Development of gene correction and supplementation therapy based on chemical modification and sequence optimized mRNA for monogenetic diseases.

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

AKM Ashiqul Haque

Dhaka/Bangladesch

Tübingen

2019

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

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1. Berichterstatter:	Prof Michael S.D Kormann
2. Berichterstatter:	Prof Hans-George Rammensee

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Abbreviations

1. Summary

Gene therapy has been considered as a viable therapeutic option for life limiting genetic diseases (e.g. Cystic Fibrosis (CF) and ß-Thalassemia) lacking conventional therapy. Over the last three decades a shift of opinion was observed in the scientific community regarding gene therapy, from US Food and Drug Administration (FDA) halting all gene therapy-based trails in 1996 to approving 9 clinical trials in the last 5 years. A similar trend can be observed for RNA. After the discovery of mRNA in 1961 it was kept almost untouched by scientists till the 21st century and 2016 saw the first mRNA based clinical trial. Originally, RNA was known for its instability and for being immunogenic. RNA can activate immune responses by interacting with various pattern recognition receptors (PRRs) like toll-like receptors (TLR3, TLR7 and TLR8) and RNA dependent Protein Kinase R (PKR). In recent years around 150 post transcriptional chemical modifications of RNA have been reported that allow for a greater variety of the 4 basic ribonucleotides. These modifications can influence both intramolecular (more stability or flexibility) and intermolecular interactions (with PRRs, less immunogenicity).

Studies conducted during my PhD provide an overview of how mRNAs can be improved for gene supplementation or gene correction therapy by introducing chemical modifications and sequence optimization. For gene supplementation therapy, cystic fibrosis transmembrane conductance regulator (CFTR) mRNA was *in vitro* transcribed with 2-Thiouridine (s2U), 5-Methylcytidine (m5C) and N1-Methylpseudouridine (m1Ψ). Chemically modified human CFTR mRNA (cmRNA^{hCFTR}) showed significantly higher CFTR protein expression and channel functionality in CF bronchial epithelial cell line (CFBE41o-) compared to unmodified h*CFTR* mRNA (mRNA$^{h\mathit{CFTR}}$) and plasmid DNA expressing h*CFTR* (pDNA$^{h\mathit{CFTR}}$). *In vivo* (*Cftr*$^{-/-}$ mouse model), cmRNA$^{h\mathit{CFTR}}$ together with Chitosan-coated PLGA (poly-D, L-lactide-co-glycolide 75:25 (Resomer RG 752H)) nanoparticles (NPs) can drastically improve the lung function. Forced expiratory volume (FEV, the most important parameter considered for CF patients to determine disease progression) was notably improved by cmRNA$^{h\mathit{CFTR}}$ compared to groups treated with pDNA$^{h\mathit{CFTR}}$ and unmodified mRNA$^{h\mathit{CFTR}}$ regardless the route of administration (intravenous (i.v.) and intratracheal (i.t.)). cmRNA$^{h\mathit{CFTR}}$ also exhibits reduced immune responses in *ex vivo* and *in vivo* experiments.

The second study investigated mRNA encoding clustered regularly interspaced short palindromic repeat (CRISPR) associated protein 9 (Cas9) for gene correction of a common ß-thalassemia splicing variant *HBB*$^{IVS1\text{-}110}$. Cas9 was observed to be the superior programmable site-specific endonuclease in comparison to Zinc-finger nucleases (ZFNs) and Transcription

activator like effector nucleases (TALENs). Cas9 when delivered in a plasmid form did not achieve sufficient amount of double strand breaks to result in significant gene correction by homology directed repair (HDR). Chemically modified *Cas*9 mRNA (cmRNA^{Cas9}) induced higher amount of double stranded breaks and subsequently HDR in K562 (immortalized myelogenous leukemia cell line) and bone marrow-derived CD34^{+} hematopoietic stem cells (HSCs).

The final study provided a new angle on increasing efficiency and reducing immunogenicity of mRNA by taking advantage of the degeneracy of the genetic code to exchange specific nucleotides (especially depleting Uridine in the mRNA transcript) without altering the amino acid composition. Sequence engineering of *Cas*9 mRNA can increase the functionality of CRISPR/Cas system (by producing more double strand breaks in HBB gene) in CD34^{+} HSCs compared to non-sequence engineered *Cas*9 mRNAs. The reduction of immunogenicity was achieved by sequence engineering and introduction of 5-methoxyuridine (5moU) further decreased the level of measured cytokines.

All the findings during the PhD thesis contributed towards a review article putting the outcomes into perspective of the current state of mRNA research. The article signifies the role of target specific components like route of administration and carriers of mRNA and how it can be formulated together. Finally, the review article reflects the importance of chemical modification and sequence engineering as milestones in development of mRNA therapeutics.

2. Zusammenfassung

Die Gentherapie wird als mögliche Therapieoption lebensverkürzender Erkrankungen (wie z.B. Cystische Fibrose (CF) oder β-Thalassämie) erwogen, welche bisher als unheilbar gelten. In den letzten 30 Jahren hat sich die Meinung über Gentherapie in der wissenschaftlichen Gemeinschaft stark gewandelt, vom zeitweiligen Stopp aller gentherapie-basierten klinischen Studien durch die US Food and Drug Administration (FDA) bis hin zu 9 erfolgreichen klinischen Studien in den letzten 5 Jahren. Ähnliches kann auch zum Thema RNA beobachtet werden: nach ihrer Entdeckung im Jahre 1961 galt RNA lange Zeit als zu instabil und zu immunogen für therapeutische Anwendungen. In der Tat kann RNA Immunreaktionen durch die Aktivierung sog. Pattern Recognition Rezeptoren (PRRs) wie Toll-like Rezeptoren (TLR3, TLR7 und TLR8) oder Protein Kinase R (PKR) hervorrufen. Hierzu wurden in letzten Jahren etwa 150 posttranskriptionelle chemische Modifikationen der 4 Basen der RNA beschrieben, die für eine höhere Variabilität und Einsatzfähigkeit von RNA sorgen. Diese Modifikationen beeinflussen sowohl die Interaktionen innerhalb der RNA (erhöhte Stabilität oder Flexibilität) als auch Interaktionen mit anderen Molekülen, wie z.B. PRRs (geringere Immunogenität). Auf Grund dieser Weiterentwicklungen konnte im Jahr 2016 die erste mRNA-basierte klinische Studie durchgeführt werden.

Die Untersuchungen im Rahmen meiner Doktorarbeit geben einen Überblick über die Möglichkeiten wie mRNA für Gensupplementtherapien und Genkorrektur eingesetzt und zu diesem Zweck angepasst (mittels chemischer Modifikation und Sequenzoptimierung) werden kann. Als Beispiel für Gensupplementtherapie wurde ein mRNA Transkript des Cystic Fibrosis Transmembrane Regulator (CFTR) Gens mithilfe von *in vitro* Transkription erstellt. Zusätzlich wurden Transkripte unter Verwendung von 2-Thiouridin (s2U), 5-Methylcytidin (m5C) und N1-Methylpseudouridin (m1Ψ) chemisch modifiziert. Chemisch modifizierte h*CFTR* mRNA (cmRNA$^{h\mathit{CFTR}}$) zeigte eine signifikant höhere CFTR Expression und Funktionalität in CF Bronchialepithelzellen (CFBE41o-) im Vergleich zu unmodifizierter mRNA (mRNA$^{h\mathit{CFTR}}$) und Plasmid-DNA (pDNA$^{h\mathit{CFTR}}$). Im *in* vivo Cftr$^{-/-}$ Mausmodell konnte cmRNA$^{h\mathit{CFTR}}$ in Verbindung mit Chitosan-beschichteten poly-D, L-lactide-co-glycolide 75:25 (Resomer RG 752H) (PLGA) Nanopartikeln (NPs) eine drastische Verbesserung der Lungenfunktion erzielen. Die Einsekundenkapazität (FEV, bei Mäusen 0,1s, einer der wichtigsten Lungenfunktionsparameter zur Verlaufskontrolle bei CF Patienten) konnte im Vergleich zu mRNA$^{h\mathit{CFTR}}$ und pDNA$^{h\mathit{CFTR}}$ durch cmRNA$^{h\mathit{CFTR}}$ deutlich gesteigert werden. Dabei spielte der Administrationsweg (intravenös (i.v.) oder intratracheal (i.t.)) keine Rolle. Wie erwartet konnte

außerdem eine Verminderung der Immunantwort bei cmRNA^{hCFTR} in *in vivo* und *ex vivo* Experimenten beobachtet werden.

In einer zweiten Studie wurden mRNA Transkripte vom Clustered Regularly Interspaced Palindromic Repeats (CRISPR) associated Protein 9 (Cas9) untersucht. CRISPR/Cas9 wurde hierbei zur Genkorrektur der Spleißvariante *HBB*$^{IVS1\text{-}110}$ des β-Globin Gens, welche zur β-Thalassämie führt, genutzt. Im Vergleich zu anderen sequenzspezifischen Endonukleasen, Zink-Finger-Nukleasen (ZFN) und Transcription activator-like effector nucleases (TALENs), konnte eine höhere Aktivität bei Cas9 gemessen werden. Auch wenn Cas9 in Form von Plasmid-DNA noch keinen ausreichenden Prozentsatz an DNA-Doppelstrangbrüchen induzieren konnte, um Genkorrektur durch homologe Rekombination zu ermöglichen, konnte eine Reparatur des Gendefekts mithilfe von cmRNA^{Cas9} in K562 Zellen (immortalisierte myeloische Leukämie Zelllinie) und CD34^{+} hämatopoetischen Stammzellen (HSCs) nachgewiesen werden.

Der Einfluss von Modifikationen auf die Effizienz von *Cas*9 mRNA wurde in einer weiteren Studie tiefergehend beleuchtet. Unter Zuhilfenahme der Degeneration des genetischen Codes wurde eine Sequenzoptimierung und insbesondere eine Verminderung des Uridingehaltes innerhalb des *Cas*9 mRNA Transkriptes vorgenommen ohne dabei die Aminosäuresequenz des Proteins zu ändern. Die Sequenzoptimierung der *Cas9* mRNA konnte die Induktion von DNA-Doppelstrangbrüchen im HBB Gen in CD34^{+} HSCs im Vergleich zur nicht-sequenzoptimierten Form weiter steigern. Außerdem konnte auch die Immunogenität der mRNA durch Sequenzoptimierung verringert und durch Verwendung von 5-Methoxyuridin (5moU) noch weiter minimiert werden.
Die Ergebnisse dieser Doktorarbeit konnten innerhalb eines Review-Artikels in den wissenschaftlichen Kontext eingeordnet werden. Darüber hinaus weist dieser Artikel auf weiter zu beachtenden Faktoren, wie Administrationsformen, Eigenschaften von Transportmolekülen (NPs) und deren Formulierung hin. Außerdem zeigt der Artikel in diesem Zusammenhang die Bedeutung von chemischen Modifikationen und Sequenzoptimierung von mRNA als wichtiger Meilenstein in der Entwicklung von mRNA-basierten Therapien auf.

3. Publications

3.1. Original Publications

1. Sriram Vaidyanathan, Krist T. Azizian, **AKM Ashiqul Haque**, Jordana M. Henderson, Ayal Hendel, Sabrina Shore, Justin S. Antony, Richard I. Hogrefe, Michael S. D. Kormann, Matthew H. Porteus, Anton P. McCaffrey. "Uridine Depletion and Chemical Modification Increase *Cas9* mRNA Activity and Reduce Immunogenicity without HPLC purification". ***Molecular Therapy-Nucleic Acids***. 2018. PMID: 30195789

2. **AKM Ashiqul Haque**, Alexander Dewerth, Justin S Antony, Joachim Riethmüller, Georg Schweizer, Ngadhnjim Latifi, Petra Weinmann, Hanzey Yasar, Nicoletta Pedemonte, Elvira Sondo, Brian Weidensee, Anjali Ralhan, Julie Laval, Patrick Schlegel, Christian Seitz, Brigitta Loretz, Claus-Michael Lehr, Rupert Handgretinger, Michael S. D. Kormann. Chemically modified h*CFTR* mRNAs recuperate lung function in a mouse model of cystic fibrosis. ***Nature Scientific Reports***. 2018. PMID: 30425265

3. Justin S Antony, Ngadhnjim Latifi, **AKM Ashiqul Haque**, Andrés Lamsfus-Calle, Alberto Daniel-Moreno, Sebastian Gräter, Praveen Baskaran, Petra Weinmann, Markus Mezger, Rupert Handgretinger and Michael S. D. Kormann. Gene correction of HBB mutations in $CD34^{+}$ hematopoietic stem cells using *Cas9* mRNA and ssODN donors. ***Molecular and Cellular Pediatrics***. 2018. PMID: 30430274

4. Itishri Sahu, **AKM Ashiqul Haque**, Brian Weidensee, Petra Weinmann, Michael S. D. Kormann. Recent developments in mRNA-based protein supplementation therapy to target lung diseases. ***Molecular Therapy***. 2019. PMID: 30905577

5. Al Mamun Bhuyan, **AKM Ashiqul Haque**, Itishri Sahu, Hang Cao, Michael S.D. Kormann, Florian Lang. Inhibition of Suicidal Erythrocyte Death by Volasertib. ***Cellular Physiology and Biochemistry***. 2017. PMID: 29035889

6. Justin S. Antony, **AKM Ashiqul Haque**, Andrés Lamsfus Calle, Alberto Daniel Moreno, Markus Mezger, Michael S.D. Kormann. CRISPR/Cas9 system: A promising technology for the treatment of inherited and neoplastic hematological diseases. ***Advances in Cell and Gene therapy***. 2018.

7. Justin S. Antony, Alexander Dewerth, **AKM Ashiqul Haque**, Rupert Handgretinger, Michael S.D. Kormann. Modified mRNA as a new therapeutic option for paediatric respiratory diseases and hemoglobinopathies. ***Molecular and Cellular Pediatrics***. 2015. PMID: 26589812

3.2. Oral Presentations

Presentation on "Topical and Systemic Administration of Chemically Modified hCFTR mRNA Restores Lung Function in a Mouse Model of Cystic Fibrosis" at 20th annual meeting of American Society of Gene and Cell therapy, Washington, D.C.(US) 2017.

Presentation on "Chemically modified hCFTR mRNAs-Chitosan-coated PLGA nanoparticle recuperate lung function in a mouse model of cystic fibrosis" at Biobarrier 2018, Saarbrücken (Germany) 2018.

Presentation on "Transcript Therapy with hCFTR mRNA to normalize lung function in CF mouse model" on behalf of Michael Kormann at 20th Deutsche Mukoviszidose Tagung Würzburg (Germany) 2017.

Presentation on "Chemically modified CFTR mRNA normalizes critical lung function parameters *in vivo*" at 5th international mRNA health conference, Berlin (Germany) 2017

3.3. Poster Presentations

Presentation on "*In vivo* Genome editing using chemically modified *Cas*9 mRNA in SP-B deficient mouse model" at CRISPR: From Biology to Technology and Novel therapeutics, Sitges (Spain) 2017

Presentation on "Optimizing the use of *Cas*9 based on expression and kinetics analysis of chemically modified mRNA *in vivo*" at 5th international mRNA health Conference, Berlin, (Germany) 2017

Presentation on "Utilizing human whole blood to predict *in vivo* immune responses against *in vitro* transcribed chemically modified *Cas*9 mRNA" at 20th annual meeting of American Society of Gene and Cell therapy, Washington, D.C.(US) 2017

Presentation on “Optimizing the utilization of chemically modified *Cas*9 mRNA / sgRNA against the underlying genetic defect in surfactant protein B deficiency based on expression kinetics” 22nd annual meeting of American Society of Gene and Cell therapy, Washington, D.C.(US) 2019

Presentation on “Optimizing the utilization of chemically modified *Cas*9 mRNA / sgRNA against the underlying genetic defect in surfactant protein B deficiency based on expression kinetics” 6th lung health conference, Nice (France) 2019

Erklärung nach § 5 Abs. 2 Nr. 8 der Promotionsordnung der Math.-Nat. Fakultät
-Anteil an gemeinschaftlichen Veröffentlichungen-
Nur bei kumulativer Dissertation erforderlich!

Declaration according to § 5 Abs. 2 No. 8 of the PhD regulations of the Faculty of Science
3.4. Collaborative Publications

For Cumulative Theses Only!

Last Name, First Name: Haque, AKM Ashiqul

List of Publications

1. Sriram Vaidyanathan, Krist T. Azizian, **AKM Ashiqul Haque**, Jordana M. Henderson, Ayal Hendel, Sabrina Shore, Justin S. Antony, Richard I. Hogrefe, Michael S. D. Kormann, Matthew H. Porteus, Anton P. McCaffrey. "Uridine Depletion and Chemical Modification Increase *Cas9* mRNA Activity and Reduce Immunogenicity without HPLC purification". ***Molecular Therapy-Nucleic Acids***. 2018. PMID: 30195789

2. **AKM Ashiqul Haque**, Alexander Dewerth, Justin S Antony, Joachim Riethmüller, Georg Schweizer, Ngadhnjim Latifi, Petra Weinmann, Hanzey Yasar, Nicoletta Pedemonte, Elvira Sondo, Brian Weidensee, Anjali Ralhan, Julie Laval, Patrick Schlegel, Christian Seitz, Brigitta Loretz, Claus-Michael Lehr, Rupert Handgretinger, Michael S. D. Kormann. Chemically modified h*CFTR* mRNAs recuperate lung function in a mouse model of cystic fibrosis. ***Nature Scientific Reports***. 2018. PMID: 30425265

3. Justin S Antony., Ngadhnjim Latifi, **AKM Ashiqul Haque**, Andrés Lamsfus-Calle, Alberto Daniel-Moreno, Sebastian Graeter, Praveen Baskaran, Petra Weinmann, Markus Mezger, Rupert Handgretinger and Michael S. D. Kormann. Gene correction of HBB mutations in $CD34^{+}$ hematopoietic stem cells using *Cas9* mRNA and ssODN donors. ***Molecular and Cellular Pediatric***. 2018. PMID: 30430274

4. Itishri Sahu, **AKM Ashiqul Haque**, Brian Weidensee, Petra Weinmann, Michael S. D. Kormann. Recent developments in mRNA-based protein supplementation therapy to target lung diseases. ***Molecular Therapy***. 2019. PMID: 30905577

EBERHARD KARLS
UNIVERSITÄT
TÜBINGEN

Mathematisch-Naturwissenschaftliche Fakultät

Nr.	Accepted publication yes/no	List of authors	Position of candidate in list of authors	Scientific ideas by the candidate (%)	Data generation by the candidate (%)	Analysis and Interpretation by the candidate (%)	Paper writing done by the candidate (%)
1	Yes	Sriram Vaidyanathan, Krist T. Azizian, **AKM Ashiqul Haque**, Jordana M. Henderson, Ayal Hendel, Sabrina Shore, Justin S. Antony, Richard I. Hogrefe, Michael S. D. Kormann, Matthew H. Porteus, Anton P. McCaffrey	First Author (Shared, Position 3)	40%	50%	40%	30%
2	Yes	**AKM Ashiqul Haque**, Alexander Dewerth, Justin S Antony, Joachim Riethmüller, Georg Schweizer, Ngadhnjim Latifi, Petra Weinmann, Hanzey Yasar, Nicoletta Pedemonte, Elvira Sondo, Brian Weidensee, Anjali Ralhan, Julie Laval, Patrick Schlegel, Christian Seitz, Brigitta Loretz, Claus-Michael Lehr, Rupert Handgretinger, Michael S. D. Kormann	First Author	70%	80%	70%	60%
3	Yes	Justin S Antony., Ngadhnjim Latifi, **AKM Ashiqul Haque**, Andrés Lamsfus-Calle, Alberto Daniel-Moreno, Sebastian Graeter, Praveen Baskaran, Petra Weinmann, Markus Mezger, Rupert Handgretinger and Michael S. D. Kormann	Second Author (position: third)	30%	25%	20%	20%
4	Yes	Itishri Sahu, **AKM Ashiqul Haque**, Brian Weidensee, Petra Weinmann, Michael S. D. Kormann	First Author (Shared, position 2nd)	40%	40%	20%	30%

I confirm that the above-stated is correct.

I/We certify that the above-stated is correct.

Date, Signature of the candidate

Date, Signature of the doctoral committee or at least of one of the supervisors

3.5. Contribution to the publications in thesis

Uridine Depletion and Chemical Modification Increase *Cas9* mRNA Activity and Reduce Immunogenicity without HPLC purification.

Prior to performing the experiments, the integrity of the Cas9 mRNA was analysed by Bioanalyzer. I collected serum from whole blood transfected with Cas9 mRNA and conducted various *ex vivo* human immunological assays (ELISA). Additionally, the mRNA in nanocarrier complex was tested *in vivo* by intravenous (i.v.) injection in murine model and blood collection for immunological study at various time points by me. I further contributed towards data analysis, graphical presentation of collected data and writing the paper.

Chemically modified h*CFTR* mRNAs recuperate lung function in a mouse model of cystic fibrosis.

I designed the outline of the paper, performed experiments, analyzed data, made graphics and wrote the manuscript with the help of other authors. In particular, the *in vitro* experiments including the production of cmRNA$^{h\mathit{CFTR}}$ followed by expression analysis of hCFTR protein by flow cytometry, Western blot and Immunofluorescence as well as the *ex vivo* whole blood assay to check the immunogenicity of the mRNAs were conducted by me. For the *in vivo* study, I treated the mouse model with cmRNA$^{h\mathit{CFTR}}$ by intratracheal and intravenous administration, collected saliva for determination of chloride concentration and measured the lung mechanics via FlexiVent with the help of Alexander Dewerth, Ngadhnjim Latifi and Georg R. Schweizer.

Gene correction of *HBB* mutations in CD34$^+$ hematopoietic stem cells using *Cas9* mRNA and ssODN donors.

The *in vitro* experiment to optimize K562 cell electroporation and compare different endonucleases for gene correction were performed together with Ngadhnjim Latifi. I contributed towards the meticulous preparation of the manuscript.

Recent developments in mRNA-based protein supplementation therapy to target lung diseases.

I contributed towards the literature research, designing graphics and writing the paper with emphasis on post transcriptional mRNA modifications. The paper structure was designed by Itishri Sahu, Brian Weidensee, Michael Kormann and me.

4. Introduction

"Victory has 1000 fathers; defeat is an orphan."-Count Ciano.

4.1. Gene Therapy

Forty-seven years ago, in 1972, Theodore Friedmann proposed a prophetic account regarding the potential advantages of gene therapy along with the downsides[1]. But "Gene therapy" was coined to separate the term from George Orwell's proposed dystopia based on "human genetic engineering". The term used by Orwell was influenced by "Genetic engineering", which was first used at the Sixth International congress of Genetics held in 1932[2]. Gene therapy is by no means a new concept and the early history of gene therapy is a combination of missteps, attempt of experiments before the necessary tools were available, extravagant dreams of numerous dreamers. Just as the rudimentary flying machine made by Wright brothers led to the sophisticated supersonic jets, development of recombinant DNA technology by Herbert W. Boyer, Stanley N. Cohen, and Paul Berg opened a whole new chapter for curing untreatable conditions[3].

4.1.1. Molecular genetics and Gene therapy (Early age)

Genes can be transferred as nucleic acid (in *Pneumococci*) was first observed by Avery, Macleod and McCarthy. This proves to be a vital point of reference for molecular genetics[4]. Further studies provided evidence of viruses containing the capacity to transmit genes[5] and becoming an enduring part of cellular genome[5,6]. Rous sarcoma virus (RSV) can successfully transduce cells in culture to reproduce virus, providing the first evidence of viral gene mediated cell transduction. Similarly, Simian Virus 40 (SV40)can integrate viral DNA in SV40-transduced cells[7-9]. Two more important discoveries, structure of DNA along with its function[10] and central dogma (genetic information flow; DNA to RNA to protein) provided fuel for advancement in the area of Gene therapy[11].

