNA transfection is especially suited for applications that do not require a long-term expression of a protein. For this reason mRNA delivery has mainly been considered for vaccination purposes [166]. Nevertheless, there are many other possible applications for mRNA, such as the expression of “suicide genes”, growth factors, protein hormones, and proteins that modulate immune or stem cells.

The potential of mRNA delivery urged us to compare the performance of mRNA and pDNA containing nanoparticles in respiratory cells both *in vitro* and *in vivo*. Many different non-viral gene carriers have been used for pDNA delivery to the respiratory system. However, cationic liposomes based on the GL67 lipid are still considered as the “golden standard” in non-viral respiratory gene transfer. Indeed, their therapeutic potential, their low toxicity and safety have been extensively demonstrated in many pre-clinical and clinical trials [153]. Therefore, in this paper we evaluated the GL67:DOPE:DMPE-PEG5000 (GL67-lipid formulation) as a carrier for the delivery of mRNA to respiratory cells. We first studied the physicochemical properties of mRNA/GL67 complexes and identified the optimal ratio between mRNA and GL67. After these experiments we compared the expression kinetics of mRNA and pDNA complexed with GL67 liposomes. Additionally, the efficacy of mRNA and pDNA complexed with GL67 was studied in dividing and non-dividing cells. Finally, mRNA/GL67 and pDNA/GL67 complexes were administered to the lungs of mice and the expression of the luciferase reporter protein was determined via *in vivo* optical imaging.

MATERIALS AND METHODS

Reagents

HEPES, MOPS and Roscovitine were purchased at Sigma (Bornem, Belgium). Lipofectamine2000 and Ultra Pure Agarose were from Invitrogen (Merelbeke, Belgium). Vials containing GL67:DOPE:DMPE-PEG5000 (1:2:0.05 molar ratio) as a lyophilized powder were obtained from Dr. Seng Cheng (Genzyme Corporation, Framingham, MA, USA). The amount of GL67 lipid in GL67-lipid formulation in one vial is 4 µmol. 2X Formamide-Loading Dye and RiboRuler™ RNA Ladder (High Range) were purchased at Fermentas (St. Leon-Rot, Germany). D-Luciferin was from Caliper Life Sciences (Teralfene, Belgium).

Plasmids

The pBlue-LucA50 containing the cDNA of firefly luciferase was used for the *in vitro* transcription (IVT) of mRNA. This pDNA was a kind gift of Dr. Peter Ponsaerts (University of Antwerp, Antwerp, Belgium) and was previously described by Sheets *et al.* [167]. Messenger RNA encoding GFP was obtained via IVT from pGEM4Z[eGFP]A64. The latter was provided by Prof. dr. Smita Nair (Duke University Medical Center, NC, USA). All the DNA templates have the T7 RNA polymerase promoter site upstream of the sequence to be transcribed.

pCpG-hCMV-Luc, which contains a reduced number of immunostimulatory CpG-islands was a generous gift from Prof. Ernst Wagner and dr. Manfred Ogris (Ludwig-Maximilians-University, München, Germany) and was previously described by Navarro *et al*. [168]. eGFP-N1 plasmid coding for mutant *Aequorea victoria* green fluorescent protein (GFP) was purchased from Clontech Laboratories (Mountain View, CA, USA). The pDNAs were isolated and purified from *Escherichia coli* using Qiagen Plasmid Giga Kit (Qiagen, Venlo, Netherlands). The pDNA used in the *in vivo* experiments was purified using the EndoFree Plasmid Giga Kit (Qiagen, Venlo, Netherlands). After purification, the DNA concentration was determined spectrophotometrically by the measurement of the UV absorbance at 260 nm. Purity was confirmed by checking the 260 nm/280 nm ratio as well as by 1 % agarose gel electrophoresis.

In vitro transcription of mRNA

pBlue-LucA50 was sequenced on a 3130xl DNA Analyzer with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Halle, Belgium) and confirmed to contain a luciferase gene (firefly) and a poly(A)-tail of 50 adenosines. Prior to *in vitro* transcription pBlue-LucA50 and pGEM4Z[eGFP]A64 were linearized downstream of the insert with DraI and SpeI restriction enzyme (Promega, WI, USA), respectively, and examined on an agarose gel. mRNA was transcribed with a mMESSAGE mMACHINE kit according to the manufacturer's instructions (Ambion, Austin, TX, USA). mRNA was dissolved in RNase-free water (Ambion, Austin, TX, USA). The concentration was determined by measuring the absorbance at 260 nm and its purity was assessed by measuring the 260/280 nm absorbance ratio. Additionally, the purity and size of mRNA was checked by formaldehyde gel electrophoresis. Briefly, 1 g of Ultra Pure Agarose (Invitrogen, Merelbeke, Belgium) was dissolved in 100 ml of 1x MOPS-Buffer prepared in RNase-free DEPC-treated water, containing 18 ml of 37 % formaldehyde (Sigma, Bornem, Belgium). As a reference, a RiboRuler™ High Range RNA Ladder was used (Fermentas, St. Leon-Rot, Germany).

Preparation and characterization of mRNA/GL67 and pDNA/GL67 complexes

The GL67:DOPE:DMPE-PEG5000 liposomes (1:2:0.05; molar ratios) were prepared by adding 2.667 ml of RNAse-free water (Ambion, Austin, TX, USA) to the vials containing 4 µmol GL67, 8 µmol DOPE and 0,2 µmol DMPE-PEG5000. mRNA/GL67 complexes were prepared at different ratios by mixing 3 µg of mRNA dissolved in 12.5 µl RNase-free water with different amounts of GL67-liposomes dispersed in 12.5 µl RNase-free water. After mixing, the complexes were incubated for 15 min at 30°C. pDNA/GL67 complexes with a molar ratio of 1.33 [169] were prepared in a similar way. The mRNA/GL67 or pDNA/GL67 molar ratios were calculated based on the nucleotide concentration in the mRNA or pDNA solutions using an average nucleotide molecular mass of 340 g/mol (for pDNA we used 330 g/mol) as well as the molar concentration of GL67 lipid in the liposome formulation. The lipoplexes were used immediately after preparation. The average hydrodynamic size and zeta potential of the complexes were determined on basis of dynamic light scattering and laser Doppler electrophoresis using a Zetasizer Nano ZS (Malvern, Worcestershire, UK). Prior to the measurement

the complexes were dissolved in Hepes buffer 20 mM, pH7.4 and prewarmed up to 37°C to mimic the conditions during *in vivo* delivery. A gel retardation assay was performed to determine to what extent mRNA was bound to cationic liposomes.

In vitro transfection and protein expression measurements

The human alveolar type-II-like cell line A549 (ATCC #CCL-185) was cultured in 75 cm^2 flasks in DMEM supplemented with 10 % fetal bovine serum (FBS), 50 µg penicillin/ml, 50 µg streptomycin/ml and 2 mM L-glutamine at 37°C in a humidified atmosphere containing 5 % CO_2. One day before transfection the cells were plated onto 24 well plates (24WPs). At the moment of transfection their confluency was around 80 %. Lipoplexes were prepared right before transfection. If not mentioned differently, the transfections were made in the reduced-serum medium OptiMem (Invitrogen, Merelbeke, Belgium). After 4 h of incubation, the complexes were removed and regular culture medium was added to the cells. Transfection efficiency of lipoplexes containing mRNA or pDNA encoding GFP was determined by flow cytometry (FACSCalibur, Becton Dickinson, Erembodegem, Belgium). To that end, A549 cells were washed with PBS and subsequently resuspended in a flow buffer (BD FACSFlow). Percentages of GFP positive cells and their mean fluorescence intensity were used for analysis. 10000 cells per sample were analyzed. Data analysis was performed with CellQuest software (Becton Dickinson). The expression of luciferase was examined by the Luciferase Assay (Promega, WI, USA) 8 hours after adding the complexes on the cells for mRNA/GL67 complexes and 24 hours for pDNA/GL67 complexes. The luciferase activity was expressed as relative light units (RLU). Cell viability was measured 8 or 24 hours post-transfection using an MTT Cell Proliferation Kit (Roche, Vilvoorde, Belgium).

Mice and pulmonary delivery of the complexes

BALB/c mice were obtained from Janvier (Le Genest St Isle, France). The mice were housed in individually ventilated cages with a 12:12 h dark-light cycle. Access to food and water was maintained *ad libitum*. All experiments were carried out with the approval of the local Ethics Committees of the Vrije Universiteit Brussel and Ghent University.

Mice were anesthetized with 4 % isoflurane and 80 µg of mRNA/GL67 (ratio 2) or pDNA/GL67 (ratio 1.33) divided in 2 doses of 40 µg, with a delay of 2 hours, were instilled intranasally. Animals that received mRNA/GL67 and pDNA/GL67 complexes were imaged 6 hours and 24 hours after instillation, respectively. The mice were shaved before imaging.

In Vivo Bioluminescence Imaging (BLI)

Prior to imaging, mice were anesthetized with isoflurane (3 % induction and 2.5 % maintenance) with oxygen as carrier gas. Mice were imaged after intraperitoneal administration of D-luciferin at a dose of 150 mg/kg body weight. Subsequently, 30 µl of D-luciferin (15 mg/ml) was also instilled intranasally ten minutes before imaging. The emitted photons were measured for 2 minutes using the IVIS Lumina II (Caliper Life Sciences) at binning 4 and f-stop 1. The data analysis was performed with the Living Image software (Caliper Life Sciences).

Statistical Analysis

All obtained data had a normal distribution what was checked by the Kolmogorov–Smirnov test. An independent-samples t-test was performed in order to compare two groups. ANOVA followed by the Bonferroni test were conducted for multiple group analysis. The differences were considered significant when $p<0.05$. The results are presented as the mean ± standard deviation (SD).

RESULTS

Physicochemical characterization of mRNA/GL67 complexes prepared at different ratios

To gain insight into the capacity of the GL67-lipid formulation to form self-assembled nanoparticles with mRNA we prepared mRNA/GL67 complexes at different mRNA/GL67 molar ratios and determined their physicochemical properties. The extent of mRNA complexation by the GL67-lipid formulation is shown in Figure 3.1. The GL67-lipid formulation was able to complex all the mRNA up to an mRNA/GL67 ratio of 4.

When the amount of mRNA was 5 times higher than the amount of GL67-lipids, a fraction of unbound mRNA was clearly visible. The free mRNA band was located between 1.5 and 2.0 kb, which is in agreement with the calculated length of the Luc-mRNA, i.e. ≈1.7 kb.

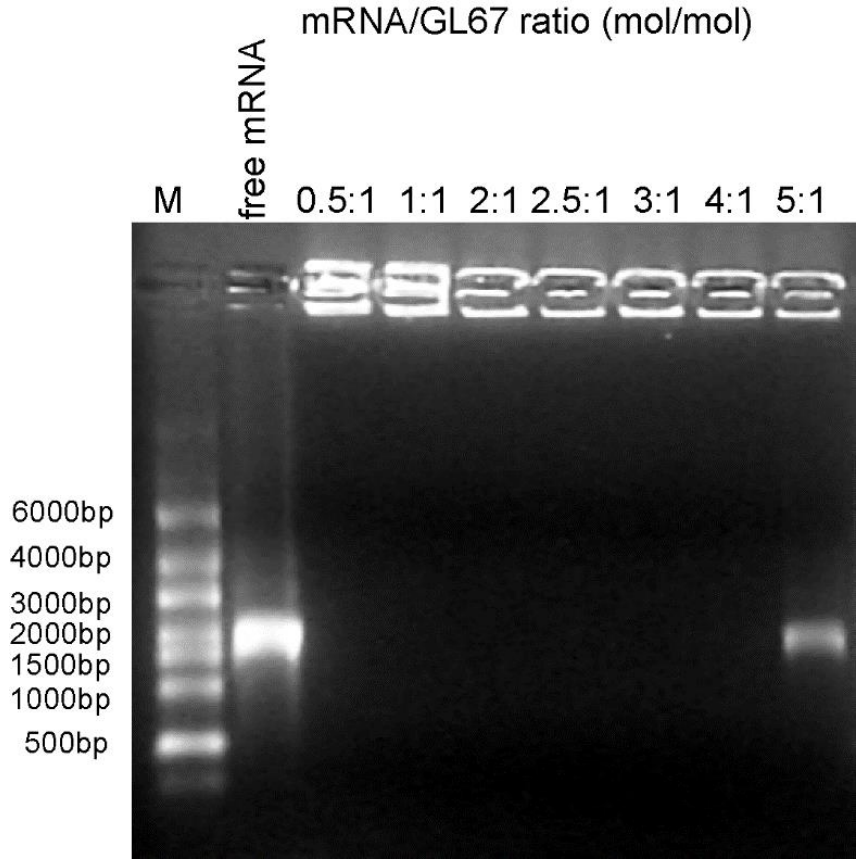

Figure 3.1. Gel retardation assay of mRNA/GL67 complexes prepared at different ratios. mRNA/GL67 complexes, containing 1 µg mRNA, were prepared at different mRNA/GL67 ratios and then loaded on a formaldehyde-agarose gel. A RNA ladder was run in a lane M. Next to the RNA ladder, 1 µg of mRNA was run as a reference.

We next determined the size and zeta potential of the mRNA/GL67 complexes. Figure 3.2 shows that upon hydration of the lyophilized GL67-lipid formulation liposomes are formed. Their mean diameter and zeta potential were 280 nm ± *10 nm* and 17.0 mV ± *0.4 mV*, respectively. When mRNA and GL67 vesicles were mixed at an mRNA/GL67 molar ratio 0.5, the zeta potential of the complexes decreased sharply to almost zero. The complexes became slightly negative at ratios ≥ 2. The complex sizes varied between 350 and 750 nm, reaching a maximum at an mRNA/GL67 ratio of 2.

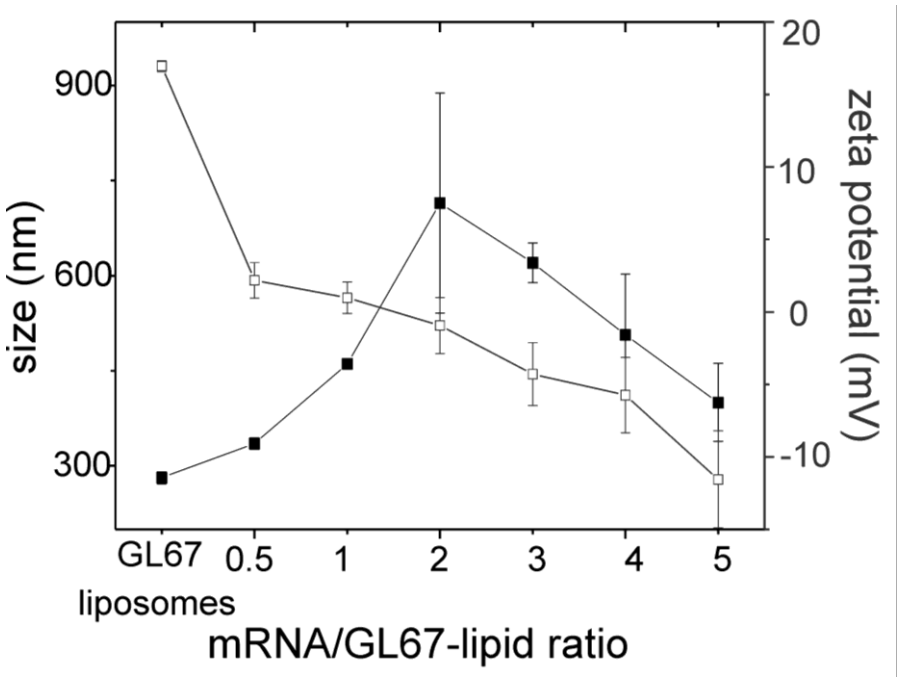

Figure 3.2. Size (closed squares) and zeta potential (ζ; open squares) of mRNA/GL67 complexes. The size and ζ of mRNA/GL67 complexes prepared at different molar ratios was measured after dilution of mRNA/GL67 complexes containing 1 µg of mRNA in 1ml of Hepes buffer (pH 7.4). The size and ζ of the GL67-liposomes diluted in the same buffer is shown at the left. The results are represented as the mean of 3 measurements ± SD.

Transfection efficiency and cytotoxicity of the mRNA/GL67 complexes at different ratios in alveolar cells

To determine the optimal ratio for transfection, type II lung epithelial cells (A549) were transfected with mRNA encoding eGFP complexed with the GL67-lipid formulation at different ratios. The transfection efficacy was studied on a single-cell basis using flow cytometry.

The percentages of GFP-positive cells as well as the mean fluorescence intensity (MFI) of the transfected cells were quite comparable for all the tested ratios (Figure 3.3). Nevertheless, a higher number of positive cells were found when the mRNA/GL67 complexes were prepared at a ratio 2 (31 %). The experiment was also performed using luciferase-encoding mRNA and the ratio 2 was confirmed to give the highest reporter gene expression (data not shown).

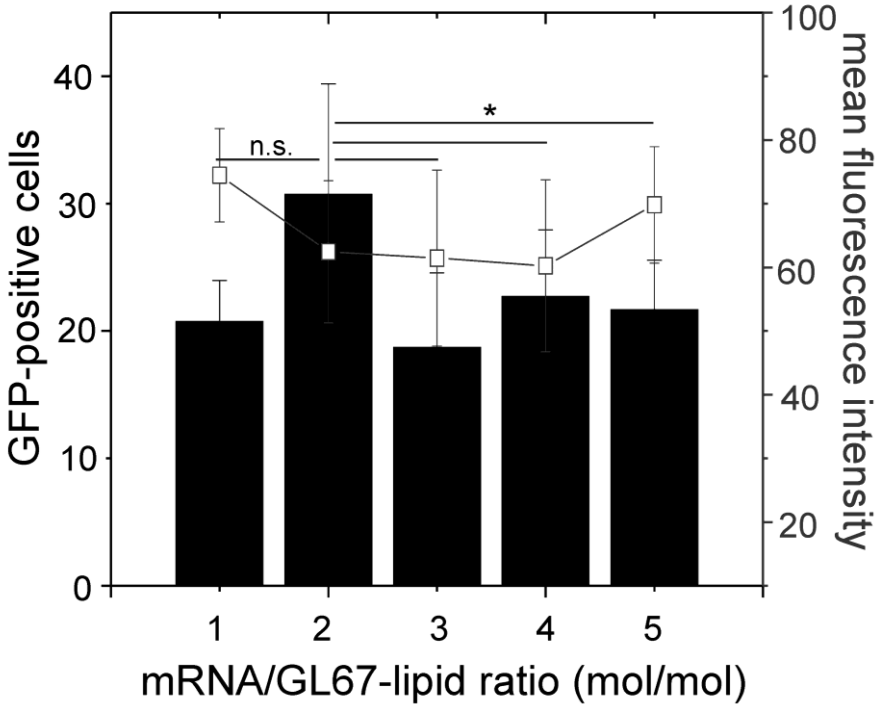

Figure 3.3. Determination of the most optimal mRNA/GL67 ratio. A549 cells plated in 24-well plates were transfected with 500 ng of mRNA/GL67 complexes prepared at different ratios. The percentage of GFP-positive cells (bars) and the mean fluorescence intensity (line) were measured 4 hours after addition of the complexes on the cells by flow cytometry. The results are presented as the mean ± SD (n≥4; * indicates p<0.05 and n.s. indicates non-significant; ANOVA).

In order to assess the cytotoxicity of mRNA/GL67 complexes, the MTT test was performed 8 hours after adding the complexes on the cells. None of the tested formulations caused a significant drop in the cell viability in reference to the untreated control (Figure 3.4). Based on these results, we prepared the mRNA/GL67 complexes at their optimal ratio, i.e. 2 in all subsequent experiments. Additional transfection experiments at higher mRNA doses resulted in a significant drop of the cell viability in comparison to a dose of 500 ng/well and hence confirmed that this amount provides a balance between toxicity and transfection efficacy (see supplementary Figure SB.1).

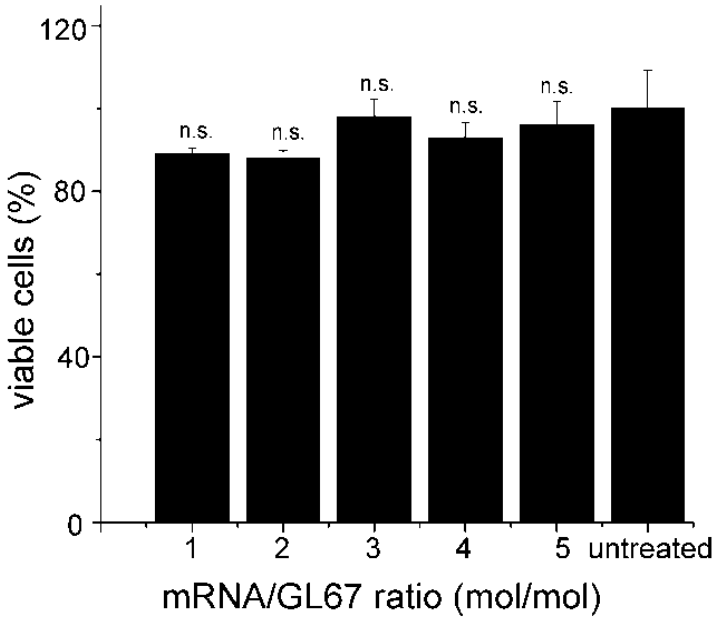

Figure 3.4. Cell viability following transfection of A549 cells plated in 24-well plates were transfected with 500 ng of mRNA/GL67 complexes prepared at different ratios. Cell viability was assessed 8 hours after adding the complexes to the cells with an MTT assay. Viability of untreated cells was set as 100 %. The data are presented as the mean ± SD (n=3) and considered significant, if $p<0.05$ compared to the untreated control (n.s. indicates non-significant; ANOVA).

Comparison of expression kinetics of mRNA/GL67 and pDNA/GL67 complexes

Subsequently, we studied the expression kinetics after transfection of A549 cells with mRNA/GL67 and pDNA/GL67 complexes prepared at their optimal ratios, i.e. 2 and 1.33, respectively. It has been shown earlier that the 1.33 ratio ensures the highest transfection efficiency of pDNA/GL67 complexes [170]. A549 cells were incubated with the complexes for 4 hours. Transfection efficiency was evaluated 4, 8, 24, 48 and 72 hours after adding the complexes to the cells by flow cytometry. As shown in Figure 3.5A, transfection with mRNA resulted in a very rapid production of GFP. The highest percentage of GFP-positive cells was achieved 8 hours after addition of the mRNA/GL67 complexes to the cells. At this time point 37 % of the cells were GFP-positive. At later time points the number of GFP-positive cells progressively dropped to about 20 %. The MFI of the GFP-positive cells followed more or less the same profile: the MFI was maximal 24 hours after adding the complexes and showed a

strong drop at later time points. In case of pDNA transfection, the maximal levels of transfection were reached much later than with mRNA (Figure 3.5B). The number of GFP-positive cells was maximal 24-48 hours after adding of pDNA/GL67 complexes, which agrees with previous reports [104]. At the 72 hour time-point, the number of GFP-positive cells slightly decreased. The MFI of the cells transfected with pDNA peaked 24 hours after addition of the complexes to the cells and gradually dropped at later time points.

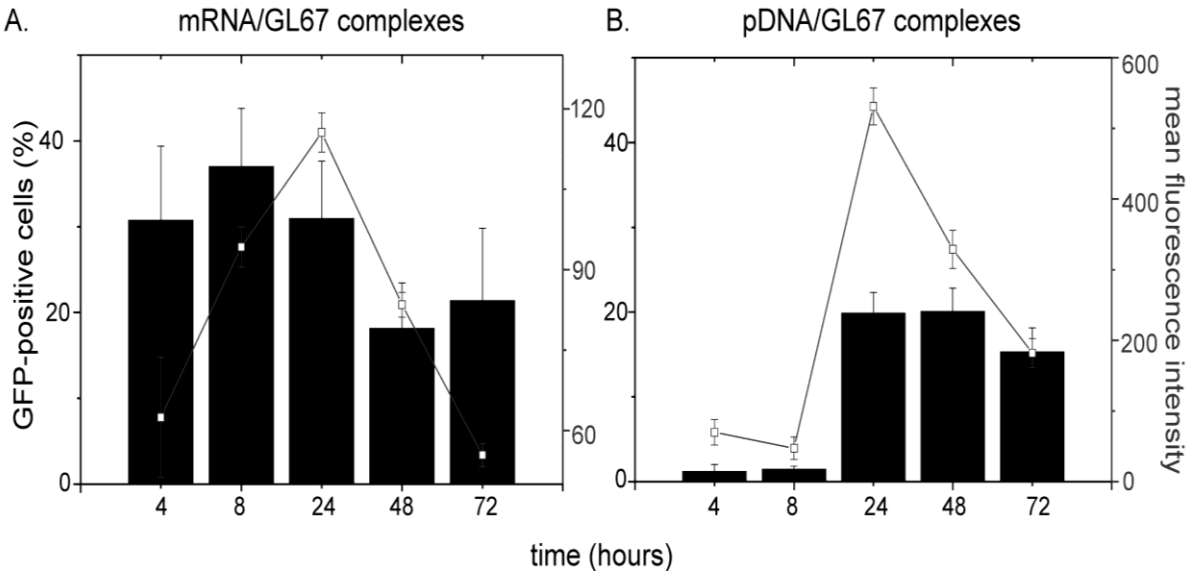

Figure 3.5. Expression kinetics after transfection of A549 cells with mRNA/GL67 complexes (A) and pDNA/GL67 complexes (B). The A549 cells were transfected with mRNA/GL67 complexes or pDNA/GL67 complexes containing 500 ng mRNA or pDNA. The GFP expression kinetics were followed over 72 hours via flow cytometry by measuring the percentage of GFP positive cells (bars) as well as their mean fluorescence intensity (line). The results are presented as the mean ± SD (n≥5).

Impact of cell division on transfection efficiency of mRNA/GL67 and pDNA/GL67 complexes

The ability of transfecting both dividing and non-dividing cells would be one of the strongest advantages of mRNA over pDNA. In order to confirm that mRNA unlike pDNA could efficiently transfect both dividing and non-dividing cells, we compared the transfection efficiency of mRNA and pDNA (encoding GFP) in dividing and non-dividing A549 cells. To arrest the cell cycle, A549 cells were treated with roscovitine,

which is a cell permeable reversible selective inhibitor of cyclin-dependent kinase 1, 2 and 5 [171]. Transfection efficacy was determined 8 and 24 hours after addition of the complexes by flow cytometry. As shown in Figure 3.6A, at the 24 hour time-point the percentages of GFP-positive cells after mRNA transfection were comparable both in dividing (~31 %) and non-dividing cells (30 %). In contrast, after pDNA transfection only 3 % of the non-dividing cells were GFP-positive. In dividing cells, pDNA transfection resulted in 20 % of GFP-positive cells. Interestingly, after mRNA transfection the MFI in proliferating cells is always lower than in cell cycle-arrested cells (Figure 3.6B). This is probably due to a dilution of both the mRNA and the expressed GFP in the daughter cells after cell division. The expression data after 8 hours in Figure 3.5 further confirm the observation that mRNA transfection results in a much faster production of the reporter protein than pDNA transfection.

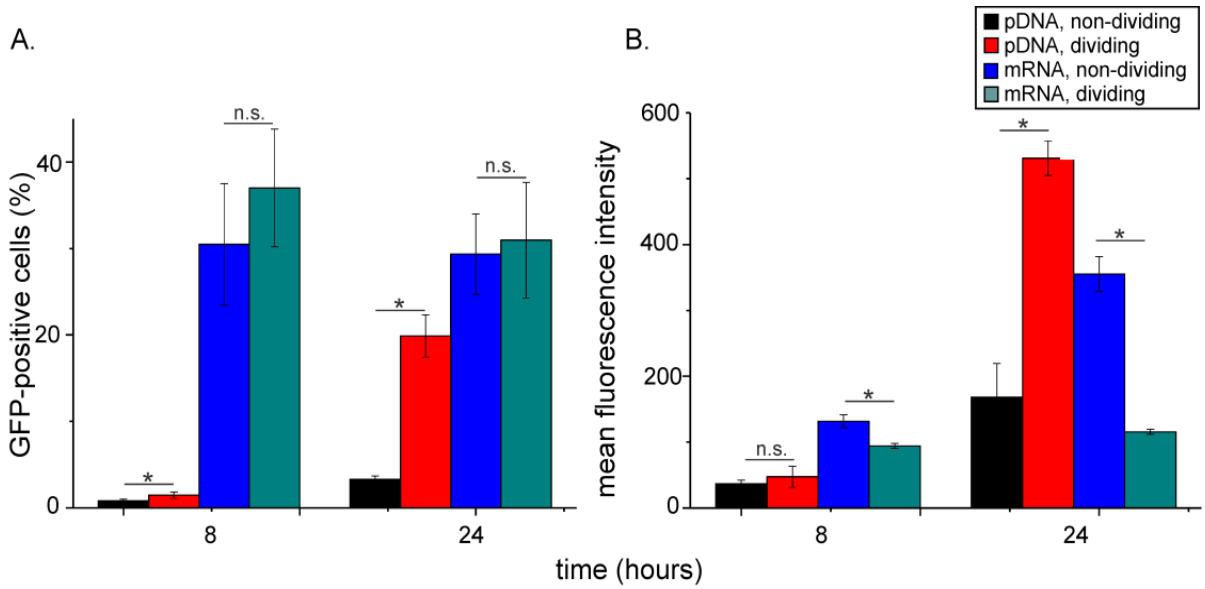

Figure 3.6. Impact of cell division on the transfection efficiency of mRNA/GL67 and pDNA/GL67 complexes in A549 cells. The A549 cells were transfected with mRNA/GL67 complexes or pDNA/GL67 complexes containing 500 ng mRNA or pDNA. The cell-cycle was arrested by addition of 15 mM roscovitine. The percentage of GFP-positive cells (A) and the mean fluorescence intensity of the cells (B) were determined by flow cytometry 8 and 24 hours after adding the complexes. The results are presented as the mean ± SD (n≥5;* if p<0.05, independent-samples *t*-test).

The mRNA concentration during preparation of the complexes affects the transfection efficiency

The concentration of the mRNA/GL67 complexes prepared for the *in vitro* tests was too low for *in vivo* application. Therefore, we prepared mRNA/GL67 complexes at higher concentration and evaluated their gene expression in A549 cells before setting the experiment *in vivo*. The complexes were prepared at five concentrations at their optimal ratio: 0.08, 0.2, 0.4, 0.6 and 0.8 µg/µl of complexed mRNA in a final volume of 25 µl (Figure 3.7). Surprisingly, the expression level of the mRNA/GL67 complexes increased when they were prepared at higher concentration. The highest expression was achieved when the complexes were prepared at an mRNA concentration of 0.8 µg/µl. The possibility to formulate the mRNA/GL67 complexes at such high mRNA concentrations is an important advantage for their use *in vivo*.

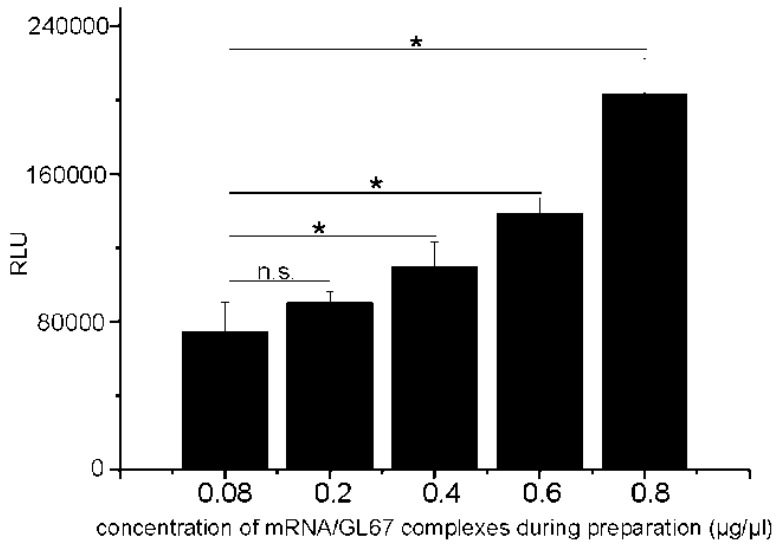

Figure 3.7. Impact of the mRNA concentration during preparation of mRNA/GL67 complexes on the transfection efficiency. The luciferase expression of mRNA/GL67 complexes prepared at five different concentrations was evaluated on A549 cells. The cells were treated with the same amount of complexes (500 ng mRNA/well). The best expression was obtained with the complexes prepared at the highest concentration. The results are presented as the mean ± SD (n≥4; * if p<0.05; ANOVA).

In an effort to elucidate the reason for the differences in transfection efficacy observed in Figure 3.7, we measured the size and zeta potential of the complexes formulated at

the lowest and the highest concentration of mRNA. It appeared that the complexes prepared at an mRNA concentration of 0.8 µg/µl have a zeta potential of -5.8 mV and a size of 346 nm, while the zeta potential and size of the complexes prepared at a 10 times lower mRNA concentration are -2.2 mV and 773 nm, respectively (Table 3.1). Complexes prepared at a higher mRNA concentration have thus a much smaller size than the complexes prepared at a lower mRNA concentration. It has been shown by Ross et al. [172] and Rejman et al. [173] that smaller complexes are taken up faster and are more efficient than larger complexes. Consequently, the higher transfection efficiency of mRNA/GL67 complexes prepared at higher concentrations may be due to their smaller particles size.

mRNA concentration during complex formation	Zeta Potential (mV)	Z-average diameter (nm)
0.08 µg/µl	-2.2 ± 0.1	773 ± 88
0.8 µg/µl	-5.8 ± 0.2	346 ± 14
0.08 µg/µl + 10% serum	-15.1 ± 0.5	250 ± 25
0.8 µg/µl + 10% serum	-11.5 ± 0.2	204 ± 10

Table 3.1. Size and zeta potential of mRNA/GL67 complexes prepared at a low and a high concentration. The mRNA/GL67 complexes were prepared at two mRNA concentrations, i.e. 0.08 and 0.8 µg/µl and their size and zeta potential were measured after dilution in Hepes buffer. Additionally, the complexes were also incubated with 10 % serum for 30 minutes at 37°C. Subsequently, these particles were diluted in 1 ml of Hepes buffer (pH 7.4) and their size and zeta potential was measured. The measurements are represented as the mean of 3 measurements ± SD.

In vivo transfection efficiency

We subsequently compared the performance of the mRNA/GL67 and pDNA/GL67 complexes *in vivo* using mRNA and pDNA encoding firefly luciferase. The used pDNA contained a reduced number of CpG-islands. The mRNA/GL67 and pDNA/GL67 complexes were administered to the lungs of the mice via intranasal instillation and the luciferase production was determined in the mice by *in vivo* bioluminescence imaging

(BLI) after 6 hours and 24 hours for mice instilled with the mRNA/GL67 and pDNA/GL67 complexes, respectively. Additionally, the signal in mice that received pDNA/GL67 complexes was also determined after 48 hours post-administration. A clear bioluminescence signal was observed at all time points in the lungs of the four mice that received pDNA/GL67 complexes (Figure 3.8A and 8C). A positive signal was also detected in the noses of 3 mice. Additionally, pDNA/GL67 complexes prepared at a ratio 4 were also administered to mice as it has been reported that their optimal ratio for intranasal application is 4, while 1.33 is more suitable for aerosol delivery [174]. The luciferase expression after intranasal delivery of the pDNA/GL67 complexes with a ratio 4 was 2.3-fold higher than with a ratio 1.33 (see supplementary Figure SB.2 and SB.3). Surprisingly, none of the mice that received the mRNA/GL67 complexes did show a clear signal in their lungs or noses (Figure 3.8B).

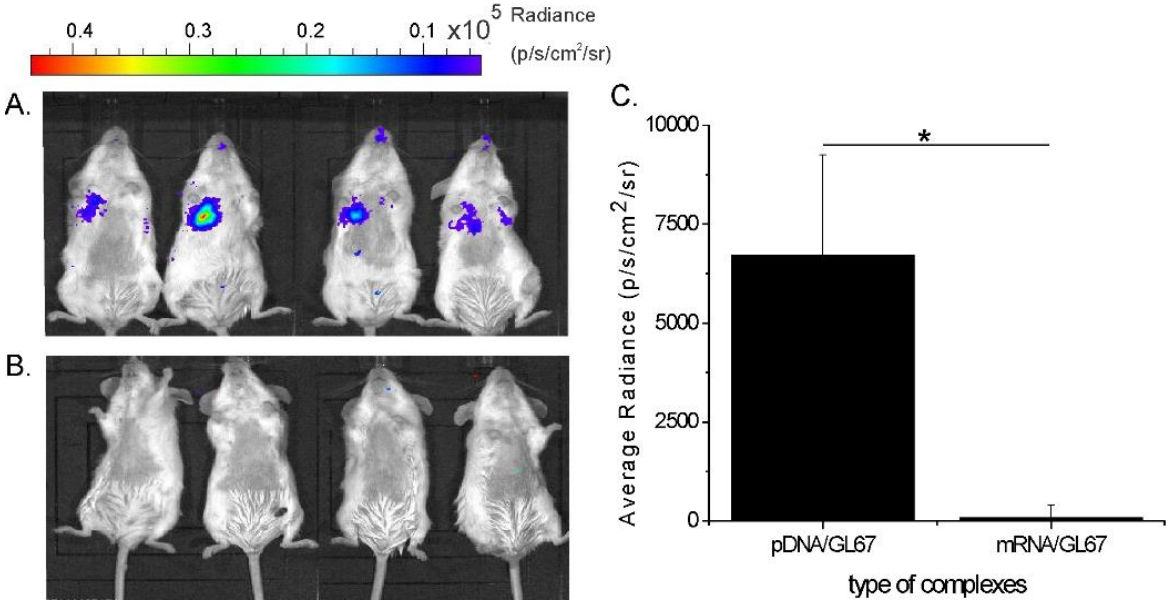

Figure 3.8. Visualization of the *in vivo* luciferase production after intrapulmonary delivery of (A) pDNA/GL67 complexes (n=4) or (B) mRNA/GL67 complexes (n=4), and (C) the average bioluminescence of four mice that received either pDNA/GL67 or mRNA/GL67 complexes. The complexes, which contained 80 µg of mRNA or pDNA, were administered to the lungs of anesthetized mice via intranasal instillation. Animals that received pDNA/GL67 and mRNA/GL67 complexes were imaged 24 hours and 6 hours after instillation, respectively. The amount and localization of the bioluminescent light was recorded via *in vivo* bioluminescence imaging. The data in graph C are obtained after subtracting the average bioluminescence signal of untreated mice (background) from the signals measured in panels A and B. The results

are presented as the mean ± SD (n=4;* if p<0.05, independent-samples *t*-test).

