Synthetic carriers for gene therapeutics – from pDNA to mRNA to Cas9/sgRNA

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ABBREVIATIONS

°C	degree Celsius
µg, µL	microgram(s), microliter(s)
Ag(s)	antigen(s)
ALT	alanine aminotransferase
ASO(s)	antisense oligonucleotide(s)
AST	aspartate aminotransferase
BUN	blood urea nitrogen
CAR T cell	chimeric antigen receptor T cell
Cas9	CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) associated protein 9
Crea	creatinine
ctrl	control
DBCO	dibenzocyclooctyne
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
e.g.	*exempli gratia* (for example)
EMA	European Medicines Agency
FACS	fluorescence activated cell sorting
FDA	Food and Drug Administration – U.S. federal agency of the Department of Health and Human Services
FELASA	Federation of European Laboratory Animal Science Associations
FolA	folic acid
FRα	folate receptor alpha

G	Birmingham gauge (parameter for needle diameter)
h	hour(s)
HBG	HEPES buffered glucose
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
i.e.	*id est* (that is)
i.t.	intratumoral(ly)
i.v.	intravenous(ly)
IVIS®	*in vivo* imaging system
KO	knockout
LAF	lipo amino fatty acids
LAR buffer	Luciferase Assay Reagent buffer
LPEI	linear polyethyleneimine
Luc	luciferase
mg, mm	milligram(s), millimeter(s)
miRNA	microRNA
mRNA	messenger RNA
MTT assay	colorimetric assay for assessing cell metabolic activity
n	number of samples
N/P	polymer nitrogen to nucleic acid phosphate ratio
NA(s)	nucleic acid(s)
nM, mM	Nanomolar, millimolar
OAA	oligoaminoamide
p.i.	*post injectionem* (after injection)
PBS	phosphate buffered saline
PD-L1	programmed death-ligand 1 (CD274)
pDNA	plasmid DNA

PEG_{24}	polyethylene glycol
PET/MRI	Positron emission tomography / magnetic resonance imaging
pH	potential of hydrogen
Ph.D.	Doctor of Philosophy
PVR	poliovirus receptor (CD155)
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RNP	ribonucleoprotein
s.c.	subcutaneous(ly)
S.E.M.	standard error of the mean
SD	standard deviation
sec	second
sgRNA	single guide RNA
siRNA	short interfering RNA
SOPF	specific and opportunistic pathogen free
succPEI	succinyl polyethylenimine
TIDE	tracking of indels by decomposition – an assay to determine the spectrum and frequency of targeted mutations by, e*.g.,* CRISPR/Cas9
TIGIT	T cell immunoreceptor with Ig and ITIM domains – immune-regulating receptor on T cells and natural killer cells
TLR	toll-like receptor
w/w	weight to weight ratio
WT	wild type

I. INTRODUCTION

As science rapidly evolves in the fields of medicine, genetics, pharmaceutics, and related, it is gaining a deeper understanding of diseases and developing more sophisticated treatments. Three decades ago, the enormous area of gene therapy emerged and has grown rapidly since [1, 2], leading to over 3600 gene therapy trials [3], 27 approved cellular and gene therapy products by the FDA [4], and 14 approved therapy medicinal products by the EMA [5] to date.

Gene therapy is a technique to modify a person's genes to treat or cure diseases by replacing a disease-causing gene with a healthy copy of the gene, inactivating a not functioning disease-causing gene, or introducing a new or modified gene to treat a disease, including cancer and other genetic disorders, as stated by the FDA [6]. Due to its complexity, since the beginning of gene therapy clinical trials, euphoria and alarm were present, as successful first treatments were overshadowed by occasional severe adverse effects [7]. Therefore, within its own limits, an emphasis has been placed on producing safer and more efficient therapies. Regarding its possibilities, gene therapy is considered a promising treatment option for several, otherwise often untreatable diseases, already demonstrating improvements for patients suffering from blindness, neuromuscular disease, hemophilia, and cancer [8].

Cancer, a term for a large group of genetic diseases, arises when normal cells transform into tumor cells that grow uncontrollably and thus can invade healthy nearby tissue and spread to other organs, *i.e.,* metastasize [9]. In 2020, cancer accounted for almost 10 million deaths and constituted a leading cause of death worldwide. One-third of deaths from cancer are due to unhealthy lifestyles, such as smoking, obesity, alcohol consumption, low intake of fruit and vegetables, and a lack of physical activity. As most cancers can be cured when detected early enough and treated effectively [10], many efforts are being made to come up with novel treatment options, such as chimeric antigen receptor T cell (CAR T) therapy [11], or *e.g.,* oncolytic virotherapy against advanced melanoma [12].

Besides novel gene therapeutic anti-cancer therapies, approaches in gene therapy, such as adeno-associated virus vector-based treatment against Hemophilia B [13], or Leber congenital amaurosis [14], ex vivo lentivirus-transduced hematopoietic stem cells to treat patients suffering from cerebral adrenoleukodystrophy [15], give hope that more untreatable diseases can be cured in the future.

Finally, gene therapy plays an important role in the newly emerging field of xenotransplantation, a field of research that aims at 'producing' genetically modified tissues and organs in animals to serve as donors for human patients [16]. Therefore, pigs, which are used because of their similarity in anatomy and physiology, get multiple genetic modifications to prevent hyperacute rejection and vascular rejection of organs or tissues transplanted into humans [17].

1.1. Delivery systems

One of the major hurdles of any therapy is to get the therapeutic agent, in this case nucleic acids (NAs), to the desired target location, which can maximize therapeutic benefits, minimize unwanted side effects, enhance patient compliance [18], but also combat poor biodistribution, rapid break down of the drug *in vivo*, unfavorable pharmacokinetics, toxicity, and lack of selectivity for target tissue, to name a few [19].

Amongst other ways, drug delivery was realized by nanocarriers, a tremendous and inhomogeneous group of nanoparticles utilized to transport therapeutic agents to their target locations [20]. Presently, there are two dominant classes of systemically applied nanocarriers for therapeutic nucleic acid agents: viral and non-viral vectors with their benefits and limitations, such as possible severe immunogenicity of viral vectors [21] while achieving decent transfection efficiencies, and *vice versa* for non-viral vectors being immune inert, but often lack transfection efficiency [22]. Due to their good transfection efficiency and their well-known cell tropism, viral vectors are still widely used in research but also for vaccines and gene therapy trials [23-25]. However, immunogenicity of viral vectors always

poses a risk of sever adverse reactions that have even led to treatment-induced leukemia and death of patients treated with viral vectors [7, 26].

Indeed, considering the otherwise welcomed benefits of non-viral vectors, delivery is the major obstacle to efficient therapies utilizing non-viral carriers to date. In the realm of non-viral nanocarriers, many different approaches have been made, reaching from biodegradable polymers, such as dendrimers [27], polylactic acid (PLA) [28], polyethyleneimine (PEI) [29, 30], or chitosan (CS) [31], to liposomes [32], gold nanoparticles [33], mesoporous silica nanoparticles [34], lipid nanoparticles [35] or carbon nanotubes [36, 37].

Vectors of some kind are indispensable for suitable and safe techniques to deliver genetic material into target cells, which are termed transfection, that enable protection against degradation by nucleases in bodily fluids, internalization of nucleic acids into the target cells, and release of the genetic material to its intended site inside the cell [38, 39]. Besides nucleases that degrade NAs, the obstacle of internalization is based on the big size and the negative charge of NAs and therefore their ineffective interacting with the also negatively charged cell membrane [39]. The difficulty in cargo release lies in the fact that nanoparticles have to be balanced between extracellular stability and the intracellular ability to release their cargo [40].

Synthetic, cationic polymer-based nanocarriers with (*i.e.* lipopolyplexes) or without (*i.e.* polyplexes) lipidic structures are one member of the group of non-viral vectors [41]. Due to their cationic structure, cationic lipids, polymers, and peptides can condense negatively charged NAs into self-assembling (Lipo-) polyplexes as well as, forming a cationic complex can interact with negatively charged cell membranes [42]. Upon interaction with the negatively charged cytoplasmic membranes, cellular internalization occurs in non-specific adsorptive endocytosis, most likely due to binding to ubiquitously expressed heparan sulfate proteoglycans (HSPGs) and thus triggers linker protein-mediated actin binding which leads to the particle being pulled into the cell [43], or in case of ligand-modified polyplexes, through receptor-mediated endocytosis [44, 45]. Overcoming this first intracellular barrier polyplexes, as well as lipoplexes, are entrapped in

endosomes whose task is, among others, to degrade absorbed material in the late endosomes and lysosomes by hydrolases and a low pH of < 6 [46]. In avoiding lysosomal degradation and for the purpose of translation or in the case of pDNA transcription, NAs have to get into the cytosol or nucleus, respectively. However, mechanisms of endosomal escape of polyplexes, more precisely, the interaction of polyplexes with the endosomal membrane are not yet fully understood [47, 48]. In contrast, it is proposed that lipoplexes, with the help of their cationic lipids, are able to destabilize endosomal membranes by inducing nonbilayer lipid structures [49], by formation of small holes in the lipid bilayers [50] or by initiating flip-flop of anionic lipids in the endosome, normally located on the cytoplasmic face of the membrane, leading to charge-neutralized ion pairs with the cationic lipids of the lipoplex which then releases the DNA from the complex and enables the DNA to enter the cytoplasm [51]. But compared to the efficient systems of viruses for endosomal release, in many cases the lack of efficient endosomal escape is still the main reason for unsatisfactory efficiency of gene delivery by polyplexes and lipopolyplexes [52]. Actually, Gilleron *et al.* revealed that only 1-2 % of, in this case, siRNA would be released from endosomes after LNP uptake by endocytosis [53]. Therefore, different pathways of enhancing endosomal escape were investigated, of which several mimic those of viruses, such as cell-penetrating peptides (CPPs) or polyplex-coupled fusogenic proteins that, upon acidification in endosomes, penetrate endosomal membranes which finally lead to a membrane fusion and cargo release into the cytoplasm [54, 55]. Others hypothesize, although it is highly debated, that the so-called proton sponge effect which is the ability of polycations, such as polyamines, to buffer the lysosomes low pH, would protect the genetic material from nuclease degradation and additionally, due to osmotic swelling of the lysosomes, as is also postulated for lysosomotropic agents [56], would lead to its rupture and thus providing an endosomal escape mechanism for the polyplexes [57, 58]. However, later studies revealed that transfected cells did not show ruptured endosomes nor release of intact lipoplexes or polyplexes into the cytosol, but rather a discharge of NAs and carrier from the endosomes, indicating the formation of (a) pore(s) within the endosomal membrane [59].

In our previous studies, sequence-defined oligoaminoamide (OAA) peptides, consisting of cationizable polymer backbone structures, such as succinoyl tetraethylene pentamine (Stp) or succinoyl pentaethylene hexamine (Sph), and various coupled natural and artificial amino acids to form carriers with different topologies, were used (**Figure 1**) [60, 61]. Through “chemical evolution” strategies, our working group led by Prof. Dr. Ernst Wagner (*Chair of Pharmaceutical Biotechnology; LMU, Munich*) has generated libraries of these OAAs of over 1800 sequence-defined carriers, complemented with different ligands for targeting, shielding, or uptake enhancement, leading to cargo-optimized non-viral vectors [62-67].

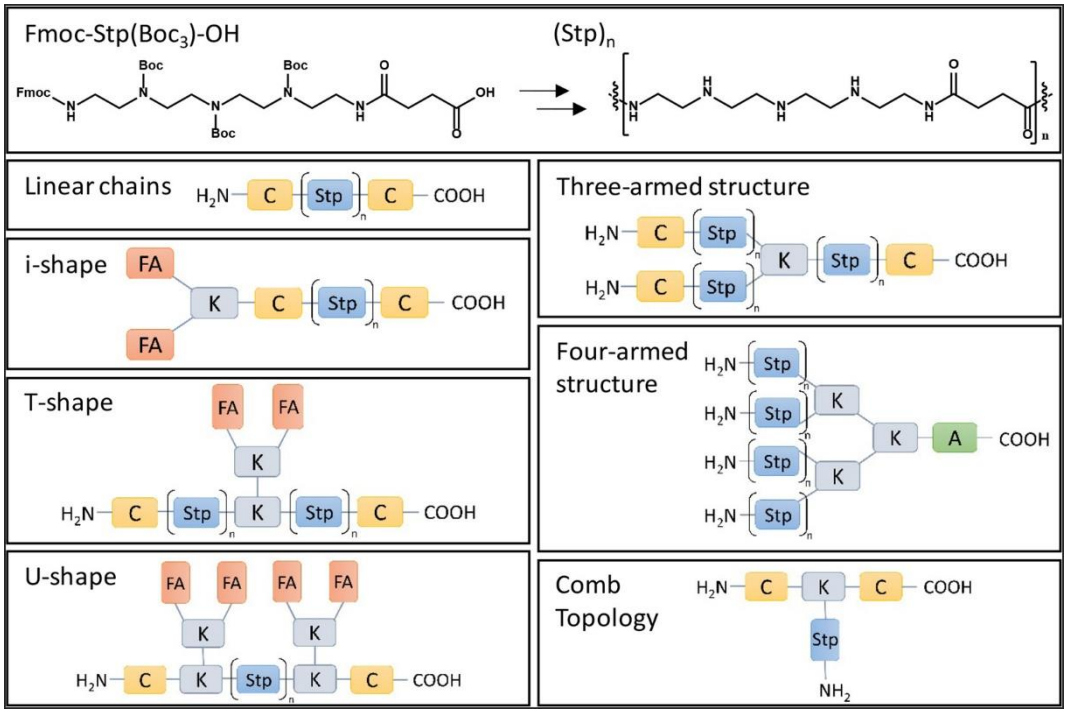

Figure 1: Protected artificial amino acid Fmoc-Stp(Boc3)-OH and sequential assembly into Stp-based oligo(ethanamino)amide (OAA) sequences with different structural topologies. Stp, succinoyl tetraethylene pentamine; C, cysteine; K, lysine; A, alanine; FA, fatty acid. Reproduced from Freitag & Wagner (Advanced Drug Delivery Reviews) with permission of the authors [62].

1.2. Cargos

Not only new delivery systems are discovered continuously, but also new cargos that can be utilized as therapeutic agents. In the early days of gene transfer, mostly DNA viruses, such as adenovirus, adenovirus-associated virus, and herpes simplex virus with their respective form of genetic material, were used in gene therapy trials [68-71].

1.2.1. Plasmid DNA (pDNA)

With the upcoming field of non-viral vectors, Felgner *et al.* introduced a lipid-based transfection technique for plasmid DNA (pDNA) called lipofection [72]. Also, direct injection of pDNA into the target location was postulated as a viable option for *in vivo* transfection, circumventing immunogenicity of viral vectors; however, owing to the technique, transfection was mainly measurable near the injection site [73, 74].

Plasmids are small, mostly ring-shaped DNA molecules in bacteria which are located extrachromosomal and can be replicated independently from the chromosomal DNA. It consists of (i) a multicloning site, where the gene of interest can be inserted, (ii) a polyadenylation site, which regulates gene transcription, (iii) an antibiotic resistance site for positive selection in the production, (iv) a promotor sequence and (v) an origin of replication site [75]. Benefits of pDNA are the ease of modification and production of recombinant pDNA, its ability to express a variety of different gene products, and its good stability, which is manifested in a shelf life of several months [76].

On the other hand, since it is DNA, it must be transported into the nucleus for transcription before translation into proteins can occur. Hence, pDNA transfection is usually ineffective for non-dividing cells [77]. Additionally, Zhao *et al.* could show that pDNA can provoke acute inflammatory responses, which is mainly, but not only, due to Toll-like receptor 9 (TLR9) recognizing CpG motifs in the pDNA sequence [78]. Furthermore, pDNA can be inserted into the host genome leading to insertional mutagenesis, which can alter gene transcription, regulation or coding sequence, potentially causing cancer when tumor suppressor genes are inactivated or,

respectively, oncogenes are activated [79].

1.2.2. Messenger RNA (mRNA)

Mature eukaryotic mRNA is built up of a Cap structure, the 5' untranslated region (5'UTR), the open reading frame (ORF) or coding sequence, the 3'UTR, and a long sequence of adenine nucleotides termed poly(A) tail [80]. Messenger RNA is a single-stranded RNA molecule that is transcribed from nuclear DNA, transported to ribosomes in the cytoplasm, and subsequently translated into proteins. Therefore, in contrast to pDNA, mRNA only needs to be delivered into the cytoplasm, which eliminates the intracellular barrier of the nucleus that pDNA has to overcome additionally.

Yet, as mRNA gets degraded after translation and because it is not inserted into the host genome, mRNA therapeutics achieve only transient therapeutic effects, *i.e.,* protein expression. Although Malone *et al.* reported successful cationic liposome-mediated mRNA transfection into murine cells in 1989 [81], and mRNA vaccinations against infectious diseases and cancer were discussed [82-84], mRNA went quiet again due to mRNA being considered too unstable. Problems of early chemically synthesized or *in vitro* transcribed (IVT) mRNA were immunogenicity due to activation of, *e.g.*, human TLR3, TLR8, murine TLR7 or double-stranded RNA (dsRNA)-activated protein kinase (PKR) [85-88], and the before mentioned instability, *i.e.,* susceptibility to degradation by ubiquitous nucleases [89]. This immunogenicity may be beneficial when utilized in vaccination but is contraindicated for repeated application in RNA-mediated protein replacement therapy [80, 90, 91].

