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mRNA-based Vaccines to Elicit CD8$^+$ T Cell Immunity

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List of Abbreviations

A	APC	Antigen presenting cell
	ARCA	Anti-reverse Cap analogue
B	BCR	B cell receptor
	BMDC	Bone Marrow derived dendritic cells
C	CCR7	CC-chemokine receptor 7
	CD	Cluster of differentiation
	CFSE	5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester
	CMV	CytoMegalo Virus
	CpG	Cytosine-phosphate-guanosine
	CPP	Cell penetrating peptides
	CTL	Cytolytic T lymphocytes
	CXCR	Chemocine receptor
	Cy3/5 label	Cyanine 3/5 label
D	DC	Dendritic cells
	DLS	Dynamic light scattering
	DNA	Desoxy ribo nucleic acid
	DOPE	1,2 dioleoyl-sn-glycero-3-phosphoethanolamine
	DOTAP	1,2 dioleoyl-3-trimethylammonium-propane
	DTT	Dithiothreitol
E	eGFP	Eukaryotic green fluorescent protein
	eIF	Eukaryotic initiation factor
	EP	Electroporation
F	FDA	Food and drug administration
	Flt3L	FMS-like tyrosine kinase 3 ligand
	fSPT	Fluorescence single particle tracking
G	GAG	Glycosaminoglycanen
	GC	Germinal centre

	GM-CSF	Granulocyte- macrophage colony-stimulating factor
	gp100	premelanosome protein
H	HIV-1	Human immunodefficienty virus-1
	HLA	Human Leukocyte antigen
	HSV	Herpes-simplex virus
I	IFN	Interferon
	IFNAR1/2	Type I interferon receptor subunit 1/2
	IgG/M	Immunoglobulin G/M
	IL	Interleukine
	IRF	Interferon regulator factor
	ISGF3	Interferon-stimulated gene factor 3
	ISRE	IFNs-stimulated response elements
	IVT	In vitro transcribed
J	JAK	Janus kinase
K	KLRG1	Killer cell lectin-like receptor G1
L	LCMV	Lymphocyte Chorio Mengitis Virus
	LN	Lymph node
	LPS	Lipopolysaccharide
M	Melan-A	Melanine-A
	MHC	Major histocompatibility complex
	miRNA	Micro RNA
	MPL	Monophosphoryl lipid A
	mRNA	messenger RNA
	MyD88	Myeloid differentiation factor-88
N	N/P	Nitrogen/Phosphate
	NF-KB	Nuclear factor –K-light chain enhancer of activated B cells
	NLR	Nodd-like receptor
	NLRP	Nucleic-binding oligomerization domain-like receptor

	NLS	Nuleus localization sequence
O	ODN	Oligodinucleotide
	ORF	Open reading frame
	OT-I	Transgenes T cell receptor for ovalbumine peptide 257-264
	OT-II	transgenes T cell receptor for ovalbumine peptide 323-339
	OVA	Ovalbumin
P	PBMC	Peripheral blood mononuclear cells
	PBS	Phosphate buffer saline
	pDC	plasmacyoid dendritic cell
	pDNA	plasmid DNA
	PEG	PEGylation
	PRR	Pattern recognition receptor
R	RBC	Red Blood cells
	RNA	Ribo nucleic acid
S	SD	Standard deviation
	SEM	Standard error of means
	siRNA	small interference RNA
	ssRNA	Single stranded RNA
	STAT	Signal transducers and activators of transcription
	SV40	Simian Virus 40
T	TAT	Transactivator of transcription
	TBK1	TRAF family-associated NF-kB activator (TANK)-binding kinase-1
	TCR	T cell receptor
	T_{EM}	Transmission electron microscopy
	T_{FH}	T follicular helper cell
	Th1/2	T helper1/2
	TLR	Toll like receptor
	TNF	Tumor necrosis factor
	TRAF	TNF receptor-associated factor
	TRIF	TIR domain-containing adaptor inducing IFN-β
	tRNA	Transfer RNA

U	UTR	Untranslated regions
V	VLP	Virus like particles
W	WHO	World Health Organization
	ψ-UTP	Pseudo Uridine Triphosphate
	5' Met-CTP	5' Methyl Cytidine Triphosphate

SUMMARY

Recently, vaccines based on messenger RNA were validated in (pre)clinical studies as prophylactic vaccines against infectious diseases as well as immunotherapeutics to treat cancer. The great potential of mRNA vaccines is based on the capacity to elicit strong cytotoxic $CD8^+$ T lymphocyte (CTL) responses against infected cells or cancer cells. The successful developments of new techniques to formulate mRNA into nanoparticles as well as the favourable safety profile of mRNA, contributed to the current great medical interest in mRNA vaccines.

Our research group used to study mRNA DOTAP/DOPE-based vaccines to elicit T cell responses against the HIV-1 antigen. During this work, vaccine-evoked type I interferons (IFNs) were addressed to have a negative impact on the mRNA vaccine efficacy. In a first section of this thesis we assessed whether type I IFNs influenced the anti-tumoral activity of the vaccine in B16 tumor model. We showed that upon prophylactic and therapeutic immunization, the induction of type I IFNs hampered vaccine-evoked tumor protection. Therefore, the primary aim of this dissertation was to address the mechanism of the inhibiting type I IFN effects and how vaccine-induced type I IFN responses could be evaded or eliminated. To this end, different strategies were tested at the level of mRNA encoding antigens as well as at the level of the vaccine carrier.

In a first endeavour, we evaluated whether type I IFN induction, due to innate immune activation, could be evaded using chemically modified mRNA. Modified mRNA is originally designed for gene therapy approaches as they are considered to bind less effective to cellular RNA sensors. However, no significant weaker IFNβ induction was measured upon the injection of modified mRNA DOTAP/DOPE lipoplexes compared to unmodified mRNA lipoplexes. In line with these results, no positive impact on the capacity of mRNA lipoplexes to elicit cytotoxic $CD8^+$ T cell responses was observed.

In a following part of this thesis, we validated a new peptide-mediated formulation of mRNA, based on the interaction between cell penetrating peptides (CPPs) and mRNA antigens. In this study we complexed mRNA to the amphipathic peptide RALA in order to form RALA mRNA nanocomplexes. RALA has a pH-dependent lytic activity, allowing to translocate the mRNA from the endosomal compartments into the cytosol. This translocation process is considered to be crucial for the processing and presentation of antigens by MHC-I molecules to $CD8^+$ T cells. Injecting RALA mRNA nanocomplexes resulted in far stronger CTL responses compared with the standard DOTAP/DOPE formulation. Furthermore, we addressed that the immunogenicity of modified mRNA RALA nanocomplexes is completely independent of type I IFNs. Further

mechanistic research proved that the immunogenic character of the modified mRNA RALA nanocomplexes is associated with the pH-dependent amphipathic character of RALA.

In a last section of this dissertation, we aimed to evaluate whether RNA can function as a Th1-skewing adjuvant for protein vaccines. To this end, we formulated non-coding RNA into PEGylated polymer-based nanoparticles. This polymer-based formulation resulted in improved RNA protection and enhanced RNA targeting to lymph node dendritic cells when compared to unformulated RNA or RNA complexed to cationic liposomes. Most importantly, we presented the RNA polymer-based nanoparticles as a potent novel adjuvant for the eliciting of effective cytotoxic $CD8^+$ T cells responses against co-delivered protein antigens.

We hope that the new realizations obtained by this doctoral research concerning the use of RNA as antigen-encoding device or as an adjuvants component opened new opportunities for future medical approaches in the field of vaccinology.

SAMENVATTING

Recent werden vaccins gebaseerd op boodschapper RNA (mRNA) gevalideerd in (pre)klinische studies enerzijds voor hun toepassing als profylactische vaccins gericht tegen infectieziekten en anderzijds als immunotherapeutische behandeling van kanker. Het potentieel van mRNA vaccinatie binnen deze twee onderzoeksgebieden berust op zijn capaciteit om sterke cytotoxische $CD8^+$ T lymfocyt (CTL) antwoorden te induceren, gericht tegen geïnfecteerde cellen of kankercellen. Het succesvol onderzoek naar nieuwe strategieën voor het formuleren van mRNA-gecodeerde antigenen in nanopartikels alsook het hoog veiligheidsprofiel van mRNA hebben bijgedragen tot de huidige medische interesse in mRNA-gebaseerde vaccins.

Binnen de onderzoeksgroep werd DOTAP/DOPE-gemedieerde mRNA vaccinatie bestudeerd voor het opwekken van T-cel immuniteit tegen HIV-1 antigenen. Daarbij werd aangetoond dat het subcutaan toedienen van mRNA DOTAP/DOPE lipoplexen een antiviraal type I interferon (IFN)-antwoord opwekt dat vervolgens remmend inwerkt op de efficiëntie van het vaccin. In een eerste luik van deze thesis werden deze bevindingen verder gevalideerd uitgaande van een experimenteel tumormodel. Aldus toonden we aan dat de negatieve inwerking van type I IFN leidt tot een verminderde bescherming tegen de ontwikkeling van tumoren bij zowel een profylactische als een therapeutische toepassing van het mRNA vaccin. Het hoofddoel van dit doctoraal werk was dan ook het mechanisme dat aan de basis ligt van deze remmende werking van type I IFN beter te begrijpen en dusdanig strategieën te ontwikkelen die toelaten deze remmende werking te omzeilen en de efficiëntie van mRNA vaccins te verbeteren. Hiertoe werden verschillende strategieën uitgewerkt, waarbij zowel de focus lag op het antigeen-coderende mRNA als op de drager van het mRNA.

In een eerste benadering, werd gepoogd om door middel van gemodificeerd mRNA de activatie van een antiviraal type I IFN-antwoord door het aangeboren immuun systeem te vermijden. Gemodificeerd mRNA werd initieel ontwikkeld in het kader van gentherapie omwille van zijn verminderde herkenning door intracellulaire RNA-sensoren, wat resulteert in een verminderde opwekking van een antiviraal cellulair antwoord. Met behulp van een IFNβ reportermuis werd echter aangetoond dat de injectie van dergelijk gemodificeerd mRNA, geformuleerd met DOTAP/DOPE lipoplexen, niet resulteerde in een verminderde opwekking van type I IFN. In lijn met deze resultaten kon er verder ook geen versterkt cytotoxische $CD8^+$ T cel antwoord worden waargenomen.

In een volgende luik van de scriptie werd gefocust op de validatie van een peptide-gemedieerde formulatie die berust op de interactie tussen *cell penetrating peptides* (CPPs) en mRNA-antigenen. Hierbij werd een partikel ontwikkeld waarin het mRNA gecomplexeerd werd met het amfipatisch peptide RALA tot vorming van RALA mRNA nanocomplexen. Het amphipatische RALA-peptide vertoont een zuurafhankelijke cellytische activiteit waardoor RALA de translocatie van het mRNA uit de endosomen naar het cytosol bevordert. Deze translocatie richting cytosol is cruciaal om de translatie van het mRNA tot het antigeen eiwit toe te laten en de daaropvolgende verwerking en presentatie door MHC klasse I moleculen aan $CD8^+$ T cellen. Injectie van RALA mRNA nanocomplexen resulteerde in de opwekking van een beduidend sterker CTL-antwoord vergeleken met de standaard lipide-gebaseerde formulering. Tevens bleek het immunogeen karakter van deze RALA gemodificeerde mRNA nanocomplexen geheel onafhankelijk te zijn van type I IFN. Verder mechanistisch onderzoek toonde aan dat het immunogene karakter van RALA mRNA nanocomplexen gekoppeld is aan het pH-afhankelijke amfipatische karakter van het RALA peptide.

In een laatste luik van het doctoraal onderzoek werd tenslotte nagegaan in hoeverre RNA naast zijn functie van boodschapper eveneens kan fungeren als adjuvans voor vaccins gebaseerd op eiwitantigenen. Ons onderzoek toonde aan dat het formuleren van niet-coderend RNA in polymeer-gebaseerde complexen cruciaal is voor de efficiëntie waarmee het RNA wordt opgenomen door migrerende dendritische cellen. Tevens konden we aantonen dat deze RNA polymeer-complexen een uitgesproken $CD8^+$ T cel antwoord opwekten tegen het toegediende eiwitantigeen.

In globo hopen we dat de nieuwe inzichten verkregen via dit doctoraal onderzoek omtrent het gebruik van RNA als antigeen-coderende entiteit of als immuunstimulerende adjuvans nieuwe wegen openen voor toekomstige medische toepassingen in de vaccinologie.

INTRODUCTION

CHAPTER 1.
VACCINES – AN INTRODUCTION

1. VACCINES AND VACCINATION – AN HISTORICAL PERSPECTIVE

The story of the history of vaccination mostly starts with the innovative contributions of Edward Jenner to the development of vaccines in 1796. But actually, the historical origins of immunization started long before the 18th century, by a primitive form of vaccination called variolation **(Figure 1).** Variolation was practiced in Africa, India and China by blowing smallpox skin scabs into the nose of healthy people who consequently contracted a mild form of the disease, but remained immune to smallpox afterwards[1]. Although variolation was not without any risk – 3 % of the persons died upon treatment – the technique reached Europe by Turkish travellers at the beginning of the 18th century and gained popularity in all layers of society[2]. In 1721, a serious epidemic broke out in Massachusetts and despite great public protest against variolation, statistics showed that only 2 % among variolated individuals died, whereas the fatality rate for the non-treated population was 14 %[3].

Even so, all credits go to Edward Jenner who performed the first real vaccination study in 1796[4]. In this study, he inoculated a young boy with pus from a cowpox lesion of a milkmaid's hand by wounding him with a lancet. Two months later the boy was inoculated with pus from a smallpox lesion and stayed unaffected. Jenner reported his findings to the Royal Society but unfortunately the paper got rejected. One year later, Jenner had strengthened his research by collecting more than twenty cases of people who had received the same inoculation procedure. He reported in 1798 his findings in a privately published booklet called '*An Inquiry into the Causes and Effects of the Variolae Vaccinae, a disease discovered in some of the western counties of England, particularly Gloucestershire and Known by the Name of Cow Pox*'[4]. In this booklet, Jenner called this new technique '*vaccinia*', the Latin word for cowpox. He further described the applied scientific methods of observation and experiments and further stressed out the importance for a safer alternative to the variolation technique. By stating that cowpox protects the human constitution from infection with smallpox, he laid the foundation of modern vaccinology[4]. Despite the fact that the first reactions from the medical community to his report were rather negative, years later, the great value of vaccination compared to variolation became clear. As a consequence, Jenner got honoured worldwide and received multiple awards for his pioneering introduction to vaccination[2,5]. And rightly so, the findings of Jenner eventually contributed to a complete eradication of small pox from nature.

Despite the successful immunizations performed by Jenner, it took more than 100 years to discover a method to create a laboratory-developed vaccine **(Figure 1).** It was Louis Pasteur, a French chemist and microbiologist, who came up with the new concept of 'attenuated vaccines' in 1880. He used attenuated *Pasteurella multocida,* the causative agent of bird cholera, to protect chickens against the disease[6]. Like many other discoveries in the earliest years of science, the merit of using attenuated pathogens was discovered rather serendipitously. Pasteur was studying cholera by injecting chickens with live bacteria and subsequently following the progression of the disease. But when his assistant injected chickens with an old stock of the bacteria, they did not die, but showed only mild symptoms. When a new fresh stock of bacteria was injected in the same chickens, they survived and, more importantly, they did not even become ill. He extended his work to the attenuation of *Baccilus Antracis* (Antrax), but always kept his methods secret until 1881. At that moment, he published a few details about how he created this 'atmospheric attenuated' vaccine[7]. During the following years, together with Koch, Ramon and Merieux, he extended his knowledge to the creation of other vaccines based on inactivated pathogens (cholera, plague, typhoid) or toxins (diphtheria and tetanus)[6,8].

In the 1950's cell culture techniques became available, allowing the development of effective techniques to create inactivated vaccines for polio[9] as well as live-attenuated vaccines against mumps, rubella and measles. In the early 1970's, a new revolution in the field of vaccination arrived with the polysaccharide-based vaccine as this vaccine format was the first non-whole organism-based vaccine approach. However, the first tests in children with polysaccharide-based vaccines revealed unsuccessful immunization efficacies against pneumococcus and meningococcus. Afterwards, polysaccharide vaccines were optimized by covalently linking them to carrier proteins in order to improve the immunogenicity. Indeed, initially only B cell activation was elicited, but upon linking the polysaccharide with carrier proteins, the vaccine format gained potential to activate $CD4^+$ T helper cells. In the late nighties, the golden age of modern biotechnology dawned[10] and many new medical techniques were developed, including a new vaccine format based on self-assembling recombinant proteins forming non-infectious viral-like particles (VLPs)[11]. The first approved VLP vaccine came on the market in 1986 to protect against the hepatitis B virus (HBV).

During Pasteur's search to treat rabies in the 19^{th} century, he introduced the use of prophylactic vaccines as a treatment. In rabies, symptoms can appear after several months or years. By immunizing a boy bitten by a rabid dog, he applied vaccines in order to prevent severe disease

after acute infection[12]. Many years later, post exposure prophylaxis was extended to the treatment of other chronic infections, such as hepatitis B virus (HBV). Based on post exposure prophylaxis, the newest revolution are without doubt the therapeutic vaccines. Therapeutic vaccination aims to treat patients with infectious disease or cancer based on stimulating the immune system. Although clinical studies regarding therapeutic vaccines showed great potential, to date, the only therapeutic vaccine approved by the FDA is Sipuleucel-T (Provenge®), which came on the market in 2010 to treat prostate cancer patients **(Figure 1).** Sipuleucel-T was an innovative patient-specific treatment based on the modification of the patient's own blood cells which are afterwards re-infused[13]. Nevertheless, five years after its launch in the USA, Dendreon, the company behind the vaccine, went bankrupt due to commercial failure of Provenge®. The cause of Dendron's bankruptcy was multi-factorial, though two issues contributed the most: the delay in securing FDA approval and the high cost of the therapy ($100.000 per patient). In addition, the product was characterized by many potential barriers to market access, such as the mechanism of action which was previously unknown to the FDA, the complex administration, the limited manufacturing capacity and no proper markers of effective treatment response[14]. During the search for new developments in the field of therapeutic vaccination, nucleic acid-based vaccines came to the forefront. The first clinical trial using DNA vaccines for the treatment of HIV-infected patients was performed in 1995 **(Figure 1).** Two years earlier, a study by Martinon and colleagues, reported for the first time the use of mRNA as antigen-encoding device for vaccines. They showed that mice vaccinated with mRNA lipoplexes encoding the influenza virus nucleoprotein (NP) induced anti-influenza cytotoxic T lymphocytes (CTL) responses[15].

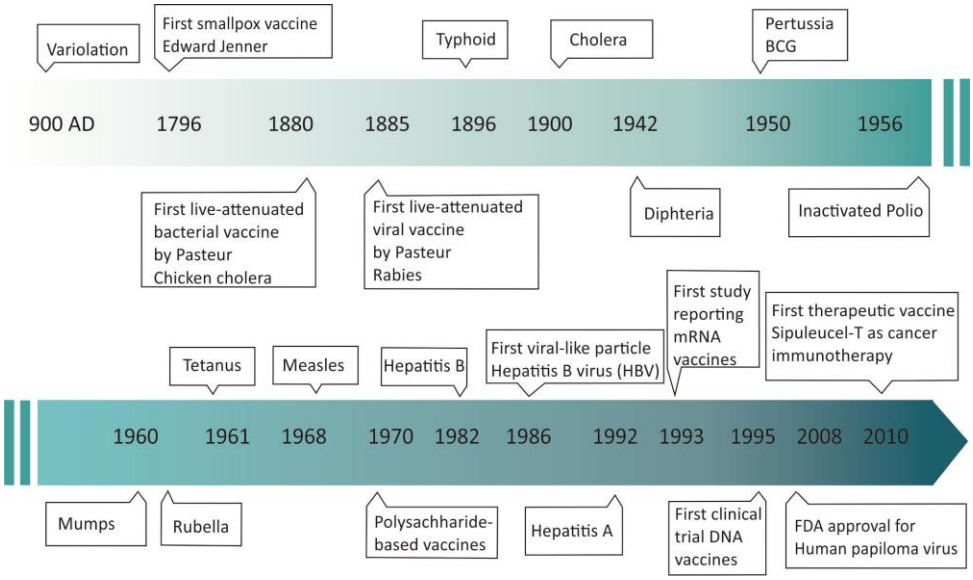

Figure 1. History of vaccines and vaccination – A time line.

2. Remarkable merits and crucial challenges

Prophylactic vaccination has significantly improved human health and continues to prevent millions of deaths worldwide. The impact of prophylactic vaccination is supported by the drastic reduction of many diseases like rabies, polio and measles and even a complete eradication of smallpox[16]. These merits makes vaccination the most relevant contribution of immunology to human health[17]. How big the impact of vaccine programs can be is clearly shown by the success of measles vaccination. Before the World Health Organization (WHO) organized worldwide vaccination programmes, measles caused more than two million deaths each year and formed one of the leading causes of death among young children. In 2014, 85 % of all children on earth received at least one vaccine dose by their first birthday, which made measles-caused death rates drop for 79 % over the last 14 years.

However, despite these significant successes, there still remain some major challenges for the development of vaccines at many different levels. First, there is the need for a new generation of vaccines that target more conserved regions of highly mutational viruses, like the influenza virus. Due to influenza antigenic variation, yearly predictions need to be made to update the vaccine antigen composition and immunize the public with the most relevant multivalent

vaccine[18]. A second major challenge in vaccinology remains the development of new adjuvants, that are able to elicit broader and sustained immune responses[19]. Improving vaccines at these different levels should make it possible to elicit immune responses of adequate strength and quality for effective protection against HIV, *Mycobacterium tuberculosis* and malaria, diseases still forming a challenge for vaccinologists.

3. VACCINE-ELICITED IMMUNE MEMORY

Prophylactic vaccination has the essential goal to induce pathogen-specific adaptive immune responses and to provide an active long-term immune protection. Most effective vaccines approved today generate protection through high-affinity antigen-specific memory B cells and memory T cells. In this chapter we will describe shortly the role of B and T cells in immune memory.

3.1 HIGH-AFFINITY MEMORY B CELLS

An effective B cell memory response is characterised by different functional isotype classes of high-affinity plasma cells and a panel of non-secreting memory B cells[20]. The process of memory B cell programming starts with antigen priming of naïve B cells. A naïve B cells binds either to soluble proteins in the lymphoid fluid or to proteins presented by dendritic cells (DCs) and macrophages. Upon binding of the antigen on a naïve B cell receptor (BCR), the antigen gets internalized, where after the B cell gets activated followed by IgM-antibody secretion[21]. Subsequent contact with antigen-specific follicular helper $CD4^+$ T cells (T_{FH}) and cytokines, like interleukin (IL)-4[22], initiate immunoglobulin class switching and differentiation of the plasma cell into non-germinal centre plasma B cells. At this phase of B cell memory development, the initially pre-germinal centre-primed B cells split up into two groups. A first part of the pre-germinal centre B cells continues differentiation into plasma cells via an extra follicular B cell pathway. A second part of the B cells migrate to the germinal centre of secondary lymph nodes[23–25]. During their stay in the germinal centre, these B cells will undergo antigen-specific clonal expansion and BCR diversification, followed by positive selection of high-affinity BCR variants, which will leave the germinal centre[20]. During this process, B cells differentiate into long-lived high-affinity antibody secreting plasma cells[26] – which house in the bone marrow - or

long-lived non-immunoglobulin secreting memory B cells – which remain in lymphoid tissue[27]. Memory B cells do remain for many years upon infection or immunization and are responsible for the recognition of identical antigens in order to quickly reply on re-exposure to the pathogen. The increased affinity of the BCR on memory cells does contribute to the sensitivity of low-dose soluble antigens.

3.2 Effector And Memory CD8+ T Cells

The activation of $CD8^+$ T cells requires three signals: TCR engagement (signal 1), co-stimulation (signal 2) and an inflammatory stimulus (signal 3). The first signal is converted by the initial interaction between the antigen presenting cell (APC) and the antigen-specific T cell. This interaction constitutes the binding between the APC MHC-I-epitope and MHC-II complex and the T cell receptor (TCR) - CD3 complex of $CD8^+$ T cells or $CD4^+$ T cells resp. In addition to this MHC-I presentation of the peptide, a co-stimulatory response, signal 2, is required to prevent anergic responses. Such co-stimulatory molecules are CD80 and CD86 expressed on the APC surface, which interact with the CD28 receptor on the T cell surface. Also the interaction between CD40-CD40 ligand is described to act as a critical signal 2 for proper T cell activation. The third signal for effector and probably also memory T cell differentiation is conveyed by the secretion of certain cytokines, which will ultimately determine the functionality of the evoked T cell response. Type I IFNs and IL-12 are the key cytokines that govern the differentiation of $CD8^+$ T cells into IFN-γ secreting and cytolytic effector cells. Although type I IFNs and IL-12 can partially compensate for each other, their relative contributions in guiding T cell differentiation depend on the nature of the infecting pathogen[28].

Upon proliferation, most of the T cells will terminally differentiate into end-stage effector cells and die off after the infection is cleared. $CD4^+$ T cells differentiate into different types of T helper cells, depending on the cytokine milieu[29,30]. In brief, Th1 skewed $CD4^+$ T cells produce mainly IFNɣ, IL-2 and TNF to directly stimulate killing of pathogen-infected cells by i.e. cytolytic effector $CD8^+$ T cells. Th2 skewed $CD4^+$ T cells and T_{FH} cells produce mainly IL-4, IL-5 and function as B cell supporting T helper cells[31,32]. Viral or intracellular bacterial infections promote differentiation of $CD8^+$ T cells into effector CTLs that mediate viral clearance and a small percentage of memory precursors cells[33–35]. The effector $CD8^+$ T cells destroy malignant or infected cells via the secretion of cytokines- such as IFNɣ and tumor necrosis factor (TNF)[36] and via the excretion of

cytotoxic granules containing perforins[37] and granzymes[38,39]. They further stimulate apoptosis via Fas/FasL[40] interaction. A small percentage of effector $CD8^+$ T cells that remain after the elimination of the pathogen will further differentiate into memory $CD8^+$ T cells. The two broadest subsets of memory T cells have been identified as effector memory T cells (T_{EM}; $CD62L^{low}CCR7^{low}$), homing to non-lymphoid tissue, and central memory T cells (T_{CM}; $CD62L^{hi}CCR7^{hi}CD27^+$), homing to secondary lymph nodes and bone marrow[41]. T_{EM} are memory cells that have lost the expression of CCR7 and CD62L. T_{EM} are characterized by rapid effector function and they produce perforins and IFNɣ within hours following antigenic stimulation[42]. T_{CM} are memory cells that, compared with naïve T cells, have higher sensitivity to antigenic stimulation, are less dependent on co-stimulation, and upregulate CD40L to a greater extent, thus providing more effective stimulatory feedback to DC and B cells. Following TCR triggering, T_{CM} produce mainly IL-2, but after proliferation they efficiently differentiate to effector cells and produce large amounts of IFN-γ or IL-4[41,43,44].

Different models exist predicting the differentiation of effector and memory T cells, which are nicely reviewed by Keach *et al*[45]. The identification of both populations and intermediate cell phases from naïve cells to terminally differentiated cells, is based on a set of cell markers: killer cell lectin-like receptor G1 (KLRG1), Interleukine 7 receptor subunit α (IL-7Rα), CXC chemokine receptor 3 (CXCR3), CD27 and CD62L. Memory $CD8^+$ T cells are maintained in absence of antigen trough IL-7 and IL-15 cytokines, promoting cellular survival and self-renewal[46].

4. VACCINE ANTIGEN FORMAT

To-date, different vaccine approaches are commercialised and in this thesis, they will be classified into four types depending on the nature and formulation of the antigen **(Figure 2).** The first group consists of whole organism vaccines, subdivided into live-attenuated vaccines and inactivated vaccines[47]. A second group, the subunit vaccines, contain selected pathogenic antigens, which can be encoded by proteins, peptides or nucleic acids[46,55]. Based on the antigen formulation format, subunit vaccines can be subdivided into VLPs, nanoparticle-based vaccines and naked nucleic acid vaccines. Next, a third group compromises toxoid vaccines[52-54], inducing protection to the secreted toxins rather than the pathogen itself. The last group presents the conjugated vaccines, which are polysaccharide-based vaccines conjugated to a carrier protein[56-57].

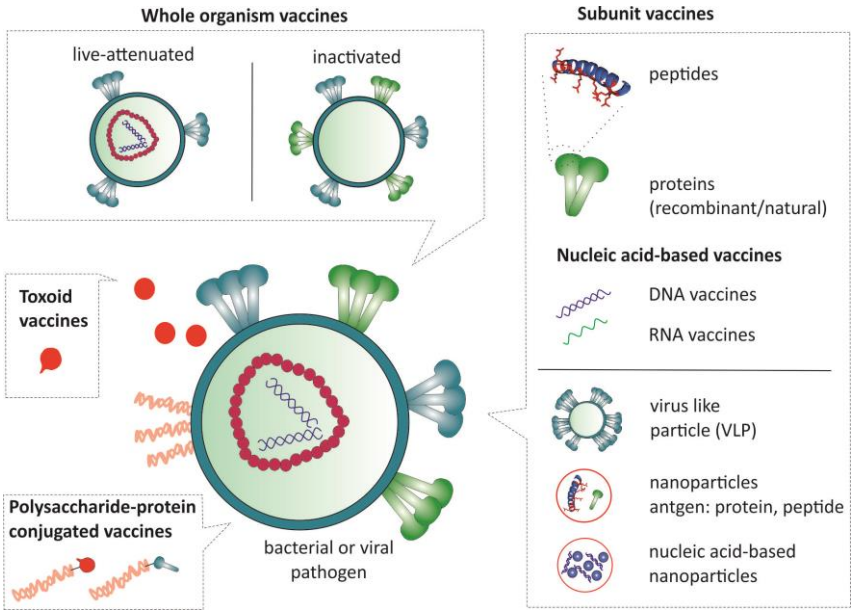

Figure 2. Based on the vaccine antigen format, four major groups of vaccines are classified. The group of whole organism vaccines can be subdivided into live-attenuated vaccines and inactivated vaccines. The subunit vaccines are subdivide into VLPs, nanoparticle-based vaccines and nucleic acid vaccines. Toxoid vaccines aim to induce humoral immunity to neutralise secreted toxins, which are responsible for illness. The group of conjugated vaccines are based on polysaccharides linked to a protein carrier.

4.1 Whole virus vaccines: Live-attenuated or inactivated

Live-attenuated vaccines consist of living bacteria or virus strains weakened to such extent that they are still able to transiently infect humans without causing disease. Due to the natural infection process, live-attenuated vaccines elicit both humoral and cellular immune responses and as a consequence, they can be considered as most immunogenic amongst all vaccine approaches. Furthermore, live-attenuated vaccines don't need to be supplemented with separate adjuvants.

Although live-attenuated vaccines form the biggest group on the market, they display an alarming safety profile and therefore, safer vaccine formats are needed **(table 1).** Risks of the use of living viruses although attenuated, include the chance on mutation to a more virulent form, causing disease especially in immune-compromised people include HIV+ subjects, transplant patients and people treated with an immune suppressive drug. Another drawback of

live-attenuated vaccines is the need for storage of living organisms at temperatures around 4-8 degrees, which imposes limitations for the use in developing countries[47].

However, in contrast to live-attenuated vaccines, inactivated vaccines based on pathogens killed by chemicals, heat or radiation, are more stable and safer than their living counterparts. In addition, they can be stored in a freeze-dry state, making them accessible to people in developing countries. Yet, although inactivated vaccines are safer than live-attenuated vaccines, they are less immunogenic due to the lack of an infection process[47].

4.2 SUBUNIT VACCINES

During the last years, significant progress has been made in the search for new approaches to circumvent the use of living organisms and to focus more on synthetic vaccine formats, namely subunit vaccines. Subunit vaccines constitute a broad collection of different vaccine formats using recombinant antigens instead of a whole organism. These synthetic antigens can be encoded either by recombinant proteins, peptides or nucleic acids. Due to the controllable production process of synthetic antigens, the safety profile of subunit vaccines scores much better than live-attenuated vaccines. When a soluble antigen is administered, the specific pathogenic composition is lost. For this reason, the immunogenicity of the selected antigen drops significantly. To deal with this problem, subunit vaccines are formulated in nanostructures or accompanied with adjuvants, whose function is to increase and skew the immunogenicity of the vaccine proteins. In here, we categorize subunit vaccines in subgroups based on the antigen-encoding device and the mechanism of formulation.

Virus Like Particles (VLPs)

First, there is the group of virus like particles (VLPs). The principle of VLPs is the spontaneous interaction and self-assembly of the capsid proteins into virion-like structures. Due to a viral mimicry, VLPs are able of inducing both humoral and cellular responses[48] **(table 1).** A well-known VLP-based vaccine on the market is the nine-valent Gardasil 9® to protect against infection by nine different human papillomavirus (HPV) strains, all causing cervical cancer and neoplasia. Gardasil 9® is composed of strain-specific major capsid proteins L1, which are produced in yeast, and aluminium hydroxide-based adjuvants.

Nanoparticle-based subunit vaccines

Besides the use of self-assembling proteins to form a virion-like structure, another strategy of antigen delivery is based on the formulating antigens into a carrier in order to obtain an immunogenic nanoparticle. Multiple carriers are studied, such as lipids and polymers, in order to deliver proteins, peptides or even RNA and DNA, all encoding antigens[49–51]. Nanoparticles have the great potential to be efficiently engulfed by APCs, which is crucial for antigen presentation to $CD4^+$ and $CD8^+$ T cells.

Based on the antigen-encoding device, nanoparticle vaccines can be subdivided into protein vaccines and nucleic acid vaccines. For the latter subgroup, the vaccine antigens are expressed in the host cell upon translation of the administered DNA or RNA molecules. This intracellular process grants nucleic acid vaccines an intrinsic adjuvant character, caused by the interaction between the nucleic acids and endosomal and cytosolic pattern recognition receptors (PRRs) **(table 1).** In addition, priming of $CD8^+$ T cell responses requires the antigen to enter the cytosolic route of antigen presentation. This route starts with the cleavage of cytosolic proteins into peptides, followed by the import of these peptides in the endoplasmic reticulum and their loading onto MHC-I molecules for presentation to $CD8^+$ T cells. As plasmid DNA (pDNA) and messenger RNA (mRNA) vaccines enable the cytosolic expression of antigens, they possess a superior potential to elicit $CD8^+$ T cell immunity compared to protein-based vaccines. Protein-based vaccines largely fail to access the cytosolic route of antigen processing as they are degraded in the endolysosomal compartments by acidic proteases followed by presentation via MHC-II to $CD4^+$ T cells.

4.3 TOXOID VACCINES

For some pathogens, illness is mainly caused by secreted toxins rather than the bacterium itself. For example, the principal toxin causing tetanus, tetanospasmin, binds to specific membrane receptors located on motor nerve cells to get internalized by the nervous system[52]. Once in the nervous system, the toxin blocks the glycine metabolism, which is essential for the working mechanism of neurons, and directly leads to the typical tetanus muscle spasms. To protect people from illness caused by pathogen-secreted toxins, toxoid vaccine were developed. A toxoid vaccine is based on the inactivation of toxins by formaldehyde-treatment in order to create minor molecular conformational changes without losing the physicochemical character of

the natural toxin. A prime and boost immunization promote humoral immunity driven by the induction of neutralizing cross-reactive antibodies. This means that the antibodies which are initially evoked by toxoid vaccination, will target and neutralize the natural toxins after exposure to the pathogen[53]. In general, toxoid vaccines are very effective and in addition, they are considered as a safe method to immunize the young and elderly as they do not mimic a bacterial infection **(table 1)**. Toxoid vaccines are used to protect people against the highly infectious diphtheria, botulism, and tetanus.

4.4 POLYSACCHARIDE-PROTEIN CONJUGATED VACCINES

Polysaccharide-encapsulating bacteria cause disease mainly in children during their first years of life but become less immunogenic for infants[54]. Polysaccharides are T cell independent antigens which generally stimulate short-lived B cell responses by cross-linking the B cell receptor. In order to enhance the immunogenicity of polysaccharides for immunization, they are chemically attached to a protein carrier[55]. This carrier can be a toxoid or pathogen-outer membrane protein[56]. The chemical conjugation of the polysaccharide to a carrier allows direct processing of the protein by polysaccharide-specific B cells. After processing of the carrier protein, peptides will be presented to via MHC-II molecules to protein carrier-specific $CD4^+$ T cells. For this reason, a conjugated polysaccharide vaccine induces both B cell and T cell responses, whereas the un-conjugated polysaccharide shows shortcomings for the inducing of T cell responses[55]. Examples of FDA-approved polysaccharide conjugated vaccines are the Haemophilus influenza type b vaccine and the streptococcus pneumoniae vaccine **(table 1)**.

Vaccine type	Example vaccine	Advantage	Disadvantages
Live-attenuated	Measles-Mumps-Rubella (M-M-RII, Merck)	- Due to self-replicative properties, single dose is effective - Immune response against all antigens - Broad immune response	- Since it is a living organism it may cause disease and storage at correct conditions is required - Risk of reversion to virulence by mutations - Not suitable for immunosuppressed patients
Inactivated	Hepatitis A (Havrix, GSK)	- Safe use in immunosuppressed patients. - Better safety profile: no risk for reversion; no virulence - relative simple procedure	- Less immunogenic; need for multiple doses and booster immunizations
VLP	Human papilloma virus (Gardasil9, VLP, Merck)	- Safe in immunosuppressed patients. - Cannot cause disease - Less side-effects because of purified antigens	- Most effective and suited antigens need to be identified - Less immunogenic; need for proper adjuvants
Nucleic acid-based vaccines	Hematopoietic necrosis virus (Apex-IHN, DNA-based, Novartis); approved for salmon	- Intrinsic adjuvant character - Cannot cause disease - Very effective induction of CTL responses	- Still, less immunogenic - Need to be protected from nucleases - DNA is not FDA-approved for human use
Toxoid	Tetanus, Diphteria (DTaP,DT,Td,Tdap; Sanofi Pasteur; Ltd)	- Stable - Safe in immunosuppressed patients	/
Conjugated vaccines	Haemophilus influenzae type B, Pneumococcen, Meningococcal vaccines	- Very effective - Relative easy production - Applicable for young children - Safe	/

Table 1. Vaccine formats. VLP, Virus like particle; CTL, cytotoxic T cells.

5. ADJUVANTING THE VACCINE: CARRIERS AND PRR-AGONISTS

When the focus of vaccine design shifted from whole organisms to the safer subunit format, the need for adjuvants was clear-cut. In general, all adjuvants aim to improve the immunogenicity of vaccine antigens in order to elicit effective and long lasting immune responses. To elicit an immune response, adjuvants employ one or more of the following mechanisms: i) sustained release of the antigen, ii) up-regulation of cytokines and chemokines, iii) cellular recruitment to the site of injection, iv) increased uptake and presentation of the antigens by APCs, and v) the maturation and activation of APCs followed by the migration to draining lymph nodes[57]. In this chapter, adjuvants will be classified into two groups dependent on how they fulfil their function as immune stimulators. A first group of adjuvants can be classified as antigen carriers and a second group contains the PRR-agonists **(table 2)**. Yet, important to know is that most adjuvants are a combination of both classes, as PRR-agonists benefit from being formulated into nanoparticle carriers.

5.1 ANTIGEN CARRIERS

A first group of adjuvants includes the antigen carriers. Carriers might fulfil their role as adjuvant via different mechanism, including targeting the antigenic cargo to APCs. In this way, the amount of antigen reaching the target cells increases, which directly leads to improved immune responses. Another possible mechanism includes their role as an antigen depot[58] as they encourage a slow release and prolong vaccine antigen exposure[59]. For most carriers, even to-date, there is still a lot of debate about their effective mechanisms and how they adjuvant the vaccine. In here, we give an overview of the most applied carrier-based adjuvants.

Liposome-based delivery vehicles

Lipid vesicles comprise all formulations based on a lipid bilayer encapsulating an aqueous core[60]. Multiple liposome-based delivery vehicles exist, but the most common liposomes are composed of phospholipids and cholesterol[61]. One of them is **AS01**, a liposome-based adjuvant of GlaxoSmithKline that was tested in a clinical trial for Malaria vaccination **(table 2).** The most important advantage of liposomes are their versatility and plasticity, as their size and charge can be adapted in order to create the required conditions to entrap specific antigens or adjuvants[60].

Water-soluble antigens, such as proteins, peptides and nucleic acids, are entrapped within the inner space of the liposomes, whereas lipophilic antigens or lipopeptides are located in the lipid bilayer. Liposomes, or nanoparticles in general, exert their immune stimulating effect by enhancing antigen delivery as well as by the activation of PRRs in DCs. Lipid-based vesicles can both induce Th1 and Th2 immunity, dependent on their size and cargo. Badiee and colleagues evaluated the impact of the particle size on immunity of liposomes containing a *Leishmania* surface glycoprotein. They showed that immunization with small liposomes induced Th2 immune responses, whereas larger liposomes induced Th1 immunity[62].

Immunostimulatory complexes (ISCOMs)

Another group of carrier-based formats which play a role in the adjuvant character of the vaccines, consists of immunostimulatory complexes or ISCOMS. ISCOMS are composed of saponins, cholesterol and phospholipids and form a cage-like structure in which the antigen is incorporated. It is still unclear whether ISCOMS stimulate immunity via the activations of PRRs or via the improvement of antigen uptake by DCs followed by maturation and rapid transport to the draining lymph nodes[63,64]. Upon uptake by DCs, ISCOMS have been proved to destabilize the endosomal membranes resulting in the endosomal escape of both antigen and adjuvant[63,65]. For this reason, ISCOMS are able to induce both humoral as well as cellular immunity without causing a biased Th1 or Th2 immunity **(table 2)**[63].

Aluminum salts

The most applied adjuvants for human use are the Th2-skewing alum adjuvants **(table 2)**. Alum absorbs proteins and generates antigen depots trapped at the injection site[66,67]. Alum is known to promote Th2 immune responses and differentiation of B cells, resulting in robust antibody production[57]. But surprisingly, despite the FDA-approval of alum, there is no consensus regarding the molecular mechanism behind the immunogenicity of Alum yet. To-date, several mechanisms have been put forward in order to explain the immunogenicity[68–71]. In brief, Alum has been proved to improve antibody production which might be associated with the depot effect of Alum[72]. Although, there is no direct evidence that a depot effect significantly contributes to adjuvant capacity[73]. Schijn and colleagues reported that surgical removal of the antigen-Alum depot, 2 hours after immunization, did not effected humoral or cell-mediated immunity[74,75]. A second underlying mechanism of the immunity of Alum, might be the induction

of inflammation and the recruitment of antigen presenting cells, more specifically monocyte-derived DCs[76,77]. Thirdly, as Alum has the capacity to form nanoparticle complexes upon interaction with proteins, it may enhance phagocytosis by APCs. However, how Alum activates APCs and the role herein of NLRP3 and the inflammasome remains a matter of debate[78–81]. At last, Marichal and colleagues suggested that alum-induced cytotoxicity resulted in the release of host DNA, which acts as a DAMP, and as a consequence host DNA acts as an endogenous adjuvant in alum vaccination. Further, Immunizing Nlrp3-deficient ($Nlrp3^{-/-}$) and Caspase 1–deficient ($Casp1^{-/-}$) mice resulted in the development of humoral responses comparable to those of WT mice, which was in contrast to the immunization of Irf3-deficient ($Irf3^{-/-}$) mice, showing reduced IgE antibody responses, whereas IgG1 responses remained unaffected. These results indicated that the extracellular DNA-mediated adjuvant activity of Alum was dependent on IRF3 activation, rather than on inflammasomal activation. Although the recruitment of immune cell populations at sites of alum injection did not differ significantly between WT and $Irf3^{-/-}$ mice, a reduction of inflammatory monocyte (iMono)-derived inflammatory DCs (iDCs) in the draining lymph nodes was observed upon OVA and alum–treatment of $Irf3^{-/-}$mice. Furthermore, a direct link between the recruitment of iDCs to the draining LNs and the percentage of cell death and DNA release upon Alum injection was suggested. Taken together, in this study the crucial role of IRF3 in the induction of T_h2 cell and IgE responses to Alum is correlated to the recruitment of iMonos, the precursors of iDCs to the draining lymph nodes[82].

Oil-in-water (o/w) Emulsions

Two FDA-approved examples of oil-in-water emulsions are **MF59** and **AS03 (table 2)**. Both formulations are based on squalene, a biosynthetic precursor of cholesterol and steroid hormones, completely degradable for humans. MF59 consists of squalene oil droplets stabilized by Tween80 and Span85, two non-ionic surfactants[83,61]. Calabro and colleagues reported that the combination of all three components are crucial for the adjuvanticity of MF59[84]. However oil-in-water emulsions are currently used in many human vaccines, likewise aluminum salts, the Th2-skewing mechanisms is largely unknown[65,83,85]. It has been reported that MF59 immunity is not based on a depot effect as MF59 is rapidly cleared out, independent of the vaccine-antigen[86]. The MF59-mediated cellular recruitment to the injection site is been investigated in detail[84], revealing that MF59 induces a local stimulation and recruitment of DCs, neutrophils and granulocytes, resulting in enhanced uptake of the antigen[87]. This is achieved by the upregulation

of CCR2, the receptor of chemoattractant CCL2, involved in monocyte infiltration[87,88]. Seubert and colleagues showed that, similar to Alum, MF59 recruits inflammatory Ly6C$^+$ CD11b$^+$ monocytes, which differentiate into CD11c$^+$ MHC-II$^+$ DCs upon antigen uptake, in a MyD88-dependent manner. Furthermore, they proved that MF59 did not activate any of the Toll-like receptors *in vitro*, suggesting a role for cytosolic receptors in the MyD88-dependent signalling upon MF59 injection[83].