Effect of serum on the physical properties and transfection efficacy of mRNA/GL67 and pDNA/GL67 complexes

Messenger RNA is very vulnerable to degradation by ribonucleases, which are present in all organisms. Therefore, enzymatic degradation of the mRNA and/or a release of the bound mRNA from the mRNA/GL67 complexes after contacting biological fluids may be a possible explanation for the failure of the mRNA/GL67 complexes to generate detectable amounts of luciferase after intranasal instillation. To check this hypothesis we incubated the mRNA/GL67 complexes with 10 % serum and subsequently measured their zeta potential and size (Table 2.1). mRNA complexes incubated with serum had a much lower zeta potential and size. These data urged us to further evaluate the impact of serum (0 %, 10 % and 50 %) on the transfection efficiency of mRNA/GL67 as well as pDNA/GL67 complexes. The data in Figure 3.9 show that mRNA/GL67 complexes are much more affected by serum than their pDNA/GL67 counterparts. The transfection of the pDNA/GL67 complexes dropped with only 30 % in the presence of 10 % serum, while the mRNA/GL67 complexes lost more than 90 % of their transfection capacity in 10 % serum (Figure 3.9A). At 50 % serum the changes in luciferase production were similar for both types of complexes. In order to elucidate why mRNA/GL67 complexes perform so poorly after contact with serum we performed a gel retardation assay using mRNA/GL67 complexes that had been exposed to 10 % serum for 30 minutes at 37°C. As a reference we also run mRNA/GL67 complexes that had been incubated with Hepes buffer only. In Figure 3.9B a clear detachment and degradation of the mRNA can be observed when the mRNA/GL67 complexes were incubated with serum.

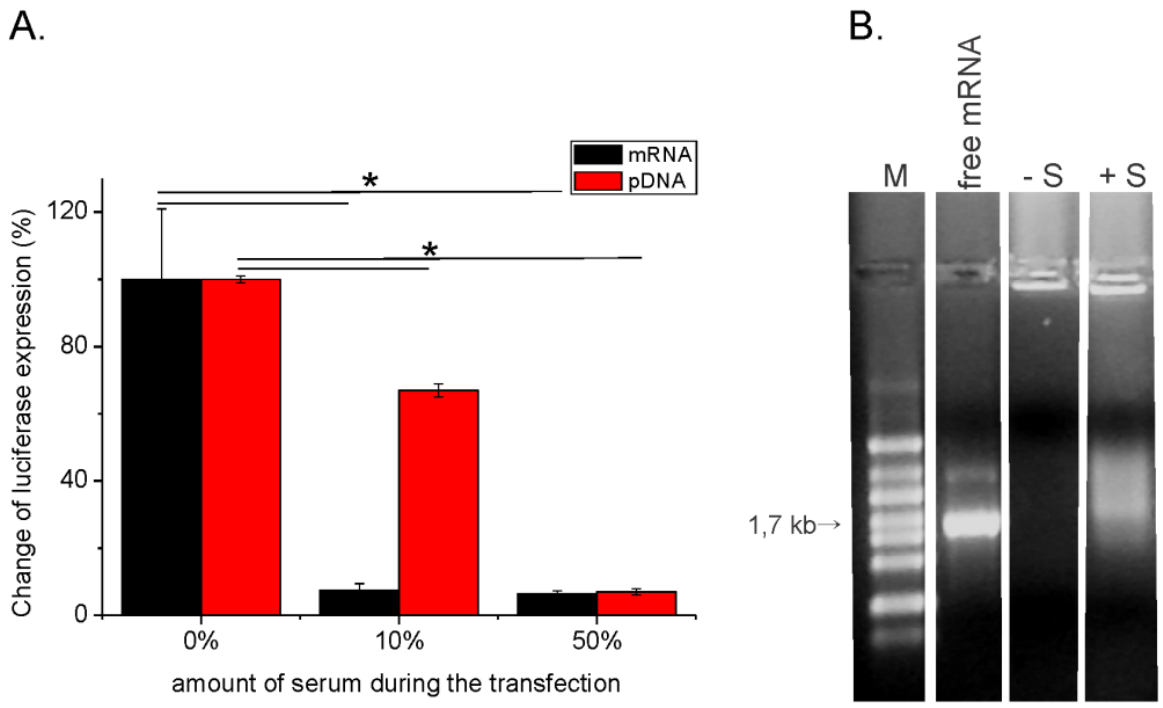

Figure 3.9. Impact of serum on the mRNA/GL67 and pDNA/GL67 complexes. To study the influence of serum on the transfection efficiency A549 cells were transfected with 500 ng of mRNA/GL67 and pDNA/GL67 complexes (prepared at their optimal ratios) in the presence of 0 %, 10 % or 50 % serum (A). The luciferase production was measured 8 hours and 24 hours after transfection with mRNA/GL67 and pDNA/GL67 complexes, respectively. The transfection efficacy in the absence of serum was set as 100 %. The results are presented as the mean of 3 measurements ± SD and considered significant, if $p<0.05$ compared to transfection in 0 % serum (ANOVA). In panel B a gel retardation assay of mRNA/GL67 complexes in the absence (-S) and presence (+S) of 10 % serum is shown (1 µg mRNA was loaded). An RNA ladder was run in lane M. Next to the RNA ladder, 1 µg of free mRNA was run as a reference.

DISCUSSION

In this work we prepared mRNA/GL67 complexes and compared their *in vitro* and *in vivo* transfection characteristics with pDNA/GL67 complexes. The GL67-lipid formulation was selected as the delivery agent because of its proven efficacy with pDNA in lung cells and its safety profile in clinical trials. The formulation contains three lipids, the GL67-lipid, DOPE and DMPE-PEG_{5000} in a molar ratio of 1:2:0.05. This lipid mixture is stored as a lyophilized powder. Upon hydration of this powder we obtained

positively charged PEGylated vesicles that can complex all the mRNA up to an mRNA/GL67 ratio of 4. The sharp drop of the surface charge of the GL67 vesicles after binding of the mRNA indicates that at least a part of the mRNA is bound to the surface of the vesicles. It has previously been shown that pDNA is also mainly bound to the surface of the GL67 vesicles [169]. *In vitro* transfection of A549 cells with mRNA/GL67 complexes prepared at different ratios revealed that the highest transfection was obtained at a ratio of 2. We have to remark that the optimal ratio for transfection seems to be cell type dependent as we observed that in HEK293 (human embryonic kidney) cells the highest transfection was obtained when the mRNA/GL67 complexes were prepared at a ratio of 4 (data not shown).

The comparison of the expression efficacy and kinetics of mRNA/GL67 with pDNA/GL67 complexes in A549 demonstrates a very fast and relatively short production of GFP after mRNA transfection. This is in agreement with the work of Zou *et al*. [104], who also found that the highest number of GFP-positive cells occurred about 8 hours following the addition of the mRNA/liposomes complexes to Chinese Hamster Ovary (CHO) cells. The highest mean fluorescence was observed after 24 hours. However, when we used mRNA encoding luciferase, instead of GFP, the maximal expression occurred after 8 hours and dropped rapidly after this time point (data not shown). Also Zou *et al*. [104] and Bettinger *et al*. [50] observed this shift in expression kinetics when using mRNA encoding luciferase. This shift can be explained by the fact, that the half-life of firefly luciferase is 3 to 6 hours [175], while the reported half-life of GFP is greater than 24 hours [176]. The low percentage of GFP-positive cells after <8 hours following pDNA-transfection indicates the importance of the cell division for pDNA-transfection. Indeed, the percentage of cells that divided after <8 hours is limited as the doubling time of A549 cells is about 22.3 hours [177]. To study in more detail the importance of cell division we compared the transfection efficiency of mRNA/GL67 and pDNA/GL67 complexes in dividing and non-dividing cells. The data in Figure 3.5 clearly demonstrate that for pDNA-transfection the breakdown of the nuclear membrane during cell proliferation is really required. In contrast, the number of GFP-positive cells after mRNA-based transfection is independent of the cell cycle. However, 24 hours after mRNA-transfection we observed that the average amount of GFP per cell is lower in dividing cells than in non-dividing cells. This is most likely due to a dilution of both the mRNA and the expressed GFP reporter in the daughter cells after cell division. This dilution effect does not seem to play an important role after pDNA transfection as

the MFI after 24 hours is the highest in dividing cells. After pDNA transfection, the GFP-positive cells probably contain many pDNA copies in their nucleus, which are divided over the daughter cells during cell division. These pDNAs in the daughter cells can continuously generate many new mRNA copies that are translated into GFPs. This phenomenon counterbalances the dilution of the produced GFP-mRNA and GFP during the first cell cycles. The faster drop in gene expression after mRNA transfection in comparison to pDNA transfection can be explained by the short half-life of mRNA and the fact, that many mRNA templates can be produced from a single pDNA molecule after reaching the nucleus.

For the *in vitro* test we prepared the mRNA/GL67 complexes at a concentration of 0.12 µg mRNA/µl. However, for intrapulmonary administration in mice a much higher concentration of mRNA/GL67 complexes is required. Indeed, to reach a dose of 50 µg of complexed mRNA per mouse we would have to administer about 400 µl of these complexes. This is far too much as it is our experience that the maximal volume that can be administered to the lungs of mice is about 80 µl/25 g body weight. Hence, more concentrated mRNA/GL67 complexes were needed. In general, the transfection efficacy of non-viral gene complexes decreases when they are prepared at high concentration due to a concentration dependent aggregation of the complexes [178]. Surprisingly, the transfection efficacy of mRNA/GL67 complexes increased when they were prepared at a higher concentration. The mRNA/GL67 complexes prepared at the highest mRNA concentration (0.8 µg/µl) were 2.5-fold more efficient, compared to the complexes prepared at the lowest concentration. A similar profile of expression efficiency was observed by Ogris *et al.* [179] when using rising pDNA concentration during preparation of pDNA/Tf-PEI complexes. The observation that mRNA/GL67 complexes do not lose their efficacy when prepared at a high concentration can be explained by the fact that the GL67-lipid formulation contains low amounts of DMPE-PEG_{5000} lipids, which, as demonstrated for pDNA/GL67 complexes [174], prevent a massive aggregation of the mRNA/GL67 complexes when prepared at a high concentration. Moreover, we showed that the higher efficiency of the mRNA/GL67 complexes prepared at the higher mRNA concentration is most likely due to their smaller size (Table 3.1). Indeed, it has been reported that smaller gene complexes have a higher cellular uptake and hence also a higher gene expression [172,173].

Our *in vitro* transfection data (Figure 3.6) clearly demonstrate that in non-dividing cells mRNA/GL67 complexes are much more effective than pDNA/GL67 complexes.

Consequently, one would expect that also after pulmonary administration the mRNA/GL67 complexes would be superior to the pDNA/GL67 complexes. However, our *in vivo* data did not confirm this hypothesis. All the mice that received pDNA/GL67 complexes showed a clear bioluminescent signal, while no bioluminescence could be detected in the mice receiving mRNA/GL67 complexes. This observation is in agreement with the data reported in a poster abstract of Painter *et al.* [180]. In this study the gene expression in the lungs after intranasal instillation of mRNA/GL67 complexes was slightly higher than the background signal, but much lower than the expression obtained after instillation of pDNA/GL67 complexes. The low efficiency of mRNA/GL67 complexes in the lungs may be due to the fact that negatively charged bio(macro)molecules in respiratory fluids caused a detachment and enzymatic degradation of the mRNA in the mRNA/GL67 complexes. This hypothesis is supported by our data in Figure 3.9. Kormann *et al*. recently demonstrated in the lungs of mice a therapeutic effect after administration of 20 µg of naked mRNA. Importantly, their mRNA contained chemically modified nucleotides, which may increase the stability and avoid the recognition of mRNA by the innate immunity [6]. Nevertheless, it is generally believed that naked pDNA and mRNA have difficulties in crossing cell membranes. It is well-known in the field that the use of distilled water gives rise to much better transfection data after pulmonary gene delivery [181,182]. Pulmonary administration of distilled water or hypotonic liquids will create a hypotonic environment in the lungs. Cells placed in a hypotonic solution tend to swell and this may induce pores in the cell membrane through which naked pDNA or mRNA can enter the cell. This hypothesis can explain how naked mRNA can enter cells after pulmonary delivery. Alternatively, a receptor for DNA and RNA may be present on the surface of lung cells [183,184]. Unfortunately, in our hands administration of 50 µg of naked and unmodified mRNA into the lungs of mice did not result in a detectable luciferase expression (data not shown). Also Su *et al.* reported a positive bioluminescent signal in the nose of the mice after instillation of firefly luciferase-encoding mRNA-loaded particles [185]. In our work luciferase expression in the nose was only observed in 3 out of 4 mice that received the pDNA/GL67 complexes. *In vivo* optical imaging was used in our study to reduce the number of animals and to comply with the 3 R's principle in animal research. However, the *in vivo* BLI method is less sensitive than an *ex vivo* luciferase assay. Therefore, it is possible that the expression of the mRNA/GL67 complexes or the naked mRNA in the lungs inside the animal is too weak to be detected. In future

experiments, the use of more stable mRNA would be interesting. The stability of the mRNA can be increased by incorporating the UTR sequences from β-globine [186] and by adding a longer poly(A)-tail [187]. Additionally, in this study we used the regular cap analog during IVT. It is known, that this cap is bound incorrectly to 50 % of the capped mRNAs. Messenger mRNAs that are not correctly capped are inactive. The incorrect incorporation of the cap can be prevented by the use of anti-reverse cap analog (ARCA) [188] or by enzymatic capping [189]. Finally, the use of modified nucleosides in the mRNA can further increase the stability and prevent that mRNA is recognized by the innate immune system [6,190]. In this study we used the same mass of mRNA and pDNA encoding firefly luciferase. As a result, the copy number of mRNAs was 5.8-fold higher than the copy number of pDNA. However, only 50 % of the mRNA transcripts are functional as half of the mRNAs are capped wrongly during IVT. On the other hand, one has to consider that each pDNA that reaches the nucleus can produce many mRNA copies.

CONCLUSIONS

In conclusion, the claimed advantages of mRNA delivery urged us to study the potential of mRNA delivery to respiratory cells. Up till now, carrier-mediated mRNA delivery to respiratory cells has not been studied in detail. In this work we demonstrated that mRNA delivery, using the GL67-lipid formulation, results in a fast and temporal expression of marker genes in alveolar cells. Additionally, in non-dividing cells the transfection efficacy of mRNA/GL67 complexes was much higher than that of pDNA/GL67 complexes. This confirms that mRNA delivery is independent of the cell cycle. Surprisingly, after pulmonary administration in mice we found a clear bioluminescent signal after administration of the pDNA/GL67 complexes but not after administration of the mRNA/GL67 complexes. We showed that mRNA/GL67 complexes undergo dissociation and degradation of the mRNA after contacting the bio(macro)molecules present in serum. Such destruction of the mRNA/GL67 complexes is also expected when they come in contact with the biofluids of the lungs and this may be one explanation for their low *in vivo* transfection efficiency.

SUPPORTING INFORMATION AVAILABLE (APPENDIX B)

SB.1. Figure depicting impact of the dose of the mRNA/GL67 complexes on the cell viability.

SB.2. Supporting figure showing comparison of the average bioluminescence after intranasal administration of pDNA/GL67 complexes prepared at a ratio 1.33 or 4.

SB.3. Visualization of the *in vivo* luciferase production after intrapulmonary delivery of pDNA/GL67 complexes prepared at ratio 4.

This information is available also free of charge via the Internet at http://pubs.acs.org/.

CHAPTER 4

Innate immune response and programmed cell death following carrier-mediated delivery of unmodified mRNA to respiratory cells

The chapter is based on the publication:

Oliwia Andries[1], Marina De Filette[1], Stefaan C. De Smedt[2], Jo Demeester[2], Mario Van Poucke[3], Luc Peelman[3], Niek N.Sanders[1];
"Innate immune response and programmed cell death following carrier-mediated delivery of unmodified mRNA to respiratory cells"; ***Journal of Controlled Release***. 2013 April 28;167(2):157-66. doi: 10.1016/j.jconrel.2013.01.033.

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INTRODUCTION

Genetic vaccination, using pDNA or mRNA, is a very attractive strategy that offers many advantages over vaccines based on proteins, polysaccharides, or inactivated pathogens [191]. Indeed, gene-based vaccines have a lower production cost, a higher pharmaceutical stability, a better safety profile and they can encode for multiple antigens [192,193]. Furthermore, the antigens expressed by genetic vaccines can be presented in a MHCI as well as a MHCII context leading to both cellular and humoral immune responses [193-195]. The use of non-viral carriers for the delivery of genetic vaccines is gaining more and more attention as they may improve the efficacy of unformulated gene-based vaccines [196]. Additionally, also mRNA vaccines recently attracted much attention. The main advantage of using mRNA is that it is translated in the cytosol, and hence does not have to cross the nuclear membrane, which is the biggest obstacle in non-viral DNA delivery [161,162]. Moreover, in contrast to pDNA, the use of mRNA excludes an important FDA safety concern, namely the risk of insertion mutagenesis [197]. Additionally, transfection of unmodified mRNA results in a rapid and short-lived expression of the encoded protein (antigen), which is long enough to give an immunological response but not too long to cause tolerance towards the antigen [50,198,199]. It has been shown that carrier-mediated delivery of mRNA in immune cells may, besides producing the antigen for the adaptive immune reaction, induce an innate immune response [122,200-202]. This response leads to the induction of cytokines that may stimulate the adaptive immune response after carrier-mediated delivery of genetic vaccines [203]. The stimulation of the innate immune system is due to recognition of *in vitro* transcribed (IVT) mRNA by pattern recognition receptors (PRRs) [204-206]. An important family of PRRs are the TLRs, which detect pathogen-associated molecular patterns (PAMPs) of various origin, such as e.g. viral dsRNA or unmethylated CpG motifs in bacterial pDNA [207]. The first reports on recognition of mRNA by PRRs found that IVT mRNA can interact with TLR3 [202] and TLR7 [122,166].

After administration of gene-based vaccines most of them might end-up in non-immune cells, and it is known that cross-presentation of the antigen by these cells plays an important role in the establishment of the adaptive immune response [196,208]. However, the cytokine signature that is associated with recognition of mRNA by PRRs of non-immune cells has not been studied in detail. Additionally, it is also not

completely clear whether the recognition of IVT mRNA by PRRs has negative effects on the viability of the transfected cells and on the translatability of the delivered mRNA. Therefore, the aim of this work was to study the effect of carrier-mediated delivery of mRNA on the innate immune response, the viability and translatability of the delivered mRNA. In this study we used lung epithelial cells because of our interest in mucosal immunization against respiratory pathogens and lung cancer [209]. Pulmonary vaccination can increase the efficacy of a vaccine as this delivery strategy may induce local immune responses that can neutralize pathogens at the entry port [210,211]. Human as well as murine lung cells were used to compare their transfection efficiency, protein expression, cytotoxicity and eventually the innate immune responses. For the delivery of the mRNA we used the GL67-lipid formulation, which is considered as the golden standard in non-viral respiratory gene transfer [153,212-214]. The therapeutic potential, the low toxicity and safety of this formulation has been extensively demonstrated in many pre-clinical and clinical trials [215,216].

MATERIALS AND METHODS

Cell culture

Human lung adenocarcinoma cells (A549 cells, ATCC n° CCL-185) and murine lung adenoma cells (LA-4, ATCC n° CCL-196) were plated onto 24-well plates one day before transfection. At the moment of transfection their confluency was around 80%. Human embryonic kidney cells (HEK293) and HEK293 stably overexpressing TLR3 (HEK293-TLR3 cells) were a generous gift from Prof. Rudi Beyaert (Department for Molecular Biomedical Research, VIB, Belgium). The HEK293 cells were seeded in the same format as the lung cells. All cells were cultured at 37°C in a humidified atmosphere containing 5 % CO2. DMEM supplemented with 10% fetal bovine serum (FBS), 50 µg penicillin/ml, 50 µg streptomycin/ml and 2 mM L-glutamine (Invitrogen, Merelbeke, Belgium) was used as culture medium. Above that, neomycin (Sigma Aldrich, Bornem, Belgium) was added to the culture medium of HEK293-TLR3 cells.

Plasmids

The pBlue-LucA50 containing a poly(A)-tail of 50 adenosines and the cDNA of firefly luciferase was used for the *in vitro* transcription (IVT) of mRNA. This pDNA was a kind gift of Dr. Peter Ponsaert (University of Antwerp, Antwerp, Belgium) and was previously described by Sheets et al. [167]. The pGEM4Z[eGFP]A64 containing a poly(A) of 64 adenosines and the cDNA of eGFP was used for IVT of mRNA encoding eGFP. The latter was provided by Prof. dr. Smita Nair (Duke University Medical Center, NC, USA). pGL2 plasmid encoding firefly luciferase was purchased from Promega (WI, USA). The plasmids were purified with QIAGEN Plasmid Giga Kit (Qiagen, Venlo, The Netherlands).

In vitro transcripton of mRNA

The modified mRNA containing pseudouridine and 5-methylcytidine nucleotides was purchased from Stemgent (Miltenyi Biotec, Leiden, The Netherlands). This modified mRNA codes for eGFP. Prior to *in vitro* transcription the pBlue-LucA50 and the pGEM4Z[eGFP]A64 were linearized downstream of the insert with respectively DraI and SpeI restriction enzyme (Promega, WI, USA) and examined on a 1 % agarose gel. mRNA was transcribed with a mMESSAGE mMACHINE kit according to the manufacturer's instructions (Ambion, Austin, TX, USA). The mRNA was dissolved in RNase-free water (Ambion, Austin, TX, USA) and the purity and size was checked by formaldehyde gel electrophoresis. The formaldehyde gel was prepared as follows. One gram of Ultra Pure Agarose (Invitrogen, Merelbeke, Belgium) was dissolved in 100 ml of MOPS/formaldehyde-buffer (20 mM MOPS, 2.1 M formaldehyde, pH 7 prepared in RNase-free DEPC-treated water) (Sigma Aldrich, Bornem, Belgium). As a reference, a RiboRulerTM High Range RNA Ladder was used (Fermentas, St. Leon-Rot, Germany). The mRNA concentration was determined by measuring the absorbance at 260 nm by NanoDrop (Thermo Scientific, DE, USA) and its purity was assessed by measuring the 260/280 nm absorbance ratio.

Preparation of complexes and transfection experiments

The GL67:DOPE:DMPE-PEG5000 liposomes were prepared by adding 2,667 ml of

RNAse-free water (Ambion, Austin, TX, USA) to the vials containing 4 µmol GL67, 8 µmol DOPE and 0.2 µmol DMPE-PEG5000. The mRNA/GL67 and pDNA/GL67 complexes were prepared by mixing the mRNA or pDNA (dissolved in RNAse-free water) with the GL67 liposomes at their optimal ratios, namely 2:1 (mRNA:GL67 lipid molar ratio) [217] and 1.33:1 (pDNA:GL67 lipid molar ratio) [169]. The liposomes and the nucleic acids were shortly incubated at 30°C before mixing them. The mRNA/GL67 or pDNA/GL67 molar ratios were calculated based on the molar concentration of the GL67 lipid in the GL67:DOPE:DMPE-PEG5000 liposome formulation and the nucleotide concentration in the mRNA or pDNA solutions. To calculate the nucleotide concentration an average nucleotide molecular mass of 340 g/mol (330 g/mol for pDNA) was used. After mixing, the complexes were incubated for 15 min at 30°C and subsequently they were further diluted in OptiMem (Invitrogen, Merelbeke, Belgium) and added to cells seeded in 24 well plates. The complexes were removed 4 hours after addition and replaced by fresh culture medium.

Protein expression measurements and viability assay

The luciferase expression was examined 24 hours post-transfection by a luciferase assay following the manufacturer's protocol (Promega, WI, USA). The measurements were carried out in a GloMax microplate luminometer (Promega, WI, USA). The luciferase activity was expressed as the number of relative light units (RLU) per µg of protein. The protein concentration was determined by a BCA assay (Thermo Scientific, DE, USA) measured on EnVision Multilabel Reader (Perkin Elmer, Waltham, MA, USA). Transfection efficiency of lipoplexes containing mRNA encoding eGFP was determined by flow cytometry (FACSCalibur, Becton Dickinson, Erembodegem, Belgium). A549 cells were washed with PBS and subsequently resuspended in a flow buffer (BD FACSFlow). Percentages of eGFP positive cells and their mean fluorescence intensity were used for analysis. 10000 cells per sample were analyzed. Data analysis was performed with CellQuest software (Becton Dickinson).
In order to check the viability of the cells, the MTT proliferation kit (Roche, Vilvoorde, Belgium) and the luminescent cell viability assay CellTiter-Glo (Promega, WI, USA) were used.

Total RNA extraction and determination of its quality and quantity

During the experiment 3 biological replicates were evaluated from A549 and LA-4 cells for each treated and untreated cells. The untreated cells were conditioned in the same manner as the treated cells, besides the step including adding the mRNA/GL67 complexes. Total RNA was isolated with RNeasy Mini Kit (Qiagen, Venlo, Netherlands) 24 hours after adding complexes on cells according to the manufacturer's protocol. The extraction included on-column treatment with DNAse. Before performing qPCR total RNA was confirmed to be free from genomic DNA by minus RT-PCR according to the following protocol: 8 min 45 s at 95ºC followed by 40 cycles of (15 s at 95ºC, 15 s at 57ºC, 30 s at 72ºC) and finally 2 min at 72ºC. 1 µl of primermix (5 µM each: ACTB +1 AGGGAAATCGTGCGTGACAT, ACTB -1 GAGCAGTAATCTCCTTCTGC ATCC) was added to 1 µL FastStart buffer (10x), 0,1 µL FastStart Polymerase (5 Units/µL), 0,2 µL dXTPs (deoxyribonucleotide triphosphates) (10 mM each) and DNA (gDNA or cDNA) or RNA. Water was added up to 10 µl per reaction. Water and genomic DNA with primers specific for beta-actin (ACTB) gene were used as the negative and positive control, respectively. The concentration was evaluated spectrophotometrically by NanoDrop (Thermo Scientific, DE, USA). The samples with the ratio 260 nm/280 nm between 1,96 and 2,21 as well as 260 nm/230 nm between 1,92 and 2,29 were further evaluated for their quality. The integrity of the total RNA was determined both by the formaldehyde gel electrophoresis and the Experion automated electrophoresis system (BioRad, Nazareth, Belgium).

cDNA first strand generation.

The cDNA first strand was generated with the RT2 First Strand Kit (SABiosciences, MD, USA) primed with random hexamers and oligo-dT, according to the manufacturer's protocol. The cDNA was synthetized out of 1 µg of total RNA. Following the reaction, the presence of cDNA in the sample was confirmed by the same PCR as for the minus RT-PCR. For one qPCR array (96 well plate), 106 µl of template was dissolved 20 times in SYBR Green Master Mix (SABiosciences, MD, USA). 20 µl of sample working solution was added per well.

TLR-related pathway qPCR array

The upregulation or downregulation of genes associated with the human (cat # 00188255) and mouse (cat # 00188196) toll-like receptor signaling pathways were evaluated with Lonza standard 96 StellARray™ qPCR arrays (Lonza, Basel, Switzerland). The StellARray system profiles 94 different TLR-associated genes (see the supplementary data S1). For both qPCR experiments RT² SYBR® Green qPCR Master Mix (SABiosciences, MD, USA) was used. The cycling conditions were: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min and 40 cycles at 95°C for 15 s and 60°C for 1 min. Melting curve was set according to a protocol: 70ºC → 95ºC; (10 s/0,5 ºC) x 50. The melting curve analysis confirmed that a single amplicon was produced. According to the company the PCR efficiency of all primers is between 90-100 %. The data analysis of the qPCR arrays was performed using the Global Pattern Recognition™ (GPR) 2.0 Analysis Tool (Lonza, Switzerland). The Global Pattern Recognition chose 18 genes for A549 and 9 genes for LA-4 unchanged in expression as normalizers (supplementary data S1). Global Pattern Recognition™ Software globally positions the expression level of each gene with respect to all genes within an experiment. For the statistical analysis, the genes that were not detectable during qPCR got a Cq value of 40.

ELISA assays

Mouse IFN-β, mouse IL-6, mouse IL-12 and mouse TNF-α ELISA kit were purchased from BioLegend (Antwerp, Belgium) and used to determine the concentration of secreted cytokines in the medium of LA-4 cells treated with mRNA/GL67 complexes, naked mRNA or GL67 liposomes only. Human IFN-β ELISA kit was purchased from Thermo Scientific (Erembodegem, Belgium). Human IL-6 and human TNF-α were obtained from BioLegend (Antwerp, Belgium). The human cytokines were measured in the medium of A549 cells treated with mRNA/GL67 complexes, naked mRNA or GL67 liposomes only. Briefly, 100 µl of the media and the cytokines' standards were added in triplicates to the wells of the 96 well microtiter plates that were pre-coated with an antibody against specific cytokine. After 60 minutes of incubation the wells were washed 3 times with the provided wash solution. Subsequently, the wells were incubated with a detecting antibody labeled with horseradish peroxidase (HRP). After

60 minutes unbound detecting antibodies were washed away as described above and the microtiter plates were incubated for 15 minutes with Tetramethyl-benzidine (TMB) substrate. The reaction was stopped by addition of stop solution and the absorbance was measured at 450 nm with an Envision Multilabel Reader (Zaventem, Belgium).

In vivo experiment

Balb/c mice were obtained from Janvier (Le Genest St Isle, France). Mice were anesthetized with 4 % isoflurane and 80 µg of unmodified mRNA/GL67 complexes were intranasally instilled to a group of 3 animals. The respective volume of dissolvent (RNAse-free water) was administered in the same way to a control group (n=3). The mice were imaged 4 and 24 hours later with *in vivo* bioluminescent imaging system (IVIS Lumina II, Caliper Life Sciences). After that, the animals were euthanized by cervical dislocation and their lungs were removed and homogenized. The samples were evaluated for IFN-β, IL-6, IL-12 and TNF-α cytokines production by ELISA assays.

Statistics

All obtained data sets had a normal distribution as assessed by the Kolmogorov–Smirnov test. An independent-samples t-test was performed in order to compare two groups. ANOVA followed by the Bonferroni test were conducted for multiple group analysis. The differences were considered significant when $p<0.05$. The results are presented as the mean ± standard deviation (SD).

RESULTS

Cytotoxicity kinetics after transfection of A549 cells with mRNA

Cytotoxic effects associated with mRNA delivery are often studied only shortly after transfection. Consequently, the cytotoxicity associated with mRNA transfection may have been underestimated. Therefore, we monitored the viability of lung epithelial cells up to three days after transfection with mRNA/GL67 complexes. Four hours after

mRNA transfection the viability dropped with 30 % (Figure 4.1). The drop in viability of the mRNA-transfected cells continued the following hours and started to level off at day 2 post-tranfection. Three days after transfection only 10 % of the mRNA transfected cells were still viable. In contrast, the viability of cells transfected with pDNA was much higher. Transfection of the cells with lower amounts of nucleic acids showed similar cytotoxicity kinetics, although the toxicity was lower (data not shown).

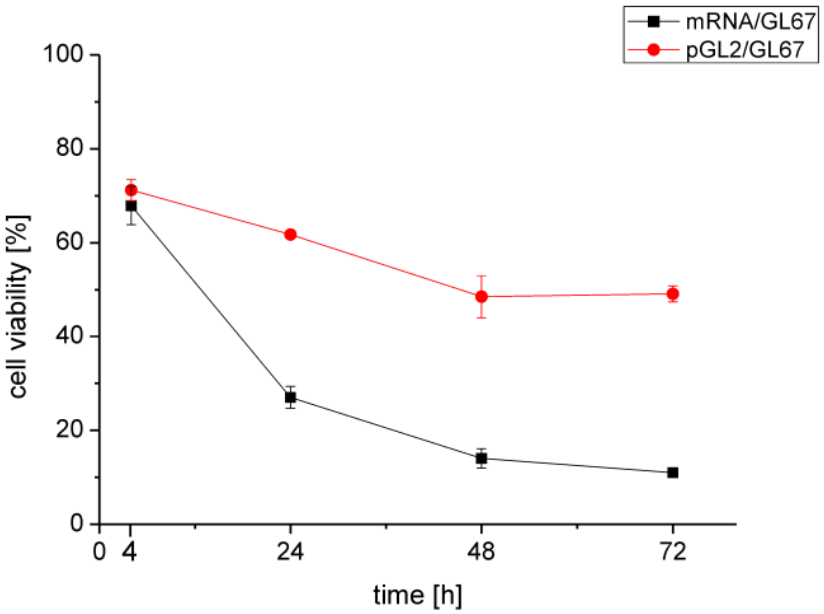

Figure 4.1. Cytotoxicity kinetics after transfection of A549 cells with mRNA/GL67 and pDNA/GL67 complexes. A549 cells were transfected with mRNA/GL67 and pDNA/GL67 complexes containing 500 ng of complexed nucleic acids. Their viability was measured 4 h, 24 h, 48 h and 72 h after addition of the complexes using an MTT assay. The cell viability was calculated relatively to the viability of untreated cells. The results are presented as the mean ± SD.

Cytotoxicity of the constituents of the mRNA/GL67 complexes

The huge drop in cell viability after mRNA transfection urged us to unravel which constituents of the mRNA/GL67 complexes were most responsible for the cell death. Therefore, the effect of mRNA/GL67 complexes, naked mRNA or empty GL67 liposomes on the viability of human lung epithelial (A549) cells was measured 2 days after transfection. This experiment was repeated with murine lung epithelial (LA-4) cells to determine the differences between the human and mouse cell line model. Interestingly, neither naked mRNA nor the GL67 liposomes alone caused a significant

reduction in cell viability (Figure 4.2A and 4.2B). In contrast, mRNA complexed to the GL67 liposomes induced a substantial and significant cytotoxicity in both cell lines (Figure 4.2A and 4.2B). These results suggest that mRNA becomes toxic for the cells when it is taken up by the cell, a process mediated by the GL67 liposomes. Figure 4.2 also shows that the toxic effects are significantly different between the two cell lines. Indeed, 2 days after mRNA transfection the viability of the murine LA-4 cells was 50 % higher than the viability of the human A549 cells. Additionally, the transfection efficiency of the mRNA/GL67 complexes was checked in both cell lines. Transfection of A549 cells with mRNA/GL67 complexes resulted, 24 hours after transfection, into more than 46 % of eGFP positive cells, while only 5 % of the transfected LA-4 cells were eGFP positive (Figure 4.3).

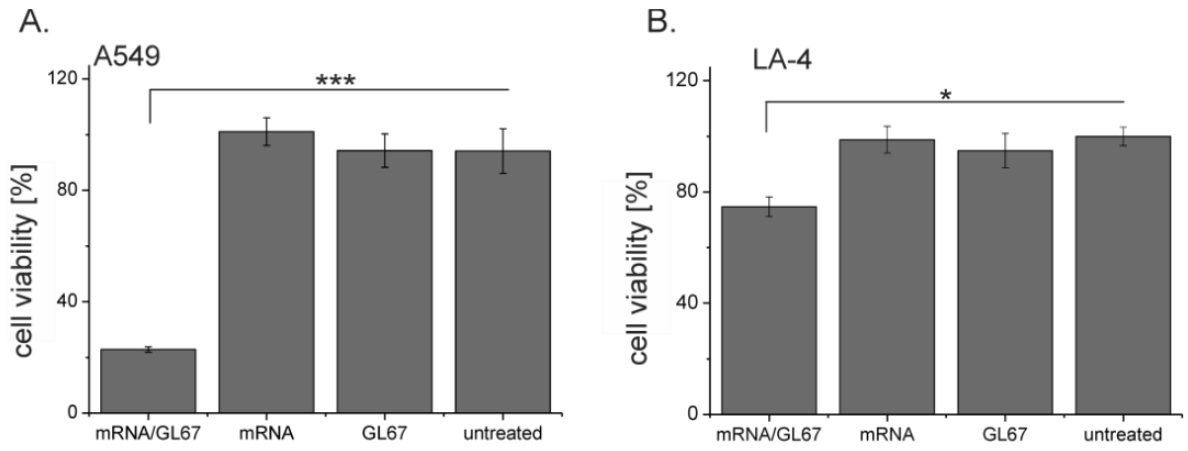

Figure 4.2. Cytotoxic effect of the constituents of the mRNA/GL67 complexes. A549 (A) and LA-4 cells (B) were transfected with 750 ng of mRNA/GL67 complexes, the same amount of naked mRNA or just GL67 liposomes. The viability of the cells was measured 48 h post-transfection using CellTiter-Glo (A549) and MTT (LA-4) assay and compared to the viability of untreated cells. The bars represent the mean ± SD (*, p<0,05; ***, p<0,001 ANOVA).

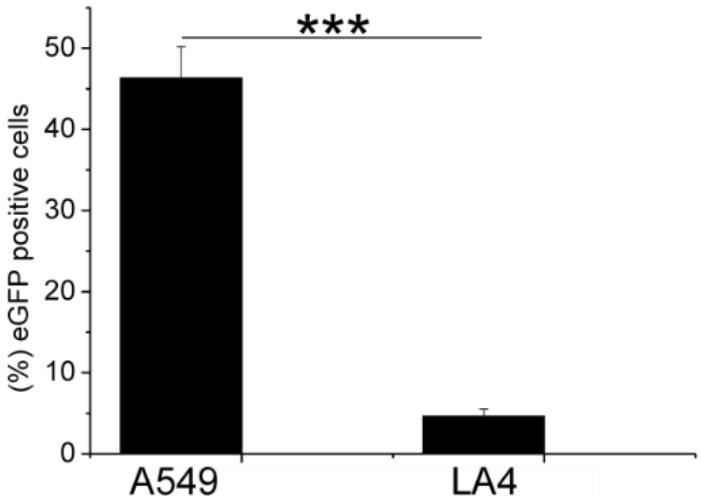

Figure 4.3. Comparison of the transfection efficiency of mRNA/GL67 complexes in human (A549) and murine (LA-4) respiratory cells. The A549 and LA-4 cells were transfected with 500 ng of complexed mRNA. Transfection efficiency was measured by flow cytometry 24 h after adding the complexes on the cells. The results are presented as the mean ±SD (n=6; ***, p<0,001, independent samples *t*-test).