However, Karikó *et al.* revealed an inverse correlation of the RNA's immunogenicity to the extent of their nucleoside modification, assuming that this is due to the mammalians immune system having evolved to recognize bacterial RNA which is less modified [92]. Besides the modification of RNA nucleosides, also unmodified sequence-engineered mRNA was reported to be a non-immunogenic alternative, actually achieving enhanced protein expression compared to nucleoside modified mRNA [91]. Furthermore, stability, along with translational efficiency, could be increased by

introducing 5'-cap analogs, which improved stability against degradation [93-95].

To date, in clinical and pre-clinical trials, mRNA vaccines have demonstrated good tolerability and the capability to induce an immune response against RSV [96], rabies [97], prostate cancer [98], and non-small cell lung cancer [99], amongst others. Warren *et al.* discovered another application of mRNA: reprogramming multiple human call types into RNA-induced pluripotent stem cells (RiPSCs) and also, as an example for tissue-engineering, directing the differentiation of RiPSCs into terminally differentiated myogenic cells [100].

A great breakthrough in mRNA therapeutics was achieved when end of 2020 and beginning of 2021, two mRNA-based vaccines against SARS-CoV-2, utilizing lipid nanoparticles (LNPs), obtained their conditional marketing authorization [101].

1.2.3. Protein

Proteins are the most versatile macromolecule in our bodies, being catalysts or receptors and channels in membranes, comprising intracellular and extracellular scaffolding support, and transport molecules within a cell or from one organ to another [102]. Therefore, proteins not properly working, due to incorrect translation or posttranslational modifications or proteins getting damaged or over- or underexpressed, often lead to diseases [103-105]. Since 1982, when the FDA approved the first recombinant protein, namely Humulin (Insulin) [106], a large number of therapeutic peptides and proteins complemented the list of approved drugs [107, 108].

However, facing the same obstacles in intracellular delivery as NAs do, these peptides and proteins could only interact with cells by extracellular receptors. Although many recombinant proteins, such as monoclonal antibodies or recombinant growth factors, have extracellular receptors, intracellular delivery would enable proteins to aim at intracellular molecular targets [109].

As pDNA and mRNA delivery is applied to correct or replace proteins in

target cells, another approach is to directly deliver proteins into the cells to bypass the translation and, if necessary, transcription processes. Besides the benefit of faster kinetics due to circumvention of transcription and translation, a direct delivery could avoid the uncontrollable timespan and level of protein expression and thus, provide more controllability to the therapy [110]. What makes protein delivery so difficult is their large size, surface charge distribution, and their complex tertiary and quaternary structure, making them labile, and prone to denaturation, degradation, and aggregation, hence creating the intricate task of developing suitable carriers that address all of the above challenges [110].

1.2.4. Other cargos

All of the above cargos serve mainly the purpose of replacing missing or defective proteins by direct replacement or introduction of protein expressing genes. In a wide filed of diseases, such as cancers, other genetic disorders, and viral infections, expression of genes can, however, be the disease-causing mechanism. Thus, silencing these genes would causally treat those diseases [111]. This approach is realized by an enzyme-mediated process called RNA interference (RNAi), a naturally occurring process found in most eukaryotes, which utilizes the endogenous RNA-induced silencing complex (RISC) to specifically target and cleave mRNA before translation, and thus, protects the cells from viruses and transposable elements [112, 113]. Thereby, for therapeutic purposes, endogenous microRNA (miRNA), synthetic short interfering RNA (siRNA), or antisense oligonucleotides (ASOs) are employed [114]. The mechanism is based on the activation of dicer RNase III upon recognition of endogenous (pre-microRNAs) or exogenous (*e.g.*, virus infection) double-stranded RNA (dsRNA). Dicer enzymatically cuts dsRNA into 22-nucleotide pieces, which are loaded into RISC, and subsequently cleaves mRNA complementary to the dsRNA's guide strand [111]. Evidence of the great potential of exploiting RNAi is the approval of 4 siRNA-based drugs by the FDA, with more in clinical trials to date [115].

1.3. Targeting

As shortly mentioned above, one method to enhance the efficiency of vectors is to utilize different ways of targeting.

Targeting of nanocarriers comprises all methods that pursue the aim to enhance specific affinity toward target cells, or more broadly, toward a pathological site [116]. This aspect not only has the advantage of higher transfection efficiency, but also minimizes off-target effects, and reduces overall therapeutic doses and, therefore, dose-dependent toxicities [116, 117]. Targeting methods include passive targeting, such as the enhanced permeation and retention (EPR) effect, which is based on the accumulation of all sorts of macromolecules into tumor tissue due to irregularly shaped, dilated, leaky or defective neovasculature and poor lymphatic drainage [118], and active targeting. Active targeting, on the other hand, utilizes surface-bound moieties, such as antibodies, aptamers, peptides, small molecules, or carbohydrates that specifically interact with receptors on the cells at the target site [117, 119].

Another strategy addresses the activation of transcription rather than aiming at specific cells, the so-called transcriptional targeting. It exploits the fact that gene expression is regulated by promoters/enhancers in a cell-type-specific manner and, thus, utilizing certain promoters, a cell-specific expression of the protein of interest is feasible [120].

1.4. Shielding

A second strategy for efficiency enhancement, termed shielding, aims at protecting systemically administered nanocarriers from plasma protein binding, fast immune clearance, toxicity, or immune recognition to prevent immunogenicity and to enable nanocarriers to reach their target site before clearance by phagocytic cells takes place. Therefore, to meet these requirements, the best shielding agents are often molecules that are normally found in the circulation, such as endogenous carbohydrates, membrane-like lipids, or proteins and polypeptides [121]. Yet, one of the most widely used shielding agents for *in vivo* application is the synthetic polymer polyethylene glycol (PEG) which, in addition to the before

mentioned, has been shown to prevent self-aggregation, erythrocyte aggregation, and induced a prolonged circulation time, and a higher stability [122-124].

2. Aims of this thesis

This thesis aimed at evaluating novel synthetic carriers and their ability to deliver pDNA, mRNA, and Cas9 RNPs *in vitro* and especially *in vivo* in a mouse model.

2.1. Transcriptional targeting of pDNA to dendritic cells *in vivo*

Immunotherapy, a big field of therapies based on (re-)activating a patient's immune system against a disease, can be realized passively by antibodies or actively by activating various immune cells, as e.g. in vaccines [125]. However, in order to achieve long lasting therapeutic effects, an active immunotherapy is necessary. Dendritic cells (DCs), which are antigen presenting cells (APCs), have the unique ability to activate naïve T cells, and are therefore highly interesting targets in immunotherapy [126]. To effectively reach DCs, different methods of targeting can be utilized to enhance transfection efficiency, reduce therapeutic doses, and minimize toxicities and off-target effects [116, 117].

In this thesis, the feasibility of transcriptional targeting DCs was evaluated. Firstly, it was to assess which of the established carriers, succPEI and LPEI, was most suitable to deliver pDNA *in vivo*, and in what organs, and to what extent therein, luciferase was expressed.

In a following experiment, the aim was to appraise in what cell types the newly developed Fascin promoter facilitated luciferase expression and thus, assess if targeting DCs was actually achievable.

2.2. Novel dynamic lipopolyplexes containing lipo amino fatty acids (LAF) for potent mRNA delivery *in vivo*

Many efforts have been made to optimize non-viral vectors to combat poor transfection efficiency, mainly resulting from insufficient endosomal escape

after endocytosis. To further refine synthetic carriers, unbiased and willful combinatorial chemistry, which we refer to as ‘chemical evolution’, was applied to generate libraries of non-viral vectors that were safe and adapted to their respective cargo [46]. One part of this chemical evolution was to couple different ligands, such as, lipidic residues, carbohydrates, membrane destabilizing or shielding agents to the cationizable polymers to overcome extra- and intracellular barriers that delivery systems face in highly complex organisms.

A new promising strategy was discovered, due to our group’s development of a new lipidic domain, termed lipo amino fatty acid, an artificial amino acid coupled to two fatty chains, creating an amphiphilic structure with a protonable tertiary amine. Thus, coupling LAFs to existing OAAs, pH-sensitive carriers were synthesized that would be protonated in the acidic environment of endosomes, resulting in endosomal escape by destabilizing the endosomal membrane.

Therefore, the first step was to evaluate the transfection efficiencies of the newly synthesized carriers *in vitro*, followed by further selection by repeating the transfection experiments after the incubation of the carriers in fetal bovine serum (FBS) to mimic conditions after systemic application.

A subsequent experiment’s aim was to assess the biodistribution and also the transfection efficiency of the nanocarriers in the different organs in a tumor mouse model, using a luciferase mRNA as cargo.

2.3. Delivery of Cas9 RNPs for dual immune checkpoint disruption in colon cancer by a folate receptor targeted synthetic carrier

To deliver Cas9 RNPs efficiently into the tumor, a suitable nanocarrier had to be developed. For that purpose, a derivative of **1105**, a structure previously identified as a potent structure for delivery of Cas9 RNPs, was post functionalized with PEG_{24}-shielding and folate receptor α- (FR α) targeting, which is a receptor highly expressed on CT26 tumors, and subsequently, its biodistribution was evaluated.

Since often inhibitory immune checkpoints are severely overexpressed in

several types of cancers, the host's immune system is not able to respond accordingly, as immune responses are strongly inhibited by the PD-L1/PD-1 and PVR/TIGIT pathways, among others. In contrast to treatment with transiently working immune checkpoint inhibiting antibodies, CRISPR/Cas systems could enable permanent genome editing. The aim of this thesis was to "reactivate" the immune system to fight CT26 tumors by a dual immune checkpoint disruption of PD-L1 and PVR with the help of CRISPR/Cas9 RNPs.

Consecutively, after proof of concept *in vitro*, it was to evaluate if immune checkpoint disruption was also seen *in vivo* upon intratumoral administration in CT26 tumor-bearing mice and if post functionalizing had benefits in knockout (KO) efficiency.

Lastly, the aim was to appraise the effect of repeated injection of sgPD-L1- and sgPVR-containing RNPs on tumor growth and the experimental animals' survival time *in vivo.*

II. MICE, MATERIALS AND METHODS

1. Mice

1.1. Mouse strains

1.1.1. BALB/c mice

Female BALB/cJRj mice were purchased from Janvier Labs (Le Genest-St-Isle, France). It is one of the most widely used inbred albino mouse strains known for its calm and easy to handle characteristics. BALB/c were used as a syngeneic CT26 tumor model. As the mice have an intact innate and adaptive immune system, SOPF animals of this strain are also used as sentinel animals in our lab for the mandatory health monitoring.

1.1.2. A/J mice

Female A/JOlaHsd mice were obtained from Envigo RMS GmbH (Düsseldorf, Germany). A/J mice, an albino inbred strain, are widely used to model cancer, cardiovascular research, and developmental biology. A/J mice tend to be very docile and easy to work with [127]. We used the mice as a syngeneic N2a tumor model.

1.2. Housing conditions

The mice were purchased at 5-8 weeks of age and were given an acclimatization time of at least seven days prior to experimentation. The animals were housed in isolated ventilated cages (IVC type II long, Tecniplast, Hohenpeißenberg, Germany) under specific pathogen-free conditions. They were kept in an air-conditioned room with a 12 h day/night interval, the light intensity not exceeding 200 Lux. The room temperature and air humidity were maintained at 24-26 °C, respectively 40-60 %, and documented daily. The cages were equipped with enrichment in form of a plastic house, a wooden tunnel, paper towels, and additionally dust-free

bedding (ABEDD Vertriebs GmbH, Vienna, Austria). On demand, or at least once a week, the cages, likewise water and feed, were exchanged. Feed was acquired from Ssniff Spezialdiäten (Soest, Germany), together with water offered *ad libitum.* Every component coming into contact with the mice, for instance, housing material, feed, and water were sterilized by an autoclave beforehand. Animal welfare was controlled and documented daily following §11 of the German Animal Welfare Act [128].

1.3. Health monitoring

Two SOPF BALB/c mice were kept in the same conditions as the experimental animals, in an isolated ventilated cage and getting used feed and bedding material from all other cages at the time of the weekly exchanges. Quarterly, 2 sentinel animals were sent in for health analysis to an external laboratory (mfd Diagnostics GmbH, Wendelsheim, Germany) according to FELASA guidelines. In all listed experiments no findings were made.

2. Materials

2.1. Cell culture

Material	Source
CT26 WT cells (murine colon carcinoma cells)	American Type Culture Collection (ATCC; Manassas, VA USA)
CT26 KO cells (murine colon carcinoma cells, with PD-L1 knockout)	Pharmaceutical Biotechnology, Ludwig Maximilian University (Munich, Germany)
Neuro-2a (N2a) cells (murine neuroblastoma cells)	American Type Culture Collection (ATCC; Manassas, VA USA)
antibiotics	Sigma Aldrich (Taufkirchen, Germany)

FBS (Fetal bovine serum)	Sigma Aldrich (Taufkirchen, Germany)
HEPES	Biomol GmbH (Hamburg, Germany)
DMEM	Sigma Aldrich (Taufkirchen, Germany)

2.2. Buffers and chemicals

PBS	136.89 mM sodium chloride, 2.68 mM potassium chloride, 8.10 mM sodium phosphate dibasic heptahydrate, 1.47 mM potassium dihydrogen phosphate, pH 7.4
HBG	20 mM HEPES, 5 % glucose
LAR buffer	20 mM glycylglycine; 1 mM MgCl2; 0.1 mM ethylene-diaminetetraacetic acid; 3.3 mM dithiothreitol; 0.55 mM adenosine 5′-triphosphate; 0.27 mM coenzyme A, pH 8-8.5

Cell culture 5x lysis buffer	Promega (Mannheim, Germany)
D-luciferin	Promega (Mannheim, Germany)
Protease and Phosphatase Inhibitor Cocktail	Sigma Aldrich (Taufkirchen, Germany)

2.3. Polyplexes

The oligomer **1445** (illustrated in **Figure 11**) was synthesized by Yi Lin (former PhD student at Pharmaceutical Biotechnology, LMU, Munich).

The oligomers **1611**, **1760**, **1719**, **1752** were synthesized by Melina Grau (PhD student at Pharmaceutical Biotechnology, LMU, Munich).

The oligomer **1621** was synthesized by Lun Peng (former PhD student at Pharmaceutical Biotechnology, LMU, Munich) and Melina Grau (PhD

student at Pharmaceutical Biotechnology, LMU, Munich).

ID	Topology	Sequence	Stp/LAF ratio
1621	Bundle 2	K[K(8Oc)2]2-Stp	1:4
1752	Bundle 2	K[K(12Bu)2]2-Stp	1:4
1611	U-shape 1	K(12Oc)-Stp-K(12Oc)	1:2
1719	U-shape 1	[K(12Oc)]2-Stp2-[K(12Oc)]2	2:4
1760	U-shape 1	[K(12He)]2-Stp2-[K(12He)]2	2:4

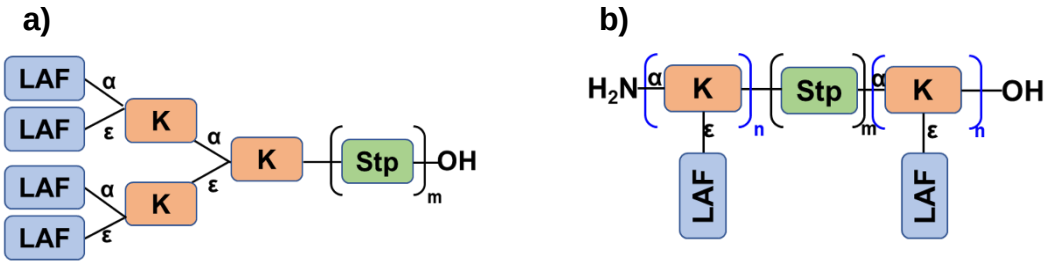

Figure 2: Characteristics of LAF-oligomers. a) Scheme of a Bundle 2 structure. **b)** Scheme of a U-shape 1 structure. (K= lysine, Stp= succinoyl tetraethylene pentamine, LAF= lipo amino fatty acid, 8Oc= LAF based on 8-aminooctanoic acid and two octyl chains, 12Bu= LAF based on 4-aminobutanoic acid and two dodecyl chains, 12He= LAF based on 6-aminohexanoic acid and two dodecyl chains). Figures 2 a and b provided by Melina Grau (PhD student at Pharmaceutical Biotechnology, LMU, Munich)

2.4. Nucleic acids

sgRNAs were chemically modified (2' O-methyl modification on the first 3 and last 3 RNA bases) and purchased from Integrated DNA Technologies (Coralville, IA USA).

sgCtrl	mG*mG*mG*rUrArArCrCrGrUrGrCrGrGrUrCrGrUrArCrGrU rUrUrUrArGrArGrCrUrArGrArArArUrArGrCrArArGrUrUrArA rArArUrArArGrGrCrUrArGrUrCrCrGrUrUrArUrCrArArCrUrU rGrArArArArArGrUrGrGrCrArCrCrGrArGrUrCrGrGrUrGrC mU*mU*mU*rU
sgPD-L1	mG*mA*mC*rUrUrGrUrArCrGrUrGrGrUrGrGrArGrUrArGrU rUrUrUrArGrArGrCrUrArGrArArArUrArGrCrArArGrUrUrArA rArArUrArArGrGrCrUrArGrUrCrCrGrUrUrArUrCrArArCrUrU rGrArArArArArGrUrGrGrCrArCrCrGrArGrUrCrGrGrUrGrC mU*mU*mU*rU
sgPVR	mG*mC*mU*rUrCrUrArArUrCrUrCrCrArCrCrGrUrArGrGrUr UrUrUrArGrArGrCrUrArGrArArArUrArGrCrArArGrUrUrArAr ArArUrArArGrGrCrUrArGrUrCrCrGrUrUrArUrCrArArCrUrUr GrArArArArArGrUrGrGrCrArCrCrGrArGrUrCrGrGrUrGrCm U*mU*mU*rU

mRNA

CleanCap® FLuc mRNA (5moU)	Trilink Biotechnologies (San Diego, CA USA)

pDNA

pCMVLuc	Plasmid Factory GmbH (Bielefeld, Germany)
pFscnLuc	Plasmid Factory GmbH (Bielefeld, Germany)

2.5. *In vivo* experiments

Isoflurane CP®	CP-Pharma (Burgdorf, Germany)
Bepanthen®	Bayer Vital GmbH (Leverkusen, Germany)
Syringes	B. Braun (Melsungen, Germany)
cannulas	Henke-Sass, Wolf GmbH (Tuttlingen, Germany); BD Diagnostics (Heidelberg, Germany)
EDTA blood sample tube	KABE Labortechnik GmbH (Nümbrecht-Eisenroth, Germany)
Cell strainer	pluriSelect Life Science UG (haftungsb.) & Co.KG (Leipzig, Germany)

2.6. Instruments

Instrument	Source
Caliper DIGI-Met	Preisser (Gammertingen, Germany)
IVIS Lumina	Caliper Life Science (Rüsselsheim, Germany)
Cordless animal shaver GT 420 ISIS	Aesculap Suhl GmbH (Suhl, Germany)
Centro LB 960 plate reader Luminometer	Berthold Technologies GmbH & Co. KG (Bad Wildbad, Germany)
Centrifuge 5415 D	Eppendorf SE (Hamburg, Germany)

Homogenizer FastPrep-24™ Classic	MP Biomedicals Germany GmbH (Eschwege, Germany)

2.7. Software

Software	Provider
Graph Pad Prism 9 software	Graph Pad Software (San Diego, CA USA)
Living Image 3.2	Caliper Life Science (Rüsselsheim, Germany)

3. Methods

3.1. Cell culture

The murine neuroblastoma cell line Neuro2a (N2a) was cultured in DMEM-low glucose (1 g L^{-1} glucose) supplemented with 10 % (v/v) FBS, 4 mM stable glutamine, 100 U mL^{-1} of penicillin, and 100 µg mL^{-1} of streptomycin.