	Adjuvant	Functional component	Innate Immune trigger	Principal immune response	Refs	
FDA approved	Alhydrogel Adju-Phos	Aliminium salts (hydroxid or phosphate)	NLRP3? Inflammasome?	Ab, Th2	68,71,89–92	Antigen carrier
	MF59, AS03	Squaleen in water emulsion	MyD88 dependent No receptors defined yet.	Ab, Th2	93–95	
	AS04	Alum and MPL	NLRP3? Inflammasome? TLR4	Ab, Th1	96–98	
Human and mice studies	AS01	Liposomes	The broad variety of cargo - different immune activating strategies	Ab, Th1, Th2	60,62	
	ISCOMs	Saponin, cholesterol phospholipids	Unclear	Ab, Th1, Th2	63	
	IFA	Mineral, paraffin oil+ surfactant	undefined	Ab, Th1 and Th2	99–101	
	CFA	IFA + peptidoglycan, trehalose dimycoltae	NOD-2; inflammasome	Ab, Th1, Th17	99,102,103	
	Poly:IC	Synthetic variant of dsRNA	TLR3, MDA5	Ab, Th1, CD8$^+$ T cells	104–106	PRR-agonists
	Imiquimod	Imidazoquinoline derivate	TLR7/TLR8	Ab, Th1, CD8$^+$ T cells	107,108	
	IC31, QB10	DNA oligo's with CpG motifs	TLR9	Ab, Th1, CD8$^+$ T cells	109–112	

Table 2. Adjuvants currently employed in human vaccines licensed for use in the US and/or Europe and adjuvants used in pre-clinical and clinical studies. TLR, toll like receptor; MPL, monophosphoryl lipid A; IFA, Incomplete Freund's adjuvant; CFA, complete Freud's adjuvant;MDA5, Melanoma differentiation-

associated protein 5; NLR, Nod-like receptor; NLRP3, NLR family pyrin domain containing 3; Ab, antibodies; Th, T helper cell response. Taken over from[113].

5.2 MOLECULAR ADJUVANTS : PRR AGONISTS

To induce a cellular immune response rather than a humoral response, antigens need to enter the cytosolic pathway to be presented by MHC-I molecules to $CD8^+$ T cells. Live-attenuated vaccines induce $CD8^+$ T cell immunity through the endogenous MHC-I pathway. However, to obtain $CD8^+$ T cell immunity upon recombinant protein vaccination, an adjuvant is needed in order to stimulate cross-presentation and push immunity towards Th1 responses. As classical adjuvants induce mainly strong Th2 responses and are less competent to induce Th1 immunity, the current challenge remains to develop adjuvants suitable for the induction of strong Th1 skewed immune responses. To this end, new adjuvants are being developed based on the natural ligands or synthetic agonists of TLRs. However, not all TLRs skew immunity towards a Th1 response. For example, TLR2 and TLR5 enhance T cell and antibody responses without altering Th1/Th2 cell balance. Of note, important to know is that TLR-ligands generally benefit from being formulated into nanoparticles as formulation i) provides protection of the adjuvant, ii) allows interaction between the antigen and the adjuvant, promotes uptake in the same cell, and at last, iii) limits biodistribution of the agonists and promotes targeting to the draining lymph nodes, so it lowers cytotoxicity. In here, we will give a short overview of the some Th1-skewing TLR-agonists. A more in depth description about how PRR receptors induce pro-inflammatory cytokines and type I IFNs upon triggering will be discussed in chapter 2.

PolyI:C. PolyI:C is a synthetic analogue of dsRNA and a ligand for **TLR3**, cytosolic retinoic acid-inducible gene-1 (**RIG-I**) and melanoma differentiation factor associated gene 5 (**MDA5**)[106,114,115]. PolyI:C is characterized as an inducer of IL-1, IL-6, IL-12 and IFNβ upon transfection of DCs and is currently evaluated in preclinical research for his capacity to promote Th1 immunity and cross presentation in $CD8^+$ T cells[106,116]. The polyI:C derivative Ampligen® (or polyI:C12U) has been under clinical investment for as a therapy for patients suffering from Chronic Fatigue Syndrome and HIV[117–119].

Monophosphoryl lipid A. Bacterial lipopolysaccharide are natural **TLR4** agonists but are considered as a high-risk for toxicity, limiting their potency for human use. In the early 80's a less toxic derivative was developed, called monophosphoryl lipid A (MPL). MLP forms a

component of the licenced adjuvant AS04, used in Cervarix® vaccines combined with Alum[97]. AS04 stimulates polarized Th1 immunity via MyD88 and TRAF6, leading to the recruitment of transcription factors, such as NF-KB to regulate the expression of pro-inflammatory cytokines (IL-1, IL-6, IL-12 and TNFα). Like almost all adjuvants, also AS04 induce DC maturation to enhance adaptive immunity[97].

TLR7/8-agonists. Uridine-rich ssRNA is identified as a natural ligand of **TLR7** and **TLR8**[107,120]. During the last years, synthetic variants have been designed with same TLR7/TLR8-binding specificity as ssRNA but improved stability. One such example is imiquimod, a profound type I IFN inducer which has recently been heavily investigated in multiple pre-clinical trials[121,122].

CpG. DNA molecules or synthetic 18-25 oligonucleotides (ODN) containing CpG motifs have been studied intensively for their binding capacity to **TLR9** and their capacity to induce Th1 skewed immunity. Further, CpG activates DC maturation (via the upregulation of CD40, CD80, CD86) and increase antigen expression (via the upregulation of MHC-II)[123]. In order to target both antigen and CpG to the same cell, both components are mostly complexed into liposomes to force co-localized uptake in APCs[109,124].

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CHAPTER 2.
NUCLEIC ACID-BASED VACCINES

1. NUCLEIC ACIDS AS ANTIGEN-ENCODING DEVICES

Because mRNA was considered too unstable for *in vivo* use, research into the nucleic acid vaccines has been strongly focused on pDNA vaccines in the past. Over the last decades, mRNA has however started to outcompete pDNA as nucleic acid format of choice to deliver antigenic information to the immune system. This paradigm shift is mainly driven by the enhanced stability of *in vitro* transcribed mRNA alongside with serious safety concerns regarding the use of pDNA. In this chapter we will provide a first impression of nucleic acid vaccines and how they elicit antigen-specific immunity.

1.1 DNA AS ANTIGEN-ENCODING DEVICE

1.1.1 Mode of action

Following intramuscular or intradermal injection, antigen-encoding plasmid DNA (pDNA) is taken up by both structural and antigen presenting cells (APCs)[1]. Upon intracellular translation, the antigenic proteins will be cleaved by the proteasome into peptides, which are presented to $CD8^+$ T cells via major histocompatibility class I (MHC-I) molecules on structural cells and professional APCs[2,3]. Secreted proteins that ended up in the extracellular matrix will be endocytosed by APCs and presented via MHC-II to activate $CD4^+$ T cells and B cells. For this reason, pDNA vaccination is able to induce both humoral and cellular immune responses. This renders DNA vaccines much more effective than current vaccines in evoking a long-term immune response[4–7]. Generally, pDNA vaccines favour Th1 responses as mostly IgG2a titres are promoted upon DNA vaccination[8,9]. Nevertheless, some parameters, such as the type of antigen and the delivery method can skew this preferential IgG2 profile towards dominated IgG1 titres. Th2 skewed immunity has been shown for pDNA vaccines encoding secreted proteins as well as for pDNA vaccines which have been delivered by gene gun technology[10,11].

pDNA vaccination provides important advantages compared to whole organism-based vaccines or protein-mediated subunit vaccines. First, pDNA vaccines seem to approach the capacity of live-attenuated vaccines to induce MHC-I-restricted $CD8^+$ T cell responses, without the risk of causing infections or mutations which might result in a virulent organism. Furthermore, in addition to a prophylactic response, pDNA vaccines can also be applied therapeutically. Second,

the production and storage of DNA-based vaccines is relative easy and cost-effective due to their high stability. Finally, full-length nucleic acid-based vaccines are not restricted by the patient's HLA type, unlike peptide-based vaccination. However, the major drawback of pDNA vaccines is the risk on genome integration by insertion mutagenesis. At last, pDNA vaccination require cell proliferation for the expression of the encoded protein as the pDNA vector needs to enter the nucleus in order to get transcribed and translated. Although it has experimentally been shown that the chance on plasmid integration is lower than the chance on spontaneous mutation[12], at this moment, DNA vaccination is not generally used for the treatment of cancer patients, although a select number of clinical trials are ongoing.

1.1.2 DNA Vector Construct

DNA vaccines consist of antigen-encoding genes cloned into a bacterial plasmid under the control of a strong eukaryotic promoter to guarantee optimal expression in mammalian cells[13]. The two most prominently used promoters are originally derived from the cytomegalovirus (CMV) and simian virus 40 (SV40) genome. The CMV promoter enables high constitutive expression levels in a wide range of mammalian tissue and does not suppress downstream read-through mechanisms[14,15]. Further, the antigen-encoded gene is flanked at the 3' end by a polyadenylation sequence to stabilize the mRNA transcript upon translation[16]. Next, a DNA vector also contains an origin of replication to allow large copy numbers and a bacterial antibiotic resistance gene (ampicillin or kanamycin) for plasmid selection during bacterial culture[17].

1.1.3 DNA Vaccine Delivery Systems

As mentioned before, a delivery system can improve vaccine immunogenicity via two ways. First, they enhance the delivery efficiency of the antigen, as a consequence, they enable larger amounts of antigen reaching the target cells. On the other hand, they promote the immunogenicity of the vaccine by activating innate immunity. For the administration of DNA as a vaccine, different approaches can be applied. First of all, some physical DNA delivery approaches have been developed, including magnetic beads, gene gun delivery[18], microneedle injection[19], electroporation and sonoporation. Sonoporation, or cellular sonification, refers to the use of ultrasound to permeabilize the cell membrane in order to deliver nucleic acids into the cell[20].

The combination of sonoporation and manose lipoplex-based delivery of DNA vaccines has been tested in a clinical study including relapsed melanoma patients[21]. This study revealed enhanced secretion of Th1 cytokines (IFNɣ and TNFα) and the effector function of cytotoxic $CD8^+$ T cells upon treatment. Besides physical approaches, a broad panel of nanoparticle-based formulation has been extensively tested in preclinical studies, including polymers, peptides and liposomes[22]. In this section we will highlight the use of electroporation and cell penetrating peptide-based delivery of DNA vaccines and describe how these strategies contribute to the improvement of DNA vaccine immunogenicity.

In Vivo Electroporation

Electroporation (EP) is the most commonly used – 45 % of all clinical trials concerning DNA vaccination deliver DNA via EP[23,24] - and most powerful delivery method for DNA vaccines[25,26]. EP-mediated delivery is based on membrane destabilization, mediated by electric pulses resulting in uptake of DNA. Mostly APCs are *in vitro* electroporated and subsequently transferred to the patient. Beside this *in vitro* approach, DNA may also be directly delivered via injection, followed by *in vivo* EP. The success of *in vivo* EP may be explained by the high transfection efficacy combined with the induction of pro-inflammatory cytokines[27] and local recruitment of immune cells[28,29]. The *in vivo* induction of type I IFNs upon electroporation is documented by Lambricht and colleagues using an IFNβ reporter mouse model[5]. They showed that the combination of both EP and plasmid (empty or coding for an antigen) injection is required for the induction of significant type I IFN responses.

Like other carrier systems, the site of administration influences both the type and the magnitude of the immune response induced by DNA vaccine electroporation[17]. Multiple delivery routes concerning the EP-based delivery of DNA vaccines have been tested in the past[4,30–33]. Vandermeulen and colleagues performed a study in which multiple antigens (ovalbumin, gp160 and P1A) encoded by pDNA were injected, followed by EP at three different sites: tibial cranial muscle, abdominal skin and ear pinna[4]. They observed the highest gene expression levels and IgG titres in mice that were intramuscularly vaccinated. However, the strongest $CD8^+$ T cell responses and IgG antibody titres were generated in mice that were electroporated in the ear pinna. Although the strongest CTL responses were generated via ear pinna electroporation, when tumor protection was compared, electroporation of muscle and ear pinna were equally

efficient in delaying P815 growth and prolonging mice survival. Taken together, this study showed the need for the validation of a specific administration route dependent on the aimed type of immunity. However, in the context of *in vivo* EP, injecting DNA intramuscularly or in the ear pinna is preferred for the induction of an effector $CD8^+$ T cell response capable of destructing infected or malignant cells.

Cell Penetrating Peptides

Cell penetrating peptides (CPPs) are peptides of 8 to 30 residues that unlike other peptides have membrane disruptive abilities, making them perfectly suitable to transport cargo molecules – like siRNA, pDNA, and proteins - across cellular membranes. There are three groups of CPPs, namely cationic, amphipathic and hydrophobic CPPs.

The first reported **cationic CPP** was the HIV-1 protein TAT, an arginine-rich peptide[34]. Futaki reported in a study about arginine-rich peptide-delivered proteins that at least eight positive charges are needed for efficient uptake of the cationic CPP-complexed cargo[35]. The uptake is mediated by the electrostatic interaction between CPPs and glycosaminoglycans (GAGs) on the cellular surface to enter the cell via endocytosis[36]. Besides endocytosis-mediated uptake, CPPs can also enter the cell directly. This direct translocation is suggested to be dependent on acid sphingomyelinase activation, followed by a change in composition of the membrane lipids[37]. Nucleus localization sequence (NLS) peptides are a well-known family of cationic peptides. NLS peptides are mainly based on arginine-, lysine- and proline-rich profiles and promote the translocation of molecules through the nuclear pore complex. Although they naturally function as a transport molecule, NLS-peptides do not efficiently disrupt membranes and only gain effective CPP features when they are covalently attached to hydrophobic sequences in order to create a chimeric amphipathic CPP. For example, Crombez and colleagues described the use of the amphipathic MPG-8 peptide to deliver siRNA to target B1 cyclin transcripts *in vivo* to prevent tumor growth. MPG-8 is composed of the fusion domain of HIV gp41 and the nuclear import sequence of SV40 T antigens[38].

The hydrophobic CPPs consist a small group of peptides, whose uptake is based on apolar residues or hydrophobic motifs. It has been shown, at least for some hydrophobic CPPs, that they can translocate directly through the membranes[39]. Direct crossing of the membrane is

advantageous for the delivery of cargo straight in the cytosol without risking to be trapped by the endosome[40].

Amphipathic CPPs can be classified into two groups based on their secondary structures; the α-helical and the β-sheet amphipathic CPPs. The α-helical group can be further subdivided into primary and secondary helixes. The primary α-helix is composed of hydrophilic and hydrophobic parts at separate faces of the helix[41], whereas secondary α-helixes have hydrophobic parts on one face, but the hydrophilic face can be either cationic, anionic or polar[41]. Although mostly all amphipathic CPPs are cationic, Scheller and colleagues proved that the membrane permeabilization character was not dependent on the charge of the peptide but rather on the amphipathic nature of the peptides[42]. Indeed, via the replacement of arginine by lysine residues, he created neutral and anionic amphipathic peptides whose cell penetrating capacities remained. Nevertheless, not all amphipathic peptides enable membrane permeabilization. So to date, the main requirements for the uptake capacities of amphipathic CPPs still remain to be determined. Besides α-helical structures, also β-sheet-structured amphipathic peptides are identified[43], but these are less studied than their helical counterparts. The different forms of amphipathic CPPs can influence the mechanism of uptake but in general, endocytosis and direct translocation are considered as the major cellular membrane crossing processes of amphipathic CPPs.

The Ph-Driven Amphipathic CPP: RALA

McCarthy and colleagues successfully optimized the use of CPPs for *in vivo* delivery of pDNA by circumventing the frequently observed endosomal trap effect. They started their study using a pH-driven CPP, called GALA. GALA assumes a random coil α-helix at pH lower than 6. Due to this conformation, GALA can oligomerize within the membrane to form pores and cause membrane leakage[40,44]. When GALA arrives in the cytosol, where pH is higher than 6, the α-helical structure is destabilized due to deprotonation. As a consequence GALA obtains a negative charge and becomes membrane inactive[45]. Nevertheless, due to its anionic nature, GALA is not suited to efficiently condense nucleic acids, a crucial feature to deliver antigen-encoding pDNA. In order to create a stronger capacity to bind nucleic acids, GALA was modified by replacing the glutamate with lysine residues, resulting in a new peptide named KALA. KALA was shown to assist oligonucleotide nuclear delivery in many cell types[46]. However, due to the replacement of

the glutamate acids with lysine residues, the increased positive charge on KALA improved KALA's membrane disruptive capacities[47]. As a result, KALA loses its endosomal specificity and causes toxicity[48]. To further optimize the perfect pH-driven CPP to deliver pDNA, the McCarthy group searched for KALA-inspired peptides able to bind nucleic acids but without losing the pH-specific endosomal disruption capacity. As arginine is found in most natural DNA binding motifs, they tested the replacement of lysine residues to arginine residues, to create the RALA peptide[47]. RALA is composed of 30 amino residues: N-WEARLARALARALARHLARALARALRACEA-C. The hydrophilic arginine (R) facilitates nucleic acid binding, whereas the hydrophobic Leucine (L) interacts with lipid membranes. These two regions are separated by alanine (A), resulting in an α-helical structure. The glutamic acids (E) at each terminus enhance solubility in water (pH 7,4). RALA peptide has been proven to efficiently condensate pDNA into nanoparticles which enable high *in vivo* antigen expression levels upon intravenous delivery[47]. Although cell-penetrating peptides have been extensively studies as delivery enhancers for nucleic acid-based vaccines, currently RALA is only validated as a DNA and siRNA delivery format in the context of nucleic acid therapeutics[49]. As far as we know, no research is performed yet concerning the use of RALA peptides as a nucleic acid carrier to evoke $CD8^+$ T cell immunity.

1.2 mRNA As Antigen-Encoding Device

1.2.1 Mode Of Action

mRNA vaccination is a potent vaccine format for the activation of antigen-specific cellular CTL responses, due to the followed intracellular track upon uptake by DCs. The intracellular track starts with the translation of mRNA antigens in the cytosol followed by antigen processing and presentation via the MHC-I molecules to $CD8^+$ T cells. Still, due to cytosolic autophagy, a fraction of the proteins will be presented via MHC-II to $CD4^+$ T cells. This $CD4^+$ T cell activation is crucial for eliciting a robust cellular immune response since $CD4^+$ T helper cells stimulate $CD8^+$ T cells by producing IL-2 and activating DCs through CD40/CD40L interaction[50]. Further, mRNA has an intrinsic adjuvant character due to its ability to trigger intracellular PRRs, whose activation leads to the secretion of inflammatory cytokines and type I IFNs.

The last two decades, much effort was put in the optimization of the mRNA molecules and the delivery formats of mRNA vaccines. Due to this progress, at present, mRNA vaccination shows

multiple benefits compared with pDNA vaccination. First, mRNA vaccines are safer than pDNA vaccines due to the negligibly small chance on genome integration causing insertional mutagenesis. Second, mRNA does not need to cross the nuclear membrane to be functional, in contrast to pDNA. As a consequence, mRNA is perfectly suitable for the transfection of slowly growing and even non-dividing cells. Third, due to the transient expression character of mRNA sequences, mRNA antigen delivery is far more controllable than pDNA-mediated delivery[51–53].

1.2.2 mRNA Constructs

In vitro transcribed (IVT) mRNA is processed by an RNA polymerase-based reaction started from a DNA template vector. Generally, this DNA template consists of a RNA-polymerase-specific promoter, untranslated regions (UTRs) flanking the gene coding sequence, and a poly(A) tail[54]. In the following paragraph, a short overview is given about the latest optimizations regarding the mRNA constructs.

All endogenous mRNA sequences do include a 5' and a 3' UTR, flanking the open reading frame (ORF) in order to enhance RNA stability and translational efficacy[55]. The use of the β 5' UTR and α 3' UTR of the globin gene of *Xenopus* is widely applied and has been proved to instigate strong translation efficiencies[56–58]. Still, in order to further enhance translational efficacy, multiple structural elements can be inserted in the 5' and 3' UTR regions[55], such as Translation Enhancer (TE) elements and Expression and Nuclear Retention Elements (ENEs). Translation enhancers are a broad group of features which all aim to increase the translation efficacy of mRNA, such as internal ribosome entry sites (IRES). Besides TE elements, the RNA transcription vector can be further optimized by the incorporation of (ENEs) as patented in 2014[59]. The ENE of nuclear restricted RNA sequences of the Kaposi's sarcoma associated Herpes virus (KSHV) is responsible for enhanced viral RNA abundance[60]. The underlying mechanism is based on specific U-rich hairpin structures interacting with the poly(A) tail of the mRNA sequences. Due to this interaction, a secondary structure is obtained resulting in the retention of RNA in the nucleus. But even more important for the application of *in vitro* transcribed mRNA, whenever the hairpin structure interact with the poly(A) tail, a 'shield' is created which protects the RNA from degradation by the host[59].

Next, the 5'methylated m^7GpppN cap structure at the 5' end of the mRNA molecule plays a crucial role in mRNA stability, RNA splicing and most importantly, in the recruitment of

ribosomes, and thus in the translation efficacy[61]. At present, the original cap structure is replaced by an anti-reverse cap analogue (ARCA). A conventional Cap can ligate to the sequence in two directions due to their ability to bind both via the methylated as well as the of unmethylated guanine residue. However, only the binding of the unmethylated guanine residue will result in a functional mRNA sequence as the eukaryotic initiation factor (eIF4A) does only recognise a methylated base at the 5' end of the mRNA[62]. Thus, using a conventional Cap, statistically only half of the produced RNA sequences are functional. In contrast, ARCA cannot bind with the 3' OH methylated guanosine to the RNA sequence, so is forced to insert with the unmethylated guanosine residue, leading to 100% functional mRNA molecules. As a consequence, by adding ARCA to the *in vitro* RNA transcription reaction, the efficacy of translation does increase dramatically[63–65].

At least, the poly(A) tail positively influences the mRNA stability by protecting RNA against nuclease activity. It has been proven that a poly(A) tail should consist of at least 20 adenosine residues[66,67]. However, the translation efficacy can be dramatically enhanced using poly(A) tails of 100 nucleotides and more[67,68]. Moreover, it has been shown that the combination of a 5' cap and 3' poly(A) tail synergistically effects the translation efficacy due to the essential formation of a cap-eIF4E-eIF4G-PAPB-poly(A) loop-structured complex, which enables the recycling of ribosomes and protects the mRNA against enzymatic degradation[55,69].

1.2.3 mRNA Vaccine Delivery Systems

In theory, exogenous RNA needs to cross one lipid bilayer to become internalized by target cells and translated into a functional antigen. This is in contrast to pDNA, which needs to enter the nucleus in order to get transcribed. Naked mRNA is spontaneously taken up by many different cell types[70–72]. It has been shown that the uptake of naked RNA by immature DCs is an active process, which involves scavenger receptor-mediated endocytosis and micropinocytosis[70,73]. Both pathways lead to endolysosomal localization, where after only a small fraction of intact RNA sequences enter the cytoplasm[72]. To deal with this, multiple formats have been designed to both target the mRNA to antigen presenting cells as well as to augment the amount of RNA reaching the cytosol after uptake. Mostly all developed approaches are based on nanoparticle formation, such as the use of liposomes, polymers and peptides. **Table 3** presents the most abundant lipid- and peptide-based formulations for mRNA vaccines which have been tested in

animal studies and clinical trials. In this section, we will shortly describe cationic lipid- and protamine-based delivery of mRNA to elicit effective $CD8^+$ T cell responses for both prophylactic as well as therapeutic vaccination strategies.

	Delivery component	mRNA-encoded Antigen	Size (nm)	Administration route	Refs.
Lipid-based delivery systems	DOTAP	OVA	/	iv. / id.	[74]
	DOTAP/DOPE	OVA HIV gag	100	iv./ sc./ id.	[74–76]
	DOTMA+DOTAP+DOPE+cholesterol	OVA influenza-HA	200-230	iv.	[77]
	Lipofectamin RNAiMAX	HPV-E7 OVA TRP2	nanosize	iv.	[78]
	PBAE+ DOTAP/DOPC Lipid + shell + DSPE-PEG2000	OVA	280	In.	[79]
	Tween80 + oil phase containing Span85 + DOTAP + squaleen	RSV-F	86	Im.	[80]
Lipid+ peptide	Lipid-protamine	HSV1-TK	/	iv.	[81]
peptide	Unifectin and protamine	β-gal	100	iv./sc./ id.	[82–84]

Table 3. Nanoparticle-based mRNA vaccine delivery systems used in animal studies and clinical trials. DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phospho ethanolamine; PBAE, Polyβ-amino ester poly-1; DOPC, L-α-Di-oleoyl phosphatidyl choline; SDPE-PEG, 1,2-Distearoyl-sn-glycero-3-phosphoethanol amine-N-amino(polyethylene glycol)-2000; OVA, ovalbumin, HIV gag, gag protein of Human immunodeficiency virus; β-gal, β galosidase; RSV-F, fusion glycoporotein of respiratory syncytial virus; TRP2, tyrosinase related protein-2; HSV-TK1, herpes simplex virus 1 thymidine kinas HPV-E7, human papilloma virus-E7; i.v, intravenous; id., intradermal; sc., subcutaneous; in., intranodal; im., intramuscular. Table adapted from[85].

Cationic Lipid-Based Delivery Of mRNA Vaccines

To complex negatively charged mRNA, cationic lipids are perfectly suited as both components spontaneously interact to form lipoplexes[85]. Lipoplex-based delivery of mRNA has two main benefits. First, the mRNA is condensed into particles within the range of micro-organisms, resulting in efficient targeting and uptake by professional APCs. Second, in a condensed state, the mRNA is less vulnerable for intracellular and extracellular enzyme-mediated degradation.

The cationic lipid 1,2 dioleoyl-3-trimethylammonium-propane **(DOTAP)** is one of the most extensively studied lipid carriers for cellular delivery of nucleic acids and may be considered as the bench mark lipid-based delivery vehicle for mRNA vaccines[75,76,86]. For multiple years, researchers have been trying to optimize DOTAP-mediated mRNA transfection. For example, DOTAP mixed with carbonate apatite – a pH-sensitive inorganic crystal with strong affinity for nucleic acids – resulted in a ten-fold increase in expression levels of mRNA luciferase due to enhanced cellular uptake, compared to DOTAP alone[87,88]. The transfection efficiency was further optimized adding 1,2 dioleoyl-sn-glycero-3-phosphoethanolamine **(DOPE)** and cholesterol to the DOTAP mRNA lipoplexes to function as neutral helper lipids. DOPE has a phosphoethanol amine head group whose size is smaller than its hydrophobic diacyl chain. This formation enables DOPE to induce membrane fusion[89]. Adding DOPE to the DOTAP lipoplexes resulted in enhanced endosomal escape of the nucleic acid cargo and consequently higher transfection efficacies. We and others showed that intradermal, subcutaneous and intravenous immunization with cationic DOTAP-based lipoplexes resulted in effective CTL responses against the mRNA encoded antigens in mice studies[74–77].

Until now, it is still not fully understood how the structural organization between mRNA and cationic lipids occurs. It appeared to be difficult to analyse the interaction due to the unstable character of mRNA and its RNAse sensitivity. Nevertheless, it has been demonstrated that DNA and cationic lipids undergo topological transformation to form compact quasi-spherical vesicles with a 100-300 nm diameter and a multi-lamellar structure[85,90]. The chance that mRNA lipid complexation might exhibit another type of supramolecular organization is quite high, as single stranded RNA forms secondary structures and therefore not behaves as supercoiled pDNA.

Protamine- based delivery of mRNA vaccines

Complexing mRNA with the membrane-translocation peptide protamine has been successfully applied by Curevac®. They demonstrated that complexing mRNA with protamine enabled the induction of Th1 responses against the mRNA-encoded antigens[84]. However, the protamine-mRNA interaction is very tight in such extent that the adjuvant effect comes at the cost of weak antigen expression levels[83]. To solve this problem, a two-component format was developed, whereby mRNA was only partially complexed to protamine. As the uncomplexed mRNA appears to be responsible for antigen expression, whereas the complexed mRNA attributes a strong immunogenic signal, it is highly suggested that both mRNA components follow distinct intracellular pathways after endocytosis by APCs[91,92]. Immunizing mice with a two-component protamine-based vaccine resulted in the induction of a strong and balanced humoral immune response consisting of high IgG1 and IgG2a antibody titres, which suggested that both Th1 and Th2 immune responses were elicited[83]. Although evoked T cell responses comprise both IFNɣ-secreting functional $CD4^+$ and $CD8^+$ T cells, only the $CD8^+$ T cell responses have further increased with repeating immunizations[83]. This protamine-format has been proven to have potential to instigate CTL responses in clinical research[93] (see 1.2.5). Besides the use of cancer models, protamine-based mRNA vaccination has further been evaluated in a prophylactic context to study its protection capacity for infectious disease. In this study, mice were immunized via the intradermal route with influenza hemagglutinin (HA) mRNA and showed effective seroconversion and presence of virus-neutralizing antibodies[94].

1.2.4 Delivery routes for mRNA vaccines

Local delivery – intradermal and subcutaneous

Since the skin serves as a first line of protection against invaders, macrophages, DCs, pDCs, Langerhans cells and T cells are present. For this reason, intradermal and subcutaneous delivery of mRNA has formed the focus of mRNA vaccine delivery for many years. The uptake mechanisms upon intradermal delivery of 'free' mRNA are still not fully understood. Probst and colleagues showed that uptake of i.d. injected mRNA in Ringer lactate was dependent on the presence of calcium in the injection solution[73]. They further showed that this dermal uptake of 'free' mRNA was mediated by an active mechanism specific for nucleic acids. The question

remains if RNA in Ringer lactate can be considered as free mRNA, as the need for calcium suggests that some form of aggregation might occur, creating particulate structures which are known to enhance the uptake of mRNA. Besides the formulation of mRNA in Ringer lactate, also protamine-based and lipid-based delivery of mRNA has been described to lead to high antigen expression levels upon dermal injection[82,85]. The potential of intradermal delivery of mRNA-based vaccines is supported by the successful outcome of many (pre)-clinical studies[82,92,95].

Targeted delivery – Intranodal

Another rewarding route for mRNA vaccines includes direct intranodal injection. The strength of intranodal injection is based on the targeted delivery of mRNA to resident DCs in the draining lymph nodes. Indeed, a bio distribution study showed that a 100-fold increase of RNA reaching the lymph node occurred after intranodal delivery compared with injection near by the lymph node[96]. The mechanisms of uptake upon intranodal delivery is partially cleared out by the Diken and colleagues who reported that mRNA injected into the lymph node is directly taken up by the resident $CD11c^+$ DCs in a macropinose-mediated way, resulting in the activation of the DC[70]. More specific, Van Lint and colleagues showed that intranodal injection of mRNA in Ringer lactate or Ca^+ containing balanced salt solution showed far stronger and longer expression profiles compared to mRNA in phosphate buffered saline[97]. These data are in line with the ion-dependent uptake of 'free mRNA' after intradermal injection, described by Probst[73]. The main drawback of intranodal delivery to mice, is the need for surgery to make a precise injection possible. During this step, it is hard to circumvent minor bleedings, which are ruinous for unprotected free mRNA due to their high susceptibility to nucleases in the serum. Furthermore, in view of applicability to treat patients, it is obvious that the easy practicability of intradermal injections are preferred over intranodal injections which require ultrasound-mediated visualization of the lymph nodes. However, new strategies are under investigation to target the mRNA to the draining lymph nodes after subcutaneous administration via ultrasound-guided microbubble-assisted drug delivery[98].

Systemic delivery – intravenous

Recent reports demonstrated that systemic delivery of lipid-based mRNA particles resulted in strong and effective CTL responses[77,78]. Kranz and colleagues showed that the injection of

intravenously administered mRNA-DOTMA/DOPE lipoplexes induced strong effector and memory T-cell responses, and mediate potent IFNα-dependent rejection of progressive tumours in mice. The authors suggested that the intravenously delivered mRNA lipoplexes are targeted to APCs in the lymphoid compartments, solely by adjusting the negative charge of the particle[77]. Furthermore, Broos and colleagues showed that systemically delivered cationic mRNA lipoplexes (encoding three different tumor associated antigens) elicited strong antigen-specific T cell responses. Moreover, in line with the results of Kranz, they showed that the uptake and translation of the mRNA-encoded protein in the spleen and liver occurred predominantly by $CD11c^+$ cells upon intravenous delivery[78].

1.2.5 Currently completed and ongoing clinical trials

In contrast to the many clinical trials concerning the adoptive transfer of RNA-modified DCs (not discussed in this thesis)[99–101], so far, only a few trials have been performed regarding the direct administration of mRNA vaccines[92,102]. The first clinical study concerning mRNA vaccination was based on the intradermal injection of total tumor mRNA extracts of melanoma patients[103]. Although enhanced humoral and cellular responses were demonstrated, no clinical regression was observed. In a follow-up study, a cocktail of protamine-complexed mRNA sequences encoding six different melanoma antigens (Melan-A, Tyrosinase, gp100, Mage-A1, MA-age-A3 and survinin) was administered intradermally in 21 metastatic melanoma patients, followed by the injection of GM-CSF 24 hours after immuniztion[93]. In this study a decrease in myeloid suppressor cells was registered in peripheral blood of patients after the treatment. Further, an increase of antigen-specific T cells was detected in a subset of treated patients. Still, in general, the few number of patients and the individual differences made the clinical outcome uncertain. Nevertheless, one out of seven stage IV patients showed a complete clinical response[93]. At the same time, the lab of Brossart performed a phase I/II trial to assess feasibility, safety and immunological responses to mRNA-based vaccines in 30 patients with stage IV renal cell cancer. Also in this study GM-CSF was applied as an adjuvant and a mixture of 6 different tumor associated antigens (TAAs; MUC1, CEA, her-2/neu, telomerase, surviving and MAGE-A1) was injected intradermally[104]. No severe side effect of the vaccine was detected and in addition, the induction of $CD4^+$ and $CD8^+$ T cell responses for several TAAs increased in all patients. Furthermore, 7 out of 30 patients showed clinical response upon vaccination. In 2013 a clinical study started to test IVAC MUTANOME, a poly-epitopic RNA vaccine which targets patient-

specific mutations[105]. In this study, all melanoma patients received an individual vaccine composition via direct intranodal injection. As this study is planned to end in December 2016, no data are available yet. Further, Curevac® has started a study to test the intradermal delivery of the protamine-two-component mRNActive® vaccine in non-small cell lung cancer (NSCLC) patients[106]. In this study patients were injected 5 times in a 15 week interval with self-adjuvanted mRNA molecules encoding five NSCLC-associated tumor antigens. In 65 % of the patients, antigen-specific immune responses against at least one antigen were detected (39 % cellular and 49 % humoral). Still, in general, the clinical studies concerning direct mRNA vaccination to treat cancer patients, have been showing clearly mixed achievements. In general, suboptimal antitumor immune responses have been elicited, a disappointing observation in view of the promising pre-clinical data. One of the major reasons for the limited clinical success of cancer vaccination is the influence of the immunosuppressive tumor microenvironment. In view of this limitation, more recent studies have been focussing on the administration of immunosuppressive blocking antibodies in combination with cancer vaccines. For example, ipilumumab, an FDA-approved therapy based on the anti-cytotoxic T lymphocy-associated antigen (CTLA)-4 inhibitory antibody, showed clinical response in treated cancer patients[107]. CTLA-4 is homologue to B7 molecules and also binds to CD28, like B7. However, CTLA-4 is a negative regulator for T cell proliferation and is naturally upregulated upon T cell activation to create a negative feedback-loop. As a future perspective, the co-administration of anti-CTLA-4 (and anti-PD-1) antibodies with mRNA vaccines might lead to highly efficient vaccine formats with great clinical responses[108–110].

Besides the use of RNA-based therapeutic vaccines for the treatment of cancer, in the field of prophylactic vaccines for preventing infectious diseases, Curevac® is currently testing the RNActive® approach for prophylactic vaccination in a first clinical phase[111]. Preclinical data revealed that immunization of mice and pigs with non-replicating rabies virus glycoprotein (RABV-G) encoding messenger RNA (mRNA) resulted in both specific $CD4^+$ T cell as well as $CD8^+$ T cell responses[112].

2. Nucleic Acids As Adjuvants

2.1 The Immunogenicity Of DNA Vaccines

In contrast to studies in small mammalian animals, which proved that DNA vaccination induced strong humoral and cellular immune responses, studies in patients showed much weaker immunogenicity[113,114]. In order to enhance the immunogenicity of DNA vaccines, two different strategies have been worked out. First, as described before, multiple carriers and delivery methods have been optimized in order to promote and skew the induction of immune responses to DNA vaccination. Second, currently almost all DNA vaccines are co-formulated with an adjuvant. Importantly, the currently benchmark-followed strategy includes the combination of a carrier and an adjuvant in order to gain efficacy. In this section we give an overview of the intrinsic adjuvant features of pDNA and the different strategies to enhance the immunogenicity of DNA vaccines.

2.1.1 Intrinsic Adjuvant Characteristics

Cytosine-phosphate-guanosine (CpG) motifs in pDNA vectors are the main contributors to the immunogenicity of DNA vaccines[7,9]. It is clearly proven that intrinsic CpG motifs acts as an adjuvant by triggering TLR9 in the endosomes of pDCs and B cells[115–118] and signals via the downstream myeloid differentiation factor-88 (MyD88) pathway, resulting in the expression of pro-inflammatory cytokines, such as IL-6, IL-12 and TNF-α, regulated by the transcription factor IFN regulator factor 7 (IRF7; **Figure 3).**

Since it was demonstrated that TLR9 knock-out mice still responded to DNA vaccination[119], Babiuk and colleagues suggested that the TLR9 function was redundant and can be taken over by cytosolic sensors[120]. Indeed, DNA motifs in the cytosol can be recognized by multiple receptors, including DNA-dependent activator of IFN-regulatory factors (DAI), the cytosolic GAMP synthase (cGAS), PHYN IFN-inducible protein 16 (IFI16) and DDX41[7,8,24,121–127]. Two signalling pathways are involved, one resulting in the induction of type I IFNs and the other leading to induction of NF-κB. The first involves DAI-mediated phosphorylation of TBK1 via the stimulator of interferon genes (STING) and subsequent activation of the transcription factor IRF3. IRF3 will migrate to nucleus to start transcription of type I IFNs[128]. The second pathway

requires phosphorylation of the receptor interacting protein-1 kinase (RIPK1), leading to phosphorylation of IκB-α and subsequent activation of the transcription factor NF-κB, which regulates the expression of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), IL-6 and IL-12 **(Figure 3).** Further, *in vitro* studies in a human pDC line revealed an important role for aspartate-gutamate- any amino acid-aspartate/histidine (DExD)-box helicase 9 (DHX9) and DHX36 receptors as cytoplasmic sensors of CpG. Both DHX36 and DHX9 directly interact with the receptor domain of MyD88. They are both associated with IRF7 nuclear translocation and IFNα production, whereas DHX9 appeared to be crucial for TNFα and IL-6 production via NF-κB activation[129].

At last, binding of dsDNA to absent in melanoma (AIM2) leads to the formation of an inflammasome complex together with ASC to activate caspase-1 and NF-κB[130] **(Figure 3).** Although AIM2 is known for their role in the ASC-dependent inflammasome-mediated catalytic cleavage of pro-forms of IL-1β and IL-18, it was recently suggested that AIM2 is also indirectly involved in type I IFN production in response to DNA vaccination[131].

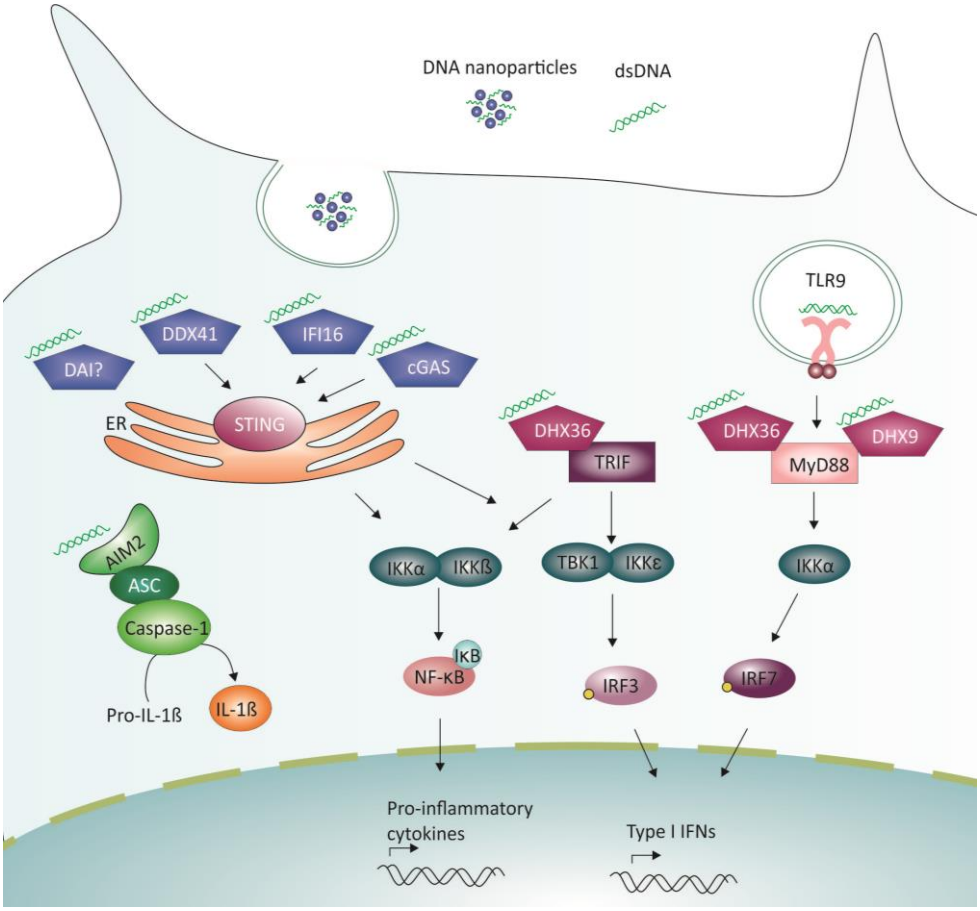

Figure 3. Innate sensing of DNA sequences. dsRNA, double stranded RDA; TLR, Toll-like receptor; TBK1, TRAF family-associated NF-kB activator (TANK)-binding kinase-1. TRIF, TIR domain-containing adaptor inducing IFN-β; MyD88, myeloid differentiation factor-88; IRF, interferon regulatory factor; DDX, DExD/H helicase box; IFI16, PHYN IFN-inducible protein 16; STING, stimulator of interferon genes; DAI, activator of IFN-regulatory factors; cGAS, cytosolic GAMP synthase; AIM2, absent in melanoma. Figure based on[118].

2.1.2 Improving the immunogenicity of DNA vaccines

Since the immunogenicity of DNA vaccines appeared to be suboptimal, multiple studies have been performed regarding the use of conventional adjuvants to enforce the immunogenicity. The use of physical delivery systems - such as *in vivo* electroporation[5,24], nanoparticle formulation[30,47] or a combination of those strategies[24,25,30] - to stimulate immunity has showed mixed successes depended on the experimental set up and conditions. An overview of these

studies is provided by Saade and colleagues[14]. The most important strategy to enhance DNA vaccine immunity is the co-delivery of molecular adjuvants, such as CpG, cytokines, chemokines, co-stimulatory molecules and heat-shock proteins[20]. In this section we describe how the co-delivery of immune stimulatory cytokines, IL-2 and GM-CSF can enhance the immunogenicity of pDNA vaccines.

The co-delivery of immune stimulatory cytokines

If cytokine induction remains rather low upon vaccination, one possibility is to co-deliver them with the antigen, both encoded by the same pDNA vector[125]. This approach has multiple advantages, such as the non-complex vaccine composition as a simple insertion of an extra gene in the DNA vector is sufficient. Moreover, as both the antigen as well as the adjuvant is encoded by the same vector, they will be both targeted to the same cell. Furthermore, the production and purification of cytokines is expensive, so the *in vivo* expression of cytokines is also cost-effective. A large number of studies have been focussing on the incorporation of Th1-inducing cytokines such as IFNɣ, GM-CSF, IL-2, IL-12 and TNF-α to stimulate immunity of pDNA-based cytotoxic T-lymphocyte-inducing vaccines[125].

IL-2. The co-delivery of DNA plasmids encoding the pro-inflammatory cytokine IL-2 has been proven to be very potent in multiple pre-clinical studies[132,133]. IL-2 enhances Th1 responses by stimulating T cell IFNɣ-production. Interestingly, to increase the half-life time of IL-2 from approximately ten minutes to two days, IL-2 has been fused to an immunoglobulin Fc-segment. This prolonged half-life of IL-2 resulted in significantly increased memory T cell responses[132,134]. A pDNA-based vaccine encoding the HIV-related Gag–Pol–Nef antigens and IL-2/Ig conjugates has been successfully evaluated in a clinical trial[135]. Importantly, the timing of IL-2-encoding plasmid injection relative to the antigen, is a crucial mediator for the different types of evoked immunity. It has been shown that the delivery of IL-2/Ig resulted in decreased humoral and CTL responses when injected prior to, or simultaneously with the DNA vaccine. However, these results are in sharp contrast to the increased responses observed when IL-2/Ig was injected five days after DNA antigen vaccination[125,134].

GM-CSF. GM-CSF is a pro-inflammatory cytokine, crucial for DC development and maturation[136]. Furthermore, GM-CSF is also directly linked to anti-tumoral activities. These activities of GM-CSF were shown to be based on increased DC density in the draining lymph node, and increased

frequency of antigen-specific T cells secreting IFN-ɣ. In addition, GM-CSF has the capacity to increase antigen-induced immune response and to alter the Th1/Th2 cytokine balance[136]. The co-delivery of GM-CSF has been shown to promote priming of potent antigen-specific $CD4^+$ T cell responses followed by increased migration. Santana and colleagues showed that pGM-CSF co-immunization improved both the magnitude and IFNɣ-production of the $CD4^+$ T cell response to HIV-vaccination[137]. Of note, also the capacity of GM-CSF to stimulate immunity is dependent on the relative timing of injection. Likewise IL-2, the administration time of GM-CSF-encoding plasmid relative to the time of antigen administration affects the type of the elicited immune responses[125]. Kusakabe and colleagues showed that injection of GM-CSF encoded by pDNA three days prior to DNA vaccination induced mainly Th2 immune responses and increased IgG levels, whereas the injection of GM-CSF three days after DNA vaccine administration, enhanced Th1 immunity[138]. Importantly, these findings seem to differ dependent on the setting as Barouch and colleagues showed rather reduced humoral responses when injecting GM-CSF-encoding DNA five days before the DNA vaccine[134].

2.2 The immunogenicity of mRNA vaccines

Likewise pDNA vaccination, the immunogenicity of RNA vaccines is established on one hand by the intrinsic adjuvant character of the RNA molecule and on the other hand by the applied delivery method. In this chapter we will describe how both features affect the immune stimulatory character of mRNA vaccines and how the immunogenicity can be further enhanced by the co-delivery of co-stimulatory molecules.