Activation of TLR-related pathways and innate immune responses in respiratory cells after liposome-mediated delivery of IVT mRNA

The observation that only mRNA/GL67 complexes were toxic for the cells and not their constituents may indicate that the intracellular delivery of mRNA triggers cell death. Previous experiments in HEK cells and immune cells have shown that transfected IVT mRNA is recognized by several TLRs, such as TLR3 [202] and TLR7 [122,166], which are mainly localized in endosomes. It is well known that nucleic acid containing nanoparticles are taken up by cells via endocytosis. Consequently, carrier-mediated delivery of mRNA may bring the mRNA to these endosomal TLRs and promote TLR signaling, which, as shown for poly(I:C), may induce cell death [218]. Therefore, we measured the upregulation of TLR associated genes after liposome-mediated delivery of IVT mRNA. We first set out to determine the expression level of all known TLRs in untreated A549 and LA-4 cells (Figure 4.4A and 4.4B). Figure 4.4 presents the Cq values at which the cDNA encoding each TLR was detected. Ten different TLR typical for human cells (TLR1-10) were evaluated in A549 cells. TLR6 was expressed at the highest level in untreated human cells (Cq<30). A low expression was observed for TLR1, 3 and 4 (30<Cq>35). Non-detectable or almost non-detectable amounts were

found for TLR2, 5 and 7-10 (Cq>35). The extremely low expression of TLR7 and the lack of TLR8 expression in A549 agrees with previous report of Tissari et al., who also noted no expression of these TLRs in A549 [219-221]. In case of murine LA-4 cells, thirteen different TLRs (TLR1-13) typical for murine cells were evaluated. The highest expression in non-treated cells was observed for TLR1 and TLR7 (Cq<30). TLR3, 4 and 6 were expressed at low levels (30<Cq>35), while TLR2, 5, 8-13 were expressed at extremely low to undetectable levels (Cq>35).

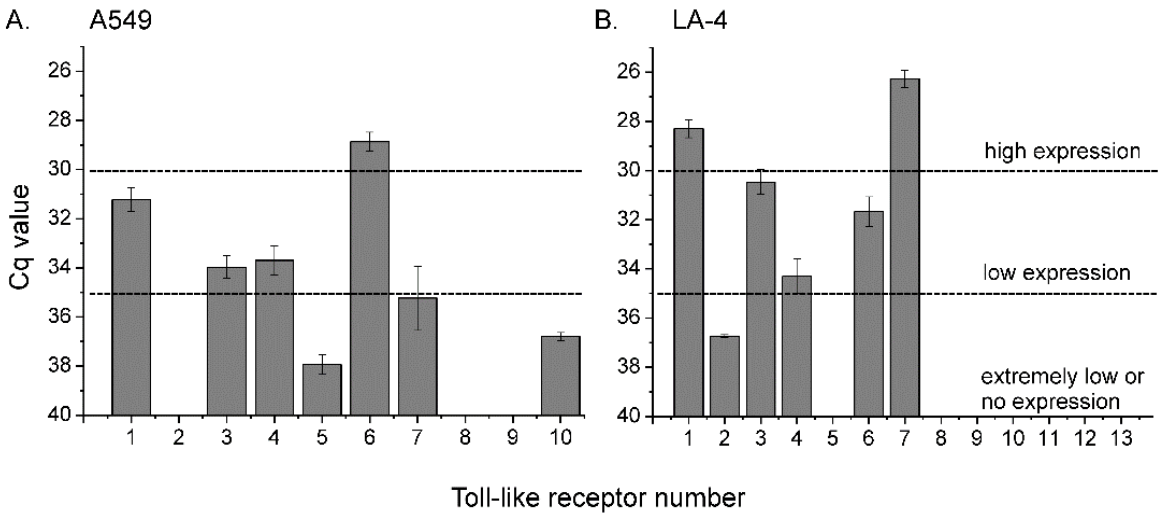

Figure 4.4. The expression level of TLRs in untreated A549 and LA-4 cells. The Cq value at which the cDNA of each TLR was detected in untreated human A549 (A) and murine LA-4 (B) epithelial cells were determined via qPCR. A gene is considered to be highly expressed when its Cq value is lower than 30. A Cq value between 30 and 35 stands for a low expression, while a Cq value higher than 35 indicates that the expression is extremely low or nihil. Notice that the scale of the Y-axis is inverted so that higher bars represent higher expression levels. The data are presented as mean values ± SD (n=3).

Next, we evaluated whether liposome-mediated delivery of IVT mRNA resulted in an activation of TLR pathways. Therefore, the expression levels of 94 TLR-associated genes were determined in A549 and LA-4 cells 24 hours after exposure to mRNA/GL67 complexes or medium only (an overview of the analyzed genes can be found in the supplementary data, table SC.1 and SC.2). The time-point of 24 hours was chosen as the most suitable to evaluate the activation of cytokines connected to TLRs signaling [222]. At this time, the cells do not achieve the highest cytotoxicity level yet but they

express the specific factors responsible for the cell death. The genes that were significantly upregulated or downregulated after liposome-mediated delivery of IVT mRNA are summarized in Table 3.1. Transfection of A549 cells and LA-4 cells with mRNA caused an upregulation of 27 and 14 TLR-associated genes, respectively. Interestingly, in A549 cells not only more TLR-associated genes are induced upon mRNA transfection, but also the extent of upregulation of these genes is much higher than in LA-4 cells. Indeed, the four most induced genes were upregulated more than 4000-fold in A549 cells, while only a 20-fold induction was detected for the four highest upregulated genes in LA-4 cells. Nine genes were substantially upregulated by both cell lines after carrier-mediated mRNA delivery, i.e. IFN-β, CCL5 (also called RANTES), CXCL11, CCL4 (also called MIP-1β), IL-6, IRF-7, CXCL10 (also called IP-10), TNF-α and TLR3. Remarkably, IFN-α and caspase-1 were significantly and highly induced by mRNA delivery in human A549 cells only.

A. A549

No.	Gene	P-value	Fold Change
1	CCL5	0.00049	8921.59
2	IFNB	0.00018	6421.93
3	CXCL11	0.00287	6214.77
4	CXCL10	0.00004	4147.09
5	IFNA	0.00005	653.22
6	IL6	0.00255	498.08
7	CASPASE 1	0.00491	384.31
8	IRF7	0.00014	140.17
9	CCL4	0.00013	99.56
10	CCL3L1	0.00016	83.41
11	PTX3	0.00021	67.38
12	CXCL9	0.00095	57.87
13	CIITA	0.00072	53.85
14	SOCS1	0.00023	42.29
15	TLR3	0.00054	29.67
16	TNF	0.00277	26.22
17	IRF1	0.00210	22.52
18	STAT1	0.00134	8.01
19	MYD88	0.00246	5.50
20	IRAK2	0.00205	4.77
21	IL12A	0.00339	4.57
22	MUC1	0.01181	3.84
23	TRAFD1	0.01250	2.90
24	JUN	0.01361	2.80
25	TICAM1	0.01588	2.71
26	RIPK2	0.01198	2.17
27	TLR1	0.03338	2.07
28	CNPY4	0.02836	-2.17
29	CD14	0.00627	-3.18

B. LA-4

No.	Gene	P-value	Fold Change
1	CCL4	0.027	22.93
2	IFNB	0.034	22.73
3	TNF	0.011	21.10
4	IRF7	0.026	20.22
5	CXCL11	0.012	16.79
6	IL6	0.018	15.30
7	CCL5	0.021	13.08
8	TLR3	0.006	10.76
9	CXCL10	0.013	10.24
10	STAT1	0.014	5.96
11	IRF1	0.035	4.37
12	TRAFD1	0.017	4.31
13	SOCS1	0.024	3.65
14	MYD88	0.040	2.23

Table 4.1: Effect of carrier-mediated delivery of mRNA on TLR associated genes. Overview of TLR associated genes that were significantly up- or down-regulated after carrier-mediated delivery of mRNA in human A549 (A) and murine LA-4 cells (B) in comparison to untreated cells. The cells were transfected with mRNA/GL67 complexes and 24 h later the total RNA was extracted and checked for quantity and quality. Subsequently the expression level of TLR associated genes was determined in mRNA transfected and untreated cells (n=3). The fold change in gene expression induced by carrier-mediated mRNA delivery was calculated using the global pattern recognition analysis tool as described in Materials and Methods.

Secretion of IFN-β and IL-6 by respiratory cells after liposome - mediated delivery of IVT mRNA

In general, gene upregulation is reflected in a higher production of the encoded protein. IFN-β and IL-6 were highly upregulated in LA-4 as well as in A549 cells after carrier-mediated delivery of mRNA (Table 4.3). IFN-β is expressed and secreted after recognition of a danger pattern by TLR3 in order to *(1)* sensitize the cells against viral infection, *(2)* inhibit the viral proliferation, *(3)* promote Th1 response by increasing the synthesis and expression of MHC-I as well as the release of other cytokines [223]. IL-6 is considered an activator of acute phase responses and a lymphocyte stimulatory factor [224]. Therefore, as these cytokines play an important role in the TLR3 signaling pathway we decided to investigate, if a similar upregulation of IFN-β and IL-6 is also observed at the protein level. Figure 4.5 shows the extent of the cytokines secretion by respiratory cells after incubating them with the mRNA/GL67 complexes and the single constituents of the complexes. Additionally, complexes with mRNA encoding luciferase (1.7 kb) and eGFP (0.7 kb) were compared, as we previously observed differing viability pattern between them (data not shown). Longer luciferase mRNA bound to GL67 liposomes gave typically rise to a higher amount of measured cytokines (besides hIL-6), what agrees with their stronger cytotoxic effect. It might confirm the assumption, that longer mRNA chains may be responsible for more frequent interactions with PRRs. Moreover, longer mRNA contains statistically higher number of unmodified nucleotides responsible for innate immunity recognition [225]. The IFN-β and IL-6 cytokines were not detected in a medium of untreated cells and neither after treatment with naked mRNA nor the GL67 liposomes. On the contrary, carrier-mediated delivery of mRNA to both A549 and LA-4 cells provoked a substantial and significant IFN-β and IL-6 secretion. The data in Figure 4.5 mirrors the effect of mRNA/GL67 complexes and its constituents on the cell viability (Figure 4.2) and complements the qPCR data in Table 4.1.

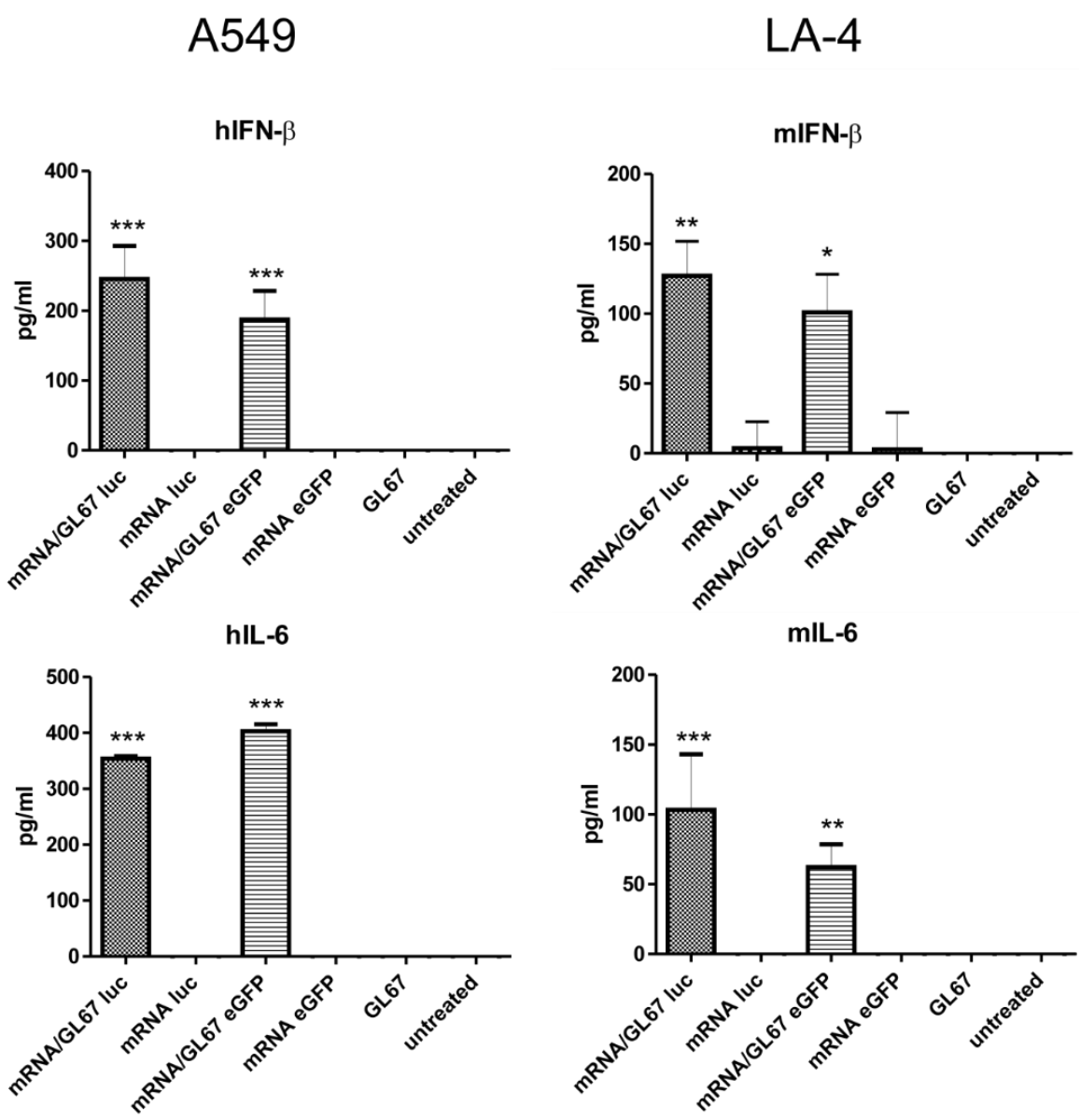

Figure 4.5. IFN-β and IL-6 production after treatment of A549 and LA-4 with mRNA/GL67 complexes and their constituents. The cells were transfected with either mRNA/GL67 complexes containing 750 ng of complexed mRNA, the same amount of naked mRNA or just GL67 liposomes. 24 h post-transfection the secretion of the cytokines by the cells was measured using an ELISA assay. The bars represent the mean ± SD (n=3, *, p<0,05; **, p<0,01; ***, p<0,001 ANOVA).

Impact of mRNA mediated activation of TLR3 on the transla-tion efficiency of the delivered mRNA

Based on the observed upregulation of TLR3 and its downstream signaling molecules,

we can conclude that TLR3 plays an important role in the induction of the innate response after carrier-mediated delivery of mRNA to respiratory cells. It has been shown that the type I interferons that are produced during signaling through TLR3 cause a global suppression of translation [226-228]. Therefore, we decided to explore the impact of TLR3 signaling on the translation efficiency of the delivered mRNA. For this purpose, GL67 liposomes with mRNA encoding luciferase were used to transfect HEK293 and HEK293-TLR3 cells, which overexpress TLR3. 24 hours after transfection we determined the amount of luciferase produced by the cells and the cell viability. In case of HEK cells overexpressing TLR3, the luciferase levels were about 25-fold lower than in regular HEK cells. Additionally, cells overexpressing TLR3 demonstrated higher cell death after mRNA transfection (Figure 4.6).

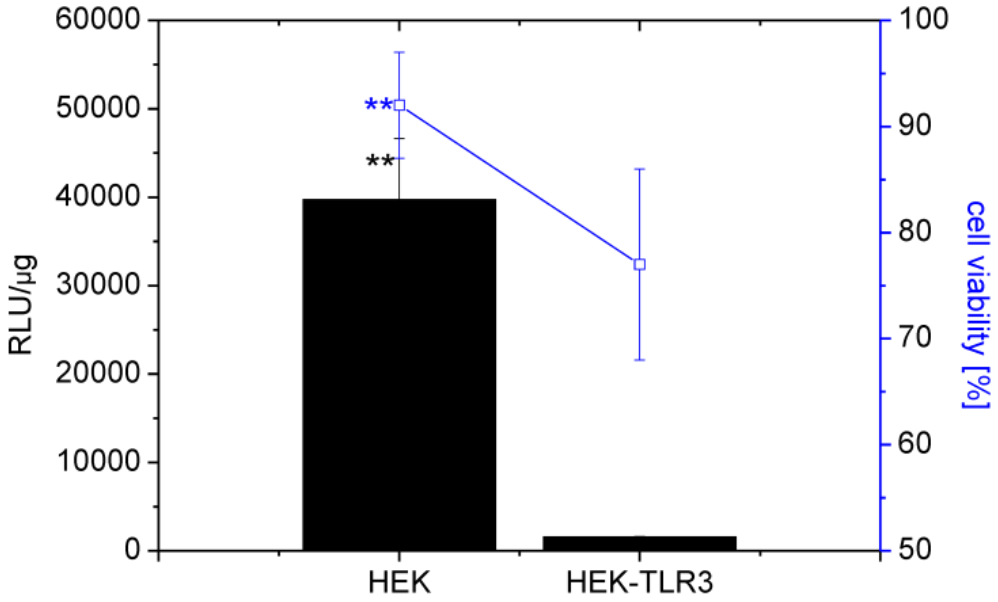

Figure 4.6. Impact of TLR3 on recombinant protein expression and viability after carrier-mediated delivery of mRNA. HEK cells and HEK cells overexpressing TLR3 were transfected with 750 ng of mRNA/GL67 complexes. 24 h post-transfection the luciferase expression was measured (bars). The viability of cells was measured after 48 h with MTT assay (line). The results are expressed as the mean ± SD (n=3, ** for p<0.01; independent-samples *t*-test).

Effect of modified mRNA on the cell viability and transfection efficacy

It has been described in the past that the use of modified mRNA can decrease the

activation of the innate immune system [6,229]. Therefore, we determined whether incorporation of modified nucleotides in the mRNA could also prevent the cytotoxic effect caused by mRNA mediated stimulation of the innate immune system. We used modified mRNA that contained pseudouridine and 5-methylcytidine nucleotides. Both A549 and LA-4 cells were transfected with mRNA/GL67 complexes containing either unmodified or modified mRNA. Twenty hours after transfection the viability of the A549 cells transfected with unmodified mRNA was below 30 %, while the viability of the cells transfected with modified mRNA was above 80 % . The viability of LA-4 cells 24 hours after adding the complexes with unmodified mRNA was at the level of 64 % where the modified mRNA/GL67 complexes gave the result of 88 % (Figure 4.7). Next, we evaluated whether the use of modified mRNA can also increase the gene transfer efficacy. Surprisingly, as shown in Figure 4.8 the use of modified mRNA does not increse the number of transfected cells. However, the level of gene expression in the eGFP positive cells was much higher with the modified mRNA.

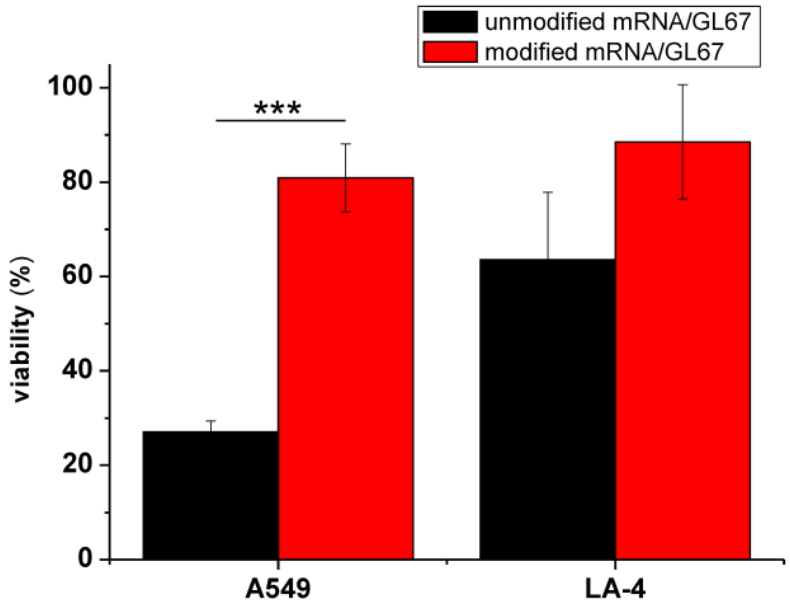

Figure 4.7. Cytotoxicity kinetics after transfection of A549 and LA4 cells with mRNA/GL67 complexes containing unmodified or modified nucleotides. The modified mRNA contains both pseudouridine and 5-methylcytidine. The viability was measured 24 h after addition of the complexes using an MTT assay. The cell viability was calculated relatively to the viability of untreated cells. The results are presented as the mean ± SD (n=3, ***, p<0,001, independent samples *t*-test).

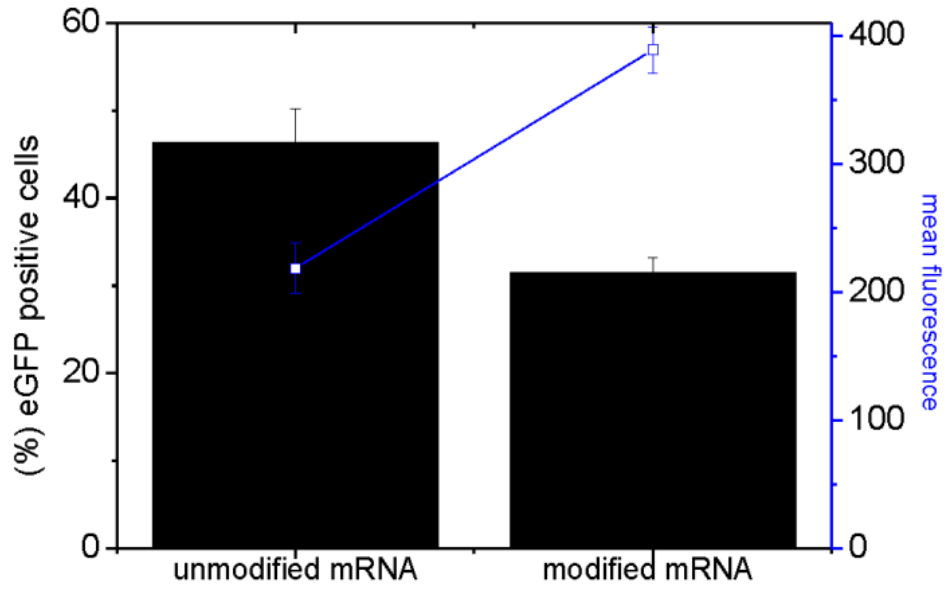

Figure 4.8. Effect of modified mRNA on the transfection efficiency of mRNA/GL67 complexes. A549 cells were transfected with mRNA/GL67 complexes containing 500 ng of unmodified or modified mRNA. The modified mRNA contained both pseudouridine and 5-methylcytidine. Transfection efficiency (bars) and mean fluorescence (line) was measured by flow cytometry 24 h after adding the complexes on cells. The results are presented as the mean ± SD (n=6).

Cytokine expression after intrapulmonary administration of mRNA/GL67 complexes in vivo

Transfection efficiencies and hence immunological effects *in vitro* may differ *in vivo*. Residing pulmonary APCs, such as macrophages or dendritic cells, are specialized in phagocytosis of any self and unself molecules and production of a proper innate immune response followed eventually by an adaptive immune response specific for the antigen. This system is also employed in pulmonary vaccination. A pilot *in vivo* study was carried out and 80 µg of complexed unmodified mRNA (dissolved in RNAse-free water) was instilled intranasally to lungs of mice (n=3). In order to evaluate a pure effect of the lipoplexes, as the control group we treated mice (n=3) with the same volume of RNAse-free water. Both groups of the animals were imaged 4 and 24 hours later by *in vivo* bioluminescence imaging system, however no signal was detected. After removing lungs from the animals the ELISA assays were performed and the concentration for following cytokines was measured: IFN-β, IL-6, IL-12 and TNF-α

(Figure 4.9). After intrapulmonary treatment with unmodified mRNA/GL67 complexes, the mice developed significant overexpression of inflammatory cytokines, such as IL-6 and TNF-α as well as cytokine typical for professional APCs: IL12. Production of IL-12 and GM-CSF (not shown semi-quantitative data) confirms the hypothesis, that the complexes are phagocytized by the professional APCs residing in the lungs, what also prevents the transfection of pulmonary epithelial cells and eventually expression of the encoded proteins.

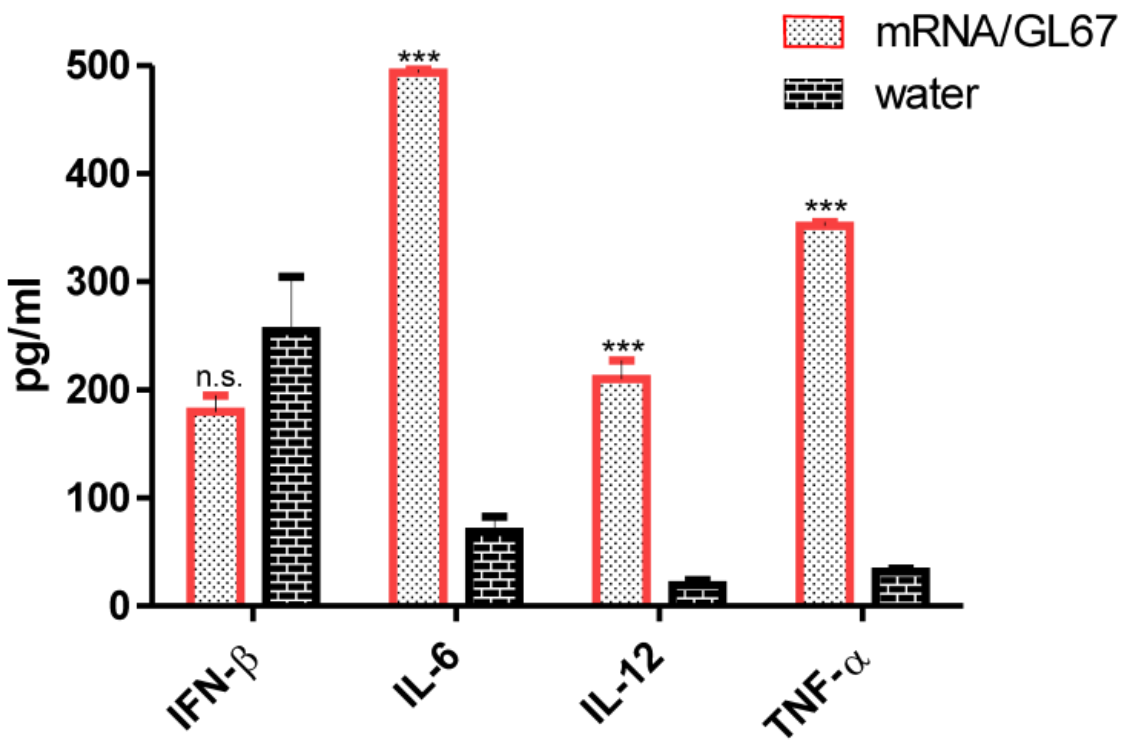

Figure 4.9. Inflammatory cytokines expression after unmodified mRNA/GL67 intrapulmonary delivery *in vivo*. 24 hours after intranasal instillation of unmodified mRNA/GL67 complexes in water (n=3) or adequate volume of just water (n=3) the lungs of mice were removed and homogenized. After that ELISA assay were performed for IFN-β, IL-6, IL-12 and TNF-α. The bars represent the mean ± SD (*, p<0.001 ANOVA).**

DISCUSSION

The presented study demonstrates a powerful stimulation of the innate immune system after carrier-mediated delivery of mRNA in respiratory cells. Most of the genes that were upregulated after mRNA transfection can be brought back to the TLR3 signaling

pathway and its downstream effectors, i.e. type I interferons as well as inflammatory cytokines. Figure 4.10 schematically depicts these upregulated genes in the TLR3 signaling pathway. The clear upregulation of TLR3 and its adaptor protein TICAM1, also called TRIF, indicates that mRNA is recognized by TLR3, which is one of the PRRs of the innate immune system responsible for interaction with dsRNA, usually of viral origin. One would expect that the delivered mRNA interacts with TLR7 and TLR8, which are known to interact with ssRNA. However, they were not upregulated after mRNA transfection. Moreover, TLR8 was not expressed in respiratory cells and only LA-4 cells showed a clear expression of TLR7. Although mRNA is transcribed as a single strand, it often contains double stranded regions. This may explain the recognition of mRNA by TLR3. We confirmed the presence of such secondary structures in our mRNA using the RNAfold Website predictor software (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

Almost all of the upregulated genes in Table 1 are directly linked to the TLR3 signaling pathway (Figure 4.10). TICAM-1, IRF3 and 7 are responsible for the induction of type I interferons (IFN-α and IFN-β) [230,231] and IL-12 [232] following TLR3 activation. These type I interferons subsequently activate, via STAT1, the CXCL and CCL chemokines. The downstream TLR3 signaling to NF-κB can explain the upregulation of IL-6 [231], IL-12 [233] and TNF-α [231]. The upregulated genes SOCS1, MUC1, TRAFD1, IRAK2 and MyD88 are known as negative regulators of TLR3 signaling. The induction of these negative regulators after TLR3 activation has also previously been reported and it is believed that they prevent an overstimulation of the innate immune system [234-237].

All the cytokines that are induced after carrier-mediated delivery of mRNA are known to support the adaptive immune response. Indeed, type I interferons, which are highly induced after carrier-mediated mRNA delivery, are strong vaccine adjuvants as they increase the expression of MHCI [238], tumor antigens [239], activate NK cells [240,241] and facilitate the cross-priming [242]. Their function in bridging innate with adaptive immunity is being unraveled [223,243-245]. Furthermore, the massive production of TNF-α, CXCL and CCL chemokines may also increase the immune response after mRNA vaccination because these chemokines will attract immune cells to the injection spot and activate them [246-249]. CCL5 or RANTES has gained much attention as it recruits dendritic cells (DCs) and induces a cytokine cascade in these cells [250]. Therefore, CCL5 is currently evaluated as a vaccine adjuvant [251]. Moreover, a recent study showed that CCL5 is essential for sustaining a CD8+ T cell response during infection [252]. The two

upregulated interleukins, i.e. IL-6 and IL-12, play pivotal roles during the transition from innate to antigen-specific adaptive immunity. IL-6, which was highly upregulated after mRNA delivery is responsible for the attraction of monocytes and T-cells after the acute inflammation phase. Furthermore, it inhibits TGFβ mediated differentiation of T cells into regulatory T cells and skews T cell differentiation towards Th2 cells or, when also TGFβ is present, towards Th17 cells [253]. IL-12, which was slightly induced by mRNA transfection, skews T cell differentiation towards Th1-cells, stimulates cytotoxic T cells and NK cells, and induces IFN-γ production by these cells and DCs [232,254].

Surprisingly, carrier-mediated delivery of mRNA caused, in contrast to pDNA delivery, an extensive and delayed cell death. The cytotoxicity of mRNA was much more pronounced in the human A549 cells than in the murine LA-4 cells. This is in line with the data both on mRNA and protein level, which show that the innate immune response after mRNA delivery is much higher in the human than in the murine respiratory cells. It is well known that type I interferons exhibit antiproliferative and apoptotic effects [255,256]. As discussed above, type I interferons were heavily upregulated after mRNA delivery. Consequently, it is very likely that they play a role in the observed cytotoxicity. Additionally, based on Kubo et al. [257], who studied the dose-dependent effect of IFN-β on the viability of melanoma cells, we can conclude that the amount of IFN-β produced by the LA-4 cells after mRNA transfection (see Figure 4.5) is enough to affect their viability. Interestingly, in A549 cells, but not in LA-4 cells, caspase-1 is hugely upregulated together with RIPK2, which is involved in the processing of pro-caspase-1. Therefore, we may conclude that carrier-mediated delivery of mRNA in A549 cells results in pyroptosis, i.e. a caspase-1 mediated form of programmed cell death [258,259]. IRF1, which is especially upregulated in A549 cells, is known to induce the transcription of the caspase-1 gene [260]. Caspase-1 was also slightly, although not significantly, upregulated in LA-4 cells transfected with mRNA (see supplementary table SC.1). It has been reported that such a small upregulation of caspase-1 can stimulate lipid production and prevent cell death, especially in epithelial cells [261]. When caspase-1 overexpression passes the critical threshold, as observed in the A549 cells, pyroptosis occurs. Activation of caspase-1 can potentially increase vaccination efficacy. Indeed, pyroptosis is accompanied with IL-18 secretion and cell lysis, which will result in the release of the produced antigen in the extracellular space [259].

We cannot exclude that the observed innate immune response after carrier mediated delivery of mRNA is also partly due to detection of the mRNA by cytosolic receptors

such as the RIG-I-like receptors or the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). It is even very likely that the delivered mRNA was sensed by NLRs (also called inflammasomes). Indeed, activation of caspase-1 is a typical hallmark of inflammasome stimulation by DAMPs or PAMPs [258,262]. Consequently, our data indicate that IVT mRNA may be a new stimulant of the inflammasome.

The stimulation of the innate immune system may potentially also have negative effects on the vaccination efficacy. Indeed, we found that TLR3 recognition of mRNA decreases the translation of the mRNA. Type I interferons are known to induce protein kinase R (PKR) and 2′,5′-oligoadenylate synthetase (OAS) [263]. Activated PKR inhibits translation by phophorylating the alpha subunit of the eukaryotic translation initiation factor 2 (eIF2a) [228,264], while OAS activates RNase L which causes an extensive cleavage of cytosolic RNA [265]. Also Fotin-Mleczek et al. attributed the drop in protein expression of mRNA:protamine complexes, prepared at high mRNA:protamine ratios, to their capacity to stimulate the innate immune system. Interestingly, these authors also found that, *in vivo*, naked mRNA resulted in a higher protein expression than mRNA:protamine complexes. Therefore, they suggested a two-component mRNA vaccine that contains both free mRNA, which produces the antigen, and mRNA:protamine complexes, which induce the innate immune response. The stimulation of the innate immune system by their mRNA:protamine complexes was essential to obtain a good anti-tumor vaccination effect with their two-component vaccine. Nucleic acid complexes based on protamine are known to induce a slow and inefficient endosomal release of the nucleic acids [266]. Consequently, at the moment that the mRNA is released in the cytosol, the cell probably already turned off its protein expression. Therefore, carriers which cause a rapid release of the mRNA may enable a sufficient protein production before the innate immune system starts to suppress the translation activity of the cell. A second concern one may have is the observation that carrier-mediated delivery of mRNA caused a much higher innate immune response and cell death in human respiratory cells, than in murine respiratory cells (Figure 4.2 and Table 4.1). However, this difference in innate immune response and toxicity is not necessarily a species-specific effect. Indeed, the mRNA transfection efficacy, i.e. the number of eGFP positive cells, was much lower in the murine than in the human lung cells (see Figure 4.3). Therefore, the intracellular concentration of the transfected mRNA was most likely much lower in the murine cells. A massive production of cytokines (so called “cytokine storm”) in the respiratory tract can be life-threatening [267].

Moreover an increased mortality associated with a cytokine storm has recently been reported in mice after peptide vaccination [268]. The risk of a too strong innate immune response and the negative effects of this immune response on the translatability of the delivered mRNA brings us to the question whether mRNA for vaccination purposes should not be made non-immunogenic. This question can only be answered by a comparative vaccination study using immunogenic and non-immunogenic mRNA. We showed that the cytotoxic effects of the mRNA disappear when they contain modified nucleotides. This is in agreement with previous reports that showed that modified mRNA is much less recognized by the innate immune system. Besides modified nucleotides [229] also a long poly(A) tail (i.e. > 150 adenosines) [269] is known to reduce the immune stimulatory capacity of mRNA.

It has been reported that type I interferons, IL-6, IL-12, and CXCL10 can suppress tumor growth in mice and/or humans [270-272]. In our study we found that carrier-mediated mRNA delivery heavily induced the production of these cytokines. IL-6, IL-12 and TNF-α were additionally significantly ($p<0,001$) overexpressed *in vivo* after intrapulmonary administration of the complexes (Figure 4.9). Therefore, inclusion of a control mRNA vaccine to enable discrimination between real vaccination effects and “off-vaccine” effects, caused by the induced cytokines, is recommended. The “off-vaccine” effects may also be a wanted side-effect in case of tumor vaccination. Therefore, to increase the effect of the induced cytokines by carrier-mediated delivery of mRNA one could consider intratumoral injection of mRNA vaccines. A substantial production of IL-12 and GM-CSF after mRNA/GL67 administration *in vivo* suggests phagocytosis of the complexes by professional APCs residing in the lungs, such as macrophages or dendritic cells.