The murine colon carcinoma cell line CT26 was cultured in DMEM supplemented with 10 % (v/v) FBS, 100 U mL^{-1} of penicillin, and 100 µg mL^{-1} of streptomycin.

Before inoculation into mice, cells were passaged in antibiotic free medium.

3.2. *In vivo* experiments

For tumor experiments, cells were suspended in 150 µL PBS and subsequently injected subcutaneously into the left flank of the mice using a 25G cannula. Tumor cell injection was performed under isoflurane inhalation anesthesia (3 mL isoflurane / 100 mL air for induction and 2,5 mL

isoflurane / 100 mL air for maintenance with an oxygen rate of flow of 2,5 L/min). The tumor size was measured by caliper, and the volume was calculated with the well-established formula [$V= (W^2 \times L)/2$] [129]. Intratumoral injections were performed under a short isoflurane inhalation anesthesia with a maximum volume of 50 µL using a 30G cannula.

Intravenous injections were performed into the lateral tail vein by fixing the mice in a restrainer and injecting a maximum volume of 200 µL. For easier injection the tail vein can be hold in lukewarm water (max. 45 °C) for 10-20 sec. to achieve vasodilation.

All experimental animals were weighed and checked daily for their well-being. Mice were euthanized when termination criteria occurred, such as a tumor size ≥12 mm in diameter or severely affected well-being (e.g., continuous weight loss, pain symptoms, apathy or automutilation) by cervical dislocation.

All animal experiments were approved by the district government of Upper Bavaria (file number: ROB-55.2-2532.Vet_02-19-19, ROB-55.2-2532.Vet_02-19-20) and were consistent with the guidelines of the German Animal Welfare Act [128].

3.2.1. Transcriptional targeting of pDNA to dendritic cells *in vivo*

3.2.1.1. Biodistribution of pCMVLuc pDNA and pFscnLuc pDNA expression with succPEI and LPEI

Six-week-old BALB/c mice were randomly split into four groups (n=5) and intravenously injected with 200 µL of two different carriers, namely LPEI (N/P=9) and succPEI (w/w=1.5), with 60 µg pCMVLuc pDNA or pFscnLuc pDNA, respectively. Twenty-four hours *p.i.* the animals were euthanized, and the organs lungs, liver, and spleen, were dissected, washed in PBS, and subsequently frozen overnight at -80 °C. The next day, tissue samples of 100-500 mg were homogenized by a FastPrep-24™ instrument in 500 µL of cell culture lysis buffer 1x, supplemented with 1 % (v/v) protease and phosphatase inhibitor cocktail, followed by freezing overnight to ensure a complete lysis of cells. In a next step, the samples were thawed and centrifuged for 10 min at maximum speed (~13,000 rpm) and 4 °C.

Luciferase activity in 25 µL of cell lysate was measured for 10 secs by a Centro LB 960 plate reader luminometer after the addition of 100 µL LAR buffer supplemented with 5 % (v/v) of a mixture of 10 mM luciferin and 29 mM glycylglycine. Transfection efficiency was calculated for the tissue sample and presented as relative light units (RLU) per g of organ.

3.2.1.2. Comparison of pCMVLuc pDNA and pFscnLuc pDNA expression on a cellular level

Six-week-old BALB/c mice were randomly split into three groups (n=5) and intravenously injected with 200 µL of succPEI (w/w=1.5) containing 60 µg pCMVLuc pDNA or pFscnLuc pDNA. Group 3 served as an untreated negative control. Twenty-four hours *p.i.* the animals were euthanized, and the organs lungs, liver, spleen, and inguinal lymph nodes were dissected, put in ice-cold PBS, and sent to Dr. Matthias Bros and his team at the work group led by Prof. Stefan Grabbe (*Johannes Gutenberg University; Mainz, Germany*), where single cell suspensions of the respective organs were stained immunohistochemically for antigens CD11c (dendritic cells), MAC (macrophages), F480 (Kupffer cells), and luciferase.

3.2.2. Novel dynamic lipopolyplexes containing lipo amino fatty acids (LAF) for potent mRNA delivery *in vivo*

3.2.2.1. Biodistribution of luciferase expression by LAF carriers

6-week-old A/J mice were inoculated subcutaneously with 1 x 10^6 N2a cells into their left flanks. When tumors reached a size of 250-500 mm^3, mice were divided into groups of n=5 and treated with a single intravenous injection of 150 µl of different LAF carriers encapsulating either 1, 3, or 10 µg of luciferase mRNA. Animals were euthanized 24 hours, respectively six hours *p.i.,* and the organs: brain, heart, lungs, liver, spleen, kidneys, and the tumor were dissected, washed in PBS, and frozen at -80 °C. The next day, the tissue samples were homogenized in 500 µL cell culture lysis buffer 1x and subsequently again frozen overnight. Afterward, luciferase activity was measured, as described in **3.2.1.1..**

3.2.2.2. Evaluation of clinical blood parameters

Tumor-free nine-week-old A/J mice were divided randomly into groups of n=4 and subsequently injected intravenously with 150 µL of either **1611** (3 µg luciferase mRNA), **1752** (1 µg luciferase mRNA), or HBG (n=2). Untreated animals, as well as the HBG group served as reference. Six hours *p.i*. animals were euthanized and subsequently blood was collected into EDTA-coated tubes. Blood was centrifuged (Eppendorf Centrifuge 5415 D) at 3000 rpm for seven min to obtain plasma, which was consecutively analyzed in the *Clinic of Small Animal Medicine (Faculty of Veterinary Medicine, LMU Munich)* for liver parameters (*i.e*., alanine aminotransferase, aspartate aminotransferase) and renal parameters (*i.e.,* creatinine, blood urea nitrogen).

3.2.3. Delivery of Cas9 RNPs for dual immune checkpoint disruption in colon cancer by a folate receptor targeted synthetic carrier

3.2.3.1. Pre-experiment: i.v. biodistribution of 1445 structures

Four animals, inoculated with the CT26 WT cell line, were intravenously injected with 150 µL of either "naked" Cas9 RNP, **1445** + Cas9 RNP, PEG_{24}-**1445** + Cas9 RNP or FolA-PEG_{24}-**1445** + Cas9 RNP. All injections contained 125 µg ATTO740-labeled Cas9 + 25 µg sgRNA. Using an IVIS Lumina device, mice were imaged at different time points (0 min, 15 min, 30 min, 1 h, 2 h, 4 h, 24 h) after an intraperitoneal injection of luciferin (100 mg/kg solved in 150 µL PBS) under isoflurane inhalation anesthesia.

3.2.3.2. Molecular confirmation of gene knockout *in vivo*

Six-week-old female BALB/c mice were inoculated subcutaneously with 5 x 10^5 CT26 WT cells into their left flanks, and the injection sites got marked. Three days *p.i.* the mice were randomly divided into five groups (n=6) and got subcutaneously injected a volume of 50 µL of either **1445**, FolA-**1445,** or FolA-PEG_{24}-**1445** Cas9 RNPs + sgRNA (12,5/12,5 mg sgPD-L1/sgPVR). Injections of HBG and FolA-**1445** Cas9 RNPs + sgCtrl served as negative control groups. The therapy consisted of three subcutaneous/intratumoral injections on days three, six and nine after tumor inoculation. The tumor size was measured and documented daily. All animals were euthanized on day 16, and the tumors were dissected.

Tumor tissue was homogenized using a FastPrep-24™ Instrument (MP Biomedicals Germany GmbH, Eschwege, Germany), passed through 100 µm and subsequently, 40 µm cell strainers, and washed with PBS to obtain single-cell suspensions. The flow cytometry analysis was performed by Yi Lin (former PhD student at Pharmaceutical Biotechnology, LMU, Munich)

3.2.3.3. Therapeutic study

1 x 10^5 CT26 WT cells were inoculated in the left flanks of eight-week-old BALB/c mice and injection sites were marked. Mice were then randomly divided into five groups (n=6). Treatment consisted of six injections in total and were performed on days 4, 7, 11, 18, 21 and 25. The animals were injected with FolA-PEG_{24}-**1445** + Cas9 RNPs with 25 µg of sgCtrl or 12,5 µg of sgPD-L1 and sgPVR each or 12,5 µg of sgCtrl in combination with 12,5 µg of either sgPD-L1 or sgPVR. An untreated group served as a negative control. Injections were performed under isoflurane inhalation anesthesia with an injection volume of 50 µL. Daily, tumors were measured by caliper, and the well-being of the animals was checked and documented. Mice were euthanized when tumors reached a size of ≥12 mm in diameter or when the well-being of mice was severely affected.

3.3. Statistical analysis

Statistical analysis was performed with unpaired students t-test using the software GraphPad Prism 9. P-values < 0.05 were determined as significant (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns = not significant). Results are expressed as mean + S.E.M. unless indicated otherwise.

III. RESULTS

1. Transcriptional targeting of pDNA to dendritic cells *in vivo*

Of all antigen-presenting cells (APCs), dendritic cells (DCs) stand out, as they can stimulate B cells but also initiate APC-dependent antigen-specific T cell expansion and maturation [130]. In addition to major histocompatibility complex (MHC) I molecules that present mainly intracellular proteins to immune cells, DCs also express MHC II molecules which, upon antigen (Ag) capture, present parts of the Ags in order to prime naïve CD4+ T cells [131]. However, DCs also induce tolerance toward self-antigens by deletion of self-Ag affine T cells (negative selection), hence inhibiting autoimmune responses [132, 133]. Taken together, the DCs ability to activate T cells and, on the other hand, to induce T cell tolerance which is exploited by some tumors [131], makes them a highly interesting target for immunotherapy.

In 2003 Bros et al. reported on the human Fascin promoter, which contains a stage-specific enhancer region that elicits high promoter activity only in mature immunostimulatory DCs. This insight provided a promising way for transcriptional targeting of DCs [126]. Later, the same laboratory designed pFscn plasmid vectors for DC–selective gene expression driven by the Fascin promotor-enhancer construct. In our laboratory (Simone Berger, PhD student at Pharmaceutical Biotechnology, LMU), a well-established luciferase pDNA, driven by the cytomegalovirus (CMV) promoter, which has binding sites to several ubiquitously expressed transcription factors [134], was compared to Luc pDNA, driven by the newly developed Fascin (Fscn) promoter, and demonstrated favorable expression in DCs *in vitro*. As these encouraging experiments were performed *in vitro*, an animal experiment was conducted to evaluate the feasibility and efficacy of transcriptional targeting *in vivo*.

1.1. Biodistribution of pCMVLuc pDNA and pFscnLuc pDNA expression with succPEI and LPEI

For an initial comparison of the cytomegalovirus (CMV) promoter with the Fascin (Fscn) promoter, mice were intravenously injected with 60 µg of luciferase pDNA either with the CMV or the Fscn promoter, encapsulated in succinylated PEI (succPEI), or linear PEI (LPEI), respectively. Twenty-four hours *p.i.*, the animals were euthanized, and the organs lungs, liver, and spleen were dissected, and processed further for an *ex vivo* luciferase assay. Upon analysis, LPEI showed an overall higher luciferase expression in the lungs compared to succPEI. Interestingly, within the LPEI group, pFscnLuc demonstrated a reduced (10-fold) luciferase expression in lungs and liver but a constant value in the spleen. In the succPEI group, on the other hand, pFscnLuc showed significantly higher (12-fold) RLU values for the spleen with constant values in lungs and liver. These findings led to the assumption that pFscnLuc, indeed, illustrates higher transcriptional activity in DCs.

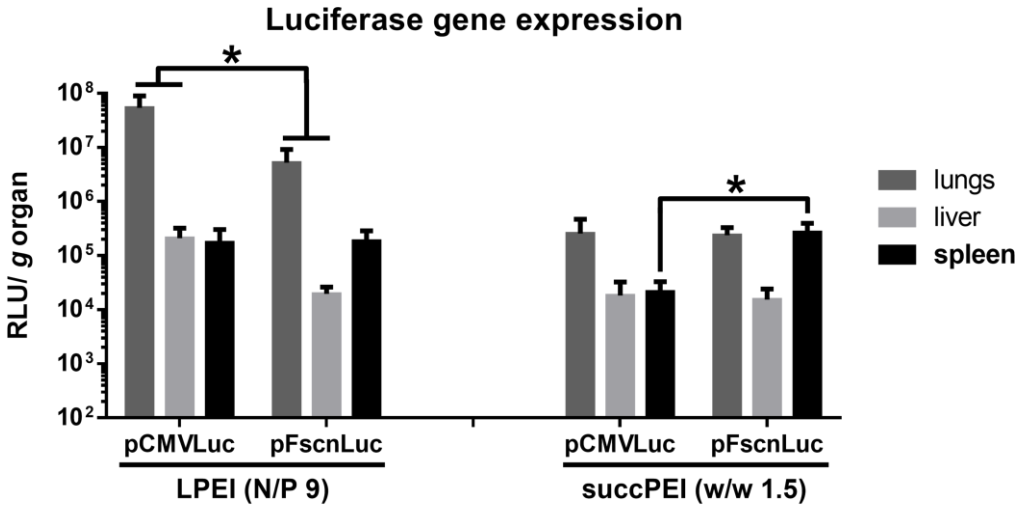

Figure 3: *In vivo* performance of pFscnLuc in comparison to pCMVLuc. The performance of 60 µg intravenously applied Luc-pDNAs (pCMVLuc and pFscnLuc) with either succPEI or LPEI was evaluated via an *ex vivo* luciferase assay of the organs lungs, liver, and spleen of tumor-free BALB/c mice 24 h *p.i.*. (n=5; mean + SD; *p ≤ 0.05). Figure provided by Simone Berger (PhD student at Pharmaceutical Biotechnology, LMU, Munich).

1.2. Comparison of pCMVLuc pDNA and pFscnLuc pDNA expression on a cellular level

In a subsequent experiment it was to evaluate in what types of cells luciferase was expressed. Therefore, succPEI was chosen for this comparison as it showed similar expression values for the spleen but lower values for the liver and lungs, suggesting being the more specific carrier for spleen delivery. Tumor-free mice were divided randomly into groups of n=5 and subsequently treated with 60 µg of pDNA containing either the CMV or the Fscn promoter via intravenous injection. Twenty-four hours *p.i.*, animals were euthanized, organs were dissected, and sent to Dr. Matthias Bros and his team at the work group led by Prof. Stefan Grabbe (Johannes Gutenberg University; Mainz, Germany) for immunohistochemical staining.

The ratio of luciferase-positive DCs was compared to luciferase-positive MACs to verify the specificity of the Fascin promoter for DCs. Therefore, the mean fluorescent intensity (MFI) of luciferase expressing DCs and MACs, which were set in relation to the negative control ([MFI] fold ctrl), were put into proportion. **Figure 4** depicts a ~1.5- to 4-fold higher expression level in DCs over macrophages utilizing the Fascin promoter. Compared with the strong CMV promoter, a slight but non-statistical advantage of the Fascin promoter regarding luciferase expression in DCs (spleen: p=0,483; lymph nodes: p=0,1942; lungs: p=0,0908; liver: p=0,3514) was observed.