2.2.1 Intrinsic adjuvant characteristics

The innate immune activating capacity of RNA is based on their interaction with intracellular PRRs, resulting in the expression of pro-inflammatory cytokines or inflammasome activation **(Figure 4).** The first identified and best characterized group of PRRs are the membrane-bound TLRs[139]. In the endosomal compartments, TLR3 and TLR7/ TLR8 respond to double-stranded and single-stranded RNA, respectively. TLR3 signals via the TIR domain-containing adaptor inducing IFN-β (TRIF) pathway. The TRIF pathway signals mainly through tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3) and TRAF6. TRAF activation leads to the formation of a complex composed of IKKε and TRAF family-associated NF-kB activator (TANK)-binding kinase-1

(TBK1), to activate the interferon regulatory factor 3 (IRF3) and IRF7 transcription factors (**Figure 4**). Sensing of RNA by the TLR7 receptor activates NF-κB downstream the myeloid differentiation primary response-88 (MyD88) pathway. In pDCs, MyD88 signalling upon the activation of TLR7 and TLR8 leads to the IRF7 dependent expression of type I IFNs[118,140]. It has been shown for both protamine- and lipoplex-based vaccines to support immune stimulation via TLR7-dependent type I IFN induction[77,78]. Further, Wagner and colleagues showed that simultaneous activation of both TLR3 an TLR7 signalling cascades synergized with respect to DC activation and induce superior cytotoxic T cell responses *in vivo*[141].

Less research is reported about the activation of cytosolic receptors upon IVT mRNA vaccination. To our knowledge, only nucleic binding oligomerization domain-like (NOD)-like receptors (NLRs) have been described to sense IVT mRNA, namely the NLRP3 and NOD2[142–144]. They interact both with cytosolic dsRNA and signal through the adaptor molecule ASC to form a complex with caspase-1[145]. The activation of caspase-1 mediates catalytic cleavage of proIL-β and proIL-18 in order to create the functional pro-inflammatory IL-β and IL-18 **(Figure 4).** Caspase-1-mediated pyroptosis can potentially increase the vaccination efficacy as cell lysis results in the release of the produced antigens in the extracellular space. Furthermore, cross-presentation will occur upon uptake of the apoptotic antigen-containing cells by freshly recruited APCs.

It is highly assumable that IVT mRNA might be detected by cytosolic RNA-specific aspartate-glutamate-any amino acid-aspartate/histidine (DExD/H)-box helicases, such as retinoic acid-inducible gene- I (RIG-I) and melanoma differentiation–associated protein 5 (MDA5) receptors[146–148] **(Figure 4)**. Although the binding criteria for RIG-I are not fully clear yet, it appears to recognise an uncapped triphosphate group at the 5' end[149]. As IVT mRNA solutions consist of partially uncapped RNA molecules, IVT mRNA might be a possible target of RIG-I. The cytosolic RNA sensors RIG-I and MDA5 rely on the adaptor mitochondrial antiviral signalling protein (MAVS; or IPS1) for the activation of the IRF3, IRF7 and NF-κB transcription factors in order to express pro-inflammatory cytokines. Downstream MyD88, TRIF and the cytosolic signalling pathways, the phosphorylated and activated IRF3 and IRF7 transcription factors translocate to the nucleus to regulate the expression of type I IFN genes. NF-κB binds to the promoter region of other pro-inflammatory cytokines, like IL-6, IL-12 and TNFα[79,150] . These inflammatory cytokines are forming the basis of the immunogenicity of RNA vaccines, as they are crucial for DC activation, T cell proliferation and differentiation. To be precise, besides RIG-I

and MDA5, other (DExD/H)-box helicases DDX1, DDX21 and DHX36 were reported to be triggered by polyI:C **(Figure 4)**[151].

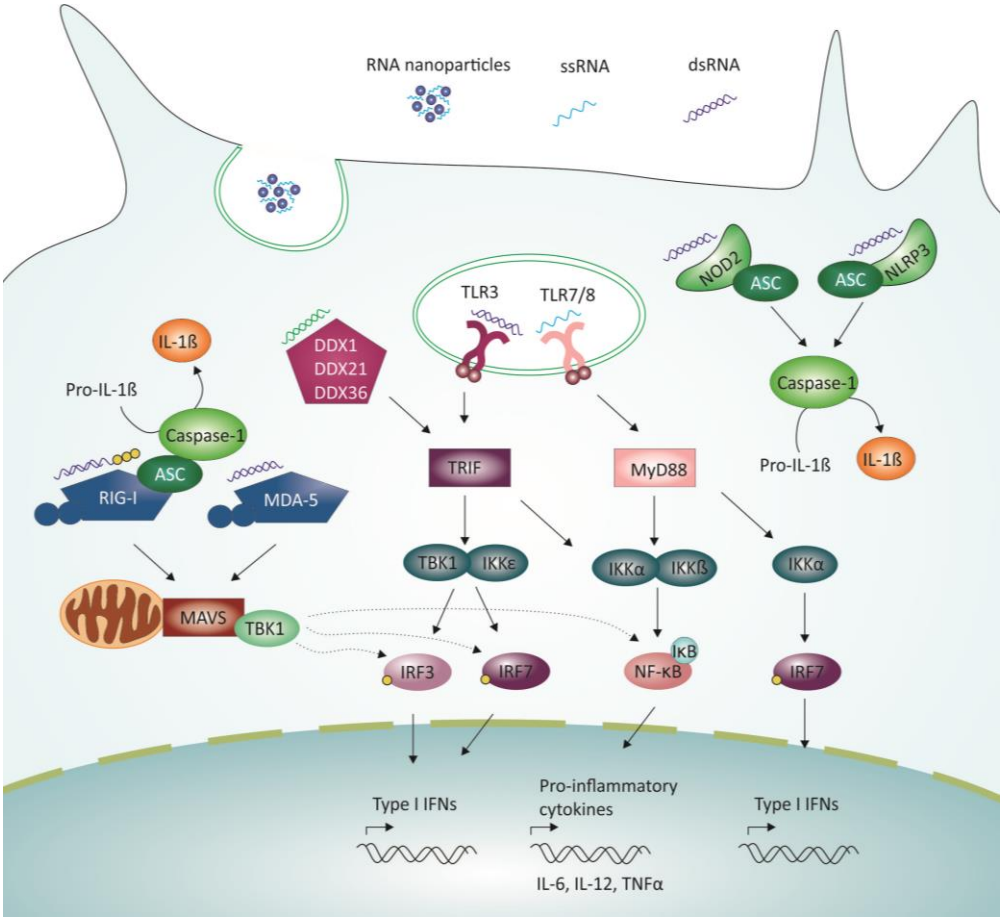

Figure 4. Innate sensing of RNA sequences via different pattern recognition receptors. RNA-specific PRRs include TLR3 and TLR7/8 in the endosome and RIG-I and MDA5 in the cytosol. Activation leads downstream phosphorylation and activation of transcription factors IRF3, IRF7 and NFκB which bind to the promoter region of type I IFN and other pro-inflammatory genes. Activation of the NOD-like receptors lead to the maturation of pro-Ilβ and pro-IL-18. ssRNA, single stranded RNA; dsRNA, dubble stranded RNA; TLR, Toll-like receptor; NOD2, nucleic-binding oligomerization domain-like receptor 2; RIG-I, retinoic acid-inducible gene- I; MDA5, melanoma differentiation – associated protein 5, MAVS, mitochondrial antiviral signalling protein; TBK1, TRAF family-associated NF-kB activator (TANK)-binding kinase-1. TRIF, TIR domain-containing adaptor inducing IFN-β; MyD88, myeloid differentiation factor-88; IRF, interferon regulatory factor; NLRP3, Nacht, LRR, PYD domain containing protein. Figure based on[150].

2.2.2 Improving the immunogenicity of mRNA vaccines

As mentioned above, mRNA sequences feature an intrinsic adjuvant character as they do activate PPRs in the endosomal and cytosolic compartments. Nevertheless, injecting uncomplexed mRNA seems to be insufficient to efficiently activate and mature DCs[92]. Nanoparticle-mediated delivery affects the immunogenicity of mRNA vaccines due to their ability to target the mRNA cargo to the endosomes where it can bind efficiently to TLRs. Indeed, Scheel and colleagues claimed that a mixture of partially protamine-complexed RNA is most optimal for immunization as free mRNA is responsible for the expression of the antigen and complexed RNA is needed for innate activation via triggering of endosomal TLRs[84]. Furthermore, the nanoparticle material itself may also cause immunity. This observation has been made by Chen and colleagues, who reported the upregulation of CD80 and CD86 co-stimulatory molecules upon DOTAP treatment of DCs in absence of nucleic acids or CpG motifs[155]. The researchers claimed that the cationic lipid DOTAP alone serves as an efficient vaccine, based on the activation of mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/ERK), chemokine induction and reactive oxygen species (ROS)[155]. However, we showed that the injection of DOTAP liposomes in the absence of nucleic acids induces no pro-inflammatory cytokines[75,76].

To improve the immunogenicity of mRNA vaccines, the complexation of mRNA into nanoparticles is often combined with the co-delivery of additional adjuvants, such as cytokines and co-stimulatory molecules. In the most elegant way, these adjuvant molecules are encoded by mRNA rather than being added as a protein. A first study about the co-delivery of mRNA encoded adjuvants showed stimulatory effects of GM-CSF, IL-2 and CD80 on evoked $CD8^+$ T cell responses. Nevertheless, despite the enhanced CTL responses, the formulation appeared to be insufficient to overcome tolerance in a tumor model[74].

The TriMix adjuvant consists mRNA encoding three adjuvant components; namely CD40 ligand, CD70 and constitutive active (ca)TLR4[97]. The potential of TriMix vaccines can be explained by the individual role of the co-stimulatory molecules in the activation of innate immunity. First, the CD40 receptor is expressed on DCs and interacts with the CD40 ligand on $CD4^+$ T cell surfaces. Simulating DCs with soluble CD40 ligand, induces co-stimulatory molecules and cytokines[156]. Signalling via CD40-CD40 ligand results in Th1 activation of $CD4^+$ T cells, which enforce the generation of primary $CD8^+$ T cell responses. Furthermore, it has been proved for peptide and

protein-based vaccines that the co-delivery of CD40 ligand-stimulating antibodies results in enhanced tumor protection due to improved $CD4^+$ and $CD8^+$ T cell responses[157,158]. Next, the co-stimulatory molecule CD70 is located on B cells, T cells and APCs and interacts with CD27, which is expressed on activated B cells and a subset of memory T cells[97,159]. Mice studies revealed an important role for CD70-CD27 signalling in the expansion of $CD8^+$ T cells and formation of effector and memory immunity[159,160]. At last, LPS-triggered TLR4 signalling has been proven to mature DCs and enhance specific T cell responses by blocking regulatory T cell activity[161]. Unfortunately, due to its high toxic character, LPS-based adjuvants are not suitable for clinical use. For this reason, the expression of a constitutive active TLR4 receptor instead of the administration of LPS constitues a safer alternative[162].

Earlier, TriMix has been evaluated as in a clinical trial via *in vitro* electroporation of DCs (TriMixDC-MEL), followed by intradermal[99] and later mixed intradermal and intravenous transfer to melanoma patients[163]. In here, DCs were *ex vivo* electroporated with mRNA encoding multiple tumor associated antigens (MAGE-A3, MAGE-C2, tyrosinase and gp100) and TriMix adjuvant. The latter study revealed objective tumor responses in 4 out of 15 patients (27%). Two of these patients showed complete responses and two patients showed partial responses. These four tumor responders, and one patient with a disease stabilization, were progression free at least 28 months after the treatment[163,164]. Skin infiltrating T lymphocytes were observed in 6 out of 12 patients, whereas tumor-specific $CD8^+$ T cells were detected in the blood of 4 out of 5 studied patients. Although these clinical validation proved that TriMixDC-MEL is safe and results in clinical responses, the development of a patient-specific vaccine is expensive and therefore direct administration of mRNA offers a big advantage. In a more recent study, TriMix was evaluated upon direct intranodal immunization.

However, the development of a patient-specific vaccine is expensive and therefore direct administration of mRNA offers a big advantage. In a more recent study, TriMix was evaluated upon direct intranodal immunization. In here they showed that the uptake and translation of antigens was inhibited by the co-delivery of classical maturation stimuli, like MPL, LPS and PolyI:C, whereas TriMix did not inhibited uptake and translation[97]. This success is associated with the relative timing of antigen uptake and adjuvant activity. It is generally accepted that the maturation of DCs abrogates uptake and translation of exogenous mRNA. In here, the adjuvant needs to be translated to become functional, thus after antigen uptake and translation, causing

a delay of DC activation, allowing the antigen-encoding mRNA to be efficiently engulfed and translated.

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CHAPTER 3.

THE JANUS FACE OF TYPE I INTERFERONS UPON NUCLEIC ACID-BASED VACCINATION

The Janus face of type I IFNs upon nucleic acid-based vaccination

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Abstract: Nucleic acid-based vaccines show superior potency to induce cytolytic T cell responses to destroy malignant or infected cells. The intrinsic adjuvant character of nucleic acid-based vaccines appears to be based on their capacity to evoke type I IFNs. However, recently opposing results have been reported about the role of type I interferons in regulating T cell immunity to nucleic acid-based vaccines. In this review, we will attempt to reconcile recent discrepant observations regarding the role of type I IFNs in steering T cell immunity to mRNA vaccination. Moreover, we will discuss the growing body of evidence indicating that the impact of type I IFN induction on T cell immunity is determined by its kinetics of induction and its anatomical distribution.

1. INTRODUCTION

Over the past decade, interest in using nucleic acids as a mean to encode antigenic information or as adjuvant for protein vaccines has exponentially boomed. Vaccines that use nucleic acids to encode antigenic information display a far superior potential to elicit cytolytic T cell responses compared to protein-based vaccines and are thus intensively explored to combat cancer[1,2]. Whereas historically the nucleic acid vaccination field has been dominated by pDNA, mRNA vaccines have now clearly taken the lead with numerous clinical trials ongoing[3–6]. The paradigm shift from pDNA to mRNA as means to deliver antigenic information has been driven mainly by the superior safety profile of mRNA alongside technical breakthroughs that have improved the *in vivo* stability of IVT mRNAs[7,8]. Non-coding nucleic acids and their synthetic analogues in turn have been explored as adjuvants for protein vaccines and as immunotherapeutics for cancer due to their capacity to skew $CD4^+$ T cell responses towards a Th1 oriented phenotype[9–11].

Overall, the efficacy of any vaccine to instigate T cell immunity is determined by the early interplay between vaccine and innate immune cells and the nature of the inflammatory environment hereby created. In terms of the inflammatory environment they produce, all nucleic acid containing vaccines have a common theme: the generation of a potent type I IFN response[11–14]. Type I IFNs are highly pleiotropic cytokines that on top of their well-known direct antiviral effects also profoundly impact T cell responses. The consequences of type I IFNs on T cell responses are highly diverse and can be both stimulatory and inhibitory. Much akin to the role of type I IFNs in the context of infectious disease, the effects of type I IFNs on the T cell response elicited by nucleic acid-based vaccines are complex and context dependent. In this review, we will elaborate on the functional relevance of type I IFN induction on the vaccine elicited T cell response. Important discrepancies between pDNA and mRNA-based vaccines will be highlighted. In addition, we will attempt to reconcile recent conflicting observations regarding the role of type I IFNs in steering T cell immunity to mRNA vaccination. Finally, we will discuss the growing body of evidence indicating that the impact of type I IFN induction on T cell immunity is determined by its kinetics of induction and its anatomical distribution.

2. THE OMNIPOTENT TYPE I IFNS: LESSONS LEARNT FROM CANCER AND INFECTIOUS DISEASE

The type I IFN family is composed of multiple IFNα variants, IFNβ and the poorly characterized IFNε, IFNτ, IFNκ, IFNω, IFNδ and IFNζ. Type I IFN production is triggered upon sensing of microbial nucleic acids by various pattern recognition PRRs located in endosomal membranes and the cytosol. All type I IFNs signal through the heterodimeric IFNα receptor (IFNAR) composed of IFNAR1 and IFNAR2, except for IFNβ, which binds to IFNAR1 homodimers[15]. IFNα/β binding to their transmembrane receptor leads to the activation of the janus kinase (JAK) – signal transducers and activators of transcription (STAT) pathway[16–18], which induces the transcription of interferon stimulated genes (ISGs) (***Box 1***)[16,19].

Although type I IFNs originally were described as host components interfering with viral replication[20], it became evident that type I IFNs also regulate anti-viral T cell responses through modulation of DC function or through direct signalling in T cells, thus modulating T cell expansion, differentiation and survival. Over the past years, type I IFNs have been identified as critical host factors that instigate anti-tumor T cell immunity.

BOX 1: **IFNAR signalling; creating broad variety of outcomes**

The canonical type I IFN signalling pathway is the JAK-STAT pathway, named after the involved Janus kinases (JAKs), which phosphorylate signal transducers and activators of transcription (STATs). The type I IFNs receptor IFNAR is composed of a low affinity IFNAR1 and a high affinity IFNAR2. The IFNAR1 subunit receptor interacts with tyrosinase kinase (Tyk2), whereas the IFNAR2 subunit interacts with JAK1. The activation of JAK kinase results in the recruitment, the phosphorylation and dimerization of STAT proteins, controlling different gene-expression programmes. The first STAT dimer is composed of STAT1 and STAT2, which binds with IRF9 to form the interferon-stimulated gene factor 3 (ISGF3) complex. This complex will translocate to the nucleus and bind to the IFNs-stimulated response elements (ISREs) to initiate transcription of interferon-stimulated genes (ISGs). Furthermore, also STAT1-STAT2 homodimers are formed in response of IFNα. They bind to gamma-activated sequences (GASs) for the transcription of pro-inflammatory genes. In total, six different STAT proteins have been described, which means a great variety of homo- and hetero-dimer STAT complexes can be formed in response to type I IFNs[21–25]. This combination of different STAT complexes in turn will determine the transcriptional and functional consequences of type I IFNs. Moreover, it is becoming increasingly evident that activation of the JAK-STAT pathway is not sufficient for the transcription of all type IFNs inducible genes. In fact, many 'alternative' pathways are activated by IFNAR signalling. Some of these pathways interact with the different STATs, but others function fully independent of STAT proteins[26].

2.1 TYPE I IFNS REGULATE DENDRITIC CELL FUNCTION

DCs are the most potent antigen presenting cells and have a unique potential to activate naïve T cells. Type I IFNs influence multiple aspects of DC function including antigen presentation, co-stimulation, cytokine secretion and DC turnover. Treatment of *in vitro* differentiated DCs with type I IFNs upregulates co-stimulatory ligands and promotes antigen presentation **(Figure 5)**[27–31]. The strongest evidence that type I IFNs act through DCs to promote T cell immunity derives from the cancer immunology field. DNA released from dying tumor cells has been shown to result in the release of IFNβ from tumor cells and DCs in a stimulator of IFN genes (STING) dependent fashion[32,33]. In 2011, the Schreider and Gajewski labs independently demonstrated that spontaneous T cell responses against tumors strictly rely on IFNAR triggering in the $CD8\alpha^+$ DC subset[34,35]. Ever since, the vital role of type I IFNs in mediating anti-tumor T cell responses has been extended to various cancer treatment modalities, including radiotherapy, chemotherapy and intra-tumoral injections with nucleic acids[36,37]. Type I IFNs have also the ability to regulate DC turnover. Strong type I IFN responses elicited upon systemic viral infection or upon injection with TLR agonists inflict apoptosis in plasmacytoid DCs (pDCs)[38] and $CD8\alpha^+$ DCs[39]. Such transient DC loss might be a mean to avoid excessive immune activation by dampening T cell immunity. Conversely, antigen-containing apoptotic DCs may be efficiently cross-presented by newly recruited DCs and thereby elicit T cell immunity. With the consequences of DC apoptosis still being a matter of debate, clear-cut cases have been described where the actions of type I IFNs on DCs are malicious to T cell immunity. Bacterial sepsis has been shown to cause a profound type I IFN release that blocks antigen presentation by DCs and thereby weakens T cell responses[40]. Also, infection with Plasmodium parasites curtails DC function and the priming of Th1 immune responses. T cell immunity in this setting was restored in mice lacking IFNAR on DCs. Chronic infection with LCMV causing a sustained expression of type I IFNs similarly imposes an immune suppressive phenotype in DCs, a feature that could be reversed by treatment with IFNAR blocking antibodies restored DC function and improved T cell immunity [41].

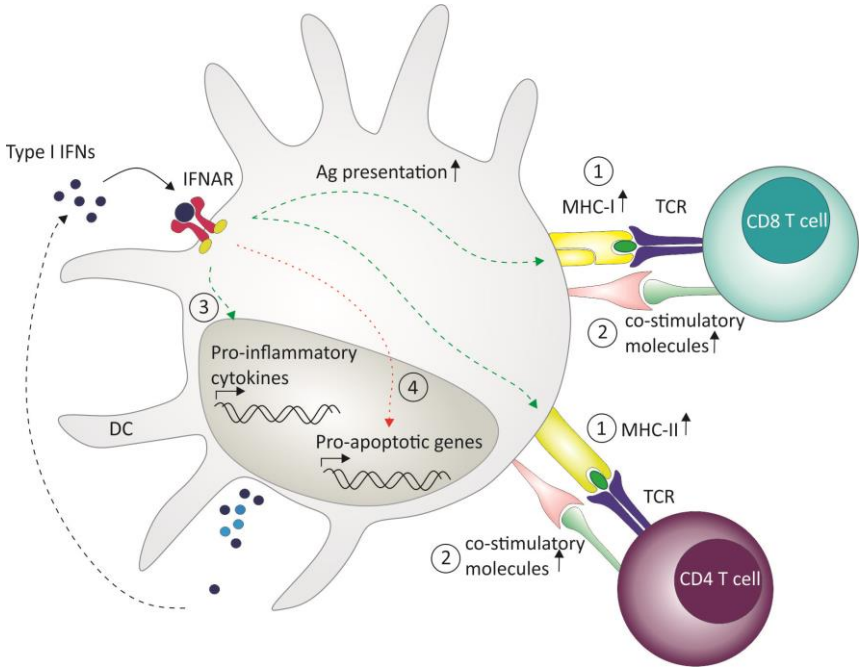

Figure 5. Type I IFNs regulate DC function. IFNα/β binding on IFNAR receptor promotes antigen presentation by the upregulation of major histocompatibility molecules-I and –II (1) and the expression of co-stimulatory ligands (2). Further, they will also stimulate the expression of pro-inflammatory cytokines, including type I IFNs (3). Strong type I IFN responses inflict apoptosis by enhancing the expression of pro-apoptotic genes (4). *Ag, antigen; DC, dendritic cell; IFN, interferons; IFNAR, IFNα/β receptor; MHC major histocompatibility complex; TCR, T cell receptor*

2.2 TYPE I IFNS REGULATE T CELL FUNCTION: A MATTER OF TIMING?

The complex consequences of type I IFN signalling in $CD8^+$ T cells in response to viral infection have been extensively reviewed by Crouse[42]. In brief, type I IFNs can exert either stimulatory or inhibitory actions on antigen experienced T cells dependent on the timing of IFNAR signalling relative to TCR signalling. If TCR signalling shortly precedes or co-incides with IFNAR signalling, type I IFNs function as true signal 3 cytokines promoting the expansion and subsequent differentiation of antigen primed $CD8^+$ T cells into cytolytic effector cells[43–45]. Proof that type I IFNs also suport memory T cell formation is less strong. In LCMV infected mice, type I IFNs promote the expansion of antiviral $CD8^+$ T cells by increasing their survival, which might enlarge the final memory T cell pool generated. Nonetheless, high levels of type I IFNs might drive antigen primed T cell into terminally differentiated effector cells rather than into long-lived

memory T cells[46]. In case IFNAR signalling precedes TCR signalling, the inhibitory properties of type IFNs on T cell immunity seem to prevail[21,23,47]. Out of sequence exposure of T cells to type I IFNs imposes an anti-proliferative status[21,23,47] and activates a pro-apoptotic program[48–50]. On a molecular level, this differential outcome can be traced back to a distinct profile of STATs that get activated **(Figure 6)**. Upon TCR stimulation, $CD8^+$ T cells upregulate STAT4 whilst maintaining low levels of STAT1. Subsequent IFNAR triggering mainly activates STAT4, resulting in a proliferative and anti-apoptotic program. If IFNAR signalling precedes TCR stimulation, signalling through STAT1 dominates, instigating a pro-inflammatory yet anti-proliferative and pro-apoptotic program[23,25,51,52].

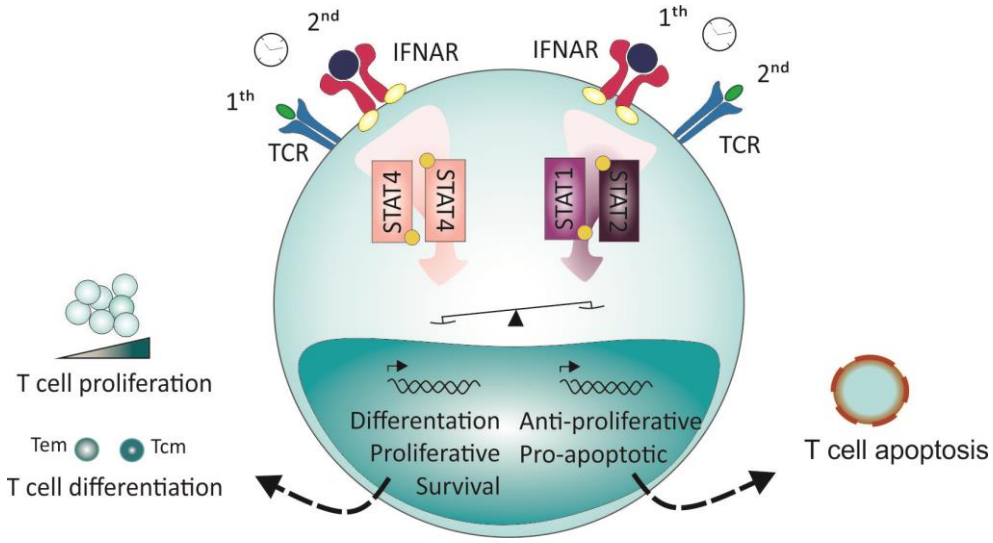

Figure 6. Type I IFNs regulate T cell function. In case T cell receptor signalling precedes with type I IFN signalling, type I IFNs may function as signal 3 cytokines, promoting expansion and subsequent differentiation of $CD8^+$ T by expression a specific cluster of genes, regulated by STAT4 activation (left). Signalling of type I IFNs prior to T cell receptor activation imposes an anti-proliferative status and activates a pro-apoptotic program, characterized by STAT1 activation (right); *TCR, T cell receptor; IFNAR, IFNα/β receptor; STAT, signal transductors and activators of transcription*. Figure based on[28].

3. TYPE I IFNS AND THE REGULATION T CELL IMMUNITY TO ANTIGEN ENCODING NUCLEIC ACID VACCINES

3.1 TYPE I IFNS AND PDNA VACCINES: A SETTLED CASE?

Administration of pDNA vaccines is characterized by high levels of type I IFNs[53–55]. In a pioneering study, Tudor *et al.* demonstrated that ifnar$^{-/-}$ mice respond to DNA vaccination with lower IgG2a/IgG1 antibody ratios and weaker CD8^{+} T cell responses[55]. These findings were confirmed by other reports showing that DNA vaccinated ifnar$^{-/-}$ mice fail to generate cytolytic T cell responses[55–57]. Although the beneficial role of type I IFNs in pDNA immunogenicity is uncontested, the underlying PRRs and signaling pathways are still a matter of debate. Over 20 years ago, TLR9 was the first PRR described to recognize pDNA vaccines[58]. Triggering of TLR9 by unmethylated CpG motifs in the pDNA vector induces a MyD88 dependent signaling cascade that culminates in the release of inflammatory cytokines including type I IFNs[59–61]. Nonetheless, pDNA vaccinated Tlr9$^{-/-}$and MyD88$^{-/-}$ mice mount adaptive immune responses comparable to wild type mice pDNA vaccination, arguing against a major contribution of TLR9[62]. Instead, pDNA immunogenicity appears to largely rely on DNA sensing by cytosolic DNA sensors that activate type I IFN responses via the stimulator of IFN genes (STING) and the non-canonical IkB kinase, TANK binding kinase-1 (TBK1)[63,64]. The exact nature of the initial cytosolic sensor mediating DNA recognition remains ill defined. Multiple proteins have been described as cytosolic DNA sensors that activate STING, including DAI[65], cGAS[66] and Ifi16[53], but none of these sensors has been conclusively linked to pDNA immunogenicity. Also inflammasomes have been implicated in steering pDNA immunogenicity through modulation of cell death. Mice lacking AIM2 inflammasomes display decreased numbers of IFNγ secreting T cells upon pDNA vaccination against Influenza HA[67,68]. This effect was shown to be independent of the caspase1 targets IL-1β and IL-18. Moreover, Aim2$^{-/-}$ mice showed decreased levels of IFNα/β at the immunization site[67]. Although suggestive, the authors did not address whether this deficit in type I IFN production is at the basis of the decreased adaptive immune response. The failure to narrow pDNA immunogenicity down to a single cytosolic DNA sensor best agrees with a mechanism in which multiple cytosolic DNA sensors coexist that all can recognize the vaccine pDNA and induce type I IFNs[63]. In such a setting, knocking down a single DNA sensor will not strongly affect pDNA immunogenicity, as other DNA sensors will compensate for its function.

3.2 Regulation of T cell immunity to mRNA vaccines: type I IFNs show their Janus face

Like pDNA vaccines, mRNA vaccines elicit profound type I IFN responses[12–14]. Unlike the unisonous reports on the beneficial impact of type I IFNs on pDNA vaccines, the role of type I IFNs in regulating T cell immunity to mRNA vaccines is however ambivalent. Earlier, type I IFNs were shown to negatively interfere with the induction of IFNγ-secreting T cells upon subcutaneous immunization with Gag-encoding mRNA lipoplexes[69]. More recently, these observations were extended to intradermal and intranodal vaccination with mRNA lipoplexes[14]. Immunization studies in Ifnar$^{-/-}$ mice revealed an increased initial priming of vaccine-specific CD8^{+} T cells in the absence of IFNAR signalling. Furthermore, vaccine-primed Ifnar$^{-/-}$ T cells acquired full cytolytic effector function. Interference with type I IFN signalling through co-administration of an IFNAR blocking antibody further confirmed the finding made with Ifnar$^{-/-}$ mice, thus resulting in amplified cytolytic T cell responses and an increased vaccine-elicited melanoma control[14]. These findings are in sharp contrast with two recent studies which have demonstrated that type I IFNs actually promote T cell immunity to systemic immunization with mRNA lipoplex vaccines. Kranz *et al*. observed a profound secretion of type I IFNs by splenic pDCs and macrophages upon intravenous injection of mRNA lipoplex vaccines [12]. Preventing IFNAR signalling by the administration of an IFNAR blocking antibody, did not affect the magnitude of the vaccine antigen-specific CD8^{+} T cell response. Yet, CD8^{+} T cells primed under IFNAR deficient conditions failed to acquire effector function indicating type I IFNs act as true signal 3 cytokines in this setting. In line with these findings, Broos and colleagues independently identified the vaccine mRNA elicited type I IFNs as necessary for instigating cytolytic T cell responses upon intravenous immunization with mRNA lipoplexes[13].

Reconciling these discrepancies - type I IFNs counteracting versus supporting cytolytic T cell responses to the vaccine mRNA-encoded antigen – is a challenging task **(Figure 7)**. Whereas differences in the exact composition of the mRNA lipoplexes applied may be involved, the main discriminating factor between the aforementioned studies clearly is the route of vaccine administration: local versus systemic. Without doubt, the different routes of vaccination affect the type of cells that encounter the vaccine, express the encoded antigen and secrete type I IFNs. Upon intradermal mRNA injection, mRNA gets ingested by non-leukocytes and by DCs[70]. Upon intravenous injection, DCs are the dominant cell type internalizing and expressing the

mRNA[12,13]. The route of lipoplex administration also alters the sequence of antigen expression and type I IFN secretion. Broos *et al.* showed peak antigen expression levels in splenic DCs already at one hour post intravenous injection, meaning antigen presentation and TCR stimulation can occur very rapidly[13]. IFNα titres in serum became detectable at two hours and peaked at six hours. Similar kinetics of IFNα induction were reported in the parallel study by the Sahin lab[12]. After intradermal administration of mRNA lipoplexes, we found that type I IFNs peak with similar kinetics but antigen expression now takes longer to unfold. In addition, upon intradermal injection, skin DCs that take up the mRNA lipoplexes need to migrate to the draining lymph node in order to present the antigen[70]. The bulk of antigen presentation is thus likely occured after T cell exposure to type I IFNs. Addressing the functional relevance of these differential kinetics will require experimental effort, but one may speculate that intravenous injection causes concomitant T cell stimulatory TCR and IFNAR signalling whereas intradermal injection rather results in a T cell inhibitory out-of-sequence signalling.

In contrast to pDNA vaccines, TLR recognition appears to be indispensable for the efficacy of mRNA vaccines. Similar to viral ssRNA, IVT mRNA complexed to protamine or to cationic liposomes activates TLR7/8[12,71,72]. $Tlr7^{-/-}$ mice showed reduced but not totally abrogated IFNα titers upon intravenous injection of mRNA lipoplexes[12]. TLR3, which recognizes dsRNA, was not implicated in IFNα production. Unfortunately, no data on the quality and magnitude of the cytolytic T cell response in mRNA vaccinated $Tlr7^{-/-}$ mice have been reported. As IFNα was still induced in $Tlr7^{-/-}$ mice – albeit to a much lower level – additional RNA sensors must be involved in the recognition of mRNA lipoplexes. As IVT mRNAs mimic in many aspects viral ssRNAs, also cytosolic RNA sensors are probably implicated in the type I IFN response to mRNA vaccines. Nonetheless, firm experimental data to support a role for cytosolic RNA sensors in the type I IFN response or in the regulation of the ensuing T cell response upon vaccination are currently lacking.

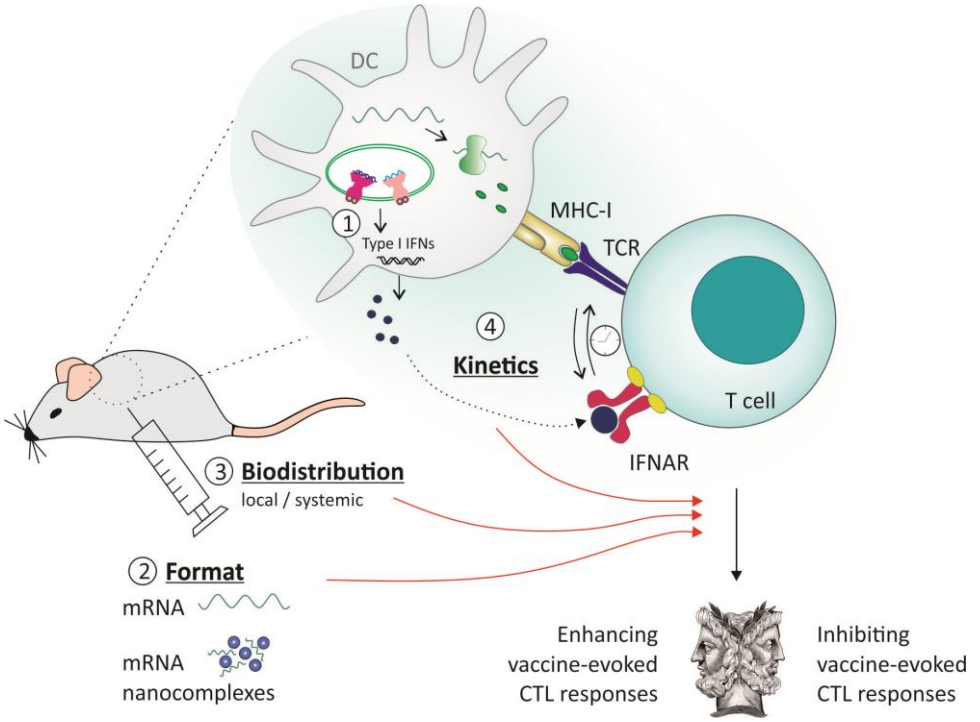

Figure 7: Parameters that govern type I IFN- mediated regulation of T cell immunity to mRNA-based vaccines. mRNA vaccines elicit profound type I IFN responses upon pattern recognition receptor activation (1). The role of type I IFNs in regulating T cell immunity to mRNA vaccines is ambivalent, illustrated here by the Janus face. Plausible causes may be the composition of the mRNA nanoformulations (2) or the route of injection, which is directly linked to the bio distribution of evoked type I IFNs (3). Bio distribution might influence the relative timing of T cell receptor activation and type I IFN signalling (4), directly leading to the activation of different T cell gene clusters resulting in pro-apoptotic processes or pro-proliferative and differentiating programmes.

3.3 Non-coding nucleic acids as adjuvants and immunotherapeutics: keeping type I IFNs local?

Non-coding nucleic acids promote Th1 and cytolytic T cell responses and are intensively evaluated as adjuvants for protein vaccines or as immunotherapeutics for cancer treatment. Oligo's containing unmethylated CpG motifs trigger TLR9 and potently stimulate IFNα secretion by pDCs. PolyI:C and polyA:U are synthetic dsRNA that trigger TLR3 in the endosomes. In addition, polyI:C is also recognized by melanoma differentiation associated protein (MDA5) in the cytoplasm. As an adjuvants for protein vaccines, polyI:C induces durable Th1 $CD4^+$ T cell and

cytolytic $CD8^+$ T cell responses, which depend on type I IFN secretion by hematopoietic and by stromal cells[31]. Direct IFNAR signaling was found to be crucial for DC activation. Expansion of $CD8^+$ T cells to primary immunization with soluble antigens and polyI:C depended largely on TLR3 activation in DCs, although MDA5 also contributed to effector T cell differentiation. Remarkably, MDA5 activation and type I IFN secretion by stromal cells were found crucial to boost memory $CD8^+$ T cell formation[73]. Intratumoral injection of polyI:C promotes anti-tumor immunity in an IFNAR dependent way, which can be further improved by injection of FMS-like tyrosine kinase 3 ligand (Flt3L) that recruits DCs to the tumor bed[37]. Similarly, intra-tumoral injection of polyA:U required IFNAR signaling to yield anti-tumor T cell responses[74]. TLR7 and TLR8 naturally recognize ssRNAs present in endosomal compartments and elicit a potent type I IFN response upon activation by their ligands. Synthetic ssRNAs stabilized by a phosphorothioate backbone[75] or by complexation to cationic carriers[76] have been developed as adjuvants that promote Th1 and cytolytic T cell responses against co-delivered antigens. Besides synthetic RNAs, also small molecule components of the imadozoquinoline family (e.g. imiquimod) activate TLR7/8. Topical application of imidazoquinolines into tumors activates pDCs[77] and provokes systemic anti-tumor T cell responses[78]. Direct involvement of IFNAR in this process was demonstrated using IFNα/β blocking serum. Besides TLR agonists, also synthetic cyclic dinucleotides (CDNs) that activate STING have also entered the immunotherapy arena, based on the recognition that triggering of the STING pathway in CD8α+ DCs is required to raise spontaneous anti-tumor immunity[79]. Intra-tumoral injection of these synthetic CDNs was demonstrated to induce tumor rejection in multiple murine tumor models and required IFNAR signaling[80].

Although these nucleic acid-based adjuvants possess the capacity to promote cellular immunity, they suffer from one major drawback: the induction of a systemic type I IFN response that causes inflammatory toxicity and interferes with clinical application. To resolve inflammatory toxicity, various research groups have been formulating nucleic acids or imidazoquinolines in such a way that inflammation is restricted to the injection site and its draining lymph nodes[81–84]. Strikingly, such formulations also dramatically improved the strength of the evoked T cell response. Coupling of the TLR7 agonist imiquimod to nanogels restricted the type I IFN response to the intradermal injection site and its draining lymph node, whereas soluble imiquimod provoked systemic type I IFN responses. When admixed with the *Mycobacterium tuberculosis* antigen PPE44, superior Th1 immunity was elicited using the nanogel coupled imiquimod[11]. A

similar system was developed by Seder group, who showed that polymer coupled imiquimod resulted in a prolonged DC activation in the draining lymph node and in superior adjuvant capacity[83]. Imiquimod formulated as a cream (Aldara) outperforms soluble imiquimod in the priming of melanoma specific $CD8^+$ T cells[85]. Along the same line, particulate polyI:C[86] and CpG[87] outperformed their soluble counterparts in eliciting cytolytic T cells against a co-injected antigen.

The anatomical distribution of type I IFNs (local versus systemic) might be linked to the capacity of these nucleic acid-based adjuvants to promote T cell responses. Remarkably, we demonstrated that type I IFNs do play a beneficial role when RNA-encoding antigens are administered systemically, whereas the administration of nucleic acid adjuvants seemed to benefit from being formulated in order to create a local type I IFN induction and inflammation. Whether and how the presence of local versus systemic type I IFNs alter the expansion and survival of antigen-primed T cells in an apparently opposing way compared to antigen-encoding mRNA, remains to be established. Strong and systemic type I IFNs might drive antigen-primed T cells into the short-lived effector pool rather than into the memory pool, which will be detrimental for the outcome of vaccination[88]. Alternatively, limiting inflammation to the vaccine draining lymph nodes might focus the relevant immune cells to the place antigen resides and where TCR triggering is initiated. Conversely, systemic inflammatory responses will divert resources and immune cells to non-antigen containing lymph nodes.

4. CONCLUDING REMARKS

Nucleic acids-based vaccines and adjuvants have shown great promise in pre-clinical and clinical studies but have yet to fulfil their potential in real clinical practice. To achieve this, improvements in efficacy and safety are required. Deciphering the cellular network and the critical host factors that determine potency and inflammatory toxicity should pave the way for a more rational design of nucleic acid-based vaccines and boost their clinical development. Type I IFNs are the most prominent cytokines elicited by nucleic acid-based vaccines. The impact of type I IFNs on T cell immunity can be beneficial or detrimental, depending on their kinetics of induction, intensity and anatomical distribution. For pDNA vaccines, the outcome of IFNAR signalling is univocally beneficial. For mRNA vaccines, the situation is more ambiguous with type I IFNs exerting profound stimulatory effects upon intravenous injection yet potent inhibitory

effects upon topical injection. The mechanisms behind this differential outcome are still unresolved. Given the important safety issues associated with intravenous injection – which is unseen within the vaccination field – it will be essential to design topically applied mRNA vaccines that are either capable of harnessing the positive effects of type I IFNs or that avoid type I IFN induction and activate the innate immune system in an alternative way. Finally, nucleic acid-based adjuvants and immune-therapeutics clearly require type I IFNs for their immune-potentiating effects. Nonetheless, in this case the capacity of type I IFN responses in promoting T cell immunity might depend on the anatomical distribution of type I IFN induction. Localized type I IFN responses appear to be linked to superior T cell immunity induction when compared to systemic type I IFN responses, but the real causalities between the anatomical distribution of type I IFNs and T cell immunity remain to be established.

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Research Aims

mRNA-based vaccines have a great potential to elicit antigen-specific cytotoxic $CD8^+$ T cell (CTL) responses and therefore are under intensive (pre)clinical investigation. mRNA has the advantage to possess a high safety profile due to a low risk on genomic integration and transient antigen exposure. Nevertheless, for a long time mRNA was considered to be too unstable for direct *in vivo* administration. As a consequence, most applications were based on adoptive transfer of *in vitro* mRNA-modified dendritic cells rather than on direct injection of the mRNA into a patient. However, during the last years, novel encapsulation and targeting techniques came to the forefront, rendering a direct administration of mRNA vaccines a feasible alternative. The overall aim of this thesis therefore was to explore new strategies and mechanisms to increase the efficacy of mRNA-based vaccines, administered directly to the patient.

Previously, our research group has shown that subcutaneous injection of mRNA lipoplexes induces type I IFNs which negatively interfered with the immunogenicity of mRNA lipoplex vaccines **(chapter 4).** In this thesis we further aimed (i) to understand how this vaccine-induced type I IFN response negatively affects the efficacy of the mRNA-based vaccine and (ii) to develop new strategies that may circumvent this negative effect of type I IFNs and thereby increase the strength of mRNA vaccine-elicited CTL responses. Different approaches to reach both goals were tested, based on either modifying the mRNA itself or modifying the mRNA carrier. The impact of modifying the vaccine mRNA, by replacing cytidine and uridine with chemically modified variants, on vaccine efficacy and type I IFN induction, was assayed. The results obtained are described in **chapter 5** and **6** of this thesis. Next to modifying the mRNA, we validated in **chapter 6** a RALA peptide-based delivery format as an alternative to the mRNA lipoplex vaccine approach. Our mechanistic studies not only highlighted the importance of the amphipathic nature of the RALA peptide for the efficacy of the vaccine but also demonstrated the absence of type I IFNs. This absence of inhibiting type I IFNs may underlie the higher efficacy of this RALA peptide-based vaccine strategy compared to the type I IFN-sensitive conventional lipoplex format.

As a last objective of this thesis, we addressed the question whether RNA, besides a role as antigen-encoding device, may also function as a Th1 immunity skewing adjuvant. This proposition was based on several reports describing a role for non-coding RNA in the activation of innate immunity. Here, we aimed to develop a nanoparticulate formulation that not only protects the RNA from degradation, but also efficiently delivers to RNA to lymph node dendritic

cells. We further investigated the efficacy of these RNA complexes as novel adjuvant for the eliciting of cytolytic $CD8^+$ T cell responses against co-delivered protein antigens **(chapter 7).**

RESULTS

CHAPTER 4.

TYPE I INTERFERONS INTERFERE WITH THE CAPACITY OF mRNA LIPOPLEX VACCINES TO ELICIT CYTOLYTIC T CELL RESPONSES.

Type I Interferons Interfere With The Capacity Of mRNA Lipoplex Vaccines To Elicit Cytolytic T Cell Responses.

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Author contributions

A.D.B. designed, performed and analyzed experiments (Fig.2, Fig.4, Fig.5, Fig.S1, Fig.S2, FigA1). C.P. designed, performed and analyzed experiments. S.V.L., L.V.H., K.R., M.D.S., V.K.K., T.N. provided technical assistance. N.S. provided technical equipment, S.L. and S.W. provided mice, X.S., G.V., J.G. designed research and G.V., J.G. supervised the project. S.D.K., designed research and edited the manuscript.