Arthur Kornberg achieved replication of DNA in a test tube[12] which further helped Waclaw Szybalski transfer a mammalian gene for the first time[13]. From the late 1960s and early 1970, gene therapy became an important part of the scientific community. This lead Martin Cline of the University of California Los Angeles to conduct the first human gene therapy but without obtaining approval from any regulatory body. The recombinant DNA advisory committee (RAC) was already working with the US Food and Drug Administration (FDA) to regulate gene therapy and created a new committee called the Human Gene therapy working group (now

known as the Human Gene Therapy Subcommittee (HGTS)) to avoid unregulated gene therapy. In 1990, the HGTS approved the first T lymphocyte-directed gene therapy for two children with Adenosine deaminase (ADA) mediated severe combined immunodeficiency (SCID). The trial was not a success as transduced peripheral blood T cells cannot elevate the level of ADA significantly[14]. In 1993, Hematopoietic Stem cells (HSCs) based gene therapy was conducted by CellPro on ADA SCID patients with no significant outcome but this paved the way for stem cell therapy[15]. Stem cell-based gene therapies were conducted in 1999 on human immunodeficiency virus (HIV) patients and the study produced valuable insight on stem cell therapy but failed to be clinically significant[16].

The downsides of gene therapy were exposed when Jesse Gelsinger was reported dead 4 days after being administered a high dose of Adenoviral vector for the treatment of ornithine transcarboxylase (OTC) deficiency[17]. Stem cell therapy also came under scrutiny when two of the ten children treated for X-linked SCID developed leukemia-like conditions[18]. FDA sent out the "March 6 letter" to all the investigator working on gene therapy to implement new scrutiny and Europe halted all gene therapy for re-review.

4.1.2. A New age of Gene Therapy

In 1996, the National Institute of Health (NIH) concluded that based on ongoing gene therapy trials, there is a lack of knowledge of the disease, the viral vector, target cells and tissues were the source of disappointing results[19]. But the last 10 years have been monumental for gene therapy as a better understanding and improvement of delivery vectors and gene expression resulted in significant clinical advancement. Till now, few gene therapies have received approval in the US and Europe (Table1) to be used as medicine.

Table 1: Approved gene therapies in western world

Drug name	Disease	Vector/transgene	Approval committee	Year
Glybera®	-Familial lipoprotein lipase deficiency	AAV based	EMA	2012
IMLYGIC®	-Lesion in patients with melanoma	Modified herpes simplex virus type I	FDA	2015
STRIMVELIS®	-Adenosine deaminase (ADA) deficiency-SCID	γ-retrovirus	EMA	2016
KYMRIAH®	-Non-Hodgkin lymphoma	CD-19 directed CAR-T cell	FDA	2017
YESCARTA®	-Non-Hodgkin lymphoma -Lymphoblastic Leukemia	CD-19 directed CAR-T cell	FDA	2017
LUXTURNA®	-Retinal dystrophy	AAV based	FDA	2017
AVXS-101	-Spinal muscular atrophy	AAV based	FDA	2016
SCAAV2/8-LP1-HFIXCO	-Hemophilia B	AAV based	FDA/EMA	2014/2017
SPK-9001	-Hemophilia B	AAV based	FDA/EMA	2016/2017
AMT-061	-Hemophilia B	AAV based	FDA/EMA	2017/2017
SB-FIX	-Hemophilia B	Zinc finger nuclease-AAV2/6	FDA	2017

European Medicines Agency (EMA)

US Food and Drug Administration (FDA)

Along with the commercialized gene therapy, there are numerous clinical trials in progress for multiple diseases based on T cell, Hematopoietic Stem and Progenitor Cells (HSPCs) and *in vivo* administration (Supplement Table 2). Some of the trials gained FDA or EMA approval during different phases of clinical trial for orphan diseases lacking alternative cures.

The advancement of Gene therapy is also based on better understanding of the cellular mechanism and establishment of three approach towards gene therapy; A) Gene supplementation, B) Gene suppression/inhibition and C) Gene correction (Figure 1).

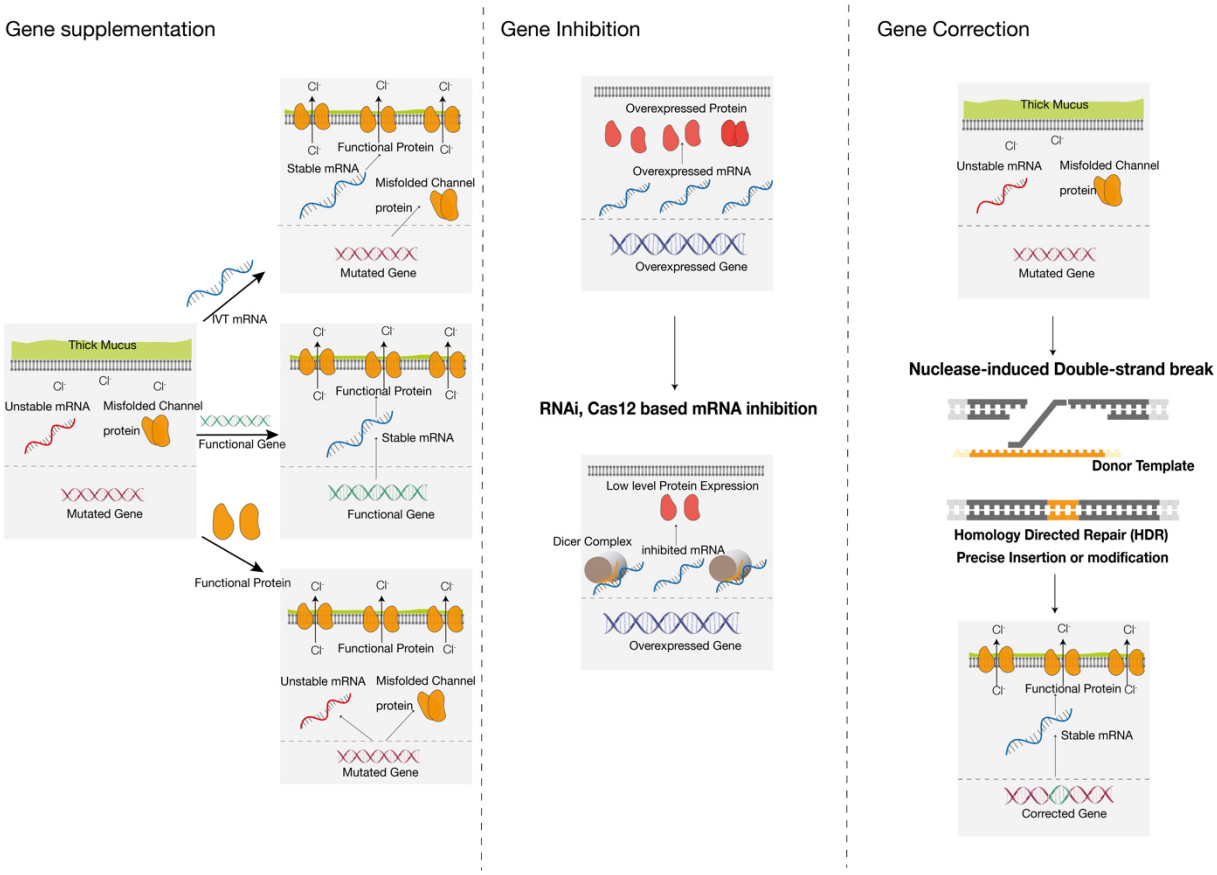

Figure 1: The fundamental approach of Gene therapy

Gene supplementation therapy restores function without alteration in genomic level. Gene inhibition by RNAi or Cas12 can reduce the expression of protein temporally. Gene editing or correction can have long lasting effects in stem cells.

Gene supplementation restores normal cellular function without affecting the disease gene by providing a functional copy of gene in trans. Functional protein[20], *in vitro* transcribed mRNA (IVT mRNA)[21,22], and plasmid DNA[23] have been studied for gene supplementation therapy and proved to be very efficient. Gene suppression or inhibition is used to reduce the expression of mutated genes to stop harmful accumulation of a faulty protein by RNA interference (example: Huntington's disease)[24] or anti-sense oligonucleotide[25]. Gene correction provides a site-specific correction by DNA double-strand break at the target site (by CRISPR/Cas9, ZFN or TALEN) and repair of the target site by homology directed repair (HDR) or Non-homologous end joining[26-28]. Progressions in the field of gene therapy has made diseases like Cystic fibrosis and ß-Thalassemia perfect candidates to be investigated.

4.2. Cystic Fibrosis (CF)

CF is an autosomal-recessive disease, with limited therapeutic options available, affecting about 80,000 people worldwide[29]. It is caused by different mutations within the gene encoding

for the CF transmembrane and conductance regulator (*CFTR*). Those mutations result in compromised anion secretion and hyper-absorption of sodium ions across epithelia[30,31] (Figure 2). *CFTR* consists of 27 exons and is located on chromosome 7q31.2[32]. The final transcript of *CFTR* (6.5 kb) has been reported for over 2000 mutations and CF can be connected to about 250 mutations[33,34]. Based on the impact on CFTR gene and protein, all the mutations have been categorized in six classes (Figure 2).

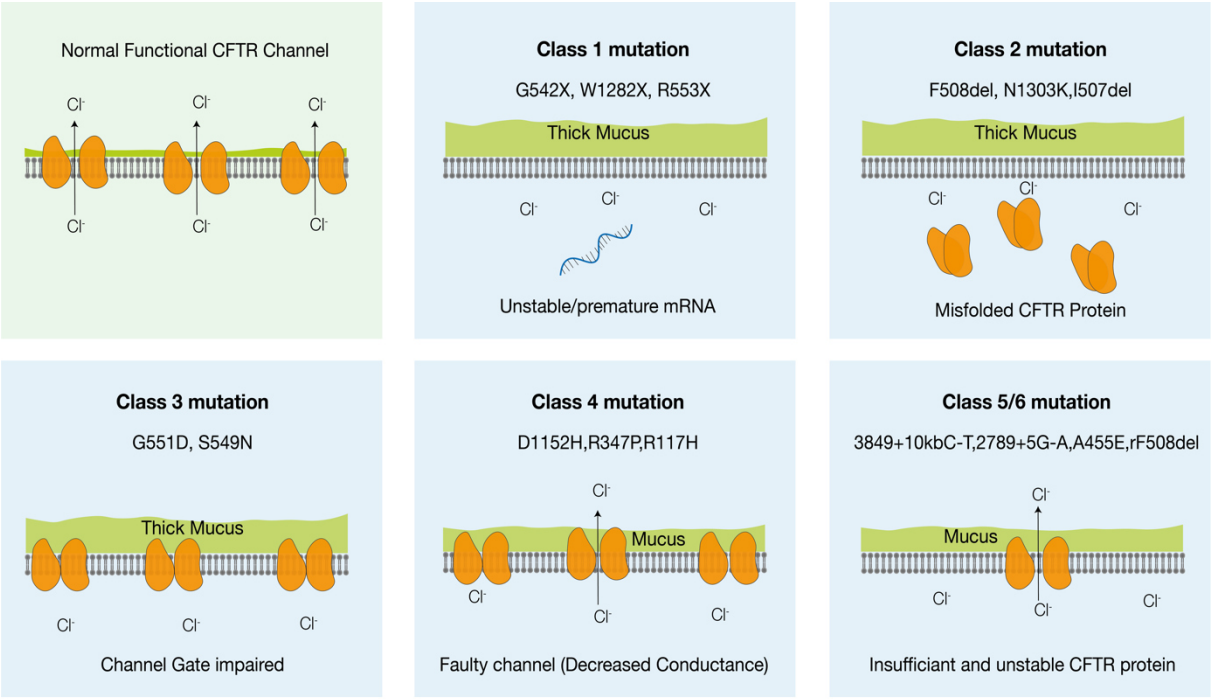

Figure 2: Normal CFTR channel function and CFTR mutation classes

Normal function of CFTR channel and effect of different mutation classes at the physiological level.

4.2.1. CF Therapies

CF therapy so far based on symptomatic control and consists of antibiotic treatment, supplementation of pancreatic enzymes and fat-soluble vitamins[35]. The symptomatic approach was inadequate and targeted therapy based on specific mutation of the *CFTR* was called for. Ivacaftor (Kalydeco®) was announced as a potentiator to increase the open possibility of CFTR channel and therefore an exclusive drug for patients with class III mutations. Patients with conductance mutation (class IV) also reported to have a beneficial effect[36,37]. Lumacaftor (VX-809) was the second line of potentiator but unfortunately can only be used as a combination therapy with ivacaftor (Trade name Orkambi®) for moderate improvement in lung function of patients with F508del mutation[38]. The approach using the potentiator is difficult to implement for each class of CFTR mutation and that makes mRNA-based gene supplementation therapy a viable option for CF patients regardless of the mutation.

4.3. ß-Thalassemia

Major adult hemoglobin (HbA) consists of α-globin chain (chromosome 16) and ß-globin chain (chromosome 11). Absence of partial or complete α-globin chain or ß-globin chain can result in α-Thalassemia or ß-Thalassemia, respectively[39]. Imbalance in α-globin and ß-globin chain determines the severity of disease and can result in erythropoietin overexpression which leads to bone marrow proliferation and variety of growth and metabolic abnormalities. Over 200 mutations in ß-globin gene have been identified to cause disease ranging from no clinical symptoms (silent mutations) to complete absence of ß-globin chain synthesis[40].

4.3.1. Therapy for ß-Thalassemia

Red blood cell (RBC) reduction is the main clinical symptom presented by ß-Thalassemia patients. Normal clinical therapy of chronic anemia patients is RBC transfusion and can heavily impair the quality of life of the patients. Iron overdose in the vital organs of the patients, recurring infections, and immune reactions caused by the transfusion can cause morbidity and mortality[41]. Removal of excess iron by iron chelators are in clinical use (Deferoxamine, Deferasirox and Deferiprone). However, these can cause adverse effects like nausea, abdominal pain, diarrhea, liver dysfunction, kidney dysfunction, neutropenia and agranulocytosis. The complications of the current clinical treatment call for gene therapy-based applications.

4.4. mRNA based therapy

Recently, *in vitro* transcribed (IVT) messenger RNA came into focus as substantial efforts have been made for engineering mRNA with diverse pharmacokinetic properties[26,42]. Instability and immune response against IVT mRNA were the main issues. To avoid these downsides, modification of structural elements such as 5′ Cap, 5′- and 3′- untranslated region (UTR), poly(A) tail and the coding region were implemented[21,43-46] (Figure 3).

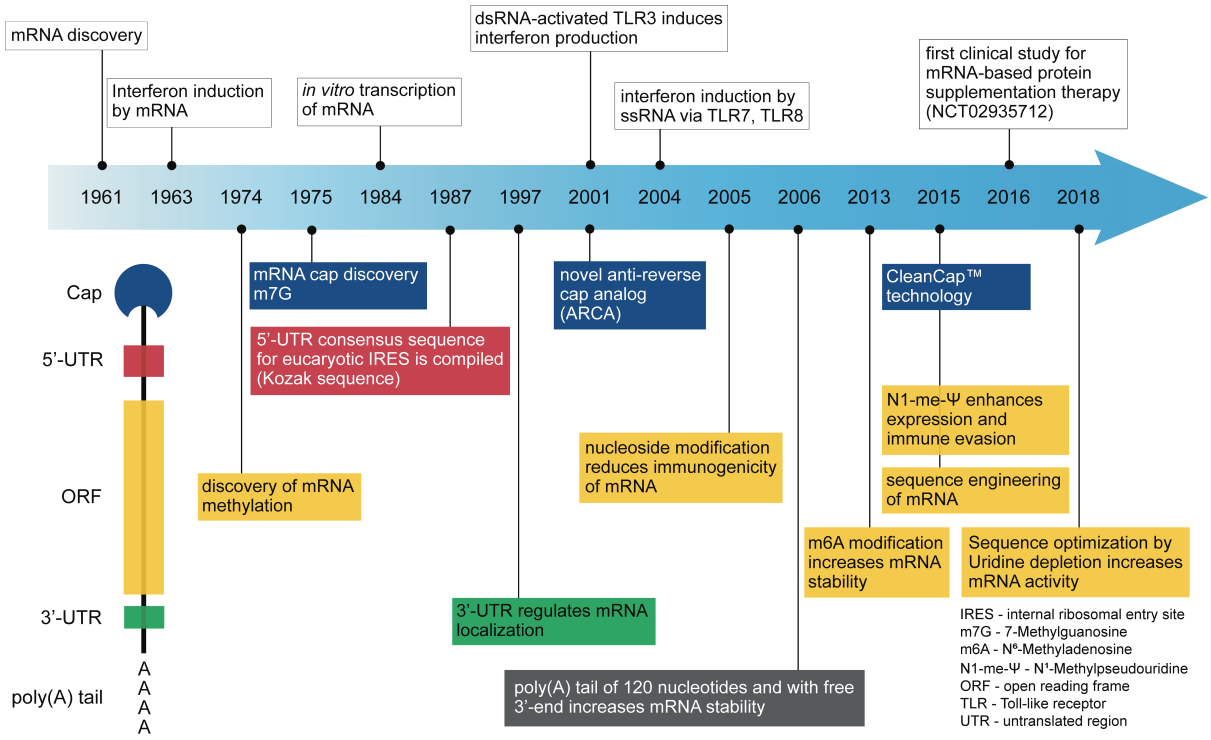

Figure 3: IVT mRNA development timeline

Adapted from Sahu I, Haque A, Weidensee B et al 2019 © Molecular Therapy publishing group.
Overview of milestones in development of *in vitro* transcription of mRNA. White boxes: important milestones for development of mRNA therapy[47]. Blue boxes: evolution of different cap structures (Cap 0 and Cap 1)[21,48,49]. Red, green and grey boxes represent development in the area of 5′-UTR, 3′-UTR, poly(A) tail, respectively[43,50,51]. Yellow boxes represent nucleoside modifications and sequence optimizations in development for mRNA-based therapy[52-57].

4.4.1. Chemical modifications to increase mRNA effectiveness

RNA can be modified post-transcriptionally and over 150 chemical modifications are listed by different studies[58]. In mRNA, only a small subset of these naturally occurring modifications are reported to be capable of reducing innate immune responses and improving pharmacokinetic properties[21,53,59]. The first generation of modified IVT mRNAs contain 5-Methylcytidine (m5C) or Pseudouridine (Ψ) to reduce innate immune responses and enhance translation[22,60]. TLR3, TLR7, TLR8, and retinoic acid-inducible gene I (RIG-I) activation was reduced by m5C, *N*^{6}-methyladenosine (m6A), 5-Methyluridine (m5U), Ψ-UTP and 2-Thiouridine (s2U) [21,22,53,61] modified nucleosides. RNA-dependent protein kinase (PKR) which mediates immune responses and translation inhibition (by phosphorylating the alpha subunit of translation initiation factor 2 (eIF-2α)) can be escaped using Ψ-UTP or m5C modified nucleosides[62]. m5C is explicitly recognized by the mRNA Aly/REF export factor (ALYREF) and increases mRNA-binding affinity and associated mRNA export out of the nucleus[63]. N1-methylpseudouridine (m1Ψ) is the most used chemical modification in recent studies and

showed remarkable expression compared to Ψ substituted mRNA even when delivered by different routes *in vivo*[55]. m1Ψ initiates a tight binding to RIG-I and blocks RIG-I signaling[61,64].

4.4.2. Seqeunce engineering to increase mRNA effectiveness

Codon optimization or sequence engineering of mRNA uses the flexibility of the genetic code to substitute specific nucleosides of a mRNA sequence without altering the resulting amino acid composition. Several recent studies have described codon optimization of unmodified and Ψ-UTP-modified mRNA through enriching guanosine/cytosine (G/C) can result in superior expression[56,59].

4.5. Programmable site-specific nucleases

A new era for whole genome sequencing is paving the way towards revolutionizing basic science and personal medicine. Zinc-finger nuclease (ZFNs), transcription activator like effector nucleases (TALENs) and clustered regulatory interspaced short palindromic repeats (CRISPR) are advancing gene therapy based on the advancement of whole genome sequencing. The resourcefulness of ZFNs and TALENs gives the option to customize the DNA binding domain to identify practically any sequence[65]. On the other hand, the CRISPR/Cas system is distinct from ZFNs and TALENs as it can target any DNA sequence by CRISPR RNA (crRNA) and work as an RNA-guided DNA endonuclease. All the programmable site-specific nucleases can be conducted by all RNA approach and various studies, including those from our lab, have shown that remarkable efficiency can be achieved[21,26,27].

4.5.1. Zinc-finger nuclease (ZFNs)

Zinc finger (ZF) is composed of approximately 30 amino acids with a zinc atom bound to Cys_2 and His_2. Klug *et al*. first described repetitive zinc-binding domains from *Xenopus-oocytes*[66]. The Cys_2-His_2 domain is the most common type of DNA binding domain with a conserved $\beta\beta\alpha$ configuration[67]. The α-helix structure of ZF can naturally contact 3 bp of major grove of DNA, however, the DNA recognition was advanced to 9-18 bp by constructing synthetic zinc finger protein (ZFP) with highly conserved linker sequences[68]. The attractive framework of providing tailor made sequence specificities by ZFPs was used to create designer nucleases. Two ZF proteins, Sp1-QNR and CP-QDR, were fused with the *Fok*I cleavage domain of Sp1-QNF-F_N and QDR-F_N respectively and were termed zinc finger nucleases (ZFNs)[69](Figure 4).

4.5.2. Transcription activator like effector nucleases (TALENs)

A recent discovery of TALE DNA binding motif (33-35 amino acids) from *Xanthamonas* bacteria provided an alternative platform for designing programable DNA binding proteins[70,71]. ZF can recognize 3-4 bases but TALE can recognize a single nucleotide by repeat variable diresidue (RVD, Amino acid NI recognize A, HD recognize C, NG and HG recognize T and NN recognize G or A). TALENs were designed by fusing *Fok*I cleavage domain with TALE motifs and have been demonstrated to have similar cutting efficiency like ZFNs with lower cytotoxicity[26,72](Figure 4).

4.5.3. Clustered regulatory interspaced short palindromic repeats (CRISPR)

Bacteria and Archaea have adapted a defense mechanism by integrating DNA fragment into clustered regularly interspaced short palindromic repeats (CRISPR) to fight against reoccurring viral and plasmid DNAs[73]. In the simplest form of the CRISPR system, CRISPR repeats are transcribed into long RNA that are further processed to contain a part of both the repeat sequence and a single spacer known as crRNA[74-76.] CRISPR locus also contain sequences to produce transactivating crRNA (tracrRNA). crRNA and tracrRNA complexes can associate with Cas9 protein. The tripartile structure can detect specific DNA targets by in proximity to a specific short sequence known as Protospacer-adjacent motif (PAM). The Cas9 protein from *Streptococcus pyogenes* (most commonly used) contain two nuclease active sites and can initiate a double strand break 3 bp upstream of the PAM sequence[77](Figure 4).