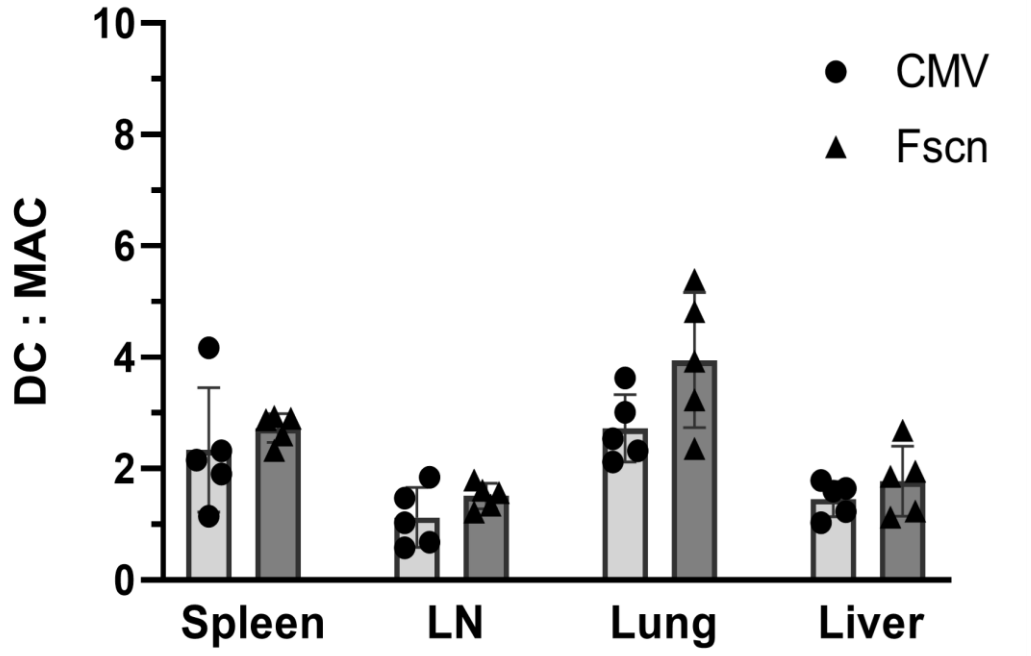

Figure 4: Ratio of luciferase positive dendritic cells and macrophages after an i.v. application of 60 µg Luc-pDNA (pCMVLuc or pFscnLuc) with succPEI was evaluated via immunohistochemical staining and subsequent FACS of single cell suspensions of the organs lungs, liver, spleen, and inguinal lymph nodes of tumor-free BALB/c mice 24 h *p.i.*. (n=5; mean + SD). Figure provided by Yanira Zeyn (PhD student at University Medical Center Mainz, Johannes Gutenberg University, Mainz).

2. Novel dynamic lipopolyplexes containing lipo amino fatty acids (LAF) for potent mRNA delivery *in vivo*

As gene delivery, especially endosomal escape, still constitutes the major bottleneck of non-viral carriers *in vivo*, working groups all over the world investigate tirelessly to develop new strategies. As more detailed in the introduction, our working group has generated OAA libraries with different topologies and different ligands and residues.

Recent studies in our working group revealed that the lytic activity and, thus, transfection efficiency was influenced by the chain length of the coupled fatty acids. The lytic potential was highest for chain lengths of C10 and C14 and lowest for C2, C6, and C18. Simultaneously, chain length also had an impact on stability: the longer the fatty acids, the more stable the polyplexes were, indicating strong hydrophobic interactions [40]. Furthermore, studies showed that unsaturated or modified C18 fatty acids exhibit higher lytic activity than unsaturated C18 fatty due to less stable polyplex formation [135]. Combining these two findings, a reversibly cationizable tertiary amine, placed in the middle of a longer hydrophobic chain, was considered to enhance stability in blood pH levels and to increase its lytic potential by structural transformation in an acidic pH level upon endocytosis. In the literature, several approaches are presented that use different pH-responsive mechanisms to enhance stability, biocompatibility, and endosomal release, thus increasing transfection efficiency [136, 137]. Therefore, as shown in **Figure 5**, by replacing normal fatty acids with these newly formed lipo amino fatty acids, our library of OAAs was extended in the hope of exploiting the advantages of these double pH-responsive carriers in terms of better transfection efficiency and simultaneously better biocompatibility, *i.e.*, less cytotoxicity [137].

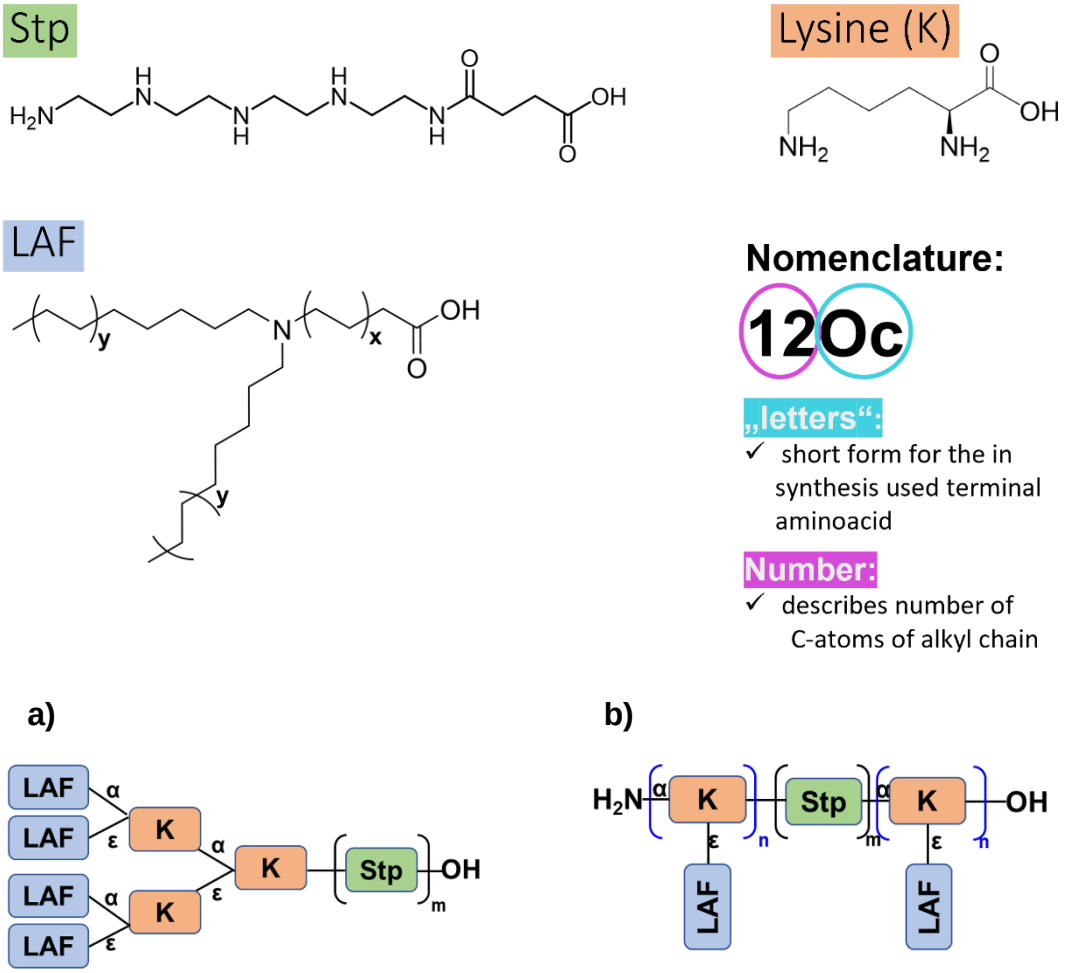

Figure 5: Schematic structure of LAF oligomers. Building blocks: Stp= succinoyl tetraethylene pentamine, K= lysine, LAF= lipo amino fatty acid, and nomenclature of LAF. **a)** Bundle 2 structure. **b)** U-shape 1 structure (other topologies not shown). Figures provided by Melina Grau (PhD student at Pharmaceutical Biotechnology, LMU, Munich).

As depicted in **Figure 6**, better biocompatibility, as well as enhanced endosomal escape, is based on the hypothetical pH-dependent structural transformation of the LAFs leading to stable, non-toxic nanoparticles in neutral pH, as given in the blood. In the endosomal acidic milieu, the LAFs get protonated, which leads to structural transformation, thus losing their ability to form stable polyplexes. Consequently, protonated tertiary amines might interact with the partially negatively charged endosomal membrane

lipids, leading to potential membrane fusion or pore formation [51, 59].

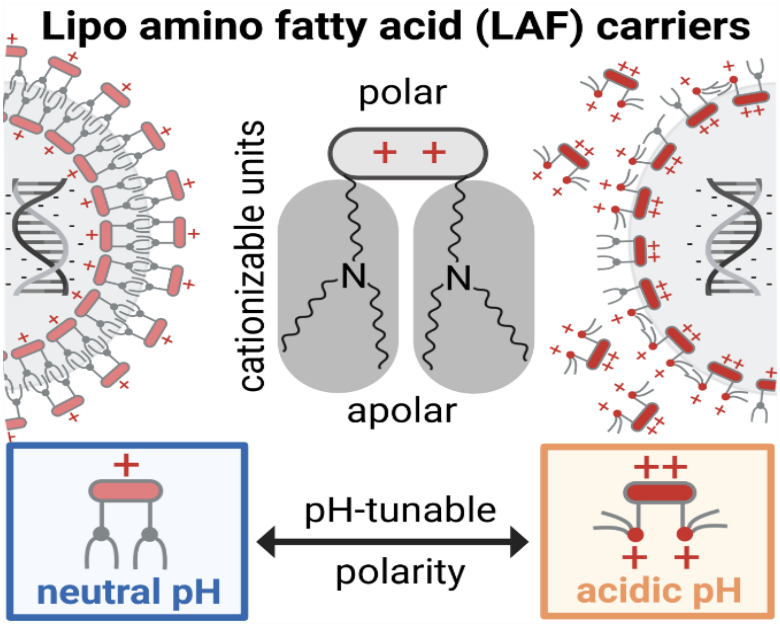

Figure 6: Hypothetical pH-responsive mechanism of the LAF carrier's structural transformation. Upon acidification in endosomes, the tertiary amine gets protonated which might lead to structural transformation of the LAF carrier potentially resulting in disintegration of the lipid bilayer. Figure from Thalmayr *et al.* [138]

In vitro screening for suitable LAF carriers

In a first *in vitro* screening, performed by Sophie Thalmayr (PhD student at Pharmaceutical Biotechnology, LMU, Munich), mRNA polyplexes were evaluated regarding their transfection efficiency after incubation in fetal bovine serum (FBS) or HBG, respectively. This way, potential carriers could be determined for subsequent *in vivo* studies.

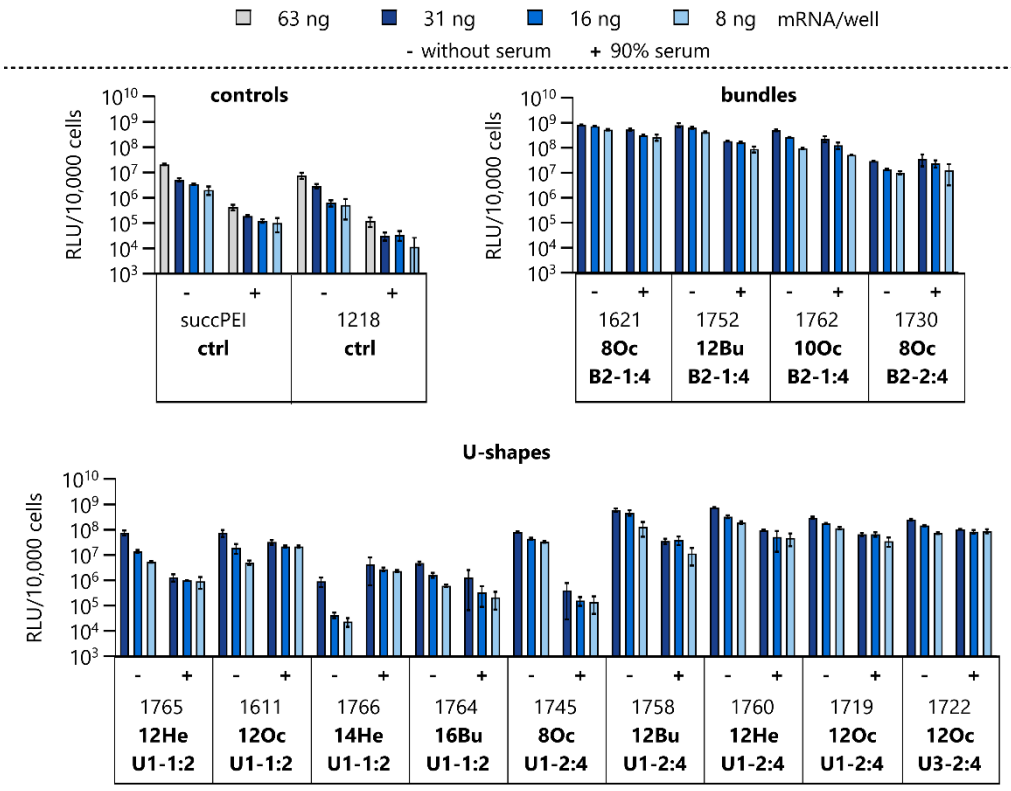

Figure 7: *In vitro* luciferase assay in the presence of FBS. The transfection efficiency of mRNA polyplexes, incubated in 90 % (v/v) FBS for 2 h at 37 °C, at indicated doses on N2a cells in comparison to respective polyplexes diluted in HBG. Luciferase expression was evaluated 24 h after transfection after 1:10-dilution. **1218** N/P 12; succPEI w/w 4; **1745**, **1758**, **1760**, **1719**, **1722** N/P 12; **1621**, **1752**, **1730**, **1765**, **1611**, **1766** N/P 18; **1762, 1764** N/P 24 (n=3; mean ± SD). This experiment was performed by Sophie Thalmayr (PhD student at Pharmaceutical Biotechnology, LMU, Munich). Figure adapted from Thalmayr *et al.* [138]

2.1. Biodistribution of luciferase expression by LAF carriers

Considering the findings of the full serum pre-experiments, the best performers were chosen for the *in vivo* biodistribution study, *i.e.,* **1621** (8Oc–B2–1:4) and **1752** (12Bu–B2–1:4), representatives of the bundle topology and **1611** (12Oc–U1–1:2), **1719** (12Oc–U1–2:4), and **1760** (12He–U1–2:4), carriers of the U-shape topology.

A first *in vivo* screening of these carriers, containing 10 µg of luciferase mRNA, was performed. Therefore, mice were subcutaneously inoculated with 1x 10^6 N2a WT cells, and when tumors reached a size of 250-500 mm^3, divided into groups of n=5. Subsequently, mice were treated intravenously with the respective polyplexes. Twenty-four hours *p.i.*, mice were euthanized and an *ex vivo* luciferase assay was performed (**Figure 8a**). Both, the positive control succPEI and the U-shape topology carriers led to high expression values, especially in tumor, lungs, liver, and spleen. Comparing the U-shape carriers amongst each other, **1611** stood out as it produced overall the highest values and did not show any toxicity, which was contrary to the prediction of the U-shape carriers **1716** and **1760** with an Stp/LAF ratio of 2:4 (**1611**: Stp/LAF ratio of 1:2) were considered to form more stable mRNA polyplexes, which could have been beneficial for *in vivo* application. **1621** and **1752**, on the other hand, showed high toxicity leading to euthanasia of all animals treated with **1621** and two out of three animals of the **1752** group.

Further *in vitro* results indicated fast kinetics of the LAF-carriers, and hence, the second part of the experiment was performed with a read-out time of six hours *p.i.* (**Figure 8c**). Therefore, positive control succPEI and the best performer **1611** were applied in doses of 3 and 10 µg mRNA/mouse. Additionally, **1752**, the less toxic of the bundle structures, was administered in a lower dosage of 1 µg mRNA/animal to combat toxicity. SuccPEI as well as **1611,** showed a dose dependency. However, less pronounced in **1611**. Thus, comparing the low dose to the high one of **1611** and succPEI, only a 15-fold compared to a ~200-fold reduction of luciferase expression in the tumor, an 11-fold compared to a ~130-fold reduction in the lungs, a 45-fold vs. ~70-fold reduction in the spleen and only a 26-fold vs. ~890-fold reduction of RLU values in the kidneys was detected. It is worth

emphasizing that the expression values of **1752** in a dose of 1 µg mRNA were mostly in the same log range as those of **1611** in a dosage of 10 µg mRNA.