Abstract

Given their high potential to evoke cytolytic T cell responses, tumour antigen-encoding mRNA vaccines are now being intensively explored as therapeutic cancer vaccines. mRNA vaccines clearly benefit from wrapping the mRNA into nano-sized carriers such as lipoplexes that protect the mRNA from degradation and increase its uptake by DCs *in vivo*. Nevertheless, the early innate host factors that regulate the induction of cytolytic T cells to mRNA lipoplex vaccines have remained unresolved. Here, we demonstrate that mRNA lipoplexes induce a potent type I IFN response upon subcutaneous, intradermal and intranodal injection. Regardless of the route of immunization applied, these type I IFNs interfered with the generation of potent cytolytic T cell responses. Most importantly, blocking type I IFN signalling at the site of immunization through the use of an IFNAR blocking antibody greatly enhanced the prophylactic and therapeutic anti-tumour efficacy of mRNA lipoplexes in the highly aggressive B16 melanoma model. As type I IFN induction appears to be inherent to the mRNA itself rather than to unique properties of the mRNA lipoplex formulation, preventing type I IFN induction and/or IFNAR signalling at the site of immunization might constitute a widely applicable strategy to improve the potency of mRNA vaccination.

1. INTRODUCTION

The induction of strong cytolytic $CD8^+$ T cell responses capable of killing transformed cells is considered vital for the success of therapeutic cancer vaccines[1]. As $CD8^+$ T cells guard the intracellular proteome, their efficient induction typically requires the presence of antigens in the cellular cytosol, where they can enter the classical route of proteasome degradation and MHC-I mediated antigen presentation. In contrast to protein based vaccines, vaccines based on messenger RNA (mRNA) enable protein expression inside the cytosol of transfected cells and thus show great potential to evoke cytotolytic T cell responses[2]. Due to the limited stability of early *in vitro* transcribed (IVT) mRNAs, mRNA vaccines have been predominantly delivered in the format of *ex vivo* electroporated DCs for most of the time[3]. Over the past years, technical improvements in the way IVT mRNA is prepared (5' Cap modifications, optimized GC content, improved polyA tails, stabilizing UTRs) have increased the stability of IVT mRNA to such extent protein expression can now be achieved for days after direct *in vivo* administration of the mRNA[4-6]. These breakthroughs have revolutionized the mRNA vaccination allowing direct

injection of antigen-encoding mRNA to be explored for the treatment of patients with prostate cancer, non-small lung cell carcinoma and melanoma[7-13].

When applied directly *in vivo*, mRNA vaccines strongly benefit from wrapping the mRNA into nano-sized carriers. Within this context, our group previously demonstrated that condensing mRNA into cationic lipoplexes increases the potency of the mRNA vaccine evoked T cell response by several orders of magnitude[14]. One of the typical hallmarks of IVT mRNAs condensed into nano-formulations is their capacity to elicit intense secretion of Type I IFNs in murine and human DCs[14,15]. Indeed, IVT mRNA appears to mimic viral RNA in its capacity to trigger a variety of cellular endosomal and cytosolic RNA sensors that all induce a signalling cascade culminating in the release of type I IFNs[14-17]. Type I IFNs are highly pleiotropic cytokines that can either promote or inhibit T cell responses dependent on the context. Type I IFNs can augment T cell immunity by activating DCs and increasing antigen presentation.

Conversely, the antiviral actions of type I IFNs - production of RNAses and instigation of translation arrest – might interfere with the expression of the mRNA encoded antigen and therefore negatively impact T cell immunity. Type I IFN signalling on antigen experienced T cells can promote T cell proliferation, survival and differentiation into effector cells[18]. Nevertheless, type I IFN exposure prior to T cell receptor activation can induce anti-proliferative and apoptotic programmes in T cells[18-21]. How type I IFNs impact the characteristics of the T cell responses to mRNA lipoplex vaccines and their efficacy to control tumour growth is therefore far from forgone conclusion and constitutes the main goal of this study.

Using an IFN-β reporter mouse strain, we were able to demonstrate that mRNA lipoplexes instigate profound type I IFN responses upon subcutaneous, intradermal and intranodal injection. In sharp contrast to the beneficial role of type I IFNs in protein and peptide based vaccines[22-25], type I IFNs severely hampered priming of vaccine specific T cell responses and the generation of anti-tumour immunity to lipoplex based mRNA vaccination. Preventing type I IFN induced signalling through co-administration of an IFNAR blocking antibody at the site of mRNA vaccination amplified the cytotolytic T cell response and significantly strengthened vaccine elicited tumour control in prophylactic and therapeutic settings.

2. RESULTS

2.1. MRNA LIPOPLEXES INDUCE A POTENT TYPE I IFN RESPONSE *IN VIVO*

Cationic liposomes have been reported to increase T cell responses to mRNA encoded antigens[26]. In this study, liposomes composed of the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and the neutral helper lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were used to condense mRNA into lipoplexes. Preliminary research was done to determine the nitrogen/phosphate ratio most suited for *in vivo* application and is shown as additional data **(Figure S1)**. We evaluated two N/P ratios that give yield to mRNA lipoplexes of similar size (± 300-400 nm) but opposite charge, namely lipoplexes at N/P1 had a negative zeta-potential of -18 mV and N/P10 lipoplexes displayed a positive charge of +32 mV **(Figures S1a,b)**. Further, we addressed mRNA lipoplexes of ratio N/P1 as most suited to yield high expression levels of the delivered mRNA **(Figure. S1c)** and to induce proper induction of IFN-ɣ producing $CD8^+$ and $CD4^+$ T cells upon subcutaneous injection **(Figure S1d).** As a consequence, N/P1 was selected in all further experiments aimed at addressing the impact of type I IFNs on the efficacy of mRNA lipoplexes to yield T cell immunity.

Previously, we have demonstrated that DOTAP-based mRNA lipoplexes elicit strong type I IFN secretion upon incubation with bone marrow derived DCs *in vitro*[14]. To address to which extent mRNA lipoplexes would trigger type I IFNs *in vivo* upon subcutaneous injection, we used an IFN-β reporter mouse in which a firefly luciferase encoding sequence has been placed under the control of the IFN-β promoter **(Figure 1a)**[27]. As type I IFN production is regulated by self-enforcing feedforward loops, heterozygous reporter mice (IFN-$\beta^{+/\Delta\beta\text{-luc}}$) were used to allow signal amplification by early induced IFN-β. Mice were injected subcutaneously with respectively DOTAP liposomes (no mRNA), unformulated mRNA or mRNA lipoplexes. *In vivo* bioluminescence imaging revealed a strong induction of the IFN-β promoter to injection of naked mRNA and of mRNA lipoplexes, but not to liposomes without mRNA **(Fig. 1b,c).** Strikingly, naked OVA mRNA elicited the most prominent induction of type I IFNs, clearly indicating that type I IFN induction to mRNA is inherent to the mRNA itself rather than to unique features of the mRNA lipoplexes.

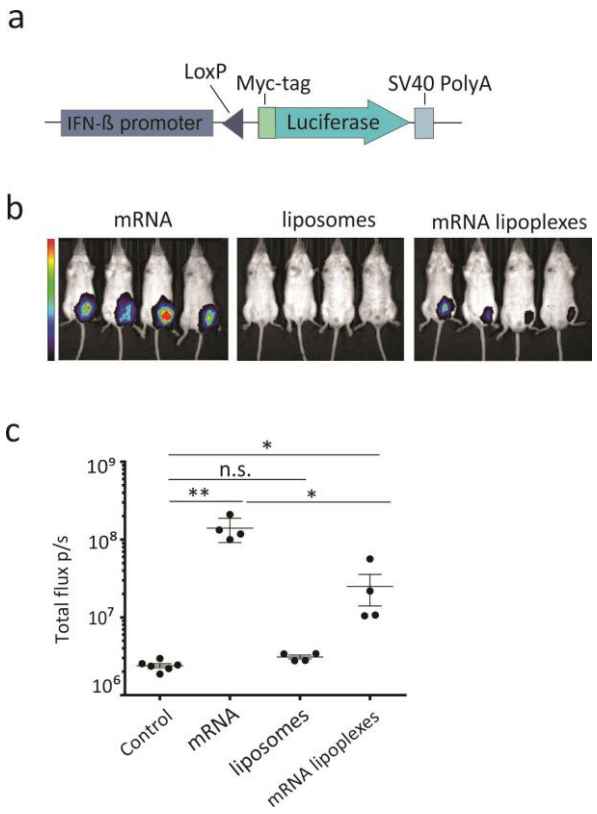


Figure 1. mRNA lipoplexes induce a potent type I IFN response *in vivo*. (a) Graphical scheme of the IFN-β reporter construct. The myc-tagged luciferase gene is brought under the control of the IFN-β promoter by the Cre-Lox system. **(b-c)** IFN-$\beta^{+/\Delta\beta\text{-luc}}$ mice were s.c. injected with 10 ug of OVA mRNA, mRNA lipoplexes and liposomes. Luminescence was measured 6 hours post-injection. Data are shown as mean ± SD of 4 mice. ** $p < 0{,}001$. * $p < 0{,}05$ (Mann- Whitney test). Control = 5% glucose water; liposomes = DOTAP/DOPE lipids; mRNA lipoplexes = messenger RNA complexed to liposomes.

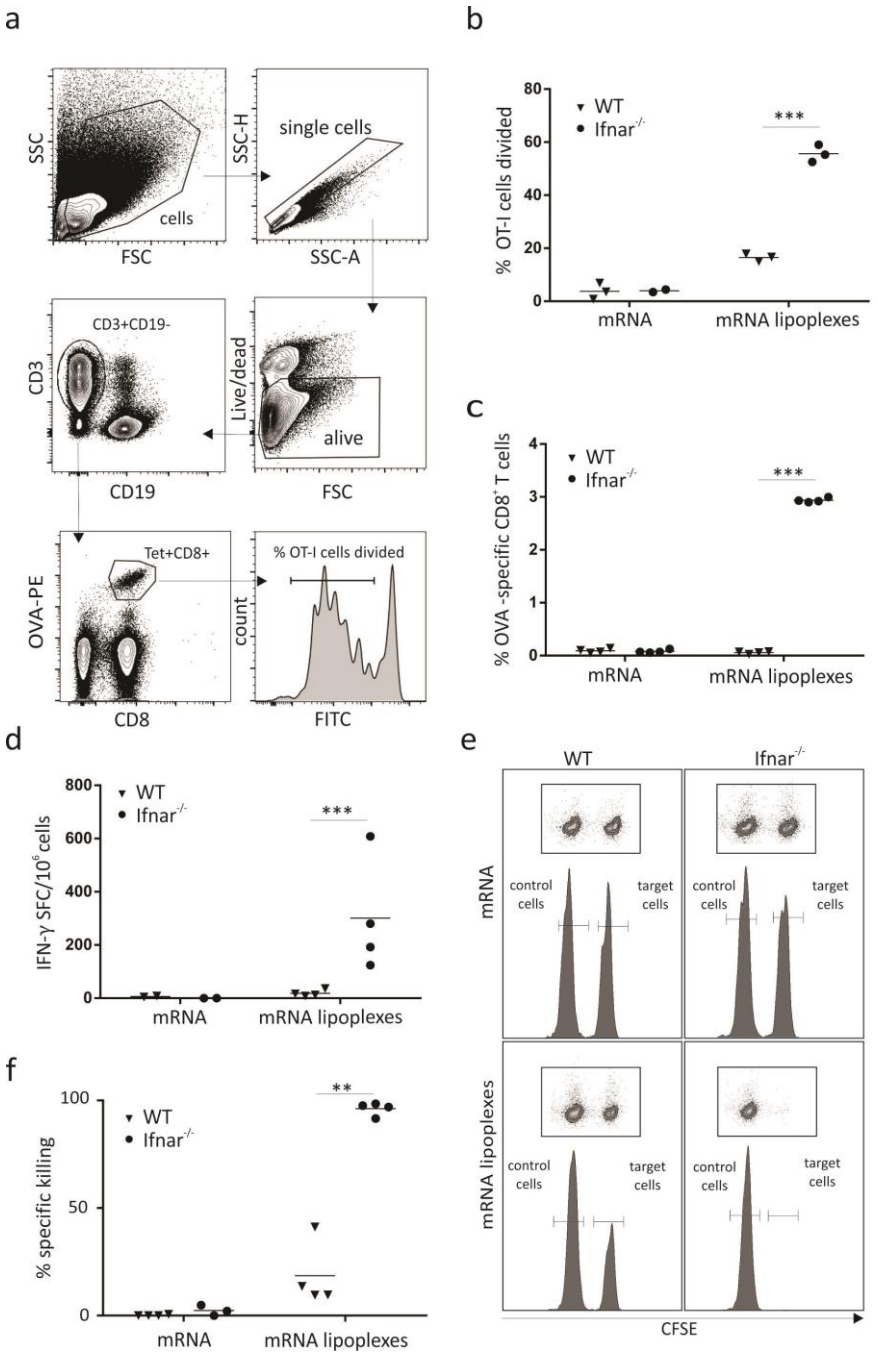
2.2 Type I Ifns Impact The Magnitude And Functional Characteristics Of The Vaccine Elicited CD8+ T Cell Response.

Depending on the context, type I IFNs have been reported to either promote or interfere with the generation of T cell responses. As a consequence, we thoroughly addressed the impact of type I IFN signalling on the magnitude and functionality of the T cell response generated by mRNA lipoplex vaccination through comparative immunization studies in wild type mice and in mice lacking he common IFN-α/β receptor IFNAR1 ($Ifnar^{-/-}$). First, we addressed the effects of

type I IFNs on the initial priming of antigen-specific T cells. To this end, CFSE labelled transgenic OVA-specific $CD8^+$ T cells (OT-I T cells) were transferred to respectively wild type and $Ifnar^{-/-}$ mice, which were subsequently immunized with OVA mRNA lipoplexes. Four days post immunization, the draining popliteal lymph nodes were dissected and OT-I T cell proliferation was analysed by flow cytometry **(Figure 2a).** As shown in **Figure 2b**, $Ifnar^{-/-}$ mice showed strongly elevated OT-I proliferation when compared to wild type mice. This negative impact of type I IFNs on the magnitude of the vaccine evoked $CD8^+$ T cell response was confirmed by quantification of vaccine elicited OVA-specific $CD8^+$ T cells in the blood of wild type versus $Ifnar^{-/-}$ mice **(Figure 2c).** Five days after boost immunization, OVA-specific $CD8^+$ T cells were hardly detectable in the blood of wild type mice but reached up to 3% of all $CD8^+$ T cells in the blood of $Ifnar^{-/-}$ mice. No significant numbers of OVA-specific T cells were detected in response to unformulated OVA mRNA. Next we analysed the impact of IFNAR deficiency on the functional properties of the vaccine induced $CD8^+$ T cell response. As type I IFNs have been reported to stimulate the differentiation of primed $CD8^+$ T cells into effector cells[20,21,28,29], the increased numbers of vaccine elicited $CD8^+$ T cell response observed in $Ifnar^{-/-}$ mice not necessarily translate into increased effector function in these mice. To address this issue, we compared OVA-specific IFN-ɣ secretion and target cell specific lysis between immunized wild type and $Ifnar^{-/-}$ mice. ELISPOT assays were performed on splenocytes two weeks after a booster immunization with OVA mRNA lipoplexes to quantify the numbers of IFN-ɣ producing OVA-specific T cells. As depicted in **Figure 2d**, immunized $Ifnar^{-/-}$ mice showed a strong increase in the numbers of OVA-specific IFN-ɣ secreting T cells. The cytolytic capacity of the evoked $CD8^+$ T cell response was analysed through an *in vivo* killing assay. In brief, two weeks following a booster immunization with OVA mRNA lipoplexes, mice were challenged with a 1:1 ratio of OVA peptide-pulsed $CFSE^{hi}$ splenocytes (target cells) and non-pulsed $CFSE^{low}$ splenocytes (non-target cells). Two days later, spleens were dissected and the ratio of target cells versus non-target cells was analysed by flow cytometry to determine the extent of killing of the target cells. Whereas immunization of wild type mice with OVA mRNA lipoplexes resulted only in a limited killing of the target cells, virtually all target cells were eliminated in immunized $Ifnar^{-/-}$ mice **(Figures 3e-f).** Taken together, these data clearly demonstrate that IFNAR deficiency increases initial T cell priming to subcutaneously administrated mRNA lipoplex vaccines and that type I IFN are not required for these antigen-experienced T cells to acquire effector function.

As we earlier observed an increase in the expression of lipoplex delivered mRNA in bone marrow derived DCs lacking IFNAR, we decided to quantify mRNA expression after subcutaneous injection of mRNA lipoplexes in wild type and Ifnar$^{-/-}$ mice. If Ifnar$^{-/-}$ mice would show strongly increased mRNA expression levels, increased antigen expression might well underlie the raise in initial T cell proliferation we observed in Ifnar$^{-/-}$ mice. To address this issue, luciferase encoding mRNA was condensed into lipoplexes at N/P1 and luciferase expression was assessed through *in vivo* bioluminescence measurement. Although luciferase expression was slightly elevated in the IFNAR deficient setting, this increase was very subtle and did not reached significance **(Figure S2)**. As a consequence, events downstream of antigen expression must be at the origin of the dramatically raised T cell responses in Ifnar$^{-/-}$ mice.

Figure 2. Type I IFNs impact the magnitude and functional characteristics of the vaccine elicited CD8$^+$ T cell response. (a) Gating strategy used for OVA- specific CD8$^+$ T cell counting and proliferation. Cells are gated based on FSC and SCC, before single cells are gated based on SSC-area and height. Living cells are selected and gated for CD3$^+$CD19$^-$ T cells. Within CD8$^+$ T cells, OVA-specificity is gated by labelling with MHC-I SIINFEKL – PE dextramer. Proliferation of CFSE positive OVA-specific CD8$^+$ T cells is shown. **(b)** Two days prior to immunization CFSE-labelled OT-I cells were adoptively transferred to wild type (WT) and Ifnar$^{-/-}$ mice. Subcutaneous (s.c.) immunization was performed at tail base with 10 µg OVA mRNA lipoplexes, naked mRNA or liposomes alone. Four days after immunization inguinal lymph nodes were isolated and CD8$^+$ T cell proliferation was analyzed by flow cytometry. Data are shown as mean of 2-3 mice. *** $p < 0.001$ (Chi-square test). **(c)** Wild type (WT) and Ifnar$^{-/-}$ mice were s.c. injected twice with 20 ug OVA mRNA lipoplexes or naked OVA mRNA as a control in a two week interval. Blood was isolated 5 days later and the percentage OVA-specific CD8$^+$ T cells was determined by dextramer staining followed by flow cytometry. Data are shown as mean of 4 mice per group. *** $p < 0.001$ (Chi-square test). **(d)** Wild type (WT) and Ifnar$^{-/-}$ mice were immunized s.c. with 20 µg OVA mRNA lipoplexes or naked mRNA as a control. Two weeks later, mice were boosted with the same formulation. Spleens were isolated two weeks after boost immunization, and the number of OVA-specific interferon-γ spot-forming CD8$^+$ and CD4$^+$ T cells (SFC) was determined by enzyme-linked immunosorbent spot (ELISPOT). Data are shown as mean of 2-4 mice per group. *** $p < 0.001$ (Chi-square test). **(e,f)** Wild type (WT) and Ifnar$^{-/-}$ mice were immunized with a two-week interval with naked OVA mRNA or OVA mRNA lipolexes. Two weeks after boost immunization, a mixture of CFSE-labelled cells pulsed with control (CFSE^{low}) or OVA peptide (CFSE^{high}) were adoptively transferred. Specific killing was measured 2 days later by flow cytometry. Data are presented as means of 100 - 100x ((CFSE^{high}/CFSE^{low})$^{immunized\ mice}$/(CFSE^{high}/CFSE^{low})$^{mock\text{-}mice}$) of 3-4 mice per group. ** $p < 0.01$ (Chi-square test). mRNA = OVA-coding messenger RNA; mRNA lipoplexes = messenger RNA complexed to DOTAP/DOPE liposomes.


a
SSC
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FSC
SSC-H
single cells
SSC-A
CD3+CD19-
CD3
CD19
Live/dead
alive
FSC
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OVA-PE
CD8
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control cells
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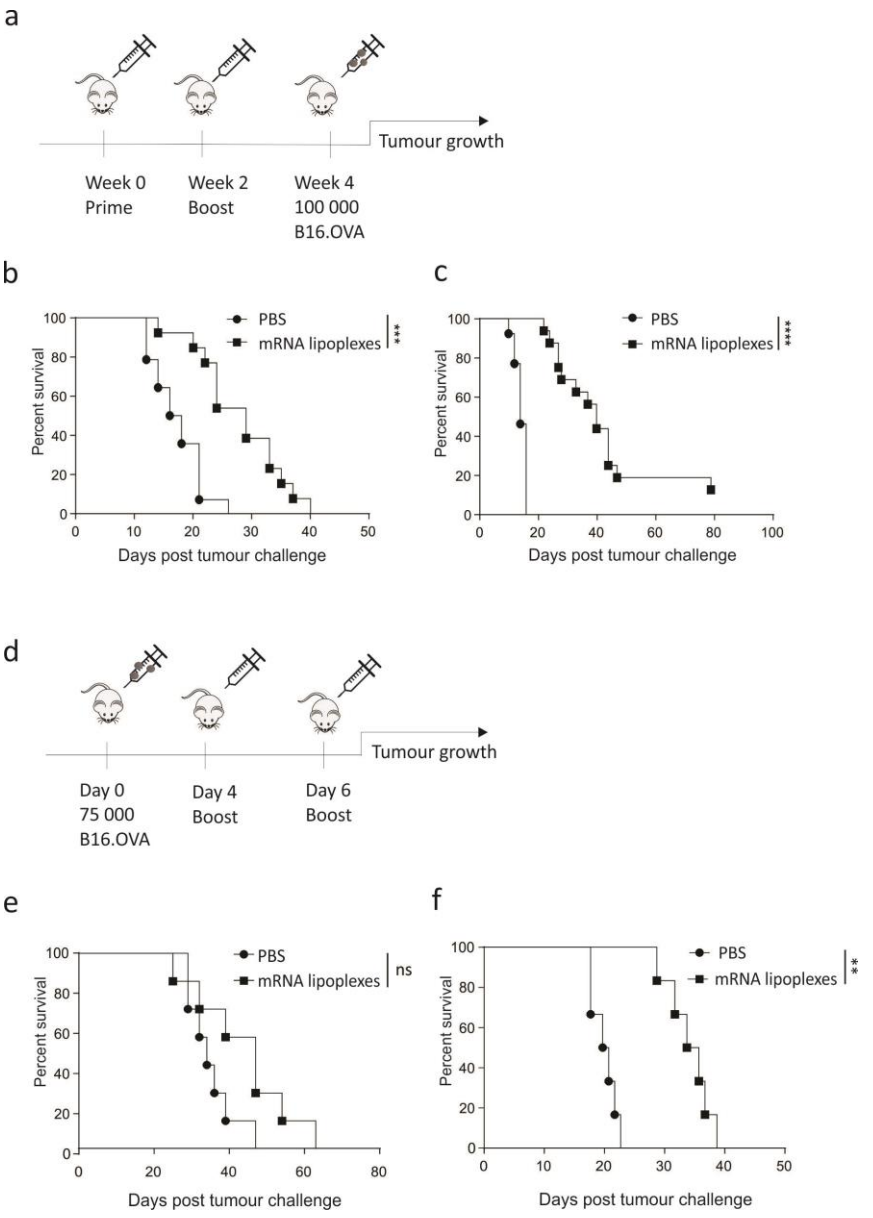
2.3 Impact Of Type I Ifns On The Efficacy Of Anti-Tumour Immunity Elicited By Mrna Lipoplex Vaccination.

The functional impact of type I IFNs on anti-tumour immunity mediated by mRNA lipoplex vaccination was addressed in the highly aggressive B16.OVA melanoma model. Mice were either vaccinated prophylactically or therapeutically according to the schedule shown in **Figures 3a,d**. In wild type mice, prophylactic vaccination significantly increased the median survival time from 17 to 29 days **(Figure 3b).** In line with their elevated vaccine elicited T cell responses, Ifnar$^{-/-}$ mice benefited even more from vaccination than wild type mice, as the median survival time increased from 14 to 40 days **(Figure 3c).** This observation is highly striking as Ifnar$^{-/-}$ mice notoriously lack spontaneous anti-tumour immune responses and succumb much faster to tumours when left untreated[28-31]. Therapeutic vaccination caused a small though non-significant improvement in median survival time from 34 to 47 days in wild type mice **(Figure 3e).** Conversely, therapeutic vaccination yielded a significant survival benefit in Ifnar$^{-/-}$ mice with an increase in median survival time from 20 to 35 days **(Figure 3f).** Nevertheless, in the therapeutic vaccination setting, vaccinated wild type mice still controlled tumours better than vaccinated Ifnar$^{-/-}$ mice, a feature that can be most likely ascribed to the lack of spontaneous anti-tumour responses in the IFNAR deficient setting.

Figure 3. Impact of type I IFNs on the efficacy of anti-tumour immunity elicited by mRNA lipoplex vaccination. (a) Prophylactic vaccination scheme. Wild type (WT) mice **(b)** and Ifnar$^{-/-}$ mice **(c)** were either mock s.c. immunized (i.e. injected with PBS only) or immunized with 20 μg of mRNA lipoplexes. Two weeks later, mice were boosted with the same formulation. At week 4, mice were inoculated with 100.000 OVA-expressing B16 melanoma cells. (n= 12-16 mice/group). **(d)** Therapeutic vaccination scheme. Wild type (WT) mice (e) and Ifnar$^{-/-}$ mice **(f)** were inoculated with 75.000 B16.OVA melanoma cells. 4 and 6 days later immunization was performed with similar preparations as in the prophylactic setting. (n = 5-6 mice/group). mRNA lipoplexes = OVA- coding messenger mRNA complexed to DOTAP/DOPE liposomes. ** $p < 0,01$; *** $p < 0,001$; ****$p < 0,0001$ (Mantel-Cox log-rank test).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Tumour growth
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Percent survival
Days post tumour challenge
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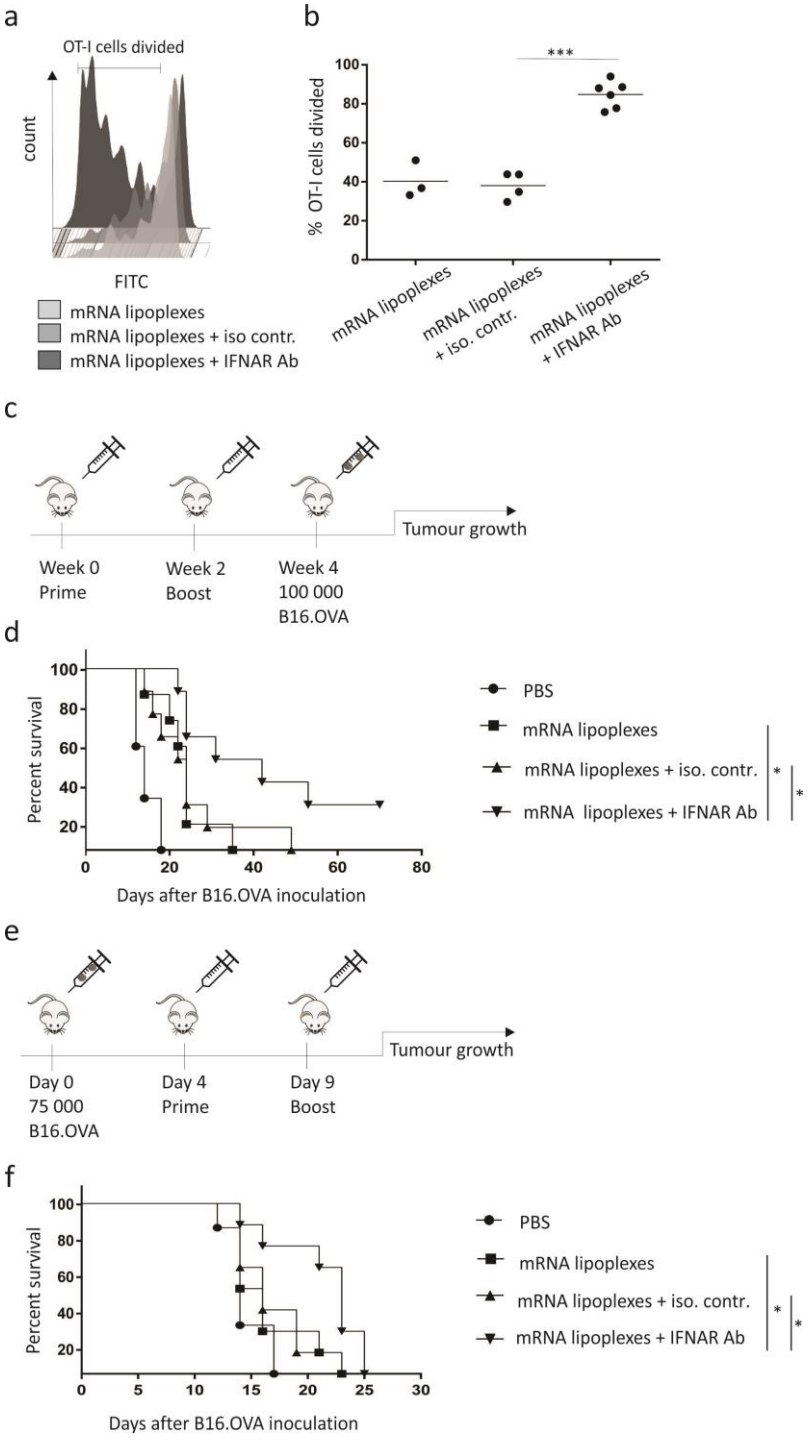
mRNA lipoplexes
**
Percent survival
Days post tumour challenge

2.4 Antibody Mediated IFNAR Blockade Improves The Efficacy Of The mRNA Vaccine Evoked Anti-Tumour Immune Response.

Results of the experiments in the previous paragraph illustrated that in immunized wild type mice tumour growth control is determined by the combined strength of the spontaneous and vaccine elicited immune responses, whereas in Ifnar$^{-/-}$ mice tumour control will entirely depend on the vaccine elicited immune response. As a consequence, direct comparisons of tumour growth rates between immunized wild type and Ifnar$^{-/-}$ mice do not allow a reliable assessment of the impact of type I IFNs on vaccine mediated tumour control. To circumvent the detrimental effect of genetic IFNAR deficiency on spontaneous anti-tumour immunity, we therefore decided to switch to antibody mediated inhibition of IFNAR signalling at the spot of vaccination in wild type mice. Local interference with IFNAR signalling should leave the spontaneous anti-tumour response intact and thereby allow us to specifically address the impact of type I IFN signalling on vaccine mediated tumour control.

First, we validated whether antibody-mediated IFNAR blockade would indeed amplify the CD8^{+} T cell response elicited by the mRNA lipoplex vaccine in wild type mice. As can be appreciated from **Figure 4a,b** co-injection of the IFNAR blocking antibody increased the proliferation of OVA-specific OT-I cells in response to mRNA lipoplexes, whilst the isotype matched antibody had no impact on OT-I proliferation. We next determined if blocking IFNAR at the site of immunization would improve the anti-tumour efficacy of the lipoplex mRNA vaccines in case of prophylactic **(Figure 4c,d)** and therapeutic vaccination **(Fig. 4e,f)** In the prophylactic vaccination setting, co-injection of the IFNAR-blocking antibody MAR1-5A3 with the OVA mRNA lipoplexes significantly improved the survival rate of immunized mice **(Figure 4d).** Importantly, the benefit of blocking IFNAR was preserved in the therapeutic vaccination setting, as mice immunized with mRNA lipoplexes in the presence of MAR1-5A3 displaying an improved outcome compared to mice receiving the same mRNA vaccine alone or combined with an isotype control antibody **(Figure 4f).** Taken together, these findings demonstrate that type IFNs induced by mRNA lipoplex vaccines negatively impact the vaccine elicit T cell response and its efficacy to control tumour growth upon subcutaneous vaccination.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% OT-I cells divided
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60
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mRNA lipoplexes
mRNA lipoplexes + iso. contr.
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Tumour growth
Week 0
Prime
Week 2
Boost
Week 4
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B16.OVA
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Percent survival
Days after B16.OVA inoculation
PBS
mRNA lipoplexes
mRNA lipoplexes + iso. contr.
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Tumour growth
Day 0
75 000
B16.OVA
Day 4
Prime
Day 9
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Percent survival
Days after B16.OVA inoculation
PBS
mRNA lipoplexes
mRNA lipoplexes + iso. contr.
mRNA lipoplexes + IFNAR Ab
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Figure 4. Antibody-mediated blocking of IFNAR improves the efficacy of the mRNA vaccine evoked anti-tumour immune response. (a,b) Two days prior to immunization CFSE-labelled OT-I cells were adoptively transferred to wild type (WT) mice. Immunization was performed in the footpad with 10 μg mRNA lipoplexes in the absence or presence of 20 μg IFNAR blocking antibody or isotype control. Four days after immunization inguinal lymph nodes were isolated and $CD8^+$ T cell proliferation was analysed by flow cytometry. Data are shown as mean of 3-6 mice per group.*** $p < 0{,}001$ (Chi-square test). (a) A representative sample out of 3-6 mice each group is presented. (c) Prophylactic vaccination scheme. (d) Wild type (WT) mice were immunized s.c with 20 μg of mRNA lipoplexes in absence or presence of 20 μg of the IFNAR blocking antibody or isotype control. Two weeks later, mice were boosted with the same formulation. At week 4, mice were inoculated with 100.000 B16.OVA melanoma cells (n= 6-8 mice/group). * $p < 0{,}05$ (Mantel-Cox log-rank test). (e) Therapeutic vaccination scheme. (f) Wild type (WT) mice were inoculated with 75.000 B16.OVA melanoma cells. Four and 9 days later immunization was performed using 20 μg of mRNA lipoplexes in absence or presence of the IFNAR blocking antibody or isotype control (20 ug) (n = 6-8 mice/group). * $p < 0{,}05$ (Mantel-Cox log-rank test). mRNA lipoplexes = OVA- coding messenger mRNA complexed to DOTAP/DOPE liposomes.

2.5 Type I IFNs Dampen Cytolytic T Cell Responses To Intradermal And Intranodal mRNA Lipoplex Vaccination.

As the route of immunization has a dramatic impact on the type of innate immune cells the mRNA lipoplexes encounter and thereby potentially also on the ensuing T cell response, we decided to evaluate the impact of type I IFNs on the cytolytic T cell response to intradermal and intranodal immunization with mRNA lipoplexes. mRNA lipoplexes also instigated a profound type I IFN response to intradermal **(Figure 5a)** and intranodal **(Figure 5c)** injection. In terms of T cell immunity, intradermal immunization with mRNA lipoplexes behaved much alike subcutaneous immunization, with the strength of the cytolytic T cell response shifting from near absent in wild type mice to virtually complete in $Ifnar^{-/-}$ mice **(Figure 5b).** In line with reports of the Thielemans[32] and Sahin[33] groups, intranodal immunization turned out to be by far the most potent route of immunization with strong cytolytic T cell responses now being evident in immunized wild type mice **(Figure 5d)**. Nevertheless, even intranodal immunization was aided by IFNAR deficiency, as the cytolytic T cell response was even further enlarged in $Ifnar^{-/-}$ mice. Taken together, these data firmly demonstrate that type I IFNs dampen the strength of the cytolytic T cell response evoked by lipoplex-based mRNA vaccination, regardless of whether the mRNA lipoplexes are delivered subcutaneous, intradermal or intranodal.

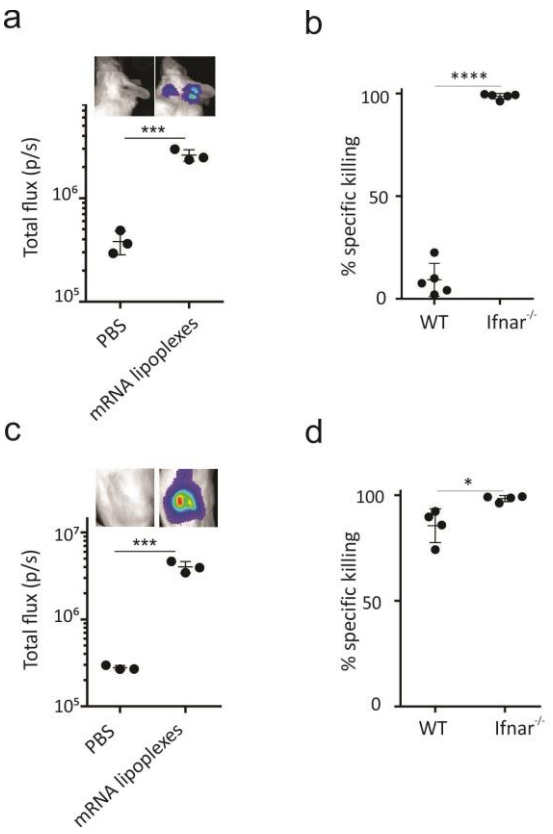


Figure 5. Type I IFNs inhibit the induction of cytolytic T cells upon intradermal and intranodal delivery of mRNA lipoplexes. (a) IFN-$\beta^{+/\Delta\beta\text{-luc}}$ mice were intradermally injected with 10 µg of OVA mRNA lipoplexes complexed or PBS. *In vivo* bioluminescence was measured 6 hours post-injection. Data are shown as mean ± SD of 3 mice. *** $p < 0{,}001$ (t-test). (b) Wild type (WT) and Ifnar$^{-/-}$ mice were immunized with a two-week interval with 10 µg of mRNA lipoplexes. Two weeks after boost immunization, a mixture of CFSE-labelled cells pulsed with control (CFSE^{low}) or OVA peptide (CFSE^{high}) were adoptively transferred. Specific killing was measured 2 days later by flow cytometry. Killing percentages were calculated with the following formula: 100 - 100x ((CFSE^{high}/CFSE^{low})$^{immunized\ mice}$/(CFSE^{high}/CFSE^{low})$^{mock\text{-}mice}$) of 5 mice per group. **** $p < 0.0001$ (t-test). (c) IFN-$\beta^{+/\Delta\beta\text{-luc}}$ mice were intranodally injected with 10 µg of OVA mRNA lipoplexes or mock treated. *In vivo* bioluminescence was measured 6 hours post-injection. Data are shown as mean ± SD of 3 mice. *** $p < 0{,}001$ (t-test). (d) Wild type (WT) and IFNAR$^{-/-}$ mice were immunized with a two-week interval with 10 µg of OVA mRNA lipoplexes and killing was performed as previously described. * $p < 0{,}05$ (t-test). mRNA lipoplexes = OVA- coding messenger mRNA complexed to DOTAP/DOPE liposomes.

3. DISCUSSION

Condensing mRNA into lipoplexes significantly improves the strength of the T cell response against the mRNA encoded antigen upon *in vivo* immunization. Nevertheless, the key innate host factors that determine the potency of lipoplex mRNA vaccines and their efficacy to instigate anti-tumour immunity have remained unresolved. Earlier, we have shown that type I IFNs are the most prominent cytokines secreted by DCs when incubated with mRNA lipoplexes[14,15]. As type I IFNs are major regulators of T cell immunity to viruses and to tumours, we decided to address their functional impact on the T cell response to mRNA lipoplex vaccines. Vaccination studies in Ifnar$^{-/-}$ mice revealed a dramatically increased priming of vaccine specific T cells in the absence of IFNAR signalling. These vaccine primed T cells acquired full effector function and efficiently eliminated target cells. When challenged with the highly aggressive B16 melanoma model, vaccinated Ifnar$^{-/-}$ mice benefited more from mRNA lipoplex vaccination compared to wild type mice in terms of increase in survival time to non-treated controls. Nevertheless, therapeutically vaccinated Ifnar$^{-/-}$ mice still succumbed earlier to B16 challenge when compared to vaccinated wild type mice. Ifnar$^{-/-}$ mice however lack spontaneous anti-tumour immunity, making direct comparisons between Ifnar$^{-/-}$ en wild type mice concerning the effects of vaccination on tumour control difficult to interpret. To avoid any confounding effects of genetic IFNAR deficiency on spontaneous versus vaccine elicited anti-tumour immunity, we therefore shifted to co-administration of an IFNAR blocking antibody at the time and spot of immunization in wild type mice.

Blocking IFNAR at the vaccination site conferred a substantial survival benefit in response to both prophylactic and therapeutic vaccination, thereby establishing type I IFNs as host factors that severely hamper the efficacy of mRNA lipoplexes as anti-tumour vaccines.

The exact mechanism by which type I IFNs exert their negative impact remains largely unresolved. Type I IFNs can affect the instigation of effector T cell immunity at multiple levels. First, as type I IFNs are potent antiviral cytokines that typically activate RNAses and block translation to prevent viral replication[35], they might hamper T cell immunity to mRNA vaccines by lowering the amount of antigen expressed, a feature we have reported on using *in vitro* BM-DCs incubated with mRNA lipoplexes[14]. Nevertheless, the impact of IFNAR deficiency on the mRNA expression level *in vivo* was very limited and thus most likely does not constitute the major factor behind the dramatically improved cytolytic T cell response in Ifnar$^{-/-}$ mice. A

potential explanation is that type I IFNs exert their negative impact directly at the level of the T cell. Indeed, whereas type IFNs can clearly act as signal 3 cytokines that promote the differentiation of antigen primed $CD8^+$ T cells into cytolytic effectors[29], they can also block T cell proliferation and even instigate T cell apoptosis[19-21]. Which of these opposing effects prevails, depends on the kinetics of T cell exposure to type I IFNs[17]. If IFNAR triggering precedes TCR triggering, the T cell inhibitory properties prevail. In case of mRNA lipoplex vaccination, type I IFN release occurs rapidly – TLRs and other RNA sensing receptors can be triggered in the endosomal compartments even before the mRNA leaves the endosomes for translation – and most likely before DCs that have taken up the mRNA lipoplexes have reached the lymph nodes to present the antigen. Nevertheless, studies using mice selectively deficient in IFNAR in DCs or in T cells are required to shed further light at which stage type I IFNs exactly interfere with T cell immunity to mRNA lipoplexes.

In summary, we have firmly established type I IFNs as host factors that negatively regulate the capacity of mRNA lipoplex vaccines to instigate cytolytic T cells upon subcutaneous, intradermal and intranodal administration. As type I IFN induction is inherent to IVT mRNAs, our findings can likely be extended to many other nano-formulations explored for mRNA vaccination. If so, strategies to prevent or reduce type I IFNs might be of great value to improve the clinical efficacy of mRNA vaccines.

4. Materials And Methods

Mice

Female wild type C57BL/6 mice were purchased from Janvier (Le Genest Saint Isle, France). OT-I mice carrying a transgenic $CD8^+$ T cell receptor specific for the MHC I-restricted ovalbumin (OVA) peptide SIINFEKL were donated by Dr. Bart Lambrecht from Ghent University (Ghent, Belgium). $Ifnar1^{-/-}$mice were bred at the breeding facility of the Vlaams Instituut voor Biotechnolgoy (VIB, Ghent, Belgium). C57BL/6 luciferase reporter mice (IFN-$\beta^{+/\Delta\beta\text{-luc}}$) were bred at the Helmholtz Centre for Infection Research (HZI). All mice were 7-12 weeks old at the start of the experiment and maintained under specific pathogen-free conditions. Animals were treated according to the European guidelines for animal experimentation. All experiments were approved by the local ethical committee for animal experiments of Ghent University (Ghent, Belgium) or of the Helmholtz Center for Infection Research (Braunschweig, Germany).

Production of IVT mRNA

The pGEM4Z-OVA-A64 and the pGEM4Z-EGFP-A64 plasmids were kindly donated by dr. David Boczkowski from Duke University (Durham, NY). The pBluescript-luc-A64 plasmid was provided by Dr. Joanna Rejman from Ghent University (Ghent, Belgium). All plasmids were propagated in *E. coli* competent cells (Stratagene, La Jolla, CA, USA) and purified using endotoxin-free QIAGEN-tip 500 columns (Qiagen, Chatsworth, CA, USA). The pGEM4-OVA-A64 and pGEM4Z-EGFP-A64 plasmids were linearized with *Spe*I (MBI Fermentas, St Leon-Rot, Germany), whereas the pBluescirpt-luc-A64 plasmid was linearized with *Dra*I (MBI Fermentas, St Leon-Rot, Germany). Linearized plasmids were purified using a PCR purification kit (Qiagen, Venlo, The Netherlands) and RNA was transcribed using the T7 mMessage Machine Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The *in vitro* transcribed mRNA was purified by lithium chloride precipitation.

Immunizations and injections of mRNA lipoplexes

Subcutaneous immunizations were performed in C57BL/6 mice twice at tail base in a 2 week interval. According to the experiment 10 or 20 μg of OVA-encoding mRNA was complexed with DOTAP/DOPE lipids in a N/P ratio of 1 (Avanti Polar Lipids, Alabaster, AL, USA) and injected in a total volume of 40 μl of 5% glucose water (Ambion, Life technologies, USA). For intranodal delivery of mRNA, C57BL/6 mice were anesthetized with ketamine (70 mg/kg; Ceva) and xylazine (10 mg/kg; Bayer). The inguinal lymph node was surgically exposed and injected with 10 μg RNA lipoplexes in a total volume of 15 μl. Subsequently, the wound was closed. For intradermal immunization, 10 μg of mRNA lipoplexes was injected into the ear dermis in a total volume of 20 μl. Accordingly to the experiments, the total vaccine volume included 20 μg of MAR1-5A3 (anti-mouse IFNAR) or mouse IgG1 isotype control (both from Leinco Technologies, St. Louis, MO, USA). For *in vivo* measuring of mRNA expression levels wild type and Ifnar$^{-/-}$ mice were injected s.c. with 10 μg of luciferase encoded mRNA. mRNA expression levels were measured 8 hours after injection via *in vivo* biolumenescence.

Flow cytometry

All flow cytometric experiments were performed on a triple-laser (B-V-R) LSR-II (Becton Dickinson, San Jose, CA, USA) and analyzed with FlowJo (Treestar, OR). Cells were stained with α-CD16/CD32 (BD Biosciences, San Diego, CA, USA) to block non-specific FcR binding, and with Live/Dead Fixable Aqua stain (Invitrogen) to eliminate dead cells from analysis. Antibodies used

are α-CD8 PerCP, α- CD3 pacific blue, α-CD19 APC-Cy7, α-CD11c PerCP-Cy5.5, α-F4/80 APC (all BD Biosciences, San Diego, CA, USA) and MHC dextramer H-2 Kb/SIINFEKL-PE (Immudex, Copenhagen, Denmark).