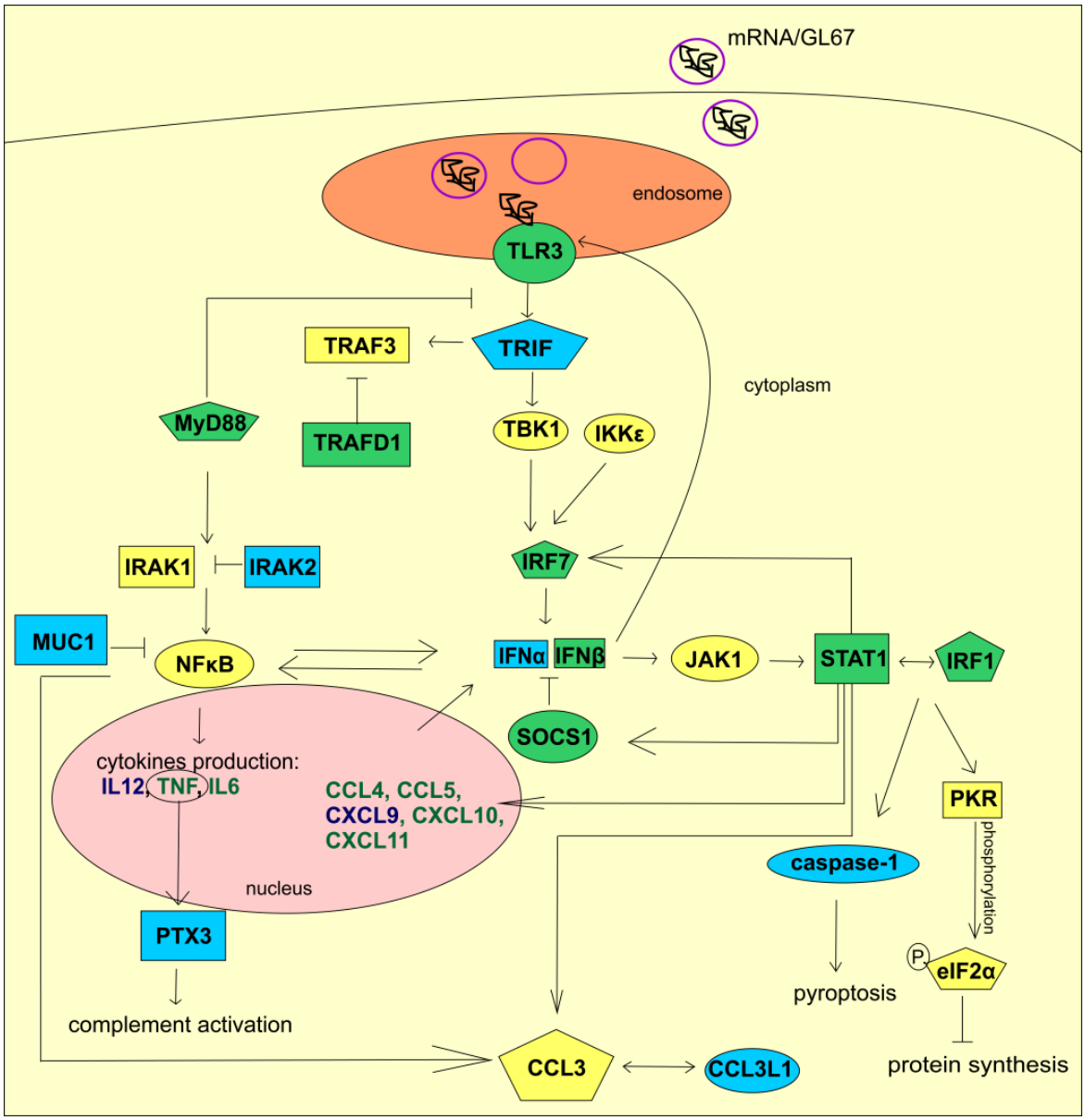

Figure 4.10. Scheme of the TLR3 signaling pathway with the most important TLR3-associated genes that are induced by carrier-mediated delivery of mRNA in lung epithelial cells. mRNA/GL67 complexes are endocytosed by the lung epithelial cells and double-stranded secondary structures of mRNA are recognized by TLR3 residing in endosomes. Following the interaction of TLR3 with its specific adaptor protein TRIF (TICAM1), the TLR3-dependent signaling pathway leads to the production of type I interferons and inflammatory cytokines. The genes presented in green (boxes) were significantly overexpressed in both human (A549) and murine (LA-4) cell lines. The genes presented in blue (boxes) were significantly overexpressed only in A549 cells and the genes presented in yellow boxes were not found to be significantly overexpressed or they were not evaluated during the qPCR experiment. → shows the positive regulation of a gene, while ⊣ represents an inhibition of a gene.

CONCLUSIONS

In this paper, we demonstrated for the first time that carrier-mediated delivery of mRNA activates TLR3 signaling in respiratory cells leading to production of type I interferons and other immunostimulating cytokines. The activation of the innate immune response was much higher in human than in murine respiratory cells. Additionally, human respiratory cells transfected with mRNA underwent a delayed cell death that exhibited features of caspase-1 mediated programmed cell death. This indicates that NOD-like receptors, which are cytosolic receptors of PAMPs and DAMPs, also recognize the delivered mRNA as caspase-1 production is regulated by NOD-like receptors. The viability of murine respiratory cells was much less affected by mRNA transfection. This was in line with the lower innate immune response and the absence of a massive caspase-1 upregulation in these cells. The induction of immunostimulating cytokines and pyroptosis in lung epithelial cells after carrier-mediated delivery of mRNA may help the residing professional antigen presenting cells in the lungs, such as macrophages and dendritic cells, to present the antigens encoded by the mRNA and to create a suitable cytokine environment to obtain the appropriate immune answer. However, the induction of the innate immune response does also decrease the translation of the mRNA. Whether this will decrease the efficacy of mRNA vaccines will dependent on the system used for mRNA delivery.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION AVAILABLE (APPENDIX C)

Supplementary Data CS.1. qPCR array for A549 cells

Supplementary Data CS.2. qPCR array for LA-4 cells

Chapter 5

N^1-methylpseudouridine-incorporated mRNA outperforms pseudouridine-incorporated mRNA by providing enhanced protein expression and reduced immunogenicity in mammalian cell lines and mice

Parts of this chapter are under preparation as a manuscript:

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INTRODUCTION

mRNA as a gene expression platform has numerous advantages over pDNA-based expression modalities. For instance, unlike pDNA, mRNA does not need to enter the nucleus to carry out its function. Therefore mRNA can immediately express proteins inside a cell, including those that are not rapidly dividing [273]. Moreover, mRNA vectors are safer than pDNA vectors in that they have virtually no risk of genomic integration and mutagenesis of critical regions of the host genome.

While the concept of using mRNA as a modality for protein replacement therapy had been originally demonstrated 25 years ago [274], this approach was not popular for a long time due to the general instability and immunogenicity of the RNA molecule. However, a series of studies initiated by Kariko et al. provided a breakthrough in the field of mRNA therapy by demonstrating that the incorporation of base modifications found in natural RNAs such as 5-methylcytidine (m5C), N^6-methyladenosine (m6A), Ψ, 5-methyluridine (m5U), and 2-thiouridine (s2U) or combinations thereof into mRNA can reduce Toll-like receptor (TLR) mediated immunogenicity of RNA [16] and increase the translational capacity and biological stability of RNA [23]. The increased translational capacity of Ψ-modified mRNA was due to 1) the diminished activation of protein kinase R (PKR) by the modified RNA and reduced phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2-α) [21,275] and 2) reduced activation of 2'-5'-oligoadenylate synthetase (OAS) by the modified RNA and reduced cleavage of the RNA by RNase L [21].

Various pre-clinical studies have demonstrated the vast potential of modified mRNA for therapeutic applications including the work of Kormann et al. [6] which used m5C/s2U-modified mRNA to treat mice with surfactant protein B (SP-B) deficiency, a lethal congenital lung disease, the work of Warren et al. [54] which used m5C/Ψ-modified mRNA to reprogram and differentiate human cells, and the work of Zangi, Lui et al. [276] which used m5C/Ψ-modified mRNA to treat a mouse model of myocardiac infarction.

Given the pre-clinical success in using mRNA with various modified bases for *in vitro* and *in vivo* therapeutic applications, we sought to identify RNA base modifications that could further reduce the immunogenicity and translational capacity of mRNA by using mRNA containing Ψ as a benchmark.

Here we demonstrate that the incorporation of m1Ψ, a modification naturally found in 18S rRNA as a precursor of 1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine

(m1acp3Ψ) [277] drastically improved the translational capacity of mRNA compared to Ψ-modified mRNA in A549 human lung epithelial cells, BJ human foreskin fibroblasts, C2C12 murine myoblast cells, HeLa human cervix epithelial cells, human primary keratinocytes from neonatal foreskin, as well as when the mRNA was injected intradermally (i.d.) or intramuscularly (i.m.) into mice. We show in the various cell lines that m1Ψ-modified mRNA had reduced cytotoxicity compared to Ψ-modified mRNA. m1Ψ-modified mRNA also had reduced activation of intracellular innate immunity. Finally, we show that the superiority of m1Ψ-modified mRNA over Ψ-modified mRNA may be due to its improved ability to evade TLR3 activation. Thus, m1Ψ-modified mRNA could be a potentially more optimal alternative to Ψ-modified mRNA for therapeutic applications.

MATERIALS AND METHODS

Cells and Reagents

Human lung epithelial cell line (A549, ATCC® CL-185™), human foreskin fibroblasts (BJ, ATCC® CRL-2522™), murine muscle cells (C2C12, ATCC® CRL-1772™), human cervix epithelial cells (Hela, ATCC® CCL-2™), human primary keratinocytes from neonatal foreskin cells (ATCC® PCS-200-010™) were purchased from ATCC and cultured according to their recommendations. Modified nucleoside triphosphates, 5-methylcytidine-triphosphate (m5C), pseudouridine-triphosphate (Ψ) and N^1-methylpseudouridine-triphosphate (m1Ψ) were purchased from TriLink (San Diego, CA, USA). Lipofectamine 2000 was obtained from Invitrogen (Merelbeke, Belgium). D-Luciferin for *in vivo* measurement of firefly luciferase activity was purchased from Gold Biotechnology (St. Louis, MO, USA).

Plasmids

Plasmids used for *in vitro* transcription of firefly luciferase and mVenus encoding mRNA were constructed using standard cloning procedures including In-Fusion PCR cloning (Clontech) and Gateway cloning (Invitrogen). The plasmids included a bacteriophage T7 polymerase promoter, the open reading frame (ORF) of interest

flanked by the 5' UTR of the *Venezuelen equine encephalitis virus* (VEEV) subgenomic RNA and two tandem repeats of the 3' UTR of VEEV subgenomic RNA, a 40 nucleotide poly(A) sequence, and a consensus recognition sequence for the I-SceI homing endonuclease. Plasmids sequences and maps are available upon request.

mRNA in vitro transcription

mRNA was produced by *in vitro* transcription (IVT) of I-SceI (NEB)-linearized plasmid DNA using the MEGAscript® T7 Transcription Kit (Invitrogen) with unmodified nucleotides or a combination of the modified nucleotides (replacing the nonmodified equivalents) described above. RNA was subsequently purified using the RNeasy® Mini Kit (Qiagen), denatured at 65 ºC, enzymatically (cap1) capped using the ScriptCap™ 2'-O-Methyltransferase Kit (Cellscript) and ScriptCap™ m7G Capping System (Cellscript), poly(A) tailed using the A-Plus™ Poly(A) Polymerase Tailing Kit (Cellscript), and purified again using the RNeasy® Mini Kit (Qiagen) following the manufacturers' protocols.

mRNA electroporation

All cell lines were electroporated in 0.2 cm gap cuvettes (BioRad, Temse, Belgium) with a square wave electroporator, BTX ECM 830 Harvard Apparatus (VWR International, Leuven, Belgium). Electroporation conditions were optimized for each cell line and are as follows: A549 (400 V, 1.4 ms, 1 pulse), BJ (250 V, 1.4 ms, 1 pulse), C2C12 (300 V, 1.4 ms, 1 pulse), HeLa (300 V, 1.4 ms, 1 pulse), primary keratinocytes (300 V, 1.4 ms, 1 pulse). Prior to electroporation, the cells were washed twice with ice-cold PBS (Gibco, Merelbeke, Belgium), counted, and resuspended in Opti-MEM I reduced serum medium (Gibco, Merelbeke, Belgium) at a concentration of 1 x 10^6 cells/ml. 100 µl of cell suspension was electroporated with 1 µg of unmodified or modified mRNA.

mRNA lipofection

mRNA was mixed with Lipofectamine 2000 at a ratio of 1:2 (µg mRNA: µl Lipofectamine 2000) in Opti-MEM I. The complexes were allowed to form for 30

minutes at room temperature and afterwards 1 µg of complexed mRNA was transferred to cells pre-seeded in 24 well plates. The complexes were removed from cells 4 hours later and Opti-MEM I was replaced with the standard ATCC recommended culture media containing serum.

ELISA assays

Cell culture supernatants were collected 24 hours after transfection with mRNA and stored at -80 ºC until performing the ELISA assays, unless stated otherwise. ELISA MAX Deluxe kits for IL-6 and CCL5, ELISA LEGEND MAX for mouse IFNβ were purchased from BioLegend (ImTech Diagnostics, Antwerp, Belgium). The human IFNβ ELISA kit - LumiKine was obtained from Life Technologies (Merelbeke, Belgium). ELISAs were performed according to the manufacturers' recommendations, as published previously [14].

Intracellular staining assays

24 hours after transfection with mRNA, cells were collected, washed with PBS and incubated in the dark, at room temperature for 1 hour in 1 x Fixation Buffer (eBioscience, Vienna, Austria). Subsequently, the fixed cells were washed twice with 1 x Permeabilization Buffer (eBioscience, Vienna, Austria). After centrifugation, the cells resuspended in 1 x Permeabilization Buffer were incubated in the dark for 30 minutes with fluorescent-dye conjugated antibodies against TLR3 (BioLegend, ImTec Diagnostics N.V. Belgium). Afterwards, the cells were washed twice to get rid of any unbound antibodies, and resuspended in PBS. Fluorescence signal was measured on an Accuri C6 flow cytometer (BD Biosciences, Erembodegem, Belgium) and analyzed as described below.

Flow cytometry assays

Flow cytometry was performed on an Accuri C6. Data were analysed using the CFlow Plus Analysis software (BD Biosciences, Erembodegem, Belgium). Live cells were gated based on forward and side scatter.

Mouse experiments

7-week-old Balb/c mice were obtained from Janvier (Le Genest St Isle, France). Mice were housed in individually ventilated cages (IVC) under the 12:12 h dark-light cycle conditions. Access to food and water was maintained *ad libitum*. All experiments were carried out with approval of the Ghent University Ethics Committee (nº EC 2014/57). Mice were anesthetized with constant flow of isoflurane during injections and intradermal (i.d.) or intramuscular (i.m.) electroporations. 50 µg of naked mRNA resuspended in PBS were injected i.d. or i.m into the *tibialis anterior* muscle. Naked mRNA injections were followed by calliper-mediated electroporation with the BTX ECM 830 Harvard Apparatus using previously optimized conditions (100 V, 40 ms, 6 pulses for i.m. and 75 V, 40 ms, 6 pulses for i.d.). A small amount of conductive gel was applied to the calliper-plates before electroporation.

In vitro firefly luciferase and viability assays

The used mammalian cells were transfected in 24 well plates with 1 µg of nonmodified or modified mRNA as described above (section: mRNA electroporation, mRNA electroporation). 24 hours after transfection, cells were lysed with 100 µl of 1 x Passive Lysis Buffer (Promega, Leiden, The Netherlands). *In vitro* firefly luciferase assay was performed with Luciferase Assay Kit (Promega) according to manufacturer's protocol. Luminescence was measured by Glomax instrument (Promega).
Viability of mRNA-transfected cells was measured 24 hours later by MTT proliferation assay according to manufacturer recommendations (Roche, Vilvoorde, Belgium). All experiments were performed in triplicates.

In vivo imaging of firefly luciferase expression

The expression level of firefly luciferase in murine tissue was measured over time using the in vivo bioluminescent imaging system, IVIS Lumina II (PerkinElmer, Zaventem, Belgium) until no detectable signal could be acquired from the injected mRNA. Mice were injected intraperitoneally (i.p.) with 50 mg/kg of D-Luciferin (Gold Biotechnology,

St. Louis, MO, USA). Luminescence was measured 10 minutes after the i.p. injection of D-Luciferin. Acquisition settings were set at f-stop: 1, binning: 8, and auto-exposure.

Statistics

The experiments are represented as the mean ± SD. Statistical analysis was performed in a GraphPad Prism 6 software. In order to check significance of the variance among different experimental groups, ANOVA test was calculated followed by ad hoc Tukey's test. The differences were considered significant when $p<0.05$.

RESULTS

m1Ψ-incorporated mRNA has a higher translational capacity than Ψ-incorporated mRNA in vitro

In order to test whether there are natural nucleobase modifications which are superior to Ψ at enhancing the translational capacity of mRNA, we incorporated m1Ψ into RNA by *in vitro* transcription to compare it to RNA containing Ψ. Ψ and m1Ψ are natural derivatives of uracil that can be distinguished by the N^1 positions of their bases (m1Ψ is methylated at N^1) (Figure 5.1).

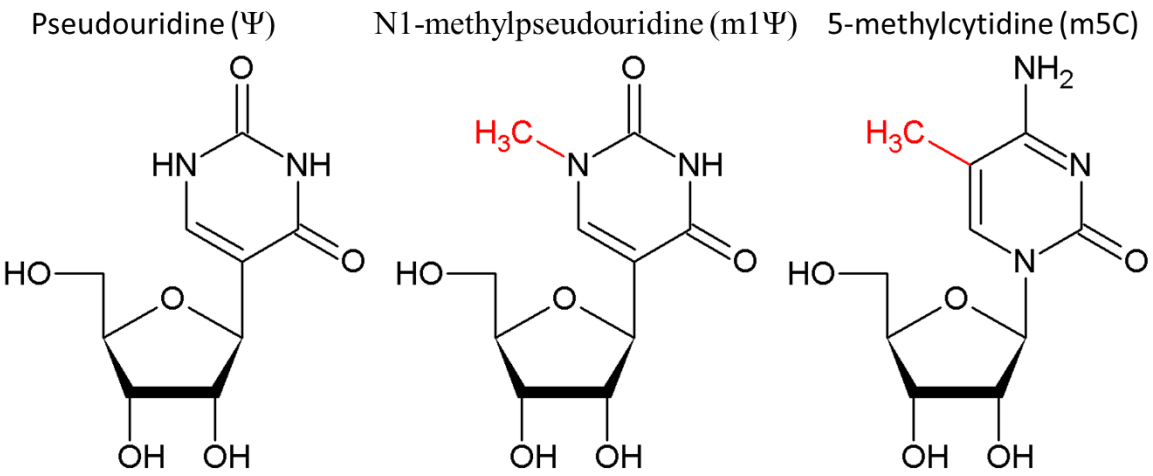


Figure 5.1. Chemical structure of nucleoside modifications used in this study. The chemical structures of pseudouridine (Ψ), N^1-methylpseudouridine (m1Ψ), and 5-

methylcytidine (m5C). Adapted from the *Modomics* database [278].

In addition to Ψ or m1Ψ single modified mRNA, we decided to compare m5C/Ψ or m5C/m1Ψ double modified mRNAs, since it had been shown previously by others that m5C (a natural derivative of cytosine; see Figure 5.1) can increase the translational capacity of Ψ single modified mRNA [54]. The RNAs used in this study contained, at the 5' end, an N^7-methyl-guanosine cap and a 2'-O-methyl at the penultimate nucleoside (i.e. a cap 1 structure), a poly(A)-tail at the 3' end, and the 5' UTR and two repeats of the 3' UTR sequence of the VEEV subgenomic RNA flanking the ORF of interest. We transfected unmodified or modified mRNAs encoding the firefly luciferase gene into several cell lines (A549 [human lung carcinoma cells], BJ [human foreskin fibroblasts], C2C12 [mouse myoblasts], and HeLa [human cervical adenocarcinoma cells]) as well as primary cells (human neonatal foreskin primary keratinocytes) by lipofection. We chose cell lines or primary cells of different cell types or derived from diverse tissues to ensure that the effects we observe are general. Luciferase assays were performed 24 hours after mRNA transfection. As shown in Figure 5.2 and Supplementary table SD.1, we observed a statistically significant difference in luciferase activity in all of the cell types transfected with the differentially modified mRNAs. In particular, the m5C/m1Ψ double modified mRNA resulted in the highest amount of luciferase activity in every cell type transfected with the exception of C2C12 cells in which m1Ψ was the highest. Similar results were obtained when mVenus-encoding mRNAs were transfected into the same group of cells Supplementary figure SD.1 and table SD.2. Therefore, we demonstrate that m1Ψ containing modified mRNA (particularly the m5C/m1Ψ combination) outperforms the previous state-of-the-art Ψ modified mRNA expression platform.

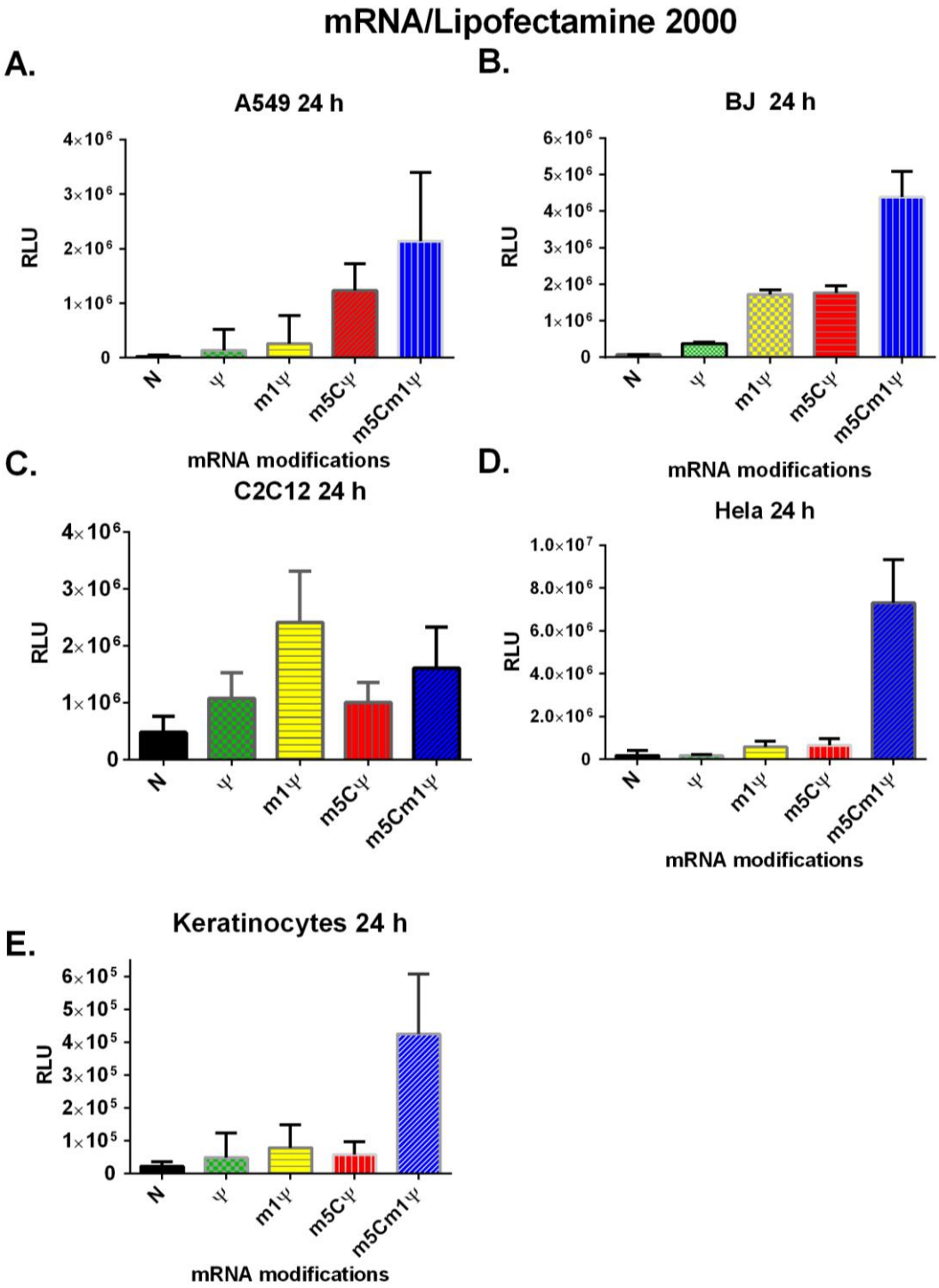


Figure 5.2. Comparison of luciferase activity 24 hours after lipofection of unmodified or modified (Ψ, m1Ψ, m5C/Ψ, m5C/m1Ψ) mRNAs encoding firefly luciferase in various cell lines. Luciferase activities for each mRNA species are shown for (A) A549 human lung carcinoma cells, (B) BJ human foreskin fibroblasts, (C) C2C12 mouse myoblasts, (D) HeLa human cervical adenocarcinoma cells, and (E) human neonatal foreskin primary keratinocytes. 1 µg of each mRNA species was transfected into each cell type by lipofection. The results are presented as the mean ± SD (n=3, ANOVA results in supplementary table SD.1).

The translational lifetime of m1Ψ-incorporated mRNA is longer than that of Ψ-incorporated mRNA in vitro

We next assessed whether the duration of protein expression from m5C/m1Ψ mRNA was longer than that of the other mRNAs by performing a time course assay for luciferase activity. For this, we lipofected the various mRNAs into the A549 cell line and measured luciferase activity at 3, 6, 12, 24, and 48 hr. As shown in Figure 5.3, there was a statistically significant difference (ANOVA, $p<0.05$) in luciferase production at each of the different time points. At each of the time points, the m5C/m1Ψ-modified mRNA outperformed the rest of the mRNAs. We observed an initial burst in luciferase activity at the 3 h time point, where m5C/m1Ψ mRNA produced ~916.7-fold more activity than unmodified mRNA, ~118.1-fold more than Ψ mRNA, 23.0-fold more than m1Ψ mRNA and 44.1-fold more than m5C/Ψ mRNA. Subsequently, the expression from the mRNAs dropped drastically between the 3 and 6 h time points after which the drop in luciferase activity was less severe. Importantly, the luciferase signal between the 24 and 48 h time points decreased the least for the m5C/m1Ψ RNA. Thus the m1Ψ outperformed the Ψ mRNA expression platform with regards to the duration of expression.

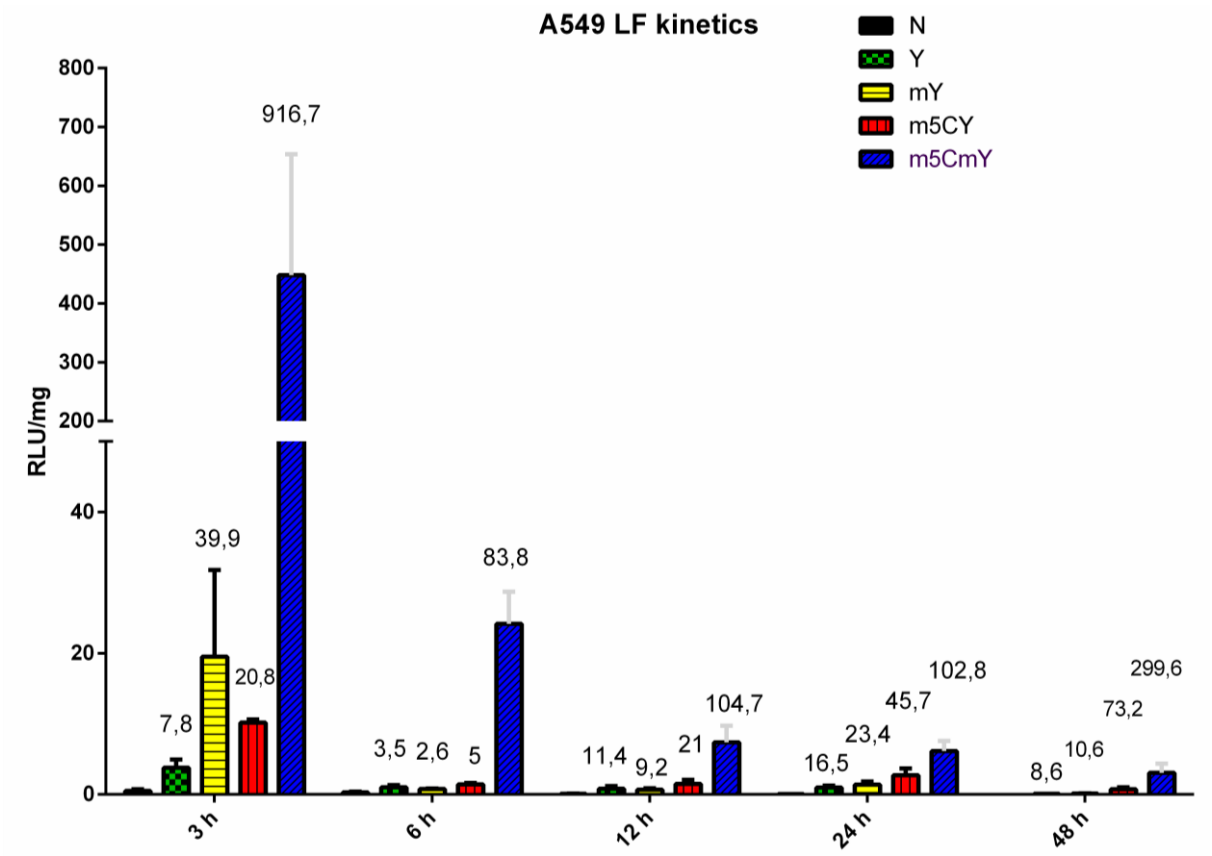


Figure 5.3. Kinetics of luciferase activity after lipofection of unmodified or modified (Ψ, m1Ψ, m5C/Ψ, m5C/m1Ψ) mRNAs encoding firefly luciferase in A549 cells. Luciferase activities for each mRNA species at 3, 6, 12, 24, and 48 h time points are graphed. Luciferase activity (RLU) was normalized to the amount of total cellular protein concentration measured by a BCA assay to correct for the differences in the number of cells at the different time points of the time series. 1 µg of each mRNA species was transfected into A549 cells. The results are presented as the mean ± SD (n=3, ANOVA $p<0.05$).

m1Ψ-incorporated mRNA is less cytotoxic than Ψ-incorporated mRNA when delivered using lipid-based carriers in vitro

We previously demonstrated that the transfection of *in vitro* transcribed mRNAs into mammalian cells can negatively affect the health of the transfected cells [14]. Since m5C/Ψ incorporated mRNA had drastically less cell death upon transfection compared to unmodified RNA, we next sought to determine how the cytotoxicity of m1Ψ mRNA compared to Ψ mRNA. For this, we first lipofected the RNAs containing either no

modification or the various combinations of modifications described above and performed an MTT assay to quantify the amount of viable cells after transfection of each RNA species into various cell lines. As shown in Figure 5.4, the effects of the various RNAs on cell viability were dependent on the cell type and delivery method. In the case of lipofection (Figure 5.4.A), all cell types except primary keratinocytes showed a statistically significant difference in the overall viability pattern. Specifically, in the A549, C2C12, and HeLa cell lines, m1Ψ was less toxic than Ψ, however, both m5C/m1Ψ and m5C/Ψ were equally non-toxic. In the BJ cell line, m5C/m1Ψ was superior to all other combinations of modifications upon lipid-based transfection (supplementary table SD.3). However, when we delivered the various RNAs into the cells by electroporation, only the A549, BJ, and HeLa cells showed a statistically significant difference in the overall viability pattern (Figure 5.4.B). Specifically, in A549 cells, m1Ψ was less toxic than Ψ. Thus, we found that the toxic effects of IVT mRNA on cells is dependent on both cell type and the delivery method. However, when we did observe a noticeable difference of base modifications on cellular viability, m1Ψ outperformed the Ψ platform.

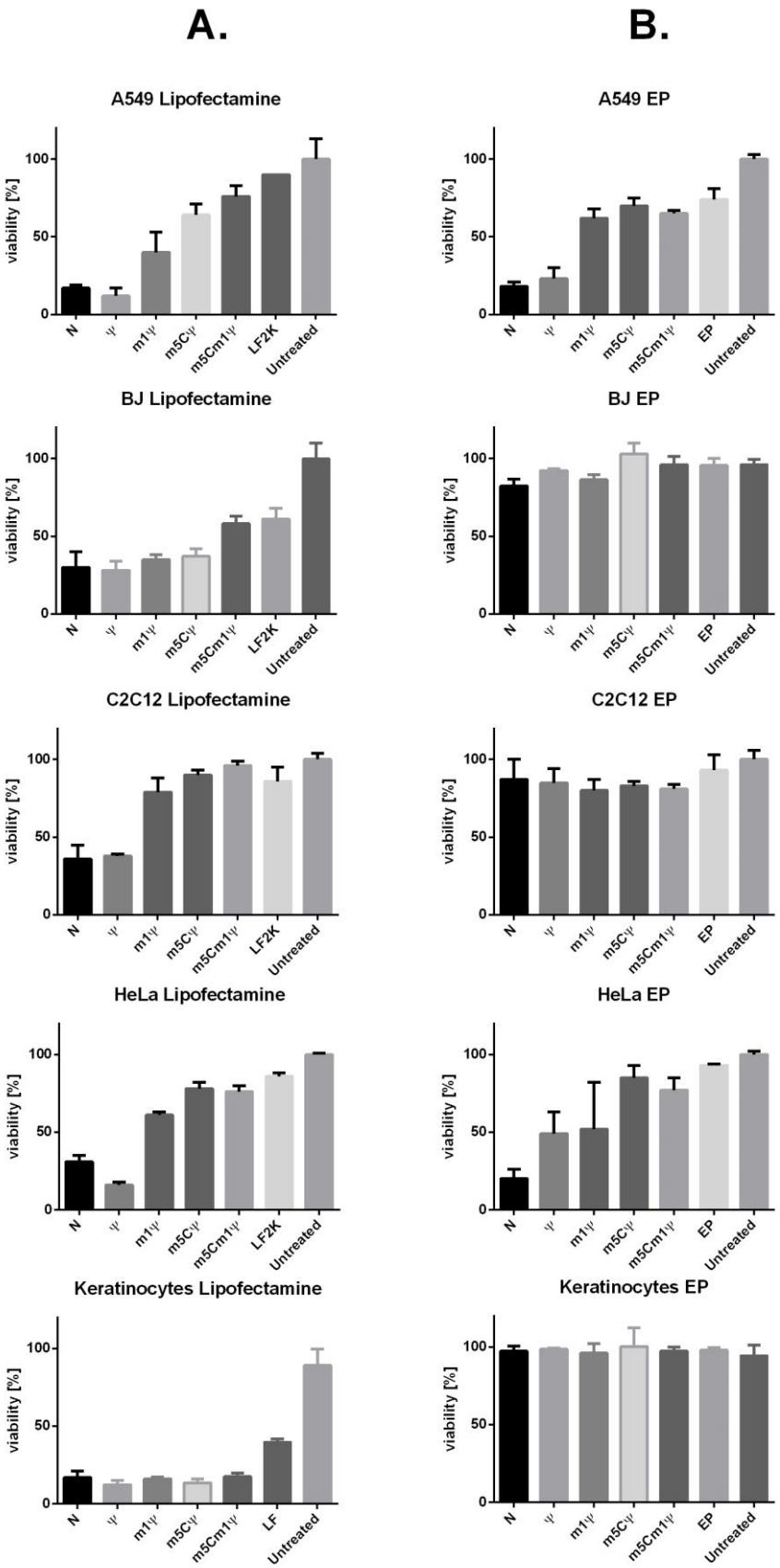


Figure 5.4. Viability of mammalian cells 24 hours after transfection of unmodified or modified (Ψ, m1Ψ, m5C/Ψ, m5C/m1Ψ) mRNAs determined using an MTT assay. 1 µg of

each mRNA species was transfected into A549, BJ, C2C12, HeLa, and primary keratinocytes by (A) lipofection or (B) electroporation. The results are presented as the mean ± SD (n=3, ANOVA results in supplementary table SD.3).

m1Ψ-incorporated mRNA stimulates intracellular innate immune signaling pathways less than Ψ-modified mRNA in vitro

Since the superior translational capacity and reduced cytotoxicity of modified mRNAs are generally known to correlate with reduced activation of the intracellular innate immune pathway, we next asked whether there was a difference in the activation of key cytokines upon transfection of the differentially modified RNAs. For this, we lipofected A549 cells with the various mRNAs and measured the levels of secreted interferon-β (IFN-β) and Chemokine (C-C motif) ligand 5 (CCL5, also known as RANTES) by ELISA. As shown in Figure 5.5, there was a statistically significant difference in the expression levels of IFN-β. Specifically, the IFN-β production from cells transfected with Ψ, m1Ψ, m5C/Ψ and m5C/m1Ψ RNAs were respectively reduced by ~3.2, 10.6, 4.3, and 13.7-fold relative to unmodified RNA (N). For CCL5, as shown in Figure 5.6, the m5C/Ψ and m5C/m1Ψ double modified mRNAs showed the lowest amount of cytokine induction and for the single modified RNAs, m1Ψ mRNA induced less cytokine expression than Ψ mRNA. Thus, overall, the m1Ψ platform was less immunogenic than Ψ when assessed by the amounts of IFN-β or CCL5 activation.