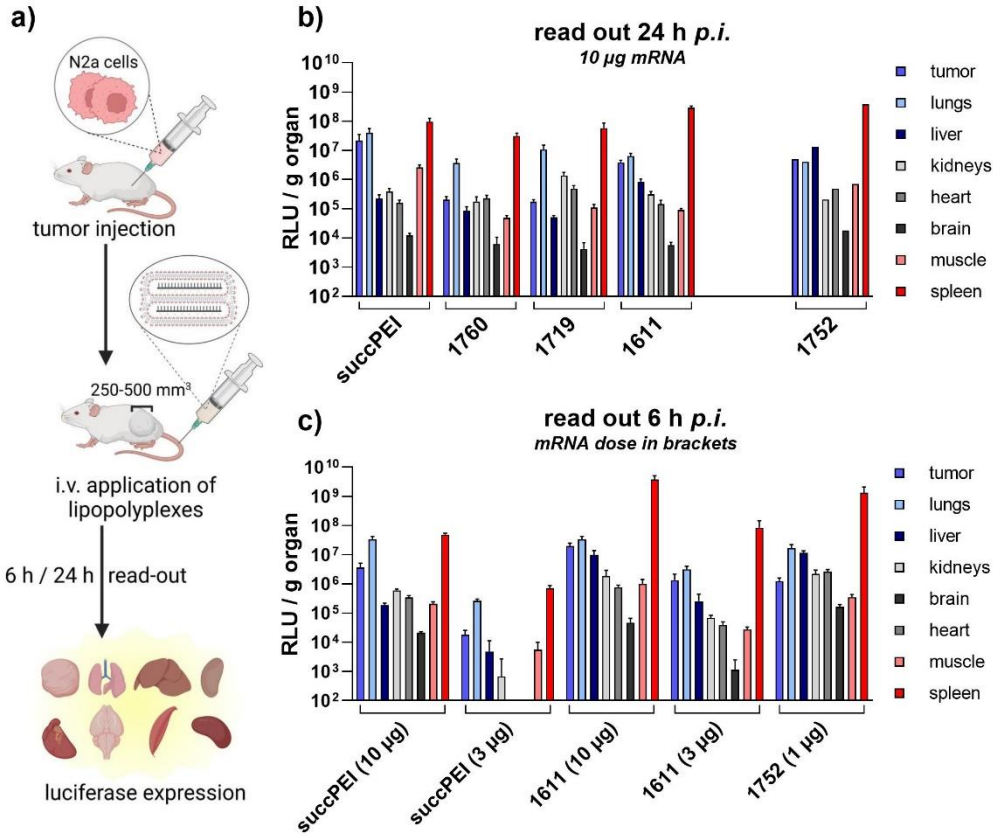

Figure 8: Screening for *in vivo* mRNA polyplexes in an N2a tumor model. a) Schematic representation of the experimental procedure **b)** Screening of succPEI (w/w 2) and **1611**/**1760**/**1719**/**1752** (N/P 18) at a dose of 10 µg mRNA/animal with a read-out 24 h *p.i.* (n=1 for **1752**; n=5 for the rest; mean + S.E.M.). **c)** Screening of succPEI (w/w 2) and **1611**/**1752** (N/P 18) at different doses of mRNA with a read-out 6 h *p.i.* (Weight in brackets refers to a dose of mRNA/animal, n=5; mean + S.E.M.). Figure 8a) was adapted from Sophie Thalmayr *et al.* [138]

Comparing the positive control succPEI to **1611** in a dosage of 10 µg mRNA/animal at six hours and 24 hours *p.i.*, **Figure 9a** demonstrates the fast kinetics of the LAF structures compared to succPEI. Luciferase expression levels of **1611** showed significantly higher values at six hours than 24 hours *p.i.* for some organs, *e.g.,* tumors and lungs. Contrarily,

succPEI showed slightly higher expression values at 24 hours compared to the values six hours *p.i.*. Nevertheless, in the same dosage as succPEI, **1611** could achieve mostly higher expression rates, particularly in the already good values of tumor and spleen.

Figure 9b illustrates the luciferase expression of **1611**, **1752,** and succPEI in their low dose, showing the high efficiency of these new LAF-containing carriers. Compared to succPEI, **1611** displayed higher RLU values in all organs. **1752** which was administered at an even lower dose, also showed significantly higher values compared to succPEI, *inter alia,* for tumor, lungs, and liver.

In summary, the U-shape carrier **1611** proved to be the best performer *in vivo*, showing high efficiency and no toxicity even at a higher dose (10 µg mRNA/animal). The bundle structures generated also high values, in case the of **1752** even higher values at its lower dose, yet it also showed toxicity at a higher dose. **1611**, as well as **1752,** showed particularly high values in the spleen, which as the main immune organ, could be addressed for further studies.

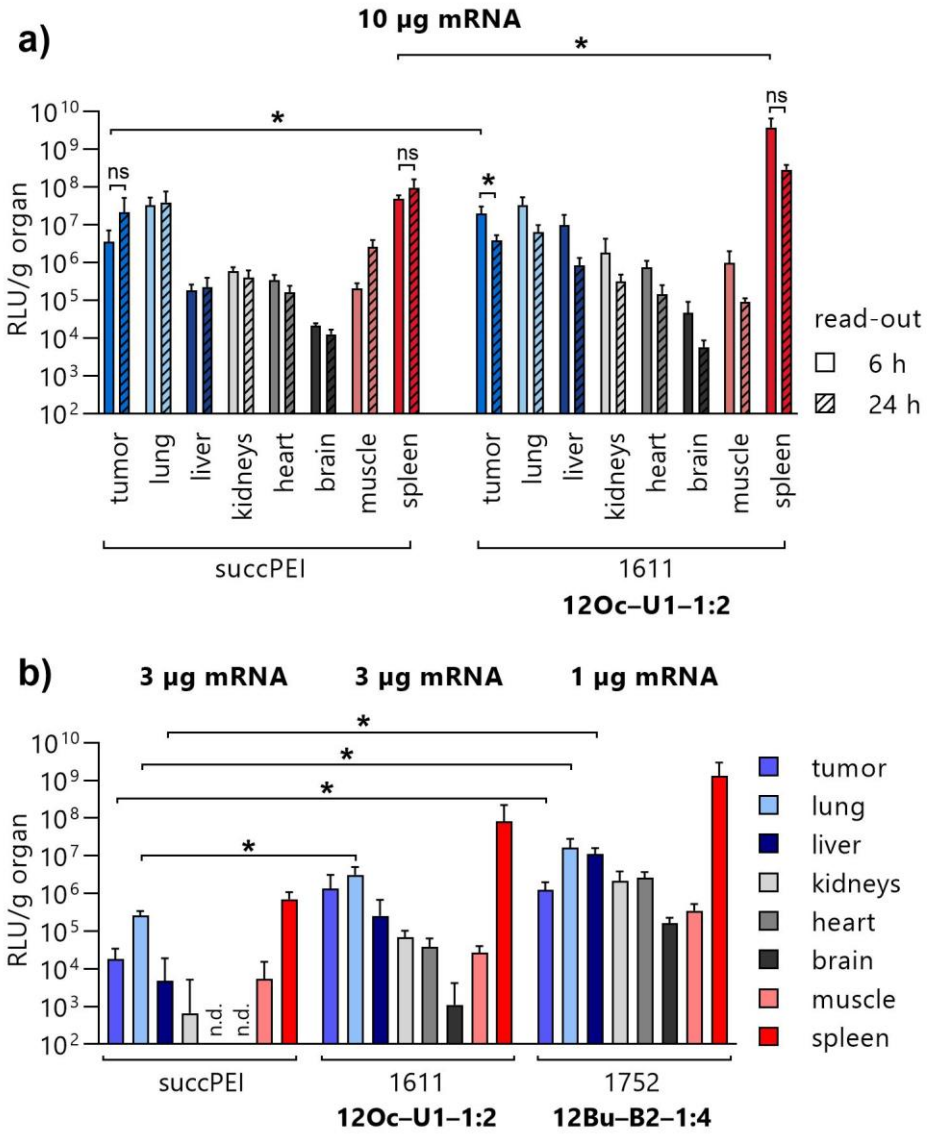

Figure 9: *In vivo* performance of mRNA polyplexes in an N2a tumor model. The performance of intravenously applied mRNA-Luc lipopolyplexes at different mRNA doses was evaluated via an *ex vivo* luciferase assay of the organs of N2a neuroblastoma tumor-bearing A/J mice (*n*=5; mean + SD, ns = not significant, *p ≤ 0.05). **a)** Comparison of succPEI (w/w 2) and **1611** (N/P 18) at two different time points (6 h, 24 h) at a dose of 10 µg mRNA/animal. **b)** Evaluation of succPEI (w/w 2) and **1611** (N/P 18) at a dose of 3 µg mRNA/animal as well as **1752** (N/P 18) at a dose of 1 µg mRNA/animal 6 h *p.i.* (n.d. = not detectable). Figure adapted from Thalmayr *et al.* [138]

2.2. Evaluation of clinical blood parameters

As systemic administration of newly developed formulations might potentially lead to adverse reactions, blood samples were analyzed in order to determine the tolerability of the applied substances. Therefore, A/J mice of the same age and with the same treatment regimen as in the biodistribution experiment were used, except that they were tumor-free, since tumors themselves can lead to aberrant blood parameters.

The best performers **1611** in a dose of 3 µg and **1752** in a dose of 1 µg were injected intravenously. Six hours *p.i.* animals were euthanized and subsequently blood was collected. Plasma samples were analyzed for liver parameters ALT (alanine aminotransferase) and AST (aspartate aminotransferase) and renal parameters creatinine and BUN (blood urea nitrogen). **Figure 10** illustrates the biochemical parameters of the two treated groups compared to an untreated group and a group treated with HBG, which is the formulations solvent. All parameters are in the range of the two control groups, thus indicating good tolerability of the tested LAF-carriers.

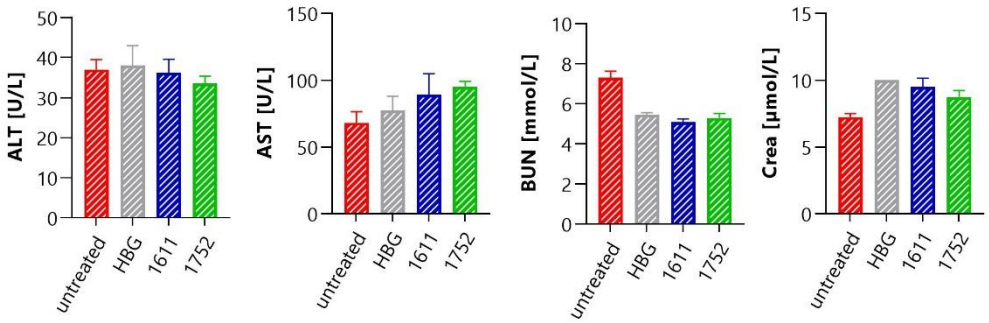

Figure 10: Clinical biochemistry parameters. EDTA-plasma of tumor-free mice injected with the best performers **1611** (N/P= 18; 3 µg mRNA) and **1752** (N/P= 18; 1 µg mRNA) was compared to that of an HBG group and an untreated group. (AST= aspartate aminotransferase, ALT= alanine aminotransferase, BUN= blood urea nitrogen; n=2 for HBG group, n=4 for other groups; mean + S.E.M.)

3. Delivery of Cas9 RNPs for dual immune checkpoint disruption in colon cancer by a folate receptor targeted synthetic carrier

This chapter presents an experimental series that utilizes gene therapy in the form of CRISPR/Cas-facilitated genome editing for anti-cancer therapy.

A giant step in genome editing, and therefore in the development of innovative gene therapies, was made when clustered regularly interspaced short palindromic repeats (CRISPR/CRISPR-associated (CAS) systems), an evolved adaptive immune defense of bacteria and archaea to degrade foreign nucleic acids, has been engineered to function as a specific genomic loci-targeting genome editing tool in eukaryotic cells [139, 140]. This discovery led to several new approaches in the development of therapies against genetic diseases [141-144] and the first trials against cancer [145].

In the latter, mostly cancer immunotherapy is exerted and realized, *e.g.,* by chimeric antigen receptor T (CAR T) cell therapy [146], disruption of endogenous inhibitory immune checkpoints on T cells [147, 148], both *ex* vivo techniques, or by monoclonal antibodies against those immune checkpoints *in vivo*, such as *e.g.* the programmed cell death protein 1 pathway (PD-1/PD-L1) [149-151]. The second immune checkpoint of interest, the Poliovirus receptor (PVR/CD155), a representative of the nectin-like molecules that play an important role in cells adhesion, movement, contact inhibition, proliferation [152], and immune response [153, 154], has recently been focused on for immune checkpoint inhibitor based anti-tumor therapy [155, 156]. Interestingly, it was shown by Zheng *et al.* that even the knockdown of CD155 in colon cancer cells on its own resulted in significantly increased cancer cell apoptosis [157]. In sum, immune checkpoints are regulatory pathways in cells, consisting of corresponding integral membrane proteins on normal and on immune cells, maintaining self-tolerance (*i.e.,* prevention of autoimmunity) and modulating immune responses of immune cells to protect healthy tissue from collateral damage when antigens are attacked [158, 159]. Both PVR and PD-L1 are overexpressed on several types of tumor cells [154, 160-162], and thus induce immune evasion by, among others, inhibitory mechanisms of PD-

1/PD-L1 through apoptosis of activated tumor-reactive T cells [163] or PVR/TIGIT which inhibits T cell activation and NK cell mediated killing of tumor cells, respectively [155, 164]. In contrast to the well-established immunotherapy via antibodies inhibiting immune checkpoints, knockout by Cas9 systems could disrupt them permanently [165], and therefore immune checkpoint knockout *in* vivo could be an auspicious long lasting therapeutic method.

To date, there are three different options for CRISPR/Cas delivery, of which each has its benefits and limitations: pDNA is relatively stable against exonucleases, but on the other hand, has to be transported into the nucleus for transcription. Messenger RNA can be translated directly and thus leads to faster kinetics of protein synthesis within a few hours, yet it is prone to degradation by nucleases. And lastly, when a suitable delivery system is available, fully built proteins which provide instantaneous genome editing function [166]. To conduct this experimental series, **1445** (**Figure 11**) was chosen to deliver Cas9 RNPs, a sequence-defined hydroxystearic acid containing oligo(ethylenamino) amide (OAA) [167], and derivative of **1105**, which had proven its ability to efficiently deliver RNPs [168].

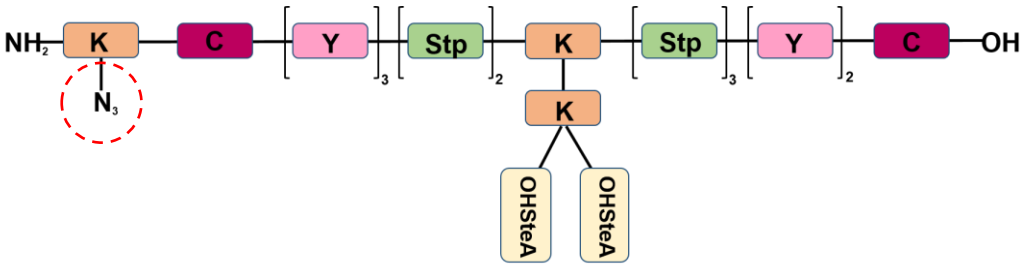

Figure 11: Schematic structure of the T-shape oligomer 1445 (K= lysine, C= Cysteine, Y= Tyrosine, Stp= succinoyl tetraethylene pentamine, OHSteA= hydroxystearate, encircled in red: azido group for post functionalization). Figure provided by Melina Grau (PhD students at Pharmaceutical Biotechnology, LMU, Munich).

To increase transfection efficiency and limit side effects as well as toxicity, targeting ligands, *e.g.,* receptor ligands, are used to enhance receptor-mediated intake of nanocarriers [169, 170]. As folate receptor α is highly expressed in colorectal cancers, folate is a popular targeting ligand for

fluorescent intra-operative imaging, targeted PET/MRI, and selective cytotoxic drug delivery [171, 172].

In this work, we present a folate receptor α targeted, non-viral vector-transduced, *in vivo* dual immune checkpoint disruption of PD-L1 and PVR in a CT26 tumor mouse model.

Molecular confirmation of gene knockout *in vitro*

Firstly, dual PD-L1/PVR disruption was evaluated *in vitro* in CT26 WT cells by Yi Lin (former PhD student at Pharmaceutical Biotechnology, LMU, Munich). Therefore, CT26 cells were treated with an RNP dose of 75 nM encapsulated into the differently modified **1445** polyplexes at an equimolar ratio, containing Cas9/sgPD-L1 and Cas9/sgPVR. 48 hours after a four-hour incubation, the cells' viability was determined via MTT assay. Notably, the FRα-targeted and PEG_{24}-shielded nanocarrier diminished cell viability to 23 %, whereas the unmodified carrier could reduce cell viability only to about 40 % and the PEG_{24} shielded to about 60 %, emphasizing the high efficacy of the targeted nanocarrier (**Figure 12a)**. KO efficiency of PD-L1 and PVR was evaluated by flow cytometry and confirmed by TIDE analysis (data not shown). The former showed in the FolA-PEG_{24}-**1445** group a combined KO efficiency of ~59 %, of which a good 54 % consists of a dual KO, ~2 %, and ~3 % of single KO of PD-L1, and PVR, respectively. The unmodified nanocarrier achieved only a combined KO level of ~38 %: ~30 % dual KO, ~2 % of PD-L1 KO and ~6 % of PVR KO. Even less efficient, the PEG_{24}-shielded polyplex induced editing events in only 28 % of the cancer cells (**Figure 12b**).