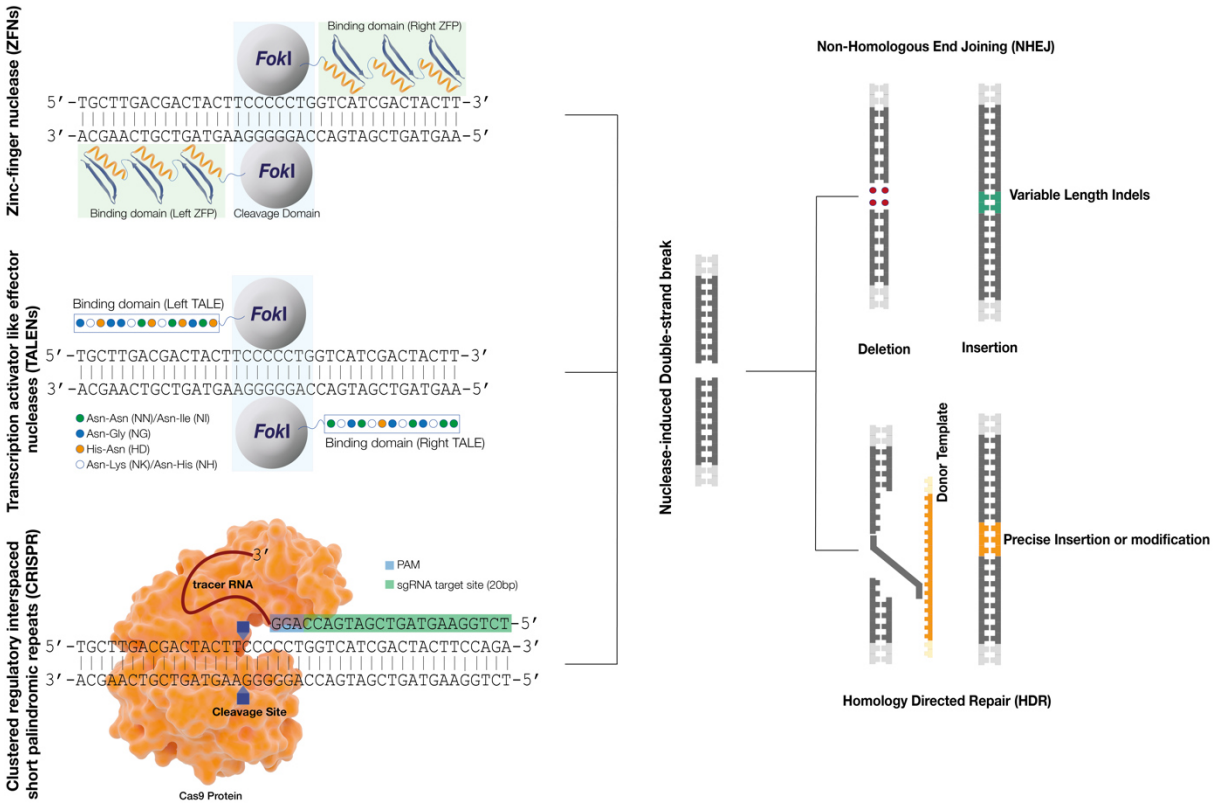

Figure 4: Programmable site-specific nucleases to achieve precise gene editing

ZFN, TALEN and CRISPR/Cas9 based gene editing. Each of the programmable nuclease is comprised of different approach for targeting specific genomic areas and can produce double strand breaks to initiate gene correction by NHEJ or HDR.

4.5.4. Gene editing by programmable site-specific nucleases

Site specific nucleases possess a powerful system for generating a DNA double strand break. The double strand break gives an opportunity for gene editing to modify DNA at a specific locus on the basis of repair. Double strand breaks are repaired either by non-homologous end joining (NHEJ) or by homology directed repair (HDR). NHEJ occurs more frequently, no template is required and closes the break without adding to the DNA sequence[78]. NHEJ give programmable site-specific nucleases the ability to induce indels (insertion or deletion), to knockout specific genes by frameshift mutations or premature stop codon. On the contrary, HDR is based on homologous recombination. A homologous strand is needed and HDR can only occur in S or *G2* phase of the cell cycle[79]. HDR can repair a single mutation or insert a sequence of interest in targeted locus. For this purpose, the repair template can be given with non-integrating viral vectors (e.g. Adeno-associated viral vectors) or by using single-stranded oligodeoxynucleotides (ssODNs)[26,27] (Figure 4).

5. Aim of the study

Recent advancements in understanding the underlying mechanisms of gene therapy, *in vitro* transcribed (IVT) mRNA and CRISPR based correction system pave the way to study monogenetic diseases such as cystic fibrosis and ß-Thalassemia. The goal of the study was to obtain better insights about using chemical modifications and sequence optimization to improve IVT mRNA for efficient, less immunogenic gene therapy.

One of the targets was to produce chemically modified h*CFTR* mRNA (cmRNA$^{h\mathit{CFTR}}$) which can produce a functional CFTR protein and evade immunological response. Using various *in vitro*, *ex vivo* and *in vivo* models of cystic fibrosis, cmRNA$^{h\mathit{CFTR}}$ was tested rigorously and compared against wildtype CF models for restoration of functionality without immune response. The second target was to improve *in vitro* transcribed (IVT) *Cas*9 mRNA to achieve a high level of personalized gene-correction therapy for β-thalassemia. The study also includes a comparison of established programmable nucleases such as CRISPR/Cas9, TALENs, and ZFNs to identify the most promising gene correction approach. The first two studies indicated the potential IVT mRNA holds for protein supplementation and as a transiently expressed endonuclease for gene correction. This line of thought was extended by investigating different chemical modifications and sequence engineering to further enhance the usability of IVT mRNA.

6. Results and Discussion

6.1. Chemically modified h*CFTR* mRNAs recuperate lung function in a cystic fibrosis mouse model (paper 1)

Though the underlying genetic defect of Cystic Fibrosis has been identified, restoration of robust CFTR function in patients suffering from cystic fibrosis remains unfulfilled. Small molecule agents (modulator/potentiator) such as Ivacaftor (for G551D mutation) or Orkambi (Lumacaftor-Ivacaftor, for F508del mutation) are available to CF patients but are limited to specific mutations in the CFTR gene. Unfortunately, Orkambi did not reach the predicted expectations for the benefit of patients with F508del mutations, the most common mutation among CF patients[80]. Attempts were made to supplement CFTR in the form of plasmid DNA providing promising data *in vitro* but exhibiting limitations in *in vivo* application[23]. In this study, cmRNA$^{h\mathit{CFTR}}$ was vigorously tested for mRNA therapy with a focus on restoring *in vitro* protein

expression and *in vivo* lung function. The unique formulation can be applied both targeted (intratracheally) and systemically (i.v.), having a profound effect on the lung function parameters.

6.1.1. *In vitro* functional restoration and detection of CFTR protein in CFBE41o– and CFTR null A549 cells

Cystic Fibrosis Bronchial Epithelium cells (CFBE41o-) are a well-documented cell line as *in vitro* CF model. The cell line was created from the bronchial epithelium of a CF patient homozygous for F508del mutation[81]. CFTR protein quantification by flow cytometry using a CFTR specific antibody (596) in CFBE41o- cells exhibits around 5-fold higher protein expression for cmRNA^{hCFTR}, compared to unmodified mRNA^{hCFTR} (24 hours). Flow cytometry data were substantiated by both Western blot and immunofluorescence analysis. Western blot image indicates a glycosylated band (160 kDa) after treatment with cmRNA^{hCFTR}, a confirmation of functional CFTR protein. Immunofluorescence analysis also provides clear evidence of presence of CFTR protein when compared to untreated CFBE41o- cells. Halide-sensitive Yellow fluorescent protein-based (YFP) assay in CFTR null A549 and CFBE41o– cells also corroborate the findings from flow cytometry and Western blot analyses by showing a significant increase in Iodide (I^-) influx by functional CFTR channels and quenching of YFP 48h post transfection.

6.1.2. *Ex vivo* immune response

The whole blood assay (WBA, *ex vivo*) was described by Coch and colleagues to have the potential to reflect broad aspects of *in vivo* cytokines and is used in various pre-clinical studies[82]. IFN-α, TNF-α and IL-8 levels were measured to predict activation of the immune system as reaction to RNA. pDNA^{hCFTR} and unmodified mRNA^{hCFTR} showed increased cytokine levels whereas cmRNA^{hCFTR} did not show a significant elevation. This experiment provided the direction of eliminating unmodified mRNA^{hCFTR} in mouse model as it potentially exhibits unnecessary immune responses. pDNA^{hCFTR} (as it had been used in various study to produce functional CFTR) and two chemically modified CFTR mRNAs were used for further *in vivo* lung function restoration studies.

6.1.3. *In vivo* restoration and detection of CFTR protein in CF knockout mice to determine clinical potential of cmRNA$^{h\mathit{CFTR}}$.

Gut-corrected CFTR knock out mouse model (based on CFTR$^{tm1Unc/J}$) is a useful mouse model for cystic fibrosis. Due to the promoter specific expression of *CFTR* in the intestine, the mouse model does not die from intestinal obstruction (ileum or large intestine). The mouse model does not demonstrate the typical lung phenotype seen in CF patients, however, various groups have reported findings such as the acid mucopolysaccharide on the bronchial surface by scanning electron microscopy, reduced airway compliance and increased resistance typical to CF in comparison to wild type mice[83,84]. The model is also reported to show similar symptoms of CF such as age dependent pulmonary inflammation, death of respiratory epithelial cells and severe persistent *Pseudomonas aeruginosa* infection[85]. The clinical potential of CFTR-encoded cmRNAs were tested in the mouse model by two different routes of administration. Intratracheal administration allows for a targeted delivery to the respiratory system while intravenous (i.v.) route was chosen for systemic delivery.

To assess the impact of cmRNA$^{h\mathit{CFTR}}$ on lung function, an evaluation of clinically relevant lung mechanics was conducted using the FlexiVent® system. A significant difference was observed in three important parameters (airway compliance, resistance and $FEV_{0.1}$) between *Cftr*$^{-/-}$ and healthy wild-type mice. I.t. administration of cmRNA$^{h\mathit{CFTR}}$ improved the compliance significantly although relatively less than i.v. administration. Resistance was also improved significantly by both i.v. and i.t. administration of cmRNA$^{h\mathit{CFTR}}$. FEV_1 percentage (for mouse or small animal $FEV_{0.1}$) is related to survival and the most important physiological parameter for CF patients. $\%FEV_1$ has become a significant parameter to check in clinical setup as a reduction of more than 30%FEV_1 (compared to healthy individuals) can indicate mortality witin 2 years[86]. A strong variance amid *Cftr*$^{-/-}$ controls and mock-treated *Cftr*$^{-/-}$ mice compared to homozygous wild-type mice (*Cftr*$^{+/+}$) mice had been perceived in the case of $FEV_{0.1}$. I.v. injection of 40µg cmRNA$^{h\mathit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ significantly improved the $FEV_{0.1}$ by 23 percentage points. pDNA$^{h\mathit{CFTR}}$ when administered via the i.t. route showed improving parameters of lung function measurements including $FEV_{0.1}$, but in lower levels compared to cmRNA$^{h\mathit{CFTR}}$. Interestingly, it was observed that both i.v. and i.t. administration of cmRNA$^{h\mathit{CFTR}}_{s2U_{0.25}/m5C_{0.25}}$ positively compensated most of lung function parameters.

Sweat chloride concentration is a recognized procedure to assess the treatment effects in CF patients. Based on this, salivary chloride assay has been described as an analogous method

in mice[87]. In our study, substantial decrease in salivary Chloride (Cl^-) content of cmRNA^{hCFTR} and pDNA^{hCFTR} treated mice were observed regardless the route of administration, indicating a restoration of CFTR in the duct compartment of salivary glands and thus leading to an improved Cl^- absorption.

Along with the functional parameters, the expression of functional protein and disposition of cmRNA^{hCFTR} in mouse lungs was tested by hCFTR ELISA and RT-qPCR. Both methods indicated that cmRNA$^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ lead to a higher protein expression and as well as mRNA disposition in the lung. The administration route of cmRNA$^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ had little effect on the ability to express functional protein. The special role of this chemical modification has to be further investigated for improving most of the lung function parameters including its effects on kinetics and expression patterns of mRNA.

An extensive *in vivo* immune assay has been conducted to rule out immune reactions caused by either NPs (Chitosan-coated PLGA (poly-D, L-lactide-co-glycolide 75:25 (Resomer RG 752H)) or the cmRNA^{hCFTR} itself. Positive control (*E. coli* total mRNA) provided immunostimulatory effects *in vivo* but a response from NPs or the cmRNA^{hCFTR} was not detectable at the endpoint analysis which is in line with our previous data[26]. Systemic delivery has also been reported to have no impact on proinflammatory cytokine secretion[88].

This study is the first proof of concept for the efficient application of NPs-cmRNA^{hCFTR} *in vivo* to restore lung function in a *Cftr*-deficient mouse model with undetected immune responses *in vivo* and *ex vivo*. CFBE41o- cells when transfected with cmRNA^{hCFTR} efficiently produced functional protein which can be detected by Western blot, flow cytometry, immunofluorescence and YFP assay. cmRNA^{hCFTR} could efficiently restore lung function in *Cftr*$^{-/-}$mice. In addition, the study produced a comprehensive comparison of two well-known mRNA modifications with pDNA^{hCFTR} and of two different delivery routes, demonstrating that systemic administration of cmRNA^{hCFTR} targets lung cells more efficiently at lower dosages. This study provides a strong base for cmRNA^{hCFTR} transcript supplementation therapy for CF patients independent of *CFTR* mutations.

6.2. Gene correction of *HBB* mutation in CD34$^+$ hematopoietic stem cells using *Cas9* mRNA (paper 2)

Studies had been conducted by various groups to target HBB gene using CRISPR/Cas9 system in HSCs and mostly focused on HBB gene addition or targeting sickle cell disease mutations[89,90]. To best of our knowledge, this study is the first study to target the common ß-thalassemia splicing variant *HBB*$^{IVS1\text{-}110}$. The knowledge gathered from our previous study about optimization of expression by chemical modification of mRNAs was implemented to design a *Cas9* mRNA to achieve higher indel induction and correction efficiency.

6.2.1. Comparing ZFNs, TALENs and CRISPR/Cas9

ZFNs, TALENs and CRISPR/Cas9 were designed to target splicing variant *HBB*$^{IVS1\text{-}110}$ and efficacy was determined by T7 endonuclease-I (T7EI) assay in HEK293 cells. CRISPR/Cas9 displayed an indel efficiency of approximately 60% in comparison to TALENs with an efficiency of about 35-40% and ZFNs reaching not over 10%. Interestingly, CRISPR/Cas9 yielded similar amounts of indels regardless of the tested concentrations.

6.2.2. Plasmid vs. all-RNA approach of CRISPR/Cas9

A modified pX330 plasmid (pX330.sg *HBB*$^{IVS1\text{-}110}$) encoding sgRNA sequence (to guide Cas9 protein to specific mutation site) and SpCas9 was designed. Several ssODNs (to produce homology directed repair, HDR) were designed with homology arms varying in length and symmetry around the mutation site. pX330.sg *HBB*$^{IVS1\text{-}110}$ based CRISPR/Cas9 system in K562 cells provided a mean indel frequencies of 44±18% for target loci and resulted in around 20% HDR. In bone marrow-derived CD34$^+$ hematopoietic stem cells (HSCs) this combination of pX330.sg *HBB*$^{IVS1\text{-}110}$ and ssODNs produced relatively low indel and HDR rates (~30% indel and 3% HDR, determined by TIDE analysis). To increase the effectiveness of correction, pX330.sg *HBB*$^{IVS1\text{-}110}$ was substituted with an all-RNA approach. Chemically modified *Cas9* mRNA (cmRNA^{Cas9}) and sgRNAs were synthesized to increase the protein expression of Cas9 and induction of indels as reported earlier[57,91,92]. The all-RNA approach yielded higher indels ranging from 65% to 87% and up to 11% HDR in CD34$^+$ HSCs. Absolute quantification of *Nhe*I tag integration by next generation sequencing (NGS) reached around 8% when chemically modified *Cas9* mRNA, sgRNA and ssODNs were used. These results indicate the potential of RNA-based CRISPR/Cas9 systems for *ex vivo* gene correction in CD34$^+$ HSCs. Furthermore, the flexibility CRISPR/Cas9 is advantageous and yields higher efficiency over other site-specific endonucleases like ZFNs and TALENs. Although gene correction of 8% in

$CD34^+$ HSCs was achieved a further improvement of HDR could result in translation into clinics.

6.3. Uridine depletion and chemical modification increase *Cas9* mRNA activity and reduce immunogenicity without HPLC purification (paper 3)

In both studies concerning cystic fibrosis and ß-thalassemia chemical modifications of IVT mRNA helped to increase the efficacy of gene supplementation and gene correction. To further optimize IVT mRNA, we investigated three design parameters to improve the activity of Cas9 protein. These parameters cover sequence engineering by Uridine depletion, HPLC purification and testing of different Cap structures.

6.3.1. Sequence engineering (Uridine Depletion) improve Cas9 activity and reduce immunogenicity

In order to replace particular nucleotides (especially Uridine), the degeneracy of the genetic code was utilized. Sequence engineering of the mRNA could be performed without changing the amino acid configuration. The Uridine-depleted *Cas*9 mRNA (with or without chemical modifications) yielded higher indel rates (77%-87%) compared to first generation chemically modified *Cas9* mRNA (approximately 60%). The indel formation by Cas9 Ribonucleoprotein (RNP), which is used frequently by various groups for higher indel efficiency, provided 67% indel activity at the same target. Chemical modification of sequence engineered *Cas*9 mRNA did not play any significant role in elevating the indel activity. On the contrary, chemical modification (5moU and Ψ) played an important role in immune evasion. Uridine depletion alone can successfully reduce TNF-α and IL-6 level compared to most of the chemically modified *Cas9* mRNA. Addition of 5moU and Ψ on the sequence engineered *Cas9* mRNA lowered the level of TNF-α and IL-6 to a non-detectable level in whole blood assay.

6.3.2. Influence of High-performance liquid chromatography (HPLC) purification on Cas9 mRNA

Uridine depletion in combination with chemical modification could reduce IL-6 and TNF-α responses but was not successful in reducing IFN responses (U-depleted wild type and U-depleted Ψ *Cas9* mRNAs). HPLC purification of mRNA had been reported to reduce dsRNA impurities and hence can avoid activation of innate immune sensors (TLRs, PKR, or MDA5)[93].

HPLC purification effectively reduced IFN responses of U-depleted wild type and U-depleted Ψ *Cas9* mRNAs. In the context of functionality of Cas9, HPLC purification played no significant role with the exception of unmodified *Cas9* mRNA (wt).

6.3.3. Capping strategy to influence *Cas*9 mRNA activity and immunogenicity

mRNA contains a 5' capping system which initiates translation by binding with eIF4E and eIF4G (eukaryotic translation initiation factors) and reduces deterioration by binding with Dcp1, Dcp2 and DcpS (mRNA decapping proteins)[94,95]. The natural occurring 5'-Cap is a 7-methylguanosine (m7G) connected by a 5'-to-5' triphosphate bridge with the first nucleotide (Cap 0). A modification of Cap 0, Cap 1, (2′-ribose position of the first cap-proximal nucleotide is 2′O-methylated) is used to reduce immune responses as previously reported[96,97]. Cap 1 and Cap 0 structures for U-depleted *Cas*9 mRNA with 5moU modification were compared and no significant difference was observed in indel induction or levels of tested cytokine expressions.

The testing of diverse modalities of *Cas9* mRNA modifications showed a vast difference in functionality. Among these, Uridine depletion came up as the promising candidate by inducing up to 87% indel rate, a significant accomplishment towards achieving gene correction. Unfortunately, both HPLC purification and methylation on the 2′-ribose position of the first cap-proximal nucleotide could not increase the indel induction by Cas9. Chemical modifications (5moU and Ψ) reduce immune response measured by IL-6, IL-12 and TNF-α. Moreover, the combination of chemical modification of Uridine and Uridine depletion taken together surpassed the results of 5moU or Ψ alone and can be recommended for further gene correction studies.

6.4. Recent Developments in mRNA-Based Protein Supplementation Therapy to Target Lung Diseases (paper 4)

The study focusses on three aspects: respiratory diseases, barriers of the lung for drug delivery, and recent advancement in IVT mRNA therapies. Understanding the complexity of these aspects is the key towards a clinically relevant protein supplementation therapy for the lung. The various cell populations which are associated with different lung diseases play a vital role in establishing a therapeutic target. Especially differentiated cells, like ionocytes and ATII cells, or stem cells like bronchioalveolar stem cells (BASC) and myoepithelial cells (MEC) provide a strong target for mRNA therapy. Based on the selected target, nanocarriers need

to be designed with specific properties for delivery to specific cells and resistance against defense systems like respiratory mucus and alveolar fluids. The substantial size and charge of naked mRNA limits the cellular uptake, but nanocarriers can also here facilitate the transport across the cellular membrane. Finally, chemical modifications and sequence engineering of the mRNA itself improve the therapeutic ability by reducing immunogenicity and increasing protein expression. This study provides a critical overview of how to find a formulation considering the aspects of target, delivery and modification of mRNA.

7. Concluding remarks

mRNA has been sidelined for a long time as an unstable molecule. Recent advancements in the field of mRNA bring the true potential of this molecule to light. In three separate studies we could show the potential of cmRNA in protein supplementation therapy for cystic fibrosis, its potential for gene correction using CRISPR/Cas9 for ß-thalassemia and strategies to further customize IVT mRNA technology. I believe that these studies could contribute towards the advancement in the field of mRNA therapy and bring it a step further to clinical translation.

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10. Supplement

Table 2: Gene therapy-based product development land marks for ex vivo and in vivo therapies

Therapy type	Disease	Vector/transgene	Clinical trials
Hematopoietic Stem and Progenitor Cells (HSPCs)	ß-Thalassemia	-Lentivirus (LV) ß-hemoglobin	-NCT02453477 -NCT01639690
	Wiskott-Aldrich syndrome (WAS)	-Lentivirus WAS	-NCT01515462
	Adenosine deaminase deficiency (ADA)	-Lentivirus ADA	-NCT02999984
	Human Immunodeficiency virus (HIV)	-Zinc finger nuclease CCR5 electroporated (*ex vivo)*	-NCT02500849
	Metachromatic leukodystrophy	-Lentivirus Arylsulfatase A (ARSA)	-NCT01560182
	Adrenoleukodystrophy	-Lentivirus ABCD1	-NCT03727555
	Sickle cell anemia	-Lentivirus anti-sickling ß-hemoglobin	-NCT02151526 -NCT02140554 -NCT02247843
T cell	Acute /chronic Lymphoblastic Leukemia (ALL/CLL)	-UCART19 -γ-Retrovirus CD19 (CD28) CAR-T	-NCT02746952 -NCT02348216
	Multiple myeloma	-γ-Retrovirus BCMA (CD28) CAR-T -γ-Retrovirus BCMA (4-1BB) CAR-T -Lentivirus-BCMA CAR-T	-NCT02215967 -NCT03070327 -NCT03090659
	Human Immunodeficiency Virus (HIV)	--Zinc finger nuclease CCR5 electroporated (*ex vivo)*	NCT00842634
In vivo	Hemophilia A	AAV5-Factor VIII	NCTO2576795
	Retinal dystrophy	AAV2-RPE65	NCT00643747
	Parkinson's disease	AAV2-AADC	NCT03562494
	Aromatic l-amino acid decarboxylase deficiency	AAV2-AADC	NCT02926066

11. Curriculum Vitae

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Education

PhD Researcher
University clinic Tuebingen (2015-Present)
-Chemically modified h***CFTR*** mRNAs as gene supplementation therapy to recuperate lung function in a mouse model of cystic fibrosis. (See publication)
- Effect of Uridine Depletion and Chemical Modification to Increase Cas9 mRNA Activity and reduced Immunogenicity. (See publication)
- Surfactant protein B (SP-B) deficiency causing mutation,121ins2 correction by state of art Cas9/sgRNA system in humanized mouse model of SP-B.