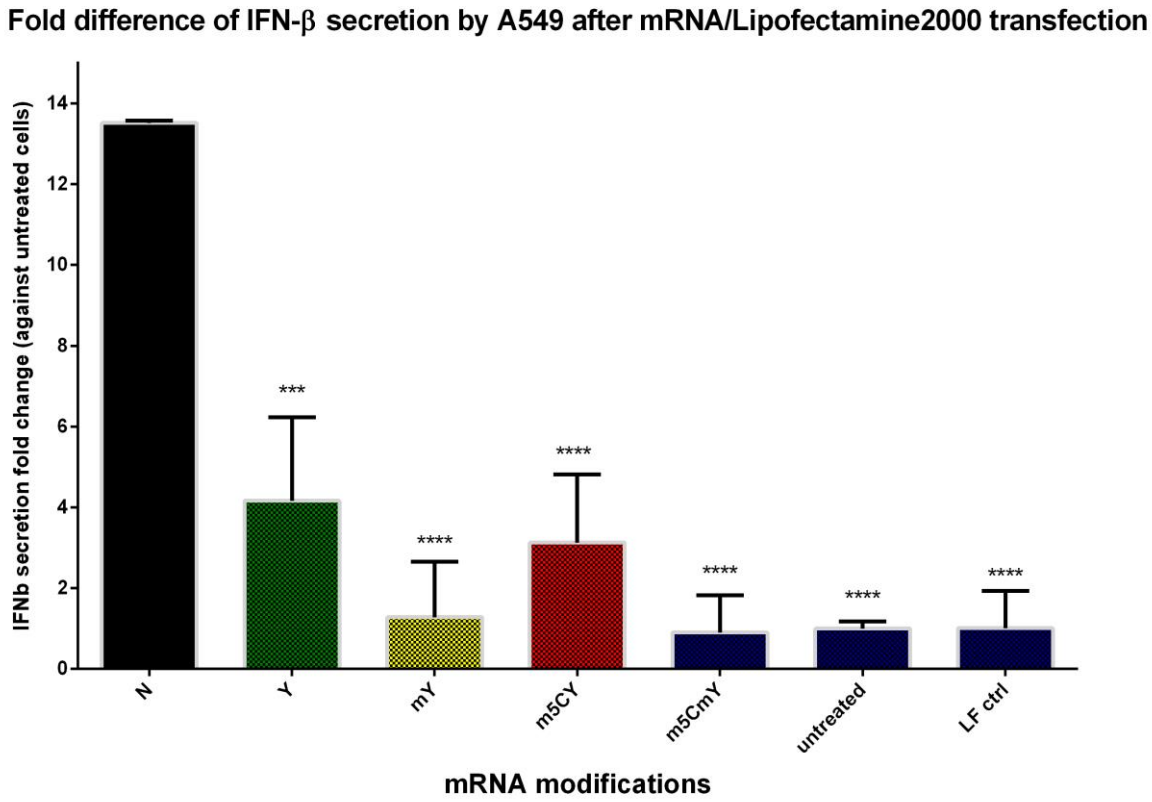
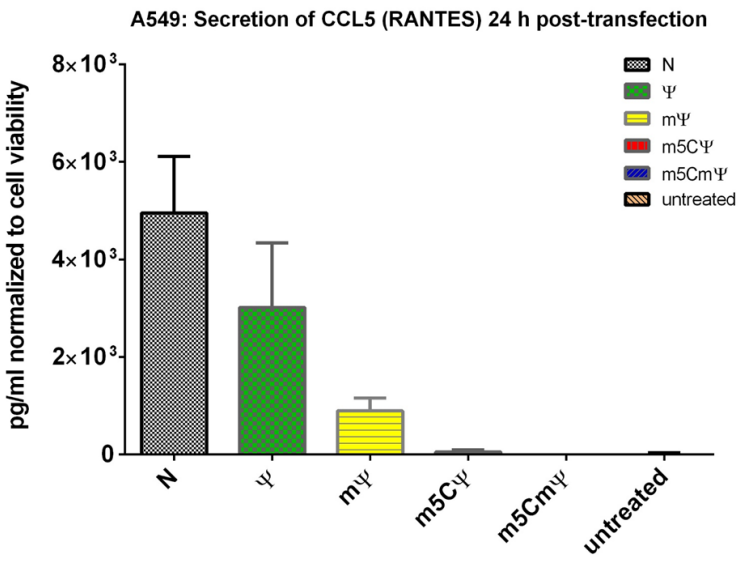


Figure 5.5. Levels of secreted IFN-β measured by ELISA 24 h after lipofection of unmodified or modified (Ψ, m1Ψ, m5C/Ψ, m5C/m1Ψ) mRNAs into A549 cells. 1 µg of each mRNA species was lipofected into A549 cells and the supernatants were subjected to ELISA to detect IFN-β. The results are presented as the mean ± SD (n=3, ANOVA)

A.



B.

F	24.14
P value	< 0.0001
P value summary	****
Tukey's multiple comparisons test	CCL5
N vs. Ψ	ns
N vs. mΨ	***
N vs. m5CΨ	****
N vs. m5CmΨ	****
N vs. untreated	****
Ψ vs. mΨ	*
Ψ vs. m5CΨ	**
Ψ vs. m5CmΨ	**
Ψ vs. untreated	**
mΨ vs. m5CΨ	ns
mΨ vs. m5CmΨ	ns
mΨ vs. untreated	ns
m5CΨ vs. m5CmΨ	ns
m5CΨ vs. untreated	ns
m5CmΨ vs. untreated	ns

Figure 5.6. Levels of secreted CCL5 (RANTES) measured by ELISA 24 hours after lipofection of unmodified or modified (Ψ, m1Ψ, m5C/Ψ, m5C/m1Ψ) mRNAs into A549

cells. A) 1 µg of each mRNA species was lipofected into A549 cells and the supernatants were subjected to ELISA to detect CCL5. B) The results are presented as the mean ± SD (n=3, p<0.0001, ANOVA).

TLR3 overexpression is sufficient to convert HEK cells from being not modification sensitive to preferential expressers of m1Ψ-incorporated modified mRNA

We next sought to address the mechanism by which cells preferentially translate m1Ψ-incorporated mRNA over Ψ-mRNA. Since base modifications such as Ψ are known to reduce intracellular innate immune activation by evading TLR signaling, we asked whether TLR signaling could explain the difference in translation. To test this hypothesis, we took advantage of the HEK cell line, which normally does not express endosomal TLRs (supplementary figure SD.2). We transfected unmodified and various modified mRNAs encoding luciferase into normal HEK cells and HEK cells ectopically expressing TLR3. In normal HEK cells, we did not observe a difference in luciferase activity between the different RNAs. Strikingly, the HEK cells overexpressing TLR3 showed a statistically significant difference in expression where m1Ψ expressed 5.6-fold more luciferase activity than Ψ incorporated RNA (Figure 5.7). The data are consistent with the hypothesis that differential activation of the TLR3 signaling pathway may explain why the m1Ψ platform has superior translational capacity and less innate immune activation compared to the Ψ mRNA platform.

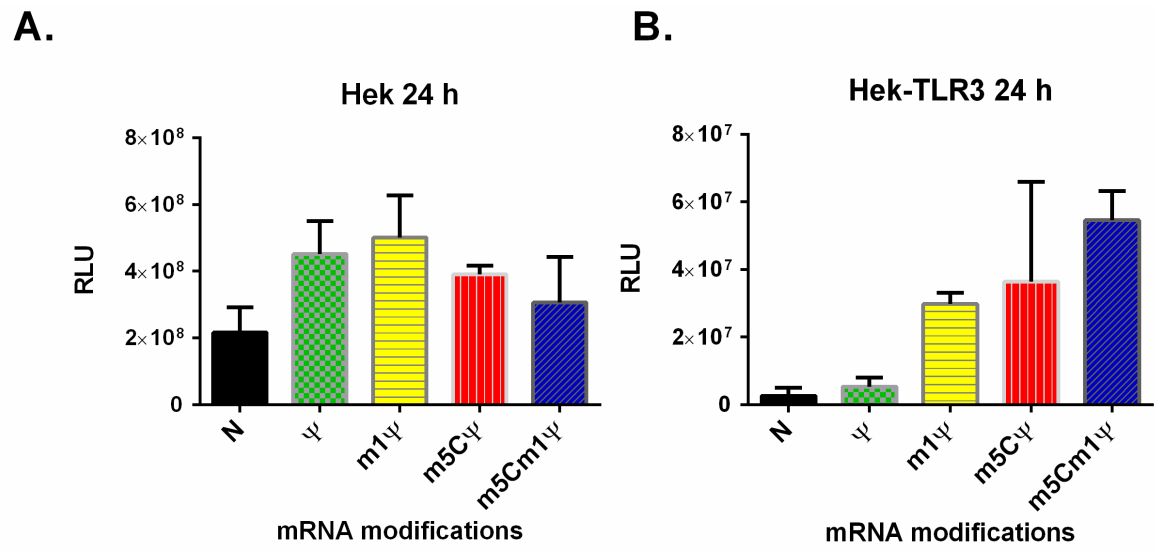


Figure 5.7. Comparison of luciferase activity 24 hours after lipofection of unmodified

or modified (Ψ, m1Ψ, m5C/Ψ, m5C/m1Ψ) mRNAs encoding firefly luciferase in HEK and TLR3 overexpressing HEK (HEK-TLR3) cells. Luciferase activities for each mRNA species are shown for (A) HEK cells and (B) HEK-TLR3 cells. 1 µg of each mRNA species was transfected into each cell type by lipofection.

m1Ψ-incorporated mRNA has a higher translational capacity than Ψ-incorporated mRNA in mice in vivo

Finally, we assessed whether m1Ψ mRNA had superior translational effects over Ψ when injected *in vivo*, into mice. For this, we delivered naked (uncomplexed) luciferase mRNAs encoding firefly luciferase by i.d. or i.m. injection into mice and immediately electroporated the injection area. The kinetics of expression was then followed by bioluminescent imaging (BLI) over a period of 42 days. As shown in Figure 5.8A,B, as expected, the luciferase signal after e.p. decayed over time. Importantly, as shown in Figure 5.8C,D, the RNA modifications affected the total amount of protein expressed *in* vivo (as measured by quantifying the area under the curve of each series in Figure 5.8A,B). Specifically, the m5C/m1Ψ double modified mRNA expressed the best followed by m5C/mΨ, mΨ, Ψ, and lastly, unmodified mRNA (N). Thus the m1Ψ platform has a translational advantage over the Ψ platform *in vivo*.

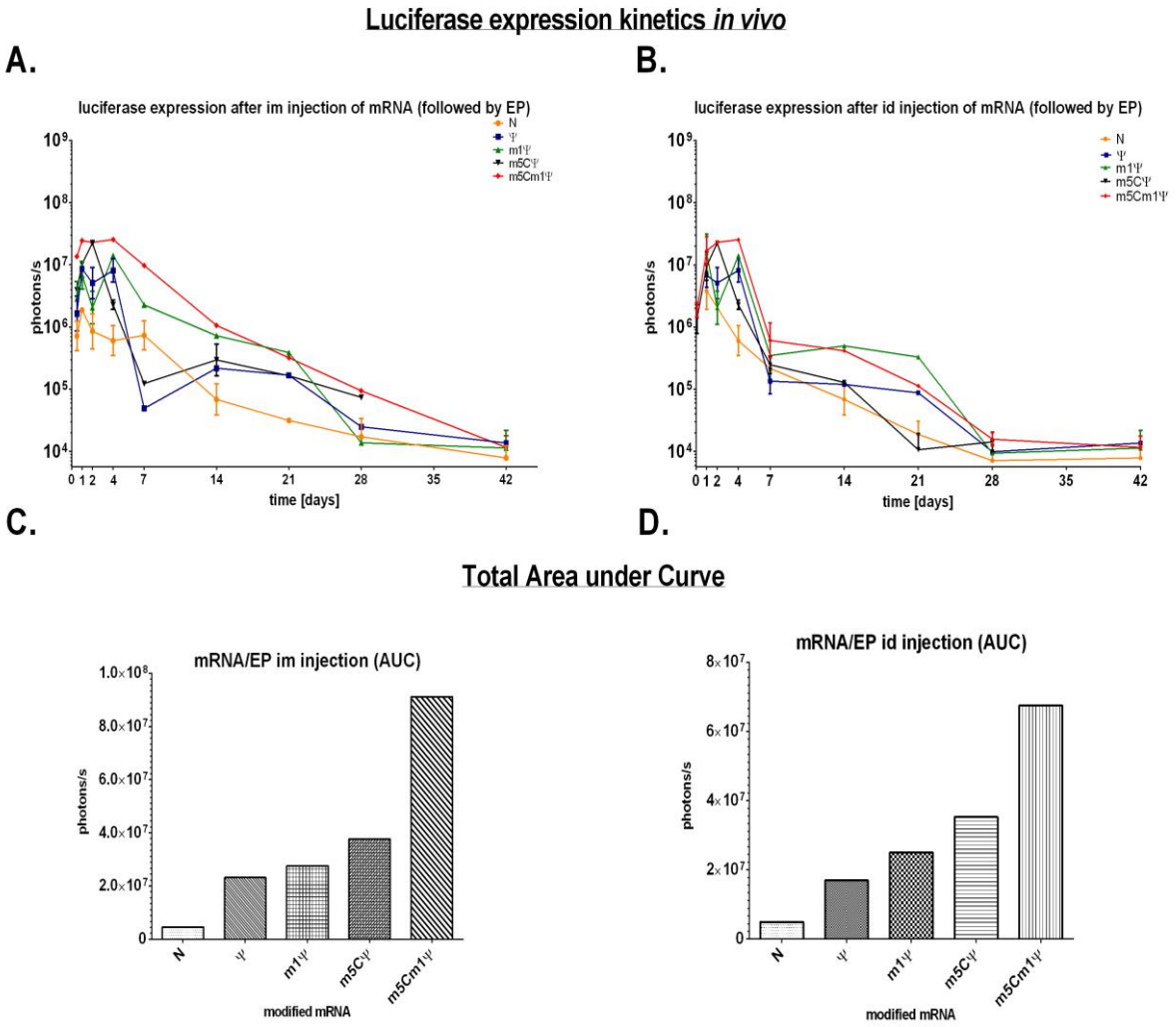


Figure 5.8. Comparison of *in vivo* luciferase activity following injection of unmodified (N) or modified (Ψ, m1Ψ, m5C/Ψ, m5C/m1Ψ) mRNAs administered through i.d. or i.m. routes by e.p. Luciferase activities measured by BLI for each mRNA species followed over a course of 42 days (A) and (B). The results are presented as a mean ± SD (n ≥ 4). The area under the curve of each series was calculated and plotted to estimate the total amount of protein expression per series (C) and (D). 50 µg of each mRNA species was administered to mice for each delivery method and route.

DISCUSSION

A decade has passed since Kariko et al. discovered that incorporation of modified bases into mRNA can reduce the innate immunogencity of RNA [16]. The subsequent demonstration that modified mRNA enhances the translational capacity and stability of

RNA [23] inspired a series of successful pre-clinical studies in which mRNAs with different combinations of modifications were used for various therapeutic applications [6,54,276,279,280].

In this manuscript, in light of the enormous therapeutic potential of modified mRNA demonstrated in these previous studies, we sought to identify naturally existing base modifications that may enable further improved translational capacity and reduced immunogenicity of mRNA beyond the current state-of-the-art Ψ-modified mRNA platform. Indeed, we find that m1Ψ-modified mRNA can express reporter genes at levels more than an order of magnitude higher than Ψ-modified mRNA in multiple cell lines and in mice. m1Ψ-modified mRNA also had reduced cytotoxicity and immunogenicity compared to Ψ-modified mRNA. These superior properties of m1Ψ-modified mRNA in comparison to Ψ-modified mRNA may be due to the ability of m1Ψ-modified mRNA to more effectively evade endosomal TLR receptors such as TLR3.

Previously, the use of chemical modified nucleotides that do not naturally exist in nature has been explored for the purpose of antiviral therapy [281]. Unnatural chemical base modifications could also be used in theory to enhance the properties of mRNA. However, great safety precautions must be taken when doing so as the administration of unnatural modified nucleotides into human patients had previously resulted in mitochondrial toxicity, liver failure, and death during clinical trials [282]. Furthermore, unlike native modifications, unnatural modifications may elicit an adaptive immune response against the RNA.

Therefore, a more prudent strategy may be to restrict the investigation of mRNA enhancing modifications to those that exist in nature. Currently, 66 nucleoside modifications have been demonstrated to be post-transcriptionally incorporated into eukaryotic RNA, 51 of which are incorporated into tRNA, 23 in rRNA, 13 in mRNA, 11 in snRNA [283]. The current state-of-the-art mRNA modification Ψ is the most prevalent nucleoside modification found in nature and was originally thought to be only incorporated into tRNA, rRNA, and snRNA mainly to stabilize the structure of the RNA [284]. However, recent genome-wide mapping studies have demonstrated that Ψ is also naturally incorporated into mRNA as well as snoRNA [285,286]. Incorporation of Ψ into mRNA was upregulated by cellular stress conditions such as heat shock or nutrient deprivation thus implicating Ψ as a possible native regulator of mRNA function. While the function of m1Ψ, a precursor of m1acp3Ψ in 18S rRNA [277], is not entirely known, it would be interesting to test whether it is also naturally incorporated into native cellular

mRNA.

Future studies may address the mechanisms by which m1Ψ-modified mRNA provides further enhanced translational capacity and reduced immunogenicity compared to Ψ-modified mRNA. Our results implicated that m1Ψ may be able to evade the endosomal TLRs more efficiently than Ψ. However, it is also possible that m1Ψ could evade retinoic acid-inducible gene-I (RIG-I)-like receptors or PKR more efficiently, is more resistant to RNase L, or has a generally increased rate of ribosomal translation. Moreover, as it had previously been shown that Ψ-containing stop codons have an increased rate of translational readthrough [287,288], this may be true for m1Ψ as well. Nevertheless, in this manuscript, we showed that m1Ψ-containing mRNA is more superior than Ψ-containing mRNA in its capacity to produce protein and also its ability to reduce the intracellular innate immune response. Future work may demonstrate the enhanced capability of m1Ψ-containing mRNA for applications such as protein replacement therapy.

SUPPLEMENTARY DATA (Appendix D)

Figure SD.1. Comparison of mVenus expression level 24 hours post-transfection from unmodified and modified mRNA in various cell lines

Figure SD.2. flow cytometry data TLR3 and RIG-I ICS

Table SD.1. ANOVA statistics followed by *ad hoc* Tukey's multiple comparisons test after transfection of luciferase-encoding mRNA/Lipofectamine 2000

Table SD.2. ANOVA statistics followed by *ad hoc* Tukey's multiple comparisons test after transfection of mVenus-encoding mRNA/Lipofectamine 2000

Table SD.3. One-way ANOVA statistics followed by *ad hoc* Tukey's multiple comparisons test based on results in Figure 5.4 depicting viability

Table SD.4. Characterization of complexes – Zeta Potential.

CHAPTER 6

GENERAL DISCUSSION, CONCLUSIONS and SUMMARY

GENERAL DISCUSSION

In this Ph.D. dissertation, I conducted a comprehensive investigation of messenger RNA as a potent and safe gene-based therapeutic modality to identify its advantages and understand the source of its weaknesses. The properties (half-life, expression level, innate immune-stimulatory activity) of the mRNA-based pharmaceutical can be defined by 1) its fundamental structure: the cap, the polyA-tail, sequences of the 5' UTR, 3' UTR, and ORF, 2) its fundamental building blocks: the ribonucleotides, their nucleobase modifications, and respective ratios, and 3) intracellular physical and chemical interactions: inter-RNA interactions, interactions between RNAs and intracellular ions, and interactions between RNAs and proteins responsible for translation, degradation, and recognition of PAMPs and DAMPs as reviewed in Chapter 2 of this dissertation. As mRNA has the potential to become a cost-effective and exceptionally safe therapeutic modality for combating an array of diseases, such as cancer, infectious diseases, genetic disorders, metabolic disorders, or allergies, it is critical for the scientific community to gather the multidisciplinary knowledge required to realize these goals and also to understand the possible pitfalls for using mRNA as a drug.

My doctoral research was initiated by first characterizing and comparing the transfection efficiencies of lipocomplexed DNA and RNA *in vitro* and in *vivo*. While the transfection efficiency of formulated RNA was higher than that of DNA *in vitro*, it did not give rise to a detectable reporter signal *in vivo* (Chapter 3). This promoted us to question whether the exogenously produced RNA was triggering an innate immune response *in vivo*. Indeed, we discovered that the RNA was activating an immune response through TLR3 (Chapter 4). This innate immune "alert" mediated by endosomal TLRs or cytoplasmic RLRs, which in nature is indicative of a potential viral infection, can serve a self-adjuvanting function during mRNA vaccination or immunotherapy. The prompt production and secretion of type I interferons, followed by other proinflammatory cytokines, such as IL-6, IL-28, RANTES or IL-12 (Chapter 4 and 5), creates a chemoattractive microenvironment for immune cells. Moreover, this cytokine profile based on type I interferons subsequently orchestrates an adaptive immune response that enhances CD8+ and Th1 CD4+ cells with the additional help of NK cells. This type of immune response is especially desirable in the treatment of

cancer or vaccination against intracellular pathogens, such as viruses (see Figure 6.1).

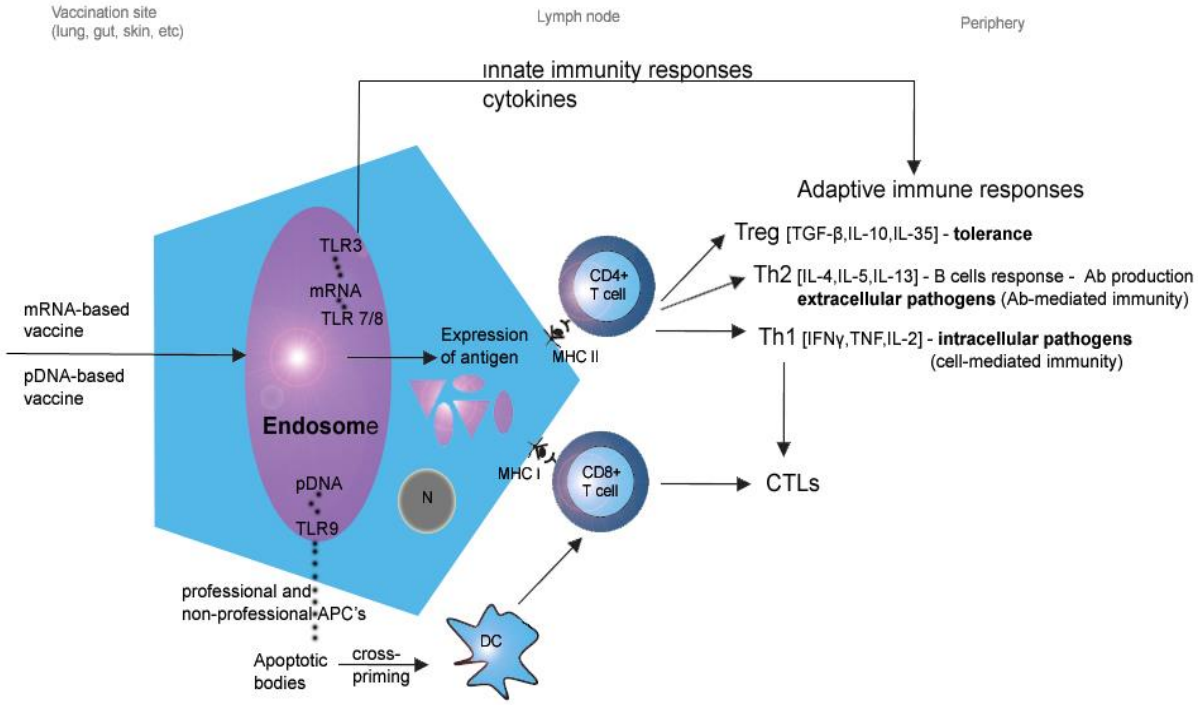


Figure 6.1: Schematic representation of the impact of innate immunity on gene-based vaccination.

While the immunogenic effect of mRNA is an apparent advantage for the purpose of vaccination, it should be noted that in reality there is a trade-off between self-adjuvancy and diminished antigen expression or "translational shutdown" (Chapter 4). Such innate immune stimulation, which reduces protein expression from RNA or even induces cell death (Chapter 4) is certainly undesirable for protein replacement therapies. To overcome this problem, we characterized how the incorporation of various different natural nucleoside modifications into RNA would affect the immunogenicity of the RNA (Chapter 5). We found that mRNA with novel combinations of base modifications including N1-methylpseudouridine (m1Ψ) outperformed the current state-of-the-art pseudouridine (Ψ)-modified mRNA platform by providing up to 44-fold or 13-fold reporter gene expression upon transfection into cell lines or intradermal (i.d.) injection into mice, respectively. We showed that m1Ψ-modified mRNA resulted in reduced intracellular innate immunogenicity and improved cellular viability compared to pseudourudine-modified mRNA upon transfection *in vitro*. The enhanced capability of m1Ψ-modified mRNA to express proteins may be due to an increased ability of the RNA

to evade activation of endosomal Toll-like receptor 3 (TLR3) and downstream innate immune signalling. We believe that the combination of modifications presented here may serve as a new standard in the field of modified mRNA-based therapeutics.

The results obtained in this dissertation suggest that the properties of the mRNA can be carefully adapted for each application to balance protein production and immunostimulation. As discussed in Chapter 2, one possible solution to achieving the highest level of protein production while still obtaining an adjuvant effect may be to co-express cytokines such as IL-12 encoded on pDNA or mRNA or in the form of a recombinant protein along with an antigen. However, many reports have described that these cytokines may manifest a severe toxic effect upon systemic delivery if the protein concentration in the body becomes too high. Hence, m1Ψ modified mRNAs that could express such potent but toxic therapeutic proteins in a regulatable manner would provide additional safety measures. Such ON/OFF switches that can be controlled using small molecules and genetic circuitry can be engineered using the latest advances in the field of synthetic biology as discussed in Chapter 7. I believe that the creation of such RNA "smart vaccines" from which the levels of antigen and adjuvants can be controlled on-demand will be the next endeavor of RNA-based vaccines.

GENERAL CONCLUSIONS

mRNA-based drugs are among the most promising therapeutic modalities in the fight against cancer, metabolic disorders or even allergies. Thus, the general goal of the proposed project was to further develop this safe and potent protein expression platform. Chapter 2 of this dissertation reviews the strengths and weaknesses of the current state-of-the-art mRNA pharmaceutical and emphasizes how its inherent safety features may enable it to surpass the more traditional pDNA- or viral DNA-based gene therapeutic. This critical feature of RNA motivated me to identify the molecular mechanisms underlying any potentially undesirable effects of mRNA (e.g. immunostimulation or cytotoxicity) as well as to optimize *in vivo* delivery and search for more effective solutions to enhance the stability and protein production capacity of RNA. To this end, as described in Chapter 3, I initiated my studies by comparing the *in vitro* and *in vivo* performance of unmodified mRNA and pDNA upon lipofection. I was able to clearly demonstrate, *in vitro*, an important advantage of mRNA in transfecting slowly- or non-dividing cells (similar to cells in a living organism). However, I also realized that unmodified RNA caused cellular cytotoxicity and did not express proteins for a long period of time. Thus, in Chapter 4, I sought to understand the molecular mechanisms that were behind this shortcoming. I confirmed that carrier-mediated delivery of mRNA resulted in the endosomal recognition of exogenous mRNA by TLR3, followed by type I interferon overexpression/secretion and subsequent expression of proinflammatory cytokines. Additionally, I detected significant overexpression of caspase-1 and cell death, which indicated the activation of pyroptosis, a type of programmed cell death. Finally, in Chapter 5, I investigated how incorporating different nucleoside modifications into RNA affects the various properties of the therapeutic modality (i.e. expression level, duration, immunostimulatory effects). I discovered that m1Ψ modified RNA was superior to the current state-of-the-art modification (Ψ) with regards to cellular viability and lack of innate immune stimulation, and level and duration of protein expression both *in vitro* and *in vivo*.

Thus in summary, through my doctoral studies, after characterizing how to formulate and deliver RNA *in vitro* and *in vivo*, I identified the molecular mechanisms underlying the disadvantages of RNA as a therapeutic and ultimately was able to discover a method to counteract this shortcoming by further improving upon the state-of-the-art

mRNA molecule. Thus, I believe that my dissertation has made a significant contribution towards the mRNA therapeutics community.

Future perspectives

Any therapeutic that is approved by regulatory agencies must not only be effective but also safe. A current problem related to mRNA drugs is that we do not have full control over the exact amount of protein production in a patient upon administration of the therapeutic. Hence, the effective total therapeutic protein dose cannot be reliably predicted, raising the concern of overdosing. Despite all of the efforts in the mRNA therapeutics industry to optimize and modulate the quantity and duration of protein expression from RNA, interpatient and intrapatient variation in protein production from RNA calls for a mechanism to fine-tune the expression levels of a protein post-administration of the RNA drug. The RNA-based genetic devices and regulatory circuits described in Chapter 7 of this dissertation may enable doctors to control the amount of protein production in a patient depending on how the patient is responding to the RNA drug. Furthermore, genetic circuits that can distinguish different cell types by sensing the gene expression pattern of different cells can also be used in addition to delivery-based cell-type specific targeting techniques as a method to reduce the toxic side-effects of protein expression in unwanted cell types. I predict that the future of RNA therapeutics will involve the use of such sophisticated mechanisms to enhance the efficacy and safety of RNA as a drug modality.

Summary

For many years, the instability of RNA had raised doubts as to whether it was possible to effectively use mRNA for gene therapy. However, rapid advances in messenger RNA-based technologies in the last decade have transformed mRNA into an increasingly popular therapeutic modality, especially in the field of vaccination against cancer and viral infections. Today, mRNA is considered a safer alternative to pDNA-based therapeutics, as it does not pose the risk of genomic integration, unlike DNA. Furthermore, mRNA-based approaches offer immediate expression of a protein of interest even in non-dividing cells.

In Chapter 2 of this dissertation we reviewed the general properties and advantages of RNA as a therapeutic modality. Moreover, we discussed specific attributes, limitations and benefits of unmodified, modified and self-replicating mRNA platforms. Additionally, this chapter also provides insights into the instability of the mRNA molecule and strategies to improve the delivery efficiency of *in vitro* transcribed (IVT) mRNA. We discussed how the inclusion of modified nucleotides, such as 5-methylcytidine (m5C) or pseudouridine (Ψ), can increase the half-life and translatability of IVT mRNA or decrease its immunogenicity, where necessary. Furthermore, this chapter gave an in-depth overview of the various techniques and vehicles used for intracellular mRNA delivery including electroporation, gene gun injection, and lipo- and polyplex based methods that have been exploited by us and other groups, mostly for the purpose of mRNA-based vaccination.

In Chapter 3 of this dissertation, we compared DNA and RNA-based strategies for heterologous gene expression using cationic liposomes as a delivery vehicle. We showed that transfection of human lung adenocarcinoma cells with mRNA complexes results in much faster expression compared to pDNA complexes. While the efficacy of mRNA complexes is independent of the cell cycle, pDNA complexes result in weak expression in nondividing cells. Thus, these data demonstrate that the nuclear barrier is a crucial obstacle for pDNA but not for mRNA. However, when mRNA and pDNA complexes encoding luciferase were administered intranasally to the lungs of mice, only the pDNA complexes gave rise to a detectable bioluminescent signal. This is likely due at least in part to the differences in the stability of the complexes as we showed that mRNA complexes are less stable in biological fluids compared to DNA complexes. However, as described in the next chapter (Chapter 4), the innate immune response

of the cells in the mouse lungs is also likely to be a major cause of the reduced expression from mRNA. Regardless, these results demonstrated the functional limitations of the traditional unmodified mRNA platform and encouraged us to develop a more stable and efficient RNA platform for mammalian cells applications as we described in Chapter 5.

In Chapter 4, we showed that carrier-mediated delivery of mRNA may activate TLR3 signaling in respiratory cells. Carrier-mediated delivery of mRNA following intranasal instillation caused activation of the innate immune system and massive production of immunostimulatory cytokines such as IL-6 or TNFα *in vitro* as well as in mice. Additionally, significant production of IL-12, typically expressed from immune cells, was detected 24 hours after instillation of mRNA complexes in murine lungs. Overexpression of the immunostimulatory cytokines was most likely caused by immune cells residing in the lung including antigen-presenting cells (APCs) such as dendritic cells (DCs) or macrophages, which are capable of phagocytosing the administered mRNA complexes. Furthermore, the data demonstate that the recognition of mRNA by the innate immune system is also associated with cell death, which proceeds in human respiratory cells via pyroptosis, a form of programmed cell death mediated by overexpression of caspase-1. This indicates that the transfected mRNA also activates the NOD-like receptors, which in turn regulate caspase-1 production. Finally, we showed that recognition of the delivered unmodified mRNA by the innate immune system had a negative effect on mRNA translation by comparing unmodified mRNA with innate immune-evading double modified 5-methylcytidine and pseudouridine (m5C/Ψ) mRNA.

Finally, in Chapter 5 of this dissertation, with the lessons learned in the previous two chapters in mind, we advanced the state-of-the-art modified RNA expression platform by discovering that incorporation of N1-methylpseudouridine (m1Ψ) into mRNA enables stronger and more sustained gene expression compared to pseudouridine (Ψ)-modified mRNA. The impact of this modification on the level and duration of gene expression, cellular viability, and the innate immune response was evaluated *in vitro* in different cell types as well as *in vivo* in mice. While endocytosis-dependent delivery (lipofection) of unmodified mRNA caused overexpression of TLR3 in respiratory cells, electroporation of the RNA into the same cell types resulted in a reduced innate immune response and less *in vitro* cytotoxicity.

Nevertheless, mRNA therapeutics still have limitations that we are aware of and should

be addressed in future research. Chapter 7 (Appendix A) provides a thorough review of the latest advances in synthetic biology, which may contribute to overcoming the existing challenges in the mRNA therapeutics field.

Samenvatting

Jarenlang had de instabiliteit van RNA twijfel gezaaid over de vraag of het mogelijk is om mRNA effectief te gebruiken voor gentherapie. Snelle vooruitgang in mRNA-gebaseerde technologieën in het laatste decennium heeft mRNA echter omgezet in een steeds populairdere vorm van behandeling, vooral op het gebied van vaccinatie tegen kanker en virale infecties. Tegenwoordig wordt mRNA beschouwd als een veiliger alternatief voor pDNA-gebaseerde therapieën omdat er geen risico op genomische integratie is, in tegenstelling tot pDNA. Bovendien biedt de mRNA-gebaseerde aanpak een onmiddellijke expressie van het eiwit dat van belang is, zelfs in niet-delende cellen.

In Hoofdstuk 2 van dit proefschrift beoordeelden we de algemene eigenschappen en voordelen van RNA als een therapeutische modaliteit. Bovendien hebben we gesproken over de specifieke attributen, beperkingen en voordelen van niet-gemodificeerde, gemodificeerde en zelf-replicerende mRNA platformen. Voorts geeft dit hoofdstuk ook inzicht in de instabiliteit van de mRNA-molecule en strategieën om de efficiëntie van de transfectie van *in vitro* getranscribeerde (IVT) mRNA te verbeteren. Eerst hebben we besproken hoe de opname van gemodificeerde nucleotiden, zoals 5-methylcytidine (m5C) of pseudouridine (Ψ), de halfwaardetijd en de translatie van IVT mRNA kan verhogen alsook de immunogeniciteit kan verlagen, indien nodig. Bovendien geeft dit hoofdstuk een diepgaand overzicht van de verschillende technieken en non-virale afgiftesystemen voor intracellulaire levering van mRNA, waaronder elektroporatie, gene-gun injectie en lipo- of poly-plex gebaseerde methoden, die door ons en andere groepen geëxploiteerd werden, vooral ten behoeve van mRNA-gebaseerde vaccinatie.

In Hoofdstuk 3 van dit proefschrift vergeleken we experimenteel pDNA- en mRNA-gebaseerde strategieën voor heterologe genexpressie met behulp van kationische liposomen als afgiftesysteem. We hebben aangetoond dat een transfectie van menselijke long adenocarcinoom cellen met mRNA complexen in veel snellere expressie resulteerde in vergelijking met pDNA complexen. Terwijl de werkzaamheid van mRNA complexen onafhankelijk van de celcyclus is, resulteren pDNA complexen in zwakke expressie in niet-delende cellen. Deze gegevens tonen dus aan dat de nucleaire barrière een cruciaal obstakel is voor pDNA, maar niet voor mRNA. Toen mRNA en pDNA complexen die *firefly* luciferase codeerden echter intranasaal in de

longen van muizen toegediend werden, zorgden enkel de pDNA complexen voor het ontstaan van een detecteerbaar bioluminescent signaal. Dit komt waarschijnlijk, ten minste gedeeltelijk, door de verschillen in de stabiliteit van de mRNA complexen. We hebben namelijk aangetoond dat mRNA complexen minder stabiel zijn in biologische vloeistoffen, vergeleken met pDNA complexen. Zoals beschreven in het volgende hoofdstuk (Hoofdstuk 4), is de aangeboren immuunrespons van de cellen in de muizenlong echter waarschijnlijk ook een belangrijke oorzaak van de verminderde expressie van mRNA. Desalniettemin toonden deze resultaten de functionele beperkingen van de traditionele ongemodificeerde mRNA platformen, wat ons aanspoorde om een meer stabiel en efficiënt mRNA platform te ontwikkelen voor zoogdiercellen toepassingen, zoals wij beschreven in Hoofdstuk 5.

In Hoofdstuk 4 hebben we getoond dat carrier-gemedieerde levering van mRNA de TLR3 signalering kan activeren in de longcellen. Carrier-gemedieerde afgifte van mRNA veroorzaakte *in vitro* zowel als bij muizen, na intranasale indruppeling, een activering van het aangeboren immuunsysteem, wat gepaard ging met een massale productie van immuunstimulerende cytokinen, zoals IL-6 en TNFα. Daarnaast werd een significante overexpressie van IL-12, typisch voor immuuncellen, in muriene longen gedetecteerd 24 uur na de toediening van mRNA complexen. Dit zou een betrokkenheid suggereren van de antigen-presenterende cellen (APC's) aanwezig in de longen, zoals dendritische cellen (DCs) of overvloedig aanwezige macrofagen, die de toegediende mRNA complexen fagocyteren en adaptieve immuunreacties aansturen. Bovendien wijzen de gegevens erop dat de erkenning van mRNA door het aangeboren immuunsysteem ook geassocieerd is met celdood, wat in menselijke ademhalingscellen via pyroptosis verloopt, een vorm van geprogrammeerde celdood gemedieerd door overexpressie van caspase-1. Dit geeft aan dat het getransfecteerde mRNA waarschijnlijk ook de NOD-achtige receptoren activeert die caspase-1 reguleren. Tot slot hebben we getoond, dat de erkenning van het geleverde ongemodificeerde mRNA door het aangeboren immuunsysteem een negatief effect had op de translatie van mRNA door het vergelijken van de dubbele gemodificeerde 5-methylcytidine en pseudouridine (m5C/Ψ) mRNAs, die het aangeboren immuunsysteem ontwijken.

Tenslotte, in Hoofdstuk 5 van dit proefschrift, de ervaringen uit de voorbije twee hoofdstukken indachtig, verbeterden we het state-of-the-art gemodificeerde RNA expressie platform. We hebben namelijk ontdekt dat de incorporatie van N1-

methylpseudouridine (m1Ψ) in mRNA voor een sterkere en constante genexpressie zorgt, in vergelijking met pseudouridine (Ψ)-gemodificeerde mRNA. De impact van deze wijziging op de omvang en duur van genexpressie, cellulaire levensvatbaarheid en de aangeboren immuunrespons werd *in vitro* bestudeerd op verschillende celtypes maar ook *in vivo* in muizen. Terwijl endocytose-afhankelijke afgifte (lipofectie) van ongemodificeerd mRNA overexpressie van TLR3 veroorzaakte in respiratoire cellen, resulteerde de elektroporatie van mRNA in dezelfde celtypen in een verminderde aangeboren immuunrespons en minder *in vitro* cytotoxiciteit.