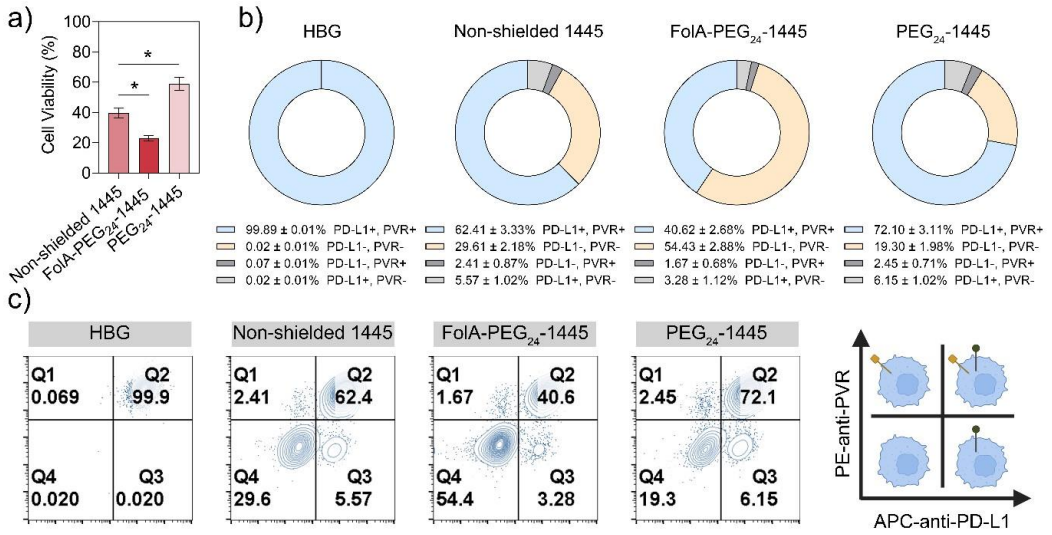

Figure 12: Dual PD-L1/PVR disruption *in vitro*. a) Cell viability and **b)** dual PD-L1/PVR knockout efficiency in CT26 WT cells treated with HBG or nanocarriers containing 37.5 nM Cas9/sgPD-L1 and 37.5 nM Cas9/sgPVR RNP (0.75 eq modification). Cas9 RNP nanocarriers were incubated with the cells for 4 h followed by medium change. Respectively, MTT assay and flow cytometry were performed 48 h and 72 h after the treatment. (*$p \leq 0.05$; mean ± SD; n = 3). **c)** Flow cytometry scatter plots showing the PD-L1 and PVR expression of CT26 WT cells after 4 h of treatment with HBG buffer, or nanocarriers (37.5 nM Cas9/sgPD-L1 and 37.5 nM Cas9/sgPVR RNP, 0.75 eq modification). This experiment was performed by Yi Lin (former PhD student at Pharmaceutical Biotechnology, LMU, Munich). Figure adapted from Yi Lin *et al.* [173]

3.1. Pre-experiment: i.v. biodistribution of 1445 structures

The prerequisite for KO in cancer cells *in vivo* is to reach those cells with the therapeutical agent, in this case Cas9 ribonucleoproteins. Therefore, an intravenous biodistribution of the lipo-oligoaminoamide (lipo-OAA) **1445,** an azido-containing analog of the potent structure for intracellular delivery of Cas9 RNPs, **1105** [168], was performed. Shielding and tumor targeting was enabled by post-functionalization with DBCO reagents, namely PEG_{24}-DBCO and FolA-PEG_{24}-DBCO, via strain-promoted azide-alkyne cycloaddition (SPAAC) [173].

Figure 13 depicts the biodistribution of ATTO740-labeled Cas9 RNP in naked form, along with **1445,** respectively PEG_{24}- and FolA-PEG_{24}-modified **1445**. In comparison to the **1445** encapsulated RNP, the naked RNP showed a faster accumulation in the liver. Nevertheless, together with the unmodified **1445** group, it also had a faster clearance than the PEG_{24} shielded groups.

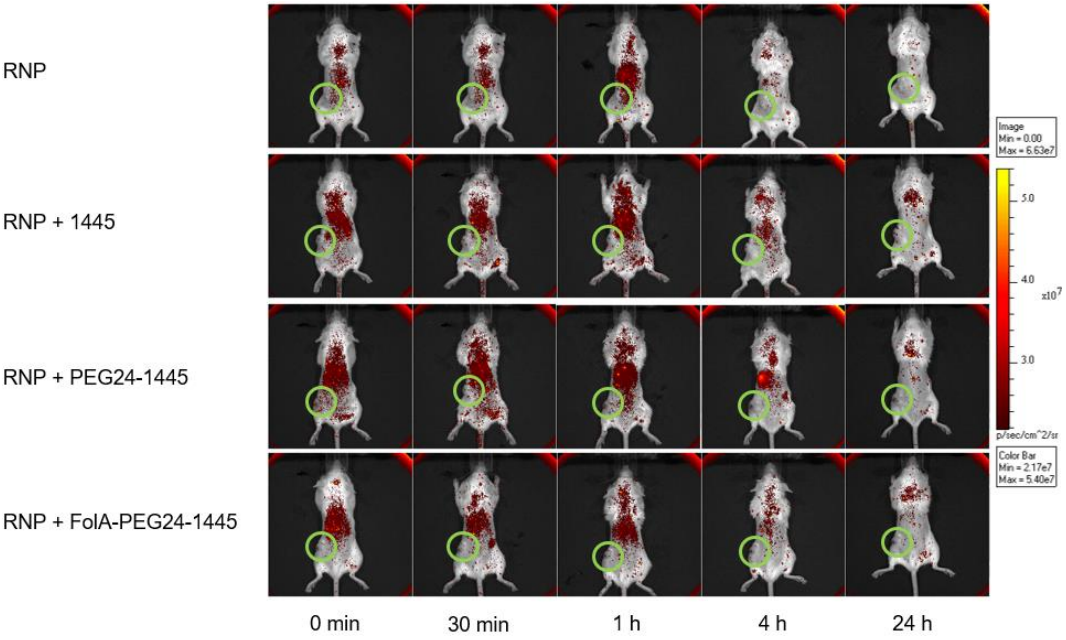

Figure 13: Biodistribution of 1445 in CT26 tumor bearing mice. 'Naked' RNPs as negative control (1.row), RNPs with unmodified **1445** (2.row), PEG_{24}-modified **1445** (3.row) or FolA-PEG_{24}-modified **1445** were injected intravenously and subsequently NIR fluorescence imaging was performed at different time points throughout 24 h. All injections contained 125 µg ATTO740-labeled Cas9 + 25 µg sgRNA. The color scale (efficiency) with a minimum of 2.2 e^{-7} and a maximum of 5.4 e^{-7} fluorescent photons/incident excitation photon. Tumors are highlighted by light green circles.

In conclusion, the PEG_{24} shielding prolonged circulation time in both modified groups. However, an accumulation in the tumor was not achieved. Thus, an intratumoral treatment was chosen for the following experiments.

3.2. Molecular confirmation of gene knockout *in vivo*

In view of the findings of the biodistribution and encouraged by the good *in vitro* results, the capability of dual KO of PD-L1 and PVR of intratumorally applied RNP nanocarriers *in vivo* was assessed. Therefore, 5 x 10^5 CT26 WT cells were inoculated subcutaneously into the left flank of BALB/c mice, and subsequently the animals were divided into five groups. On day three *p.i.* the mice got injected intratumorally or subcutaneously in the respective skin area, with RNPs containing 25 µg of sgRNA (25 µg of sgCtrl, or 12,5 µg of sgPD-L1 and sgPVR) encapsulated in **1445**, respectively its derivatives FolA-PEG_{24}-**1445** and PEG_{24}-**1445**, for three times in total. Mice were euthanized on day 16, seven days after the last injection, and single cell suspensions of the tumor tissue were prepared, and finally analyzed in a flow cytometer by Yi Lin (former PhD student at Pharmaceutical Biotechnology, LMU, Munich).

As **Figure 14** depicts, the KO efficiency of the FolA-PEG_{24}-modified **1445** polyplex was roughly 40 %, of which ~25 % comprises a dual KO, ~8 % PD-L1, and ~6 % PVR KO. In comparison, the unmodified polyplex reached a combined KO level of ~24 %, only around two thirds of the efficiency of the FRα-targeted carrier, emphasizing the targeting benefits. PEG_{24} shielded **1445**, in contrast to the other two groups, had very low efficiency, in fact only ~4 % of the cells showed any KO (**Figure 14b**). The second analysis, also performed by Yi Lin (former PhD student at Pharmaceutical Biotechnology, LMU, Munich), evaluated whether a dual immune checkpoint KO leads to an infiltration of cytotoxic T cells into the tumor tissue, which would indicate stimulation of the immune system, the essential part of combating cancer and the underlying idea of immune checkpoint therapy [174]. Supporting this concept, the data confirm a significantly increased infiltration of cytotoxic T cells into the tumor, in correlation with KO efficiency (**Figure 13c**). The more KO took place, the more CD8+ T cells infiltrated the tumors microenvironment.

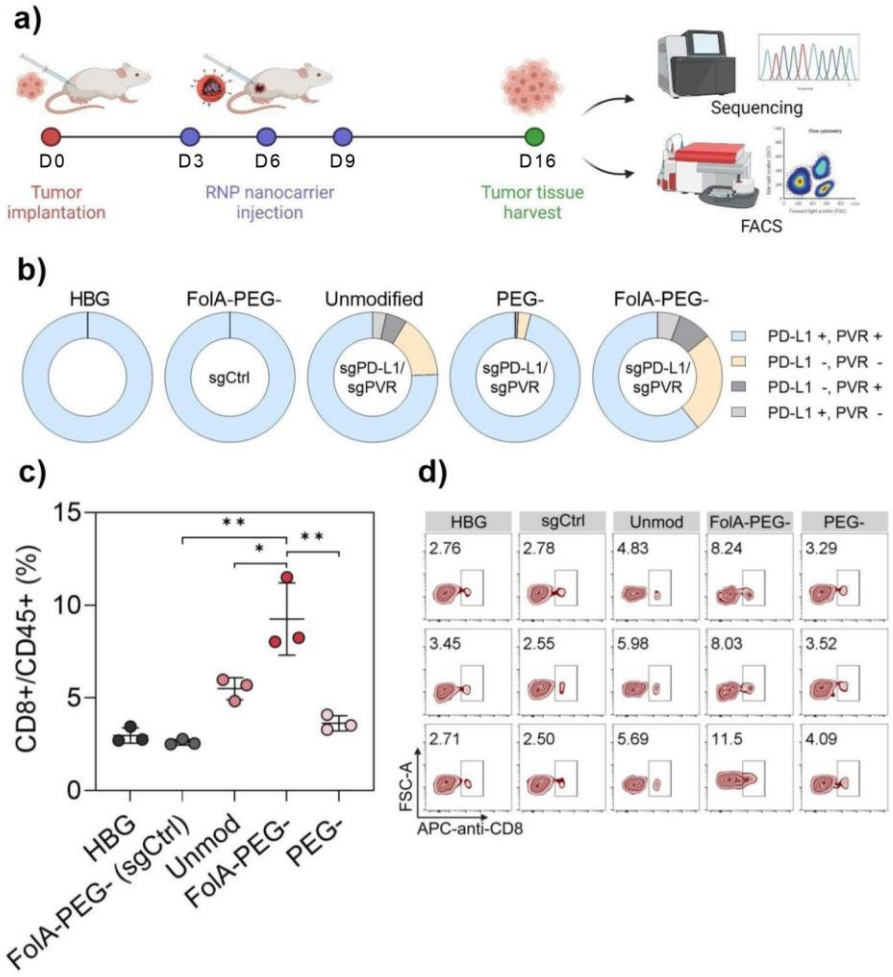

Figure 14: Flow cytometry analysis of tumor tissue. Dual PD-L1/PVR knockout efficiency in CT26 WT tumors. **a)** Mice treated i.t. with either HBG or **1445** polyplexes containing 25 µg of sgRNA (25 µg of sgCtrl, or 12,5 µg of sgPD-L1 and sgPVR) trice, on days 3, 6 and 9. (n=3, mean + SD, *p ≤ 0.05, **p < 0.01). **b)** KO efficiency in dissected tumor tissue. **c)** CD8+/CD45+ ratio for the determination of CD8+ T cell infiltration of the tumors. **d)** Flow cytometry scatter plots for quantification of CD8+ T cells. Figure adapted from Yi Lin *et al.* [173]

Despite the good KO levels, there was no macroscopically notable regression of tumors in the FolA-PEG$_{24}$-modified nanocarrier group, nevertheless the slight swelling of tumor sites in this group on days four to six indicated an immune response by the host (**Figure 15a**). The similar

weight development (**Figure 15b**) of mice proved a good tolerability of the Cas9 RNP carrier formulations.

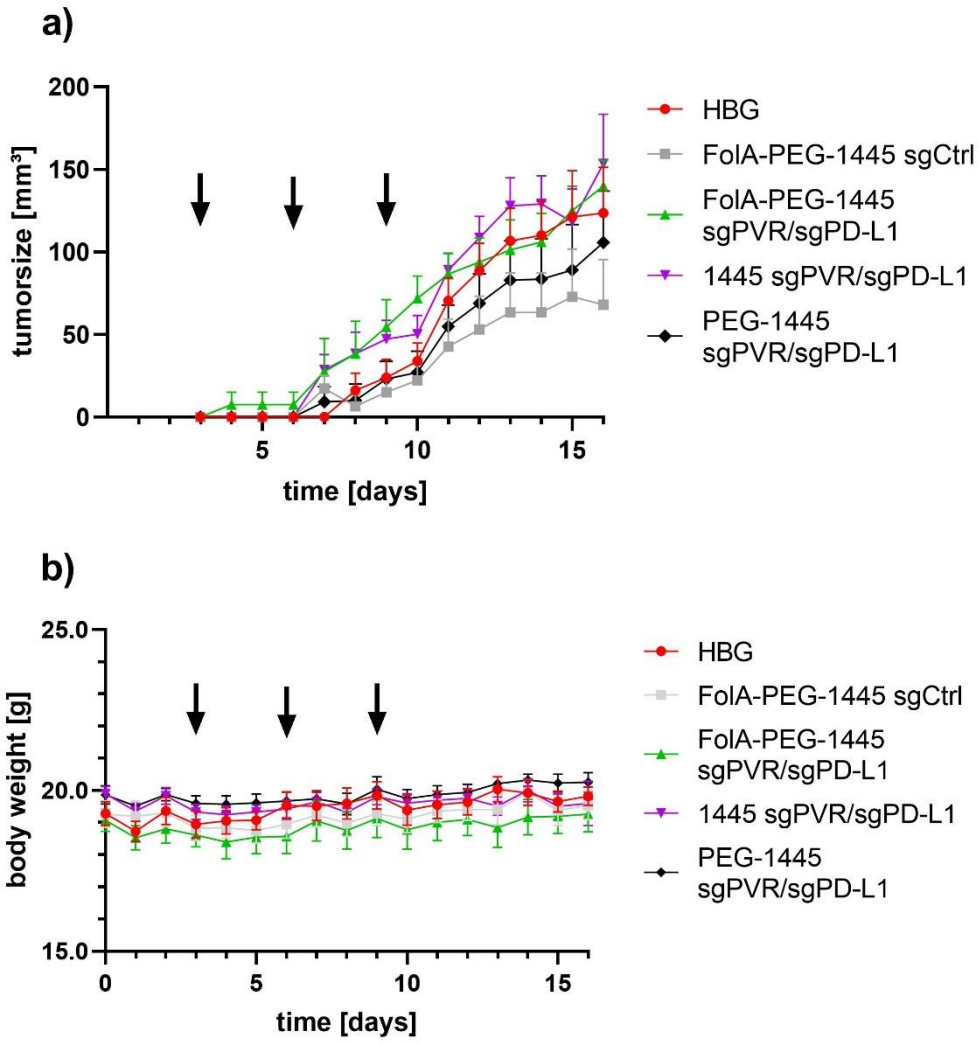

Figure 15: Tumor growth and weight development. CT26 tumor-bearing mice treated i.t. with 50 µL of either HBG, FolA-PEG$_{24}$-**1445** with sgCtrl or differently modified **1445** with sgPD-L1 + sgPVR on days 3, 6 and 9 (indicated by black arrows). The animals were euthanized on day 16 for flow cytometry analysis of PD-L1/PVR KO. Day 0 represents the day of tumor cell inoculation (n=6, mean + S.E.M.). **a)** Tumor growth of the subcutaneous CT26 tumors. **b)** Body weight of mice throughout the experiment.

3.3. Therapeutic study

To evaluate the before mentioned immune response, a therapeutic study was implemented. Therefore, BALB/c mice were inoculated with CT26 WT cells and subsequently divided into five groups. Considering the findings of **3.2.** (Molecular confirmation of gene knockout *in vivo*), experimental setup of the therapeutic studies was adapted accordingly. Instead of 0.5 million, this time 0.1 million cells were inoculated which served two purposes: firstly, tumor growth is slower and thus the treatment time in which tumor sizes are below 12 mm in diameter is longer, and secondly, the i.t., respectively s.c. treatment reaches a higher percentage of injected cancer cells. Additionally, a higher number of treatments, six injections in total, were performed. Starting on day four *p.i.*, mice got i.t. treated with FolA-PEG_{24}-**1445** RNP polyplexes, containing 25 µg of sgRNA (sgCtrl, or sgCtrl with sgPD-L1 or sgPVR, or sgPD-L1 and sgPVR in a ratio of 1:1). With the exception of the dual treatment group, i.e., sgPD-L1/sgPVR, tumors were measurable beginning with day eight, and all of them showed very similar growth characteristics. Hence, none of these mentioned groups showed any tumor growth inhibition which led to the fact that by day 21, all groups already had at least one animal that had to be euthanized due to tumor size. On the other hand, the dual treatment group showed strongly retarded tumor growth, with tumors starting to be measurable on day 14 (**Figure 16b**). On day 20, the last day all animals were alive, comparison of the mean tumor sizes showed a significant difference. Thus, the dual treatment group showed a 2.9-, 3.7-, 5.0- and 5.4-fold smaller tumor size than the groups sgCtrl, sgPD-L1/sgCtrl, sgPVR/sgCtrl, and the untreated group, respectively (**Figure 16d**).