University of Leeds (2014-2015)
M.Sc. in Infection and Immunology, Leeds, United Kingdom
-Expressing CDC20 promoter region in pancreatic tumor cells using Adenovirus to regulate the cell cycle checkpoint

University of Leeds (2011-2014)
B.Sc. in Biochemistry, Leeds, United Kingdom
-Influence of detergent size and Protein detergent complex on membrane protein crystallization and X-ray crystallography

Publications

First author:
-Vaidyanathan S, Azizian KT, **AKM Ashiqul Haque**, Henderson JM, Hendel A, Shore S, Antony JS, Hogrefe RI, Kormann MSD, Porteus MH, McCaffrey AP. "Uridine Depletion and Chemical Mod-ification Increase Cas9 mRNA Activity and Reduce Immunogenicity without HPLC purification".***Molecular Therapy Nucleic Acids***. 2018

-**AKM Ashiqul Haque**, Dr. Alexander Dewerth, Justin S Antony, Joachim Riethmüller, Georg Schweizer, Ngadhnjim Latifi, Petra Weinmann, Miss Hanzey Yasar,Nicoletta Pedemonte , Dr. Elvira Sondo , Brian Weidensee, Anjali Ralhan, Julie Laval, Patrick Schlegel, Christian Seitz, Brigitta Loretz, Claus-Michael Lehr, Rupert Handgretinger, Kormann MSD. Chemically modified hCFTR mRNAs recuperate lung function in a mouse model of cystic fibrosis. ***Nature Scientific Reports***. 2018

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Patent

-Holding an European patent on delivery of cmRNA^{hCFTR} complexed with nanoparticles (17169561.2-1401).

12. Appendix

SCIENTIFIC REPORTS

OPEN

Chemically modified h*CFTR* mRNAs recuperate lung function in a mouse model of cystic fibrosis

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Gene therapy has always been a promising therapeutic approach for Cystic Fibrosis (CF). However, numerous trials using DNA or viral vectors encoding the correct protein resulted in a general low efficacy. In the last years, chemically modified messenger RNA (cmRNA) has been proven to be a highly potent, pulmonary drug. Consequently, we first explored the expression, function and immunogenicity of human (h)CFTR encoded by cmRNA^{hCFTR} *in vitro* and *ex vivo*, quantified the expression by flow cytometry, determined its function using a YFP based assay and checked the immune response in human whole blood. Similarly, we examined the function of cmRNA^{hCFTR} *in vivo* after intratracheal (i.t.) or intravenous (i.v.) injection of the assembled cmRNA^{hCFTR} together with Chitosan-coated PLGA (poly-D, L-lactide-co-glycolide 75:25 (Resomer RG 752 H)) nanoparticles (NPs) by FlexiVent. The amount of expression of human hCFTR encoded by cmRNA^{hCFTR} was quantified by hCFTR ELISA, and cmRNA^{hCFTR} values were assessed by RT-qPCR. Thereby, we observed a significant improvement of lung function, especially in regards to $FEV_{0.1}$, suggesting NP-cmRNA^{hCFTR} as promising therapeutic option for CF patients independent of their *CFTR* genotype.

Cystic fibrosis (CF), the most common life-limiting autosomal-recessive disease in the Caucasian population (1/2,500 newborns), affects more than 80,000 people worldwide[1]. It is caused by different mutations within the gene encoding for the CF transmembrane conductance regulator (CFTR). Those mutations result in impaired anion secretion and hyper-absorption of sodium ions across epithelia[2,3]. Chronic lung disease and slow lung degradation are the major contributing factors to both mortality and strongly reduced quality of life[4,5]. With currently available therapies, the mean survival is between 35 and 45 years[6,7]. Since the CFTR gene was first cloned in 1989, many efforts have been made to deal with the mutations at a cellular and genetic level[8,9]. Gene therapy approaches made it quickly to the clinic aiming to deliver viral CFTR-encoding vectors (such as adenoviruses (Ad) or adeno-associated viruses (AAV)) to CF patients[10]. However, none of the clinical studies and current treatments seem to provide sufficient human (h)CFTR expression to prevent the ultimately lethal CF symptoms in the respiratory tract of CF patients. Furthermore, repeated administration of viral vectors or DNA may lead to the development of unwanted immune reactions, mainly due to viral capsids and vector-encoded proteins[10–12].

Newly designed viral vectors circumvent those problems and can be administered repeatedly, but from a clinical perspective, the field is still in need of a therapeutic tool that combines efficient expression in lungs and other (affected) organs and cells while avoiding immunogenicity and genotoxicity completely[13–15]. The non-viral

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CFTR-encoding plasmid–liposome complex pGM169/GL67A has been one of the most promising therapeutical approach used in clinical trial by Alton's group. Beside some encouraging results, the trial only managed to modestly improve forced expiratory volume in 1 s (FEV_1) after repeated administration along with no improvement in patient's quality of life[16,17]. Recently, *in vitro* transcribed (IVT) chemically modified messenger RNA (cmRNA) came into focus, which has the potential to combine striking advantages in a single-stranded molecule[18,19]. Chemically modified mRNA has been tested for repeated administration, without developing immune responses or losing efficacy, presenting $cmRNA^{hCFTR}$ complexed with biodegradable chitosan-coated PLGA nanoparticles (NPs) as a promising therapeutic for the treatment of CF patients[19–21]. Versatile delivery options of mRNA ensure the unique possibility to utilize NP-$cmRNA^{hCFTR}$ in early infants as well as adults, independent of the underlying *CFTR* mutation. To the best of our knowledge, we provide the first *in vivo* studies delivering $cmRNA^{hCFTR}$ to the lungs of CFTR deficient mice ($Cftr^{-/-}$) by intravenous (i.v.) and intratracheal (i.t.) administration, complexed with NPs. We provide a proof of concept of NP- $cmRNA^{hCFTR}$ mediated, ELISA quantified, hCFTR expression in the lungs of $Cftr^{-/-}$ mice, leading to significantly reduced chloride secretion and, more importantly, restored criticial lung function parameters, including the most important parameter to evaluate mortality and morbidity of CF patients, the forced expiratory volume (FEV) in 1s or 0.1s in small animals, respectively[22–24].

Materials and Methods

mRNA production. h*CFTR* was PCR amplified from pcDNA3.hCFTR with primers adding *Nhe*I (Fwd: 5′-TTAGCTAGATGCAGAGGTCGCCTC-3′) and *Kpn*I (Rev: 5′-GCGGGTACCTATCTTGCATCTCTTCT -3′) restriction sites to each end. The PCR product was cloned into a poly(A)-120 containing pVAX (pVAX.A120, www.lifetechnologies.com) by sticky-end ligation using the mentioned restriction sites. pVAX.A120 containing h*CFTR* is referred as $pDNA^{hCFTR}$ throughout this study. For control experiments, DsRed reporter protein was sub-cloned into pVAX.A120 vector from its original vector pDsRed (www.clontech.com). For *in vitro* transcription (IVT), the plasmids were linearized downstream of the poly(A) tail with *Xho*I (www.neb.com). IVT reaction was carried out using MEGAscript T7 Transcription kit (www.ambion.com) with an anti-reverse CAP analog (ARCA) at the 5′ end (www.trilink.com). To produce chemically modified mRNA, the following chemically modified nucleosides were added to the IVT reaction in the indicated ratios: uridine-tri-phosphate (UTP) and cytidine-tri-phosphate (CTP) were fully replaced by N1-Methylpseudo-UTP and 5-Methyl-CTP, abbreviated to $cmRNA^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$ and partly replaced by the incorporation of 25% 2-Thio-UTP and 25% 5-Methyl-CTP, respectively, abbreviated to $cmRNA^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ (www.trilink.com). The $cmRNA^{hCFTR}$ and $cmRNA^{DsRed}$ were purified using the MEGAclear kit (www.ambion.com) and analyzed for size and concentration using an RNA NanoChip 6000 for Agilent 2100 Bioanalyzer (Supplement, Fig. S1) (ww.agilent.com).

Cell culture and Transfection. CFBE41o− and 16HBE14o- cells (from Gruenert's lab) were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 until they reached 80–90% confluency. Cell lines were washed with cold, sterile PBS and detached by Trypsin-EDTA. Trypsinization was stopped by adding minimum essential medium (MEM; www.thermofisher.com) containing 10% fetal calf serum. Cells were collected and spun down at 500 × g for 5 minutes before resuspension in fresh MEM. One day before transfection, 250,000 cells/well/1 ml were plated in 12-well plates and grown overnight in MEM without antibiotics. At confluence of 70–90%, cells were then transfected with 1000 ng (c)$mRNA^{hCFTR}$ or equivalent (in nmol) $pDNA^{hCFTR}$ using Lipofectamine 2000 (www.invitrogen.com) following the manufacturer's instructions and after changing the media to the reduced serum media, Opti-MEM (www.thermofisher.com). After 5 hours, the complexes were removed by replacement with fresh culture medium. Cells were kept for 24 h and 72 h before further analyses.

Flow cytometry analyses. All flow cytometry analyses were performed using a BD LSR Fortessa X-20 SORP (www.bdbioscience.com). For detection of hCFTR protein in 16HBE14o- and CFBE41o− cell lines, cells were transfected as described above and subsequently prepared for intracellular staining using a Fixation/Permeabilization Solution Kit as directed in the manufacturer's instruction (www.bdbioscience.com). As primary antibody mouse anti-human hCFTR clone 596 (1:500, kindly provided by the cystic fibrosis foundation therapeutics Inc.) has been used. As secondary antibody served Alexa Fluor 488 goat anti-mouse IgG (1:1,000, www.lifetechnologies.com). At least 20,000 gated cells per tube were counted. Data were analyzed with FlowJo software, version 10.

Western blot analysis. Protein isolated from cell lines was separated on Bolt NuPAGE 4–12% Bis-Tris Plus gels and a Bolt Mini Gel Tank (all from www.lifetechnologies.com). Immunoblotting for hCFTR was performed by standard procedures according to the manufacturer's instructions using the XCell II Mini-Cell and blot modules (www.lifetechnologies.com). After blocking for 1 h in 5% dry milk at room temperature, primary antibody against hCFTR clone 596 (1:500, kindly provided by the cystic fibrosis foundation therapeutics Inc.) or anti-GAPDH (1:1000) (www.scbt.com) was incubated overnight, horseradish peroxidase–conjugated secondary antibodies (anti-mouse from www.dianova.com) were incubated for 1 h at room temperature. All blots were processed by using ECL Prime Western Blot Detection Reagents for 30 min exposure time (www.gelifesciences.com). Semiquantitative analysis was performed using the ImageJ software and overexposure has been avoided as per as digital image and integrity policies.

Immunofluorescence. CFBE41o− and 16HBE14o- were plated on a cell culture insert (0.75×10^6 cells per insert) containing a PET membrane (0.4 μm pore size) (www.corning.com) to provide an air-liquid interface. Cells were transfected 12 h after plating with 5000 ng $cmRNA^{hCFTR}$ or equivalent (in nmol) $pDNA^{hCFTR}$ using Lipofectamine 2000 (www.invitrogen.com) according to manufacturer's instructions. Membranes were cut out from the insert 24 h after transfection, fixed with 4% PFA, blocked with 0.1% BSA and Fc blocker. Blocking was

followed by overnight incubation with hCFTR clone 596 (1:250, kindly provided by the cystic fibrosis foundation therapeutics Inc.). As secondary antibody served Alexa Fluor 594 goat anti-mouse IgG (1:250, www.abcam.com, (ab150116)). Membranes were mounted on a coverslip and images were acquired by Zeiss Confocal Laser Scanning Microscope (CLSM) 710 NLO with Zen software.

YFP-based functional assay. CFTR activity following transient transfection of (c)mRNA^{hCFTR} in A549 and CFBE41o− cells was determined using the halide-sensitive yellow fluorescent protein YFP-H148Q/I152L[25]. CFTR deficient A549 or CFBE14o- cells stably expressing the YFP were plated in 96-well microplates (50,000 cells/well) in 100 µl of antibiotic-free culture medium and, after 6 h, transfected (in case of CFBE14o- reverse transfected) with either plasmids carrying the coding sequence for CFTR or different (c)mRNA^{hCFTR}. For each well, 0.25 µg of mRNA or plasmid DNA and 0.25 µl of Lipofectamine 2000 were pre-mixed in 10 µl of Opti-MEM (www.invitrogen.com) to generate transfection complexes that were then added to the cells. After 24 hours, the complexes were removed by replacement with fresh culture medium. The CFTR functional assay was carried out 24, 48 or 72 h after transfection. For this purpose, the cells were washed with PBS and incubated for 20–30 min with 60 µl PBS containing forskolin (20 µM). After incubation, cells were transferred to a microplate reader (FluoStar Galaxy; www.bmg.labtech.com) for CFTR activity determination. The plate reader was equipped with high-quality excitation (HQ500/20X: 500 ± 10 nm) and emission (HQ535/30M: 535 ± 15 nm) filters for yellow fluorescent protein (www.chroma.com). Each assay consisted of a continuous 14-s fluorescence reading (5 points per second) with 2 s before and 12 s after injection of 165 µl of a modified PBS containing 137 mM NaI instead of NaCl, resulting in a final 100 mM NaI concentration in the well). To determine iodide (I^-) influx rate, the final 11 s of the data for each well were fitted with an exponential function to extrapolate initial slope. After background subtraction, cell fluorescence recordings were normalized for the initial average value measured before addition of I^-. Maximum slopes were converted to rates of variation of intracellular I^- concentration (in mM/s) using the equation: $d[I^-]/dt = K_I[d(F/F_0)]/dt$, where K_I is the affinity constant of YFP for I^-, and F/F_0 is the ratio of the cell fluorescence at a given time vs. initial fluorescence[25].

Whole blood assay. Ethical approval for using whole blood from healthy donor was obtained from Ethics Commission University Clinic of Tuebingen, Germany (349/2013BO2) and experiments were conducted in accordance with relevant guidelines and regulations. Informed consent form (following WHO guideline) was signed by each volunteer (healthy donor) and kept safely by principal investigator for privacy requirement. Blood samples from three healthy donors were collected in EDTA collection tubes (www.sarstedt.com). For each treatment group, 2 ml of EDTA-blood was transferred into 12-well plates and treated accordingly. R848 (Resiquimod, www.sigmaaldrich.com) was added at a concentration of 1 mg/ml to the respective blood positive controls. cmRNA^{hCFTR} and pDNA^{hCFTR} (7 picomol each) were complexed to NPs at a ratio of 1:10. The samples were kept at 37 °C incubator maintaining 5% CO_2. At 6 h and 24 h, 1 ml of whole blood was transferred into microtubes containing serum gel (www.sarstedt.com) and spun down at 10,000 × g for 5 min to obtain serum. Sera were stored at −20 °C for further cytokine measurement analyses. Serum was used to conduct IFN-α, TNF-α and IL-8 ELISA at manufacturer's instruction (www.thermofisher.com).

Animal experiments. All animal experiments were approved by Regierungspräsidium Tübingen, Baden-Württemberg and carried out according to the guidelines of the German Law for the Protection of Animals (File Number: 35/9185.81-2/K3/16). *Cftr*$^{-/-}$ mice (CFTR^{tm1Unc}) were purchased from Jackson Laboratory (www.jax.org) at the age of 6 to 8 weeks and were maintained under standardized specific pathogen-free conditions on a 12 h light-dark cycle. Food, water as well as nesting material were provided *ad libitum*. Prior to i.t. spray applications, mice were anesthetized intraperitoneally (i.p.) with a mixture of medetomidine (0.5 mg/kg), midazolam (5 mg/kg) and fentanyl (50 µg/kg). *Cftr*$^{-/-}$ mice received 40 µg or 80 µg of cmRNA^{hCFTR} or an equivalent amount (calculated using nmols) pDNA^{hCFTR} complexed with chitosan-coated PLGA nanoparticles [Chitosan (83% deacetylated (Protasan UP CL 113) coated PLGA (poly-D,L-lactide-co-glycolide 75:25 (Resomer RG 752 H)) nanoparticles; short: NPs] (Full details of nanoparticles are provided in Supplement Table T1 and Fig. S5) by intratracheal (i.t.) spraying (n = 4), and intravenous (i.v.) injection (n = 4–7) into the tail vein. Mock-treated control *Cftr*$^{-/-}$ mice received 40 µg or 80 µg cmRNA^{DsRed} complexed to NPs (n = 5) by i.v. or i.t administration, respectively, or just 200 µl NPs by both i.v. and i.t. delivery. An antidote with a mixture of naloxone (1.2 mg/kg), flumazenil (0.5 mg/kg) and atipamezol (2.5 mg/kg) was used against anesthetizing reagents. For both interventions, NP-cmRNA and NP-pDNA complexes were administered in a total volume of 200 µl, twice at an interval of 3 days (day 0 and day 3). After 6 days, mice were sacrificed for endpoint analyses. A detailed description of the i.t. procedures are explained in previously published study[26].

Pulmonary mechanics. Lung function for each group was evaluated using a FlexiVent® equipped with FX1 module and NPFE extension and was operated by the flexiWare v7.2 software (www.scireq.com). Prior to tracheostomy, mice were anesthetized intraperitoneally as described above. After anesthesia, a 0.5 cm incision was performed in rostral to caudal direction. A flap of skin was retracted, the connective tissue was dissected, and the trachea was exposed. The trachea was then cannulated between the second and third cartilage ring with a blunt-end stub adapter. The mouse was connected to the FlexiVent® system and quasi-sinusoidally ventilated[27] with a tidal volume of 10 ml/kg. A breathing frequency of 150 breaths per min was maintained with an inspiratory to expiratory ratio of 2:3.

Airway resistance (Rn), which is dominated by the resistance of the large conducting airways was considered in this study when the coefficient of determination of the model fit was ≥0.9. Compliance (Cst) was calculated straight from deflating arm of the pressure volume (PV) loops and ramp style pressure-driven maneuver (PVr-P). For obtaining $FEV_{0.1}$ data a NPFE maneuver was performed which results in FV loops and FE-related parameters.

The mice lung was inflated by a pressure of +30 cmH_2O over 1.2 s and rapidly deflated to a negative pressure of −55 cmH_2O to generate an imposed negative expiratory pressure gradient.

Salivary assay. Prior to tracheostomy, anesthetized mice were injected with 50 µl of 1 mM acetylcholine (ACh) in the cheek to stimulate the production of saliva. The fluid was collected via glass capillaries and a chloride assay was performed using the Chloride (Cl^-) Assay Kit according to the manufacturer's protocol (www.sigmaaldrich.com). Briefly, saliva was diluted at a ratio of 1:100 with water in a total volume of 50 µl and subsequently 150 µl chloride reagent was added. After 15 min incubation at room temperature in the dark, absorbance was measured at 620 nm using an Ensight Multimode plate reader (www.perkinelmer.com).

Enzyme-linked immunosorbent assays (ELISAs). To detect protein levels of hCFTR after i.t. or i.v. injection of differently modified cmRNA^{hCFTR} in *Cftr*$^{-/-}$ mice (CFTR^{tm1Unc}), the lungs were isolated at day 6 (experimental endpoint), homogenized and lysed in 600 µl RIPA-buffer and 5 µl protease inhibitor cocktail with tubes of the Precellys Ceramic Kit 1.4/2.8 mm at 6,500 rpm for 10 s for a total of three cycles, each interrupted by a 15 s break in a Precellys Evolution Homogenizer for protein isolation (all from www.peqlab.com). Subsequently, supernatants were kept on ice and additionally homogenized 10 times with a 20G needle and incubated for 20 min (www.bdbioscience.com). Lysates were spun down for 20 min at 13,000 × g and 4 °C. The supernatant was collected and stored at −20 °C for further use. Prior to hCFTR ELISA detection, protein concentration was measured using the Pierce BCA protein assay kit (www.thermofisher.com). For each sample, an equal amount of 15 µg whole protein lysate was used. A human CFTR ELISA kit (www.elabscience.com) was used for hCFTR detection according to manufacturer's instructions.

Real-time RT-PCR. After i.t. or i.v. injection of cmRNA^{hCFTR} the lungs were isolated at day 6 (experimental endpoint), homogenized and lysed with tubes of the Precellys Ceramic Kit 1.4/2.8 mm at 5,000 rpm for 20 s in a Precellys Evolution Homogenizer for subsequent RNA-isolation (all from www.peqlab.com). Reverse transcription of 200 ng RNA was carried out using an iScript cDNA synthesis kit (www.bio-rad.com) and 1:20 dilution of the cDNA product had been used for further experiment. Detection of mRNA^{hCFTR} was performed by SYBR-Green based quantitative Real-time PCR in 15 µl reactions on a ViiA7 (www.lifetechnologies.com). In all involved procedures, we strictly followed the MIQE protocols for RealTime experiments[28]. Pre- and post-reaction rooms were strictly separated. Reactions were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 2 min at 50 °C (annealing and extension), followed by standard melting curve analysis. The following primer pairs were used:

hCFTR fwd 5′-GAGATGCTCCTGTCTCCTGG-3′, rev 5′-CCTCTCCCTGCTCAGAATCT-3′; 18S rRNA fwd 5′-GGGAGCCTGAGAAACGGC-3′, rev 5′-GACTTGCCCTCCAATGGATCC-3′. Differences in mRNA expression between groups were analyzed by pair-wise fixed reallocation randomization tests with REST 2009 software after collection of the data from Viia7.

Immune response *in vivo*. To assess immune responses to (c)mRNA^{hCFTR} and pDNA^{hCFTR}, C57BL/6 (Jackson Laboratory (www.jax.org)) mice (n = 4 per group) were treated as described for *Cftr*$^{-/-}$ mice. As positive controls a group of mice received two administrations of *E. coli* mRNA-NPs (20 µg) i.v. or i.t. C57BL/6 mice received two injections of 20 µg cmRNA^{hCFTR} complexed to NPs i.v. or i.t. After 6 h, 24 h, and 72 h of second injection mice were sacrificed and blood was collected. For cytokine measurement, blood from mice was used to obtain serum using a serum separator (www.sarstedt.com) and tested for IFN-α and TNF-α production as directed in the manufacturer's instructions (www.thermofisher.com).

Statistics. All analyses were performed using the Kruskal-Wallis test with GraphPad Prism Version 6 (www.graphpad.com). Most of the data are represented as mean ± SD; box plot data are represented as a mean ± minimum to maximum values. $P \leq 0.05$ was considered statistically significant.

Results

(c)mRNA^{hCFTR} and hCFTR protein quantification *in vitro*. To evaluate the influence of chemical nucleoside modification, we first conducted a set of *in vitro* analyses to characterize the expression and functionality of hCFTR protein. First, we compared the expression profile of plasmid-encoded hCFTR (pDNA^{hCFTR}), unmodified h*CFTR* mRNA (mRNA^{hCFTR}) and two well-defined nucleoside modifications (cmRNA$^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ and cmRNA$^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$) which have been described to exert state-of-the-art stability/expression *in vitro* or lung-specific cell contexts *in vivo*[21,29–31]. Flow cytometry analyses 24 h after transfection of human cystic fibrosis bronchial epithelial (CFBE41o−) cells with pDNA^{hCFTR}, mRNA^{hCFTR}, cmRNA$^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ and cmRNA$^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$, showed hCFTR positive cells (marked as black dots) ranging from 15.8% (pDNA^{hCFTR}) to 49.6% (cmRNA$^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$) ($P \leq 0.01$; Fig. 1A, lower panel). At 24 h, hCFTR positive cells and hCFTR median fluorescence intensities (MFIs, marked as columns) of (c)mRNA^{hCFTR} were significantly higher compared to pDNA^{hCFTR} ($P \leq 0.05$; Fig. 1A, lower panel). At 24 h the total hCFTR expression, defined as median fluorescent intensity (MFI) multiplied by the transfection efficiency, of cmRNA$^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$ was significantly higher compared to pDNA^{hCFTR} and mRNA^{hCFTR} ($P \leq 0.01$; Fig. 1A, upper panel). In contrast, after 72 h (c)mRNA^{hCFTR} expressed significantly lower compared to pDNA^{hCFTR} transfected cells, reflected in the percentage of positive cells, MFI and in total hCFTR expression ($P \leq 0.05$; Fig. 1B).