Toch hebben de mRNA geneeswijzen nog steeds beperkingen waar we van bewust zijn en waarnaar er in de toekomst verder onderzoek moet worden gevoerd. Dat is de redenering om onze visie en voorspelling voor mRNA's toekomst in Hoofdstuk 7 (Appendix A) te presenteren. Dit hoofdstuk omvat een grondige beschrijving van de nieuwste ontwikkelingen van de synthetische biologie waar we RNA apparaten voor eiwitexpressie-controle bespreken met het oog op mRNA-gebaseerde vaccinatie.

III. APPENDIX

CHAPTER 7

Appendix A

Synthetic biology devices and circuits for RNA-based “smart vaccines”: future outlook

The chapter is based on the publication:

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Synthetic biology devices and circuits for RNA-based “smart vaccines”: a propositional review.; *Expert Review of Vaccines* (SPECIAL FOCUS | RNA-Based Vaccines)

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ABSTRACT

Nucleic acid vaccines have been gaining attention as an alternative to the standard attenuated pathogen or protein based vaccine. However, an unrealized advantage of using such DNA or RNA based vaccination modalities is the ability to program within these nucleic acids regulatory devices that would provide an immunologist the power to control the production of antigens and adjuvants in a desirable manner by administering small molecule drugs as chemical triggers. Advances in synthetic biology have resulted in the creation of highly predictable and modular genetic parts and devices that can be composed into synthetic gene circuits with complex behaviors. With the recent advent of modified RNA gene delivery methods and developments in the RNA replicon platform, we foresee a future in which mammalian synthetic biologists will create genetic circuits encoded exclusively on RNA. Here, we review the current repertoire of devices used in RNA synthetic biology and propose how programmable "smart vaccines" will revolutionize the field of RNA vaccination.

INTRODUCTION

Synthetic biology is a radically new style of genetic engineering in which living organisms are "programmed" using genetic circuits to systematically engineer novel and useful biological properties. The earliest accomplishments in the field included the construction of simple genetic circuits such as oscillators [289] and toggle switches [290] in bacterial species using mathematical modeling and rational network design. Since then, increasingly more complex circuits have been engineered in prokaryotes as well as in mammalian systems using principles of synthetic biology [291-301]. This process typically involves the top-down decomposition of the high-level behavior (sensing-processing-actuation) of a genetic circuit followed by the physical implementation of the circuit via bottom-up assembly of categorized or novel biological devices with standardized functions [302,303]. The construction of synthetic gene circuits has been greatly facilitated by drastic improvements in our ability to assemble large DNA constructs as well as by the increase in the number of well characterized devices from which we can build such circuits.

By combining regulatory devices that function according to transcriptional, translational or post-translational logic, we and others have created various therapeutic circuits that operate in mammalian systems. These include circuits that selectively kill cancer cells [304], treat the symptoms of metabolic disorders [305-308], or profile allergies of people [309]. An attractive area of application for such RNA circuits is the emerging field of RNA vaccination. While RNA-based vaccines are completely synthetic, provide compositional control, and cost five to ten times less to manufacture than protein-based therapeutics [25], the creation of effective and universal nucleic acid-based prophylactic solutions is still challenging. Additionally, researchers aim to create vaccines that would simplify the process of immunization and increase accessibility around the globe by offering effective one-shot injections, as booster injections can pose a challenge to communities with limited means of access to vaccination clinics. We propose here that "smart vaccines" with programmable adjuvant expression and prime-boost behavior could provide a solution to these problems.

Devices for post-transcriptional gene regulation

Devices that can be used in RNA-based genetic circuits include: RNA binding proteins (RBPs), synthetic riboswitches, devices that modulate the RNAi machinery, devices that modulate protein stability and devices that sense the environment (see Table 7.1). Some of these devices, including a few widely used RBPs and their cognate binding motifs, were transferred from other species (e.g. phage, archaea and bacteria) in their original form into mammalian systems, whereas others such as aptamers were engineered from scratch. The majority of these devices function by inhibiting translation initiation or inducing RNA degradation.

Device	Origin	Function(s)	(Potential) vaccine application	Ref.
RNA binding proteins				
L7Ae	*Archaeoglobus fulgidus*	•Translational regulation	•ON/OFF switch for expression of antigens and adjuvants	310-313
		•RNP nanostructure	•Immunomodulation	314,315
		•shRNA processing regulation	•Immunomodulation	316
MS2-CP	Bacteriophage MS2	•Translational regulation	•ON/OFF switch for expression of antigens and adjuvants	317,318
TetR	*E. coli*	•Translational regulation	•ON/OFF switch for expression of antigens and adjuvants	319-322
		•RNA-localization regulation	•Immunomodulation	323
PUF	Eukaryotes	•Splicing regulation	•Cell fate regulation of immune cells	324
		•RNA cleavage	•ON/OFF switch for expression of antigens and adjuvants	325
		•Translational regulation	•ON/OFF switch for expression of antigens and adjuvants	326-330
Synthetic riboswitches				
Aptamer	Synthetic	•Translational regulation	•ON/OFF switch for expression of antigens and adjuvants	331-340
		•Splicing regulation	•Cell fate regulation of immune cells	341-343
		•Viral RNA replication regulation	•ON/OFF switch of vaccine circuit	344
		•shRNA processing regulation	•Immunomodulation	345,346
		•Receptor targeting	•Immunomodulation, antigen delivery to APCs and *de novo* antigen presentation	347,348* and reviewed in 349*

Aptazyme	Synthetic	•Translational regulation •shRNA processing regulation	•ON/OFF switch of vaccine circuit •Immunomodulation	350-354 355
RNAi modulators (other than those listed above)				
Oligonucleotide	Synthetic	•Drosha inhibition	•Immunomodulation	356
Small molecule	Synthetic	•Dicer or Drosha inhibition	•Immunomodulation	357
miRNA sponges	Synthetic	•Endogenous miRNA sequestration	•Immunomodulation	358
Protein (de)stabilization domains				
DD	Synthetic	•Protein stability regulation	•Immunomodulation	359-362
LID	Synthetic	•Protein stability regulation	•Immunomodulation	363
Sensors				
miRNA target site	Synthetic	•miRNA sensing	•Cell type specific vaccine circuit activation	304,364-367
mRNA strand displacement	Synthetic	•mRNA sensing	•Cell type specific vaccine circuit activation	368
Protein aptamer	Synthetic	•Protein sensing	•Detection of immune cell activity	369
Kinase translocation reporter	Synthetic	•Kinase activity sensing	•Detection of immune cell activity	370

*References in which devices were used for vaccination.
RNP: ribonucleoprotein; shRNA: short hairpin RNA; TetR: Tet repressor; PUF: Pumilio and FBF homology; APC: Antigen presenting cell; RNAi: RNA interference; miRNA: microRNA; DD: destabilizing domain; LID: ligand-induced degradation.

Table 7.1. Devices for post-transcriptional gene regulation.

However, other devices may regulate splicing, modulate innate immune activation, control protein stability or act as an interface module between the environment and other regulatory devices. Figure 7.1 provides a summary of the representative mechanisms by which these devices function.

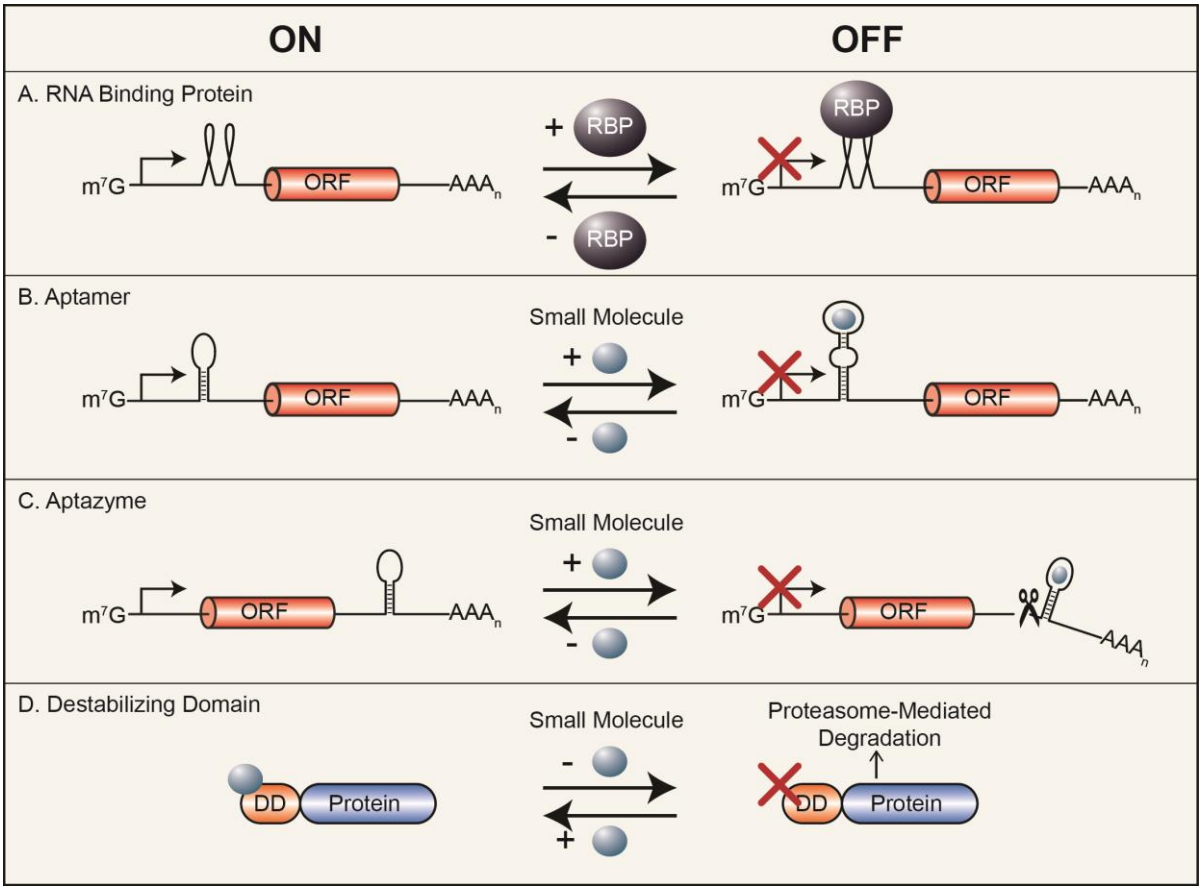


Figure 7.1. Devices for post-transcriptional gene regulation and their modes of action. Operation of (A) RNA binding proteins, (B) aptamers (C) aptazymes and (D) destabilizing domains.
m^7G: 7-methyl-guanosine; ORF: open reading frame; AAA_n: poly(A) tail; RBP: RNA binding protein; DD: destabilizing domain.

Apart from the advantages discussed above, post-transcriptional devices have additional benefits such as their fast response time (they directly modulate the expression of proteins) and their resource-friendliness (they bypass the use of cellular metabolites and energy involved in transcription). Furthermore, RNA-based devices can be versatile compared to DNA as they can carry the information of a protein output as well as form three-dimensional structures with enzymatic activities [371] or even rearrange into higher order assemblies [372]. However, general disadvantages of RNA include its inherent instability and immunogenicity, although moderate levels of innate immune stimulation by the RNA may be beneficial for certain applications such as cancer vaccination. In the following sections, we discuss in more detail the properties

of these RNA encoded devices and how they have been used to regulate RNA related processes.

RNA binding proteins

Many RNA binding protein (RBP)-based devices discussed in this section function by inhibiting translation initiation. Thermodynamically stable secondary structures within the 5' UTR of an mRNA have been shown to be inhibitory for translation [373]. Thus, this provides an opportunity for RBPs to regulate translation by binding to the 5' UTR of mRNAs to prevent scanning of ribosomes through steric hindrance, secondary structure formation or both.

L7Ae

The archaeal ribosomal protein L7Ae binds with high affinity to RNA motifs known as kink-turns (K-turns) and K-loops [374,375]. L7Ae was first used to regulate translation by Saito and colleagues who inserted a K-turn motif into the 5' UTR region of a reporter gene in HeLa cells [310]. Similarly, insertion of the K-loop motif, which binds L7Ae with slightly lower affinity, can also be inserted into the 5' UTR of a gene for repression [311]. The level of repression by L7Ae can be increased by positioning the K-turn or K-loop motifs closer to the 5' end of the mRNA or by increasing the number of motifs inserted into the 5' UTR [312]. The L7Ae/K-turn system can also be inverted to an ON switch by coupling it with the nonsense-mediated decay (NMD) pathway in which mRNAs with premature termination codons (PTCs) are rapidly degraded [313]. This ON switch was created by inserting an NMD "bait ORF" with PTCs upstream of an IRES and a reporter gene. While this mRNA is normally rapidly degraded by NMD, if the bait ORF is translationally repressed by L7Ae, then the PTCs are no longer recognized by the NMD pathway. Thus, the mRNA remains intact, and the reporter gene can be translated.
L7Ae can also be used to create interesting ribonucleoprotein (RNP) nanostructures with therapeutic potential [314,315]. Binding of L7Ae to a K-turn motif is known to bend the RNA at an angle of ~60º [376]. Saito and colleagues used this property to design an equilateral triangular RNP nanostructure containing a dsRNA circle with three K-turn motifs bound by three L7Ae proteins. Formation of the triangular structure was confirmed by atomic force microscopy (AFM) [314]. Formation of this RNP nanostructure

provides enhanced stability to the RNA when incubated in serum [315]. By incorporating a fusion protein between L7Ae and a HER2 receptor affibody (a 6 kDa engineerable three-helix peptide affinity motif) into the triangular nanostructure labeled with Alexa-647, the RNP was able to function as a detector of HER2-positive cancer cells. Finally, when the RNA strands in the nanostructure were redesigned so that three siRNA modules would protrude perpendicularly from the sides of the triangular RNP, the siRNA modules were able to undergo processing by Dicer and reporter gene expression was knocked-down in HeLa cells [315].

MS2 coat protein

The coat protein of the MS2 RNA bacteriophage (MS2-CP), in its native context, is a bifunctional protein which may exist in one of two distinct higher-order structures. When MS2-CP aggregates, it becomes the bacteriophage capsid, which functions to encapsulate and protect the bacteriophage genome. However, when MS2-CP forms an anti-parallel homodimer, it binds a stem loop region within its genomic RNA that contains the start codon of the MS2 replicase gene, thereby inhibiting translation of the gene. As expression of MS2-CP is tolerated well in eukaryotic cells, the MS2-CP/stem loop system has been used extensively in the field of RNA biology to tether and study the effect of a protein of interest on reporter RNAs (reviewed in [377]). MS2-CP is also capable of directly affecting various eukaryotic RNA processes via steric hindrance. Hentze and colleagues targeted MS2-CP to the 5' UTR of a reporter gene in HeLa cells and achieved ~16-fold repression of gene expression [317]. Repression was strictly translational as the abundance of the reporter mRNA was not affected by MS2-CP binding as shown by Northern blotting and a primer extension assay. Smolke and colleagues recruited MS2-CP to various locations within the introns of a three exon-two intron mini gene RNA and showed that the inclusion/exclusion rate of the middle exon can be increased or decreased depending on where MS2-CP was recruited to [378]. Modulation of the splicing pattern was speculated to be due to decreased binding of spliceosome components or trans-acting splicing factors through steric hindrance or by recruitment of such factors by MS2-CP.

TetR

The *E. coli* Tet repressor (TetR) protein and the various TetR fusion proteins (e.g. tetracycline-controlled transactivator: tTA [379] and reverse tetracycline-controlled transactivator: rtTA [380]) are arguably the most commonly used regulatory devices for creating synthetic gene circuits on DNA. Recently, the Suess group and Niles group performed SELEX (Systematic evolution of ligands by exponential enrichment [381,382]) and independently identified RNA aptamers that tightly bound TetR (Kd in the low nM range in the absence of tetracycline derivatives) [319-321]. The aptamers shared a similar stem loop structure with two stems and an inner loop. The inner loop portion of the identified aptamers contained conserved sequence motifs that were shown to directly interact with the TetR protein using in-line probing [319]. It was shown using site-directed mutagenesis that, not surprisingly, the aptamer binding domain of the TetR protein was located within the DNA binding domain of TetR (the N-terminal helix-turn-helix motif). Using rational design and functional testing, the Niles group engineered a minimal TetR aptamer that could repress translation when placed in the 5' UTR of several genes in *S. cerevisiae* in the presence of TetR [322]. Translational repression was relieved when a tetracycline derivative such as doxycycline was added to the culture media. Thus this system provides a general mechanism for small molecule regulated control of gene expression using an RNA binding protein.

PUF proteins

The Pumilio and FBF homology (PUF) proteins are a family of highly conserved eukaryotic translational regulators that play a role in a wide array of processes including differentiation, mitochondrial biogenesis, cell cycle regulation and memory formation (reviewed in [383]). In the native context, PUF proteins are recruited to the 3' UTRs of target mRNAs through their RNA binding domains (Pumilio homology domain: PUM-HD). By doing so, PUF proteins exert their effects as repressors or activators by interacting with or influencing the binding of other proteins such as decapping enzymes, deadenylases and possibly poly(A)-polymerases [383]. The RNA binding PUM-HD consists of eight α-helical PUM repeat motifs which assemble into a "half-doughnut" shaped structure [384]. PUF proteins are attractive targets for engineering due to their highly modular nature: each of the eight PUM repeats within a PUM-HD

recognizes a single nucleotide base of an RNA sequence according to a simple RNA recognition "code" [385]. Thus, using this code, it is possible in theory to engineer PUF proteins that target any arbitrary eight-nucleotide RNA sequence. Wang and colleagues demonstrated the potential for using PUF proteins as targeting domains for regulation of RNA related processes by fusing them to glycine-rich splicing repressors and arginine/serine-rich splicing activators [324]. When targeted to specific exons, these PUF-splicing activator/repressor fusion proteins were capable of promoting/suppressing exon skipping or influencing alternative splicing of reporter mRNAs in 293T cells. Strikingly, by engineering PUF-splicing repressor fusion proteins that bind to an exon within the cancer related *Bcl-X* pre-mRNA, the authors were able to facilitate splicing of the pro-apoptotic Bcl-xS isoform of the mRNA. This induced apoptosis of the HeLa, MDA-MB-231 (breast cancer) and A549 (lung cancer) cell lines. Subsequently, Wang and colleagues also fused a RNA endonuclease to a PUF protein to create synthetic RNA "restriction enzymes" [325]. Wickens and colleagues demonstrated the use of PUF-deadenylase or poly(A) polymerase fusion proteins for downregulation or upregulation of reporter/endogenous gene expression in *Xenopus* oocytes [326] and human cells [327]. Similarly, Schaffer, Kane and colleagues repressed translation of reporter genes by using PUF to cause steric hindrance or activated translation by recruitment of a PUF-eIF4E (i.e. an eukaryotic translation initiation factor) fusion protein [328]. Furthermore, by connecting eIF4E and PUF to CRY2 and CIB1 (components of a light inducible heterodimerization system) the authors were able to activate translation of a reporter gene using light. Other efforts to facilitate the use of PUF proteins as RNA devices include work from Zhao and colleagues who created a PUM repeat library for high-throughput cloning of synthetic PUF proteins [329] using Golden Gate cloning [386] and work from Rackham and colleagues who engineered synthetic PUF proteins with 16 PUM repeats to increase targeting specificity [330].

Another family of RNA binding proteins with great engineering potential is the pentatricopeptide repeat (PPR) protein family. PPR proteins are highly modular RNA binding proteins made up of an array of 2-30 modular PPR repeats. Like the PUM repeats of PUF proteins, each PPR motif can recognize a base of one nucleotide within a target RNA sequence. While the underlying RNA recognition code for PPR proteins was only recently elucidated [387,388], the potential for using PPR proteins as versatile tools for manipulating RNA has been recognized and reviewed elsewhere [389].

Synthetic riboswitches

Natural riboswitches, frequently found in bacteria, are RNA based molecular switches with a defined three-dimensional structure that undergo conformational changes upon intracellular metabolite binding and affect the outcome of specific biological processes including transcription, translation, and RNA processing (reviewed in [390]). Unlike most other RNA based regulators, riboswitches do not require additional protein factors to sense metabolites of interest or influence downstream biological processes. Synthetic riboswitches work in a similar manner except that they have been artificially engineered by combining synthetic small molecule binding aptamers with various RNA devices such as ribozymes. Thus by creating synthetic riboswitches that respond to non-toxic exogenous small molecules, orthogonal control of RNA based processes can be achieved.

Engineering small molecule binding aptamers

RNA aptamers are short highly structured RNA motifs that can bind with high affinity and selectivity to specific ligands. Using SELEX, hundreds of aptamers that can bind to a wide variety of molecules such as metal ions, nucleotides, carbohydrates, amino acids, peptides, proteins, and antibiotics have been engineered (reviewed in [391]). However, while SELEX has been successful in discovering aptamers that bind to molecules of interest *in vitro*, very few of these aptamers can be engineered into riboswitches that function *in vivo*. Recently, Suess and colleagues compared the thermal stability and conformation of various neomycin-binding aptamers (some that are functional *in vivo* and others that are non-functional) in the presence or absence of ligand using UV melting analysis and NMR [331]. Indeed, they found that high ligand-binding affinity and thermal stability upon ligand binding is required but not sufficient for the aptamer to serve as a functional switch. Instead they showed that the functional aptamers are those that have a destabilized basal unbound state and undergo extensive conformational changes upon ligand binding. Another issue related to the use of aptamers is the often cytotoxic high ligand concentration required for regulatory activity. It has been speculated that this may be due to discrepancies between the intracellular environment and the experimental conditions of SELEX [392]. For instance, folding or accessibility of an aptamer may be disrupted by RBPs inside a cell or the

ionic concentrations *in vivo* may be different from SELEX conditions. Thus, ultimately, to engineer an aptamer that functions *in vivo*, functional screening must be performed in cells [332].

Non-catalytic synthetic riboswitches

Despite the challenges described above, aptamers have been used successfully to modulate cellular processes. Green and colleagues inserted an aptamer for Hoechst 33258 upstream of a beta-galactosidase reporter gene and showed that small molecule dependent repression can be achieved in eukaryotic cells using aptamers [333]. Subsequently, Pelletier and colleagues definitively demonstrated this concept in wheat germ extracts and *Xenopus* oocytes by inserting aptamers for biotin or theophylline in the 5' UTR of reporter genes [334]. Translational inhibition was due to reduced 40S ribosome complex binding as well as 80S ribosome complex assembly. Similarly, Suess and colleagues developed a synthetic riboswitch that responded to the cell permeable and non-toxic small molecule tetracycline [335]. The tetracycline riboswitch functioned in a dose and position dependent manner by blocking 43S initiation complex formation when inserted in the proximity of the cap or by blocking ribosome scanning when positioned close to the AUG initiation codon [335,336]. The strength of repression increased as more aptamers were inserted in the 5' UTR [337]. Smolke and colleagues rationally designed trans-acting RNA sequences termed "antiswitches" that hybridized to regions encompassing the initiation codon of a reporter mRNA in yeast [338]. These antiswitches contained aptamer domains and were designed so that the portion of the antiswitch that hybridizes to the reporter mRNA would only be exposed upon small molecule binding to the aptamer. They were able to engineer antiswitches that repressed reporter gene translation in the presence of theophylline or tetracycline. Furthermore, they were also able to design an "on" antiswitch that responded to theophylline in the reverse manner (repressed translation in the absence of theophylline). More recently, using a rational design approach, Ogawa showed that internal ribosome entry site (IRES) mediated translation can also be regulated with small molecules using a theophylline aptamer [339]. Ogawa accomplished this using a Plautia stali intestine virus (PSIV) IRES by first inserting an anti-IRES (aIRES) sequence within the IRES that forms an aberrant hybrid and disrupts its function. He then inserted an anti-anti-IRES (aaIRES) sequence into the

IRES so that the aaIRES hybridizes with the aIRES and restores the function of the IRES. Finally, he inserted an aptamer between the aIRES and aaIRES so that in the presence of theophylline, the aIRES-aaIRES hybrid will preferentially form, thereby facilitating theophylline dependent translation from an IRES. Ogawa has also modulated a phenomenon known as "ribosome shunting" observed in certain viruses such as the cauliflower mosaic virus (CaMV). Ribosome shunting is a process by which a ribosome translates an upstream short ORF (sORF) and is then shunted to a downstream ORF (dORF) after encountering a properly positioned rigid stem structure. By modifying the CaMV 35S RNA and replacing the rigid stem structure with a theophylline aptamer, Ogawa achieved ~14 fold induction of a reporter dORF in a theophylline dependent manner [340].

Aptamers have also been used to regulate RNA related processes other than translation in a small molecule dependent manner. Gaur and colleagues showed *in vitro* that a theophylline aptamer inserted near a 3' splice site of a pre-mRNA can inhibit splicing [341] and that one inserted near the branch point can inhibit splicing *in vitro* or *in vivo* [342] in a theophylline dependent manner. Similarly, Suess and colleagues demonstrated that a tetracycline aptamer positioned near the 5' splice site of a pre-mRNA in yeast inhibited splicing in a tetracycline dependent manner [343]. The theophylline aptamer has also been used to control the replication of the positive strand RNA virus, tombusvirus. By replacing a stem loop structure whose stability is required for replication with a theophylline aptamer, White and colleagues were able to induce replication of the viral RNA by ~10-fold using theophylline [344].

Finally, Fussenegger and colleagues created an aptamer that was a fusion between the TetR aptamer (that binds the TetR protein) described above and a theophylline aptamer [393]. This TetR-theophylline fusion aptamer enabled proper folding of the TetR aptamer portion only when the theophylline aptamer portion was stabilized by theophylline. This fusion aptamer enabled disruption of tTA mediated transcriptional activation in a theophylline or doxycycline dependent manner, by inhibiting tTA binding to the promoter of a reporter gene by blocking the DNA binding domain with the TetR aptamer (by theophylline administration) or by inducing a conformational change in the structure of TetR (by doxycycline administration), respectively. In theory, this fusion aptamer could also be used to regulate TetR mediated translational regulation of a reporter RNA using two small molecule inputs.

Aptazymes

Another type of synthetic riboswitch, which combines small molecule sensing and cleavage of RNA, is the aptazyme. Aptazymes are allosteric ribozymes that undergo self-cleavage based on whether or not a small molecule is bound to the aptamer domain. The first aptazyme was created by Breaker and colleagues who modified a minimal hammerhead ribozyme (a ribozyme which consists of an 11 nucleotide conserved core sequence flanked by three stem regions) by replacing stem II of the ribozyme with an aptamer that binds ATP [394]. Depending on the "connector" sequence between the aptamer and core region of the aptazyme, ATP binding to the aptamer either inhibited or induced self-cleavage activity presumably by causing steric hindrance or stabilizing folding of the aptazyme, respectively. However, activity of a minimal hammerhead ribozyme requires a Mg^{2+} concentration much higher than that inside a cell. Thus for intracellular operation, the full-length hammerhead ribozyme which contains additional sequence elements that stabilize folding of the structure through tertiary interactions must be used [395]. Smolke and colleagues modified a hammerhead ribozyme from tobacco ringspot virus (TRSV) satellite RNA to create such an aptazyme that could function in yeast. Theophylline or tetracycline aptamers were embedded within loop II of the ribozyme so that binding of a small molecule to the aptamer would either disrupt or facilitate the proper formation of loop II and influence folding of the entire aptazyme. The aptamer sequences were rationally designed so that the aptazyme would be turned ON or OFF upon ligand binding via "strand-displacement" or "helix-slipping" based mechanisms. Insertion of these aptazymes into the 3' UTR of an mRNA enabled small molecule induction of gene expression in yeast [350]. Subsequently, by inserting two ON or OFF aptazymes that respond to different or identical small molecule inputs in the 3' UTR of a reporter mRNA, the authors were able to regulate reporter gene expression according to AND or NOR logic using theophylline and tetracycline [351]. They were also able to induce reporter gene expression when theophylline was within a certain concentration range but not higher or lower that that range (bandpass filter). Furthermore, by simultaneously inserting two different aptamers in loop I and loop II of the same hammerhead ribozyme or by connecting two aptamers in tandem in loop II, NAND or OR logic gates were, respectively, created. More recently, Hartig and colleagues created a theophylline responsive aptazyme based on the *Schistosoma mansoni*

hammerhead ribozyme that functions as an OFF switch in mammalian cells [352], and Smolke and colleagues adapted their TRSV hammerhead aptazymes to engineer T cells by expressing IL-2 or IL-15 in a small molecule dependent manner in mice [353]. Finally, most recently, Yokobayashi and colleagues created a genomic hepatitis delta virus (HDV) aptazyme OFF switch which can repress reporter gene expression ~30-fold in mammalian cells upon guanine administration [354].

RNAi modulation

Since its original discovery over two decades ago, RNA interference as a technology has transformed into one of the most predictable and effective tools to silence gene expression (reviewed in [396]). Most commonly, RNAi based silencing is induced by either delivery of small interfering RNA (siRNA) duplexes which consist of ~20-30 nucleotide long RNAs characterized by perfect base-pairing or in the form of primary miRNAs (pri-miRNAs; long single RNA molecules which contain characteristic stem loop structures) or short hairpin RNAs (shRNAs; engineered single RNA molecules which consist of minimal stem loop structures that resemble either pri-miRNAs or precursor miRNAs [pre-miRNAs] with perfectly base-paired stems) expressed from a vector. The unique stem loop structures of pri-miRNAs can be divided into four modular domains: the terminal loop, the upper stem, the lower stem, and the basal segments (5' and 3' single stranded RNA regions) [397]. Whereas siRNA duplexes are loaded directly onto the RNA induced silencing complex (RISC) with the "guide strand" retained by RISC as siRNA, pri-miRNAs and shRNAs must first undergo processing by the endogenous miRNA biogenesis machinery. Pri-miRNA stem loop structures are first recognized by the Microprocessor complex (Drosha/DGCR8) and then cleaved between the upper and lower stems to produce pre-miRNAs. Subsequently, the Dicer endonuclease recognizes the pre-miRNA structure and clips off the terminal loop region from the pre-miRNA. Finally, the miRNA duplex (typically containing a 1 bp mismatch or "bulge") originating from the upper stem region of the miRNA is loaded onto RISC, and the guide strand is selected as the mature miRNA to silence its target mRNA (reviewed in [398]).

Relatively recently, several groups have engineered regulatory devices based on aptamers, aptazymes and RBPs, or have just used small molecules to regulate gene expression by modulating shRNA or miRNA processing. Yokobayashi and colleagues

replaced the loop region of an shRNA with a theophylline aptamer and showed that processing of shRNA by Dicer was inhibited upon administration of theophylline, preventing Dicer mediated generation of siRNAs in HEK293 cells using theophylline inhibited reporter gene silencing (ON switch) [345]. Subsequently, Yokobayashi's group attached a theophylline aptazyme (a hammerhead ribozyme derived from *Schistosoma mansoni* with an aptamer inserted into stem III) to the 5' end of an shRNA so that Drosha processing of the shRNA would be inhibited due to base paring in the 5' portion of the basal segment. Upon theophylline administration to HEK293 cells expressing this aptazyme-shRNA fusion, the aptazyme cleaved itself away from the shRNA thus enabling production of an siRNA duplex and knockdown of reporter gene expression (OFF switch) [355]. Similarly, the same group attached a stem loop structure to an shRNA to prevent Drosha processing but this time dissolved the base pairing of the stem and enabled processing by transfecting a modified oligonucleotide that competes with the stem. Using this oligonucleotide induced OFF switch, they demonstrated reporter and endogenous gene knockdown in HEK293 cells [356]. Smolke and colleagues also modulated Drosha processing by inserting aptamers into the basal region of an shRNA. They showed using three aptamers (theophyline, tetracycline, and hypoxanthine) that small molecule binding to the aptamers inhibited shRNA processing by Drosha and prevented knockdown of reporter genes (ON switch) [346]. Saito and colleagues replaced the terminal loop of an shRNA with a K-turn motif and demonstrated that steric hindrance caused by L7Ae binding to the terminal loop can prevent siRNA processing by Dicer [316]. They used this ON switch to control reporter genes as well as expression of the pro-apoptotic *Bim* and anti-apoptotic *Bcl-xL* genes to regulate cell fate. Disney and colleagues used a computational approach termed Inforna to predict that a heterocyclic aromatic compound benzimidazole may bind the Drosha cleavage site of miR-96, a miRNA upregulated in cancer, and prevent processing [357]. Briefly, the Inforna pipeline uses a combination of experimentation and computation to identify RNA sequence motifs that may bind small molecule compounds of interest. The experimental part consists of a small molecule-RNA motif interaction screen, termed two-dimensional combinatorial screening (2DCS) [399]. In 2DCS, RNA hairpin structures with short randomized internal loops (e.g. six random nucleotide loops) are hybridized to small molecule ligands immobilized on an agarose microarray. Following gel extraction and sequencing of the RNA hairpins bound to a ligand of interest, the random nucleotide loop sequences are analyzed using the RNA

Privileged Space Predictor (RNA-PSP) program for statistically enriched motifs by calculating Z-statistics for each motif [400]. Strikingly, when the experimentally determined binding affinities of RNA hairpin loops to a small molecule were plotted against the sum of the Z-statistics for the statistically enriched motifs identified by RNA-PSP included within that specific internal RNA loop sequence, the data points could be fit well to a simple inverse first-order equation ($R^2 = 0.85$) [401]. This method termed Structure-activity relationships through sequencing (StARTS) was then used to successfully predict the binding affinities of various RNA hairpin loops (that were not captured by 2DCS) to the small molecule of interest. Finally, *in silico* folding of all human pri-miRNA sequences in miRBase [402] was performed using the RNAstructure program [403], and all secondary structural elements within the pri-miRNAs were extracted and queried against the 2DCS data for various small molecule compounds using StARTS or RNA-PSP v. 2.0 [357]. This Inforna platform predicted that benzimidazole would inhibit processing of miR-96. Indeed, when tested in primary cells, benzimidazole inhibited miR-96 processing by 90 %. Importantly, benzimidazole inhibition of miR-96 in the MCF7 cancer cell line caused upregulation in the protein levels of *FOXO1* (Forkhead box protein O1), a target of miR-96, and induced apoptosis. This demonstrates the potential of this method for identifying drugs that could treat diseases by intervening with RNA related processes. Finally, an alternative way to modulate miRNA activity has been described previously by Sharp and colleagues who showed that miRNA target sites themselves (with perfect or mismatch complementarity to the miRNA), when overexpressed, could act as “sponges” that titrate away endogenous mature miRNAs and prevent them from degrading their exogenous or native RNA targets [358].

Post-translational regulatory mechanisms

Synthetic biology devices for RNA vaccination need not directly act on RNA but may function at the post-translational level. Post-translational devices are capable of actuating even more rapidly than devices that regulate RNA. An example of such a device is the destabilizing domain (DD) developed by Wandless and colleagues [359]. A DD is comprised of a small-molecule ligand binding domain and a degron domain, which targets proteins for degradation in an ubiquitin and proteasome-dependent manner. When a DD is fused to a protein of interest, the half-life of the protein is

dramatically decreased. However, binding of a ligand to the DD induces a conformational change that masks the degron thus preventing subsequent ubiquitination and degradation. (De)stabilization occurs in a reversible manner, and the stability of the protein can be tuned by adjusting the concentration of the cognate ligand. The first DD developed (referred to here as DDf) was based on the human FKBP12 protein and was stabilized by a synthetic small molecule Shield-1 [359]. When a vaccinia virus harboring a fusion protein between DDf and the cytokine IL-2 was systemically delivered to tumor bearing mice, administration of Shield-1 to the mouse led to stabilization of IL-2 and a reduction in the size of the tumor [360]. Later, DDs that respond to the FDA-approved small molecule drug trimethoprim (TMP) and 4-hydroxytamoxifen (4-OHT; the active metabolite of another FDA-approved drug, tamoxifen citrate) were engineered using E. coli dihydrofolate reductase (DDd) and human estrogen receptor (DDe) [361,362]. Since TMP can traverse the blood-brain barrier, DDd-fluorescent reporter proteins delivered to the brain of a rat using lentiviruses were capable of being stabilized by TMP administration. Thus, there is potential for using this system for clinical applications related to the brain. More recently, using the same FKBP protein, Wandless and colleagues developed a ligand-induced degradation (LID) domain, which operates in the opposite manner as a DD [363]. LIDs induce degradation of a protein by exposing a cryptic degron upon binding of a ligand. DDs and LIDs are useful devices for simple protein (de)stabilization. However, another way to use these domains would be to fuse them to RBPs such as L7Ae, TetR and MS2 to regulate translation in a small molecule dependent manner as proposed later.

Sensor modules

Biological sensor modules sense endogenous or environmental signals such as small molecules, proteins, miRNAs, mRNAs, or enzymatic activity and relay information to other devices within a circuit. Thus, sensor modules are the interfaces between input signals and insulated processing modules of a circuit. For instance, Fussenegger and colleagues have developed sensor modules which use G protein-coupled receptors (GPCRs) to detect small molecules such as dopamine [308] and histamine [309] or changes in the pH [404] and communicate this information to downstream actuation devices through the cAMP signaling pathway. Smolke and colleagues adapted their MS2-CP based splicing modulation device described above to sense the p50 or p65 subunits

of NF-κB or the β-catenin protein of the Wnt signaling pathway [378]. However, since these sensors are connected to processing modules which actuate through transcription of a transgene (for GPCR/cAMP signaling based sensors) or splicing, they cannot be directly integrated into RNA encoded circuits (at least in their current form).

In contrast, one type of device that can be easily embedded into an RNA encoded circuit is a miRNA sensor. The basic unit of a miRNA sensor consists of a miRNA target site inserted into the 3' UTR of an mRNA. Using this simple setup, Naldini and colleagues demonstrated the proof of concept that tissue specific miRNAs such as miR-142-3p could be exploited to suppress gene expression in undesirable cell types using a lentiviral gene therapy vector in mice [364]. More recently, tenOever and colleagues applied the same concept to modulate the host tropism of an influenza A virus [365]. They incorporated into the viral genome a target site for a miRNA (miR-192) that is differentially expressed in different host species so that transmission of a virus would occur in ferrets but be attenuated in mice (or humans, in theory). The concept of using RNAi for complex Boolean logic evaluation was demonstrated by Benenson and colleagues in collaboration with our group [366]. In the study, logic gates were created by incorporating up to five different siRNA target sites into 3' UTRs of two reporter mRNAs or alternatively, by incorporating siRNA target sites into lacI or lacI-KRAB fusion repressor-encoding mRNA(s) which in turn repressed a reporter mRNA. Subsequently, Benenson and colleagues demonstrated that such Boolean logic gates can similarly be implemented in mammalian cells using artificial miRNAs embedded within the introns of genes regulated by transcriptional activators or repressors [367]. Finally, Benenson and colleagues and our group created a miRNA-classifier circuit which "senses" the distinct miRNA expression pattern of certain types of cells and identifies them based on evaluation of the following Boolean logic function: miR-21 AND miR-17/miR-30a AND NOT(miR-141) AND NOT(miR-142-3p) AND NOT(miR-146a) [304]. This was implemented using a combination of six miRNA target sites regulating three distinct ORFs encoding repressors, activators, and reporter or actuator proteins. This circuit was used to distinguish a HeLa cancer cell from a HEK cell and selectively kill the HeLa cell by expression of the pro-apoptotic BAX (Bcl2-associated X protein) gene.