In vivo imaging of IFNß induction

Heterozygous luciferase reporter mice (IFN-$\beta^{+/\Delta\beta\text{-luc}}$) were injected subcutaneously, with PBS, 10 µg of OVA-mRNA complexed with DOTAP/DOPE liposomes at an N/P ratio of 1 (Avanti Polar Lipids, Alabaster, AL, USA), DOTAP/DOPE alone or naked OVA-mRNA in a total volume of 20 µl 5% glucose water. Intradermal or intranodal injections were performed with 10 µg of mRNA lipoplexes at an N/P ratio of 1 (Avanti Polar Lipids, Alabaster, AL, USA), DOTAP/DOPE alone or naked OVA-mRNA in a total volume of 10-20 µl 5% glucose water. IFNβ induction was measured at 0, 3 and 6 hours after injection via *in vivo* biolumenescence.

In vivo bioluminescence imaging

For *in vivo* imaging, mice were injected intravenously with 150 mg/kg of D-luciferin (PerkinElmer, Waktham, MA, USA) in PBS and monitored using an IVIS lumina II imaging system. Photon flux was quantified using the Living Image 4.4 software (all from Caliper life sciences, Hopkinton, MA, USA).

ELISPOT

C57BL/6 mice were immunized twice with 20 µg of DOTAP/DOPE-complexed OVA-encoding mRNA in a two week interval. Two week after the boost immunization, spleens were isolated and passed through 70 µm nylon strainers (BD Biosciences, San Diego, CA, USA) to obtain single cell suspensions. Red blood cells were lysed using ACK red blood cell lysis buffer (BioWhittaker, Wakersville, MD, USA) and 2.5 x 10^5 cells were cultured for 24 hours on IFN-γ (Diaclone, Besançon, France) pre-coated 96-well plates in the presence of 10 µg/ml OVA peptides (Anaspec, Fremont, CA, USA). To quantify the amount of OVA-specific $CD8^+$ and $CD4^+$ T cells we pulsed the splenocytes with resp. 10 µg/ml MHC-I and MHC-II OVA peptides. Spots were analyzed according to the manufacturer's instructions using ELISPOT reader.

$CD8^+$ T cell dextramer staining

Mice were immunized twice with 20 µg of DOTAP/DOPE complexed OVA-encoding mRNA as described previously. Five days later, blood samples were taken and red blood cells were removed using ACK lysis buffer (BioWhittaker, Wakersville, MD, USA). Cells were stained with α-

CD16/CD32 (BD Biosciences, San Diego, CA, USA), Live/Dead Fixable Aqua stain (Invitrogen), α-CD8 PerCP, α- CD3 pacific blue, α-CD19 APC-Cy7 (all BD Biosciences, San Diego, CA, USA) and MHC dextramer H-2 Kb/SIINFEKL-PE (Immudex, Copenhagen, Denmark).

In vivo T cell proliferation assay

Two days before immunization OT-I cells were labelled with 5 µM carboxyfluorescein diacetate succinimedyl ester (CFSE; Invitrogen, Merelbeke, Belgium). Two million CFSE-labelled OT-I cells were i.v. injected into wild type and Ifnar$^{-/-}$ mice two days before immunization. Immunization was performed as previously described. Four days after immunization draining lymph nodes were isolated and CD8^{+} T cell division was analysed by flow cytometry. Cells were stained with α-CD16/CD32 (BD Biosciences, San Diego, CA, USA), Live/Dead Fixable Aqua stain (Invitrogen), α-CD8 PerCP, α- CD3 pacific blue, α-CD19 APC-Cy7 (all BD Biosciences, San Diego, CA, USA) and MHC dextramer H-2 Kb/SIINFEKL-PE (Immudex, Copenhagen, Denmark).

In vivo killing assay

Splenocytes from female wild type mice were pulsed with 1 µg/ml of MHC-I OVA peptide or HIV-1 Gag peptide as a control before labeling with 5 µM or 0,5 µM CFSE (Invitrogen, Merelbeke, Belgium), respectively. Labelled cells were mixed at a 1:1 ratio, and a total of 1,5 x 10^7 mixed cells were adoptively transferred into immunized mice two weeks after boost. Splenocytes from host mice were analyzed two days later by flow cytometry after staining with α-F4/80 (BD Biosciences, San Diego, CA, USA) to exclude auto-fluorescent macrophages. Percentage antigen-specific killing was determined using the following formula: 100 – 100* ((% CFSE^{hi} cells / % CFSE^{low}cells)$^{immunized\ mice}$ /(% CFSE^{hi} cells / % CFSE^{low} cells)$^{non\text{-}immunized\ mice}$.

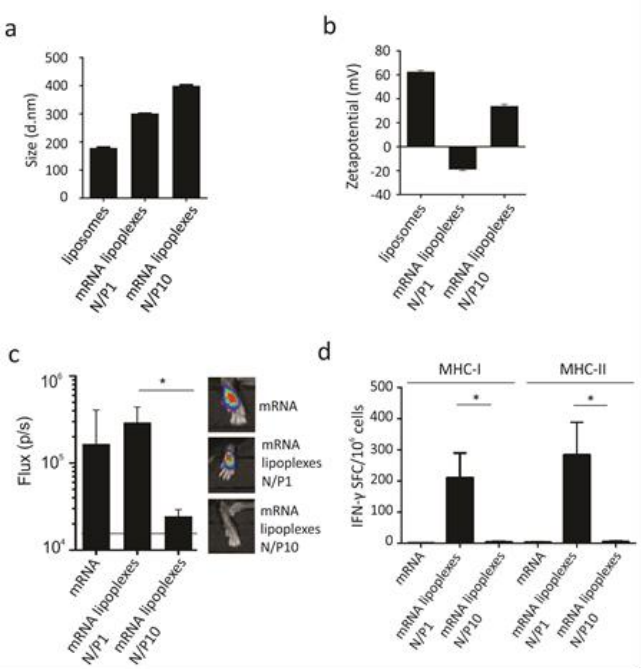
Tumour challenge

For the prophylactic tumour experiments, immunized mice were inoculated s.c. in the flank with 10^5 B16-OVA melanoma cells (VIB cell bank) in 200 µl PBS two weeks after boost immunization. Immunizations were performed as described above. Tumour growth was followed by measuring the tumour size index (TSI), i.e. the product of the largest perpendicular diameters, with a caliper. For assessment of therapeutic efficacy, 7.5 x 10^4 B16-OVA melanoma cells in 200 µl PBS were administered 4 days prior to immunization. Boost immunizations were given 2-5 days after priming.

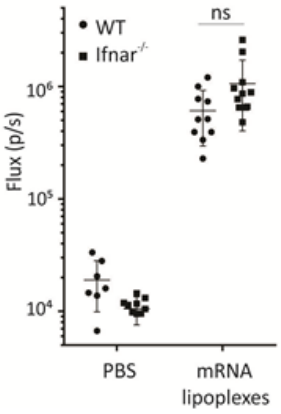
5. ACKNOWLEDGMENTS

This work was supported by grants from the Interuniversitary Attraction Pole consortium IUAPVII/63-120C07812W-B/12917/01IUAP, the UGhent Concerted Research Consortium BOF12/GOA-B/12424/01, the Fund for Scientific Research Flanders project G.0226.10, the Strategic Basic Research Program of the Flanders government project 80016, the Europrise (European vaccines and microbicides Enterprise) project 037611, the UGhent Methusalem project BOF09/01M00709, and the SOFI-B (Secondary Research Funding) at the Institute of Tropical Medicine. De Beuckelaer A. acknowledges IWT (Agentschap voor innovatie door wetenschap en technologie, Vlaanderen) for a PhD scholarship. Pollard C. was supported by a PhD scholarship from SOFI-B. Roose K. is supported by EC FP7 project FLUNIVAC. De Koker S. acknowledge FWO Flanders for funding and Van Lint S. acknowledge BOF-UGhent. The authors declare no conflict of interest.

6. SUPPLEMENTARY DATA



Supplementary data S1. (a-b) Particle size and zetapotential of DOTAP liposomes and OVA-encoding mRNA lipoplexes at ratios nitogen/phosphate of 1 (N/P1) and N/P10 were measured using zetasizer software. **(c)** Luciferase expression was measured in wild type mice after s.c injection of 10 ug luciferase-encoding mRNA and luciferase-encoding mRNA lipoplexes at ratios N/P1 and N/P10. 8 hours after injection luciferase accumulation was measured via *in vivo* bioluminescence. Data are shown as mean ± SD of 3 mice. * $p < 0,05$ (unpaired t-test). **(d)** Wild type mice were immunized with 20 µg OVA-encoding mRNA or OVA-encoding mRNA lipoplexes at both ratios N/P1 and N/P10. Two weeks later, mice were boosted with the same formulation. Spleens were isolated two weeks after boost immunization, and the number of OVA-specific interferon-γ spot-forming $CD8^+$ and $CD4^+$ T cells (SFC) was determined by enzyme-linked immunosorbent spot (ELISPOT). Data are shown as mean of 4 mice per group. * $p < 0.05$ (Chi-square test). liposomes = DOTAP/DOPE lipids; mRNA lipoplexes = messenger RNA complexed to liposomes; N/P = Nitrogen/phosphate ratio.



Supplementary data S2. Luciferase expression was measured in wild type (WT) en Ifnar$^{-/-}$ mice after s.c injection of 10 µg luciferase encoding mRNA lipoplexes at ratio N/P1. 8 hours after injection luciferase accumulation was measured via *in vivo* bioluminescence. Data are shown as mean ± SD of 10 mice; unpaired t-test. mRNA lipoplexes = OVA- coding messenger mRNA complexed to DOTAP/DOPE liposomes

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9. Additional Data

Type I Ifns Exert Their Negative Impact On Vaccine-Evoked CD8+ T Cell Responses At The Level Of Hematopoietic And Structural Cells.

The exact mechanism by which type I IFNs exert their negative impact on the instigation of effector T cell immunity remains largely unresolved. Up to now, our research was performed in Ifnar$^{-/-}$ mice, which show IFNAR deficiency in all cell types. Although it is highly assumable that the negative effects of IFNAR signalling occur at the level of DCs and T cells, this has not been established yet. To verify what cell type - hematopoietic versus non-hematopoietic cells – are implicated in the T cell immunity dampening activity of type I IFNs, we created bone marrow chimera mice between Ifnar$^{-/-}$ and wild type mice. As a control, bone marrow was transferred from WT to WT mice and from Ifnar$^{-/-}$ to Ifnar$^{-/-}$ mice. Eight weeks after bone marrow transfer to the acceptor mice, the chimeric mice were vaccinated twice with OVA mRNA lipoplexes. Four days later, the percentages of OVA-specific CD8^{+} T cells were determined in the blood by tetramer staining **(Figure A1)**. Control WT to WT and Ifnar$^{-/-}$ to Ifnar$^{-/-}$ chimeric mice displayed a similar profile as their WT and Ifnar$^{-/-}$ counterparts, with Ifnar$^{-/-}$ to Ifnar$^{-/-}$ mice showing significantly higher percentages of tetramer positive cells compared to WT to WT mice. Remarkably, WT to Ifnar$^{-/-}$ and Ifnar$^{-/-}$ to WT chimeric mice both displayed intermediate levels of OVA-specific CD8^{+} T cells, thus indicating that the negative impact of type I IFNs on the T cell response is exerted at the level of non-hematopoietic structural cells as well as hematopoietic cells. Further studies using T cell and DC conditional Ifnar$^{-/-}$ mice should help to further pinpoint the cell types that are targeted by the vaccine induced type I IFN.

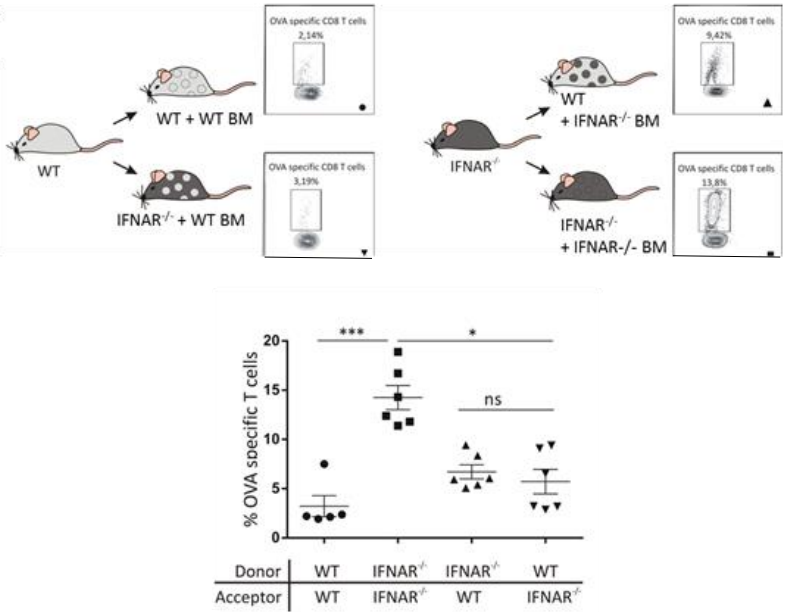

Figure A1. Bone marrow chimeras between WT and Ifnar$^{-/-}$ mice show the contribution of IFNAR signalling both at the level of hematopoietic cells and non-hematopoietic cells. WT and Ifnar$^{-/-}$ mice were irradiated and reconstituted with bone marrow of either WT or Ifnar$^{-/-}$ mice. Eight weeks after bone marrow transfer, mice received a prime and boost vaccination. Four days after the boost immunization, blood was collected and analysed to determine the percentages of OVA-specific CD8^{+} T cells. Means ± SD of 5 mice/group, * $p < 0,05$; ** $p < 0,001$ One way ANOVA.

CHAPTER 5.

EVADING TYPE I IFN RESPONSES BY LIPID-BASED DELIVERY OF MODIFIED ANTIGEN-ENCODING mRNA

Evading Type I IFN Responses By Lipid-Based Delivery Of Modified Antigen-Encoding mRNA

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Unpublished data

Author contribution

A.D.B. designed, performed and analyzed experiments (Fig.2, Fig.3, Fig.4, Fig.5). J.G. supervised the project. J.G and S.D.K., designed research

1. INTRODUCTION

1.1 REDUCING TYPE I IFN INDUCTION AT THE IMMUNIZATION SITE

Earlier, we have shown that induced type I IFN signalling upon lipoplex-based immunization hampered antigen-specific $CD8^+$ T cell priming[1]. Moreover, in the absence of type I IFN signalling, the evoked $CD8^+$ T cells acquired full effector function and efficiently eliminated target cells. However, using a tumor model to validate these findings, it became clear that losing the endogenous anti-tumor activity of type I IFNs masked the negative effects of type I IFNs on the strength of the vaccine elicited anti-tumor response. To address the problem concerning the loss of crucial endogenous type I IFN activity, an IFNAR blocking antibody was co-administered at the site of immunization, resulting in prolonged survival rates in both a prophylactic as well as a therapeutic setting[1]. In the present study, we tested alternative strategies to circumvent type I IFN induction at the site of immunization without compromising the endogenous anti-tumor activity of type I IFNs. To this end, we modified the mRNA by the replacement of uridine and cytidine with pseudouridine and 5' methylcytidine respectively. The incorporation of modified nucleotides in mRNA sequences has been shown to reduce the binding capacity of RNA to PRRs, resulting in weakened induction of inflammatory cytokines.

1.2 PSEUDOURIDINE AND 5' METHYLCYTIDINE

Pseudouridine-5' triphosphate (ψ-UTP) is found in structural RNAs such as transfer RNA (tRNA) and ribosomal RNA. ψ-UTP is an isomer of UTP in which the uracil is attached via carbon to carbon instead of a carbon to nitrogen bound **(Figure 1a,b)**. 5-methyl-cytidine-5'-triphosphate (5-met-CTP) is a common post transcriptional modified CTP, present in many mRNA, micro RNA (miRNA) and tRNA in which the 5' carbon is attached to an extra methyl group **(Figure 1d)**. The insertion of both nucleotides into IVT mRNA has been evaluated in order to suppress innate immunity as well as to increase mRNA stability and translation efficacy[2–4].

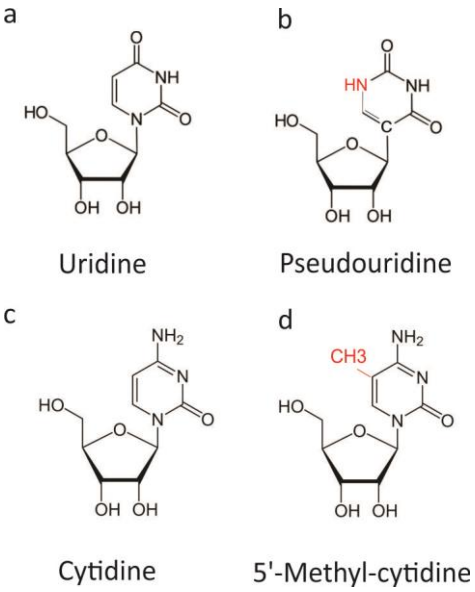

Figure 1. Chemical modification of uridine and cytidine. a) Uridine; **b)** Pseudo (ψ) uridine in which the uracil is attached via carbon to carbon instead of a carbon to nitrogen bound, resulting in a free amine group (red); **c)** Cytidine; **d)** 5' Methyl-cytidine is a derivate of cytidine in which the cytosine is attached to an extra methyl group at the 5' carbon.

1.2.1 The incorporation of modified nucleotides reduces the intrinsic adjuvant character of mRNA vaccines.

Due to the intrinsic adjuvant character of mRNA vaccines, clinical applications have so far been limited to therapeutic vaccination. However, recent studies showing that modified mRNA is less immunogenic initiated the use of mRNA for gene therapy[2,5–7]. Thus, replacing uridine and cytidine with 2'-thiouridine and 5'-methyl-cytidine synergistically decreased RNA recognition by TLR3, TLR7, TLR8 and RIG-I in human peripheral blood mononuclear cells (PBMCs)[2,6]. In line with this *in vitro* results, upon intravenous administration of modified mRNA, lower levels of pro-inflammatory cytokines (IFNɣ, IL-12 and IFNα) were detected in the blood compared to unmodified RNA. This data confirmed that insertion of the modified nucleotides resulted in a weaker activation of the innate immune system upon *in vivo* administration[2].

1.2.2 The incorporation of modified nucleotides results in increased antigen levels.

Besides a reduced immune stimulatory capacity, the injection of modified mRNA obtained higher antigen levels compared to the injection of unmodified mRNA[8,9]. It is highly suggested that the increased antigen levels observed after transfection with modified mRNA are caused by enhanced stability rather than by improved translation efficacy. This hypothesis is supported by an *in vitro* study using a cell-free translation system revealing a decreased translation efficiency for all the tested mRNA modifications while the stability of the modified mRNA generally increased. Indeed, a higher resistance to hydrolysis and an increased base stacking was observed when uridine was replaced by pseudo-uridine[10]. This observation might explain the prolonged half-live of modified mRNA molecules[2]. Strikingly, at a cellular level, the translation efficiency appears to be influenced by the mRNA elicited anti-viral response. Thus, in contrast to the use of a cell-free systems, *in vitro* cell transfection data revealed increased translation levels for modified mRNA when compared to unmodified mRNA. This difference was caused by a reduced RIG-I and protein kinase R (PKR) activation by the modified mRNA[8,11]. Activation of PKR results in the phosphorylation of the eukaryotic translation initiation factor, eIF-2α, inhibiting translation initiation. In that way, making mRNA invisible for PKR might lead to an enhanced translation efficacy[11].

2. RESULTS

Of note, earlier immunizations reported in this thesis were performed by subcutaneous injection. From this chapter on, the experiments will focus on intradermal injection as this immunization route showed stronger effector $CD8^+$ T cell responses in a comparative study (data not shown). Furthermore, all experiments were performed using mRNA from a commercial source (TriLink Biotechnologies, USA) instead of homemade mRNA as used in chapter 4. This switch was made to guarantee mRNA purity and standard composition between all experiments. Preliminary data confirmed that the impact of type I IFNs on the $CD8^+$ T cell responses to mRNA lipoplex vaccination was equal for subcutaneous and intradermal immunization. Furthermore, the negative impact of type I IFNs was shown to be fully independent of the source of mRNA (home-made or commercial).

2.1 TYPE I IFN INDUCTION BY MODIFIED MRNA LIPOPLEX VACCINE

In order to verify the effect of mRNA modification (fully substitution of pseudo-UTP and 5'methyl-CTP) on type I IFN responses, IFN-$\beta^{+/\Delta\beta\text{-luc}}$ reporter mice were intradermally injected with 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) / 1,2-dioleoyl-*sn*-glycero-3-phospho-ethanol amine (DOPE) lipoplexes comprising modified or unmodified mRNA. Three hours and six hours after injection, IFNβ induction was visualized via *in vivo* bioluminescence **(Figure 2)**. A significant increase in IFNβ induction was observed in mice injected with unmodified mRNA lipoplexes. Unexpectedly, mice injected with modified mRNA lipoplexes also showed significant induction of type I IFNs, although visualy this induction is slightly weaker. However, no significance between modified and unmodified mRNA lipoplexes is quantified (p = 0,1574), although a visible decline is observed between both groups.

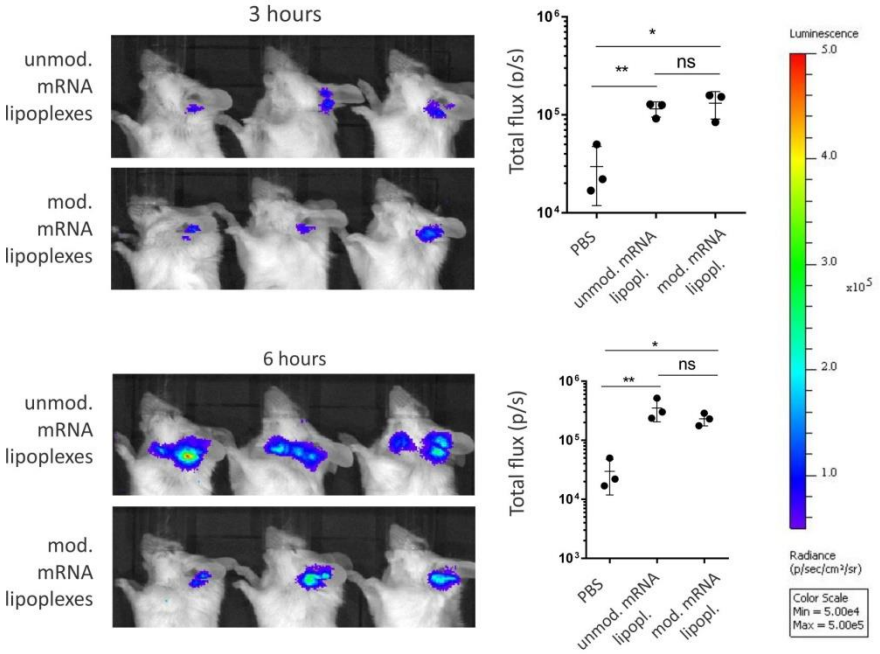

Figure 2. Effects of unmodified and modified mRNA lipoplexes on type I IFN responses. IFN-$\beta^{+/\Delta\beta\text{-luc}}$ reporter mice were i.d. injected with 7 µg of ovalbumine-mRNA lipoplexes or PBS in a total volume of 20 µl. 3 hours (upper panel) and 6 hours (lower panel) after injection, type I IFN induction was imaged using *in vivo* bioluminescence. Data are shown as mean ± SEM of 3 mice. * $p < 0,05$; ** $p < 0,01$; ns. 3h p = 0,50; ns. 6h p = 0,1574 (One-way Anova test, post-hoc Fisher- LSD). Unmod., unmodified; mod., modified; p/s, photon/sec

Of note, as depicted in **Figure 3,** injection of free mRNA resulted in ten-fold IFNβ induction relative to equal amounts of mRNA complexed into mRNA lipoplexes. These data suggest that rather free mRNA is responsible for the induction of type I IFNs than the complexed part.

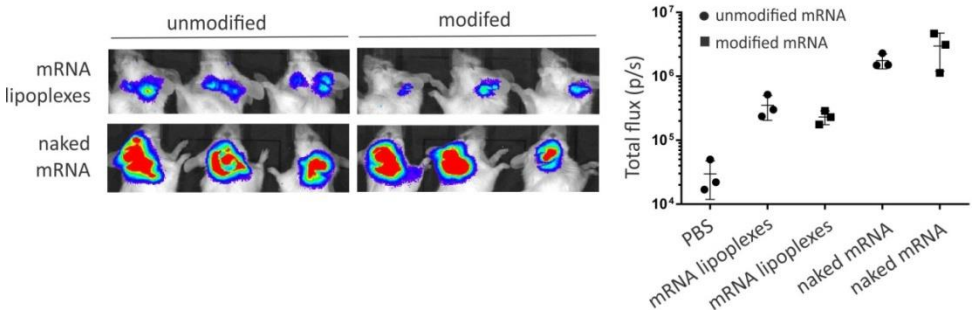

Figure 3. Injection of uncomplexed mRNA induces ten-times strong IFNβ responses than lipoplex-formulated RNA. IFN-$\beta^{+/\Delta\beta\text{-luc}}$ reporter mice were i.d. injected with 7 µg of unmodified and modified ovalbumine-mRNA lipoplexes or 7 µg naked (uncomplexed) mRNA in a total volume of 20 µl. 6 hours after injection, type I IFN induction was imaged using *in vivo* bioluminescence. Data are shown as mean ± SEM of 3 mice.

2.2 Antigen Expression Levels Produced By Modified mRNA Lipoplex Vaccine

To verify whether injection of modified mRNA (fully substitution of pseudo-UTP and 5'methyl-CTP) lipoplexes results in improved antigen expression levels compared to their unmodified counterparts, luciferase-encoding mRNA lipoplexes were i.d. injected into the ear pinna. Eight hours later, the antigen levels were visualized via *in vivo* bioluminescence. As depicted in **Figure 4,** injecting modified mRNA lipoplexes resulted in significantly higher antigen levels compared to the injection of unmodified mRNA.

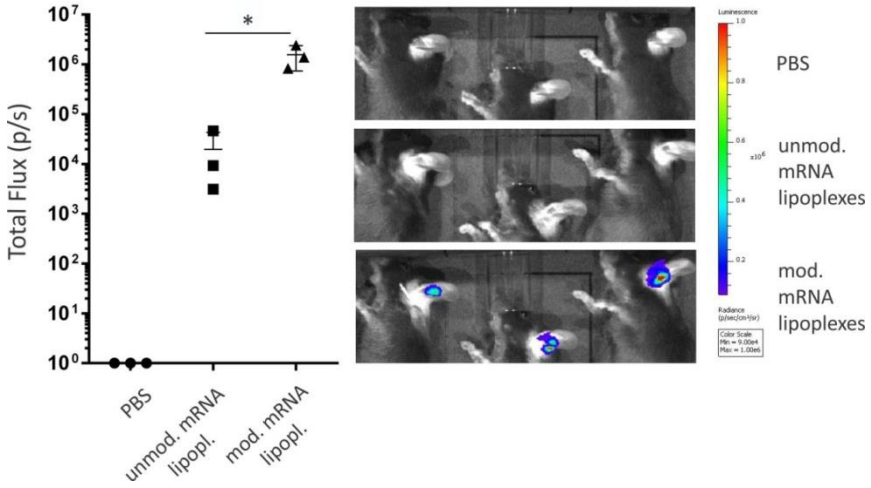

Figure 4. Modifying the antigen-encoding mRNA improves antigen expression levels. Mice were i.d. injected with 7 µg of luciferase-encoding mRNA (modified or unmodified) complexed to DOTAP/DOPE or PBS (mock). 8 hours after injection, expression levels are measured using *in vivo* bioluminescence. Data are shown as mean ± SD of 3 mice. * < 0,05 (One-way Anova test). Unmod., unmodified; mod., modified; p/s, photon/sec.

2.3 CD8+ T Cell Response Induced By Modified mRNA Lipoplex Vaccine

Considering the enhanced antigen expression levels generated by modified mRNA lipoplex formulations, we verified to what extent these properties would translate into an increased induction of CTL responses. In a first instance, we assayed the initial priming of antigen-specific $CD8^+$ T cells via an OT-I proliferation assay. To this end, CFSE-labelled OT-I T cells were adoptively transferred to wild type recipient mice, which were subsequently immunized with unmodified or modified mRNA lipoplexes. Three days post immunization, the draining lymph nodes were dissected and OT-I T cell proliferation was analysed by flow cytometry **(Figure 5a).** As shown in **Figure 5b**, both formulations evoked strong $CD8^+$ T cell proliferation. However, no significant differences were observed between the two immunized groups.

In a next step, we addressed the functional properties of the induced $CD8^+$ T cell response via an *in vivo* killing assay. To this end, two weeks after the booster immunization, mice were challenged with a 1:1 ratio of OVA peptide-pulsed $CFSE^{hi}$ splenocytes and non-pulsed $CFSE^{low}$ splenocytes. Two days later, the spleens were dissected and the ratio of target cells versus non-

target cells was analysed by flow cytometry. As shown in **Figure 5c,** moderately weak CTL responses were observed with both formulations, so no improvement for modified mRNA lipoplexes was measured.

Based on the results of the reporter mice, showing strong – however, slightly reduced – IFNβ induction in response to the injection of modified mRNA lipoplexes, enhanced CTL responses in Ifnar$^{-/-}$ mice, relative to wild type mice might be expected. To further evaluate this hypothesis, an *in vivo* killing assay was performed, showing stronger killing capacities in Ifnar$^{-/-}$ mice, compared to wild type mice in response of intradermal immunization of modified mRNA lipoplexes **(Figure 5e)**. Of note, when compared to unmodified mRNA **(Figure 5d),** the difference between wild type mice and Ifnar$^{-/-}$ mice immunized with modified mRNA, tends to be smaller. However, a direct competitive experiment needs to be performed to draw any conclusion about this. Still, we can conclude that we did not achieve an improvement of the vaccine efficacy of mRNA lipoplexes by modifying the mRNA encoding device.

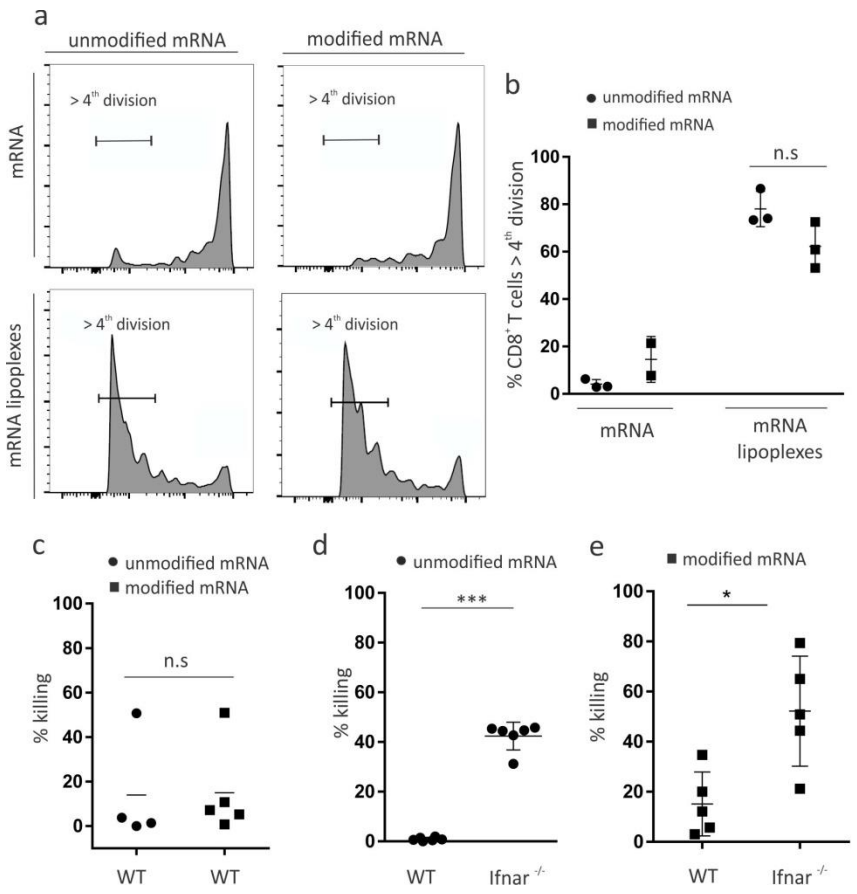

Figure 5. Modifying mRNA has no positive impact on CD8 T cell priming or effector function. a-b) Two days prior to immunization CFSE-labelled OT-I cells were adoptively transferred to wild type mice. Intradermal immunization was performed at the ear pinna with 7 µg of unmodified or modified ovalbumine (OVA) mRNA lipoplexes or free mRNA as a negative control. Three days after immunization, inguinal lymph nodes were isolated and $CD8^+$ T cell proliferation was analysed by flow cytometry. Percentage CD8 T cells above the 4^{th} division is displayed. Data are shown as mean of 2-3 mice (One-way Anova). **c)** Wild type (WT) mice were immunized with a two-week interval with 7 µg unmodified and modified OVA mRNA lipoplexes. Two weeks after boost immunization, a mixture of CFSE-labelled cells pulsed with control ($CFSE^{low}$) or OVA peptide ($CFSE^{high}$) were adoptively transferred. Specific killing was measured 2 days later by flow cytometry. Data are presented as means of 100 -100x $((CFSE^{high}/CFSE^{low})^{immunized\ mice}/(CFSE^{high}/CFSE^{low})^{mock\text{-}mice})$ of 4-5 mice per group. (One-way Anova) **d)** Wild type (WT) and $Ifnar^{-/-}$ mice were immunized with a two-week interval with modified OVA mRNA lipoplexes. Specific killing was measured as in (c). Data are shown of mean ± SD of 5 mice per group.* $p < 0,05$ (One-way anova). **e)** Wild type (WT) and $Ifnar^{-/-}$ mice were immunized

with a two-week interval with unmodified OVA mRNA lipolexes. Specific killing was measured as in (c). Data are shown of mean ± SD of 5 mice per group.* $p < 0,05$ (One-way anova).

3. Discussion

We reported earlier that type I IFN induced by mRNA lipoplex vaccination hampers the efficacy of the mRNA vaccine[1]. In here, we analysed the impact of modifying the vaccine mRNA, by fully replacing UTP and CTP with the chemically modified pseudo-UTP and 5'methyl-CTP, on vaccine efficacy and type I IFN induction. Our results showed that quite unexpectedly the administration of modified mRNA lipoplexes did not significantly reduce the induction of type I IFNs, however a tendency could be observed. One possible explanation could be that the amount of free mRNA in the mRNA lipoplex formulation is too big to be masked by modification. Although we detected significantly enhanced antigen expression levels due to the modification of the mRNA, no impact on the capacity to elicit $CD8^+$ T cell responses was measured. Our analysis of the induced $CD8^+$ T cell response in wild type and $Ifnar^{-/-}$ mice confirmed that in spite of the modified mRNA format, the vaccination efficacy is still hampered by induced type I IFNs.

4. Materials And Methods

Mice

OT-I mice carrying a transgenic $CD8^+$ T cell receptor specific for the MHC I-restricted ovalbumin (OVA) peptide SIINFEKL were donated by Dr. Bart Lambrecht from Ghent University (Ghent, Belgium). $Ifnar1^{-/-}$ mice, $Ifnar1^{+/+}$ mice and balbc luciferase reporter mice (IFN-$\beta^{+/\Delta\beta\text{-luc}}$) were bred at the breeding facility of the Vlaams Instituut voor Biotechnolgoy (VIB, Ghent, Belgium). All mice were 7-12 weeks old at the start of the experiment and maintained under specific pathogen-free conditions.

mRNA lipoplexes

mRNA was purchased from TriLink Biotechnologies (Sandiego, USA) : 5-MeC-ψU (modified) Firefly luciferase, L-6107; (unmodified) Firefly luciferase, L-6307; OVA (unmodified) L-6328; 5-MeC-ψU (modified) OVA, L-3128. Both unmodified and modified RNA is ARCA capped. The UTP and CTP nuclotides in the modified mRNA molecules are fully substituted by pseudo-UTP and 5'-

methyl-cytidine. mRNA was complexed to 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol-amine (DOPE) into lipoplexes at Nitrogen/Phosphate 1 (Avanti Polar Lipids, Alabaster, AL, USA). Lipoplexes were injected in 5% glucose water.

In vivo imaging of IFNβ induction

Heterozygous luciferase reporter mice (IFN-$\beta^{+/\Delta\beta\text{-luc}}$) were injected intradermally, with PBS, 7 µg of OVA-mRNA (L-6328 en L-3128, TriLink) complexed with DOTAP/DOPE liposomes in a total volume of 20 µl 5% glucose water. IFNβ induction was measured at 0, 3 and 6 hours after injection via *in vivo* biolumenescence. For *in vivo* imaging, mice were injected intravenously with 150 mg/kg of D-luciferin (PerkinElmer, Waktham, MA, USA) in PBS and monitored using an IVIS lumina II imaging system. Photon flux was quantified using the Living Image 4.4 software (all from Caliper life sciences, Hopkinton, MA, USA).

In vivo expression

Ifnar$^{+/+}$ mice were intradermally injected with 7 µg of Luciferase encoded mRNA (6107 and L-6307, TriLink) complexed with DOTAP/DOPE liposomes in a total volume of 20 µl 5% glucose water. Luciferase expression levels were measured 8 hours after injection via *in vivo* biolumenescence. For *in vivo* imaging, mice were injected intravenously with 150 mg/kg of D-luciferin (PerkinElmer, Waktham, MA, USA) in PBS and monitored using an IVIS lumina II imaging system. Photon flux was quantified using the Living Image 4.4 software (all from Caliper life sciences, Hopkinton, MA, USA).

In vivo CD8$^+$ T cell proliferation

Two days before immunization OT-I cells were labelled with 5 µM carboxyfluorescein diacetate succinimedyl ester (CFSE; Invitrogen, Merelbeke, Belgium). Two million CFSE-labelled OT-I cells were i.v. injected into Ifnar$^{+/+}$ mice two days before i.d. immunization with 7 µg of OVA-mRNA (L-6328 en L-3128, TriLink) complexed with DOTAP/DOPE liposomes in a total volume of 20 µl 5% glucose water. Three days after immunization draining lymph nodes were isolated and CD8$^+$ T cell division was analysed by flow cytometry. Cells were stained with α-CD16/CD32 (BD Biosciences, San Diego, CA, USA), Live/Dead Fixable Aqua stain (Invitrogen), α-CD8 PerCP, α- CD3 pacific blue, α-CD19 APC-Cy7 (all BD Biosciences, San Diego, CA, USA) and MHC dextramer H-2 Kb/SIINFEKL-PE (Immudex, Copenhagen, Denmark).

In vivo killing assay

Splenocytes from female wild type mice were pulsed with 1 µg/ml of MHC-I OVA peptide or HIV-1 Gag peptide as a control before labeling with 5 µM or 0,5 µM CFSE (Invitrogen, Merelbeke, Belgium), respectively. Labelled cells were mixed at a 1:1 ratio, and a total of 1,5 x 10^7 cells mixed cells were adoptively transferred into immunized mice two weeks after boost. Immunization was performed by injection of 7 µg of OVA-mRNA (L-6328 en L-3128, TriLink) complexed with DOTAP/DOPE liposomes in a total volume of 20 µl 5% glucose water. Splenocytes from host mice were analyzed two days later by flow cytometry after staining with α-F4/80 (BD Biosciences, San Diego, CA, USA) to exclude auto-fluorescent macrophages. Percentage antigen-specific killing was determined using the following formula: 100 – 100* ((% $CFSE^{hi}$ cells / % $CFSE^{low}$cells)$^{immunized\ mice}$ /(% $CFSE^{hi}$ cells / % $CFSE^{low}$ cells)$^{non\text{-}immunized\ mice}$.

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CHAPTER 6.

ARGININE-RICH PEPTIDE BASED MRNA NANOCOMPLEXES EFFICIENTLY INSTIGATE T CELL IMMUNITY

PART 1.

The Efficacy Of Arginine-Rich Peptide Based mRNA Nanocomplexes To Instigate T Cell Immunity Crucially Depends On The Amphipathic Organization Of The Peptide

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Article in revision at Advanced Healthcare Materials

Author contribution

A.D.B. designed, performed and analysed *in vivo* data (Fig.3 and Fig.4), edited figures and schemes (fig.1-7) and wrote the manuscript. V.K.K and J.M.C. performed and analysed *in vitro* data. C.M.M., J.L.K, D.V. provided *in vitro* data. L.V.H., K.R., K.D., B.D.G. provided technical assistance. J.G., H.O.M., S.D.K designed research. H.O.M and J.G. supervised the project. S.D.K. edited the manuscript.

Abstract

To-date, the mRNA delivery field has been heavily dominated by lipid-based systems. Reports on the use of non-lipid carriers for mRNA delivery in contrast are rare in the context of mRNA vaccination. In this paper, we have explored the potential of a CPP containing the amphipathic RALA motif, to deliver antigen- encoding mRNA to the immune system. RALA condensed mRNA into nanocomplexes that displayed acidic pH-dependent membrane disruptive properties. RALA mRNA nanocomplexes enabled mRNA escape from endosomes and thereby allowed expression of mRNA inside the DC cytosol. RALA mRNA nanocomplexes elicited potent cytolytic T cell responses against the antigenic mRNA cargo and showed superior efficacy compared to a standard lipid-based mRNA vaccine formulation.

1. INTRODUCTION

$CD8^+$ cytolytic T cells have the unique potential to destroy virus infected cells and cancer cells. Cytolytic T cells are typically elicited against antigens present in the cytosol, which are processed by the proteasome and loaded onto nascent MHCI complexes for presentation to $CD8^+$ T cells. Vaccines based on recombinant protein antigens fail to access this cytosolic route of antigen processing and thereby yield poor cytolytic T cell responses. Conversely, nucleic acid-based vaccines display a high potential to elicit cytolytic T cells provided they support the expression of adequate amounts of antigen in the cytosol of DCs, the most potent antigen presenting cells. Within the field of nucleic acid-based vaccination, mRNA has now outcompeted pDNA as the molecule of choice to deliver antigenic information due to its superior safety profile, its higher capacity to transfect non-dividing cells and its amenability to GMP production[1-5]. Over the past years, preclinical and clinical studies have highlighted the potential of antigen-encoding mRNA to elicit T cell immunity upon intradermal[6] and intranodal immunization[7,8]. Nonetheless, these studies have also unmasked the limitations of naked mRNA vaccines and have emphasized the necessity to develop formulation strategies that can improve the potency of current mRNA vaccines. Packing the vaccine mRNA into nanoparticulate carriers constitutes an interesting avenue to achieve this goal as nanocarriers can protect the mRNA from fast degradation as well as selectively target DCs. To date, the mRNA delivery field has been heavily dominated by lipid-based systems[6,9-12]. Reports on the use of non-lipid carriers for mRNA delivery in contrast are scarce. Moreover, virtually all polymeric carriers have been developed to deliver plasmid DNA

(pDNA) or small interfering RNA (siRNA) and perform very poor in the context of mRNA vaccination[9,13]. This failure of cationic polymers to deliver mRNA has been attributed to a too stringent binding of the mRNA to the cationic polymer along with an ineffective endosome-to-cytosol translocation. In a pioneering paper, Bettinger *et al*. demonstrated that the use of low molecular weight cationic polymers diminishes the strength of interaction between carrier and mRNA to such an extent that efficient mRNA expression can be achieved provided an endosome disrupting agent is added[14]. Carriers suited for mRNA vaccination thereby should meet two major requirements. First, they need to bind mRNA with such strength that nanocomplexes are formed but ribosomal binding upon cytosolic delivery is not compromised. Second, they need to be endowed with endosome disrupting properties to translocate their mRNA cargo to the cytosol. Based on these premises, we speculated that cationic CPPs might constitute excellent vehicles for mRNA delivery as they combine lower charge densities with excellent membrane disruptive abilities. CPPs have been intensively exploited to deliver drugs, proteins, pDNA and siRNA to the cellular cytosol[15-17] but have remained remarkably unexplored to deliver mRNA. Within the broad variety of CPPs, peptides containing the amphipathic RALA motif have emerged as particularly promising for systemic delivery of pDNA and siRNA because they selectively attack endosomal membranes and thereby display reduced toxicity[18-20].

In this study, we have explored the potential of RALA to deliver antigen-encoding mRNA to the immune system. RALA condensed mRNA into nanocomplexes that displayed pH-dependent membrane disruptive properties and efficiently translocated their mRNA cargo from endocytic vesicles to the cytosol. RALA mRNA nanocomplexes elicited potent cytolytic T cell responses *in vivo* against the antigenic mRNA cargo and showed superior efficacy in doing so compared to a standard lipid-based mRNA vaccine formulation.

2. Results

2.1 Characterization Of RALA Mrna Nanocomplexes

The sequence and secondary structure of RALA are shown in **Figure 1a.** At acidic pH, RALA displays an amphipathic α-helical structure with all hydrophilic arginine residues facing one side and all hydrophobic leucine residues facing the other side. This amphipathic organization enables RALA to complex nucleic acids electrostatically and to intercalate into endosomal

membranes[19]. To address the capacity of RALA to condense mRNA into particles, we mixed increasing amounts of RALA with a fixed amount of mRNA and determined particle size by dynamic light scattering (DLS) and particle ζ-potential by electrophoretic mobility measurements, therefore we tested tree different N/P ratios. The N/P ratio represents the molar ratio of positively charged nitrogen atoms in the peptide to negatively charged phosphates in the mRNA backbone. At N/P 1, a mean hydrodynamic diameter of 144 nm was measured while at higher N/P ratios, the hydrodynamic diameter decreased to 89 nm (N/P 5) and 91 nm (N/P 10) **(Figure 1b)**. The ζ-potential shifted from a negative value of – 6.6 at N/P 1 to respectively +14.6 and +26.3 at N/P 5 and at N/P 10, in line with an increasing amount of RALA incorporated into the complexes at higher N/P ratios **(Figure 1c)**. Nanoparticle formation was confirmed by transmission electron microscopy (TEM) **(Figure 1d)**. The amount of mRNA incorporated into the RALA-mRNA nanocomplexes increased with increasing N/P ratios, ranging from a mere 5 % at N/P 1, up to 88 % at N/P 10 **(Figure 1e)**.