The constant weight development, depicted in **Figure 16c**, demonstrated the good tolerability of the applied formulations. The descending end of the curves clearly showed severe affected well-being of the mice caused by tumor-induced cachexia, and therefore mice were euthanized at this stage even if they had not necessarily reached a tumor size of ≥ 12 mm in diameter.

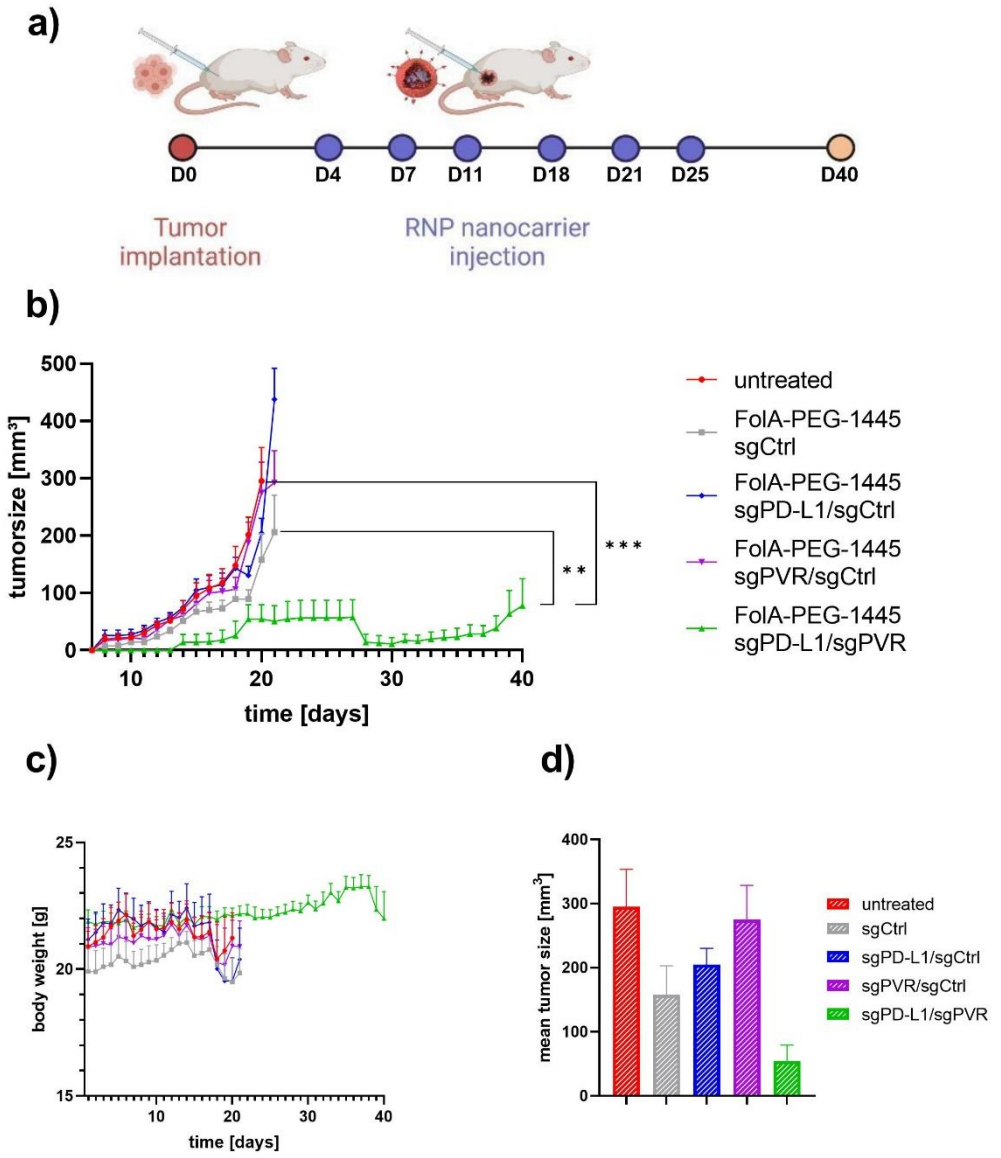

Figure 16: Tumor growth and weight development. Intratumoral application of 50 µL of different Cas9 RNP carriers in CT26 syngeneic tumor model on days 4, 7, 11, 18, 21 and 25. Day 0 represents the day of tumor cell inoculation. Data shown until first animal of the respective group was euthanized (n=5 for sgPD-L1/sgPVR, n=6 for other groups; mean + S.E.M., **p < 0.01, ***p < 0.001). **a)** Treatment schedule. **b)** Tumor growth of subcutaneous CT26 tumors. **c)** weight development of the animals during the study. **d)** comparison of the mean tumor size on day 20 after tumor cell inoculation. Figure a) adapted from Yi Lin *et al.* [173]

Figure 17 demonstrates the prolonged survival time of the dual treatment

group. Notably, on day 31, when in all other groups, at least five out of six animals had been euthanized, all animals of the dual treatment group were still alive. Even on day 43, when also the remaining mice of the other groups had been euthanized, still four out of five animals of the sgPD-L1/sgPVR group were alive (**Figure 17a**). Also, the mean survival time of mice depicts a clear picture of a significantly prolonged survival time of the dual treatment group of almost double the survival time compared to all other groups (**Figure 17b**).

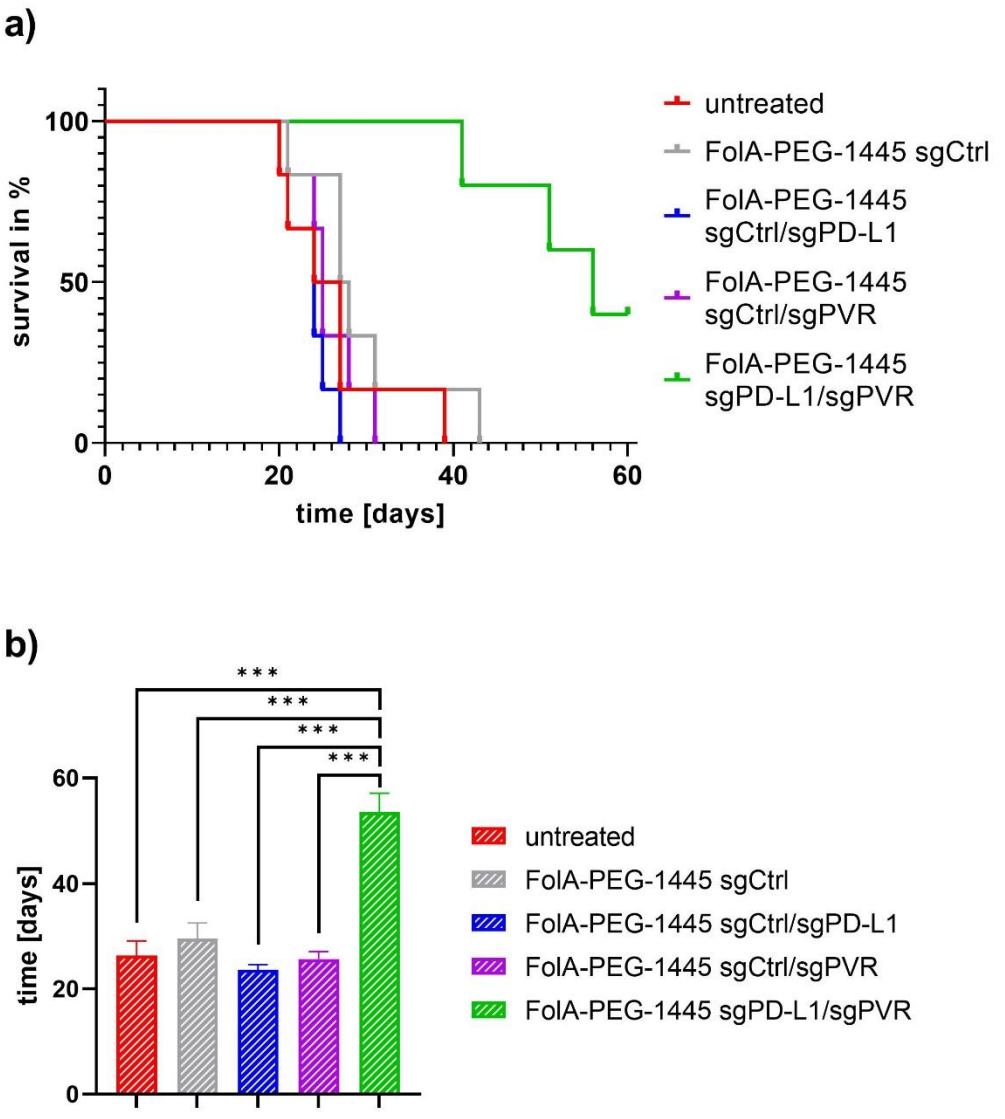

Figure 17: Survival of CT26 tumor-bearing mice treated i.t. with 50 µL of FolA-PEG$_{24}$-**1445** with different sgRNAs on days 4, 7, 11, 18, 21, and 25. Day 0 represents the day of tumor cell inoculation. (n=5 for sgPD-L1/sgPVR, n=6 for other groups) **a)** Kaplan-Meier survival analysis. **b)** Mean survival time (values included till day 60, mean + S.E.M., ***p < 0.001)

In sum, a highly capable, FRα-targeted, and PEG_{24}-shielded Cas9 RNP nanocarrier has been developed, which did not only induce efficient disruption of immune checkpoints PD-L1 and PVR *in vitro* but also *in vivo*. Analysis of harvested tumors showed a dual gene knockout of ~25 % and a significant infiltration of CD8+ T cells into the tumor microenvironment. The therapeutic study demonstrated that this led to effective inhibition of tumor growth and therefore, prolonged survival time of mice.

IV. DISCUSSION

Since its first clinical trial in humans, gene therapy has been considered the most promising way of treating otherwise untreatable diseases. At the same time, it also bears several risks, such as off targeting effects, severe adverse immune response, or insertional mutagenesis, that already led to cancer formation and, in some cases, even death [7, 26, 79]. Nevertheless, the prospect of possible treatments for, *inter alia,* cancer, other genetic disorders or severe infectious diseases outweighs the possible risks, especially for patients who already suffer from deadly diseases.

However, poor pharmacokinetics of therapeutic NAs and proteins makes it necessary to implement delivery systems to shield the therapeutics from degradation by nucleases or proteases, respectively, and to enable internalization and subsequent endosomal escape in the target cells [19, 20, 40, 56, 62]. The two dominant delivery systems to date can be divided into viral and non-viral, of which viral vectors are more efficient in delivery but also entail disadvantages, namely immunogenicity and the risk of insertional mutagenesis [38, 79]. Therefore, non-viral vectors are considered the safer and better alternative in gene delivery [175]. Unfortunately, a lack of efficiency in these delivery systems still constitutes the main reason for often slow progress in drug development [176, 177]. Thus, among others, our working group investigates potent, safe, and efficient vectors, generating a library of over 1800 sequence-defined oligoaminoamides suitable as carriers for NAs and proteins.

1.1. Transcriptional targeting of pDNA to dendritic cells *in vivo*

In order to successfully and perseveratively treat patients via immunotherapy, it is important to effectively activate a patient's immune system. In view of the unique ability of DCs to activate naïve T cells and their important role in regulation of immune response or immunological tolerance, respectively [131, 132], DCs are considered interesting primary targets in immunotherapy [126]. The aim was to specifically induce protein expression only in DCs. Therefore, a newly developed Fascin promoter was

utilized that drives translation of the used Luc pDNA.

1.1.1. Biodistribution of pCMVLuc pDNA and pFscnLuc pDNA expression with succPEI and LPEI

In fact, pDNA driven by the newly developed Fascin promoter could show an overall higher specificity for the spleen. With LPEI as delivering carrier, pFscnLuc induced a similar expression value in the spleen, but lower values for the liver and lungs, compared to the strong and ubiquitously expressed CMV-promoter-driven pCMVLuc. On the other hand, with succPEI, pFscnLuc showed similar lung and liver values but a higher expression in the spleen, identifying succPEI as the most suitable carrier with a higher ratio of expression in the spleen.

1.1.2. Comparison of pCMVLuc pDNA and pFscnLuc pDNA expression on a cellular level

As an *ex vivo* luciferase assay is only capable of determining in which organ the protein is expressed in but not in what cell types, a subsequent experiment was performed to detect cell-specific expression via immunohistochemical staining of single cell suspensions. The Fascin promoter mediated good luciferase expression in DCs, but so did the CMV promoter, which is a widely used and strong promoter that has binding sites to ubiquitously expressed transcriptional factors [134]. Nevertheless, the benefit of the Fascin promoter is its specificity for DCs. The ratio of luciferase expressing DCs to MACs (1.5- to 4-fold enhanced intensity) in relation to their respective negative controls also showed a non-statistical advantage of the Fascin promoter over the CMV promoter in DCs. Thus, the concept of transcriptional targeting DCs could be confirmed *in vivo*.

1.2. Novel dynamic lipopolyplexes containing lipo amino fatty acids (LAF) for potent mRNA delivery *in vivo*

To date, although non-viral vectors represent the safer and therefore

preferred alternative, they still lack transfection efficiency tremendously compared to viral vectors [35, 175]. For the most part, this is due to insufficient endosomal escape leading to degradation of the NAs and proteins in the late endosomes and lysosomes [52].

Many working groups exploited the fact that not only the extracellular compartment differs a lot from the intracellular compartment but also the organelles within the cell in pH level, redox potential, enzyme activity, reactive oxygen species, adenosine triphosphate (ATP) levels or salt concentrations [47, 136, 178]. These findings led to the development of “smart” or bioresponsive carriers that are equipped with, *e.g.,* pH-labile masking of endosomolytic agents [179] or redox-sensitive disulfide bonds [180], which utilize the before mentioned differences to enable targeted delivery, better biocompatibility or enhanced transfection efficiency. Also, artificial alterations can be exploited, as Takeda *et al.* and Kurisawa *et al.* reported in exploratory studies a temperature-responsive carrier that, upon cooling to 20 °C, enhanced transfection efficiency [181, 182]. Taking advantage of the pH drop in endosomes, we developed a lipidic domain that, coupled with existing carriers, utilizes the acidic milieu in endosomes to augment the lytic potential of the polyplex. *In vitro* studies demonstrated excellent transfection efficiency and good stability in FBS for some of the carriers, identifying those as suitable carriers for *in vivo* studies.

1.2.1. Biodistribution of luciferase expression by LAF carriers

Compared to our old gold standard for mRNA polyplexes, *i.e.,* succPEI [183], screening these new LAF carriers proved a superior transfection efficiency of the LAF carriers, especially for lower doses. Best performers **1611** (U-shape) in a dose of 3 µg mRNA and **1752** (bundle) in a dose of 1 µg mRNA elicited similar or even higher luciferase values than succPEI in a dose of 10 µg mRNA. Furthermore, screening revealed a much lower dose dependency for these LAF structures compared to succPEI, as well as faster kinetics, indicated by the LAF’s higher luciferase values with a read-out time of six hours than the values with a read-out time of 24 hours *p.i.*. On the other hand, the bundle structures **1621** but also **1752** particularly

generated severe toxicity at higher dose. The reasons for this are not fully understood yet. Two main obvious causes are: *i)* instability of lipopolyplexes *in vivo*, resulting in aggregation and blockade of blood capillaries; *ii)* direct cytotoxicity on transfected cells by lytic activity (similar as in cell culture). The first cause was previously documented for LPEI polyplexes, which are not lytic at all but block lung capillaries after tail vein administration, resulting in severe toxicity [184]. The second case might be relevant for highly potent lytic structures such as bundle **1621**. However, other structures with higher lytic potential did not generate toxicity to that extent. For bundles **1621** and **1752** a combination of both effects may account for the observed toxicity. Indeed, the bundle structures show less stability compared to the U-shape structures [138]. Therefore, *(i)* aggregation and accumulation in critical vasculature/tissues plus *(ii)* higher lytic effects of the locally aggregated and accumulated lipopolyplexes may damage cell membranes and tissues.

Summarizing the results, our working group developed a new lipidic domain, that, when coupled with carriers from our existing library, not only showed outstanding transfection efficiency *in vitro* but also exceeded *in vivo* values of succPEI, which was considered the gold standard for mRNA polyplexes. Moreover, the explicitly high values for the spleen, which is one of the most relevant organs of the immune system, could indicate a potential utilization, for example, in immunotherapy. Meanwhile, ongoing studies in our working group reveal the high potential of these LAF carriers to not only deliver mRNA, but also other cargos, such as pDNA and siRNA (data not shown).

1.2.2. Evaluation of clinical blood parameters

As systemically applied substances always bear the risk of severe adverse effects due to activation of the immune system or cytotoxicity based on various other mechanisms, an analysis of plasma was performed. **1611** and **1752**, both best performers, in their low dose, show similar values for the liver parameters AST and ALT compared to the control groups. Variances of the BUN and creatinine values of the treated groups compared to the untreated group could be explained by expected changes in metabolism due to the injected glucose and the injection itself. In the case of BUN, HBG,

the solvent of these formulations, could be responsible by providing enough high-energy glucose, thus reducing the protein metabolism and, consequently, urea synthesis.