Other types of sensing devices that are compatible with RNA encoded circuits include an mRNA sensor developed by Benenson and colleagues which was based on an

"RNA strand displacement" mechanism [368]. In this device, input mRNA molecules release cryptic antisense strands of siRNAs from "protecting strand" RNAs through strand exchange. This results in the generation of siRNA duplexes which are loaded onto the RISC complex to knock-down downstream target RNAs. This mRNA sensing device was used to create simple Boolean logic evaluators in Drosophila extracts. Saito and colleagues developed an shRNA based protein sensing device which could potentially be integrated into RNA encoded circuits [369]. In the study, structural modeling was used to observe the amount of steric hindrance that would be generated between Dicer and a protein of interest when the terminal loop of the shRNA was replaced by an aptamer which binds the protein of interest. Based on this information, it was possible to predict aptamer configurations that would maximize inhibition of Dicer mediated processing of the shRNA. This design process was used to create a device that senses the levels of the NF-κB p50 subunit in 293FT cells. Finally, Covert and colleagues created synthetic sensors for kinase activity dubbed "kinase translocation reporters" (KTRs) [370]. KTRs have a modular structure which consists of a kinase docking site, a nuclear export signal (NES), and a nuclear localization signal (NLS). Phosphorylation of the NES and NLS moieties of the KTR enhances nuclear export and decreases nuclear localization activities, respectively. Thereby, KTRs sense kinase activity and communicate that information in the form of a nucleocytoplasmic shuttling event. KTRs were successfully engineered for the JNK, p38, ERK, and PKA kinases demonstrating the universality of this approach.

RNA circuits

The RNA devices discussed thus far with single inputs and outputs can be connected with one another to create modules with more complex behavior. A key aspect to consider when connecting devices is their "composability." For instance, in order to directly connect device 1 (which operates in the form of: input 1 -> device 1-> output 1) with device 2 (input 2 -> device 2-> output 2), output 1 of device 1 must be able to become input 2 of device 2. Thus, only devices with compatible inputs/outputs are considered composable. In over a decade, researchers in the field of synthetic biology have used composable devices to create numerous circuit modules including oscillators, toggle switches, and cascades. These modules can be assembled further

into integrated systems with more sophisticated functions.

There are two complementary approaches by which devices can be assembled into modules and modules into systems: the first approach involves the rational matching of parts based on mathematical modeling and the other involves experimental testing of many circuit configurations by screening variations of individual parts. In actuality, gene circuit optimization cannot be accomplished solely by model-based methods and still involves a significant amount of experimental trial and error. While many sophisticated circuit modules and systems have been engineered to date, to our knowledge, none have been encoded entirely on RNA for use in mammalian systems. Although Fussenegger and colleagues used the L7Ae and MS2 devices to create "mammalian biocomputers" which perform programmable calculations based on NOT, AND, N-IMPLY, and XOR logic gates, this was done by combining transcriptional regulation and L7Ae/MS2 based translational repression [318]. In the following section, we propose examples in which RNA devices could be composed into circuits and encoded exclusively on RNA for the purpose of vaccination.

Synthetic gene circuits for "smart vaccination"

Over the years, mRNA and replicating RNA have become well established as platforms for vaccination and immunotherapy (reviewed in [63-67]). RNA based devices such as aptamers or aptazymes have also been used for immunomodulation ([353] and reviewed in [349]), cell specific targeting of antigens [347] and presentation of *de novo* antigens [348]. However, such efforts to improve vaccines/immunotherapies using RNA-based tools have thus far been limited to the use of standalone devices. Here, we propose how RNA-based "smart vaccines" with complex regulatory gene circuits inside may be used to solve unmet needs in this area, highlighting their potential as an enabling technology (Figure 7.2).

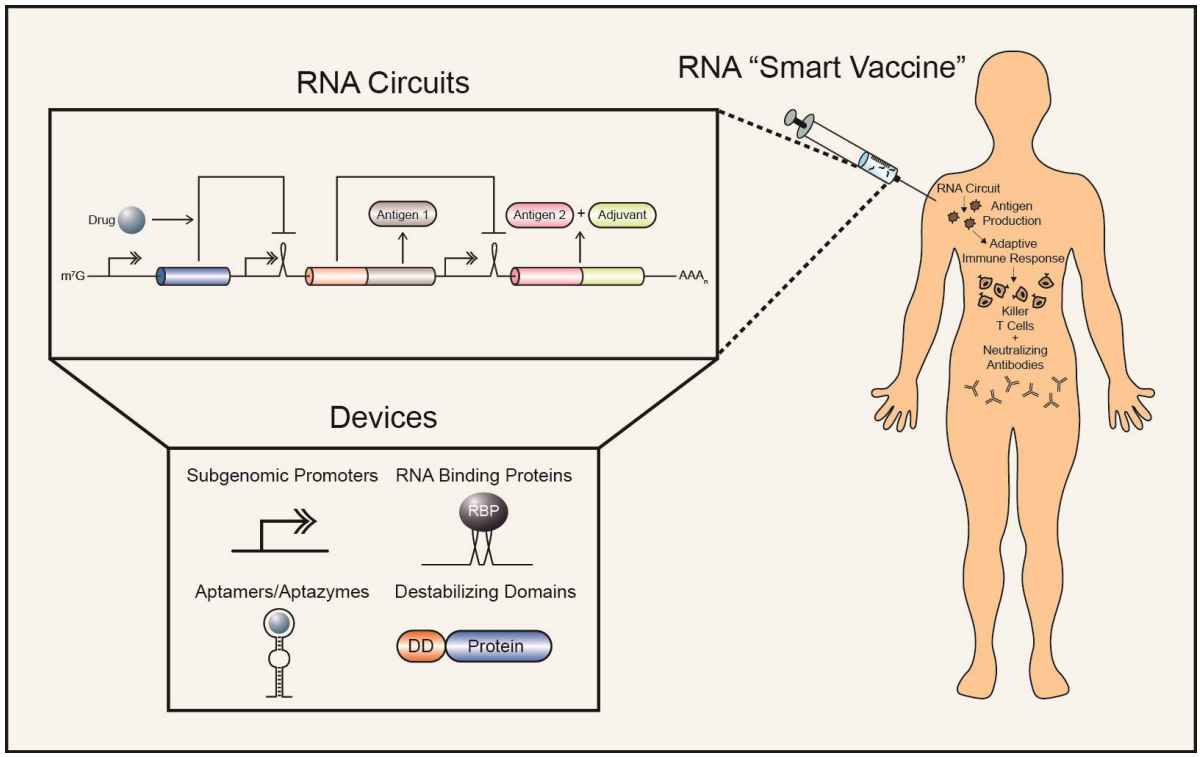


Figure 7.2. The RNA "smart vaccine" paradigm. Composable devices for post-transcriptional gene regulation can be assembled into synthetic gene circuits in the form of RNA. Such RNA circuits may be used to control the expression kinetics of antigens and adjuvants using small molecule drugs to create potent RNA "smart vaccines."

m^7G: 7-methyl-guanosine; AAA_n: poly(A) tail; Gag: group-specific antigen; IL-12: interleukin 12; RBP: RNA binding protein; DD: destabilizing domain.

"One-shot" vaccination

The development of one-shot vaccines that do not require booster shots would be particularly beneficial in communities with limited means of transportation. Chadambuka et al. reported that a significant number of children (~35 %) drop-out from vaccination programs in rural Zimbabwe due to transportation barriers [405]. Here we propose a "smart vaccine" solution to this problem in which prime-boost expression of an antigen can be achieved using a small molecule drug rather than a follow up injection of the antigen. This can be done as shown in Figure 7.3, using a replicon with two subgenomic promoters (SGPs) where one SGP expresses an RNA binding protein (RBP) fused to a DD domain and the other has a motif which binds the RBP upstream

of an antigen of interest. In this circuit, administration of a small molecule drug stabilizes DD-RBP and represses translation of the antigen. Thus, a small molecule can be used to suppress antigen expression, in effect, creating the prime and boost phases of vaccination.

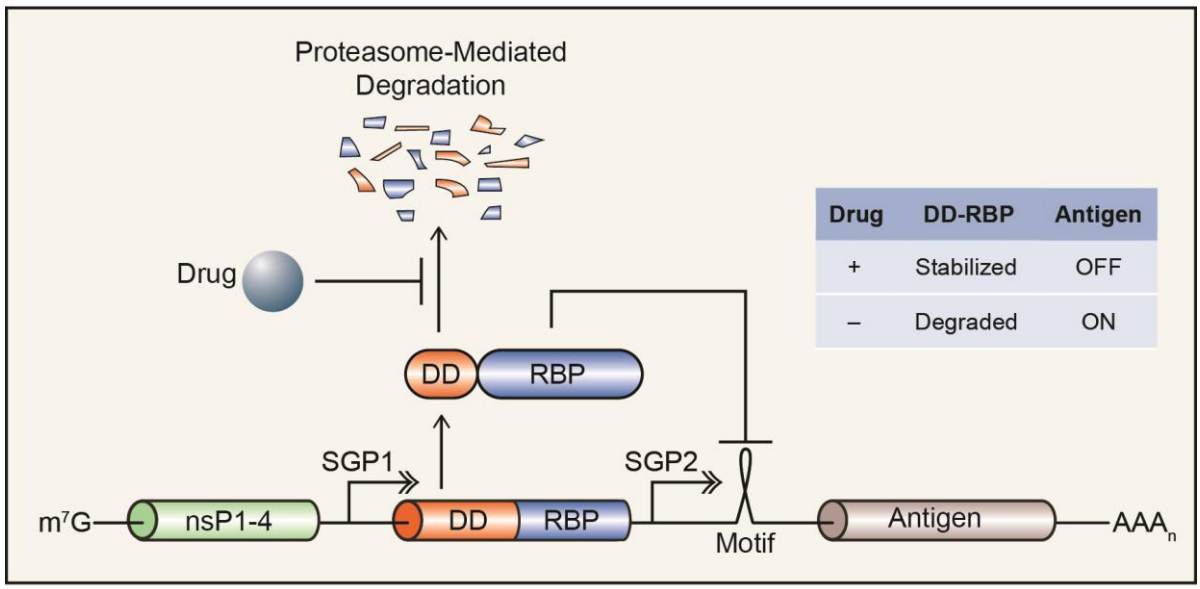
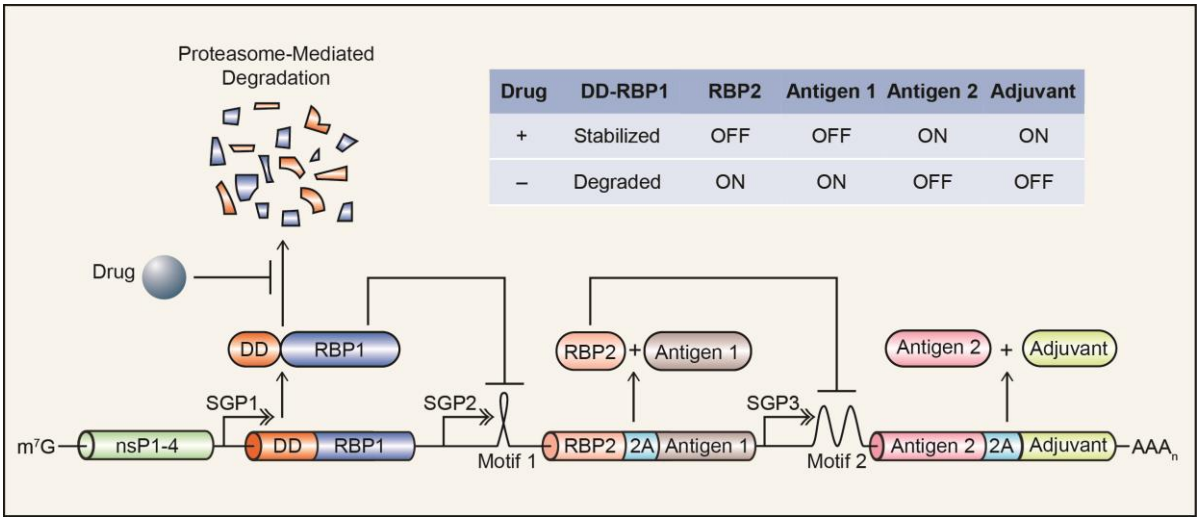


Figure 7.3. One-shot "smart vaccine" with small molecule enabled prime-boost. m^7G: 7-methyl-guanosine; nsP: nonstructural protein; SGP: subgenomic promoter; DD: destabilizing domain; AAA_n: poly(A) tail; TMP: trimethoprim.

Multivalent cancer vaccine

Intratumoral heterogeneity (the presence of many subclones of cancer cells within a tumor that are genetically different from one another) is one of the greatest hurdles in treating cancer. For cancer vaccination, heterogeneity of the tumor and the diverse gene expression pattern of individual cancer cells are a problem since not all cancer cells within a population may be targetable by immunization with a single tumor antigen. Thus, in order to attack and clear a heterogeneous population of cancer cells, it may be necessary to perform vaccination with multiple tumor antigens. However, induction of immune responses against multiple antigens by simultaneous injection/expression of antigens may be difficult for certain combinations of proteins due to "immunodominance". Immunodominance causes CD4+ and/or CD8+ T cells to

preferentially respond to the most immunogenic epitopes and leave other epitopes unattended [107]. Here, we propose a method to overcome this problem by creating a small molecule inducible sequential antigen expression cascade with additional adjuvant pulsing capabilities (Figure 7.4). This circuit is encoded on a replicon with three SGPs: the first SGP expresses DD-RBP1, the second SGP contains a binding motif for RBP1 and expresses RBP2 connected to Antigen 1 via a 2A "ribosome skipping" peptide [406] which enables co-translational separation of the antigen from RBP2, and the last SGP contains a binding motif for RBP2 followed by Antigen 2 fused to an adjuvant by a 2A peptide. In the absence of a DD-stabilizing small molecule drug, DD-RBP1 is degraded and allows expression of RBP2 and Antigen 1 (Antigen 2 is repressed by RBP2). Upon administration of the drug, DD-RBP1 is stabilized and represses RBP2-2A-Antigen 1 thereby allowing expression of Antigen 2 and the adjuvant. Here, an additional benefit of the cascade is that the potent adjuvant, which may be highly toxic when delivered systemically, is only expressed when the DD stabilizing drug is administered to the body.



Drug	DD-RBP1	RBP2	Antigen 1	Antigen 2	Adjuvant
+	Stabilized	OFF	OFF	ON	ON
–	Degraded	ON	ON	OFF	OFF

Figure 7.4. Multivalent cancer "smart vaccine" with small molecule induced antigen cascading and adjuvant pulsing.

m^7G: 7-methyl-guanosine; nsP: nonstructural protein; SGP: subgenomic promoter; DD: destabilizing domain; AAA_n: poly(A) tail.

CONCLUSIONS

Roughly a decade and a half has passed since the first synthetic gene circuits created in *E. coli* launched a field of research that has now come to be known as synthetic biology. By creating and cataloging standardized genetic parts and devices that can be assembled into modules and systems for reprogramming living organisms, synthetic biologists have transformed the field of biotechnology into a rigorous engineering discipline. In particular, mammalian synthetic biology has been experiencing rapid expansion over the past few years with successful implementations of genetic circuits in cell culture as well as in model organisms. However, we believe that mammalian synthetic biology is in fact at a crossroads. Ultimately, therapeutic gene circuit applications must graduate from the academic proof-of-concept phase and find a place in the real-world. Will mammalian synthetic biologists be able to identify society's pressing needs and deliver gene circuit solutions that can withstand the public field test? Efforts aimed in the right direction could indeed make this happen. One absolute requirement for this would be that synthetic gene circuits for therapeutic purposes be safe. Encoding genetic circuits on RNA using the emerging modified or replicating RNA-based platforms rather than DNA-based platforms will greatly facilitate this transition. Building circuits that do not trigger unnecessary innate or adaptive immune responses against regulatory components of the circuit will also be necessary. An area of particular interest for synthetic biology applications using RNA is vaccination. The proven success of antigen-encoding RNA in eliciting protective immunity combined with the desire to control the dynamics of antigen/adjuvant expression to maximize an immune response makes vaccination an optimal target for RNA circuit applications. With the ever-expanding list of parts and devices for RNA regulation and our rapidly-developing ability to rationally compose devices into regulatory circuits, it is only a matter of time before RNA "smart vaccines" with programmable antigen/adjuvant circuits inside will deliver a solution to a real-world problem: the development of potent vaccines to protect humanity from the threats of infectious diseases. Both the selection of the best delivery method and the optimization of the mRNA molecule itself will be key to achieving these goals. Thus, mRNA delivery and optimization is the main topic of the experimental section of this dissertation.

APPENDIX B

Supplementary data from Chapter 3

Supplementary data from Chapter 3

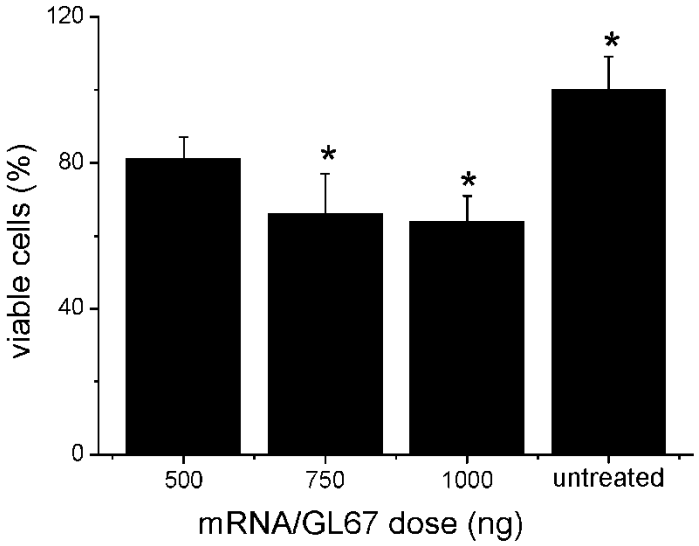


Figure SB.1. Impact of the dose of the mRNA/GL67 complexes on the cell viability. A549 cells were plated in 24-well plates and transfected with three different doses of mRNA/GL67 complexes (ratio 2), i.e. 500 ng, 750 ng and 1000 ng. Cell viability was assessed 24 hours after adding the complexes with an MTT assay. The impact of the amount of the complexes on the cellular viability was compared to untreated cells (set at 100 % viability). The results are presented as the mean ± SD (n=5) and considered significant, if $p<0.05$ compared to a dose of 500 ng/well (ANOVA).

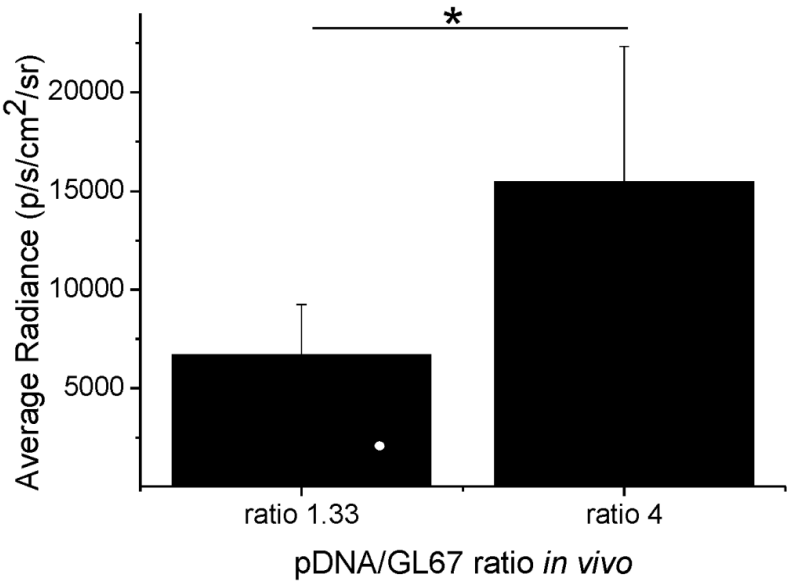


Figure SB.2. Comparison of the average bioluminescence after intranasal administration of pDNA/GL67 complexes prepared at a ratio 1.33 or 4. 80 µg of

pDNA/GL67 complexes prepared at a ratio 1.33 (n=4) or 4 (n=5) were given to anesthetized mice via intranasal instillation. 24 hours after administration the mice were imaged and the bioluminescent light was recorded via *in vivo* bioluminescence imaging. The data were obtained after subtracting the average bioluminescence signal of untreated mice (background) from the measured signals. The results are presented as the mean ± SD (* if p<0.05, independent-samples *t*-test).

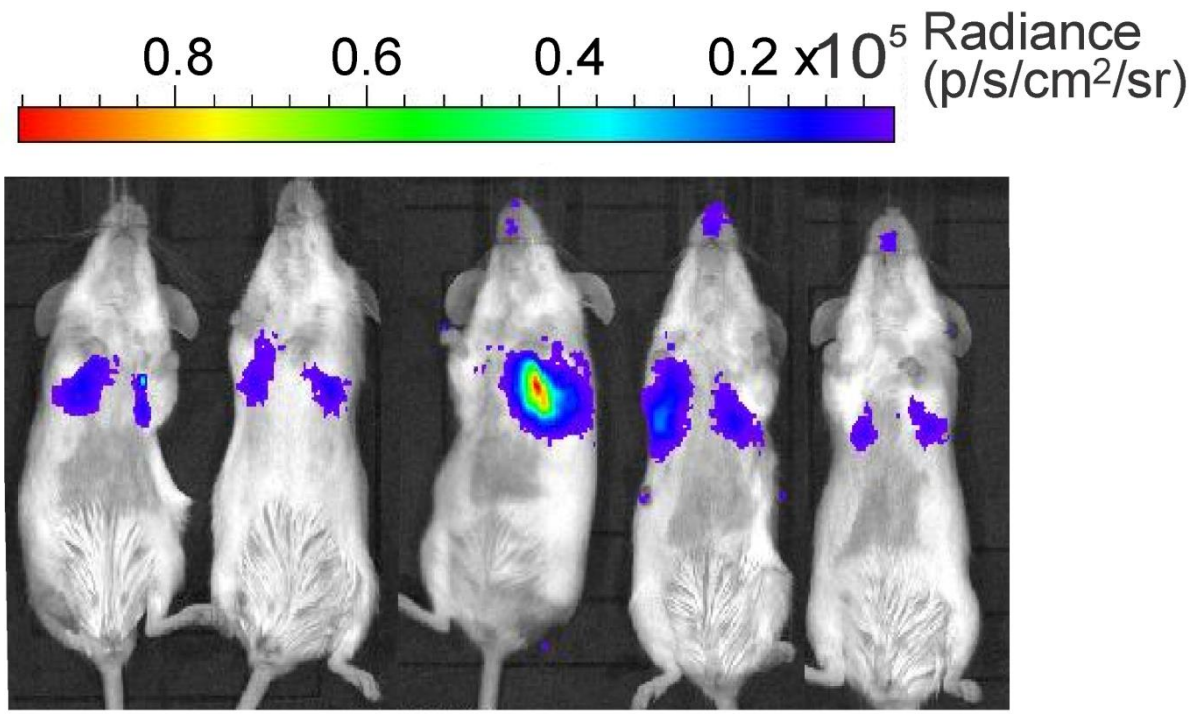


Figure SB.3. Visualization of the *in vivo* luciferase production after intrapulmonary delivery of pDNA/GL67 complexes prepared at ratio 4. Mice were anesthetized and 80 µg of pDNA/GL67 complexes prepared at ratio 4 were administered intranasally. 24 hours after instillation the mice were imaged and the bioluminescent light was recorded via *in vivo* bioluminescence imaging.

APPENDIX C

Supplementary data from Chapter 4

Supplementary data from Chapter 4

Supplementary Data CS.1. qPCR array for A549 cells

A549										
No.	Symbol	Gene Name	P-value	Fold Change	Control 1	Control 2	Control 3	Exp 1	Exp 2	Exp 3
1	CXCL10	chemokine (C-X-C motif) ligand 10	**0.000043**	**4147.88895**	**40.0**	**40.0**	**40.0**	**26.6**	**26.2**	**26.6**
2	IFNA	interferon. alpha	**0.000046**	**653.224346**	**40.0**	**40.0**	**40.0**	**29.2**	**29.2**	**30.0**
3	CCL4	chemokine (C-C motif) ligand 4	**0.000126**	**99.565208**	**40.0**	**40.0**	**40.0**	**32.3**	**32.2**	**32.9**
4	IRF7	interferon regulatory factor 7	**0.000135**	**140.165855**	**33.7**	**33.0**	**33.0**	**24.8**	**25.2**	**25.7**
5	CCL3L1	chemokine (C-C motif) ligand 3-like 1	**0.000164**	**83.408367**	**40.0**	**40.0**	**40.0**	**32.7**	**32.6**	**32.9**
6	IFNB	interferon. beta	**0.000184**	**6421.932725**	**37.2**	**38.1**	**37.1**	**23.1**	**22.8**	**23.6**
7	PTX3	pentraxin 3. long	**0.000208**	**67.379841**	**40.0**	**40.0**	**40.0**	**32.9**	**32.8**	**33.6**
8	SOCS1	suppressor of cytokine signaling 1	**0.000232**	**42.290321**	**34.8**	**33.6**	**33.7**	**27.8**	**27.7**	**28.2**
9	CCL5	chemokine (C-C motif) ligand 5	**0.000494**	**8921.590778**	**37.0**	**35.5**	**37.1**	**21.8**	**21.6**	**21.6**
10	TLR3	toll-like receptor 3	**0.000543**	**29.666660**	**34.4**	**34.0**	**33.5**	**28.7**	**28.1**	**28.4**
11	CIITA	class II. major histocompatibility complex. transactivator	**0.000721**	**53.852692**	**40.0**	**40.0**	**40.0**	**34.1**	**32.9**	**33.4**
12	CXCL9	chemokine (C-X-C motif) ligand 9	**0.000946**	**57.867356**	**40.0**	**40.0**	**40.0**	**33.6**	**32.5**	**34.1**
13	STAT1	signal transducer and activator of transcription 1	**0.001335**	**8.006063**	**27.6**	**26.6**	**26.1**	**23.5**	**23.3**	**23.1**
14	IRAK2	interleukin-1 receptor-associated kinase 2	**0.002049**	**4.767101**	**30.8**	**30.2**	**30.0**	**27.9**	**27.4**	**27.8**
15	IRF1	interferon regulatory factor 1	**0.002101**	**22.515945**	**34.0**	**32.9**	**32.5**	**27.6**	**28.4**	**28.2**
16	MYD88	myeloid differentiation primary response gene (88)	**0.002460**	**5.499716**	**31.1**	**30.1**	**29.7**	**27.4**	**27.4**	**27.5**
17	IL6	interleukin 6 (interferon. beta 2)	**0.002549**	**498.079107**	**38.5**	**40.0**	**40.0**	**29.1**	**28.5**	**29.5**

A549										
No.	Symbol	Gene Name	P-value	Fold Change	Control 1	Control 2	Control 3	Exp 1	Exp 2	Exp 3
18	TNF	tumor necrosis factor	0.002767	26.218345	40.0	40.0	38.0	34.7	33.4	33.5
19	CXCL11	chemokine (C-X-C motif) ligand 11	0.002870	6214.771533	40.0	37.1	40.0	24.5	24.0	24.6
20	IL12A	interleukin 12A (natural killer cell stimulatory factor 1. cytotoxic lymphocyte maturation factor 1. p35)	0.003389	4.568301	31.9	31.4	31.3	29.2	28.8	28.9
21	CASP1	caspase 1. apoptosis-related cysteine peptidase (interleukin 1. beta. convertase)	0.004914	384.312942	40.0	37.7	36.3	28.2	27.8	27.9
22	CD14	CD14 molecule	0.006266	-3.179524	31.0	30.9	30.8	32.6	32.6	32.6
23	TLR5	toll-like receptor 5	0.006867	4.352891	38.4	37.7	37.7	35.9	35.6	35.0
24	MUC1	mucin 1. cell surface associated	0.011806	3.838419	34.7	34.8	34.1	32.3	32.6	31.9
25	RIPK2	receptor-interacting serine-threonine kinase 2	0.011983	2.171515	32.9	31.8	31.8	31.1	30.7	30.6
26	TRAFD1	TRAF-type zinc finger domain containing 1	0.012497	2.895765	29.0	28.6	28.3	27.1	26.6	26.7
27	JUN	jun proto-oncogene	0.013613	2.801582	27.4	26.8	26.6	25.6	24.9	25.0
28	IL12B	interleukin 12B (natural killer cell stimulatory factor 2. cytotoxic lymphocyte maturation factor 2. p40)	0.014185	6.074074	40.0	40.0	38.3	36.6	36.0	36.3
29	TICAM1	toll-like receptor adaptor molecule 1	0.015880	2.711301	31.3	30.7	30.9	29.6	29.0	29.1
30	CNPY4	canopy 4 homolog	0.028357	-2.168024	30.0	29.5	29.2	31.1	30.6	30.4
31	TLR1	toll-like receptor 1	0.033384	2.072206	31.8	30.9	31.0	30.0	30.2	29.7

A549										
No.	Symbol	Gene Name	P-value	Fold Change	Control 1	Control 2	Control 3	Exp 1	Exp 2	Exp 3
32	SYK	spleen tyrosine kinase	0.050492	-3.174189	33.3	32.8	32.6	35.8	34.7	33.9
33	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.066514	1.554369	28.7	28.0	28.0	27.7	27.2	27.3
34	CASP8	caspase 8. apoptosis-related cysteine peptidase	0.067561	1.565698	27.6	26.8	26.6	26.6	25.9	26.0
35	SARM1	sterile alpha and TIR motif containing 1	0.073721	-1.576050	27.6	27.0	26.7	27.9	27.5	27.7
36	HMGB1	high mobility group box 1	0.074022	-1.512455	22.6	22.0	21.8	22.8	22.5	22.7
37	TRAF6	TNF receptor-associated factor 6	0.085535	1.432247	31.2	30.3	30.4	30.3	29.7	29.8
38	CSK	c-src tyrosine kinase	0.105771	-1.618020	31.3	30.9	30.7	31.2	31.9	31.9
39	MAPK14	mitogen-activated protein kinase 14	0.152713	-1.377923	26.7	25.5	25.2	26.5	25.8	26.2
40	CD44	CD44 molecule (Indian blood group)	0.153439	1.286669	27.7	26.8	26.5	26.5	26.4	26.5
41	IFNAR1	interferon (alpha. beta and omega) receptor 1	0.162236	-1418806	30.6	29.7	29.8	30.8	30.6	30.1
42	CNPY3	canopy 3 homolog	0.164228	-1.417384	28.7	28.2	27.9	29.0	28.5	28.6
43	IRF3	interferon regulatory factor 3	0.212622	1.190294	30.0	29.6	29.6	29.2	29.4	29.6
44	RELA	v-rel reticuloendotheliosis viral oncogene homolog A	0.217995	1.276449	26.3	25.6	25.5	25.8	25.0	25.1
45	IRAK1	interleukin-1 receptor-associated kinase 1	0.227287	-1.361350	24.7	24.4	23.9	25.1	24.8	24.3
46	UNC93B1	unc-93 homolog B1	0.227421	1.162799	27.0	26.2	25.9	26.3	25.8	25.9
47	RAC1	ras-related C3 botulinum toxin substrate 1 (rho family. small GTP binding protein Rac1)	0.251392	-1.140776	22.9	22.5	22.6	22.6	22.8	22.9

A549										
No.	Symbol	Gene Name	P-value	Fold Change	Control 1	Control 2	Control 3	Exp 1	Exp 2	Exp 3
48	CHUK	conserved helix-loop-helix ubiquitous kinase	0.253131	-1.145409	29.8	28.6	28.4	29.3	28.7	29.1
49	NR3C1	nuclear receptor subfamily 3. group C. member 1 (glucocorticoid receptor)	0.262862	1.274806	27.6	26.0	26.3	26.5	26.0	26.0
50	PYCARD	PYD and CARD domain containing	0.275406	-1.290971	35.1	34.6	34.5	34.7	35.4	35.1
51	MAPK8	mitogen-activated protein kinase 8	0.283591	-1.224079	28.8	28.1	28.0	28.8	28.3	28.4
52	HSP90B1	heat shock protein 90kDa beta (Grp94). member 1	0.297204	1.163799	27.1	26.5	26.0	26.2	26.2	26.1
53	Hs18s	18S rRNA	0.326030	-1.232284	10.1	9.7	9.7	10.2	10.0	9.9
54	IL1B	interleukin 1. beta	0.337096	2.561520	36.9	36.9	36.2	37.0	34.1	35.6
55	IRAK4	interleukin-1 receptor-associated kinase 4	0.338157	1.145744	31.4	30.8	30.7	30.9	30.6	30.4
56	AKT1	v-akt murine thymoma viral oncogene homolog 1	0.342013	-1.222811	25.6	25.1	24.7	25.7	25.1	25.2
57	TIRAP	toll-interleukin 1 receptor (TIR) domain containing adaptor protein	0.364523	-1.232323	28.8	28.5	28.4	29.1	28.6	28.6
58	MAP3K7	mitogen-activated protein kinase kinase kinase 7	0.383591	-1.153736	28.2	27.6	27.3	28.0	27.6	27.8
59	TICAM2	toll-like receptor adaptor molecule 2	0.396223	-1.083714	26.1	25.4	25.2	25.7	25.4	25.6
60	TOLLIP	toll interacting protein	0.400273	-1.080157	30.9	30.1	29.8	30.3	30.0	30.6
61	MAP3K7IP1	TGF-beta activated kinase 1/MAP3K7 binding protein 1	0.401889	-1.123047	30.2	29.5	29.4	30.1	29.6	29.6
62	TLR4	toll-like receptor 4	0.404190	1.513449	33.7	34.3	33.1	33.3	32.1	33.7
63	TLR6	toll-like receptor 6	0.406092	-1.129900	29.3	28.7	28.6	29.2	28.7	28.9

A549										
No.	Symbol	Gene Name	P-value	Fold Change	Control 1	Control 2	Control 3	Exp 1	Exp 2	Exp 3
64	SIGIRR	single immunoglobulin and toll-interleukin 1 receptor (TIR) domain	0.406340	-1.042493	25.6	25.0	25.0	25.2	25.0	25.2
65	BCL3	B-cell CLL/lymphoma 3	0.410684	-1.084639	27.5	27.0	26.7	27.1	26.9	27.2
66	CYLD	cylindromatosis (turban tumor syndrome)	0.440739	1.032445	32.6	31.7	32.0	32.3	31.7	31.9
67	DOK1	docking protein 1. 62kDa (downstream of tyrosine kinase 1)	0.453150	1.036219	32.7	32.2	32.3	32.4	31.9	32.3
68	TRAF3	TNF receptor-associated factor 3	0.453613	-1.066725	26.0	25.6	25.4	25.8	25.4	25.7
69	IRF5	interferon regulatory factor 5	0.477624	-1.091689	30.8	30.6	30.3	30.7	30.5	30.5
70	FADD	Fas (TNFRSF6)-associated via death domain	0.483384	1.087297	31.2	30.6	30.1	30.6	30.7	30.1
71	TBK1	TANK-binding kinase 1	0.489963	1.050130	27.0	26.6	26.4	26.9	26.2	26.3
72	HSPD1	heat shock 60kDa protein 1 (chaperonin)	0.492120	1.015128	24.8	24.2	23.7	24.5	24.0	23.8
73	TLR7	toll-like receptor 7	0.496414	-1.613721	34.2	36.7	34.8	40.0	34.6	34.8
74	TLR10	toll-like receptor 10	0.600137	1.309166	36.7	37.0	36.7	35.5	36.7	37.2
75	LY96	lymphocyte antigen 96	NS	-1.094964	40.0	40.0	40.0	40.0	40.0	40.0
76	TLR8	toll-like receptor 8	NS	-1.094964	40.0	40.0	40.0	40.0	40.0	40.0
77	TREM2	triggering receptor expressed on myeloid cells 2	NS	-1.094964	40.0	40.0	40.0	40.0	40.0	40.0
78	IRAK3	interleukin-1 receptor-associated kinase 3	NS	-1.094964	40.0	40.0	40.0	40.0	40.0	40.0
79	TLR2	toll-like receptor 2	NS	-1.094964	40.0	40.0	40.0	40.0	40.0	40.0
80	LY86	lymphocyte antigen 86	NS	-1.094964	40.0	40.0	40.0	40.0	40.0	40.0

A549										
No.	Symbol	Gene Name	P-value	Fold Change	Control 1	Control 2	Control 3	Exp 1	Exp 2	Exp 3
81	CD80	CD80 molecule	NS	-1.094964	40.0	40.0	40.0	40.0	40.0	40.0
82	HSGenomic	Genomic DNA control	NS	-1.094964	40.0	40.0	40.0	40.0	40.0	40.0
83	BTK	Bruton agammaglobulinemia tyrosine kinase	NS	1.579370	40.0	40.0	40.0	40.0	38.2	40.0
84	NOX4	NADPH oxidase 4	NS	1.791224	40.0	40.0	40.0	40.0	40.0	38.1
85	CD36	CD36 molecule (thrombospondin receptor)	NS	2.377010	40.0	40.0	40.0	40.0	38.2	38.2
86	IFNG	interferon. gamma	NS	1.969557	40.0	40.0	40.0	40.0	40.0	37.9
87	TLR9	toll-like receptor 9	NS	2.994596	40.0	40.0	40.0	40.0	37.4	38.3
88	CARD9	caspase recruitment domain family. member 9	NS	-1.265991	40.0	40.0	37.6	40.0	40.0	38.3
89	CD86	CD86 molecule	NS	3.977903	40.0	40.0	40.0	40.0	37.6	37.2
90	MAL	mal. T-cell differentiation protein	NS	4.869186	40.0	40.0	40.0	37.2	37.6	38.1
91	LBP	lipopolysaccharide binding protein	NS	1.046578	40.0	37.0	40.0	40.0	37.8	37.7
92	TREM1	triggering receptor expressed on myeloid cells 1	NS	5.453114	40.0	40.0	40.0	40.0	37.8	36.4
93	IL10	interleukin 10	NS	1.146065	40.0	37.3	40.0	40.0	36.9	40.0
94	CD180	CD180 molecule	NS	1.845353	40.0	40.0	37.9	36.9	40.0	40.0
95	PELI1	pellino homolog 1	NS	-2.311180	40.0	36.3	36.3	40.0	38.0	37.5
96	ATF3	activating transcription factor 3	NS	8.004811	40.0	40.0	40.0	37.5	40.0	35.8
97	Tlr11	toll-like receptor 11								
98	Tlr12	toll-like receptor 12								
99	Tlr13	toll-like receptor 13								