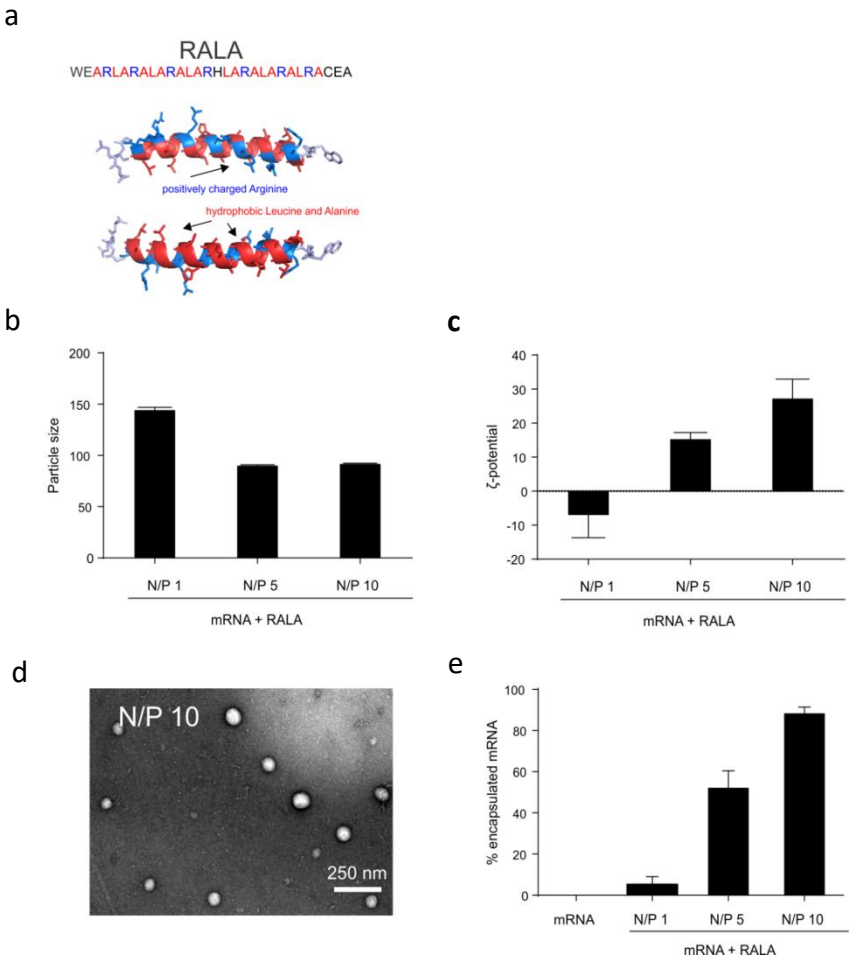

Figure 1. Characterization of RALA mRNA nanocomplexes. (a) RALA sequence and secondary structure. Arginine residues (R) are shown in blue, Leucine and Alanine residues in red. **(b)** Hydrodynamic diameter and **(c)** ζ-potential of RALA mRNA nanocomplexes at N/P 1, N/P 5 and N/P 10. **(d)** TEM images of RALA mRNA nanocomplexes at N/P 10. **(e)** Encapsulation efficiency of mRNA into RALA mRNA nanocomplexes as a function of the N/P ratio. Measurements are shown as mean (n=3) ± SD.

2.2 pH Dependent Hemolytic Activity Of RALA mRNA Nanocomplexes Enable mRNA Expression

The most appealing property of RALA is its capacity to selectively disrupt membranes at acidic pH. This feature is illustrated in **Figure 2a**, showing RALA-induced hemolysis of chicken red blood cells (RBC) at neutral pH (pH 7,4) and at acidic pH (pH 5,6). To address whether this critical

feature of RALA is retained upon mRNA binding, we performed an identical hemolysis assay, using mRNA complexed RALA. While little hemolysis was evident at N/P 1, RALA mRNA nanocomplexes did instigate significant hemolysis at N/P 5 and especially at N/P 10 **(Figure 2b).** Furthermore, levels of hemolysis were strongly elevated by lowering the pH, thereby demonstrating that RALA indeed retains its pH-dependent membrane disruptive properties even when complexed into mRNA nanocomplexes. To further verify to what extent this membrane disruptive feature of RALA mRNA complexes may enable mRNA expression inside DCs, we complexed mRNA encoding eGFP into RALA mRNA nanocomplexes. DC2.4 cells were incubated with free mRNA or with RALA mRNA nanocomplexes at respectively N/P 1, N/P 5 and N/P 10 for 4 hours. The amount of mRNA delivered was kept constant in these experiments. Flow cytometric analysis revealed no eGFP expression upon incubation of DC2.4 cells with free mRNA or with RALA mRNA nanocomplexes at N/P 1 – at which we demonstrated most mRNA to be in a free, non-complexed status. Conversely, RALA mRNA nanocomplexes supported eGFP expression in approximately 35% of all cells at N/P 5 and at N/P 10 **(Figure 2c).** Promoting endosomal disruption by addition of chloroquine – an endosomal disrupting agent did not significantly augment the transfection efficiency of the RALA mRNA nanocomplexes **(Figure 2d)**. This indicates that RALA by itself is fully competent to translocate its mRNA cargo to the cytosol.

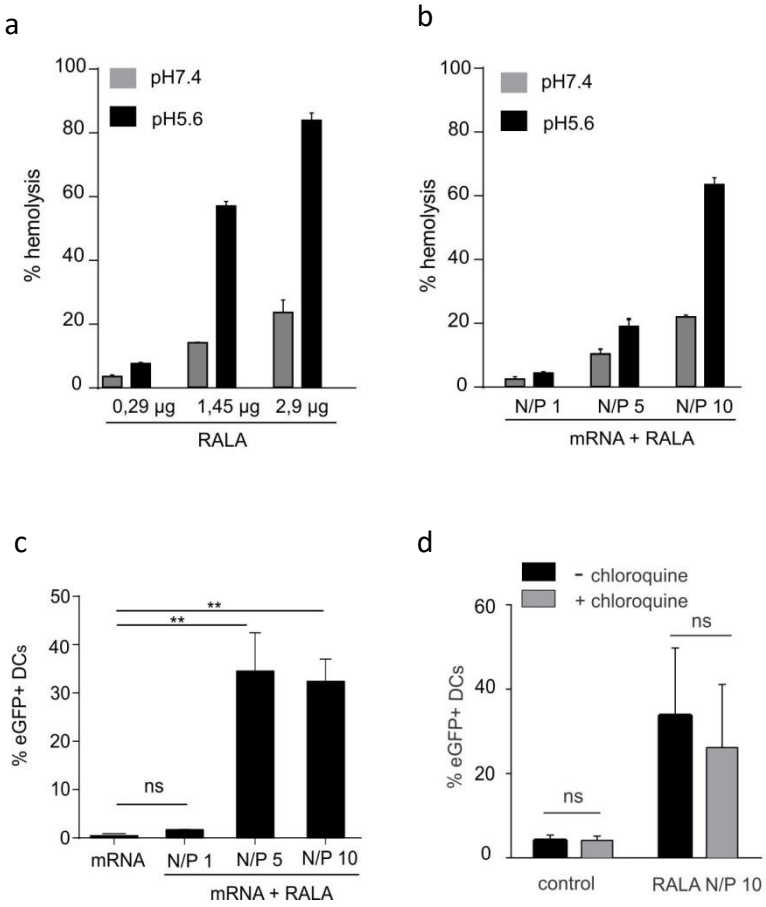

Figure 2. pH dependent hemolytic activity of RALA mRNA nanocomplexes enable mRNA expression. Hemolytic activity on chicken RBCs at respectively pH 7.4 and pH 5.6 of **(a)** different amounts of RALA, according to amount applied for the three different ratios displayed in Fig.2 b. **(b)** 200 ng RNA complexed to 0,29 µg, 1,45 µg and 2,9 µg of RALA to form polyplexes of resp. N/P 1, N/P 5 and N/P 10. Measurements are shown as mean (n=6) ± SD. **(c)** Transfection efficiencies as determined by the percentage of DC2.4 DCs expressing reporter eGFP mRNA after incubation with free mRNA or RALA-mRNA nanocomplexes at N/P 1, 5 and 10. Measurements are shown as mean (n =3) ± SD, ** $p < 0,01$. **(d)** Transfection efficiencies as determined by the percentage of DC2.4 DCs expressing reporter eGFP mRNA after incubation with free mRNA or RALA-mRNA nanocomplexes at N/P 10 in absence or presence of chloroquine. Data are shown as mean (n=3) ± SD

2.3 IMMUNOGENICITY OF RALA MRNA NANOCOMPLEXES

To determine the capacity of RALA mRNA nanocomplexes to prime $CD8^+$ T cells, an OT-I proliferation assay was performed whereby C57BL6 mice were immunized with RALA complexed to mRNA encoding the model antigen ovalbumin (OVA). T cell priming was assessed by flow cytometric quantification of the proliferation of $CD8^+$ OT-I T cells. An overview of the flow cytometry gating strategy is given in **Figure S1**. **Figure 3a** shows the flow cytometry plots and **Figure 3b** the percentages of OT-I T cells that have divided upon immunization with free mRNA or mRNA complexed with RALA at N/P 1, N/P 5 and N/P 10. Strong $CD8^+$ T cell proliferative responses were observed only upon immunization with N/P 5 and N/P 10 mRNA RALA nanocomplexes.

The incorporation of modified nucleosides, pseudo-uridine and 5-methylcytidine, into mRNA has been shown to improve mRNA expression *in vivo* and to diminish the strong innate immune activation evoked by unmodified mRNA[21,22]. To determine the optimal mRNA format in case of RALA-mediated mRNA vaccination, we compared the T cell response elicited by immunization with unmodified or pseudo-uridine/5-methyl-cytidine modified mRNA complexed to RALA. In these experiments, N/P 10 was selected as this N/P ratio allowed the best complexation of mRNA and enabled efficient $CD8^+$ T cell priming. Priming of vaccine specific T cell responses was analysed by quantifying OT-I T cell proliferation. Representative flow cytometry plots are given in **Figure 3c**. **Figure 3d** shows the percentages and **Figure 3e** the total number of OT-I T cells having divided over 4 times. These data indicated that when complexed to RALA, modified OVA mRNA is far superior in eliciting OT-I T cell proliferation compared to unmodified mRNA.

To determine whether the increased T cell proliferation following immunization also provoked cytolytic activity, an *in vivo* killing assay was performed. In such assay, immunized mice are challenged with 1/1 ratio of differentially CFSE-labelled target cells and non-target cells. Two days after challenge, the ratio of both populations represents a direct measure of the antigen-specific cytolytic T cell activity. Vaccination with RALA-complexed modified OVA mRNA killed virtually all target cells, whereas no target cell killing was observed upon vaccination with RALA-complexed unmodified OVA mRNA **(Figure 3f,g)**. This higher immunogenicity of modified mRNA over unmodified mRNA is remarkable, as modified mRNAs have been developed by the gene therapy field exactly to avoid immune responses against the encoded protein[21,22]. These data

thus indicate that RALA has intrinsic immune activating properties on its own – a feature possibly related to RALA's capacity to disturb endosomal membranes - thereby obviating the need for innate recognition of the mRNA to trigger immune activation.

In order to validate the relative strength of RALA mRNA nanocomplexes in eliciting cytolytic T cell immunity, we performed a direct comparison with a more conventional lipoplex DOTAP/DOPE-based mRNA vaccine, the current benchmark for pre-clinical lipid-based mRNA vaccination. As shown in **Figure 4**, the combination of RALA with modified mRNA resulted in a far more pronounced antigen-specific killing of target cells, compared to mRNA lipoplexes formulated with modified or unmodified mRNA.

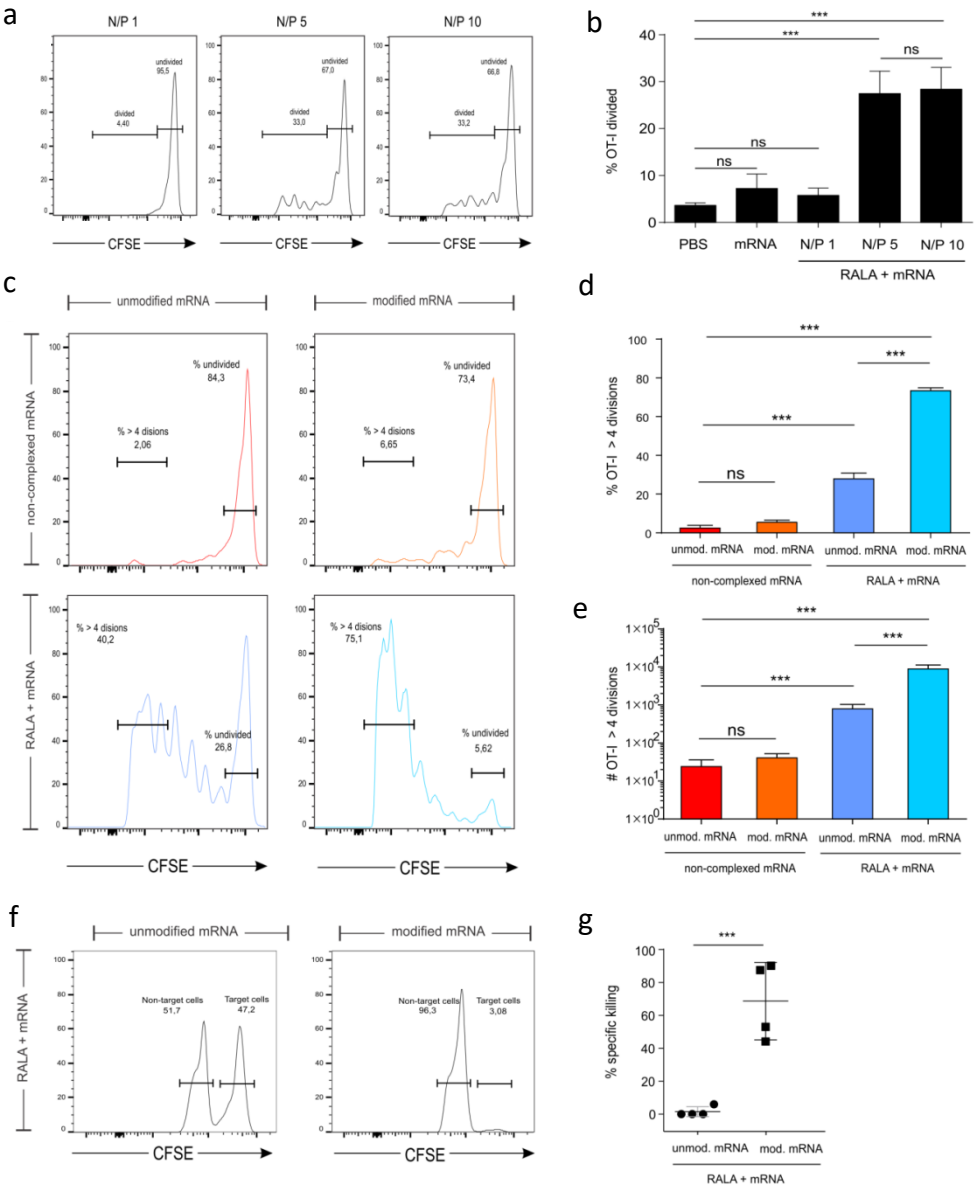

Figure 3. Immunogenicity of RALA mRNA nanocomplexes. **(a,b)** Flow cytometric analysis of OT-I T cell proliferation to immunizations with RALA mRNA nanocomplexes as a function of the N/P ratio applied. **(a)** Histograms showing the proliferation of CFSE labelled OVA-specific OT-I T cells in response to intradermal immunization with RALA OVA mRNA nanocomplexes. **(b)** Percentages of divided OT-I T cells in response to intradermal injection of PBS, non-complexed OVA mRNA and mRNA OVA nanocomplexes. Measurements are shown as mean (n =3) ± SD, *** $p < 0.001$). **(c,e)** Flow cytometric analysis of OT-I T cell proliferation in response to intradermal immunization with RALA

complexed unmodified OVA mRNA or pseudo-uridine and 5-methylcytidine modified OVA mRNA (N/P 10, 7 µg of mRNA). **(c)** Histograms showing the proliferation of CFSE labelled OVA-specific OT-I T cells. Quantification of the percentage **(d)** and absolute numbers **(e)** of dividing OT-I T cells. Measurements are shown as mean (n =4) ± SD, *** $p < 0.001$. **(f,g)** Flow cytometric analysis of antigen-specific killing of target cells in response to prime and boost vaccination with RALA complexed unmodified OVA mRNA or pseudo-uridine and 5-methylcytidine modified OVA mRNA (N/P 10, 7 µg of mRNA). **(f)** Histogram plots showing the presence of $CFSE^{hi}$ target cells and $CFSE^{lo}$ non-target cells in spleen of immunized mice. **(g)** Graph showing the percentage of antigen-specific killing of target cells. Data are reported as means (n = 4) ± SD, *** $p < 0.001$

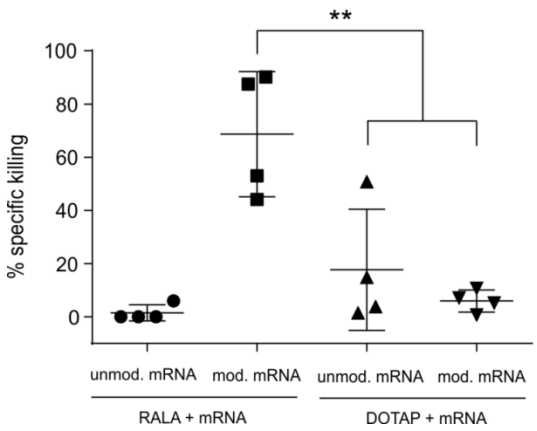

Figure 4. Cytolytic T cell responses following RALA-based versus lipid-based mRNA vaccination. Quantification of antigen specific killing induced by prime and boost vaccination with OVA mRNA complexed to RALA (N/P 10, 7 µg of mRNA) or to DOTAP/DOPE liposomes (N/P 1, 7 µg of mRNA). Measurements are shown as mean (n =4) ± SD, ** $p < 0.01$.

2.4 Non-Amphipathic Cationic Peptide Variants: Characterization Of Peptide mRNA Nanocomplexes

Having established the capacity of RALA to efficiently deliver an antigenic mRNA cargo to the immune system, we aimed to delineate to what extent the immunogenic properties of RALA mRNA nanocomplexes depend on RALA's amphipathic α-helical nature. To this end, we designed two additional arginine-rich peptides that contain identical numbers of arginine residues as RALA on a molar basis but lack RALA's hydrophobic residues. In the first peptide (named RGSG hereafter), the RALA motif was replaced by a hydrophilic RGSG motif. In the second peptide (named RRRR hereafter), the hydrophobic alanine and leucine residues of RALA were omitted,

resulting in a stretch of 7 arginine residues. Peptide sequences and predicted peptide secondary structures are depicted in **Figure 5a**. Both peptides lack RALA's amphipathic α-helical structure as they do not contain hydrophobic residues. RGSG has a random-coil like structure, while RRRR forms a short α-helical structure to minimize electrostatic repulsion between the adjacent cationic arginine residues.

First, we assessed the impact of these sequence modifications on the capacity of the respective peptides to disrupt membranes by performing a hemolysis assay. Disturbance of the amphipathic α-helical structure of RALA abrogated hemolytic activity, with RGSG and RRRR clearly failing to lyse RBCs regardless of pH **(Figure 5b)**. Yet, due to their cationic arginine residues, RGSG and RRRR were still capable of condensing the mRNA into nanocomplexes. However, when compared to RALA, the dimensions of RGSG and RRRR mRNA nanocomplexes were strongly influenced by the N/P ratio applied **(Figure 5c)**. Particle size for RGSG mRNA nanocomplexes peaked to around 600 nm at N/P 5 and subsequently declined to 150 nm at N/P 10. In case of RRRR mRNA nanocomplexes, particle sizes ranged from 140 nm at N/P 1 over 200 nm at N/P 5 up to 1050 nm at N/P 10. RGSG and RRRR mRNA nanocomplexes also showed a different surface charge evolution with increasing N/P ratios. Whereas RALA mRNA nanocomplexes robustly shifted their ζ-potential from negative to positive values at higher N/P ratios, the shift in ζ-potential was far less pronounced for RGSG and RRRR mRNA nanocomplexes **(Figure 5d)**. Instead, RGSG mRNA nanocomplexes adopted a near neutral charge at N/P 5 and at N/P 10, whereas RRRR mRNA nanocomplexes remained negatively charged even at N/P 10.

Finally, in order to compare the capacities of RALA, RGSG and RRRR to encapsulate mRNA into nanocomplexes, we measured the amount of non-encapsulated mRNA **(Figure 5e).** Also here, RALA was more efficient at encapsulating mRNA even at the low N/P 1 ratio. However, at N/P 10, all peptides succeeded in encapsulating over 75% of the added mRNA. Consequently, N/P 10 was selected for all subsequent experiments.

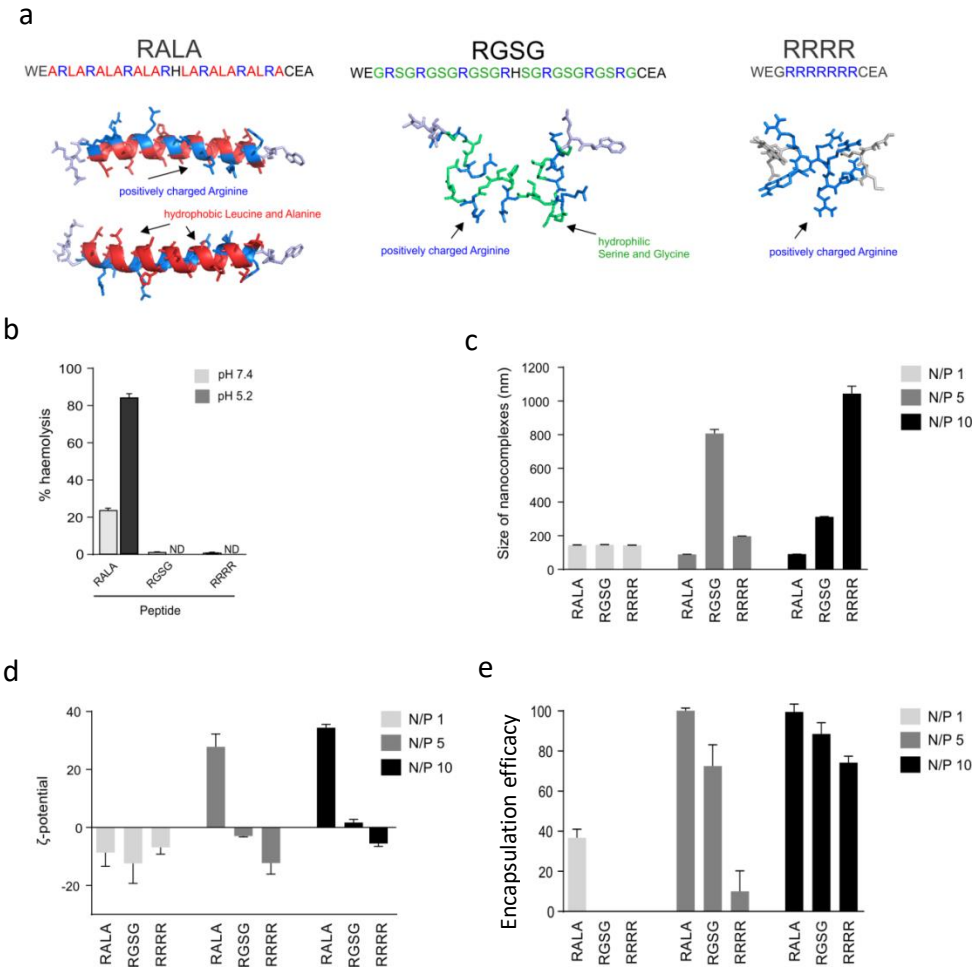

Figure 5. Characterization of RGSG and RRRR based mRNA nanocomplexes. (a) Sequences and secondary structures of RALA, RGSG and RRRR peptides, based on the *de novo* PEP-FOLD peptide prediction server. Arginine residues (R) are shown in blue, Leucine and Alanine residues in red, serine and glycine in green. **(b)** Graph depicting the hemolytic activity of equivalent amounts of RALA, RGSG and RRRR at pH 7.4 and at pH 5.6. **(c)** Hydrodynamic diameter (nm) of RALA, RGSG and RRRR peptide mRNA nanocomplexes at the indicated N/P ratios. **(d)** ζ-potential of RALA, RGSG and RRRR peptide mRNA nanocomplexes at the indicated N/P ratios. **(e)** Encapsulation efficiencies of mRNA into respectively RALA, RGSG and RRRR mRNA nanocomplexes as a function of the indicated N/P ratios. The percentage mRNA encapsulated was calculated as 100% - % free mRNA. Measurements are shown as mean (n =3) ± SD.

2.5 Lack Of T cell priming capacity Of RGSG And RRRR mRNA Nanocomplexes

To verify to what extent the loss of the amphipathic nature of RSGS and RRRR affect their capacity to prime $CD8^+$ T cell responses against the mRNA encoded antigen, we complexed modified OVA-encoding mRNA with the respective peptides. All nanocomplexes were generated at a ratio of N/P 10 as this ratio was most efficient in mRNA complexation for RGSG and RRRR as well as for RALA. The priming activity of the mRNA nanocomplexes was assayed on the basis of the *in vivo* proliferation of adoptively transferred $CD8^+$ OT-I T cells as before. RGSG and RRRR OVA mRNA nanocomplexes failed to prime substantial $CD8^+$ T cell responses **(Figure 6a),** thereby indicating RALA has unique features that mediate its effectiveness as a carrier for mRNA vaccination.

To address whether the failure of RGSG and RRRR mRNA nanocomplexes to elicit $CD8^+$ T cell reactivity might be traced back to an insufficient antigen expression in DCs, we co-incubated DC2.4 DCs with eGFP-encoding mRNA complexed to either RALA, RGSG or RRRR. The results from flow cytometry **(Figure 6b)** as well as from confocal imaging **(Figure 6c,d)** confirm the efficacy of RALA-based transfection, as well as the failure of RGSG and RRRR mRNA nanocomplexes to enable eGFP expression in DC2.4 cells.

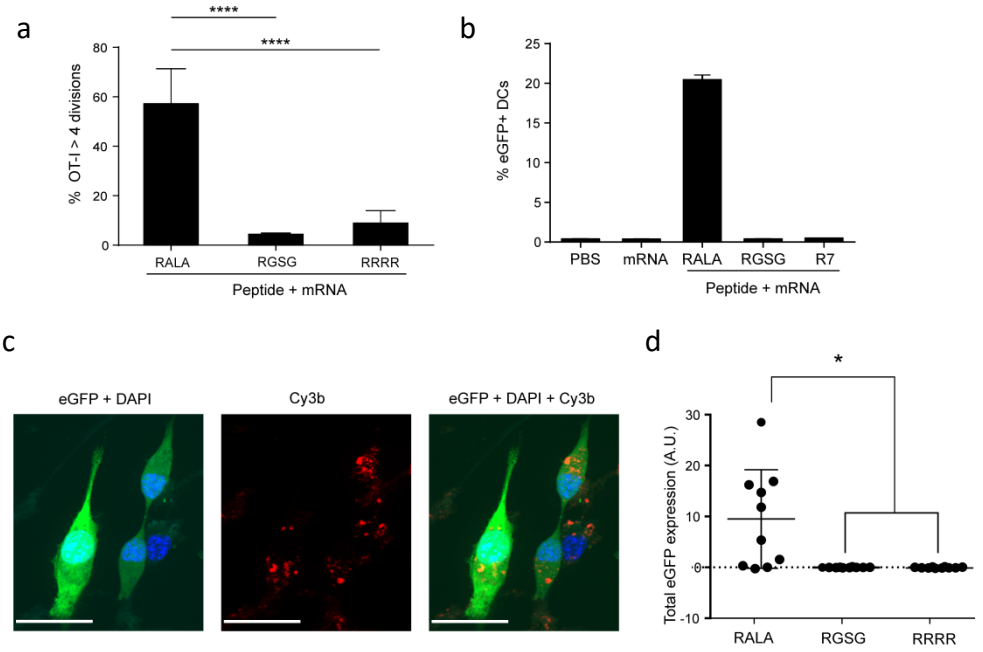

Figure 6. The lack of T cell priming capacity of RGSG and RRRR modified mRNA nanocomplexes (a) Flow cytometric analysis of OT-I T cell proliferation in response to intradermal immunization with respectively RALA, RGSG and RRRR OVA modified mRNA nanocomplexes. The graph shows the percentages of OT-I T cells having undergone over 4 divisions. Measurements are shown as mean (n =4) ± SD, **** $p < 0.0001$. **(b)** Flow cytometric analysis of eGFP expression by DC2.4 DCs incubated with non-complexed eGFP modified mRNA and eGFP mRNA complexed to respectively RALA at N/P 10. (n=5) **(c)** Confocal imaging analysis of eGFP expression in DC2.4 dendritic cells incubated with RALA eGFP modified mRNA nanocomplexes. **(d)** Quantification of eGFP expression upon transfection of DC2.4 DCs with RALA, RGSG and RRRR modified mRNA nanocomplexes. Measurements are shown as means ± SD, * $p < 0.05$.

2.6 RALA, RGSG And RRRR Differentially Facilitate The Uptake And Endosomal Escape Of The mRNA Cargo

The differential capacities of RALA, RGSG and RRRR to transfect cells and to prime $CD8^+$ T cell responses *in vivo* might reflect a differential uptake of the mRNA nanocomplexes and/or a differential capacity of the peptides to facilitate mRNA escape to the cytosol after endocytosis. In order to verify the uptake and intracellular fate of the mRNA cargo, mRNA was fluorescently labelled with Cy3b. Flow cytometric analysis showed significant differences in the percentages of DC2.4 DCs that had bound and/or internalized the respective peptide mRNA nanocomplexes

(Figure S2a). Whereas 72% of DC2.4 cells stained Cy3b positive after incubation with RALA Cy3b mRNA, this percentage dropped to a mere 7% for RGSG Cy3b mRNA and an intermediate 52% for RRRR Cy3b mRNA. Analysis of the mean fluorescence intensity revealed a similar trend with RALA enabling the strongest mRNA uptake, RGSG the lowest and RRRR an intermediate (Figure S2b). To further quantify to what extent the RALA, RGSG and RRRR peptides facilitated endosomal escape of their mRNA cargo, Mander's colocalization coefficient was calculated for colocalization of macropinosomes (ARF6), clathrin coated vesicles (Clathrin Light Chain) or caveolae (Caveolin) and mRNA nanocomplexes (Figure 7). Only in the case of RALA-mediated mRNA delivery, we could detect mRNA inside DC2.4 cells that did not co-localize with any of these vesicular markers. Conversely, mRNA delivered by RRRR and RGSG always co-localized with at least one of the vesicular markers applied. These data indicate that RALA mRNA nanocomplexes enables endosomal escape of the antigen mRNA, in contrast to RGSG and RRRR-delivered mRNA, which appears to remain trapped inside endocytic vesicles.

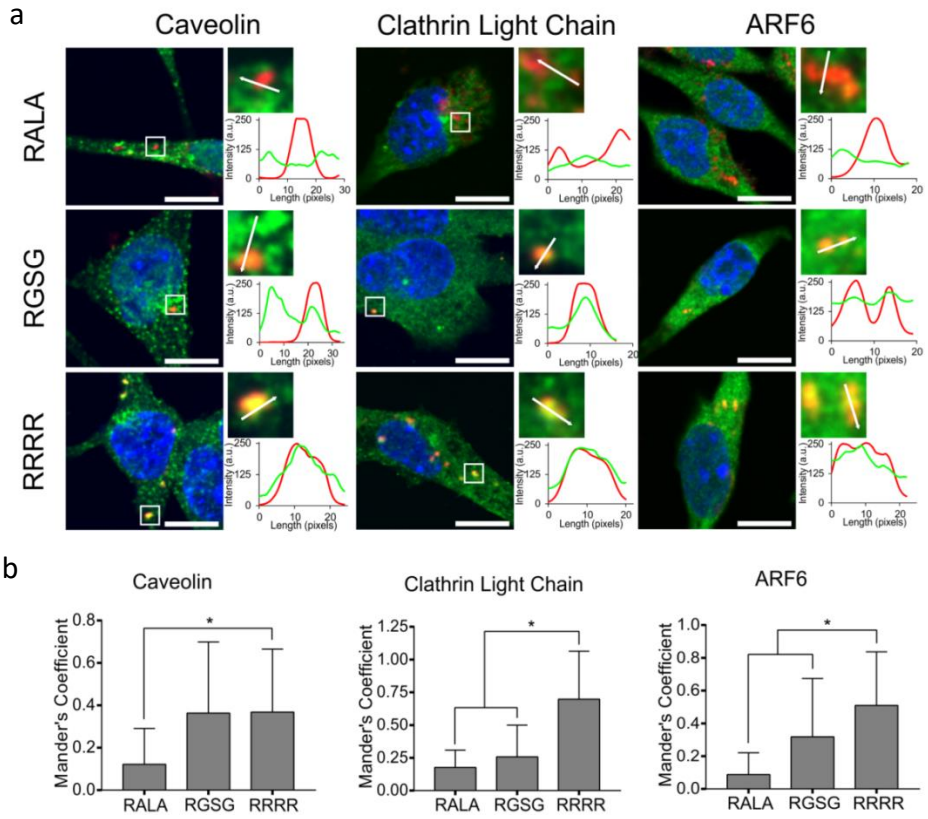

Figure 7. RGSG or RRRR delivered mRNA remains trapped in endocytic vesicles. (a) DC2.4 cells were transfected with the indicated peptide using Cy3b-labeled GFP mRNA (red). Cells were then stained for the indicated endosomal markers (green). Nuclei were stained with DAPI (blue). Cropped images are indicated by the white square. Line profiles are along the white arrow. Scale bar represents 10µm. **(b)** Bar graph summarizing the data from **(a)** that shows mean Mander's colocalization coefficient per cell between mRNA and the indicated endosomal marker. Scale bars represent standard deviation. * $p < 0.05$.

3. Discussion

CPPs have been widely explored in the context of siRNA and of pDNA delivery but have remained remarkably unexplored in the context of mRNA delivery. Here, we describe the use of the cationic, amphipathic CPP RALA to successfully deliver antigen-encoding mRNA to the immune system. We gained evidence that RALA-based mRNA delivery stimulated mRNA uptake by DCs and facilitated mRNA release from the endosomes to the cytosol, resulting in efficient

antigen expression inside the DC. Nanocomplexes of mRNA with arginine-rich peptide variants that lacked RALA's amphipathic motif showed reduced DC uptake and remained entrapped in endosomal vesicles. Furthermore, these peptide variants failed to support the expression of the mRNA-encoded antigen that is required to prime T cell responses. Although we showed some strong indications to suggest that RALA lends his unique immunogenicity to its amphipathic character, one should keep in mind that RGSG and RRRR nanocomplexes differ from RALA nanocomplexes in size, surface charge and hydrophobicity. Therefore, at this stage, we cannot exclude that these differences are not affecting the uptake of the particles, the endosomal escape and as a consequence the immunogenicity of the nanocomplexes.

In addition to a thorough assessment of the crucial features that determine the immunogenicity of RALA mRNA nanocomplexes at the peptide level, we also determined the impact of the mRNA format on the strength of the evoked T cell response at the mRNA level. *In vitro* transcribed mRNA is typically recognized by a set of immune receptors that trigger potent inflammatory cascades[23-27]. This innate immune recognition can be largely prevented through incorporation of modified nucleosides into the mRNA, which simultaneously ameliorates mRNA's stability and enhances mRNA expression levels[21,22]. In combination with RALA, modified mRNA proved to be superior in eliciting cytolytic T cells to non-modified mRNA. This observation is striking, as modified mRNAs have been developed exactly to prevent innate immune activation in the context of gene therapy. As innate immune activation is required to prime T cell immunity, our data suggest RALA possesses intrinsic immune activating properties on its own.

We have demonstrated the high potential of RALA in complexing antigen-encoding mRNA into immunogenic nanocomplexes. In terms of potency, RALA-mediated mRNA vaccination clearly outperformed a standard liposomal mRNA formulation composed of the cationic lipid DOTAP and the fusogenic lipid DOPE. Based on the combination of high immunogenicity and ease-of-production, we believe that RALA – and by extension other CPPs – constitute highly promising delivery vehicles for mRNA vaccines that merit further study.

PART 2.

RALA-Based Delivery Of Antigen-Encoding Modified mRNA Elicits Superior CD8+ T Cell Responses Independently Of Type I IFN Responses

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Ongoing research

Author Contributions

A.D.B. designed, performed and analysed experiments (Fig.8-11). J.G. supervised the project. J.G and S.D.K., designed research.

4. RESULTS

4.1 RALA-RNA VACCINES INDUCE LESS TYPE I IFNS COMPARED TO DOTAP/DOPE-RNA VACCINES.

Earlier, we have shown that the activity of mRNA DOTAP/DOPE lipoplex vaccines was hampered by vaccine-evoked type I IFNs as immunizing Ifnar$^{-/-}$ mice resulted in increased vaccine-evoked CD8^{+} T cell responses. Further on, we demonstrated that RALA mRNA vaccines are more immunogenic when formulated to modified mRNA instead of unmodified mRNA, thus suggesting that also here type I IFNs induced by unmodified mRNA may exert an inhibitory effect on the vaccine immunogenicity. Finally, we showed in chapter 4 **(Figure 4)** that immunizing wild type mice with modified mRNA-RALA vaccines elicited significantly stronger immune responses compared to modified mRNA-DOTAP/DOPE vaccines. A logical question therefore is whether this difference in immunogenicity and sensitivity to RNA modification – mRNA-DOTAP/DOPE lipoplexes are insensitive to mRNA modification – reflects a different level of type I IFN induction by both vaccine delivery formats. To find out, IFNβ$^{+/\Delta\beta\text{-luc}}$ reporter mice were injected i.d. with both RALA and DOTAP/DOPE formulations using either unmodified or modified ovalbumin-encoding mRNA. As shown in **Figure 8**, mice injected with RALA mRNA formulations showed hardly detectable IFNβ levels as opposed to mice injected with equivalent doses of DOTAP/DOPE mRNA lipoplexes.

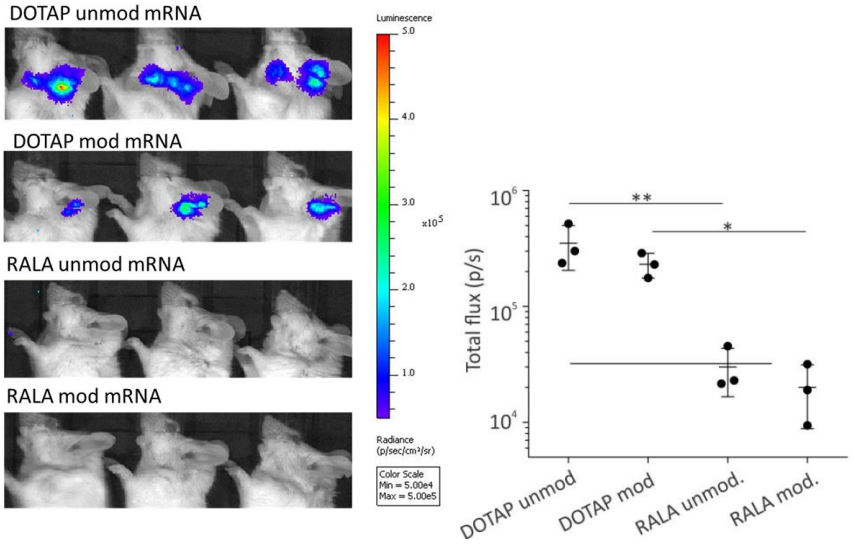

Figure 8: RALA mRNA nanocomplexes induce less IFNβ compared to mRNA lipoplexes. (a) IFN-$\beta^{+/\Delta\beta\text{-luc}}$ reporter mice were injected i.d. with 7 µg of ovalbumin-encoding mRNA DOTAP/DOPE lipoplexes of N/P 1, mRNA RALA nanocomplexes of N/P 10 or PBS (background in the graph) in a total volume of 20 µl. Six hours after injection, IFNβ induction was measured via *in vivo* bioluminescence. **(b)** Graphical representation * $p < 0,05$; ** $p < 0,01$ (One-way ANOVA, post-hoc: Tukey's). Data are shown as mean ± SEM of 3 mice. Mod, modified mRNA; unmod, unmodified mRNA.

4.2 The Efficacy Of Modified mRNA RALA Nanocomplexes Is Not Hampered By Type I Ifns.

In view of the remarkable low IFNβ levels of RALA mRNA nanocomplexes, we assessed whether the strength of the immune response elicited upon RALA mRNA nanocomplex vaccination is still hampered by type I IFNs. To this end, $Ifnar^{-/-}$ and wild type mice were immunized with RALA mRNA nanocomplexes, followed by an *in vivo* killing assay in order to quantify vaccine-elicited $CD8^+$ T cell effector functions. Strikingly, no difference was seen between $Ifnar^{-/-}$ and wild type mice immunized with modified mRNA RALA nanocomplexes, whereas unmodified mRNA RALA nanocomplexes did perform significantly better in $Ifnar^{-/-}$ mice compared to wild type mice. **(Figure 9).**

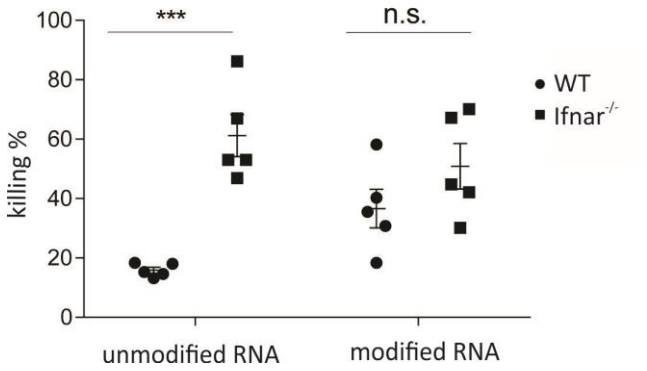

Figure 9: The efficacy of modified mRNA RALA nanocomplexes is not hampered by type I IFNs. Wild type and Ifnar$^{-/-}$ mice were immunized twice with 7 µg of unmodified and modified mRNA RALA nanocomplexes at N/P 10 in a total volume of 20 µl. Two weeks after boost, CFSE-labelled OVA-pulsed and irrelevant peptide-pulsed cells were adoptively transferred in an 1:1 ratio. Two days later, spleens were dissected and splenocytes were analysed by flow cytometry to measure the specific killing capacities of the vaccine elicited CD8^{+} T cell responses. Data are shown as mean ± 5 mice per group. *** $p < 0{,}001$ one-way ANOVA (post hoc: Tukey's test).

4.3 *In Vivo* Detectable Antigen Expression Levels Are Caused By Free mRNA

We next assessed whether the mRNA vaccine carrier affect the expression levels of the mRNA-encoded antigen. Wild type mice were injected with modified mRNA formulated into RALA- or DOTAP/DOPE-based particles. To our surprise, no signal could be detected when RALA-mRNA nanoparticles at N/P 10 – the most optimal N/P ratio for immunizations – were administered, opposed to free mRNA or DOTAP/DOPE mRNA complexes **(Figure 10a,b)**. However, when the N/P ratio of RALA was lowered to N/P 1 – meaning only 10 % of the mRNA remains encapsulated - weak expression levels were seen. As we identified RALA mRNA nanocomplexes of N/P 10 as being more immunogenic than RALA nanocomplexes at N/P 1 **(Figure 10c),** we can conclude that the quantified antigen expression levels cannot be directly related with the immunogenicity of the particles.

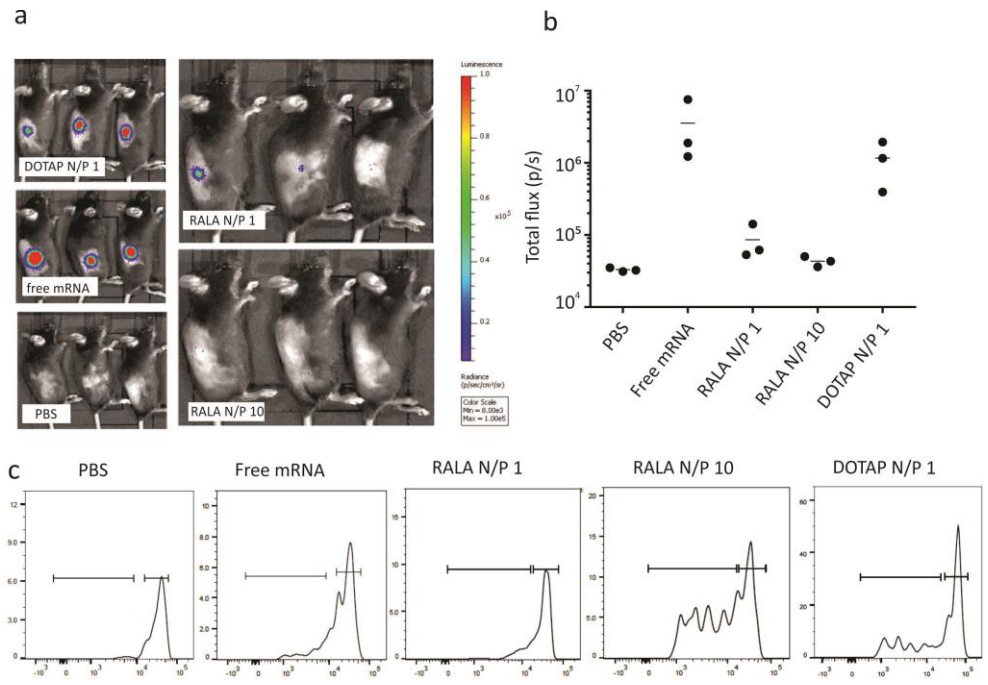

Figure 10. Quantified antigen expression levels do not reflect immunogenicity. (a-b) Wild type mice were shaved and injected with a total volume of 50 µl PBS or 20 µg luciferase- encoding modified mRNA free or complexed to RALA of DOTAP at the indicated N/P ratios. 8 hours after injection, *in vivo* bioluminescence was measured by injecting D-luciferine substrate. Data are shown as mean of 3 mice. **(c)** Graphical representation of OT-I proliferation after immunization. Two days prior to immunization CFSE-labelled OT-I cells were adoptively transferred to wild type mice. Intradermal immunization was performed at the ear pinna with 5 µg of modified ovalbumin mRNA lipoplexes of nanocomplexes at the indicated N/P ratio. Free mRNA and PBS were injected as a negative control. Three days after immunization inguinal lymph nodes were isolated and $CD8^+$ T cell proliferation was analysed by flow cytometry.