To confirm and substantiate those findings, we performed Western blot analyses of protein lysates taken from transfected CFBE41o- cells at 24 h and 72 h post treatment (Fig. 1C). As a positive control served protein lysate from untransfected 16HBE14o- cells, and GAPDH was used to normalize band intensities. At 24 h pDNA^{hCFTR}

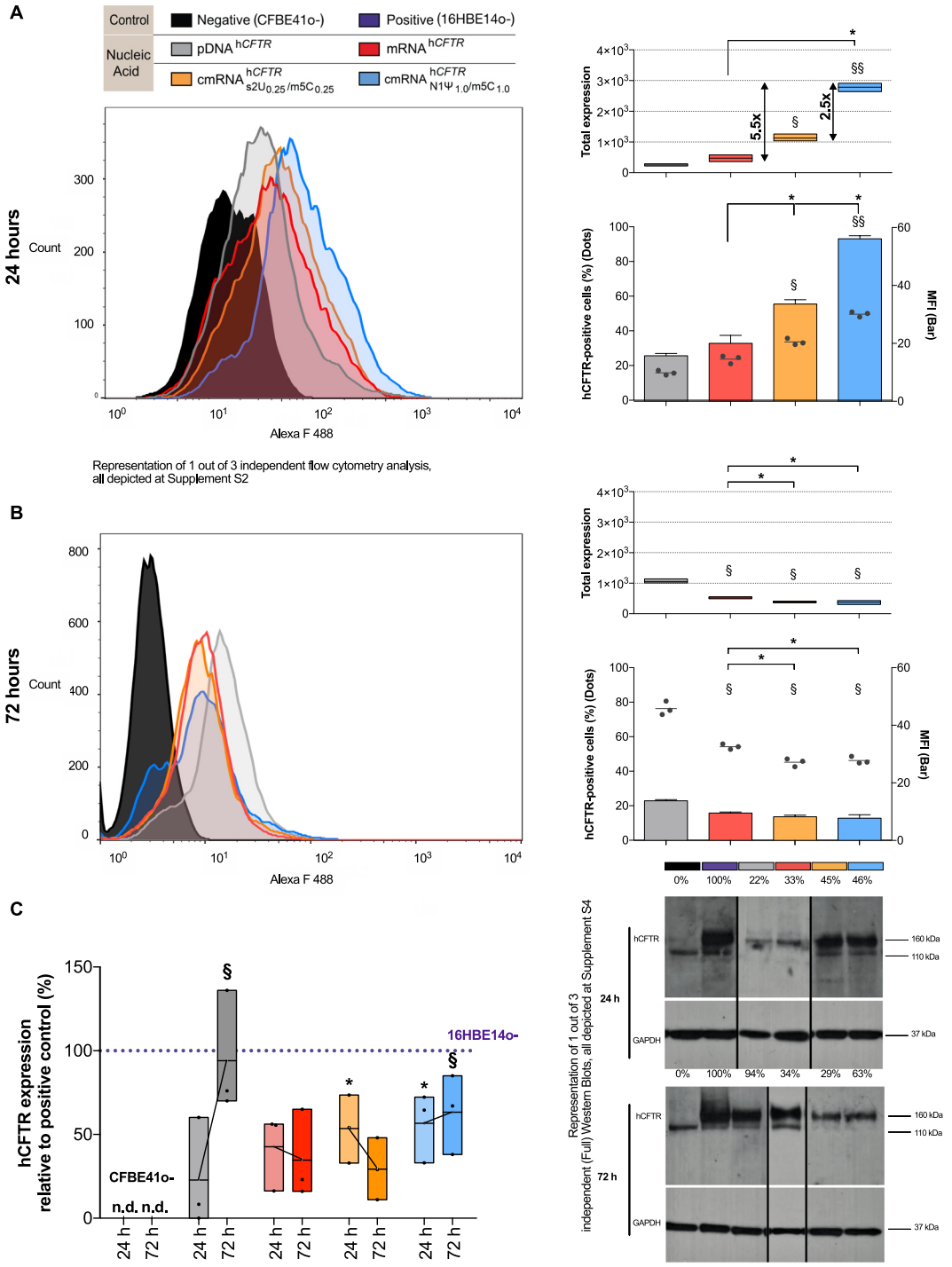

Figure 1. (c)mRNA^{hCFTR} and pDNA^{hCFTR} mediated expression of h*CFTR in vitro* (**A**) Total expression of hCFTR (calculated by multiplying positive cells (dots) and MFI (bars)) 24 h after transfection with 1 µg (c) mRNA^{hCFTR} and equivalent nmols of pDNA^{hCFTR} detected by flow cytometry. (**B**) Total expression of hCFTR 72 h after transfection with 1 µg (c)mRNA^{hCFTR} and equivalent nmols of pDNA^{hCFTR} detected by flow cytometry. (**C**) Western Blots, semi-quantifying human CFTR in transfected CFBE41o- cells, normalized to GAPDH and put relative to CFTR levels in 16HBE14o- cells. Blot section cropped from different blots are delineated with clear dividing lines (black) and full blot of same exposure time (30 mins) are depicted in Supplement Fig. S4. All bar graph data are depicted as means ± SDs while box plots data are depicted as the means ± minimum to maximum values. *$P \leq 0.05$ versus unmodified mRNA^{hCFTR}; $^{§}P \leq 0.05$ and $^{§§}P \leq 0.01$ vs. pDNA^{hCFTR}.

transfected CFBE41o- cells showed an average of 22.8% of the protein expression compared to hCFTR observed in 16HBE14o- cells, which increased 4.1-fold to 94.0% at 72 h ($P \leq 0.05$; Fig. 1C). This drastic increase of hCFTR expression after pDNA transfection goes well in line with the observations in flow cytometry. As well as the quick

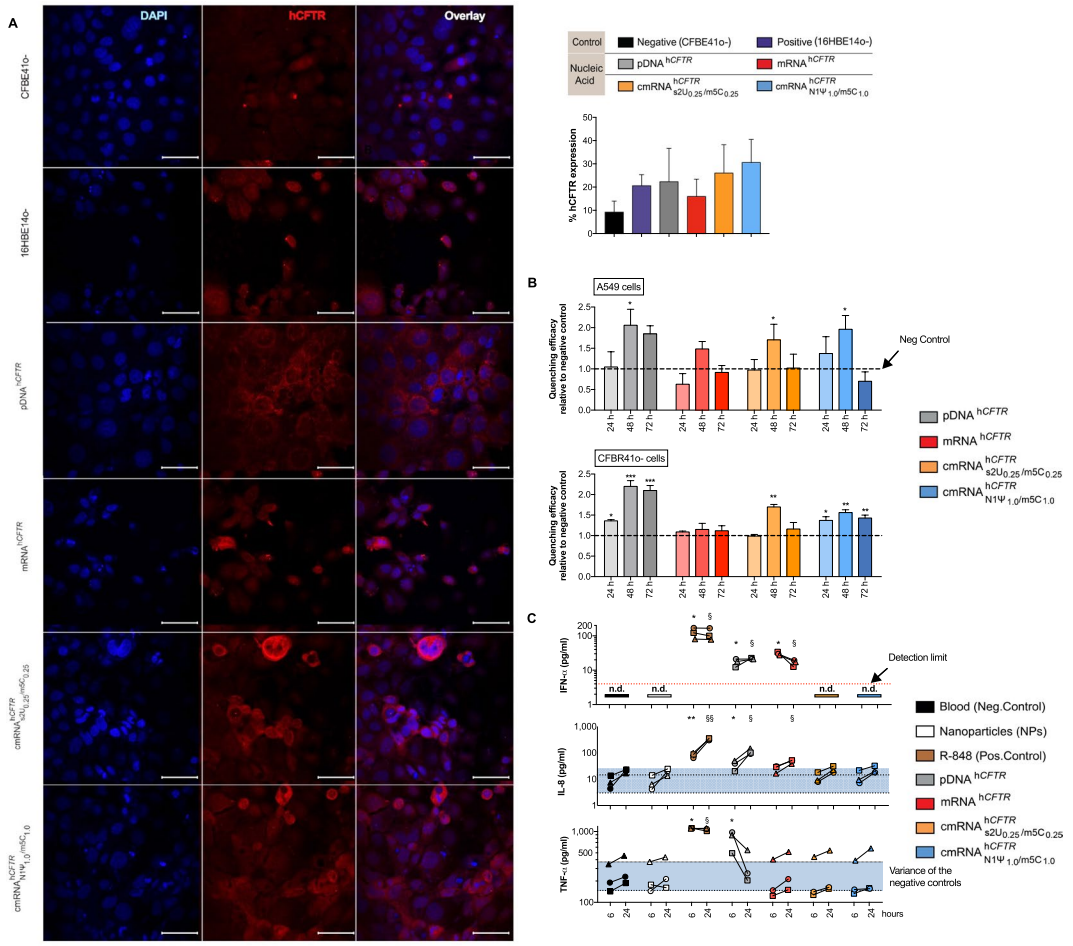

Figure 2. (c)mRNA^{hCFTR} and pDNA^{hCFTR} mediated expression of hCFTR by immunofluorescence and functional h*CFTR in vitro* and immunogenicity in human whole blood. (**A**) Detection of hCFTR protein by immunofluorescence (after 24 h), percent of hCFTR expression in pDNA^{hCFTR} or (c)mRNA^{hCFTR} transfected CFBE41o- cells compare to untransfected CFBE41o- and 16HBE14o- cells. Image J has been used for calculating means ± SDs of hCFTR positive cells; (**B**) Quenching efficacy of pDNA^{hCFTR} or (c)mRNA^{hCFTR} transfected CFBE41o- and CFTR null A549 cells relative to un-transfected controls was measured at 24 h, 48 h and 72 h post-transfection. *$P \leq 0.05$ versus un-transfected controls; (**C**) 2 ml whole blood, each from three different healthy human donors, were incubated with either R848 (1 mg/ml) or 7 pmol pDNA^{hCFTR} or 7 pmol (c) mRNA^{hCFTR} (providing the same total number of nucleic acid molecules) and NPs at a 1:10 ratio; after 6 h and 24 h the immune response was determined by ELISA in the sera; The blue area represents the variance of the negative controls which are biological replicates. n.d., not detectable and red dotted lines mark the detection limit as specified in the respective ELISA kit. All bar graph data are depicted as means ± SDs while box plots data are depicted as the means ± minimum to maximum values. *and $^{§}P \leq 0.05$ ($^{§§}P \leq 0.01$) versus control at 6 h and 24 h, respectively.

onset of hCFTR expression after (c)mRNA^{hCFTR} transfection at 24 h ($P \leq 0.05$; Fig. 1C). However, relative to the 24 h time-point, hCFTR expression after 72 h either remained nearly static (mRNA^{hCFTR} resulted in 33.8% and 34.7% expression at 24 h and 72 h, respectively), decreased (cmRNA$^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ resulted in 45% and dropped to 29.3% hCFTR expression at 24 h and 72 h, respectively) or increased (cmRNA$^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$, 46.4% at 24 h and raised to 63.3% at 72 h). Ultimately, the expression of h*CFTR* mRNA *in vitro* was strongly dependent on its chemical modification, with cmRNA$^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$ resulting in the most robust hCFTR expression among all (c)mRNA transfections (All the blots are separately provided in Supplement Fig. S4).

All *in vitro* results are also underlined by the conducted immunofluorescence imaging. All tested samples show a higher amount of hCFTR positive cells compared to the negative control (CFBE41o- cells; Fig. 2A). Additionally, transfection with unmodified mRNA^{hCFTR} produced a lower amount of hCFTR positive cells compared to both pDNA^{hCFTR} and cmRNA^{hCFTR} with the highest amount of hCFTR positive cells in the samples transfected with cmRNA$^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$ (Fig. 2A). Looking at the fluorescence image itself transfection of pDNA^{hCFTR} shows a quite dispersed appearance of hCFTR within the cells compared to cmRNA^{hCFTR} transfection seeming to have a higher abundance of hCFTR towards the cell membrane (Fig. 2A, left panel). In general, the

Immunofluorescence imaging confirms that transfection with pDNA^{hCFTR} as well as (c)mRNA^{hCFTR} leads to increased levels of hCFTR protein within the transfected cells.

hCFTR (c)mRNA functionality test *in vitro*. For functional analysis of the (c)mRNA^{hCFTR} -encoded CFTR channel, we performed a YFP-based functional assay using CFTR null A549 cells or ΔF508 CFBE41o- cells which stably express halide-sensitive YFP-H148Q/I152L[30]. Quenching of the YFP signal induced by hCFTR channel-mediated I^- influx is reciprocally proportional to hCFTR channel function[25,32]. Figure 2B shows the quenching efficacy after transfection of 250 ng (c)mRNA^{hCFTR}, for three different time points, normalized to mock-transfected cells. In pDNA^{hCFTR} transfected cells, the quenching efficacy was significantly higher after 48 h and stayed high even after 72 h ($P \leq 0.05$), while mRNA^{hCFTR} as well as modified cmRNA^{hCFTR} transfected cells revealed a single peak quenching at 48 h ($P \leq 0.05$), which was undetectable at 72 h in A549 cells. In CFBE41o- cells mRNA^{hCFTR} could not provide any detectable quenching but cmRNA$^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$ produced significant quenching at all the time points ($P \leq 0.05$) and cmRNA$^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ showed very significant quenching at 48 h ($P \leq 0.001$), which is in line with expression patterns seen in Fig. 1A,B.

(c)mRNA^{hCFTR} immunogenicity *ex vivo* by an adapted human whole blood assay. Due to lack of a reliable method to detect immune responses that therapeutic mRNAs may trigger in a living organism, we focused on an innovative approach to using whole blood from humans. Blood was collected from three healthy donors and used fresh to conduct whole blood assays. Interestingly, the negative control groups (blood only and NP only) did not raise IFN-α values above the detection limit (Fig. 2C, red dotted lines), while TNF-α and IL-8 were already measurable in human blood untreated or treated only with NPs. That is the reason why we adapted the graphical presentation, using a blue colored area that represents the variance of the negative controls, which are biological replicates. The positive control (R848) lead to a strong and significant production of IFN-α (6 h and 24 h, respectively; $P \leq 0.05$), IL-8 (6 h and 24 h, respectively; $P \leq 0.01$) and TNF-α (6 h and 24 h, respectively; $P \leq 0.05$) (Fig. 2C). All cmRNA^{hCFTR} showed a very similar result in cytokine expression as observed for negative controls: the IFN-α levels did not reach the detection limit of the ELISA; IL-8 and TNF-α responses were not statistically significant at 6 h and 24 h, respectively (Fig. 2C). Unmodified mRNA^{hCFTR} resulted in a significant increase of IFN-α at 6 h and 24 ($P \leq 0.05$), only significant increase in IL-8 at 24 hours ($P \leq 0.05$) and the TNF-α levels were in line with the negative control. While pDNA^{hCFTR} triggered high TNF-α responses at 6 h ($P \leq 0.05$), significant and detectable IFN-α and IL-8 responses after 6 h and 24 h ($P \leq 0.05$). Due to both, significantly lower expression of mRNA^{hCFTR} *in vitro* (Fig. 1) and unwanted higher immune responses of mRNA^{hCFTR}, we focused on cmRNA^{hCFTR} and pDNA^{hCFTR} in the following therapeutic studies.

Therapeutic effect of cmRNA^{hCFTR} *in vivo* in mice after i.t. and i.v. application. All *in vivo* experiments were performed with nanoparticles if not stated otherwise. Therapeutic potential of cmRNA^{hCFTR} was investigated in a mouse model of Cystic Fibrosis. *Cftr*$^{-/-}$ and *Cftr*$^{+/+}$ mice have been used in several experimental settings that are explained and color-coded in Fig. 3A. To assess the impact of cmRNA^{hCFTR} on lung function, we evaluated clinically relevant parameters using the FlexiVent® lung function measurement system. We observed significant differences between Mock controls, *Cftr*$^{-/-}$ and healthy wild-type mice for all parameters measured ($P \leq 0.05$; Figs 3 and 4B, $P \leq 0.01$; Figs 3 and 4C and $P \leq 0.001$ Figs 3 and 4D). I.v. administration of cmRNA$^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ significantly increased the compliance from 0.02 ± 0.01 ml/cmH_2O (*Cftr*$^{-/-}$ mice) to 0.03 ± 0.01 ml/cmH_2O ($P \leq 0.05$), reaching equivalent values to those measured in *Cftr*$^{+/+}$ mice (Fig. 3B). In contrast, the i.v. application of 40 µg cmRNA$^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$ or pDNA^{hCFTR} did not alter compliance significantly. Applying 40 µg of cmRNA$^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ or cmRNA$^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$ i.v. significantly lowered the resistance ($P \leq 0.01$ and $P \leq 0.05$ respectively, Fig. 3C) but pDNA^{hCFTR} did not alter the resistance at a significant level. $FEV_{0.1}$ (human equivalent of FEV_1) of *Cftr*$^{+/+}$ mice defined as projecting 100% forced expiratory volume is pointedly different compared to the $FEV_{0.1}$ value of *Cftr*$^{-/-}$ mice of only 66% of the wild-type ($P \leq 0.001$). I.v. injection of 40 µg cmRNA$^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ significantly improved the $FEV_{0.1}$ by 23 percentage points ($P \leq 0.01$) and i.v. injection of 40 µg cmRNA$^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$ provided a significant $FEV_{0.1}$ improvement of 14 percentage points compared to $FEV_{0.1}$ value of untreated *Cftr*$^{-/-}$ mice ($P \leq 0.05$; Fig. 3D). However, i.v. administration of pDNA^{hCFTR} showed no significant improvement of $FEV_{0.1}$. I.v. injected mock cmRNA^{DsRED} or nanoparticles alone encouragingly aligned with untreated groups in all determined lung function parameters. Taken together, these results demonstrate significant lung function improvement in all relevant lung function parameters of *Cftr*$^{-/-}$ mice intravenously (i.v.) treated with cmRNA^{hCFTR}.

In the i.t. treated groups, a substantial improvement in compliance and resistance could be detected when the cmRNA$^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ dose was increased to 80 µg (0.04 ± 0.01 ml/cmH_2O and 0.86 ± 0.18 cmH_2O.s/ml respectively; $P \leq 0.05$; Fig. 4B,C). However, 80 µg of cmRNA$^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$ i.t. lowered the resistance but did not improve the compliance as effectively as cmRNA$^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ ($P \leq 0.05$; Fig. 4B,C). pDNA^{hCFTR} (80 µg) i.t. treated mice also produced significant improvements of resistance and compliance ($P \leq 0.05$, Fig. 4B,C). In terms of $FEV_{0.1}$, i.t. application of 80 µg cmRNA$^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ was improved by 19 percentage points and i.t. application of 80 µg cmRNA$^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$ improved the $FEV_{0.1}$ by 12 percentage points with respect to untreated *Cftr*$^{-/-}$ mice ($P \leq 0.05$, Fig. 4D). I.t. administration of pDNA^{hCFTR} showed no significant improvement of $FEV_{0.1}$. Taken together, these results demonstrate significant lung function improvement in all relevant lung function parameters of *Cftr*$^{-/-}$ mice treated intratracheally with cmRNA^{hCFTR}.

A well-established functional test, measuring the mouse saliva chloride concentration[33] was conducted to complement the functional results observed using FlexiVent. The saliva chloride concentration detected in

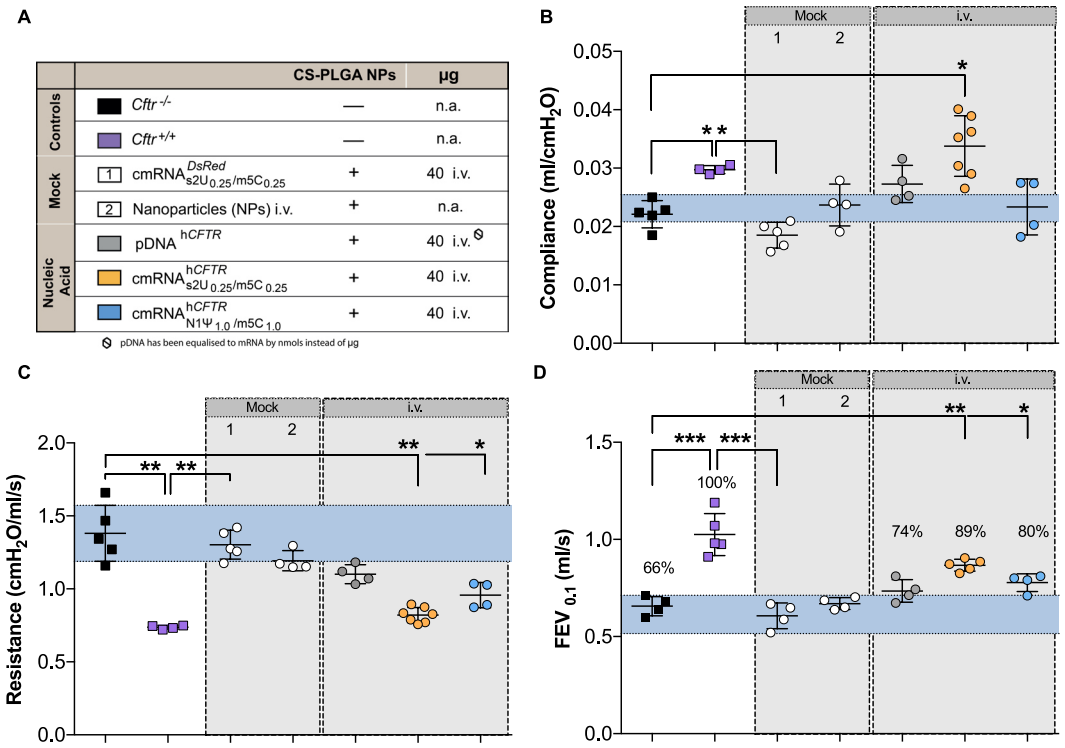

Figure 3. *In vivo* lung function measurements in cmRNA^{hCFTR} and pDNA^{hCFTR} treated *Cftr*$^{-/-}$ mice by i.v. route. All mouse groups utilized in (**B–D**) are color-coded for their treatment schemes (**A**), including dosage and application routes. (**B–D**) Precision *in vivo* lung function measurements covering all relevant outcome parameters on in *Cftr*$^{-/-}$ mice treated twice via i.v. route and measured 72 hours after the 2nd instillment; $n = 4–7$ mice per group. The blue area represents the variance of the negative controls which are biological replicates. Data represent the means ± SD on compliance, resistance and Forced Expiratory Volume in 0.1 seconds ($FEV_{0.1}$). *$P \leq 0.05$; **$P \leq 0.01$ and ***$P \leq 0.001$ versus untreated *Cftr*$^{-/-}$ mice.