LEGEND:

Control
GPR Normalizer
Genomic Contamination Levels
None The data is not compromised by the presence of genomic DNA,

In **bold** - significant changes

Supplementary Data CS.2. qPCR array for LA-4 cells

LA4										
No.	**Symbol**	**Gene Name**	**P-value**	**Fold Change**	**Control 1**	**Control 2**	**Control 3**	**Exp 1**	**Exp 2**	**Exp 3**
1	CXCL10	chemokine (C-X-C motif) ligand 10	**0.01302**	**10.24525**	**24.2**	**25.7**	**25.1**	**21.6**	**21.2**	**21.5**
2	IFNA	interferon. alpha	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
3	CCL4	chemokine (C-C motif) ligand 4	**0.02674**	**22.93084**	**28.7**	**31.9**	**30.5**	**25.0**	**25.7**	**25.4**
4	IRF7	interferon regulatory factor 7	**0.02632**	**20.22088**	**28.3**	**31.3**	**29.9**	**25.3**	**24.3**	**25.1**
5	IFNB	interferon. beta	**0.03388**	**22.73265**	**30.3**	**31.3**	**32.3**	**26.0**	**27.4**	**27.5**
7	PTX3	pentraxin 3. long	0.33584	1.13037	24.6	24.5	24.9	25.2	24.4	23.4
8	SOCS1	suppressor of cytokine signaling 1	**0.02377**	**3.64825**	**29.7**	**29.0**	**28.8**	**27.6**	**26.8**	**27.1**
9	CCL5	chemokine (C-C motif) ligand 5	**0.02073**	**13.07951**	**25.7**	**27.2**	**26.7**	**22.4**	**22.8**	**23.1**
10	TLR3	toll-like receptor 3	**0.00578**	**10.75578**	**30.6**	**30.9**	**29.9**	**27.4**	**26.4**	**26.7**
11	CIITA	class II. major histocompatibility complex. transactivator	NS	1.17025	40.0	40.0	38.3	40.0	38.1	38.3
12	CXCL9	chemokine (C-X-C motif) ligand 9	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
13	STAT1	signal transducer and activator of transcription 1	**0.01390**	**5.95756**	**24.3**	**25.4**	**25.2**	**22.7**	**21.8**	**22.0**
15	IRF1	interferon regulatory factor 1	**0.03500**	**4.37083**	**29.2**	**30.6**	**30.4**	**28.2**	**27.2**	**27.6**
16	MYD88	myeloid differentiation primary response gene (88)	**0.03955**	**2.23052**	**27.3**	**27.7**	**28.1**	**27.0**	**26.1**	**26.0**
17	IL6	interleukin 6 (interferon. beta 2)	**0.01800**	**15.30461**	**28.1**	**29.3**	**29.1**	**24.5**	**25.0**	**25.2**
18	TNF	tumor necrosis factor	**0.01054**	**21.09833**	**39.05**	**40.0**	**38.1**	**34.5**	**34.5**	**33.8**
19	CXCL11	chemokine (C-X-C motif) ligand 11	**0.01166**	**16.78909**	**26.9**	**28.0**	**29.1**	**23.6**	**23.8**	**23.7**

LA4										
No.	**Symbol**	**Gene Name**	**P-value**	**Fold Change**	**Control 1**	**Control 2**	**Control 3**	**Exp 1**	**Exp 2**	**Exp 3**
20	IL12A	interleukin 12A (natural killer cell stimulatory factor 1. cytotoxic lymphocyte maturation factor 1. p35)	NS	-1.69148	40.0	37.4	37.7	38.2	40.0	40.0
21	CASP1	caspase 1. apoptosis-related cysteine peptidase (interleukin 1. beta. convertase)	0.24295	1.82246	37.1	35.0	36.5	36.90	34.1	34.5
22	CD14	CD14 molecule	0.41695	-1.09289	30.6	30.6	30.9	32.0	29.9	30.3
23	TLR5	toll-like receptor 5	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
24	MUC1	mucin 1. cell surface associated	0.34636	-1.19550	32.2	31.4	32.4	33.6	31.6	31.2
25	RIPK2	receptor-interacting serine-threonine kinase 2	0.23490	1.18268	25.0	24.9	25.3	25.7	24.2	24.1
26	TRAFD1	TRAF-type zinc finger domain containing 1	**0.01721**	**4.30988**	**25.0**	**25.5**	**25.5**	**23.5**	**22.8**	**23.0**
27	JUN	jun proto-oncogene	0.10667	1.88638	34.7	35.1	36.0	34.8	33.7	33.8
28	IL12B	interleukin 12B (natural killer cell stimulatory factor 2. cytotoxic lymphocyte maturation factor 2. p40)	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
29	TICAM1	toll-like receptor adaptor molecule 1	0.17010	1.34264	28.6	28.6	29.0	28.8	28.1	27.5
30	CNPY4	canopy 4 homolog	0.10609	-1.48839	24.1	23.7	24.3	25.7	24.0	23.7
31	TLR1	toll-like receptor 1	0.19641	-1.27916	28.6	27.9	28.4	29.6	28.3	27.6
32	SYK	spleen tyrosine kinase	NS	2.32043	40.0	37.9	38.2	38.4	37.8	35.7
33	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.29680	1.13086	26.9	25.9	26.4	26.9	25.8	25.4

LA4										
No.	Symbol	Gene Name	P-value	Fold Change	Control 1	Control 2	Control 3	Exp 1	Exp 2	Exp 3
34	CASP8	caspase 8. apoptosis-related cysteine peptidase	0.65924	-1.03613	34.4	32.0	31.6	33.9	31.7	31.2
35	SARM1	sterile alpha and TIR motif containing 1	0.12157	-1.84926	32.6	32.1	32.7	34.9	33.0	32.0
36	HMGB1	high mobility group box 1	0.19159	-1.22410	20.3	19.8	20.3	21.3	19.9	19.6
37	TRAF6	TNF receptor-associated factor 6	0.30643	1.12400	27.7	27.5	27.8	28.0	27.2	26.8
38	CSK	c-src tyrosine kinase	0.22816	1.17157	29.5	29.5	29.7	30.1	29.0	28.4
39	MAPK14	mitogen-activated protein kinase 14	0.44544	-1.08026	25.3	24.4	25.5	25.6	25.2	24.2
40	CD44	CD44 molecule (Indian blood group)	0.36242	1.00317	31.3	30.7	31.1	31.6	30.9	30.1
41	IFNAR1	interferon (alpha. beta and omega) receptor 1	0.32739	1.04252	27.9	27.8	27.8	28.4	27.5	26.9
42	CNPY3	canopy 3 homolog	0.21233	-1.23671	25.9	25.8	26.4	27.2	25.8	25.5
43	IRF3	interferon regulatory factor 3	0.27443	1.07177	27.7	27.4	28.1	28.5	27.3	26.6
44	RELA	v-rel reticuloendotheliosis viral oncogene homolog A	0.39707	-1.12974	24.1	24.4	25.0	25.1	24.2	24.1
45	IRAK1	interleukin-1 receptor-associated kinase 1	0.20242	-1.24061	24.2	23.9	24.7	25.4	24.2	23.6
46	UNC93B1	unc-93 homolog B1	NS	-1.17942	40.0	37.2	37.4	40.0	38.1	36.4
47	RAC1	ras-related C3 botulinum toxin substrate 1 (rho family. small GTP binding protein Rac1)	NS	-1.48774	40.0	34.9	35.2	38.2	35.2	35.5
48	CHUK	conserved helix-loop-helix ubiquitous kinase	0.13579	-1.64484	26.8	26.5	26.7	28.8	26.6	26.5
49	NR3C1	nuclear receptor subfamily 3. group C.	0.35574	1.11999	27.4	26.1	26.9	27.4	26.0	25.9

LA4										
No.	Symbol	Gene Name	P-value	Fold Change	Control 1	Control 2	Control 3	Exp 1	Exp 2	Exp 3
		member 1 (glucocorticoid receptor)								
50	PYCARD	PYD and CARD domain containing	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
51	MAPK8	mitogen-activated protein kinase 8	0.26933	-1.02663	26.5	26.1	26.8	27.3	26.1	25.6
52	HSP90B1	heat shock protein 90kDa beta (Grp94). member 1	0.12283	-1.72694	19.5	19.2	19.7	21.7	19.6	19.2
53	Hs18s	18S rRNA	0.31860	-1.02378	11.4	10.9	11.1	11.7	10.8	10.5
54	IL1B	interleukin 1. beta	NS	1.56046	40.0	37.5	40.00	37.4	40.0	40.0
55	IRAK4	interleukin-1 receptor-associated kinase 4	0.24774	-1.14888	28.1	28.1	28.2	29.2	27.9	27.4
56	AKT1	v-akt murine thymoma viral oncogene homolog 1	0.26122	-1.12185	26.7	26.3	26.5	27.4	26.2	25.9
57	TIRAP	toll-interleukin 1 receptor (TIR) domain containing adaptor protein	0.65909	-1.29428	35.2	32.6	32.8	34.7	32.5	33.5
58	MAP3K7	mitogen-activated protein kinase kinase kinase 7	0.06666	-1.35195	29.2	29.0	29.3	30.5	29.3	28.8
59	TICAM2	toll-like receptor adaptor molecule 2	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
60	TOLLIP	toll interacting protein	0.24799	1.07048	24.7	24.4	24.9	25.3	24.2	23.7
61	MAP3K7 IP1	TGF-beta activated kinase 1/MAP3K7 binding protein 1	0.48254	1.04828	28.4	28.4	28.0	28.7	28.3	27.1
62	TLR4	toll-like receptor 4	0.45281	-1.18225	35.1	33.8	34.0	35.8	33.8	33.5
63	TLR6	toll-like receptor 6	0.49642	1.01439	32.3	31.1	31.6	33.3	30.9	30.5
64	SIGIRR	single immunoglobulin and toll-interleukin 1								

LA4										
No.	**Symbol**	**Gene Name**	**P-value**	**Fold Change**	**Control 1**	**Control 2**	**Control 3**	**Exp 1**	**Exp 2**	**Exp 3**
		receptor (TIR) domain								
65	BCL3	B-cell CLL/lymphoma 3	0.44535	1.26524	37.0	37.2	35.5	37.1	35.9	34.6
66	CYLD	cylindromatosis (turban tumor syndrome)	0.23020	-1.30825	31.8	30.9	31.3	32.8	31.1	30.8
67	DOK1	docking protein 1. 62kDa (downstream of tyrosine kinase 1)	0.25166	-1.10126	26.0	25.9	26.3	26.9	25.9	25.3
68	TRAF3	TNF receptor-associated factor 3	0.28324	-1.12688	29.2	28.4	28.8	29.8	28.5	28.1
69	IRF5	interferon regulatory factor 5	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
70	FADD	Fas (TNFRSF6)-associated via death domain	0.27937	1.26277	27.7	27.9	28.5	28.2	27.6	26.7
71	TBK1	TANK-binding kinase 1	0.25343	1.19439	28.2	27.4	27.7	28.2	27.0	26.8
72	HSPD1	heat shock 60kDa protein 1 (chaperonin)	0.25343	1.19439	28.2	27.4	27.7	28.2	27.0	26.8
73	TLR7	toll-like receptor 7	0.50306	1.12634	26.6	25.9	26.3	26.2	25.9	25.9
74	TLR10	toll-like receptor 10								
75	LY96	lymphocyte antigen 96	0.22188	1.17581	27.7	27.6	27.9	28.3	27.0	26.7
76	TLR8	toll-like receptor 8	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
77	TREM2	triggering receptor expressed on myeloid cells 2	NS	1.85219	40.0	40.0	40.0	40.0	40.0	37.5
78	IRAK3	interleukin-1 receptor-associated kinase 3	0.28027	2.11947	36.2	36.3	40.0	36.5	35.2	35.0
79	TLR2	toll-like receptor 2	**0.01611**	**2.47109**	**36.7**	**36.8**	**36.7**	**36.1**	**35.3**	**34.4**
80	LY86	lymphocyte antigen 86	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
81	CD80	CD80 molecule	0.19687	1.28231	32.0	31.6	31.6	31.9	31.1	30.6

LA4										
No.	Symbol	Gene Name	P-value	Fold Change	Control 1	Control 2	Control 3	Exp 1	Exp 2	Exp 3
82	HSGenomic	Genomic DNA control	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
83	BTK	Bruton agammaglobulinemia tyrosine kinase	0.06353	-3.22192	36.3	36.2	36.5	40.0	36.9	37.6
84	NOX4	NADPH oxidase 4	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
85	CD36	CD36 molecule (thrombospondin receptor)	NS	-4.53509	40.0	38.2	36.9	40.0	40.0	40.0
86	IFNG	interferon. gamma	NS	-1.59922	40.0	36.7	36.5	40.0	36.5	37.6
87	TLR9	toll-like receptor 9	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
88	CARD9	caspase recruitment domain family. member 9	0.78415	2.08796	37.0	37.3	37.2	40.0	36.8	34.0
89	CD86	CD86 molecule	NS	1.12659	40.0	36.7	36.9	38.3	36.9	36.0
90	MAL	mal. T-cell differentiation protein	NS	-1.62038	40.0	40.0	37.3	40.0	37.9	40.0
91	LBP	lipopolysaccharide binding protein	NS	1.59003	40.0	37.0	37.0	37.6	36.2	36.4
92	TREM1	triggering receptor expressed on myeloid cells 1	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
93	IL10	interleukin 10	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
94	CD180	CD180 molecule	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
95	PELI1	pellino homolog 1	0.33138	1.15791	27.2	26.8	27.2	27.3	26.5	26.3
96	ATF3	activating transcription factor 3	0.06130	4.21534	27.1	27.3	27.6	25.1	25.5	25.1
97	Tlr11	toll-like receptor 11	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
98	Tlr12	toll-like receptor 12	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0
99	Tlr13	toll-like receptor 13	NS	-1.03215	40.0	40.0	40.0	40.0	40.0	40.0

LEGEND:

Control
GPR Normalizer
Genomic Contamination Levels
None The data is not compromised by the presence of genomic DNA,

In **bold** - significant changes

APPENDIX D

Supplementary data from Chapter 5

Supplementary data from Chapter 5

SUPPLEMENTARY DATA

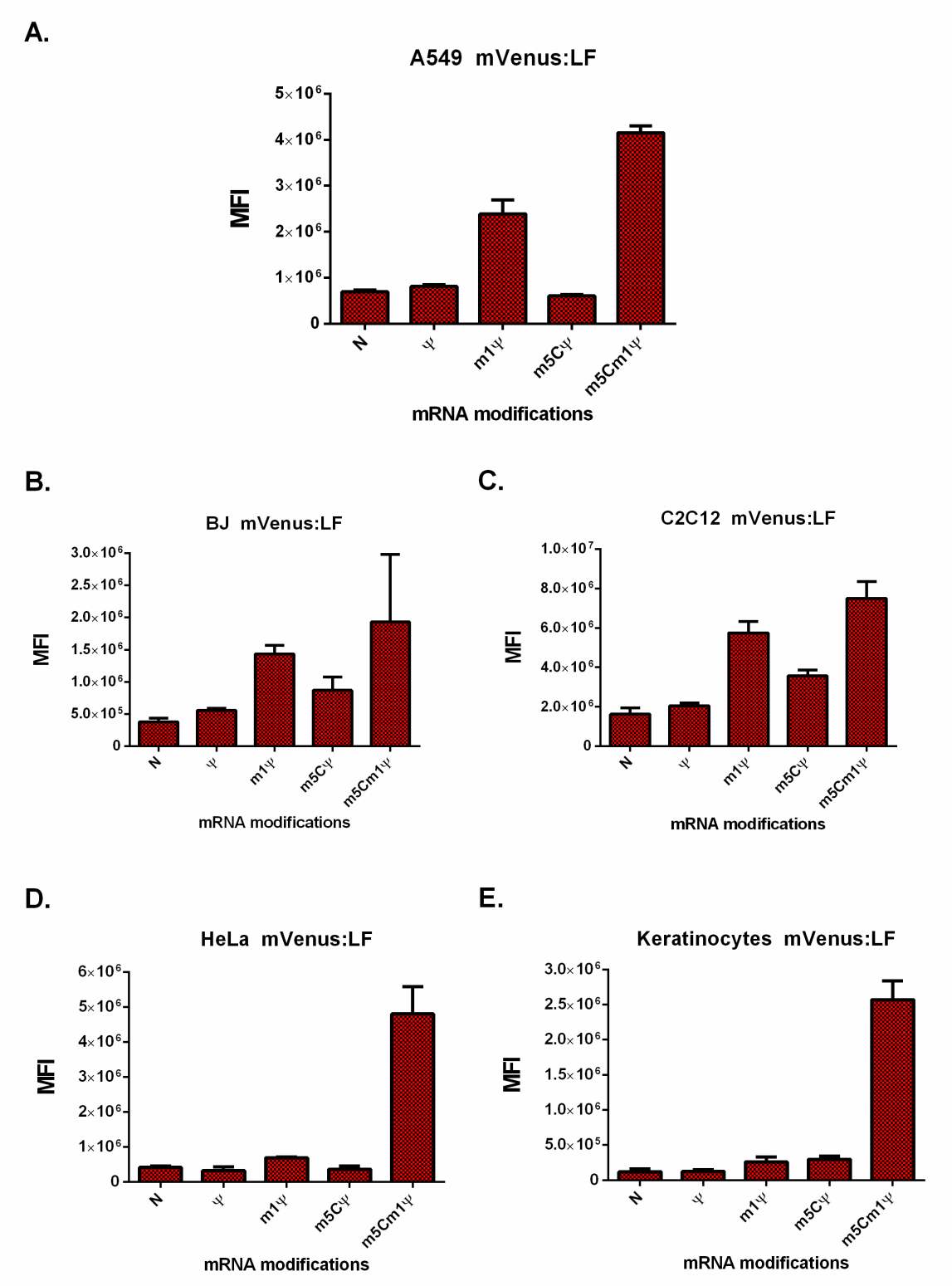


Figure SD.1. Comparison of mVenus expression level 24 hours post-transfection from

unmodified and modified mRNA in various cell lines A) human lung adenocarcinoma – A549, B) human foreskin fibroblasts – BJ, C) murine myoblasts – C2C12, D) human cervical epithelial cells – Hela and E) human primary keratinocytes (neonatal). The results are presented as the mean ± SD (n=3), statistical analysis ANOVA in the Table SD 2.

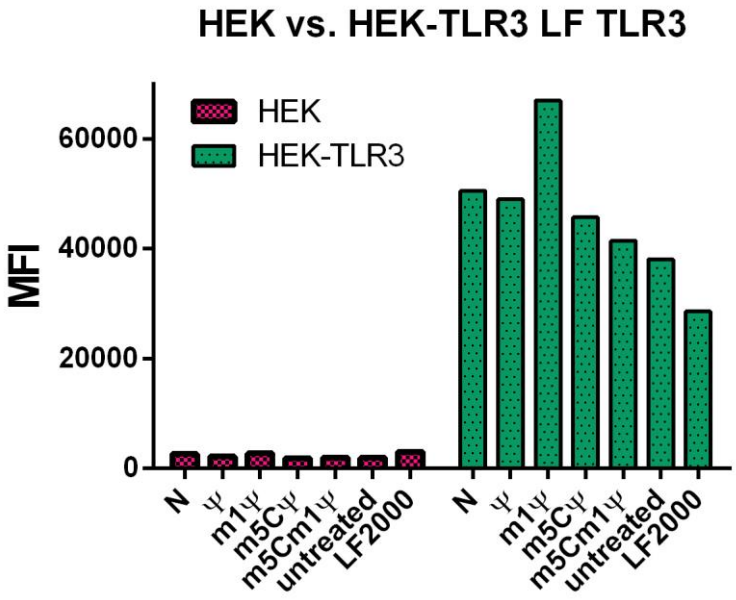


Figure SD.2. flow cytometry data TLR3 and RIG-I ICS

Luciferase							
ANOVA summary							
F	597235	80.16	4.627	33.99	8.105	3.844	9.437
P value	< 0.0001	< 0.0001	0.0225	< 0.0001	0.0047	0.0383	0.004
P value summary	****	****	*	****	**	*	**
Tukey's multiple comparisons test	A549	BJ	C2C12	HeLa	Keratinocytes	HEK	HEK-TLR3
N vs. Ψ	****	ns	ns	ns	ns	ns	ns
N vs. m1Ψ	****	***	*	ns	ns	*	ns
N vs. m5CΨ	****	***	ns	ns	ns	ns	ns
N vs. m5Cm1Ψ	****	****	ns	****	*	ns	**
Ψ vs. m1Ψ	****	**	ns	ns	ns	ns	ns
Ψ vs. m5CΨ	****	**	ns	ns	ns	ns	ns
Ψ vs. m5Cm1Ψ	****	****	ns	****	**	ns	**
m1Ψ vs. m5CΨ	****	ns	ns	ns	ns	ns	ns
m1Ψ vs. m5Cm1Ψ	****	****	ns	****	*	ns	ns
m5CΨ vs. m5Cm1Ψ	****	****	ns	****	*	ns	ns

Table SD.1. ANOVA statistics followed by *ad hoc* Tukey's multiple comparisons test after transfection of luciferase-encoding mRNA/Lipofectamine 2000 in A549, BJ, C2C12, HeLa and primary Keratinocytes cells (based on Figure 5.2); * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (ANOVA).

mVenus					
F	299.4	4.649	73.34	113.5	215.9
P value	< 0.0001	0.0261	< 0.0001	< 0.0001	< 0.0001
P value summary	****	*	****	****	****
Tukey's multiple comparisons test	A549	BJ	C2C12	HeLa	Keratinocytes
N vs. Ψ	ns	ns	ns	ns	ns
N vs. m1Ψ	****	ns	****	ns	ns
N vs. m5CΨ	ns	ns	**	ns	ns
N vs. m5Cm1Ψ	****	*	****	****	****
Ψ vs. m1Ψ	****	ns	****	ns	ns
Ψ vs. m5CΨ	ns	ns	*	ns	ns
Ψ vs. m5Cm1Ψ	****	ns	****	****	****
m1Ψ vs. m5CΨ	****	ns	**	ns	ns
m1Ψ vs. m5Cm1Ψ	****	ns	*	****	****
m5CΨ vs. m5Cm1Ψ	****	ns	****	****	****

Table SD.2. ANOVA statistics followed by *ad hoc* Tukey's multiple comparisons test after transfection of mVenus-encoding mRNA/Lipofectamine 2000 in A549, BJ, C2C12, HeLa and primary Keratinocytes cells (based on Figure SD.1); * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (ANOVA).

mRNA/Lipofectamine 2000						mRNA + ELECTROPORATION				
F	40.15	11.02	68.64	205.5	1.923	76.01	8.795	0.388	7.861	0.2034
P value	< 0.0001	0.0011	< 0.0001	< 0.0001	0.2	< 0.0001	0.0026	0.8126	0.0039	0.9308
P value summary	****	**	****	****	ns	****	**	ns	**	ns
Tukey's multiple comparisons test	A549	BJ	C2C12	HeLa	Keratinocytes	A549	BJ	C2C12	HeLa	Keratinocytes
N vs. Ψ	ns	ns	ns	**	ns	ns	ns	ns	ns	ns
N vs. m1Ψ	*	ns	****	****	ns	****	ns	ns	ns	ns
N vs. m5CΨ	***	ns	****	****	ns	****	**	ns	**	ns
N vs. m5Cm1Ψ	****	**	****	****	ns	****	*	ns	**	ns
Ψ vs. m1Ψ	**	ns	****	****	ns	****	ns	ns	ns	ns
Ψ vs. m5CΨ	****	ns	****	****	ns	****	ns	ns	ns	ns
Ψ vs. m5Cm1Ψ	****	**	****	****	ns	****	ns	ns	ns	ns
m1Ψ vs. m5CΨ	*	ns	ns	***	ns	ns	*	ns	ns	ns
m1Ψ vs. m5Cm1Ψ	**	**	*	**	ns	ns	ns	ns	ns	ns
m5CΨ vs. m5Cm1Ψ	ns	*	ns	ns	ns	ns	ns	ns	ns	ns

Table SD.3. One-way ANOVA statistics followed by *ad hoc* Tukey's multiple comparisons test based on results in Figure 5.4 depicting viability of mammalian cells 24 hours after transfection of unmodified or modified (Ψ, m1Ψ, m5C/Ψ, m5C/m1Ψ) mRNAs determined using an MTT assay.

	size (intensity mean: d. nm)			zeta potential (mV)		
	Mean	SD	N	Mean	SD	N
N/LF2K	715.47	154.21	3	-12.87	1.31	3
Ψ/LF2K	683.63	47.19	3	-21.73	1.40	3
mΨ/LF2K	733.60	29.96	3	-19.60	0.62	3
m5CΨ/LF2K	816.30	57.93	3	-21.30	0.87	3
m5CmΨ/LF2K	600.17	53.80	3	-18.97	2.01	3

Table SD.4. Characterization of complexes – Zeta Potential.

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- Ryunosuke Satoro

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As I was preparing for this defense for the past months, while exploring new challenges

as a member of the Innovation Team in Omega Pharma, I would like to thank also my new colleagues, especially **Barbara**, for your mental support, understanding and smooth onboarding me in a team! I also greatly appreciate debates with my **Omega colleagues** on the subject: “choosing a dress for a PhD defense”!

I would probably give up “everything” hundred times, if I didn’t have support from my Polish friends – **Magda, Monika, Kalina, Olga, Asia, Gosia, Ania**. Thank you very much for your love in beautiful but also more difficult times. You are my family here in Belgium, I love you and I want you to stay in my life forever.

I thank my parents, **Irena and Ryszard**, and a grandfather, **Eugeniusz**; thank you for your unconditional love and belief now, in the past and the future. Now that I am a parent myself, I understand more… *Dziękuję Wam za Waszą wiarę we mnie i bezwarunkową miłość. Teraz, jak sama jestem rodzicem, rozumiem już więcej…* Also I thank my brother, **Kamil**, for the reality checks ☺ and his respect.

Also, my love goes to my daughter **Adriana** for being cute and amazing and so smart and just perfect ☺ Ty, kochanie moje, jesteś moim największym szczęściem. Dzięki Tobie mamusia się uśmiecha I żadne mRNA mamusi z równowagi nie wyprowadza. ☺ Thank you **Nick** for her and for these years together in Belgium. En ook bedankt aan **Annie**, mijn schoonmoeder, voor om Adriana te zorgen, toen ik langer moest werken…

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Thank you!

VI. Curriculum vitae

Oliwia Andries

Education and Work Experience

2015 – present – Junior Corporate Innovation Manager - Dermatherapeutics,
Omega Pharma Corporate, Nazareth, Belgium

2008 – 2014 – PhD candidate in the Laboratory of Gene Therapy in Veterinary Medicine Sciences at Ghent University (Doctoral School of Life Sciences and Medicine)
Project: Evaluation of non-viral delivery systems and immunogenic properties of mRNA- and self-replicating RNA-based vaccine against cancer.

2003 - 2008 - Master of Science (Biotechnology)
University of Life Sciences, Poznań, Poland (2003-2006)
University of Ghent, Belgium (2006-2008; Exchange Student following the MaNaMa Programme in Molecular Biotechnology)

Core Competences: immune assays, vector-based protein expression and evaluation, cell culture assays, *in vivo* (mouse) experiments: injections, dissections, bioluminescent imaging (IVIS), electroporation, drug delivery, lipofection.

Research Experience

2008 – Present: PhD candidate in the Laboratory of Gene Therapy in Veterinary Medicine Sciences in joint collaboration with Ghent Research Group in Nanomedicines, Laboratory of General Biochemistry and Physical Pharmacy in Faculty of Pharmacy at Ghent University (Doctoral School of Life Sciences and Medicine)
Project: N*on-viral delivery systems and immunogenic properties of mRNA- and self-replicating RNA-based immunotherapeutics against cancer and infectious diseases.*
Supervision: Dr. Niek Sanders

- **Visiting Graduate Student at Ragon Institute of MGH, MIT and Harvard and Synthetic Biology Center at MIT in Cambridge, MA, USA**

June 2013 – December 2014: supported by FWO mobility grant and DARPA (Defense Advanced Research Projects Agency) PROTECT (Prophylactic Options to Environmental and Contagious Threats)

Project: *Development and evaluation of self-replicating and non-replicating mRNA for passive immunoprophylaxis against infectious diseases*.
Supervision: Dr. Galit Alter, Dr. Tasuku Kitada

2008 – Biopharmacy course at Ghent University, Faculty of Pharmacy

2003 – 2008: Master of Science (MS - Biotechnology)

- University of Ghent, Belgium (2006-2008; Exchange Student following the MaNaMa Program in Molecular Medical Biotechnology): *summa cum laude*

- **Master Thesis performed in the Protein Service Facility in VIB, Inflammation Research Center, Zwijnaarde**

March 2007 – January 2008: supported by European Union Erasmus Programme.
Project: *Production of novel recombinant antibody manifolds in Pichia pastoris.*
Supervision: Dr. Vladimir Kaigorodov, Dr. Nico Mertens

- **Research Project in the Laboratory of Prof. Dr. Jenny Rusinova in a VIB, Department of Plant Systems Biology, Zwijnaarde**

September 2006 – March 2007: supported by European Union Erasmus Programme.
Project: *Evaluation of core cell cycle proteins interactions with cyclins D by Bimolecular Fluorescence Complementation (BiFC) in Arabidopsis thaliana.*
Supervision: Dr. Jenny Rusinova, Dr. Joanna Boruc

- University of Life Sciences, Poznań, Poland (2003-2006): *summa cum laude*

Research Related Skills

- Experienced in cell culture, laboratory mouse experiments, gene transfections, drug delivery, flow cytometry, fluorescence and confocal microscopy,

PCR, qPCR, molecular cloning, *in vitro* transcription, immunological assays: ELISA, ELISPOT, intracellular cytokine staining

- Presentation skills developed through frequent data reporting in group meetings (inside and outside of lab) and experience gained by presentaing at national and international symposia
- Project Management skills developed through management of my PhD, Master theses of Pharmacy Students as well as temporally leading a collaboration project between the Ragon Institute and MIT during 7-month stay in Cambridge, MA, USA
- FELASA C-certificate – Laboratory animal sciences
- Reviewer for Journal of Controlled Release (since 2012)

Transferable Skills

1. Quality Research Skills – 2008-2009; UGent,
2. Project Management – 2008-2009; UGent,
3. Personal Effectiveness – 2008-2009; UGent,
4. Technology Transfer and Entrepreneurship; 2014; VIB.

Communication Skills

1. Networking Skills – 2012-2013, UGent,
2. Advanced Academic English: Conference Skills – 2008-2009; UGent
3. Language skills

	Understanding		Speaking		Writing
	Listening	Reading	Spoken Interaction	Spoken Production	
Polish	C2	C2	C2	C2	C2
English	C2	C2	C1	C1	C1
Dutch	B2	B2	B2	B2	B1
German	B1	B1	A1	A2	A2

Research Publications

INTERNATIONAL PEER REVIEWED PUBLICATIONS:

1. O. Andries*, T. Kitada*, N. N. Sanders, R. Weiss; Synthetic biology devices and circuits for RNA-based “smart vaccines”: a propositional review. Accepted to Expert Review of Vaccines (SPECIAL FOCUS | RNA-Based Vaccines). IF 4.217, 2014
2. M. De Filette, S. Chabierski, O. Andries, S. Ulbert and N. N. Sanders, T cell epitope mapping of the E-protein of West Nile virus in BALB/c mice. Accepted to PlosOne, IF 3.73, 2014
3. O. Andries, M. De Filette, S. C. De Smedt, J. Demeester, M. Van Poucke, L. Peelman, N. N. Sanders; Innate immune response and programmed cell death following carrier-mediated delivery of unmodified mRNA to respiratory cells. J Control Release. IF 6.499, 2013
4. O. Andries, M. De Filette, J. Rejman, S. C De Smedt, J. Demeester, M. Van Poucke, L. Peelman, C. Peleman, T. Lahoutte, N. N Sanders; Comparison of the Gene Transfer Efficiency of mRNA/GL67 and pDNA/GL67 Complexes in Respiratory Cells. Molecular Pharmaceutics. IF 4.78, 2012
5. Tavernier, G., Andries, O., Demeester, J., Sanders, N. N., De Smedt, S. C., and Rejman, J. mRNA as gene therapeutic: How to control protein expression. J Control Release. IF 7.16, 2011

*Co-First Authors

ORAL PRESENTATIONS (presenting author):

1. Oliwia Andries, Marina De Filette, Stefaan C. De Smedt, Jo Demeester, Mario Van Poucke, Luc Peelman, Niek N. Sanders

Innate immune response and programmed cell death following carrier-mediated delivery of mRNA to respiratory cells. Seminar on Inflammation and Vaccination 2012 (Merelbeke, Belgium)

2. Oliwia Andries, Cindy Peleman, Mario Van Poucke, Luc Peelman, Tony Lahoutte, Jo Demeester, Stefaan De Smedt, Niek N. Sanders. **Immunogenic potential of mRNA for genetic vaccination via pulmonary delivery**. Forum of Pharmaceutical Sciences 2011 (Spa, Belgium)

POSTER PRESENTATIONS (presenting author):

1. Oliwia Andries, Marina De Filette, Stefaan C. De Smedt, Jo Demeester, Mario Van Poucke, Luc Peelman, Niek N. Sanders, **Innate immune response and programmed cell death following carrier-mediated delivery of mRNA to respiratory cells.** Cancer Immunotherapy and Immunomonitoring Conference 2013 (Krakow, Poland) [Abstract of a poster]
2. Oliwia Andries, Marina De Filette, Stefaan C. De Smedt, Jo Demeester, Mario Van Poucke, Luc Peelman, Niek N. Sanders, **Immunostimulatory properties of unmodified mRNA in gene-based vaccination.** Immunotherapies & Cancer Vaccines Conference 2012, (Brussels, Belgium)
3. Oliwia Andries, Marina De Filette, Stefaan C. De Smedt, Jo Demeester, Mario Van Poucke, Luc Peelman, Niek N. Sanders, **Immunostimulatory properties of unmodified mRNA in gene-based vaccination.** Biopharmacy day 2012, (Utrecht, The Netherlands)
4. Oliwia Andries, Cindy Peleman, Mario Van Poucke, Luc Peelman, Tony Lahoutte, Jo Demeester, Stefaan De Smedt, Niek N. Sanders. **Immunostimulatory properties of mRNA in gene-based vaccination**. Gene Vaccination in Cancer 2011 (Ascoli Piceno, Italy)
5. Oliwia Andries, Cindy Peleman, Mario Van Poucke, Luc Peelman, Tony Lahoutte, Jo Demeester, Stefaan De Smedt, Niek N. Sanders. **Immunogenic potential of mRNA for genetic vaccination via pulmonary delivery.** Gene-Based Vaccines, 2010 (Cannes, France)
6. Oliwia Andries, Joanna Rejman, Cindy Peleman, Tony Lahoutte, Stefaan De Smedt, Jo Demeester, Niek N. Sanders. **Pulmonary delivery of mRNA: *in vitro* and *in vivo* evaluation**. The 3rd International CDTM Symposium "Cellular Delivery of Therapeutic Macromolecules", 2010 (Cardiff, UK). ***Drug Discovery Today.***
7. Oliwia Andries, Joanna Rejman, Cindy Peleman, Stefaan De Smedt, Jo Demeester, Luc Peelman, Tony Lahoutte, Niek N. Sanders. **Pulmonary delivery of mRNA: *in vitro* and *in vivo* evaluation**. The American Society Gene and Cell Therapy (ASGCT) 13th Annual Meeting, 2010 (Washington, DC, USA). *Molecular Therapy*.
8. Oliwia Najder, Joanna Rejman, Stefaan De Smedt, Jo Demeester, Mario Van Poucke, Luc Peelman, Niek N. Sanders. **Pulmonary delivery of mRNA: *in vitro* and *in vivo* evaluation.** 2nd European Summer School in Nanomedicines, 2009 (Cascais, Portugal)
9. Oliwia Najder, Joanna Rejman, Stefaan De Smedt, Jo Demeester, Mario Van Poucke, Luc Peelman, Alex Van Zeveren, Niek N. Sanders. **Delivery of mRNA via non-viral carriers. 75 Years of Veterinary Medicine at Ghent University, 2009 (Merelbeke, Belgium)**

Funding & Awards

2014 Emmanuel van der Schueren award for finalization of doctoral thesis (21.680 euros)

2013 FWO Long Term International Mobility Travel Grant

2009-2014 FWO Aspirant Fellowship

2009 Travel Scholarship for attendance of Summer School on Nanomedicines, Lisbon, Portugal.

Mentoring

1. Eline Tommelein: "Physicochemical characterization and expression efficiency of different liposome:mRNA complexes for mRNA vaccination" 2009-2010
2. Nils Jacobs: "Efficiency of mRNA and pDNA transfection *in vitro* and *in vivo* by means of square wave electroporation". 2010-2011
3. Ramona Maxim: "*In vitro* evaluation of Poly(I:C)/Dotap:Dope lipoplexes as anti-cancer agent". 2010-2011
4. Liza Heeze: "*In vitro* evaluation of innate immune responses after mRNA electroporation into BMDCs and respiratory cells." 2012-2013
5. Aaron Edwards: "Self-replicating RNA for vectored immunoprophylaxis." Ragon Institute of MGH, MIT and Harvard, 2013