4.4 The Innate Immune Activation Mechanism Of Peptide- And Lipid-Based mRNA Vaccines Differs

The low to undetectable levels of IFNβ observed upon injection of RALA mRNA nanocomplexes indicated that RALA-delivery does not trigger a pronounced type I IFN-dependent innate immune activation. In order to elucidate the molecular pathway(s) underlying innate immune activation, we performed an *in vivo* killing assay to compare vaccine-elicited CTL responses in $MyD88^{-/-}$ and $Trif^{-/-}$ mice. MyD88 plays a central role in the innate immune responses as an

adaptor molecule for signal transduction of all Toll-like receptors except TLR3[28,29]. This pathway involves the early phase of NF-KB activation, resulting in the production of inflammatory cytokines[29]. TLR3 is the only TLR receptor that is not dependent on MyD88 but signals via TRIF. The TRIF-dependent signalling pathway will result mainly in the induction of type I IFNs via the activation of the transcription factors IRF3 and IRF7. In here, we showed that modified mRNA RALA nanocomplexes completely lost their capacity to evoke functional CTL responses in Trif$^{-/-}$ mice compared to wild type, whereas MyD88 deficiency seems to slightly strengthen - although not significant - CD8^{+} T cell immunity **(Figure 11a)**. On the contrary, immunization of MyD88$^{-/-}$ and Trif$^{-/-}$ mice with modified mRNA DOTAP/DOPE lipoplexes did not result in significant stronger or weaker CTL responses relative to wild type mice **(Figure 11b)**.

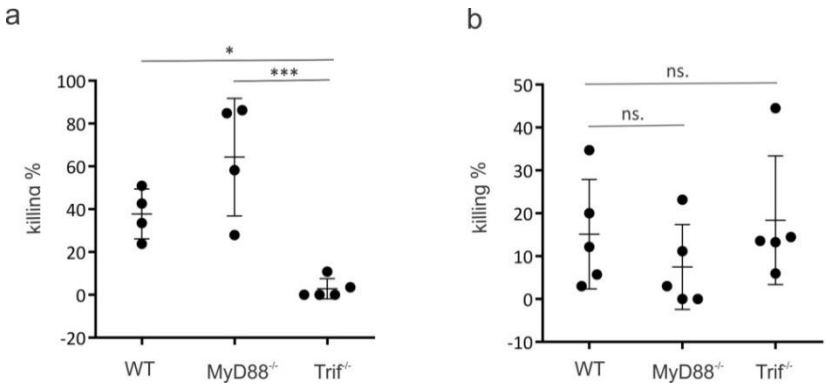

Figure 11. Different mechanisms of innate immune activation by RALA- and DOTAP-mRNA vaccines. Wild type, MyD88$^{-/-}$ and Trif$^{-/-}$ mice were immunized twice with 7 µg of modified mRNA complexed to **(a)** RALA at N/P 10 and **(b)** DOTAP at N/P 1 in a total volume of 30 µl. Two weeks after boost, CFSE labelled OVA-pulsed and irrelevant peptide-pulsed cells were adoptively transferred at a 1:1 ratio. Two days later, spleens were dissected and splenocytes were dissected to analyse the effector function of the vaccine-elicited CTL responses. Means of 4-5 mice ± SD per group are shown. * $p < 0,05$; ** $p < 0,001$; *** $p < 0,0001$; One-way Anova.

5. Discussion

In this study, we demonstrated that modified mRNA RALA nanocomplexes showed improved vaccine efficacy to unmodified mRNA RALA nanocomplexes. This result is in sharp contrast to lipid-based delivery of mRNA, showing no strengthened CD8^{+} T cell immunity in response to

modified antigen-encoding RNA delivery. Further, we have analysed whether RALA mRNA nanocomplex injection resulted in IFNβ induction, like mRNA DOTAP lipoplex vaccines do. Using an IFN-$\beta^{+/\Delta\beta\text{-luc}}$ reporter mice we revealed that RALA mRNA nanocomplexes induced significant lower IFNβ levels compared to DOTAP-based delivery. Although the injection of both unmodified and modified mRNA RALA nanocomplexes showed no detectable IFNβ induction in the reporter mice, only modified mRNA RALA nanocomplexes showed equal CTL responses in wild type and $Ifnar^{-/-}$ mice. To our surprise, unmodified mRNA RALA nanocomplexes still showed stronger CTL responses in $Ifnar^{-/-}$ mice. When interpreting these results, two main conclusions can be drawn. First, these data clearly demonstrate that RALA outcompetes DOTAP/DOPE to function as a carrier for modified mRNA-based CTL-inducing vaccines. In addition, we have provided clear evidence that modified mRNA RALA nanocomplexes are not hampered by type I IFNs, a phenomenon we earlier observed in the context of DOTAP/DOPE-based delivery.

At last, to gain some insights in how both nanoparticle formats trigger innate immunity, we performed an *in vivo* killing study using wild type, $MyD88^{-/-}$ and $Trif^{-/-}$ mice. As immunization of $Trif^{-/-}$ mice with RALA mRNA nanocoplexes showed complete lack of cytotoxic $CD8^{+}$ T cell responses, it is highly suggested that the immunogenicity of mRNA RALA nanocomplexes might be dependent on TLR3 triggering. The immunization of $Trif^{-/-}$ and $MyD88^{-/-}$ mice with mRNA DOTAP lipoplexes showed no significant differences to wild type mice. These data may indicate that lipoplex-based delivery of mRNA results in the activation of multiple cytosolic receptors and/or TLRs, resulting in a redundant effect in a specific knock-out model. As this is a preliminary experiment, further research is needed to reveal the underlying mechanisms of the immunogenicity of RALA- and DOTAP/DOPE – based vaccines.

6. Materials And Methods

Animals

Female C57BL/6 mice and OT-I mice (7 weeks old) were purchased from Janvier (Le Genest Saint Isle, France). All mice were maintained under specific pathogen-free conditions and treated according to the European guidelines for animal experimentation. All experiments were approved by the local ethical committee of Ghent University (Ghent, Belgium).

Nanoparticle preparation

RALA was produced by solid-state synthesis and supplied as an acetate salt lyophilized powder (Biomatik, USA). EGFP mRNA (L-6301) and Ovalbumin mRNA (unmod: L-6326; mod: L-6128) were purchased from TriLink BioTechnologies (San Diego, USA). RALA/mRNA complexes were prepared at N/P ratios 1, 5 and 10 by adding appropriate volumes of RALA peptide solution to a specific amount of mRNA. The N/P ratio represents the molar ratio of positively charged nitrogen atoms in the peptide to negatively charged phosphates in the mRNA backbone. For lipid-based complexation of mRNA cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were added to the mRNA at N/P ratio 1 (both Avanti Polar Lipids, Alabaster, AL, USA).

Nanoparticle characterization

For size and zeta potential measurements, particles were analyzed using a Nano ZS Zetasizer (Malvern Instruments, UK). The shape and surface morphology were observed using Jeol JEM1400plus transmission electron microscope at 30 kV.

Encapsulation assay

Quant-iT™ RNA assay kit (Life Technologies, UK) was diluted 1:200 in TAE buffer and added to an equal volume of nanoparticle prepared solution. Sample fluorescence was analyzed using a Synergy 2 Multi-Mode Microplate Reader (BioTek Instruments Inc, UK).

Hemolysis assay

Chicken red blood cells (RBCs) (Veterinary department, UGent) were resuspended in pH 5.6 and pH 7.4 buffer solutions separately. The cell suspensions were treated with the indicated formulations. 20% Triton was used as a positive control representing 100% hemolysis. After 1 hour incubation (37°C), samples were centrifuged to pellet intact RBCs. Supernatants were collected and absorbance was measured at 450nm using iMark Microplate Reader (Biorad, USA)

In vitro DC transfection

DC2.4 cells (1.2×10^5 cells/well) were incubated for 2 hours in Opti-MEM serum free media (Gibco, UK) prior to addition of 50 µl RALA-mRNA complexes (2.5µg mRNA). Four hours later, eGFP expression as analyzed on a LSRII flow cytometer (BD Biosciences).

In vivo T cell proliferation assay

OT-I T cells were labelled with 5 µM carboxyfluorescein diacetate succinimedyl ester (CFSE) (Invitrogen, Belgium) and 1 million CFSE labelled cells were i.v. injected two days prior to immunization. Mice were intradermally immunized with 7 µg of unmodified or modified mRNA nanocomplexes in a total volume of 20 µl. Three days later, lymph nodes were isolated and OT-I T cell division was analysed by flow cytometry. Cells were stained with α-CD16/CD32 (BD Biosciences, USA), Live/Dead Fixable Aqua stain (Invitrogen), α-CD8 PerCP, α- CD3 pacific blue, α-CD19 APC-Cy7 (all BD Biosciences, USA) and MHC dextramer H-2 Kb/SIINFEKL-PE (Immudex, Denmark).

In vivo cytotoxicity assay

Mice were intradermally immunized with 7 µg of unmodified or modified mRNA nanocomplexes in a total volume of 20 µl. Two weeks after prime and boost vaccination with a two week interval, splenocytes from C57BL/6 mice were pulsed with MHC-I OVA peptide (target cells) (30 µg/ml) or left unpulsed (non-target cells) for 60 minutes at 37°C, and subsequently labelled with respectively 5 µM or 0.5 µM CFSE (Invitrogen, Belgium) accordingly to the manufactur's protocol. Labelled cells were injected into immunized mice at a 1/1 ratio. Spleen suspensions were analysed 48 hours later by flow cytometry. The percentage antigen-specific killing was determined using the following formula: $1 - ((\%CFSE_{hi}\ \text{cells} / \%CFSE_{low}\ \text{cells})^{\text{immunized mice}} / (\%CFSE_{hi}\ \text{cells} / \%CFSE_{low}\ \text{cells})^{\text{non-immunized mice}})$.

mRNA labelling

Multiply labeled tetravalent RNA imaging probes (MTRIPs) were constructed as described in reference[21]. We designed four different 2'O-methyl RNA-DNA chimeric oligos containing 17-18 nucleotide regions with a short 5-7 poly(T) linker. Oligos were antisense to 4 adjacent sequences spanning the mRNA 3' UTR and contained three to four amino-modified thymidines each and containing a biotin modification (Biosearch). MTRIPs were assembled by conjugating Cy3B-NHS ester (GE Healthcare) to oligo amine groups using manufacturer protocols. Complete MTRIPs were assembled by incubation with Neutravidin (Pierce) for 1 hour at RT followed by filtration using 30 kD MWCO centrifugal filters (Millipore). mRNA was buffer exchanged into 1x PBS and heated to 70°C for ten minutes and immediately placed on ice, combined with MTRIPs in a 1:1 mRNA:MTRIP ratio for each MTRIP, then incubated overnight at 37°C. The next day, the mixture

was filtered using a 200 kD MWCO ultrafiltration unit (Advantec MFS Inc) and concentrated by 50 kD MWCO centrifugal filters (Millipore).

Co-localization studies

DC 2.4 cells were plated on glass coverslips 24 hours prior to transfection with RALA, RGSG, or RRRR along with 250 ng of eGFP mRNA labeled with cy3b MTRIPs per well. 5 hours post transfection, cells were fixed in 4% PFA and staining was performed for Caveolin (Santa Cruz Biotechnology - SCBT), Clathrin Light Chain (SCBT), ARF6 (SCBT), or a combination of CD63 (Developmental Studies Hybridoma Bank - DSHB), EEA1 (BD Biosciences), and LAMP1 (DSHB). Images were taken using a Flash 4.0 v2 sCMOS camera (Hamamatsu) with a 60x NA 1.4 Plan-Apochromat objective on an Ultraview spinning disk confocal microscope (Perkin Elmer). Imaging and analysis was performed in Volocity (Perkin Elmer). Statistical analysis and plots were performed in Prism 7 (Graphpad).

7. SUPPLEMENTARY DATA

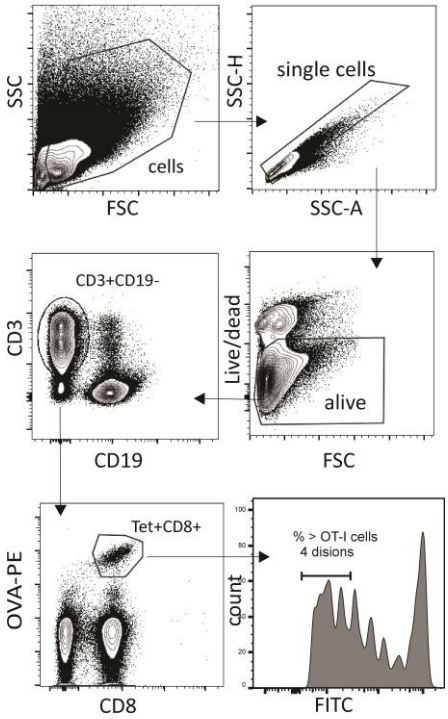

Figure S1. Gating strategy used for OVA- specific $CD8^+$ T cell proliferation. Cells are gated based on FSC and SCC, before single cells are gated based on SSC-area and height. Living cells are selected and gated for $CD3^+CD19^-$ T cells. Within $CD8^+$ T cells, OVA-specificity is gated by labelling with MHC-I SIINFEKL – PE dextramer. Proliferation of CFSE positive OVA-specific $CD8^+$ T cells is shown by counting % OT-I cells above 4^{th} division

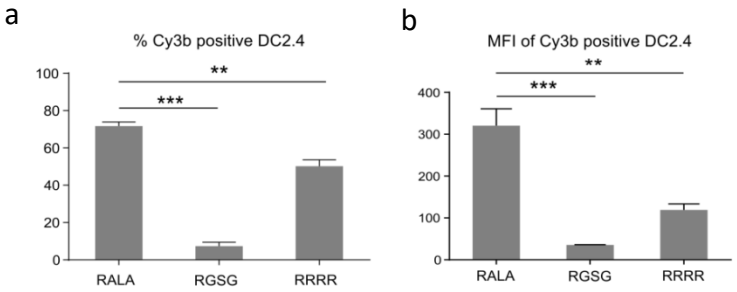

Figure S2. Effects of peptide composition on uptake of the mRNA cargo. (a,b) Flow cytometric analysis of cargo mRNA uptake after incubation of DC2.4 dendritic cells with Cy3b-labeled mRNA complexed to respectively RALA, RGSG and RRRR at N/P 10. The percentage of Cy3b positive DC2.4 cells is shown in **(a)** whereas the mean fluorescence intensity (MFI) of Cy3b-positive DC2.4 is depicted in **(b).** Measurements are reported as means (n = 4) ± SD, *** $p < 0.001$, ** $p < 0.01$.

8. Acknowledgments

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CHAPTER 7

PEG-pHDPA RNA Polyplexes, A Novel Polymer-Mediated RNA Adjuvant

PEG-pHDPA RNA Polyplexes, A Novel Polymer-Mediated RNA Adjuvant

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Ongoing Research

Author Contribution

A.D.B. designed, performed and analysed *in vivo* experiments (Fig. 4 and Fig 5.) and wrote the manuscript. L.B. and W.H. designed, produced and characterized the polymer (Fig 1-2). B.D.G. performed confocal imaging (Fig.3). G.R.D provided technical assistance. W.H., S.D.K and B.D.G designed research and supervised the project. J.G. supervised the project. S.D.K. edited the manuscript.

Abstract

Non-coding single stranded RNA (ssRNA) is being intensively evaluated as vaccine adjuvants due to their capacity to promote cytolytic $CD8^+$ T cell responses against co-delivered protein antigens. This intrinsic potential of ssRNA to promote cellular immunity is conveyed by their capacity to trigger TLRs 7/8 in the endosomal compartments of dendritic cells, leading to an inflammatory signalling cascade that culminates in the release of IL-12 and type I IFNs. Nonetheless, unformulated ssRNAs succumb to rapid degradation and largely fail to reach their TLR target in the endosomal compartment of DCs. In this study, we describe a pHPDA-based polymeric nanoparticulate system that combines efficient protection of the ssRNA with a highly effective targeting of DCs in the vaccine draining lymph node. When compared to unformulated ssRNA, pHPDA-formulated RNA drastically promoted the priming of antigen-specific $CD8^+$ T cells with cytolytic effector function against a co-administrated protein antigens.

1. INTRODUCTION

The generation of potent cytolytic $CD8^+$ T cell responses is considered to be crucial for the development of vaccines both capable of fighting insidious intracellular pathogens or cancer cells[1,2]. Unfortunately, the classical aluminium-based adjuvants fail to elicit this arm of T cell immunity[3,4], which urges the need for novel adjuvant formulations that do possess the ability to instigate cellular immunity against co-administrated antigens. Activation of T cell immunity critically depends on the prior activation of innate immune cells, which is mediated through the triggering of host PRRs by Pathogen Associated Molecular Patterns (PAMPs). Depending on the set of PRRs triggered, innate immune cells will release a distinct inflammatory cytokine profile, which will ultimately determine the characteristics of the ensuing T cell response. Viral nucleic acids are amongst the most prominent inducers of type I IFNs and IL-12, the key cytokines that govern the differentiation of antigen experienced $CD8^+$ T cells into cytolytic effector cells[5–7]. Viral ssRNA could activate TLR7 and TLR8 in specialized endosomal compartments and strongly promote cytolytic T cell responses. As a consequence, agonists of TLR7/8 have emerged as highly promising vaccine adjuvants. Imidazoquinolines were the first class of TLR7/8 agonists developed for human use[8]. The development of imidazoquinolines as antivirals for topical treatment of genital warts preceded their actual identification as TLR7/8 agonists[9]. Subsequent murine studies demonstrated that imidazoquinolines act as adjuvants that stimulate cytolytic T

cell responses against co-administrated antigens. Imidazoquinolines however rapidly diffuse from the injection site and trigger systemic inflammatory responses resulting in temporal leukocyte depletion and altered lymphoid architecture. On top, imidazoquinolines have been reported to amplify inflammation through TLR7/8 independent pathways[10].

Guanidine and uridine rich ssRNA constitute the natural agonists of TLR7/8 in the endosomes of DCs and represent less pro-inflammatory, safer alternatives to imidazoquinolines[11]. Nonetheless, to develop ssRNAs as successful adjuvants, several hurdles need to be taken. Natural ssRNAs are notoriously instable and prone to rapid degradation upon injection, a feature that interferes with their capacity to reach their endosomal TLR7/8 target. To prevent premature degradation and to increase DC uptake, ssRNAs have been complexed to cationic lipids such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)[12,13] or to cationic polymers such as polyethyleneimine (PEI)[11] and protamine[14]. Whereas such approach indeed strongly increased the adjuvanticity of the ssRNA, the amount of complexed ssRNA that actually reached the draining lymph node upon intradermal/subcutaneous injection remained highly limited[15]. As a high dose of adjuvant in the vaccine draining lymph node – the sites of antigen presentation and T cell priming – have been associated to the induction of superior T cell immunity, we aimed to develop a nanoparticulate formulation that not only protects from degradation, but also efficiently delivers the ssRNA to lymph node DCs.

To achieve these goals, we formulated ssRNA into PEGylated (PEG, poly(ethylene)glycol)- pHPDA polymeric nanocomplexes. PEGylation constitutes a well described strategy to enhance the colloidal stability of nanocomplexes by shielding their cationic charges. PEGylation has been mainly applied to increase the circulation time and half-life of nanoparticles upon intravenous administration, but PEGylation also increases lymphatic drainage of nanoparticles upon intradermal or subcutaneous injection[16]. The incorporation of PEG into polyplexes is commonly achieved by using a block copolymer consisting of cationic and PEG-domains, that simultaneously acts as a condensing agent (e.g. PEG-polylysine[17,18], PEG-poly(aspartamide)[19,20], PEG-poly(amido amines)[21,22] and PEG-PEI[23,24]). However, it has been argued that such PEG-block-polymer particles are less stable than polyplexes based on cationic polymers since the PEG-chains of block- or graft-copolymers may hinder or prevent the interaction between the cationic polymer and the nucleic acids[25,26]. An alternative method to overcome this problem is to PEGylate the surface of preformed nucleic acid-polymer complexes, called post-PEGylation.

Several papers have indicated that, compared to polyplexes formed by PEG-cationic block or graft polymers, post-PEGylated polyplexes hardly showed interactions with blood components and revealed prolonged circulation time in mice after intravenous administration[27–29].

In this paper, we have utilized copper-free click chemistry to post-modify mRNA polyplexes with PEG-Bicyclo[6.1.0]nonyne (PEG-BCN). We further show that these PEGylated polymeric formulation efficiently encapsulate the RNA cargo and moreover protect it against degradation. Moreover, the formulation of ssRNA into PEGylated polymers enhanced ssRNA targeting to lymph node DCs when compared to unformulated ssRNA or ssRNA complexed to cationic liposomes. Most importantly, we present PEG-pHDPA RNA polyplexes as potent novel adjuvant for the eliciting of potent cytolytic $CD8^+$ T cells responses against co-delivered protein antigens.

2. Results

2.1 PEG-pHDPA RNA Polyplexes; Production Process And Characterization

In this study, pHDPA polymer composed of p(HPMA-DMAE-co-PDTEMA-co-AzEMAm) was used to complex ssRNA into RNA polyplexes followed by PEGylation, hereafter named as PEG-pHDPA RNA polyplexes. A detailed description about the production process of the pHDPA polymer can be found in the material and methods section. A schematic presentation of the production process of the PEG-pHDPA RNA polyplexes is provided in **Figure 1a**. In brief, PEG-pHDPA RNA polyplexes were formed through a three-step process. The first step consists electrostatic formation of the cationic polymer pHDPA with ssRNA to form nanosized polyplexes **(Figure 1a).** To limit the amount of free polymer in the ssRNA polyplex solution a Nitrogen/Phosphate (N/P) ratio of 4 was selected. Second, the ssRNA polyplexes were post-PEGylated by adding PEG-BCN to the polyplex solution. This PEGylation step was followed by a third step in which Dithiothreitol (DTT) was added to cross-link the polyplexes via thiol-disulfide bonds exchange[30,31], yielding PEGylated, stable and nearly neutral-charged RNA polyplexes.

At N/P ratio 4, PEG-pHDPA RNA polyplexes are slightly positive to near neutral and display a diameter around 150 nm for both ssRNA formats tested; luciferase encoded mRNA and polyU **(Figure 1b).** The stability of the PEG-pHDPA RNA polyplexes was evaluated after incubation at 37

°C in the presence of 10 % serum in order to mimic *in vivo* conditions. The size distribution of the PEG-pHDPA RNA polyplexes in presence of serum was determined by fluorescent single particle tracking (fSPT)[32] and compared to polyplexes diluted in PBS. As depicted in **Figure 1c**, the particles size remained stable after 1 hour of incubation, and only slightly increased after 2 hours. Importantly, the PEG-pHDPA RNA polyplex size was similar before and after lyophilisation **(Figure 1d),** suggesting that the PEG-pHDPA RNA polyplexes can be stored in dry powder form, a great advantage since the shelf-life of RNA-based particles is rather short.

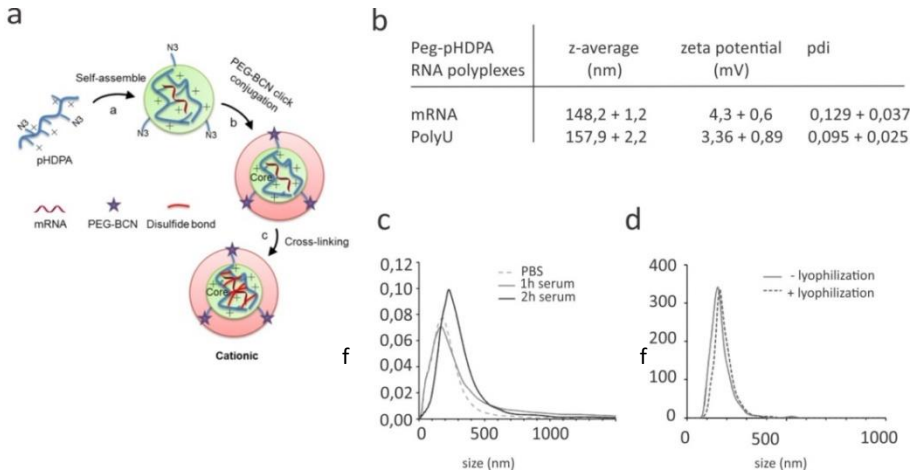

Figure 1. Schematic presentation of PEG-pHDPA RNA polyplex procedure and characterization. **a)** Schematic presentation of polyplex preparation: p(HPMA-DMAE-co-PDTEMA-co-AzEMAm) was synthesized by radical polymerization under a nitrogen atmosphere to form pHDPA polymer. (a) pHDPA polymer was mixed with ssRNA to form pHDPA RNA polyplexes (b) Next, Cyclooctynes (PEG-BCN) was added via the copper free click method using the polymer-azide binding. (c) followed by crosslinking of the polymer using Dithiothreitol (DTT), for 2 hours at pH 7.4. **b)** Size and Zeta-potential of PEG-pHDPA RNA polyplexes using ssRNA (mRNA and polyU RNA) **c)** Size distribution of PEG-pHDPA Cy5-RNA polyplexes after incubated with 10 % of serum at 37 °C for 2 hours and **d)** after lyophilisation measured by Nanosight.

2.2 PEG-pHDPA RNA Polyplex Formulation Protects Ssrna Against Degradation

ssRNA is a very fragile molecule due to its sensitivity to RNAses, resulting in a high risk for degradation after *in vivo* injection. First, to quantify the RNA release, a fluorescence correlation spectroscopy (FCS) study was performed using Cy5-labeled RNA. PEG-pHDPA RNA polyplexes

were dissolved in 10 % serum and a slow but gradual release of RNA up to 60 % after 2 hours incubation was detected **(Figure 2a)**. To further address the stability of the PEG-pHDPA RNA polyplexes and the degradation resistance of RNA polyplexes to serum, a gel retardation study was performed **(Figure 2b).** After incubation of uncomplexed RNA with 10 % serum at 37^{o}C for 30 minutes, hardly any free RNA can be detected suggesting that the RNA has been totally degraded by present nuclease enzymes **(Figure 2b, lane 9).** On the contrary, PEG-pHDPA RNA polyplexes showed partial protection of the RNA in the presence of serum **(Figure 2b, lane 5).** As a control DTT and pGA were added to destabilise the PEG-pHDPA RNA polyplexes by disturbing the cross-linking of the polymer and by expelling the RNA respectively. Remarkably, as depicted in **Figure 2b, lane 3 and 4**, most of the RNA remained attached to the polymer, indicating that the destabilization of the polyplexes was not completed. In order to check the destabilization effects of the DTT and pGA treatment, one could extend the incubation period.

To further evaluate the integrity of ssRNA upon formulation into PEG-pHDPA RNA polyplexes, translation efficacy of luciferase-encoding RNA was tested using an *in vitro* cell-free translation system. As only translation of full length RNA sequences will result in detectable signal, measuring the luciferase intensity will display the amount of qualitative, non-degraded RNA present in PEG-pHDPA RNA polyplex solution. As depicted in **Figure 2c,** in presence of serum, uncomplexed RNA showed no detectable antigen amounts, in contrast to free RNA in PBS. Whereas PEG-pHDPA RNA polyplexes resulted in clear antigen expression levels after 90 minutes of incubation in serum, indicating that the PEG-pHDPA polyplexes have the capacity to prevent breakdown of the RNA by serum proteins, leading to the maintenance of full length mRNA. The fact that the PEG-pHDPA RNA polyplexes showed lower translation levels compared to uncomplexed RNA, might attribute to RNA remained inside the polyplexes.

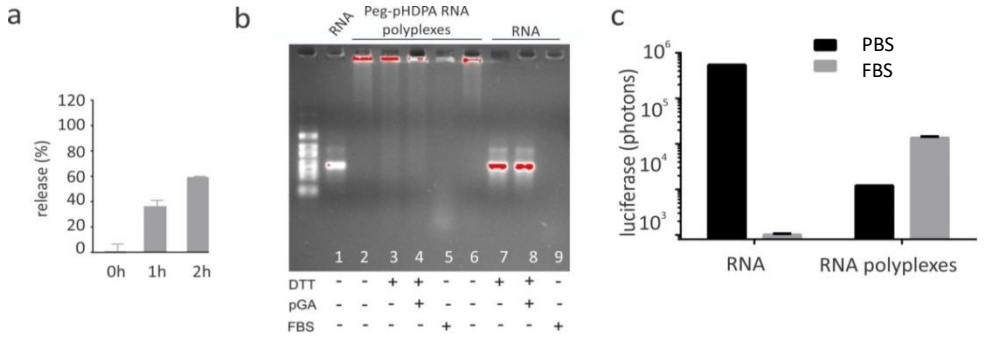

Figure 2. PEG-pHDPA RNA polyplex formulation protects ssRNA against degradation. a) The percentage of Cy5-labeled luciferase RNA released out of PEG-pHDPA RNA polyplexes in 10 % serum is analysed by fluoresence correlation spectroscopy (FCS) at 0 hour, 1 hour and 2 hours upon incubation at 37°C. **b)** Agarose gel retardation assay of PEG-pHDPA RNA polyplexes which were incubated with 10 mM DTT, polyGlutamicAcid (pGA) or 10 % serum for 0.5 hour at 37°C. **c)** Free luciferase-encoded RNA or PEG-pHDPA luciferase-RNA polyplexes were incubated in PBS or with 10 % serum (FBS) for 0.5 hour at 37 °C before *in vitro* translation was measured with a Rabbit Reticulocyte Lysate Translation Systems.

2.3 Complexation Of Ssrna Into PEG-pHDPA RNA Polyplexes Improves Ssrna Uptake By Dcs

To fulfil their role as an adjuvant, ssRNA needs to reach the endosomal compartment of DCs in order to trigger TLRs. The impact of RNA formulation into PEG-HDPA RNA polyplexes on DC uptake was analysed by incubating DC2.4 DCs with either uncomplexed or PEG-HDPA polyplexed Cy5-labeled RNA. Flow cytometry data are presented in **Figure 3a,b**. Whereas virtually no DCs internalized unformulated Cy5-labeled RNA, the vast majority of DCs became positive for Cy5-labeled RNA after incubation with PEG-pHDPA Cy5-labeled RNA polyplexes. As a control, lipofectamine-based transfection was performed resulting in similar levels of uptake compared to polymer-based transfection.

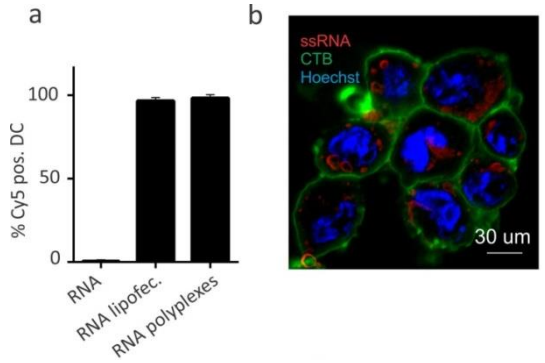

Figure 3. RNA polyplexes improve DC transfection. a) 10^5 DC2.4 dendritic cells were incubated with 0,5 µg Cy5-labeled RNA, PEG-pHDPA Cy5-labeled RNA polyplexes for 4 hours, followed by flow cytometer analysis. As a control 0,5 µg of RNA was transfected using lipofectamin (= lipofec). The means of the percentage of DCs positive for Cy5-labeled RNA are presented ± SD (n=3). **b)** Corresponding confocal microscopy images of DC2.4 cells transfected with PEG-pHDPA Cy5-labeled RNA polyplexes. *Hoechst, DNA stain;* CTB = cholera toxin subunit B- AF 488 ; ssRNA = Cy5-labeled RNA.

2.4 pHDPA SSRNA POLYPLEXES EFFICIENTLY TARGET SSRNA TO LYMPH NODE DENDRITIC CELLS *IN VIVO*

The induction of effector $CD8^+$ T cell responses relies on the presentation of antigens by activated DCs to T cells in the draining lymph nodes. As a consequence, vaccine potency is considered to benefit from strategies that augment adjuvant uptake by DCs in the draining lymph nodes. Cationic lipids or polymers have been explored to protect ssRNA from degradation and increase ssRNA uptake by DCs *in vivo*. Nonetheless, these cationic ssRNA lipoplexes and polyplexes display limited mobility *in vivo*, resulting in low ssRNA presence in draining lymph node DCs. To address whether ssRNA delivery through PEG-pHDPA RNA polyplexes would augment ssRNA uptake by lymph node DCs, we complexed Cy5-labeled RNA into our polyplex-based formula and injected them s.c in the footpad. As a control, Cy5-labeled ssRNA was formulated into DOTAP/DOPE lipoplexes, the current bench-mark delivery system for ssRNA. 24 hours after injection, the popliteal lymph nodes were isolated and analysed by flow cytometry. DCs were identified based on their expression of CD11c and further subdivided into $MHCII^{int}$ DCs and $MHCII^{high}$ DCs. As can be appreciated from **Figure 4a**, s.c. injection of PEG-pHDPA RNA polyplexes strongly increased the percentages of ssRNA-positive $MHCII^{hi}$ DCs when compared to non-complexed ssRNA or to lipoplex-based injection of ssRNA **(Figure 4a, b).** Such $MHCII^{hi}$ DCs might correspond to lymph node DCs that have become activated in the lymph node after

uptake of the ssRNA or might correspond to skin DCs that migrated to the lymph node after PEG-pHDPA RNA polyplexes uptake at the injection site. Incorporation of ssRNA in the PEG-pHDPA RNA polyplexes not only enhanced the number of Cy5-labeled ssRNA positive DCs in the vaccine draining lymph node **(Figure 4c)**, but also strongly increased the amount of Cy5-labeled ssRNA on a cell-per-cell basis as measured by the mean fluorescence intensity in the Cy5 channel **(Figure 4d).** Taken together, these data showed that formulating the RNA into PEG-pHDPA RNA polyplexes dramatically increased the amount of ssRNA in the target cell population – DCs, in the vaccine draining lymph node.

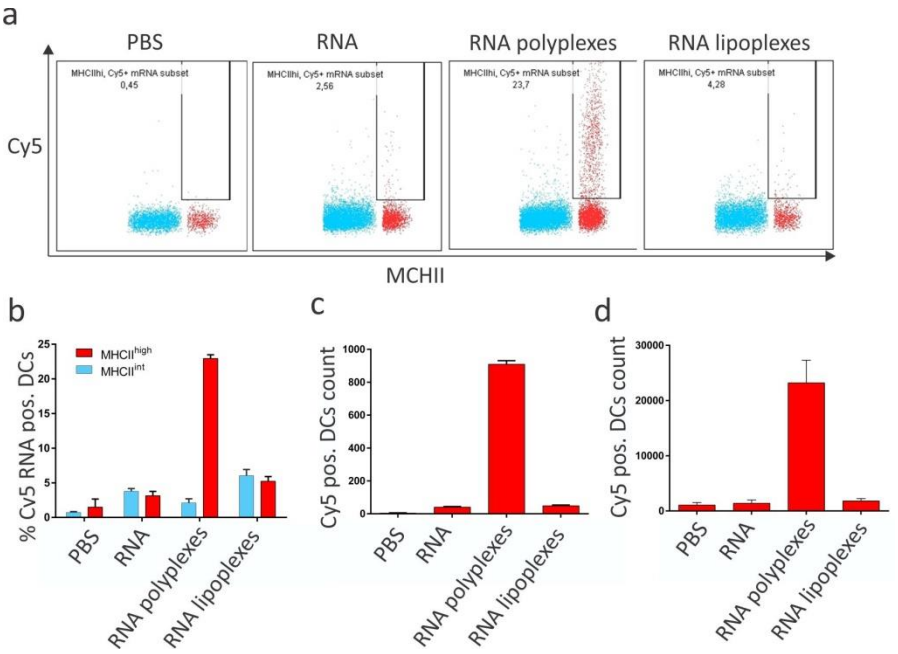

Figure 4. RNA polyplexes are present in lymph node CD11c+ MHCII^{high} DCs after 24 hours upon injection. Mice were s.c. injected in the footpad with PBS, 10 µg of uncomplexed ssRNA or the equivalent amount of ssRNA complexed in PEG-pHDPA polyplexes or DOTAP/DOPE lipoplexes. 24 hours after injection, popliteal lymph nodes were isolated and prepared for flow cytometric analysis. DCs (CD11c+) were subdivided into MHCII^{high} DCs (red population) and into MHCII^{int} DCs (blue population) and analysed for Cy5-labeled ssRNA uptake. **a)** Representative flow cytometry scatterplots. **b)** Percentage of Cy5-positive MHCII^{high} CD11c+ DCs and MHCII^{int} CD11c+ DCs. Data are presented as means ± SD of 5 mice/group. **c)** Total cell count of MHCII^{hi} CD11c+ Cy5-positive DCs. Data are presented as means ± SD of 5 mice/group. **d)** Mean fluorescence intensity (MFI) of the Cy5-signal of all MHCII^{high} CD11c+ Cy5-positive DCs. Data are presented as means ± SD of 5 mice/group. mRNA polyplex = PEG-pHDPA RNA polyplexes; mRNA lipoplex = mRNA DOTAP/DOPE lipoplexes.

2.5 PEG-pHDPA RNA Polyplexes Elicit Potent CD8+ T Cell Responses Against Co-Delivered Antigens

In view of the high percentage mRNA-positive DCs in the draining lymph nodes, we assumed that the RNA polyplexes have great potential to elicit $CD8^+$ T cell responses against co-delivered antigens. To address the impact of polyplex formulation of RNA on the magnitude and functional properties of vaccine-evoked $CD8^+$ T cell responses, an *in vivo* immunization study was performed. First, mice were s.c. immunized with PEG-pHDPA RNA polyplexes co-delivered with soluble ovalbumin (OVA) protein. A prime and boost immunization schedule was performed as presented in **Figure 5a.** As a control, soluble OVA was injected in absence and presence of the polymers or uncomplexed RNA. Six days after the booster immunization, blood was collected and the percentages OVA-specific $CD8^+$ T cells were determined via flow cytometry. As shown in **Figure 5b**, only mice injected with OVA in combination of PEG-pHDPA RNA polyplexes showed detectable vaccine-evoked $CD8^+$ T cells in the blood **(Figure 5b)**. Next, we analysed the impact of PEG-pHDPA RNA polyplexes on the effector functions of these vaccine-elicited $CD8^+$ T cell responses. Therefore, mice were challenged with a 1:1 ratio of OVA peptide-pulsed $CFSE^{hi}$ splenocytes (target cells) and non-pulsed $CFSE^{low}$ splenocytes (non-target cells), two weeks after the second boost **(Figure 5a)**. Two days later, spleens were isolated and the ratio of target cells versus non-target cells was analysed by flow cytometry to determine the killing capacity of the different vaccine formulations. Mice who received OVA co-delivered with the pHPDA polymer showed no significant improvement of killing functions compared to mice injected with soluble OVA **(Figure 5c)**. Mice receiving OVA co-delivered with uncomplexed RNA displayed a broader range of killing efficacies, resulting in a slight but significant increase compared to mice immunized with soluble OVA. Whereas immunizing mice with OVA co-delivered with PEG-pHDPA RNA polyplexes resulted in almost 100 % killing of target cells, a significant difference compared to all other control formulations **(Figure 5c)**. Taken together, these findings demonstrate that formulating RNA into PEG-pHDPA RNA polyplexes positively impact the strength of the evoked cytotoxic $CD8^+$ T cell responses against co-delivered antigens.

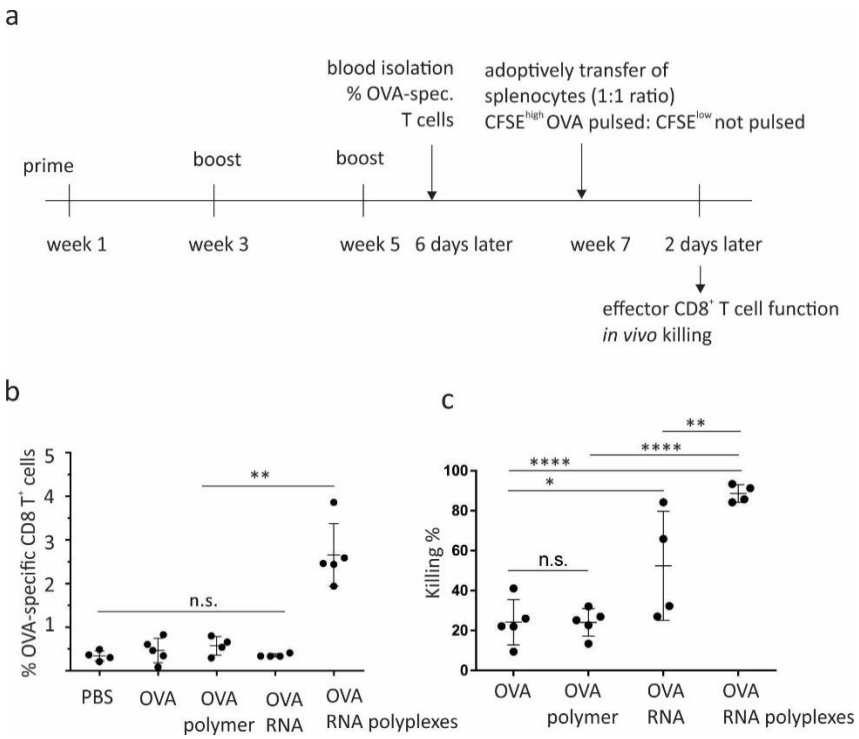

Figure 5. The co-delivery of PEG-pHDPA RNA polyplexes evoke CD8$^+$ T cell responses against ovalbumin-protein. a) Experimental set up of tetramer staining and *in vivo* killing assay. Mice were immunized three times with a two week interval. 6 days after last boost, blood was collected to determine the percentages of CD8$^+$ T cells in the blood via tetramer staining. Two weeks after boost, *in vivo* killing assay was performed. **b)** Mice were immunized with OVA co-delivered with PEG-pHDPA RNA polyplexes. As a control, mice were immunized with PBS, soluble OVA in absence or presence of polymers or uncomplexed RNA. 6 days after last boost immunization, blood was taken to determine the percentages of OVA-specific CD8$^+$ T cells via tetramer staining followed by flow cytometry. Data are shown as mean of 4-5 mice per group. ** $p < 0,01$ (One-way Anova). **c)** Two weeks after he second boost immunization, a mixture of CFSE-labeled cells pulsed with control or OVA peptide were adoptively transferred to the same mice. Specific killing was measured 2 days later by flow cytometry. Data are presented as means of 100 - 100x $((CFSE^{high}/CFSE^{low})^{immunized\ mice}/ (CFSE^{high}/CFSE^{low})^{mock\text{-}mice})$ of 4-5 mice per group. * $p < 0,05$ ** $p < 0,01$; **** $p < 0,0001$ (One-way Anova).

3. Discussion

ssRNAs have gained great interest as molecular adjuvants to evoke Th1 and cytolytic immune responses against co-delivered antigens due to their TLR7/8 activating capacities. One major drawback of ssRNA is its sensitivity to RNAses and its low efficacy in targeting DCs in the vaccine

draining lymph node upon *in vivo* administration. Complexation of ssRNA to cationic lipids or polymers can improve ssRNA stability and DC uptake. Yet, the vast majority of cationic ssRNA lipid/polymer nanocomplexes are retained at the vaccination site and show little drainage to the vaccine draining lymph nodes, resulting in suboptimal efficacy of ssRNA as adjuvants. To improve the draining capacity of RNA adjuvants, we designed a polymer-based complex which shows low cationic density in combination with a PEGylation shield. These two factors are suggested to positively impact the drainage capacity of PEG-pHDPA RNA polyplexes and thereby enhance the RNA adjuvant efficacy.

First, the PEG-pHDPA polymers were shown to protect the RNA in the presence of serum and enabled transfection of dendritic cells. Second, the formulation of RNA into PEG-pHDPA RNA polyplexes increased the amount of ssRNA in CD11c+ MHCII^{high} dendritic cells in the vaccine draining lymph nodes 24 hours after injection. These data might indicate that the PEG-pHDPA RNA polyplexes have been taken up by dendritic cells followed by active migration to the lymph nodes. However, one could not exclude that these cells are representing resident DCs which have taken up the PEG-pHDPA polymers after passive drainage. Efficient drainage to the lymph nodes is considered as a vital feature of adjuvants since it has been reported that restricting the inflammatory responses locally at the injection site and draining lymph nodes positively influenced the strength of the CTL responses[33–36]. PEGylation is a well-studied strategy to shield surface charges by creating a neutral hydrophilic shell around the polyplex and is reported for their positive impact on particle drainage[16,37]. However, to determine the need of the PEGylation in this formulation, a comparison drainage study needs to be performed using pHDPA RNA polyplexes without a PEG-shield. Furthermore, although PEGylation is beneficial for the drainage capacity of the particle, when the PEG density is too high, it might have a negative influence on the interaction between TLR7/8 and the RNA cargo and thereby interfering with the adjuvant capacity of the RNA polyplexes. For this reason, it would be interesting to perform an *in vitro* maturation study to further evaluate the optimal PEGylation density for DC activation and maturation. Besides the need to adjust the PEGylation density in order to improve TLR7/8 triggering, the strength of the polymer cross-linking should be evaluated. As the polymer seemed to be partially insensitive to DTT treatment, it might be possible that the particles are cross-linked to such a strong extent that the interaction between RNA and TLR7/8 is inhibited. Thus, as a future perspective, it would be of great value to further optimize both physical

parameters in order to improve TLR7/8 triggering and as a consequence to improve the adjuvant capacity.

4. Materials And Methods

Mice

Female wild type C57BL/6 mice were purchased from Janvier (Le Genest Saint Isle, France). OT-I mice carrying a transgenic $CD8^+$ T cell receptor specific for the MHC I-restricted ovalbumin (OVA) peptide SIINFEKL were donated by Dr. Bart Lambrecht from Ghent University (Ghent, Belgium). All mice were 7-12 weeks old at the start of the experiment and maintained under specific pathogen-free conditions. Animals were treated according to the European guidelines for animal experimentation. All experiments were approved by the local ethical committee for animal experiments of Ghent University (Ghent, Belgium).

Synthesis and characterization of pHDPA

p(HPMA-DMAE-co-PDTEMA-co-AzEMAm) was synthesized by radical polymerization under a nitrogen atmosphere. The polymers were synthesized using a monomer to initiator ratio (M/I) of 50. Different molar feed ratios of HPMA-DMAE, PDTEMA and AzEMAm were used; 70/20/10. In a typical experiment of p(HPMA-DMAE70-co-PDTEMA20-co-AzEMAm10), 200 mg (0.77 mmol) HPMA-DMAE, 56.7mg (0.22 mmol) PDTEMA (HPMA-DMAE/PDTEMA 95/5), 17 mg (0.11 mmol) AzEMAm and 3.6mg (0.022 mmmol) AIBN (M/I 50/1) were dissolved in dry DMSO (1mM) in flasks sealed with rubber septa and subjected to vacuum-N_2 cycles. The polymerization was carried at 70 °C for 48h. Next, the DMSO solutions were precipitated in cold diethyl ether, dissolved in DMF repeated 3 times. After extensive dialysis (8 kDa) against an NH_4OAc buffer of pH 5.0 (10 mM, last step 5 mM) at 4°C, the polymer was collected after freeze drying.