Cftr$^{-/-}$ mice (4084 ± 236.8 ng/µl) was significantly higher compared to *Cftr*$^{+/+}$ mice (748.8 ± 96.9 ng/µl, $P \leq 0.001$; Fig. 5A,B). The treatment of $cmRNA^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ i.v. significantly lowered the chloride concentrations in the saliva of *Cftr*$^{-/-}$ mice by more than 52 percentage points ($P \leq 0.01$; Fig. 5A) underlining the FlexiVent results. However, $cmRNA^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$ and pDNA^{hCFTR} treated mice (i.v.) only provided about 20 percentage points reduction. The treatment with $cmRNA^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ i.t. (80 µg) significantly lowered the chloride concentrations in the saliva of *Cftr*$^{-/-}$ mice by 36 percentage points ($P \leq 0.01$; Fig. 5B). $cmRNA^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$ treated mice (i.t.) abridged the chloride concentration not significantly in saliva of *Cftr*$^{-/-}$ mice but pDNA^{hCFTR} treated *Cftr*$^{-/-}$ provided a significant reduction ($P \leq 0.01$; Fig. 4B) but not as proficiently as $cmRNA^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$. The mock mRNA treated group and just nanoparticle treated group failed to decrease the chloride concentration.

cmRNA^{hCFTR} and hCFTR protein quantification in lungs after application *in vivo*. All *in vivo* experiments were performed with nanoparticles if not stated otherwise. We tested for the localization of cmRNA^{hCFTR} complexed with nanoparticle in the lungs after i.t. or i.v. application via RT-qPCR, quantified the hCFTR protein expression with hCFTR ELISA and then evaluated its immunogenicity depending on modification. In contrast to the *in vitro* data, when 40 µg $cmRNA^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ was i.v. injected into the mice, this resulted in a higher accumulation of that mRNA in the lung as compared to 40 µg $cmRNA^{hCFTR}_{N1\Psi_{1.0}/m5C_{1.0}}$ and pDNA^{hCFTR} ($P \leq 0.01$, Fig. 6C). More importantly, we wanted to analyze if there is a significant increase in hCFTR protein levels in the lungs of treated mice by hCFTR ELISA (Fig. 6B,E). These analyses confirmed that mice treated with 40 µg $cmRNA^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ i.v. had a highly significant increase of hCFTR protein in the lungs of treated mice vs. control mice ($P \leq 0.01$; Fig. 6B). Besides, we tested the effects of an increased amount (80 µg) of cmRNAs and pDNA^{hCFTR} with i.t. instillation, $cmRNA^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ and pDNA^{hCFTR} showed a clear and significant increase of hCFTR protein compared to control mice (Fig. 6E) ($P \leq 0.01$). All the mock controls used in hCFTR ELISA have proven to be not significantly different from the negative control.

cmRNA^{hCFTR} immunogenicity *in vivo* in mice after i.v. application. All *in vivo* experiments were performed with nanoparticles if not stated otherwise. First, we applied different compounds such as nanoparticles, *E. coli* extract total RNA (positive control), cmRNA^{hCFTR} and pDNA^{hCFTR} i.v. or i.t. to mice and monitored their immune reaction at three different time points. Applying 40 µg cmRNA^{hCFTR} (with any modifications used) or

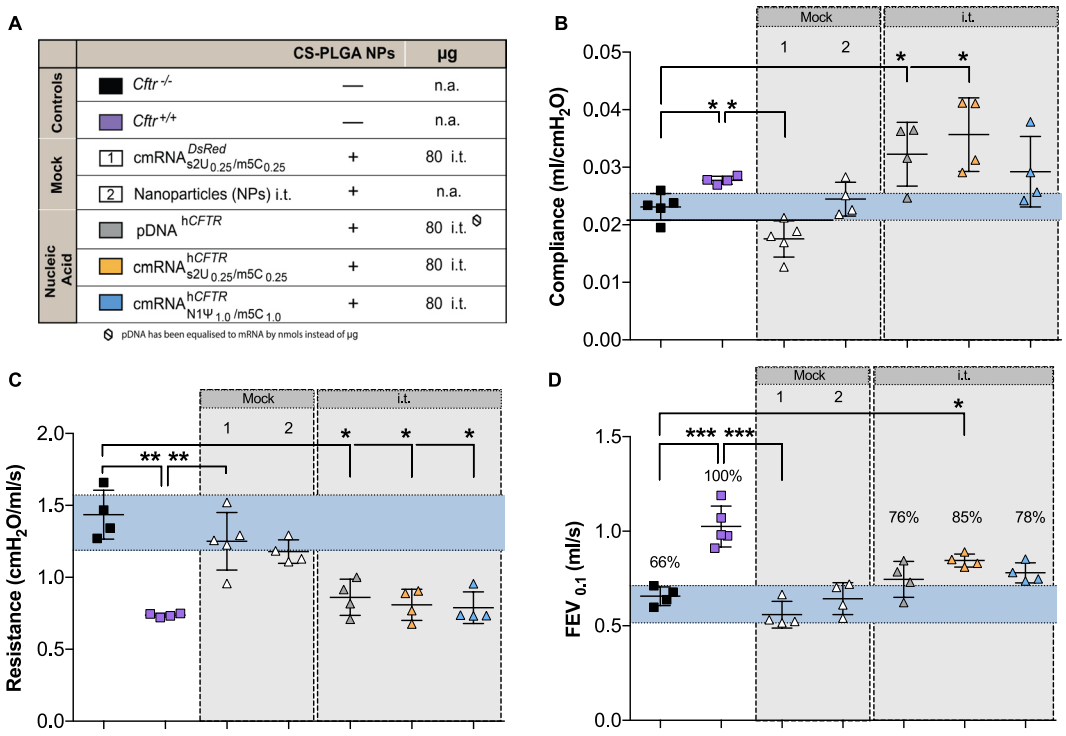

Figure 4. *In vivo* lung function measurements in cmRNA^{hCFTR} and pDNA^{hCFTR} treated *Cftr*$^{-/-}$ mice by i.t. route. All mouse groups utilized in (**B–D**) are color-coded for their treatment schemes (**A**), including dosage and application routes. (**B–D**) Precision *in vivo* lung function measurements covering all relevant outcome parameters on *Cftr*$^{-/-}$ mice treated twice via i.t route and measured 72 hours after the 2nd instillment; $n = 4–7$ mice per group. The blue area represents the variance of the negative controls which are biological replicate. Data represent the means ± SD on compliance, resistance and Forced Expiratory Volume in 0.1 seconds ($FEV_{0.1}$). *$P \leq 0.05$; **$P \leq 0.01$ and ***$P \leq 0.001$ versus untreated *Cftr*$^{-/-}$ mice.

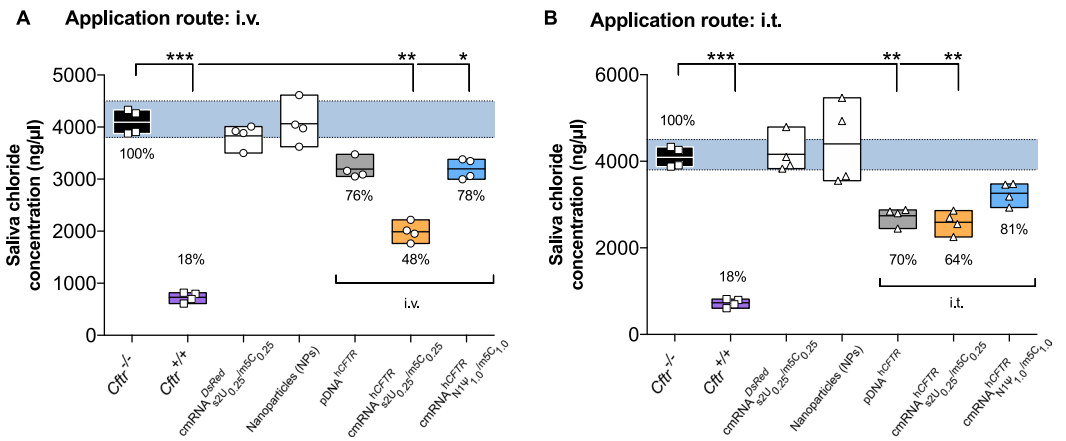

Figure 5. *In vivo* saliva chloride concentration measurement of cmRNA^{hCFTR} and pDNA^{hCFTR} treated *Cftr*$^{-/-}$ mice by i.v./ i.t. route (**A**,**B**) Functional test of reconstituted CFTR channel and reduced chloride concentration after i.v. (**A**) or i.t. (**B**) treatment of *Cftr*$^{-/-}$ mice compared to untreated *Cftr*$^{-/-}$ (black), positive controls (violet), and percentages relative to the positive control; $n = 4$ mice per group; two mock controls were included (white); boxes represent the means ± minimum and maximum values. The blue area represents the variance of the negative controls which are biological replicates. *$P \leq 0.05$; **$P \leq 0.01$ versus untreated *Cftr*$^{-/-}$ mice.

pDNA^{hCFTR} i.v. or i.t. did not lead to detectable responses of key cytokines IFN-α or TNF-α (detected by ELISA) at all three-time points (Fig. 7A)[34,35]. Nanoparticles alone (used in all *in vivo* experiments) showed no immune response over the detection limit. However, as expected the positive control (*E. coli* extract total RNA) i.v. and i.t. resulted in a significant increase of IFN-α and TNF-α at 6 h and a trend increase of IFN-α at 24 h, while an effect at 72 h was not detectable (Fig. 7A). No immune response had been observed apart from positive control in groups treated intratracheally (i.t) (Fig. 7B).

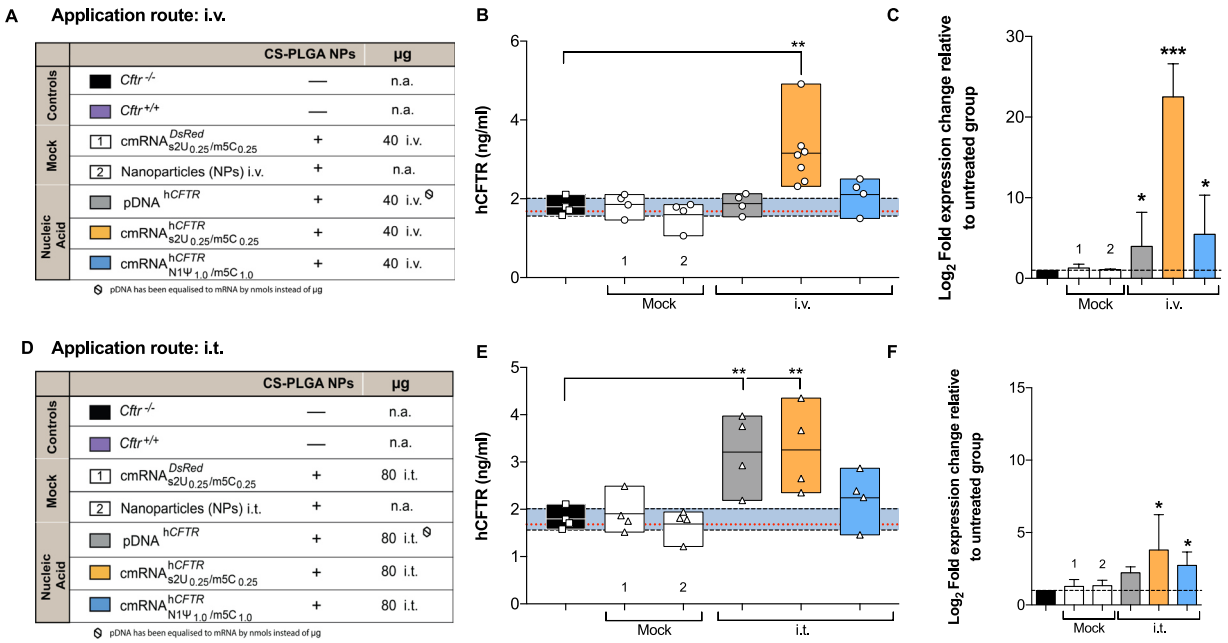

Figure 6. Expression of hCFTR protein in mouse lungs and delivery of cmRNA^{hCFTR} and pDNA^{hCFTR} in lungs. (**A**,**D**) All mouse groups, particles and particle combinations depicted in the study plan are color-coded for their treatment schemes, including dosage and application routes. (**B**,**E**) hCFTR ELISA, detecting specifically human CFTR, was performed on lung preparations at day 6 from *Cftr*$^{-/-}$ mice treated twice via i.v. (**B**) or i.t. (**E**) route and measured 72 hours after the 2nd instillment (endpoint); the same $n = 4$–7 mice per group were used. (**C**,**F**) Relative amounts of differently modified h*CFTR* mRNAs in the lungs applied i.v. or i.t., then determined by RT-quantitative PCR, compared to untreated *Cftr*$^{-/-}$ mice ($^{*}P \leq 0.05$); $n = 4$–7 mice per group. All bar graph data are depicted as means ± SDs while box plots data are depicted as the means ± minimum to maximum values. The blue area represents the variance of the negative controls which are biological replicates. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ versus untreated *Cftr*$^{-/-}$ mice.

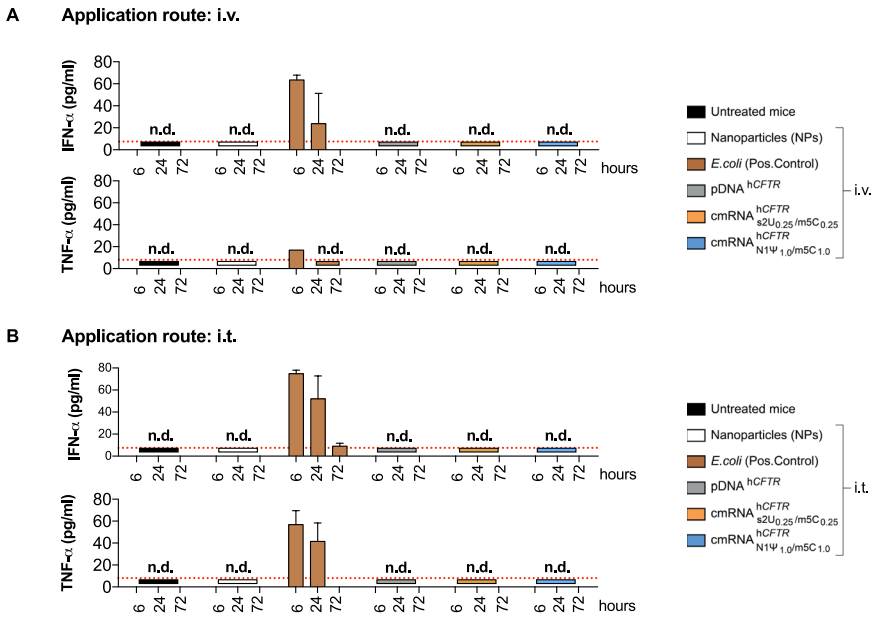

Figure 7. (c)mRNA^{hCFTR} and pDNA^{hCFTR} mediated immunogenicity *in vivo* Mice were i.v. or i.t. injected with a mix of (c)mRNA and NPs at a 1:10 ratio and ELISAs were performed post-i.v./i.t.-injection at three different time points. n.d., not detectable. The red dotted lines in (**A**,**B**) mark the detection limit as specified in the respective ELISA kit. All bar graph data are depicted as the means ± SD and box plots data are represented as the means ± minimum to maximum values.

Discussion

Although much progress has been achieved since the discovery of the CFTR gene 25 years ago, there is still a substantial need to restore robust CFTR function in patients suffering from cystic fibrosis[8]. With the recent approvals of the small molecule agents ivacaftor and lumacaftor, science has paved a possible way to overcome the hurdles caused by the disease-conferring gene. Those treatments can be more or less effectively applied to patients bearing *CFTR* mutations delF508 (Lumacaftor-ivacaftor/Orkambi) and G551D (ivacaftor)[36–39]. However, lung function, as one of the main outcome parameters probably having the most significant influence on life quality of CF patients, is rarely tested in preclinical models. In fact, actual effects of (modern) existing drugs on lung function, with forced expiratory volume in one second (FEV_1) as a key parameter, are quite low[40]. Here, by using cmRNA^{hCFTR}, we are presenting a proof of concept for a viable and potent therapeutic alternative. We have vigorously tested mRNA therapy with focus on *in vivo* lung function normalization while avoiding any possible, unwanted immune responses for a possibility of repeated dosing. The unique formulation utilized can be used both topically (intratracheally) and systemically (via i.v. injection), having in both cases a profound effect on normalizing the lung function parameters, including compliance, resistance and $FEV_{0.1}$ of treated $Cftr^{-/-}$ mice to values obtained from $Cftr^{+/+}$ mice.

In vitro, using cmRNA^{hCFTR}, CFTR protein expression in CFBE41o− cells was increased up to 5.5-fold compared to mRNA^{hCFTR}, which is consistent with previous studies obtained by us and others[18,31,41]. Incorporation of naturally occurring chemically modified nucleosides has been shown to suppress inhibitory effects on translation by avoiding detection by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) TLR3, TLR7, and TLR8[34,35]. Those receptors play a crucial role in the detection, processing, and degradation of mRNA. Interestingly, depending on the mRNA modification, kinetics of hCFTR expression varies upon the different nucleosides used. In fact, after 72 h we only observe an increased quenching of Yellow fluorescent protein (YFP) in YFP assay in CFTR null A549 and CFBE41o− cells by pDNA^{hCFTR} which would corroborate our findings from our flow cytometry and western blot analyses in CFBE41o− cells. In contrast there is a significant increase in I^- influx by functional hCFTR channels and quenching of YFP at 48 h post transfection by cmRNA^{hCFTR}. Consequently, we assume that upon different cell lines, kinetics by which the hCFTR protein is expressed varies. Earlier studies support our notion that differently modified mRNAs can have an impact on the translational effect between distinct cell lines[31,35].

To better mimic *in vivo* human conditions, we performed an *ex vivo* whole blood assay (WBA) which offers a more complex environment to test for immune responses. This assay has already been used in a number of preclinical settings, and Coch and colleagues could demonstrate that it has the potential to reflect broad aspects of the *in vivo* cytokine release caused by oligonucleotides[42]. Indeed, we could show that the small molecule Resiquimod (serving as a positive control by activating TLR7 and TLR8) lead to a substantial release of IFN-α, TNF-α and IL-8. pDNA^{hCFTR}, as well as unmodified mRNA^{hCFTR}, also showed elevated cytokine levels probably due to the activation of innate immune receptors[34,35]. In contrast, incorporation of modified nucleosides into h*CFTR* mRNA (cmRNA^{hCFTR}) abolished such responses, with no detectable amounts of IFN-α. This is in concert with previously published data, demonstrating cmRNA's limited immune responses, mainly by evading detection from receptors such as TLRs, RIG-1, MDA-5 or PKR[34,41]. Interestingly, even though TNF-α or IL-8 could be detected, it rather shows donor-dependency than effects deriving from NPs and/or cmRNA^{hCFTR} with cytokine levels being all within the variance of negative controls. Although it mirrors only the blood compartment and does not reflect the more complex *in vivo* situation, the WBA can give a prediction of how cytokines are released in the human system in response to systemically applied (c)mRNA prior to clinical testing.

To determine the clinical potential of CFTR-encoded cmRNA we compared not only different modifications *in vivo* but also two different routes of administration. I.t. application has been chosen for this study on the base of our previous findings of applying cmRNA i.t. in a surfactant protein-B deficient mouse model leading to significantly prolonged survival[26]. Given the fact that in patients suffering from CF one of the key barriers is the airway mucus layer in which inhaled particles are more likely to get trapped and removed, we sought to apply cmRNA^{hCFTR}/pDNA^{hCFTR} complexed to NPs by i.v. injection as an alternative administration route. Systemic delivery via lipid-modified polymeric nanoparticles have been already shown to target the lungs efficiently[43].

To support our notion of improved CFTR activity, we performed extensive lung function measurements using state-of-the-art technology to provide detailed *in vivo* information on different lung function parameters. There are doubts about $Cftr^{-/-}$ mice as a proper model for cystic fibrosis as it does not reflect the typical lung phenotype seen in CF patients[44]. However, the reason behind that seems to be in how deeply lungs or other affected organs had been investigated. A layer of material can be observed with characteristics of an acid mucopolysaccharide on the bronchiolar surface and is also evident in alveoli by using scanning electron microscopy in $Cftr^{-/-}$ mice, which is not evident in $Cftr^{+/+}$ mice[45]. It has also been reported $Cftr^{-/-}$ mice shows similar effect of CF patients like, age-dependent pulmonary inflammation, death of respiratory epithelial cells and high vulnerability to severe *Pseudomonas aeruginosa* infection[46]. Recent studies could demonstrate reduced airway compliance and increased resistance in comparison to wild-type mice[47,48]. Indeed, we observed significantly higher and lower levels regarding resistance and compliance, respectively, in $Cftr^{-/-}$ controls and mock-treated $Cftr^{-/-}$ mice compared to homozygous wild-type mice ($Cftr^{+/+}$) mice and demonstrated that treatment with cmRNA^{hCFTR}-NPs improved compliance and resistance significantly equal to those seen in healthy $Cftr^{+/+}$ mice. FEV_1 percentage (for mouse or small animal $FEV_{0.1}$) is related to survival in CF and a most important physiological parameter for CF patients. A previous study demonstrated that patients with a $\%FEV_1$ of <30 compared to healthy individuals had a 50% chance of mortality within 2 years and hence are regularly examined in clinical setup[49]. A strong variance amid $Cftr^{-/-}$ controls and mock-treated $Cftr^{-/-}$ mice compared to homozygous wild-type mice ($Cftr^{+/+}$) mice has been perceived in the case of $FEV_{0.1}$. Our study provides a significant improvement of $FEV_{0.1}$ due to treatment with NP-cmRNA^{hCFTR}. Interestingly, NP-pDNA^{hCFTR} when administered via i.t. route improved parameters of lung function measurements including $FEV_{0.1}$, but not as significant as cmRNA^{hCFTR}. We also observed i.v. or i.t.

administration of $cmRNA^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ to positively compensate most of lung function parameters. Overall, we could demonstrate that certain protocols, applying $cmRNA^{hCFTR}$ either i.v. or i.t. efficiently restored lung function values equal to those of wild-type. Suggesting a more even distribution through arteries and the bronchial circulation by i.v. injection, this route and formulation could lead to a very potent therapy especially for newborns and young infants. By providing functional CFTR early in life, the lungs could be protected from irreversible damage. Nevertheless, when applied intratracheally, which mimics deep inhalation of a spray or powder formulation (primary application route in adults), an adjustment in dose and/or formulation (e.g. $cmRNA^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ increased to 80 µg) might easily abrogate any negative effect of the $Cftr^{-/-}$ genetic background on lung function.

Eventually, we determined the impact of $cmRNA^{hCFTR}$ and $pDNA^{hCFTR}$ on another relevant physiological outcome such as the saliva chloride concentration to evaluate therapeutic effect and complement the lung function results. Sweat chloride concentration has become an accepted method as a diagnostic readout to assess treatment effects of CF patients[50]. As an analog, chloride concentration of β-adrenergic stimulated salivary glands of $Cftr^{-/-}$ mice can be investigated as it complies with findings in CF patients[33]. In this study, we could show a substantial difference in salivary Cl^- content of $cmRNA^{hCFTR}$ and $pDNA^{hCFTR}$ treated mice – both, i.v. and i.t. – compared to their untreated counterpart. With end point-analysis, a significant decrease in Cl^- to nearly 50% was observed, indicating a restoration of CFTR in the duct compartment of salivary glands and thus leading to an improved Cl^- absorption. Previous studies estimated that a restoration of CFTR activity to 50% could lead to sweat chloride levels to near normal levels in CF patients. Given that, it is possible that $cmRNA^{hCFTR}$ treatment has the potential to improve CFTR activity to levels that are at least similar to those in patients with a mild CF phenotype[51].