After all, all biochemical blood parameters of the **1611** and **1752** treated groups are in the range of the two control groups, indicating a good tolerability and no acute liver or kidney toxicity. The above-mentioned acute toxicity of **1752** at the higher dose, could not be detected in animal behavior or blood parameters at the lower dose, thus, reason for toxicity stays unclear, as discussed above (**1.2.1.**).

1.3. Delivery of Cas9 RNPs for dual immune checkpoint disruption in colon cancer by a folate receptor targeted synthetic carrier

There are several antibodies for anti-cancer immunotherapy on the market and in clinical trials aiming at the PD-1/PD-L1 pathway with promising therapeutic effects, yet also severe immune-related adverse effects, typical for antibody-based immunotherapies, were observed [185]. Although, in some patients, powerful and durable effects could be witnessed, the percentage of patients responding to a PD-1/PD-L1 monotherapy can be rather small due to innate and acquired resistance [186].

To realize a continuous therapeutic effect as compared to the transient effects of antibodies, we aimed at a constant immune checkpoint disruption by Cas9 genome editing. Additionally, to overcome resistance, we implemented a combined KO therapy of the PD-1/PD-L1 and the PVR/TIGIT pathway. As a delivery system, we chose **1445**, an azido-derivative of **1105** which is a T-shape lipo-oligoaminoamide with two hydroxystearic acid molecules that has already been proven to be a suitable RNP carrier by our working group [168]. This azido-group enabled post-functionalization, which we utilized for adding shielding and targeting reagents. Pre-studies performed *in vitro*, showed proof of concept that our chosen carrier **1445** not only demonstrated intracellular delivery but also capability to facilitate endosomal escape and subsequent genome editing. Also, we could reveal that FRα-targeting enhanced transfection efficiency *in vitro* to ~59 % of cells

compared to ~38 % in the unmodified group and 28 % in the PEG_{24}-shielded group.

1.3.1. Pre-experiment: i.v. biodistribution of 1445 structures

Due to frequent discrepancies between *in vitro* to *in vivo* results [187], we applied the same carriers to a CT26-tumor bearing mouse model with ATTO740-labeled Cas9 RNPs for a live imaging biodistribution assay. In line with the literature [188, 189], PEG_{24} modification showed longer circulation time, and slower clearance. Yet, it did not show visible accumulation in the tumor. The reason might be: poor vascularization of the tumor, macroscopically indicated by a pale, connective tissue rich dissected tumor, which is, however, contrary to findings from Seguin *et al.*, detecting high vascularization in CT26 tumors [190].

Regardless of the underlying cause, further modifications of this nanocarrier must be taken into account for future systemic administration of **1445** *in vivo*.

1.3.2. Molecular confirmation of gene knockout *in vivo*

Considering the findings in the biodistribution experiment, the following *in vivo* experiments were performed with intratumoral injections. Starting on day three after tumor cell inoculation, three consecutive injections were administered, since by a singular intratumoral injection of 50 µL, not all cells are considered to be reached, respectively, transfected by the carrier. On day 16, seven days after the last injection, the mice were euthanized and subsequently, the tumors were dissected, processed to single cell suspensions, and analyzed for gene KO. Similar to *in vitro* results, *in vivo* results demonstrated a clear predominance of the FolA-PEG_{24}-**1445** compared to the unmodified and the PEG_{24}-shielded **1445**, illustrating the targeting and shielding benefits for polyplexes *in vivo*, exploiting the fact of tumors overexpressing various membrane proteins and receptors.

Furthermore, by flow cytometric analysis of CD8+ T cells, we could show that infiltration of CD8+ T cells into tumor tissue was significantly higher with

the targeted and shielded polyplex than in the other two groups. As Li *et. al.* stated in their review, the infiltration of CD8+ T cells is significantly associated with a better outcome for patients treated with immune checkpoint inhibitors [191]. Therefore, detected CD8+ T cell infiltration in the FolA-PEG$_{24}$-**1445** group indicates a reactivation of the mice's immune systems and a potential anti-tumor response.

1.3.3. Therapeutic study

To evaluate whether these findings indeed influence tumor growth *in vivo*, we conducted an experiment with six consecutive injections due to no macroscopic effects on tumor growth in the experiment above, which only included three injections. Results clearly showed a significantly retarded tumor growth and a prolonged survival time in the dual KO group compared to the negative controls as well as to the single KO groups, illustrating the advantage of a combined therapy compared to monotherapy. The reason for this is presumably the synergistic effects of a combinatorial treatment, a cornerstone of cancer therapy, which circumvents the innate and acquired resistance against one part of a combined treatment [192]. In fact, in the last years, treatment with approved monoclonal Abs blocking PD-1 or PD-L1 revealed that monotherapy with PD-1/PD-L1 checkpoint blockade shows effectiveness only in some patients, and some patients develop resistance during therapy [193].

In sum, this experimental series presents a non-viral folate receptor-mediated delivery of Cas9 RNPs for a dual immune checkpoint disruption in a tumor mouse model, leading to significantly retarded tumor growth as well as prolonged survival time of treated mice. Though, for a systemic approach, the polyplex still shows insufficient delivery and needs further optimization.

V. SUMMARY

Synthetic carriers for gene therapeutics – from pDNA to mRNA to Cas9/sgRNA

Since the first successful gene therapy trial three decades ago, gene therapy has been a steadily emerging and promising field of novel therapeutic approaches with the capability of treating diseases considered to be untreatable to date. Instead of treating symptoms, gene therapy targets the causal reason for the disease by replacing a disease-causing gene, inactivating a disease-causing gene or introducing a new or modified gene. Although gene therapy has suffered some setbacks, its unique ability to already bring improvements to patients suffering from otherwise untreatable diseases is encouraging scientists around the world to relentlessly search for new, more effective, and safer treatments.

To overcome the hurdle of delivering the therapeutics, which in the case of gene therapy are mostly nucleic acids, to the target location, two dominant classes of delivery systems are utilized, namely: viral and non-viral vectors, each with their specific benefits and limitations. Due to potential adverse reactions provoked by viral vectors, non-viral vectors are considered the safer option. However, transfection efficiency of non-viral vectors is far below that of viral vectors. Therefore, many efforts have been made to optimize existing and to develop new non-viral vectors for efficient gene delivery *in vivo* while maintaining good tolerability and non-immunogenic characteristics. Over the last decade, our working group has generated libraries of over 1800 sequence-defined oligoaminoamides with different topologies and various ligands for targeting, shielding, or uptake enhancement, leading to potent cargo-optimized non-viral vectors.

In the first chapter of this thesis the feasibility of transcriptional targeting DCs was evaluated. Therefore, in the first experiment, LPEI and succPEI were assessed regarding their pDNA carrier properties. SuccPEI proved to be the more suitable carrier, as it had more specificity toward the spleen. In a consecutive experiment using succPEI as a carrier, the newly developed Fascin promoter was compared to the widely used CMV promoter to

evaluate specificity toward DCs. The immunohistochemical staining of the liver, lungs, spleen, and lymph nodes revealed a 1.5- to 4-fold higher expression in DCs compared to MACs and a (non-statistical) benefit of the Fascin promoter compared to the CMV promoter regarding their DC specificity.

In the second chapter, a newly developed lipidic domain, termed lipo amino fatty acid (LAF) was evaluated, which, coupled with existing OAAs instead of normal fatty acids, could show superior transfection efficiency compared to succPEI, the old gold standard for mRNA delivery while maintaining good tolerability in mice. Its supposed mechanism of action is based on a pH-dependent structural transformation of the LAFs leading to stable nanocarriers in neutral pH, whereas in the acidic milieu of endosomes, nanocarriers partly disintegrate and facilitate endosomal escape. In fact, in a biodistribution study using luciferase mRNA as cargo, these LAF-carriers not only showed higher transfection efficiencies *in vitro* but also, systemically applied, *in vivo* with a particularly high specificity for the spleen, indicating a potential utilization in immunotherapy. Meanwhile, ongoing studies in our working group reveal the high potential of these LAF carriers to not only deliver mRNA, but also other cargos, such as pDNA and siRNA (data not shown).

The last chapter of this thesis assessed the utilization of one of our OAAs, *i.e.,* **1445**, for Cas9-mediated dual immune checkpoint disruption in a colon carcinoma mouse model. A biodistribution study revealed insufficient delivery upon systemic application, hence following experiments were conducted, performing intratumoral injections. **1445** was functionalized via click chemistry to generate FolA-PEG_{24}- or PEG_{24}-modified nanocarriers, which were then evaluated for targeting and transfection efficiency *in vitro* identifying FolA-PEG_{24}-modified **1445** as the best performer. The same carriers, which, as in the *in vitro* study, encapsulated RNPs containing sgRNAs targeting PD-L1 and PVR were then applied intratumorally into a colon-carcinoma-bearing mouse model to appraise gene KO efficiency of PD-L1 and PVR in the tumor tissue. KO efficiency was evaluated by flow cytometry showing, according to the *in vitro* study, best results for the FolA-PEG_{24}-modified **1445** with editing events in almost 40 % of the tumor cells.

Additionally, by flow cytometry, a significantly increased infiltration of CD8+ cells into the tumor tissue could be observed demonstrating the activation of the mice's immune system. Finally, this chapter aimed to assess the effects on tumor growth, respectively survival time of tumor-bearing mice in a therapeutic study comprising six consecutive injections, revealing a significant inhibition of tumor growth and a significantly prolonged survival time of the dual KO group, compared to the control groups, as well as the single KO groups

VI. ZUSAMMENFASSUNG

Synthetische Träger für Gentherapeutika – Von pDNA über mRNA bis zu Cas9/sgRNA

Seit dem ersten erfolgreichen Gentherapieversuch vor drei Jahrzehnten ist die Gentherapie ein stetig wachsender und vielversprechender Bereich neuartiger therapeutischer Ansätze, mit denen Krankheiten behandelt werden können, die bisher als unheilbar galten. Anstatt Symptome zu behandeln, zielt die Gentherapie auf die Ursache der Krankheit ab, indem krankheitsverursachende Gene ersetzt, oder inaktiviert oder neue oder veränderte Gene eingeführt werden. Obwohl die Gentherapie einige Rückschläge erleiden musste, ermutigt ihre einzigartige Fähigkeit, Patienten bereits heute Verbesserungen zu bringen, die an ansonsten unbehandelbaren Krankheiten leiden, Wissenschaftler auf der ganzen Welt, unermüdlich nach neuen, wirksameren und sichereren Behandlungen zu suchen.

Um die Therapeutika, bei denen es sich im Falle der Gentherapie meist um Nukleinsäuren handelt, an den Zielort zu bringen, werden hauptsächlich zwei Klassen von „Delivery Systemen“ verwendet: virale und nicht-virale Vektoren, die jeweils ihre spezifischen Vor- und Nachteile haben. Aufgrund möglicher Nebenwirkungen viraler Vektoren, gelten nicht-virale Vektoren als die sicherere Option, allerdings liegt die Transfektionseffizienz nicht-viraler Vektoren weit unter der von viralen Vektoren. Daher wurden viele Anstrengungen unternommen, um bestehende nicht-virale Vektoren zu optimieren und neue zu entwickeln, die eine effiziente Genübertragung *in vivo* ermöglichen und gleichzeitig eine gute Verträglichkeit und immuninerte Eigenschaften aufweisen. In den letzten zehn Jahren hat unsere Arbeitsgruppe Bibliotheken mit über 1800 sequenzdefinierten Oligo-Aminoamiden in unterschiedlichen Topologien mit verschiedenen Liganden für „Targeting“, Abschirmung oder Aufnahmeverbesserung erstellt, die zu potenten, ladungsoptimierten nicht-viralen Vektoren führte.

Im ersten Kapitel dieser Arbeit wurde die Durchführbarkeit des transkriptionellen Targetings von dendritischen Zellen untersucht. Daher

wurden in einem ersten Experiment LPEI und succPEI auf ihre pDNA-Trägereigenschaften hin untersucht. SuccPEI erwies sich als der geeignetere Träger, da er eine höhere Spezifität für die Milz aufwies. In einem Folgeexperiment, bei dem succPEI als Träger verwendet wurde, wurde der neu entwickelte Fascin-Promotor mit dem weit verbreiteten CMV-Promotor verglichen, um sie hinsichtlich der Spezifität für dendritische Zellen zu bewerten. Die immunhistochemische Färbung von Leber, Lunge, Milz und Lymphknoten ergab eine 1.5 bis 4-fach höhere Expression in den dendritischen Zellen im Vergleich zu Makrophagen und einen (allerdings nicht statistischen) Vorteil des Fascin-Promotors im Vergleich zum CMV-Promotor hinsichtlich seiner Spezifität für dendritische Zellen.

Im zweiten Kapitel wurde eine neu entwickelte Lipiddomäne, die so genannte Lipoaminofettsäure (LAF), evaluiert, die anstelle normaler Fettsäuren an bestehende OAAs gekoppelt, im Vergleich zu succPEI, dem alten Goldstandard für den mRNA-Transport, eine höhere Transfektionseffizienz bei gleichzeitig guter Verträglichkeit in Mäusen aufweisen konnte. Der angenommene Wirkmechanismus beruht auf einer pH-abhängigen Strukturumwandlung der LAFs, die bei neutralem pH-Wert zu stabilen Nanopartikeln führt, während im sauren Milieu der Endosomen die Nanopartikel teilweise zerfallen und die Freisetzung aus den Endosomen ermöglichen. Unter Verwendung von Luziferase-mRNA als Kontrolle, zeigten diese LAF-Träger nicht nur *in vitro*, sondern nach systemischer Verabreichung, auch *in vivo* eine höhere Transfektionseffizienz mit einer besonders hohen Spezifität für die Milz, was einen möglichen Einsatz in der Immuntherapie nahelegt. Laufende Untersuchungen in unserer Arbeitsgruppe zeigen, dass diese LAF-Träger nicht nur mRNA, sondern auch andere Therapeutika wie pDNA und siRNA transportieren können (Daten nicht aufgeführt).

Im letzten Kapitel dieser Arbeit wurde die Verwendung eines unserer OAAs, d.h. **1445**, für die Cas9 induzierte duale Immuncheckpoint Blockade in einem Kolonkarzinom Mausmodell untersucht. Eine Biodistributions-Studie ergab unzureichendes „Drug Delivery“ bei systemischer Applikation, daher wurden folgende Experimente mit intratumoralen Injektionen durchgeführt. **1445** wurde mittels Click-Chemie funktionalisiert, um FolA-PEG_{24}- oder

PEG_{24}-modifizierte Träger zu erzeugen, die dann *in vitro* auf ihr „Targeting“ und ihre Transfektionseffizienz untersucht wurden, wobei sich FolA-PEG_{24}-modifiziertes **1445** als der beste Träger erwies. Dieselben Träger, welche, wie in der *in vitro* Studie RNPs mit sgRNAs enthielten, die auf PD-L1 und PVR abzielen, wurden dann intratumoral in Mäuse, eines Kolonkarzinom-Tumormodells appliziert, um die Gen-Knockout-Effizienz von PD-L1 und PVR im Tumorgewebe zu evaluieren. Die Knockout-Effizienz wurde mittels Durchflusszytometrie bestimmt und zeigte, entsprechend der *in vitro* Studie, die besten Ergebnisse für das FolA-PEG_{24}-modifizierte **1445** mit Genom-Editierung in fast 40 % der Tumorzellen. Zusätzlich konnte mittels Durchflusszytometrie eine signifikant erhöhte Infiltration von CD8+-Zellen in das Tumorgewebe beobachtet werden, was die Aktivierung des Immunsystems der Mäuse belegt. Schließlich wurden in diesem Kapitel die Auswirkungen auf das Tumorwachstum bzw. die Überlebenszeit von tumortragenden Mäusen in einer Therapiestudie mit sechs aufeinanderfolgenden Injektionen untersucht. Dabei zeigte sich eine signifikante Hemmung des Tumorwachstums und eine signifikant verlängerte Überlebenszeit der Tiere der dualen Knockout Gruppe im Vergleich zu den Kontrollgruppen sowie zu den Knockout Gruppen, in denen nur jeweils ein Gen editiert wurde

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VIII. APPENDIX

1. Publications

Lin, Y., Wilk, U., Pöhmerer, J., Hörterer, E., Höhn, M., Luo, X., Mai, H., Wagner, E., Lächelt, U., **Folate Receptor-Mediated Delivery of Cas9 RNP for Enhanced Immune Checkpoint Disruption in Cancer Cells.** Small 2023, 19, 2205318.

Köhler, B.; Dubovik, S.; Hörterer, E.; Wilk, U.; Stöckl, J. B.; Tekarslan-Sahin, H.; Ljepoja, B.; Paulitschke, P.; Fröhlich, T.; Wagner, E.; Roidl, A. **Combating drug resistance by Exploiting miRNA−200c-Controlled Phase II Detoxification.** Cancers 2022, 14,5554.

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Thalmayr, S.; Grau, M.; Peng, L.; Pöhmerer, J.; Wilk, U.; Folda, P.; Yazdi, M.; Weidinger, E.; Burghardt, T.; Höhn, M.; Wagner, E.; Berger, S. **Molecular Chameleon Carriers for Nucleic Acid Delivery: The Sweet Spot Between Lipoplexes and Polyplexes.** Advanced Materials, 2023. **35**(25): p. 2211105.

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