The molecular weights and polydisperisity (Mw /Mn) of pHDPA were determined by GPC analysis using a Viscotek-GPCmax (Viscotek, Oss, The Netherlands) light scattering (λ = 670 nm, right (90°) and low (7°) angle)/viscosimetric detection system, using a ultrahydrogel 2000 7.8 × 300 mm columns in series with a ultrahydrogel 6.0 × 40 mm guard column and 0.3 M NaAc pH 4.4,30% Acetonitrile as eluent. The flow rate was 0.8 mL/min and the run time was 40 min. PEG standards (Viscotek Benelux (Oss, the Netherlands)) was used for calibration. The copolymer

composition of the different pHDPA was determined by ^{1}H NMR analysis performed with a Gemini 400 MHz spectrometer (Varian Associates Inc., NMR Instruments, Palo Alto, CA) in D_2O. The ratio HPMA-DMAE/PDTEMA/AzEMAm was determined by comparison of the integrals at δ 4.3 ppm (bs, OCH_2CH_2,HPMA-DMAE), δ7.69ppm (bs, pyridyl group proton, PDTEMA) and δ4.08ppm(m, CH_2N_3 , AzEMAm)(δ4.3/δ7.69/δ4.08).

Synthesis of PEG-BCN: To a solution of DMSO (1.3 mL), NH2-PEG-OH (100 mg, 0.02 mmol, 1 equiv), and cyclooctyne-NHS (BCN-NHS; 9 mg, 0.024 mmol, 1.2 equiv) and trimethylamine (8.5 µL, 0.06 mmol, 3 equiv) was added, the reaction was stirred at RT overnight. The TEA and NHS byproduct was removed by precipitated into cold ether twice, then dissolved in water for dialysis 2 days, after filtration then freeze-drying get white powder. Yield 90 mg ,85.7%. 1H NMR (400 MHz, DMSO): δ = 7.05(s, 1H;OC(=O)NH), 7.05(s, 1H;C=ONH), 4.52 (t, 2H; PEG-OH),4.00 (d, 2H; BCN-CH2-O(=O)), 3.66 (t, 2H; PEG-CH2), 3.48 (br s, 440H; PEG), 3.04 (s, 2H; OCONHCH2), 2.90 (s, 2H; C=ONHCH2), 2.0-2.21(m, 4H; CH2C(=O)NH2, alkane),1.99(m,2H; NHCH2CH2),1.69-1.41 (m, 6H; alkane), 1.54-1.13 (m, 1H; alkane), 0.80-0.71 (m, 2H; alkane).

Reactivity of Cyclooctynes (PEG-BCN) toward Polymer-azide (pHDPA); Polymer pHDPA2 and PEG-BCN ((1*R*,8*S*,9*s*)-Bicyclo[6.1.0]non-4-yn-9-yl)were separately dissolved in 10 mM HEPES buffer (pH 7.4) at a concentration of 10.0 mg/mL (4.88 mM) and 20 mg/mL (3.7 mM), respectively. Polymer solution (13.2 µL) and PEG-BCN solution (17.3 µL ,molar ratio 1:1 or 8.65 µL molar ratio 2:1) were mixed and diluted with 10 mM HEPES buffer (pH 7.3) (69.5 µL or 78.2 µL). The reaction mixture was incubated at room temperature and collected at incubation times of 0, 1, 2, 3, 4, 8, 12 and 24 h. The mixture can be diluted with 10 mM HEPES buffer in different reaction concentration for reaction or been freeze-dried. The collected reaction mixtures were immediately analysed by size exclusion chromatography (SEC). The SEC analyses were conducted using an Alliance HPLC System (Waters) [column: Ultrahydrogel 1000 (Waters); eluent 0.3M NaAc, pH 4.4 with 30% Acetonitrile ; flow rate: 0.8 mL /min; temperature: 30 ^{o}C]. The conjugation ratios were calculated from the peak intensity ratio of unreacted PEG-BCN by concentration calibration curve in the SEC chart at RI detection.

Preparation of RNA polyplexes

To prepare RNA polyplexes four volumes of polymer and one volume of ssRNA were mixed. Dependent on the experiment, three different ssRNA molecules were used: Luciferase, L-6307,

TriLink Biotechnologies, USA; polyU, InVivoGen; Cy5- luc, TriLink Biotechnologies, USA) were mixed for complexation in 10 mM HEPES buffer, pH 7.4, at desired N/P ratio. Polyplexes were crosslinked by addition of Dithiothreitol (DTT) corresponding with a half molar equivalent to PDS groups of polymer for 2 hours at pH 7.4. Except mentioned otherwise, the polyplexes were prepared at N/P ratio 4, at RNA concentration of 100 µg/mL.

mRNA lipoplexes

Dependent on the experiment, three different ssRNA molecules were used: Luciferase, L-6307, TriLink Biotechnologies, USA; polyU, InVivoGen; Cy5- luc, TriLink Biotechnologies, USA). mRNA was complexed to 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol-amine (DOPE) into lipoplexes at Nitrogen/Phosphate 1 (Avanti Polar Lipids, Alabaster, AL, USA). Lipoplexes were injected in 5% glucose water.

Particle size and zeta-potential measurements

The size of the polyplexes was measured with DLS on an ALV CGS-3 system (Malvern Instruments, Malvern, UK) equipped with a JDS Uniphase 22 mW He–Ne laser operating at 632.8 nm, an optical fiber-based detector, a digital LV/LSE-5003 correlator with temperature controller set at 25 °C or 37 °C. The zeta-potential (ζ) of the polyplexes was measured using a Malvern Zetasizer Nano-Z (Malvern, UK) with universal ZEN 1002 'dip' cells and DTS (Nano) software (version 4.20) at 25 °C or 37 °C. Polyplex measurements were performed in 10 mM HEPES pH 7.4 at a mRNA/polyU concentration of 15 µg/mL.

Fuorescence single particle tracking (fSPT)

fSPT was performed to measure the size - over two time periods - of the cationic Cy5-mRNA polyplexes in 10 % serum. fSPT is a fluorescence microscopy technique that uses a fast and sensitive CCD camera to record movies of diffusion particles in fluids. These movies are analysed using in-house developed software, to obtain size distributions[32].

Gel retardation study

Polyplex (in)stability was studied by addition of dithiothreitol (DTT) (as reducing agent) and/or pGA (polyGlutamic acid) (as counter polyanion) and /or serum. Two microliters of DTT (100 mM) and/or different amount of pGA (50 mg/mL) and/ or 2.5 µL FBS were added to 25 µL of

polyplex dispersion in HBS (40 µg/mL of RNA) yielding a final concentration, 10 mM DTT and 240 µg/mL pGA, 10% serum. After 0.5 h incubation time at 37 °C, for serum protection study, the reaction was terminated with 4 µL of 0.5M EDTA (pH 8.0) and then placed on ice for 10 min. 20 µL of the sample was mixed with 3 µL 6× Loading Dye, loaded into 1% agarose gel in Tris–acetate–EDTA (TAE) buffer containing GelGreen (Biotium) at 120 V for 30 min. RNA was detected using a Gel Doc™ XR + system (BioRad Laboratories Inc., Hercules, CA) with Image Lab software.

Fluorescence Correlation Spectroscopy (FCS)

Cy5-mRNA release from PEG-pHDPA RNA polyplexes after incubation with 10% serum during 2 hours at 37°C was followed using FCS. Fluorescence correlation spectroscopy (FCS) is applied as mentioned in[38].

***In vitro* translation of RNA polyplexes**

In vitro translation was conducted using a nuclease-treated rabbit reticulocyte lysate translation system, according to the manufacture's recommendations (Promega, USA). Briefly, 12.5 µL of 0.5 µg luciferase free mRNA or polyplexed luciferase mRNA was pre-treated with 10 % serum and added to 17.5 µl Rabbit Reticulocyte Lysate. Luciferase activity was measured after 90 min incubation (37 °C) by luciferase reporter gene assay (Promega, USA), equipped with a luminescence light guide (BMG LabTech, Germany).

DC2.4 transfection

DC2.4 cells were seeded on 96-well plate (10^5 cells/ well) and incubated for 24 hours in cRMPI. Cy5-labeled luciferase-encoding mRNA was complexed to the polymers as described above. The cells were treated with polyplexes (0.5 µg Cy5 ssRNA) N/P 4 for 4 hours without serum. To quench the fluorescence of polyplexes adsorbed, the cells were incubated with 0.4 % trypan blue-containing PBS buffer for 5 min and washed with PBS. Cells were stained with Hoechst to stain DNA and CTB (cholera toxin subunit B- AF 488) to label membranes. Cellular uptake of RNA polyplexes was examined by flow cytometry (Canto II, BD).

Drainage of Cy5-labeled ssRNA polyplexes

C57BL/6 mice were s.c. injected with Cy5-labeled ssRNA (TRiLink biotechnologies) polyplexes and lipoplexes in the footpad. 10 µg of ssRNA was applied in all groups. Popliteal lymph nodes were isolated 24 hours post injection and analysed by flow cytometry. Cells were stained with α-CD16/CD32 (BD Biosciences, San Diego, CA, USA) to block non-specific FcR binding, and with Live/Dead Fixable Aqua stain (Invitrogen) to eliminate dead cells from analysis. Antibodies used were MHC-II-FITC, α-CD11c PerCP-Cy5.5, α-F4/80 PerCP (all BD Biosciences, San Diego, CA, USA). Analysis was performed on a triple-laser (B-V-R) LSR-II (Becton Dickinson, San Jose, CA, USA) followed by FlowJo (Treestar, OR) data processing.

$CD8^+$ T cell dextramer staining

3 subcutaneous immunizations were performed in C57BL/6 mice at tail base in a 2 week interval. 10 µg of OVA-encoding mRNA was complexed to polyplexes as described above or to lipoplexes using DOTAP/DOPE at N/P ratio of 1 (Avanti Polar Lipids, Alabaster, AL, USA) in a total volume of 40 µl of 5% glucose water (Ambion, Life technologies, USA). Six days after last boost, blood samples were taken and red blood cells were removed using ACK lysis buffer (BioWhittaker, Wakersville, MD, USA). Cells were stained with α-CD16/CD32 (BD Biosciences, San Diego, CA, USA), Live/Dead Fixable Aqua stain (Invitrogen), α-CD8 PerCP, α- CD3 pacific blue, α-CD19 APC-Cy7 (all BD Biosciences, San Diego, CA, USA) and MHC dextramer H-2 Kb/SIINFEKL-PE (Immudex, Copenhagen, Denmark).

In vivo killing assay

3 subcutaneous immunizations were performed in C57BL/6 mice at tail base in a 2 week interval. 10 µg of OVA-encoding mRNA was complexed to polyplexes as described above. Splenocytes were pulsed with 1 µg/ml of MHC-I OVA peptide or HIV-1 Gag peptide as a control before labeling with 5 µM or 0,5 µM CFSE (Invitrogen, Merelbeke, Belgium), respectively. Labelled cells were mixed at a 1:1 ratio, and a total of 1,5 x 10^7 cells mixed cells were adoptively transferred into immunized mice two weeks after last boost. Splenocytes from host mice were analysed two days later by flow cytometry after staining with α-F4/80 (BD Biosciences, San Diego, CA, USA) to exclude auto-fluorescent macrophages. Percentage antigen-specific killing was determined using the following formula: $100 - 100* ((\%\ CFSE^{hi}\ cells / \%\ CFSE^{low} cells)^{immunized\ mice} / (\%\ CFSE^{hi}\ cells / \%\ CFSE^{low}\ cells)^{non\text{-}immunized\ mice}$.

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General discussion

1. ANTIGEN-ENCODING MRNA VACCINES TO ELICIT CD8+ T CELL IMMUNITY

RNA vaccines are designed to mimic infectious challenges to stimulate antigen-specific humoral and cellular immunity. Thereby, they changed the vaccine paradigm that only live – or inactivated pathogens induce diverse $CD8^+$ T cell immunity. The immunogenicity of RNA vaccines is based on their intrinsic adjuvant character as RNA is a natural ligand of cellular pattern recognition receptors (PRRs). PRRs signalling results in the induction of pro-inflammatory cytokines, which are vital for the activation and skewing of Th1 immunity. Furthermore, mRNA vaccines have conceptual advantages in relation to pDNA vaccines. They cannot integrate into the genome and therefore are considered to be safer, and there is no need for mRNA vaccines to cross the nuclear core in order to be translated and processed into peptides. However, the major drawbacks of RNA vaccines are their low stability and weak capacity to be efficiently taken up by dendritic cells. Two strategies have been elaborated in the past to tackle these major limitations to make direct *in vivo* administration of mRNA an attractive alternative for *ex vivo* DC manipulation. A first achievement was the chemical and structural modification of *in vitro* transcribed (IVT) RNA, resulting in improved RNA stability and half-life and as a consequence, enhanced *in vivo* expression levels. A second strategy that improved the *in vivo* performance of RNA vaccines included the design of nanoparticle delivery vehicles, which both protected and targeted the RNA to the dendritic cells. The combination of the tremendous progress of mRNA vaccines and their attractive safety profile has propelled the RNA format to clinical trials.

Vaccine-evoked Type I IFNs. What is going on?

Earlier, it was described by Pollard *et al*. that the induction of type I Interferons (IFNs) upon mRNA lipoplex vaccination appeared to be a double edged sword[1]. They observed superior gag-specific T cell responses upon mRNA lipoplex treatment of $Ifnar^{-/-}$ DCs compared to wild type DCs. In addition, they reported that IFNAR signalling was not required for DC activation and *in vivo* induction of IFNɣ-secreting $CD4^+$ and $CD8^+$ T cells upon subcutaneous mRNA lipoplex immunization. In this thesis, we have extended this observation to intradermal and intranodal delivery of mRNA lipoplexes and evaluated the effects of type I IFNs on the vaccine-evoked $CD8^+$ T cell response in a tumor model **(Chapter 4).**

First, using IFNβ reporter mice, we demonstrated that mRNA lipoplexes induced strong IFNβ levels upon intradermal, subcutaneous and intranodal injection. Furthermore, these vaccine-evoked type I IFN responses exerted a negative impact on the priming and effector functions of the elicited $CD8^+$ T cell responses upon immunization. These findings were confirmed in a prophylactic B16.OVA tumor experiment, showing that $Ifnar^{-/-}$ mice benefited more from vaccination than WT mice, resulting in increased survival rates. This result is highly striking as $Ifnar^{-/-}$ mice completely lack spontaneous type I IFN-mediated anti-tumor responses[1,2]. However, therapeutically vaccinated $Ifnar^{-/-}$ mice succumbed earlier to B16 challenges compared with wild type mice; which is a complete reverse outcome compared with the prophylactic experimental setup. Apparently, in the therapeutic setting, the endogenous type I IFNs are vital and even overrule their negative impact on vaccine-evoked anti-tumor immunity. As it was impracticable to distinguish the endogenous type I IFN effects from vaccine-evoked type I IFN effects, the results of the therapeutic tumor experiment were hardly interpretable. In an attempt to specifically inhibit IFNAR signalling at the site of immunization instead of using completely IFNAR deficient mice, we immunized wild type mice with mRNA lipoplexes, co-delivered with IFNAR blocking antibodies. These data revealed that the co-delivery of an IFNAR blocking antibody prolonged survival rate of immunized wild type mice in both a prophylactic as well as a therapeutic setting.

Still, the **mechanism behind the negative impact of type I IFNs** on locally administered lipoplex vaccines remains unresolved. In view of the natural anti-viral functions of type I IFNs – such as the stimulation of RNAses and translation arrest through phosphorylation of eIFN2α[2,3] - it is tempting to speculate that type I IFNs might interfere with generating T cell responses by **downregulating antigen expression levels**. In addition, earlier research in our lab revealed that *in vitro* transfected $Ifnar^{-/-}$ DCs expressed enhanced antigen levels. Taken together, it was strongly indicated that type I IFNs hampered lipid-based mRNA vaccination by inhibiting or lowering *in vivo* antigen expression levels. Nevertheless, we were not able to measure significant differences between the antigen expression levels of wild type and $ifnar^{-/-}$ mice upon intradermal injection of mRNA lipoplexes. These results are in contrast to other reports that have showed increased antigen levels upon intravenous injection of mRNA lipoplexes in $ifnar^{-/-}$ mice compared to wild type mice[4]. However, in these studies the increased antigen expression levels came along with a decreased T cell response[4,5]. Thus, these findings seem to contradict

the hypothesis that type I IFNs regulate T cell immunity upon mRNA lipoplex vaccination by tuning down antigen expression levels.

Another possible mechanism explaining the inhibiting effects of type I IFNs might be based on the **relative timing between IFNAR and T cell receptor (TCR) activation.** A recent report by Crouse and colleagues, analysing the direct and indirect effects of type I IFNs on T cells, has suggested that IFNAR signalling can positively or negatively influence antiviral T cell responses, depending on the timing between IFNAR signalling and TCR activation[6]. In general, when TCR stimulation shortly precedes IFNAR signalling, the role of type I IFNs as signal 3 cytokines predominates. Conversely, when IFNAR signalling precedes TCR stimulation, the anti-proliferative and pro-apoptotic character of type I IFNs predominate[6]. The molecular mechanism supporting this hypothesis is based on the variety of pathways that could be activated upon IFNAR signalling. Indeed, IFNAR signalling can lead to the activation of 7 STATs which all initiate a different set of transcription factors and as a consequence they all might induce a totally different immune outcome. For example, it has been shown that type I IFNs act as signal 3 cytokines in mouse $CD8^+$ T survival and cytolytic function by activating STAT4[7] when a low STAT1/STAT2 state is present[8,9]. Whereas signalling through STAT1/STAT2 heterodimers in a low STAT4 environment resulted in the initiation of anti-proliferative and even apoptotic programmes[10–12]. How a $CD8^+$ T cell decides to activate STAT4 or STAT1/STAT2 and how this process is regulated appeared to be dependent on the timing of the IFNAR signalling relative to the T cell receptor (TCR) activation **(Figure 1)**[6]. An activated TCR prior to type I IFN signalling results in the phosphorylation of STAT4 instead of STAT1 and stimulates a pro-survival $CD8^+$ T cell state. Reversely, when IFNAR signalling precedes TCR activation, STAT1 is activated resulting in anti-proliferative and apoptotic situations[13–15].

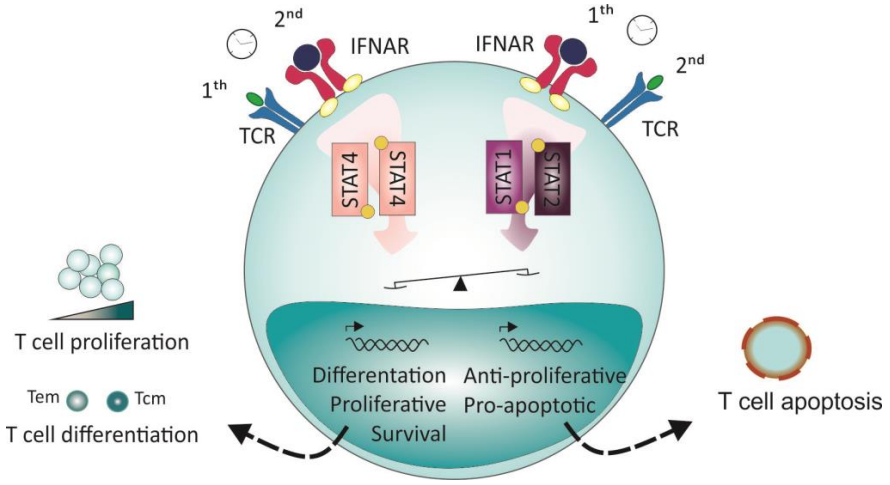

Figure 1. Illustration of how the relative timing of IFNAR and TCR activation may affect vaccine-evoked $CD8^+$ T cell responses.

In view of this theory – The impact of type I IFNs on anti-viral responses depends on the timing between IFNAR signalling and TCR activation - we believe that the inhibiting effects of type I IFNs on mRNA vaccine-evoked CTL responses might be caused by type I IFN induction prior to IFNAR signalling. After subcutaneous or intradermal administration of mRNA lipoplexes, we typically observed a rapid type I IFN induction, peaking between 3 and 6 hours after injection. Antigen expression takes longer to unfold, and peaks between 8 post injection. In addition, DCs that take up mRNA lipoplexes in the skin still need to migrate to the draining lymph node in order to present the antigen, creating an extra delay in TCR activation. Likely, due this process the bulk of antigen presentation occurs after T cell exposure to type I IFNs. Supporting this hypothesis, two recently published manuscripts have demonstrated that type I IFNs actually promote $CD8^+$ T cell immunity upon systemic delivery of similar (RNAiMAX™ and DOTMA/DOPE) mRNA lipoplexes and have identified type I IFNs as crucial signal 3 cytokines[4,5]. Here, antigen expression was reported to peak between one and four hours post injection. IFNα titres showed peak levels in circulation at six hours post immunization. As antigen expression by DCs occurs in the spleen, this means antigen presentation to splenic T cells can occur very rapidly. TCR triggering of T cells will thereby precedes or co-incides with IFNAR triggering and type I IFNs can act as true signal 3 cytokines that promote T cell differentiation. In line with the hypothesis, we could speculate that in a systemic setting, the kinetics of antigen expression and IFNAR signalling might be

reverse to a local setting. For this reason, type I IFNs support T cell immunity upon intravenous mRNA lipoplex injection rather than hampering the elicited CTL responses.

Vaccine-evoked type I IFNs. How to deal with them?

Optimizing the antigen-encoding device; Modified nucleotides

In view of the negative effects of type I IFNs on the efficacy of mRNA lipoplex vaccines, we decided to evaluate a new strategy that should dampen type I IFN induction upon immunization. Based on the non-immunogenic character of chemically modified mRNA[16–19], we evaluated whether modified mRNA lipoplexes might have an impact on the induction of type I IFNs upon subcutaneous and intradermal injection **(Chapter 5).** First, we showed that the injection of modified mRNA lipoplexes induced slightly lower IFNβ levels – although not significant - compared to unmodified mRNA lipoplexes. Despite the rather small impact on IFNβ induction, we quantified significantly higher antigen accumulation levels upon injection of modified mRNA lipoplexes relative to unmodified mRNA lipoplexes **(Table 4)**. Still, to our surprise, no improvement on the capacity to elicit $CD8^+$ T cell responses was observed using modified mRNA lipoplexes.

These data indicated that we **have not succeeded to improve the efficacy of mRNA lipoplexes via chemical modification of the antigen-encoding mRNA.** A possible explanation for this failure might rely on the fact that a lot of free mRNA is present after formulating the RNA into lipoplexes of N/P 1, the ratio applied for immunizations. We obtained evidence that the IFNβ induction appeared to be mainly induced by free mRNA, rather than complexed mRNA. To our believes, the high amount of free mRNA makes it impossible to significantly dampen the IFN induction by modifying the mRNA. Or in other words, the quantity of free mRNA might be too high to be neutralized by modification in order to evade innate immunity. This hypothesis is supported by our data showing no significant difference in IFNβ induction between unmodified and modified mRNA, although a visible trend can be detected. In line with these results, immunizing $Ifnar^{-/-}$ mice with modified mRNA lipoplexes still resulted in strengthened T cell responses. Although, dependent on the experiment, the differences between WT and $Ifnar^{-/-}$ seems to be smaller upon modified mRNA lipoplex immunization. However, a direct comparison study should produce evidence to confirm this.

It might be possible to eliminate this inhibiting effects of type I IFNs by enhancing the N/P ratio of the lipoplexes in order to limit the amounts of free mRNA. Unfortunately, immunizing mice with mRNA lipoplexes with an increased N/P ratio of 10, revealed no antigen expression and no IFNɣ-secreting $CD4^+$ or $CD8^+$ T cells.

OPTIMIZING THE DELIVERY FORMAT; RALA MRNA NANOCOMPLEXES

Besides the modification of the mRNA, we validated in **Chapter 6** a RALA-based delivery format as an alternative to the mRNA lipoplex vaccine approach. RALA is a cell penetrating peptide, composed of 32 amino acids containing 7 arginine residues separated by hydrophobic regions. First, we investigated the capacity of the amphipathic cell penetrating peptide (CPP) RALA to efficiently formulate and to deliver mRNA *in vivo*. At N/P 1, only 5 % of the RNA was encapsulated into RALA nanocomplexes. When the N/P ratio was increased up to 10, almost all the RNA was complexed. Further, these near-saturated mRNA nanocomplexes of N/P 10 instigated superior eGFP expression levels upon DC transfection. We further obtained evidence to associate the high antigen expression levels with the pH-specific membrane disruptive feature of RALA. Moreover, mice immunized with RALA modified mRNA nanocomplexes of N/P 10 evoked strong CTL responses (will be discussed later).

To further address to what extent the pH-dependent amphipathic nature of RALA was crucial for the efficient generation of antigen-specific $CD8^+$ T cell responses upon mRNA vaccination, we designed two additional peptides - named RGSG and RRRR, which did not have an amphipathic α-helical structure. Though, RALA, RGSG and RRRR contain equal amounts of arginine to ensure that the binding capacity to mRNA is not effected. We demonstrated that in contrast to RALA, both RGSG and RRRR failed to prime $CD8^+$ T cell responses *in vivo*. Although we showed some strong indications to suggest that RALA lends his unique immunogenicity to its amphipathic character, crucial for its endosomal disruptive activities, one should keep in mind that RGSG and RRRR nanocomplexes differ from RALA nanocomplexes in size, surface charge and hydrophobicity. Therefore, at this stage, we cannot exclude that these differences are not affecting the uptake of the particles, the endosomal escape and as a consequence the immunogenicity of the nanocomplexes.

The combination of RALA-based delivery and modified nucleotides; A golden marriage?

In regard to the low amounts of free mRNA when complexed to RALA nanocomplexes at N/P 10, we tested to what extent type I IFNs were induced upon injection. As we expected, IFNβ reporter mice showed remarkably lower IFNβ induction upon RALA mRNA nanocomplexes injection compared with DOTAP lipoplexes **(Table 4).**

Earlier, we suggested that the modification of mRNA failed to improve the capacity of mRNA lipoplex vaccines due to a high percentage of free mRNA. Given the fact that RALA mRNA nanocomplexes are fully functional in an almost complete saturated state (N/P 10), it might be interesting to test if the small remaining amounts of free mRNA can be masked for innate immunity by chemical modification. To reveal the impact of the mRNA modification on the vaccine efficiency of RALA nanocomplexes, wild type mice were intradermally immunized with RALA nanocomplexes. Here we showed that RALA modified mRNA nanocomplexes elicited superior CTL responses compared to unmodified mRNA nanocomplexes, an observation we could not make in the context of lipid-based mRNA vaccination **(Table 4)**. We further demonstrated that RALA mRNA nanocomplexes reached similar efficacy in wild type mice compared to Ifnar$^{-/-}$ mice, a goal we initially tried to reach by optimizing the lipid-based formulation. By accomplishing this, **we present a new format of mRNA vaccines, based on CPP-delivery of modified mRNA - which evades the inhibiting effects of induced type I IFNs, without losing the immunogenicity of the mRNA vaccine.**

The reason why immunizing mice with RALA modified mRNA vaccine reached far stronger immunity compared to the lipid-based modified mRNA vaccine might rely on the limited amounts of free mRNA **(Table 4).** Although, further research is needed to confirm this hypothesis. Either the question remains which factor is causing innate immunity when RALA modified mRNA nanocompexes are administered. One could speculate that RALA itself shows intrinsic innate immune activating properties, likely independent of the binding of RNA to TLRs. Indeed, it is highly suggested that the capacity of RALA to disrupt the endosomes is responsible for the immunogenicity of the modified mRNA RALA nanocomplexes. Although, amphipathic CPPs are described in the literature as non-immunogenic[20]. Therefore, a better understanding about the immunological mechanisms of RALA-based delivery for mRNA vaccines is required. Preliminary data revealed a total loss of RALA mRNA vaccine functionality in Trif$^{-/-}$ mice, whether

MyD88$^{-/-}$ mice revealed even slightly better outcome than wild type mice. These data might suggest that RALA modified mRNA nanocomplexes might trigger innate immunity via TLR3 signalling **(Table 4).**

	DOTAP lipoplexes	RALA nanocomplexes
Type I IFN induction	High	Low/No
Negative Impact of type I IFNs		
- If complexed to unmodified mRNA	Yes	Yes
- If complexed to modified mRNA	Yes	No
Importance of free mRNA	Yes	No
Impact of modified mRNA	No impact	Benefits from modified mRNA
In vivo Antigen expression	Detectable antigen levels	No functional expression detected
Immunogenicity	Multiple PRRs?	TRIF dependent?

Table 4. Differences between RALA and DOTAP working mechanisms.

FUTURE PERSPECTIVES

For many years, the transfer of *ex vivo* mRNA-modified DCs has dominated the area of mRNA-based vaccination. To date, successful studies led to the generally acceptance that direct *in vivo* administration of mRNA is more simplified and cost-effective alternative. In this doctoral study, we aimed to further improve the *in vivo* administration of mRNA-based vaccines and tackle the inhibitory impact of locally evoked type I IFNs on elicited CTL responses. Interestingly, we revealed that for both lipid- as well as for peptide-based delivery, the efficacy of unmodified mRNA vaccines is inhibited by type I IFNs upon local administration.

Recently we gained access to conditional Ifnar$^{-/-}$ mice, showing specific IFNAR deficiency in the dendritic cell line or in the T cell line, which might be very useful to gain some insights into the mechanism behind the inhibitory effects of type I IFNs after topical application. More specifically, they can learn us whether the inhibitory effects are mediated by direct IFNAR signalling in T cells or through modulation of DC functions. In case of the first option, one could have a further look into the our hypothesis regarding the relative timing between IFNAR and

TCR activation. In view of this hypothesis, a direct comparison between a local versus systemic delivery of RNA vaccines in Ifnar$^{-/-}$ and wild type mice would be of great value.

In a second part of this thesis, we present a new vaccine format based on the amphipathic peptide RALA which showed superior CD8^{+} T cell responses, completely independent of type I IFN induction. It would be of great interest to further validate this novel format in a prophylactic and therapeutic disease model. During many years, researchers focussed mainly on the development of mRNA-based vaccines for cancer treatment (melanoma, small cell lung cancer, prostate cancer). As more recently mRNA-based vaccines have been validated for preventing infectious disease[21,22], it would be interesting to address the capacity of RALA mRNA nanocomplexes as a cytotoxic T cell-inducing vaccine against infectious diseases caused by human deficiency virus (HIV), cytomegalovirus (CMV) and influenza virus.

To further assess a thorough preclinical evaluation of RALA mRNA nanocomplexes as a CTL-eliciting vaccine, the underlying mechanism and characteristics of RALA mRNA nanocomplexes at the level of safety and immunogenicity must be addressed. Safety is one of the major concerns of clinical applications. To achieve a safe vaccine format, the induction of pro-inflammatory cytokines should be transient and local, avoiding a systemic cytokine release. We did not detect a systemic IFNβ induction upon intradermal injection of RALA mRNA nanocomplexes, which might indicate a low risk for systemic cytokine release. Nevertheless, further research should be performed to confirm this. As RALA is designed to obtain membrane disruptive features specifically in low pH environments, like inside the endosomes, the toxicity risks of RALA mRNA nanoparticles are expected to be limited upon *in vivo* administration. Furthermore, earlier research revealed that RALA-DNA immunization did not evoke RALA-specific antibody responses (personal communication), indicating that repeated injections of RALA nanocomplexes can be applied. In addition, the RALA peptide is completely biodegradable. Taken together, we have strong indications that the local administration of RALA mRNA nanoparticles should be a safe and robust vaccine approach to initiate anti-tumor responses in a human situation. Furthermore, besides the eliciting of CD8^{+} T cells, mRNA vaccines further competence to instigate a broad Th1 immunity, including CD4^{+} T cell responses and antibody production[1,23,24]. The competences of RALA RNA nanocomplexes to induce CD4+ T cell responses and humoral immunity should be addressed.

In a pre-clinical phase, a head-to-head comparison with the protamine-delivery format (RNActive®, Curevac, Germany)[25–27] would provide clear information about the competence of RALA-based mRNA vaccination as a clinical therapeutic. Protamine is a small Arginine-rich nuclear protein which stabilizes DNA during spermatogenesis. Although the structural similarity between protamine and RALA - both form an α-helix - it is tempting to speculate that RALA-mediated delivery of mRNA antigens might be preferable to protamine-based delivery due to weaker binding interaction between the RALA-peptide and RNA. The protamine-mRNA interaction is very tight, in such extent that the adjuvant effect comes at the cost of weak antigen expression levels[26]. To solve that problem, a two-component format was developed (RNActive®), whereby mRNA was only partially complexed to protamine in order to provide an adjuvant component along with free RNA responsible for high antigen expression levels. At this point no information is obtained regarding the strength of interaction between RALA and RNA but a better balance between tight interaction and intracellular release might contribute to a simplified composition of the vaccine and improved efficacy.

mRNA VACCINES, TO BE CONTINUED…

In this thesis I focussed on the application of RNA vaccination as a cancer immunotherapy but the applicability of mRNA vaccines is far broader. Their strong Th1 immunostimulatory capacity was initially validated in a tumor model, followed by validation against infectious disease[28]. Recently, mRNA vaccines encoding hypoallergenic molecules have been presented as an attractive prophylactic vaccination strategy to prevent type I allergy based on Th1 polarization[29]. Such a hypoallergen is a modified allergen with a reduced binding capacity for pre-existing allergen-specific IgE. Roesler and colleagues showed subtle Th1 priming by RNA vaccines were sufficient for the recruitment of protective Th1 cells in a mouse model of allergy[29,30]. A prime immunization revealed to be adequate for long-term prevention due to the natural boosting procedure by allergen exposure during the pollen season. Besides the modulation of T cell responses, another reason for the success of RNA vaccines to provide protection against allergens is the induction of IgG antibodies that compete with IgE isoform for their binding sites on allergens. In summary, the concept of mRNA vaccination to combat allergies relies on the immune deviation toward a Th1 immunity, rather than the induction of regulatory T cells or tolerance[31].

Self-amplifying mRNA. Initially this strategy was designed to induce protective immunity against flavivirus infection and succeeded herein by injecting less than 1 ng of IVT genomic mRNA[32]. Self-amplifying mRNA vector systems are ideally suited for vaccine development because they provide high transient antigen expression and inherent adjuvant effects due to the intracellular replication which triggers PRRs and causes inflammatory responses. Novartis started a platform which focuses on self-amplifying mRNA (SAM®) vectors expressing the highly conserved nucleoprotein (NP) and the matrix protein 1 (M1) of the influenza virus[33]. *In vivo* lipid-based immunization of SAM® resulted in robust $CD4^+$ and $CD8^+$ T cells induction in mice. In addition, an enhanced recruitment of NP-specific CTLs was observed in the lungs of immunized mice after influenza infection, which might be associated with the reduced lung viral titters and increased survival after homologous and heterosubtypic influenza challenges[33]. These preclinical results are very promising as current hemagglutinin-based seasonal influenza vaccines induce vaccine strain-specific neutralizing antibodies which usually fail to protect against other circulating strains.

2. RNA-MEDIATED ADJUVANTS TO ELICIT $CD8^+$ T CELL IMMUNITY

Non-coding RNA is intensively evaluated as adjuvant components for protein vaccines due to their TLR activating capacities[34,35]. Likewise the application of antigen-encoding RNA, RNA adjuvants also benefit from being encapsulated into nanoparticles[34,36]. Complexation of RNA to or polymers can improve ssRNA stability and DC uptake. Yet, the vast majority of cationic ssRNA polymers are retained at the vaccination site and show little drainage to the vaccine draining lymph nodes, resulting in suboptimal efficacy of ssRNA as adjuvants. In this thesis, we investigated to what extent the formulation of ssRNA into PEGylated-pHDPA RNA polymer-based complexes had an impact on the adjuvant capacities of ssRNA to protein vaccines (Chapter 7). We showed that immunizing mice with PEG-pHDPA ssRNA polyplexes, elicited strong cytotoxic $CD8^+$ T cell responses against co-delivered ovalbumin. We further suggested that the capacity of PEG-pHDPA polyplexes to induce CTL responses is associated with an improved uptake by dendritic cells in the draining lymph nodes, the site of antigen presentation and T cell priming.

As conventionally protein vaccination is known to evoke mainly Th2 oriented humoral immune responses, due to MHC-II driven peptide presentation to $CD4^+$ T cells, PEG-pHDPA RNA

polyplexes might succeeded to promote cross-presentation, resulting in strong effector $CD8^+$ T cell responses against the co-delivered antigen, fully capable of killing antigen-specific target cells. Cross-presentation occurs mainly in professional APCs and specifically in $CD8\alpha^+$ dendritic cells and is promoted by IL-12 and type I IFNs[37–40]. For this reason it would be of great interest to analyse *in vitro* and *in vivo* to what extent PEG-pHDPA RNA polyplexes enable the induction of IL-12 and/or type I IFNs.

Earlier, we showed that coupling of the TLR7 agonist imiquimod to nanogels limited type I IFN responses to the intradermal injection site and its draining lymph node, whereas soluble imiquimod provoked systemic type I IFN responses. We further reported that a local rather than a systemic induction elicited superior Th1 immunity against co-delivered proteins[36]. In view of this, it would be interesting to get some insights in the mechanism behind the improved efficacy of formulated RNA by analyzing to what extent polyplex-based delivery of RNA results in a local or systemic type I IFN induction.

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Funding: Personal PhD scholarship for strategic Research from the Flemish Government Agentschapvoorinnovatie door wetenschapentechnologie (IWT)

Promotor: Prof. Dr. Johan Grooten (johan.grooten@ugent.be)
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Higher education

September 2010 – June 2012

Master of Science in Biochemistry and Biotechnology
Major Plant Biotechnology – Minor Medical Biotechnology
University of Ghent - Graduated cum laude

Master thesis: '*Production and characterization of VHH en VHH-Fc antibodies against plant proteins as tools for biotechnological research*'

Promotor: Prof. Dr. Ann De Picker (ann.depicker@psb.vib-ugent.be)

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September 2007 – June 2010

Bachelor of Science in Biochemistry and Biotechnology
University of Ghent

Competences and additional courses

Languages:

- Dutch: mother tongue

- English: good level of speaking and writing
  - Followed courses at UGhent:
  'Advanced Academic English: Conference Skills' 2014
  'Advanced Academic English : Writing skills' 2016

- French: basic/ competent

Computer knowledge:

- good knowledge of Word, excel and powerpoint
- good knowledge of Coreldraw/ FlowJo / FACS diva

Research tools:

- Multi-color flow cytometry
  - Followed course: 'ExCyte Flow Cytometry course (Utrecht)' 2016

- Animal (mouse) experiments:
  - Followed courses at UGhent:
  'Basic course in Laboratory Animal Science' FELASA B + C 2009 - 2010
  'Animal Experiment Leader C attestation' FELASA A 2009 - 2010
  'Working with radio actives and ionization in the lab: Safety course' 2015

- Techniques:
  Immunization (s.c., ear pinna, intra musc.)/tumor inoculation/*in vivo* bioluminescence/ iv.injection/ bone marrow transplantation/…

- General cell culture (Bone marrow cultivation/ transfection/…)
- Immunology assays (ELISA/ELISPOT/luminex/…)
- PCR and Qpcr

Leadership skills:

- Guidance of Bachelor/ Master/PhD. Students in the lab

- Leading practical course for bachelor students
  (leading a team of 3 people to organize the course)

- Tissue culture responsible in the department
  (± 30 employees; communication, leading meetings, problem solving)

Teaching skills:

2012-2016 Guidance of practical course ' Immunology'
3rd Bachelor Biochemistry and Biotechnology – Ghent University
(± 70 students; lecturing; performing experiments (ELISA, FACS,..); correcting reports)

2013 Guidance of Master 1 Project - Hannah Sanjour
'in vitro and in vivo Electroporation of mRNA'

2013- 2014 Guidance of Bachelor Thesis Student – Dicky Wullems
'Optimalisation of antigen coding mRNA'

2014- 2015 Guidance of Master 2 Thesis Student – Jolien Baert
'Comparisation of delivery methods of mRNA vaccination'

2015 Guidance of Master 1 Student – Sara Phlypo
'Effects of type I IFN on mRNA expression after in vitro electroporation'

Conference contributions

Oral and poster presentations

2015 IUAP P7/32 Meeting (Leuven, Belgium)
'*mRNA based vaccines as immunotherapy'*

2015 European Congress of Immunology, ECI2015 (Vienna, Austria)
'Inhibiting the IFNAR receptor increases antitumor potency of mRNA vac.'

Grants

2015 ECI2015 – BIS Travel Grant

Publications

De Beuckelaer Ans, Pollard Charlotte, Van Lint Sandra, Roose Kenny, Van Hoecke Lien, Naessens Thomas, Udhayakumar K Vimal, Smet Muriel, Sanders Niek, Lienenklaus Stephan, Saelens Xavier, Weiss Stephan, Vanham Guido, Grooten Johan, De Koker Stefaan. *Type I interferons interfere with the capacity of mRNA lipoplex vaccines to elicit cytolytic T cell responses.*(2016) Molecular therapy **(IF: 6,9)**

Nuhn Lutz, Vanparijs Nane, **De Beuckelaer Ans** , Lybaert Lien, Verstraete Glenn, Deswarte Kim, Lienenklaus Stefan, Nikun, M. Shukla, Salyer Alex C. D, Lambrecht Bart N., Grooten Johan, David Sunil A., De Koker Stefaan, De Geest Bruno. *pH-degradable imidazoquinoline-ligated nanogels for lymph node focused immune activation.* (2016) PNAS **(IF: 9.7)**

Lambricht Laure, Vanvarenberg Kevin, **De Beuckelaer Ans**, Van Hoecke Lien, Grooten Johan, Bernard Ucakar, Pascale Lipnik, Sanders Niek, Lienenklaus Stefan, Praet Veronique,

Vandermeulen Gaelle. *Co-administration of a plasmid encoding HIV-1 Gag enhances the efficacy of cancer DNA vaccines.* (2016) Molecular Therapy **(IF: 6.23)**

Vandermeulen Gaelle, Vanvarenberg Kevin, **De Beuckelaer Ans**, De Koker Stefaan, Lambricht Laure, Uyttenhove Catherine, Reschner Anca, Vanderplasschen Alain, Grooten Johan, Préat Veronique. (2015) *The site of administration influences both the type and the magnitude of the immune response induced by DNA vaccine electroporation.* Vaccine **(IF: 3,48)**

Smet Muriel, Pollard Charlote, **De Beuckelaer Ans,** Van Hoecke Lien, Beken, SV, Dulayymi, JR, Huygen Kris, Verschoor, J, Bairds, MS, Grooten, J. *Mycobacterium tuberculosis-associated synthetic mycolates differentially exert immune stimulatory adjuvant activity* (2016). Eur J Immunol. **(IF: 4,18)**

Li Hui, Fierens Kaat, Zhang Zhiyue, Vanparijs Nane , Martijn J. Schuijs, Van Steendam Katleen, Gracia Natalia Feiner, De Rycke Riet, De Beer Thomas, **De Beuckelaer Ans,** De Koker Stefaan, Deforce Dieter, Albertazzi Lorenzo, Grooten Johan, Bart N. Lambrecht N. Bart, and De Geest Bruno G. (2015) *Spontaneous Protein Adsorption on Graphene Oxide Nanosheets Allows Efficient Intracellular Vaccine Protein Delivery*. ACS applied materials and interfaces **(IF: 6,723)**

Naessens Thomas, Schepens Bert, Smet Muriel, Pollard Charlotte, Van Hoecke Lien, **De Beuckelaer Ans**, Willart Monique, Lambrecht Bart, De Koker Stefaan, Saelens Xavier, Grooten Johan. (2015) *GM-CSF treatment prevents respiratory syncytial virus-induced pulmonary exacerbation responses in postallergic mice by stimulating alveolar macrophage maturation.* The Journal of Allergy and Clinical Immunology **(IF: 11,476)**

De Meyer Thomas, Laukens Bram, Nolf Jonah, Van Lerberge Els, De Rijcke Riet, **De Beuckelaer Ans**, De Buck Sylvie, Callewaert Nico, Depicker Ann. (2015) *Comparison of VHH-Fc antibody production in Arabidopsis thaliana, Nicotiana benthamiana and Pichia pastoris.* Plant biotechnology journal **(IF: 5,677)**

Smet Muriel, Van Hoecke Lien, **De Beuckelaer Ans**, Vander Beken Seppe, Naessens Thomas, Vergrote K, Willart M., Lambrecht, N.B., Gustafsson, J.A., Setffensen, K.R., Grooten, J. *Cholesterol-sensing liver X receptors stimulate Th2-driven allergic eosinophilic asthma in mice* (2016) Immun. Inflamm. Dis. **(no IF)**

Submitted or under review:

Under review at Trends in Mol Med **(IF: 9,2):**
De Beuckelaer Ans, Kim Deswarte, Johan Grooten, Stefaan De Koker. *One cytokine to rule them all? Interferon governs T cell immunity to mRNA vaccines.*

Under review at Advanced Healthcare Materials **(IF 5,6)**:
De Beuckelaer, Ans, Udhayakumar V.K., McCaffrey, J., McCrudden, C.M., Kirschman, J.L., Vanover, D., Van Hoecke, L., Roose, K., Deswarte, K., De Gees B, Santangelo P.J., Grooten J., McCarthy H.O., De Koker, S. *The efficacy of arginine-rich peptide based mRNA nanocomplexes to instigate T cell immunity crucially depends on the amphipathic organization of the peptide.*

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Laure Lambricht, **Ans De Beuckelaer**, Kevin Vanvarenberg, Bernard Ucakar, Johan Grooten, Véronique Préat, Gaelle Vandermeulen. *Cancer DNA vaccine coding for a modified VSV-G is a potent inducer of antigen-specific T-cell responses,*

In preparation:
Lou, B.[*], **De Beuckelaer, Ans[*],** Grooten, J., De Geest, B., Hennink, W.E., De Koker, S. PEG-pHDPA RNA poplyplexes, a Novel Polymer-mediated RNA Adjuvant

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