In this study, by applying $cmRNA^{hCFTR}$ consecutively, both modifications were successfully delivered to the lungs with the i.v. route being more efficient at doses of 40 µg (2 mg/kg body weight) per treatment. Intriguingly, in contrast to the results obtained *in vitro*, $cmRNA^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ showed a significantly higher CFTR protein expression with higher accumulation of h*CFTR* mRNA in lung cells. Assuming differences of cmRNA-encoded transgene expression between distinct cell lines, it is plausible to consider such differences between *in vitro* versus *in vivo* applications, which is by far more complex. In this respect, the higher amount of $cmRNA^{hCFTR}_{s2U_{0.25}/m5C_{0.25}}$ found in lung cells after i.v. injection, might be due to the fact that its nucleoside composition is more favorable to evade PRRs, thus being less degraded. However, regardless of cmRNA kinetics we also observed differences in the delivery route of $cmRNA^{hCFTR}/pDNA^{hCFTR}$ -NPs. Our data suggest i.v. injection to be more efficient in delivering such complexes to the lung than topical administration. Tests of $cmRNA^{hCFTR}$ -NP's capacity of mucus penetration are in planning phase including detection of $cmRNA^{hCFTR}$ and CFTR protein (glycosylated) in a *Cftr*-deficient mouse model especially at the apical side of the bronchial epithelium. The upper airways are lined with mucus and mucociliary movements clear foreign particles immediately. In addition, the main barriers in the deeper areas are the alveolar lining, scavenger transporters and alveolar macrophages[52,53]. We, therefore, concluded that the original dosing by which cmRNA-NPs were delivered i.t. was not as efficient as using the i.v. route. Indeed, increasing the amount by doubling the dose (to 80 µg) for each treatment showed a hCFTR expression close to levels seen using the i.v. route.

To exclude immune reactions caused by either NPs or the $cmRNA^{hCFTR}$ itself, we conducted extensive immune assay tests *in vivo*. Except for the positive control (*E. coli* total mRNA), we could not detect any immunostimulatory effect *in vivo* that could arise from NPs or the $cmRNA^{hCFTR}$. These results confirm our previous studies in which we showed that NPs, as well as modified mRNA, could be administered safely to the lungs without any substantial increase in cytokines, or inflammatory-related cells such as macrophages or neutrophils[26]. Systemic delivery has also been reported to have no impact on proinflammatory cytokine secretion[29].

Taken together, this study is the first proof of concept of efficient application of NP-$cmRNA^{hCFTR}$ *in vivo* to restore lung function in a *Cftr*-deficient mouse model. Importantly, we could neither detect immune responses *in vivo* nor in a more defined setting *ex vivo*. Applying $cmRNA^{hCFTR}$ to $Cftr^{-/-}$ mice could efficiently restore lung function close to levels of healthy control mice. In addition, our study compared - apart from two well-known mRNA modifications and $pDNA^{hCFTR}$ - also two different delivery routes, demonstrating that systemic administration of cmRNA targets lung cells more efficiently at lower dosages. This study provides a proof of concept for alternative treatment of patients suffering from CF. $cmRNA^{hCFTR}$ transcript supplementation may be broadly applicable for most *CFTR* mutations, not only in adults but already in the postnatal state, thereby protecting the lungs from exacerbations from the very beginning of life.

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Author Contributions

A.H., A.D., J.S.A. and M.S.D.K. designed the experiments. A.H., A.D., J.S.A., G.R.S., P.W., N.L., N.P., E.S., A.R. and J.L. performed experiments. A.H., A.D. and B.W. analyzed the data. P.S., C.S., H.Y., B.L., C.M.L. and R.H. provided material. A.H., A.D., J.R. and M.S.D.K. interpreted the data. A.H. A.D., J.R. and M.S.D.K. drafted the paper, and A.H. and M.S.D.K. wrote the final version of the paper.

Additional Information

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Competing Interests: M.S.D.K. holds a patent on RNA modification (EP2459231B1). M.S.D.K., A.H., A.D. and J.S.A., hold a European patent on delivery of cmRNA^{hCFTR} complexed with nanoparticles (17169561.2-1401).

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SHORT COMMUNICATION Open Access

Gene correction of *HBB* mutations in CD34$^+$ hematopoietic stem cells using *Cas9* mRNA and ssODN donors

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Abstract

Background: β-Thalassemia is an inherited hematological disorder caused by mutations in the human hemoglobin beta (*HBB*) gene that reduce or abrogate β-globin expression. Although lentiviral-mediated expression of β-globin and autologous transplantation is a promising therapeutic approach, the risk of insertional mutagenesis or low transgene expression is apparent. However, targeted gene correction of *HBB* mutations with programmable nucleases such as CRISPR/Cas9, TALENs, and ZFNs with non-viral repair templates ensures a higher safety profile and endogenous expression control.

Methods: We have compared three different gene-editing tools (CRISPR/Cas9, TALENs, and ZFNs) for their targeting efficiency of the *HBB* gene locus. As a proof of concept, we studied the personalized gene-correction therapy for a common β-thalassemia splicing variant $HBB^{IVS1-110}$ using *Cas*9 mRNA and several optimally designed single-stranded oligonucleotide (ssODN) donors in K562 and CD34$^+$ hematopoietic stem cells (HSCs).

Results: Our results exhibited that indel frequency of CRISPR/Cas9 was superior to TALENs and ZFNs ($P < 0.0001$). Our designed sgRNA targeting the site of $HBB^{IVS1-110}$ mutation showed indels in both K562 cells (up to 77%) and CD34$^+$ hematopoietic stem cells—HSCs (up to 87%). The absolute quantification by next-generation sequencing showed that up to 8% site-specific insertion of the *Nhe*I tag was achieved using *Cas9* mRNA and a chemically modified ssODN in CD34$^+$ HSCs.

Conclusion: Our approach provides guidance on non-viral gene correction in CD34$^+$ HSCs using *Cas*9 mRNA and chemically modified ssODN. However, further optimization is needed to increase the homology directed repair (HDR) to attain a real clinical benefit for β-thalassemia.

Keywords: *HBB*, Beta-thalassemia, Gene correction, CRISPR/Cas9, IVS1–110, *Cas9* mRNA

Background

β-Thalassemia (OMIM: 613985) is an inherited hematological disorder caused by mutations of the human hemoglobin beta (*HBB*) gene, leading to deficient β-globin expression and severe anemia [1]. The current treatment options include allogeneic bone marrow transplantation and hematopoietic stem cell (HSC) transplantation but are limited to histo-compatible donors. However, gene therapy based on autologous transplantation of a lentiviral-transferred *HBB* gene to HSCs resulted in remarkable clinical benefit [2, 3]. Though the safety and efficacy of lentiviral-based gene therapy is positive in a treated patient, the transactivation of the proto-oncogene *HMGA2* and more than 3500 unique integration sites in tested mouse model forewarns the possibility of insertional mutagenesis [2, 4]. Earlier retroviral gene therapy studies on other inherited diseases reported the treatment-related leukemogenesis [5–7], and lentiviral

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therapy resulted in T cell lymphoma in a mouse model of X-SCID due to random integration into oncogenes [8]. Therefore, the ideal gene therapy with viral vectors must ensure targeted integration of a therapeutic *HBB* transgene in the endogenous locus. Otherwise, personalized gene-correction therapy with programmable nucleases and non-viral repair templates such as single-stranded oligonucleotides (ssODNs) must be employed as it is less likely to randomly integrate into the genome and result in a safe and precise gene editing [9]. Though several studies targeted *HBB* gene with ZFNs, TALENs, and CRISPR/Cas9, no study has ever compared all the three gene-editing platforms simultaneously. Therefore, in the present study, we compared these three approaches for their target efficiency in parallel. Here, we tested the gene correction efficiency of strategically designed ssODNs as repair templates to target *HBB* gene. This is an important measure while editing the highly proliferating stem cell population to avoid clonal selection and thereby triggering oncogenesis.

Correspondence/findings

First, we designed ZFNs, TALENs, and CRISPR/sgRNA for targeting the promoter region of the *HBB* gene (Additional file 1: Figure S1). The *HBB* gene-targeting efficacy of designed ZFNs, TALENs, or CRISPR/Cas9 was determined by T7 endonuclease-I (T7EI) assay in HEK293 cells. Interestingly, CRISPR/Cas9 exhibited higher indels for all three different concentrations (0.5 μg, 1.0 μg, and 1.5 μg) compared to ZFNs and TALENs (Fig. 1a; Additional file 1: Figure S1). Similar results were observed earlier for the $HBB^{IVS2-654}$ mutation where gene-targeting efficiency of CRISPR/Cas9 was superior to TALENs [10].

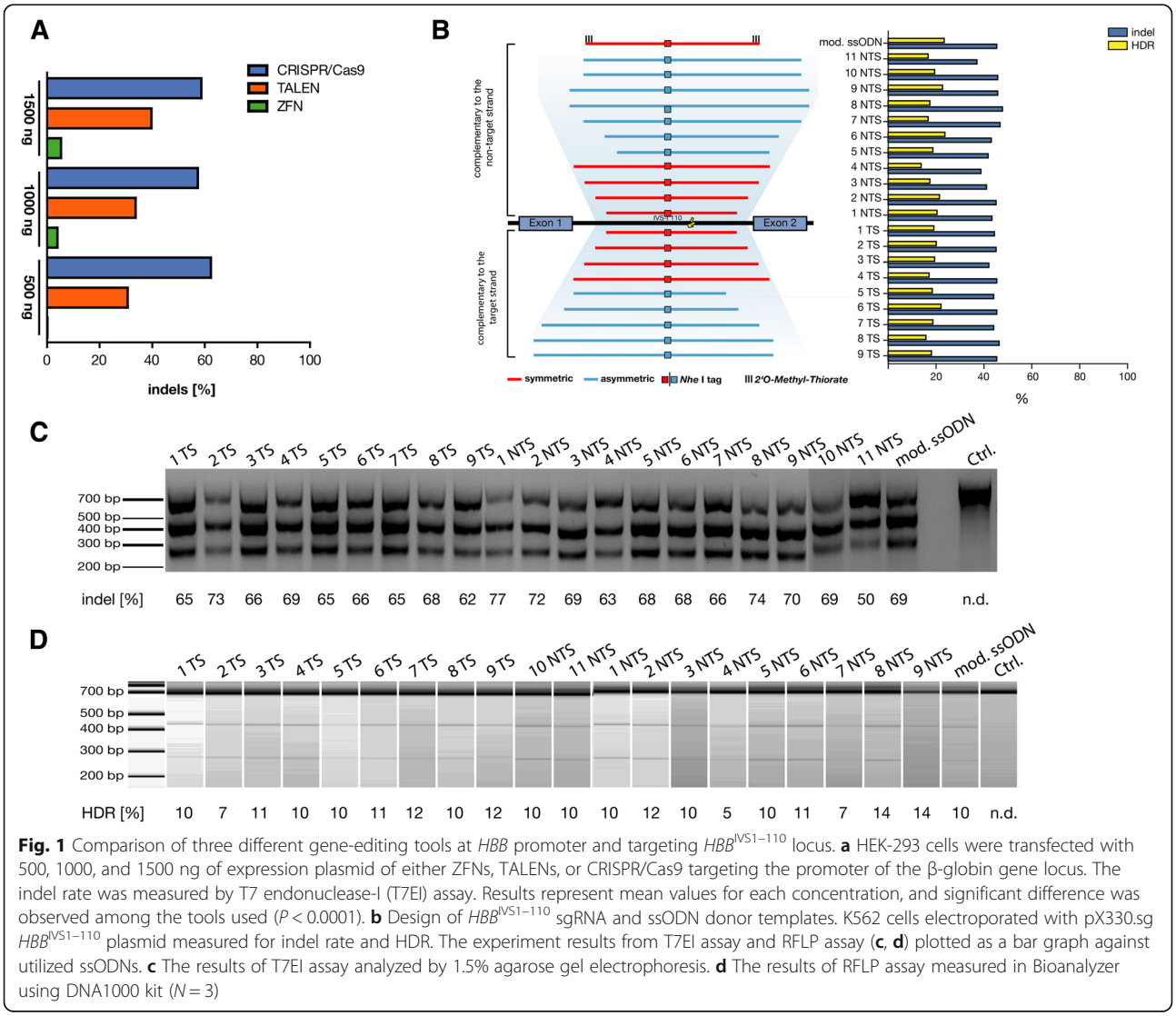

Fig. 1 Comparison of three different gene-editing tools at *HBB* promoter and targeting $HBB^{IVS1-110}$ locus. **a** HEK-293 cells were transfected with 500, 1000, and 1500 ng of expression plasmid of either ZFNs, TALENs, or CRISPR/Cas9 targeting the promoter of the β-globin gene locus. The indel rate was measured by T7 endonuclease-I (T7EI) assay. Results represent mean values for each concentration, and significant difference was observed among the tools used ($P < 0.0001$). **b** Design of $HBB^{IVS1-110}$ sgRNA and ssODN donor templates. K562 cells electroporated with pX330.sg $HBB^{IVS1-110}$ plasmid measured for indel rate and HDR. The experiment results from T7EI assay and RFLP assay (**c**, **d**) plotted as a bar graph against utilized ssODNs. **c** The results of T7EI assay analyzed by 1.5% agarose gel electrophoresis. **d** The results of RFLP assay measured in Bioanalyzer using DNA1000 kit ($N = 3$)

Therefore, we focused on CRISPR/Cas9 to examine the gene correction efficiency of non-viral repair templates. Several studies have targeted the *HBB* gene using CRISPR/Cas9 system in HSCs, induced pluripotent stem cells, and human embryos [11–16]. Most of these studies were either focused on *HBB* gene addition or targeting sickle cell disease mutation. To the best of our knowledge, this is the first study that attempted to target a common β-thalassemia splicing variant $HBB^{IVS1-110}$ (rs35004220), which leads to an alternative splice site and reduced β-globin expression with a non-viral strategy [17].

To target the $HBB^{IVS1-110}$ locus, we designed a sgRNA and several ssODNs (Fig. 1b; Additional file 1: Figure S1) with varying lengths of homology arms, symmetrical difference, and chemical modifications and complimentary to the strand targeted or not targeted by the guide-RNA [18]. To evaluate the ability of CRISPR/Cas9 to correct the $HBB^{IVS1-110}$ mutation by an exogenous DNA sequence, we introduced an *Nhe*I-tag (restriction site) into the ssODNs that can be stably integrated upon successful homology-directed repair (HDR) (Fig. 1b). We electroporated pX330.sg $HBB^{IVS1-110}$ plasmid and ssODN donors harboring *Nhe*I-tag using a Neon system into K562 cells and evaluated the frequency of indels by T7EI assay and HDR-driven *Nhe*I integration by restriction fragment length polymorphism (RFLP) assay (Figs. 1c/d). Mean indel frequencies for the targeted loci were 44 ± 18%. We next determined whether any of the rationally designed ssODNs could stimulate gene targeting by HDR and found that most of the tested ssODNs resulted in similar HDR frequencies with the median of 20% (range 5–31%).

To assess our non-viral correction strategy with bone marrow-derived $CD34^+$ HSCs, we co-delivered pX330.sg $HBB^{IVS1-110}$ plasmid and several ssODNs. Unlike our results with K562 cells, the indel frequencies in HSCs were relatively low with a median of 30% (range 0–56%) and only one ssODN (5TS) exhibited 3% HDR rate in TIDE analysis (Fig. 2a; Additional file 1: Figure S3.A). We next sought to determine HDR rate for 5TS-ssODN by a semi-quantitative single-colony sequencing and found that 2% (3/172) of clones showed *Nhe*I insertions (Fig. 2b). We observed that pDNA resulted in lower transfection rate and higher cell death in HSCs (data not shown). Due to the low indel rate achieved by pDNA-encoded Cas9, we aimed to increase the efficiency and viability by using mRNA-encoded *Cas*9 as others and ourselves earlier reported the superiority of mRNA over pDNA [19–21]. In addition, transiently expressed *Cas*9 mRNA resulted in reduced off-targets compared to long-term Cas9 expression systems [22]. Therefore, we delivered *Cas*9 mRNA and chemically modified sgRNA with four different ssODNs (chemically modified, 2 PAM-depleted, 5TS) using a 4D-Nucleofector. Genomic analysis with the T7EI assay resulted in high indels ranging from 65% (mod. ssODN) to 87% (11 NTS), and the RFLP assay showed up to 11% integration of *Nhe*I tag at the $HBB^{IVS1-110}$ locus (Fig. 2c). Our results clearly imply the superiority of *Cas*9 mRNA over pDNA (Additional file 1: Figure S3.B). We noticed significant enrichment of 6-bp insertion (range 2–9%) by *Nhe*I integration at the target locus for tested ssODNs (Fig. 2d; Additional file 1: Figure S4).

However, the absolute quantification of site-specific insertion events of the *Nhe*I tag at the $HBB^{IVS1-110}$ locus by next-Generation sequencing showed 8% correction for modified ssODN (Fig. 2e; Additional file 1: Figure S5). All the analyses spotted improved gene correction for chemically modified ssODN in HSCs as reported earlier [23]. No detectable off-target cleavage was found in six of the in silico predicted off-targets (Additional file 2: Table S4; Additional file 1: Figure S6) for the sgRNA-targeted $HBB^{IVS1-110}$ locus**.** We found that CRISPR/Cas9 greatly facilitates targeted genome modification compared to TALENs and ZFNs, and introduction of new DNA sequences in HSCs using *Cas*9 mRNA and ssODN is feasible without viral vectors. However, correction of 8% in HSCs at ex vivo setting is sub-optimal. Therefore, further improvement on HDR efficacy and selection of corrected cells is needed to attain a meaningful gene correction of *HBB* mutations to treat β-thalassemia and other related genetic diseases.

Online methods

Design of gene-editing tools

The targeting efficacy at the promoter of the *HBB* gene locus (200 bp upstream of the transcription start site) between three different gene-editing tools (CRSIPR/Cas9, TALENs, and ZFN) was evaluated (Additional file 1: Figure S1). The ZFN constructs targeting the promoter were ordered from Sigma-Aldrich (http://www.sigmaaldrich.com). The full amino acid sequences of the ZFN pair are shown in Additional file 2: Data S1. The targeting TALEN pair was designed with the help of the online tool ZiFiT Targeter Version 4.2 (http://zifit.partners.org) and assembled by fast ligation-based automatable solid-phase high-throughput (FLASH). Plasmids encoding TALE repeats harboring different repeat variable di-residues (RVDs) with their FLASH IDs are summarized in Additional file 2: Table S1. The sgRNAs for promoter (5′-AGCCAGTGCCAGAAGAGCC A-3′) and $HBB^{IVS1-110}$ (5-GGGTGGGAAAATAGACCAA T-3′) were designed using CRISPR design tool. The respective oligo pairs encoding the 20-nt guide sequences are annealed and ligated into a pX330 vector consisting of a *Sp*Cas9 expression cassette and a U6 promoter driving the expression of chimeric sgRNA. The chemically modified sgRNA for $HBB^{IVS1-110}$ mutation was synthesized by incorporating 2-*O*-methyl 3′phosphorothioate

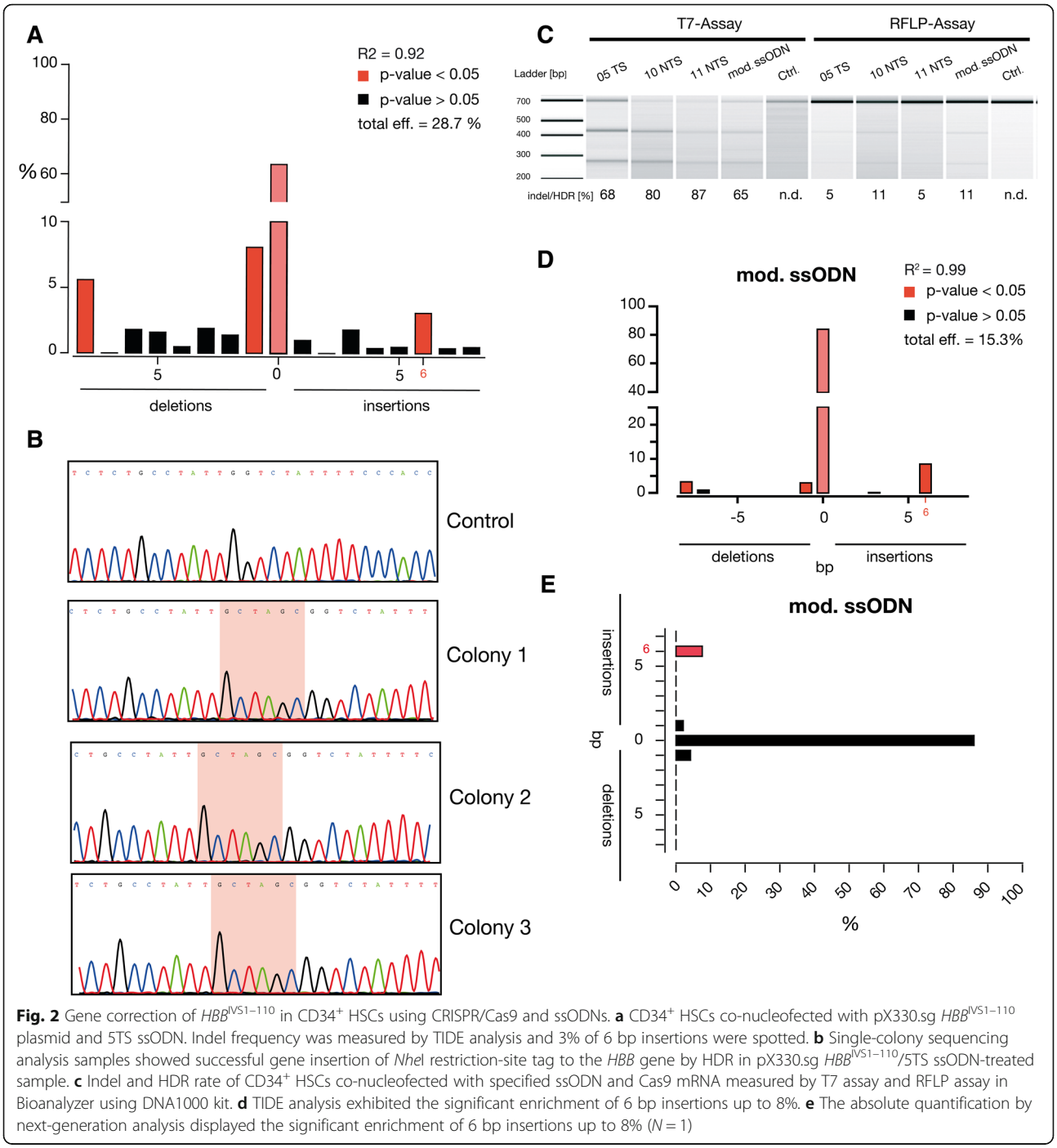

Fig. 2 Gene correction of *HBB*$^{IVS1-110}$ in CD34^{+} HSCs using CRISPR/Cas9 and ssODNs. **a** CD34^{+} HSCs co-nucleofected with pX330.sg *HBB*$^{IVS1-110}$ plasmid and 5TS ssODN. Indel frequency was measured by TIDE analysis and 3% of 6 bp insertions were spotted. **b** Single-colony sequencing analysis samples showed successful gene insertion of *Nhe*I restriction-site tag to the *HBB* gene by HDR in pX330.sg *HBB*$^{IVS1-110}$/5TS ssODN-treated sample. **c** Indel and HDR rate of CD34^{+} HSCs co-nucleofected with specified ssODN and Cas9 mRNA measured by T7 assay and RFLP assay in Bioanalyzer using DNA1000 kit. **d** TIDE analysis exhibited the significant enrichment of 6 bp insertions up to 8%. **e** The absolute quantification by next-generation analysis displayed the significant enrichment of 6 bp insertions up to 8% ($N = 1$)

(MS) modification at three terminal nucleotides at both the 5′ and 3′ ends.

Cell culture and transfection

HEK-293 cells were cultured in DMEM (https://www.thermofisher.com) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO_2. In HEK-293 cells, pDNA-encoded gene-editing tools (CRSIPR/Cas9, TALENs, and ZFN) were transfected using Lipofectamin 3000 at three different concentrations of 0.5, 1, and 1.5 μg. K562 cells were cultured in RPMI 1640 (https://www.thermofisher.com) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C with 5% CO_2. In K562 cells, 200 ng of pX330-Chimeric vector targeting *HBB*$^{IVS1-110}$ loci was co-electroporated with 10 pmol of different ssODN

using Neon Transfection System (https://www.thermofisher.com). Bone marrow-derived $CD34^+$ HSCs from healthy donors were cultured in StemSpan™ serum-free medium II (SFEMII) containing StemSpan™ Cytokine Cocktail 110 (https://www.stemcell.com). 1×10^5 $CD34^+$ HSCs were electroporated with 1.2 μg pX330 vector targeting $HBB^{IVS1-110}$ mutation and 100 pmol of specified ssODN repair template using Lonza© 4D-Nucleofector™ (Program: EO 100). The similar amount of p.Max eGFP pDNA (1.2 μg) was electroporated as a transfection control. For Cas9 mRNA experiments, 1×10^5 $CD34^+$ HSCs were co-electroporated with 5 μg chemically modified sgRNA, 10 μg Ca9 mRNA vector, and 100 pmol of specified ssODN repair template using Lonza© 4D-Nucleofector™ (Program: EO 100).

Repair templates

The ssODNs were synthesized by Metabion, Germany (www.metabion.com). The ssODNs were designed with an insertion site harboring an *Nhe*I recognition site (GCTAGC). The ssODNs were complimentary to either the strand targeted or not targeted by the gRNA, asymmetric or symmetric to the *Nhe*I tag. The chemically modified ssODN for $HBB^{IVS1-110}$ mutation was synthesized by incorporating 2-*O*-methyl 3′phosphorothioate (MS) modification at three terminal nucleotides at both the 5′ and 3′ ends with 72 bp homology arms. The complete details can be found in Additional file 2: Table S2.

T7 endonuclease assay and RFLP assay

Five days post transfection, genomic DNA was isolated using Merchery-Nagel NucleoSpin Tissue Kit following the manufacturer's instructions. The promoter region was amplified using the primer pair Prom-For_5′-GTAGACCACCAGCAGCCTAA-3′ and Prom-Rev_5′ TGGAGACGCAGGAAGAGATC-3′, and the region covering $HBB^{IVS1-110}$ mutation was amplified using the primer pair IVS1–110-For_5′-GGGTTTGAAGTCCAACTCCTAA-3′ and 3′UTR-For_5′-AGAAAACATCAAGCGTCCCATA-3′. The target regions were amplified using the AmpliTaq® Gold 360 MasterMix (https://www.thermofisher.com). The cycling parameters for both amplicons were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min. PCR products were purified by ethanol precipitation, and 1 μg of PCR product was used for T7 endonuclease assay. Likewise, 1 μg of PCR product was used for the RFLP assay where amplicons bearing the *Nhe*I tag upon successful gene correction will result in a specific cleavage after the treatment with the *Nhe*I restriction enzyme. The readout of the T7 endonuclease assay and the RFLP assay were determined on a 1.5% agarose gel and on a Bioanalyzer chip using DNA1000 kit. The band intensities were quantified using ImageJ embedded in Fiji software (www.fiji.sc).

TIDE analysis and single-colony and next-generation sequencing

For analyzing allele modification frequencies with non-enzymatic assays, we used TIDE (Tracking of Indels by Decomposition) analysis by Sanger-sequencing the purified PCR products used for T7 assay and examined with the online TIDE software (http://tide.nki.nl.) with the respective control sample. To precisely measure the events of site-specific insertion of *Nhe*I tag at $HBB^{IVS1-110}$ loci in $CD34^+$ HSPCs, we performed two different approaches: (i) Sanger-based single-colony sequencing and (ii) next-generation sequencing. For single-colony sequencing, the $HBB^{IVS1-110}$ region was PCR amplified from 5TS ssODN gene-corrected $CD34^+$ HSCs and cloned into pJET1.2 vector using CloneJET PCR Cloning kit (https://www.thermofisher.com) and transformed into Top10 competent cells using standard cloning techniques. Closely, 192 (two 96-well plates) single colonies were processed for Sanger sequencing with pJET1.2 forward sequencing primer (5′-CGACTCACTATAGGGAGAGCGGC-3′) and analyzed for the presence for *Nhe*I recognition site (GCTAGC) using Geneious-R6 software. Three of 192 clones resulted in *Nhe*I insertion (1.5%). In case of next-generation sequencing (NGS), new primers were designed with the amplicon length of 150 bp to be sequenced with the Illumina platform (Forward 5′-AGAAACTGGGCATGTGGAGA-3′; Reverse 5′-CCATAACAGCATCAGGAGTGG-3′). Further, barcode-tagged PCR primers were used to multiplex samples (Additional file 2: Table S3), and are adapter ligated, size selected, and bridge amplified and proceeded with amplicon sequencing in HiSeq 4000 system (http://www.illumina.com). The standard R-Package was used for NGS analysis where the sequencing reads were pre-filtered for low-quality reads and mapped to the reference sequence using a BWA tool. Further, the number of indel-carrying sequence reads was calculated using SAM tools. The distribution plot was generated by calculating the size of the indels in sequence and calculating the median percentage for each indel class.

In vitro transcription (IVT) of Cas9 mRNA

The open reading frame of SpCas9 was PCR amplified from the pX330 vector and sub-cloned into the pVAX.120 vector consisting of a T7 promoter and 120 bp length of a poly-A tail using standard molecular biology techniques. The IVT reaction was performed in linearized plasmid using T7 RNA polymerase in MEGAscript T7 kit (https://www.thermofisher.com). All mRNAs were produced with an anti-reverse CAP analog (ARCA; [m7G(5′)G]) at the 5′ end (https://www.trilinkbiotech.com/). The IVT-mRNAs were made with following chemical modifications in the indicated ratios: 100% Pseudo-UTP and 25% s2-thio-UTP/5-methyl-CTP

(https://www.trilinkbiotech.com/). All IVT mRNAs were purified using the MEGAclear kit (https://www.thermofisher.com) and quantified with nano-photometer and bioanalyzed for quality using the RNA6000 kit in Agilent 2100 Bioanalyzer (https://www.agilent.com).

Statistics

Kruskal-Wallis or Wilcoxon-Mann-Whitney rank-sum tests were applied wherever appropriate to analyze the differences in indel induction among the gene-editing technologies and comparison between encoded pDNA Cas9 and *Cas*9 mRNA using Graphpad Prism v.6.0d (https://www.graphpad.com).

Additional files

Additional file 1: Figure S1. Strategy for targeting the promoter and IVS1–110 mutation of the *HBB* gene. A) The promoter region of *HBB* gene targeted with three different gene-editing tools, $HBB^{IVS1-110}$ targeted with CRISPR/Cas9. B) The design of three different gene-editing tools at sequence level. **Figure S2** Comparison of three different gene-editing tools at *HBB* promoter. The complete raw data of Fig. 1a. **Figure S3** Gene correction of $HBB^{IVS1-110}$ in $CD34^+$ HSCs using pX330.sg $HBB^{IVS1-110}$ and ssODNs. A) $CD34^+$ HSCs nucleofected with pX330.sg $HBB^{IVS1-110}$ plasmid and ssODNs and measured for indel rate by T7 assay and HDR by TIDE analysis. Only 5TS resulted 3% HDR rate in TIDE analysis (as in Fig. 2a). B) Gene-editing capacity of pDNA-encoded Cas9 and mRNA-encoded *Cas*9 were compared, and superiority of Cas9 mRNA was observed ($P < 0.0001$). **Figure S4** TIDE analysis-gene correction of $HBB^{IVS1-110}$ in $CD34^+$ HSCs using Cas9 mRNA and ssODNs. TIDE analyses of four different ssODNs resulted in varying levels of 6 bp insertions that rely with the ssODN design. Modified ssODN resulted up to 8% HDR rate. **Figure S5** NGS analysis-gene correction of $HBB^{IVS1-110}$ in $CD34^+$ HSCs using Cas9 mRNA and ssODNs. The absolute quantification of *Nhe*I insertion by NGS analyses for four different ssODNs showed distinct rate of 6 bp insertions and correlate with ssODN design. Modified ssODN resulted up to 8% HDR rate. **Figure S6** Off-target analysis for the in silico predicted sites. The indel rate was measured by T7 endonuclease-I (T7EI) assay for six different off-target sites predicted through in silico (Additional file 2: **Table S4**) in K562 cells. We preselected top three hits in intronic and three hits in an exonic region. (PDF 2219 kb)

Additional file 2 Data S1 The complete amino acid sequences of the *HBB* targeting ZFNs. **Table S1** TALE FLASH IDs and RVDs targeting the promoter of *HBB* gene. **Table S2** Details of ssODNs (sequence, symmetry, and length of homology arms). Table S3 Barcode and sample details of next-generation sequencing. **Table S4** Details of off-target position, primer details, and indel frequency. (PDF 601 kb)

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Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its Additional files).

Authors' contributions

NL, JSA, RH, and MSDK conceived and designed the experiments. NL, JSA, AH, ALC, ADM, SG, and PW performed the experiments. JSA, NL, and PB analyzed the data. MSDK, MM, and RH contributed reagents/materials/analysis tools. JSA, NL, and MSDK wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval for using $CD34^+$ hematopoietic stem cells from healthy donors was obtained from Ethics commission, University Clinic of Tuebingen, Germany (829/2016BO2).

Consent for publication

Not applicable

Competing interests

M.S.D.K. is listed as main inventor on a patent application related to Nuclease encoding modified mRNA. M.S.D.K. is a main inventor on a patent licensed to the biopharmaceutical company, Ethris GmbH. M.S.D.K., A.D., A.H., and J.S.A. are inventors (with M.S.D.K. as main inventor) on a patent application related to modified mRNA encoding hCFTR.

Publisher's Note

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Molecular Therapy
Nucleic Acids
Original Article

Uridine Depletion and Chemical Modification Increase *Cas9* mRNA Activity and Reduce Immunogenicity without HPLC Purification

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The Cas9/guide RNA (Cas9/gRNA) system is commonly used for genome editing. mRNA expressing Cas9 can induce innate immune responses, reducing Cas9 expression. First-generation *Cas9* mRNAs were modified with pseudouridine and 5-methylcytosine to reduce innate immune responses. We combined four approaches to produce more active, less immunogenic second-generation *Cas9* mRNAs. First, we developed a novel co-transcriptional capping method yielding natural Cap 1. Second, we screened modified nucleotides in *Cas9* mRNA to identify novel modifications that increase Cas9 activity. Third, we depleted the mRNA of uridines to improve mRNA activity. Lastly, we tested high-performance liquid chromatography (HPLC) purification to remove double-stranded RNAs. The activity of these mRNAs was tested in cell lines and primary human CD34+ cells. Cytokines were measured in whole blood and mice. These approaches yielded more active and less immunogenic mRNA. Uridine depletion (UD) most impacted insertion or deletion (indel) activity. Specifically, 5-methoxyuridine UD induced indel frequencies as high as 88% (average ± SD = 79% ± 11%) and elicited minimal immune responses without needing HPLC purification. Our work suggests that uridine-depleted *Cas9* mRNA modified with 5-methoxyuridine (without HPLC purification) or pseudouridine may be optimal for the broad use of Cas9 both *in vitro* and *in vivo*.

INTRODUCTION

The Cas9/guide RNA (Cas9/gRNA) system, which is derived from the type II bacterial CRISPR adaptive immune system, is a powerful tool for manipulating genomes.[1–4] The Cas9/gRNA system consists of an RNA-guided nuclease (Cas9) and a single short gRNA. Upon delivery of these components to the nucleus of a cell, the guide strand directs the Cas9 protein to a specific chromosomal location, and Cas9/gRNA generates site-specific DNA double-strand breaks (DSBs), which are repaired by endogenous cellular mechanisms. Two major genome-editing events arise from the Cas9/RNA-induced DSBs: (1) a specific site can be mutated via mutagenic non-homologous end joining (NHEJ) by creating insertions or deletions (indels) at the site of the break, or (2) an exogenously introduced donor template can mediate a precise genomic sequence change via homologous recombination.[5]

Various methods have been described for delivery of the Cas9 protein into the nucleus. These include expression of Cas9 protein from a plasmid[6] or viral vectors,[7] transfection of recombinant Cas9 protein complexed to a gRNA (ribonucleoprotein or ribonucleoprotein [RNP] complex),[6,8,9] or expression from a transfected *Cas9* mRNA.[6] Expression of Cas9 protein from a plasmid or viral vector may be problematic because it risks integration of the promoter and/or *Cas9* gene cassette at the double-stranded break site, a feature of all double-stranded DNA vectors, or random integration of the DNA vector into the genome.[10] By way of contrast, Cas9 protein and mRNA do not pose the risk of *Cas9* gene integration, and they also induce limited off-target effects due to transient expression.[5,11,12] Although nanoparticle delivery of Cas9 protein has been reported, the most common approach to deliver transgenes into cells *in vivo* involves the use of mRNAs complexed with nanoparticles. This makes *Cas9* mRNA an attractive tool for genome editing in hard-to-transfect cells or tissues.

An ideal *Cas9* mRNA should mimic a fully processed mRNA and not activate innate immune pathways. Activation of these receptors induces inflammation, leads to translational inhibition, and causes mRNA degradation.[13–16] Our goal was to design and produce mRNAs that do not activate, or minimally activate, these RNA-sensing pathways. Exogenous mRNA can activate innate immunity pathways when various pattern recognition receptors (PRRs), present

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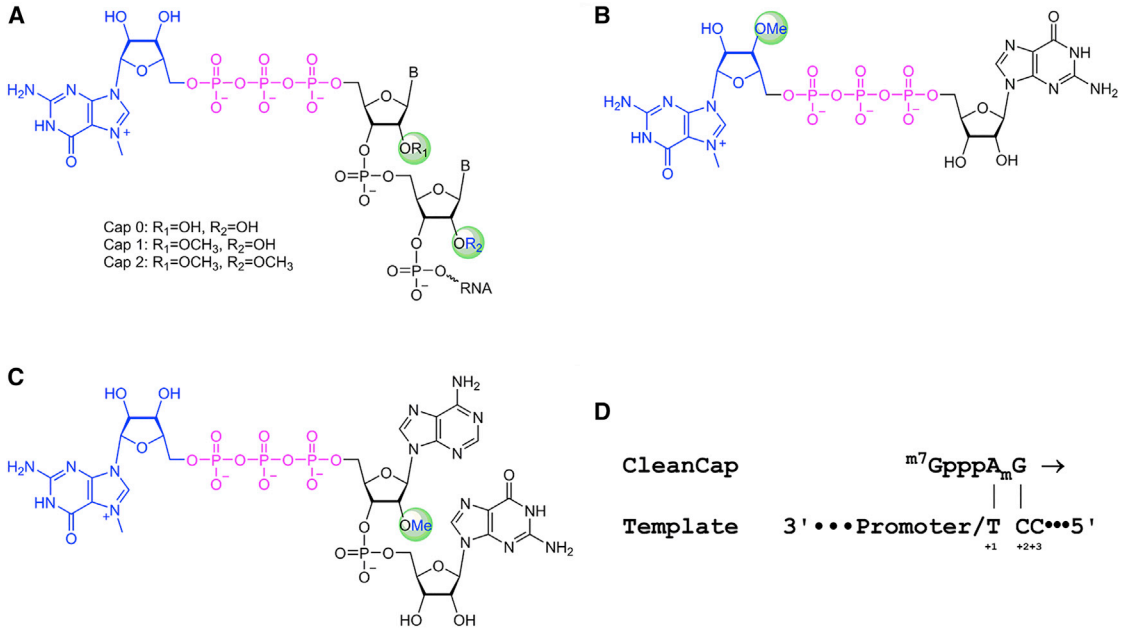

Figure 1. Eukaryotic Cap Structures and Cap Analogs

(A) Eukaryotic cap structure. Presence of 2′-O-methyl groups at R_1 and R_2 determine if a cap structure is Cap 0, Cap 1, and Cap 2 as indicated. (B) Structure of anti-reverse cap analog used in standard co-transcriptional capping. (C) Structure of CleanCap AG Cap1 Trimer. (D) Proposed mechanism of CleanCap co-transcriptional initiation in which the AmG dimer portion of CleanCap docks onto the +1 and +2 template nucleotides. Initiation occurs when CleanCap couples to an NTP occupying the +3 position.

in both endosomes and the cytosol, recognize pathogen-associated molecular patterns (PAMPs) associated with exogenous RNA (viral or transfected RNA). Specifically, Toll-like receptors (TLRs) 7 and 8, which recognize single-stranded RNA (ssRNA),[17,18] and TLR3, which recognizes double-stranded RNA (dsRNA),[19] must be avoided, since the activation of TLRs leads to inflammation and the inhibition of translation.[17–20] In addition, cytosolic PRRs to be avoided by *Cas9* mRNA include retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5), protein kinase R (PKR), and the interferon (IFN)-induced tetratricopeptide repeat (IFIT) proteins. RIG-I recognizes 5′ triphosphate (5′ppp)[21,22] or diphosphate,[23] panhandle structures of viral genomic RNA (reviewed in Weber and Weber[24]), and uridine-rich sequences.[25–28] MDA5 is activated by binding very long dsRNA,[13,29] while PKR recognizes dsRNA stretches of at least 33 nt.[30] Lastly, IFITs sense aberrant cap structures.[31] Therefore, *Cas9* mRNA must avoid both endosomal TLRs and cytosolic PRRs to achieve maximal translation and protein activity. In this study, we tested the ability of cap structure modifications, chemical modifications, sequence engineering (uridine depletion), and high-performance liquid chromatography (HPLC) purification to reduce immune activity and increase *Cas9* mRNA activity.

RESULTS

Eukaryotic Cap Structures

Eukaryotic RNAs are capped with a 7-methylguanosine (^{m7}G) connected by a 5′-to-5′ triphosphate bridge to the first nucleotide. This structure is referred to as Cap 0. Cap 0 is important for the recruitment of translational initiation factors, and it prevents degradation of the mRNA. In higher eukaryotes, the 2′ ribose position of the first cap-proximal nucleotide is methylated to form a Cap 1 structure (^{m7}GpppN$_{2'Om}$ N), and, in ~50% of transcripts, the second cap-proximal nucleotide is 2′ O-methylated to form Cap 2 (^{m7}GpppN$_{2'Om}$N$_{2'Om}$) (Figure 1A).[32]

While the presence of Cap 1 and Cap 2 in eukaryotic RNAs has been known since the 1980s, the function of these modifications has remained largely unknown. Cytoplasmic viruses frequently possess mechanisms to acquire a Cap 1 structure (reviewed in Decroly et al.[33] and Hyde et al.[34]). Many of these viruses are attenuated when their methyltransferases are inactivated, suggesting that cap structure may play an important role in self versus non-self-recognition. Cap 1 methylation has been shown to modulate binding or activation of innate immune sensors. For example, the binding affinity of IFIT-1 for Cap 1 and Cap 2 is much weaker than for 5′ triphosphate or Cap 0 RNAs, and IFIT-1 binding to non-2′-O methylated RNAs competes with the translational initiation factor EIF4E to prevent translation.[35,36] Cap 0 and 5′-triphosphate bind RIG-I with similar affinities, while Cap 1 modification abrogates RIG-I signaling.[37] Similarly, Cap 1 prevents detection by MDA5.[38]

Frequently, synthetic mRNAs are co-transcriptionally capped by including a cap analog in excess in the transcription reaction. The current state of the art is co-transcriptional capping with anti-reverse cap analog (ARCA), which is a capped dimer with the chemical structure shown in Figure 1B. ARCA results in a Cap 0 mRNA. ARCA possesses a 3′-O-methyl group on the sugar adjacent to the ^{m7}G, which

Table 1. List of *In Vitro*-Transcribed Modified *Cas9* Cap 1 mRNAs Made with CleanCap

Abbreviation	Full Name	Uridine Depleted	Cap Form	Screen
WT UD	wild-type bases	yes	Cap 1	full
5moU UD	5-methoxy uridine	yes	Cap 1	full
Ψ UD	pseudouridine	yes	Cap 1	full
WT	wild-type bases	no	Cap 1	full
5moU	5-methoxy uridine	no	Cap 1	full
Ψ	pseudouridine	no	Cap 1	full
5meC/Ψ	5-methyl cytidine/pseudouridine	no	Cap 1	full
5meU	5-methyl uridine	no	Cap 1	full
N1-me-Ψ	N1-methyl pseudouridine	no	Cap 1	full
5meC	5-methyl cytidine	no	Cap 1	full
5hmC	5-hydroxymethyl cytidine	no	Cap 1	full
N1-et-Ψ	N1-ethyl pseudouridine	no	Cap 1	initial
me1-Ψ /5meC	N1-methyl pseudouridine/5-methyl cytidine	no	Cap 1	initial
5moC	5-methoxy cytidine	no	Cap 1	initial
5camU	5-carboxy methyl ester uridine	no	Cap 1	initial
10% 5meC/2sU	5-methyl cytidine/2-thio uridine	no	Cap 1	initial
25% 5meC/2sU	5-methyl cytidine/2-thio uridine	no	Cap 1	initial
ARCA 5meC/Ψ	5-methyl cytidine/pseudouridine	no	Cap 0	initial

prevents it from incorporating in the incorrect orientation, although this is not a naturally found Cap 0 modification. In general, T7 transcripts initiate at the +1-transcript position with guanosine triphosphate (GTP) incorporated opposite a +1-template cytosine. The ARCA cap analog is provided at a 4:1 excess over GTP in the transcription reaction such that, when competing with GTP, ARCA incorporates at the +1-transcript position ~70% of the time leaving a Cap 0 structure (Figure 1A). Therefore, ~30% of the time ARCA transcription initiates with GTP to yield an mRNA with a 5′ triphosphate. Our previously published work utilized an ARCA Cap 0 *Cas9* mRNA that was fully substituted with pseudouridine (Ψ) and 5-methylcytidine (5meC).[6]

Recently, we developed a co-transcriptional capping method called CleanCap that utilizes an initiating capped trimer instead of ARCA. Co-transcriptional capping with our CleanCap Cap1 AG trimer yields a naturally occurring Cap1 structure. In this study, we tested the ability of our newly developed CleanCap Cap1 AG trimer to improve *Cas9* mRNA activity or reduce its immunogenicity. We utilized T7 RNA polymerase to generate *in vitro*-transcribed mRNAs. The structure of a CleanCap Cap 1 AG trimer is shown in Figure 1C. With the CleanCap Cap 1 AG trimer, the +1 and +2 template nucleotides are thymidine and cytosine, respectively (Figure 1D). Our hypothesis is that the CleanCap Cap 1 AG trimer initiates by occupying the +1 and +2 transcript positions and elongation occurs when the CleanCap trimer couples to the nucleoside triphosphate (NTP) occupying the +3 position. We also tested an anti-reverse CleanCap trimers with a 3′-O-methyl group on the sugar of the ^{m7}G (3′ O-methyl CleanCap AG) to prevent incorporation in the opposite orientation, but we found this to be unnecessary both in terms of yielding Cap1 structures and for indel formation (data not shown). A more extensive discussion of the CleanCap method will be presented elsewhere. To determine if we could improve on our previously published ARCA Cap 0 Ψ/5meC mRNA, we used CleanCap to generate a series of Cap 1 mRNAs that contained either wild-type (WT) bases or completely substituted with one or two modified bases (Table 1).

Chemical Modification of mRNA

Although over 100 post-transcriptional modifications are found in RNA,[39] only a subset are found in mRNAs. These mRNA modifications include N6-methyladenosine (m^6A), inosine, N1-methyladenosine (m^1A), Ψ, 5meC, and 5-hydroxymethylcytosine (5hmC).[40–42] Such chemical modifications have been shown to reduce innate immune responses and improve mRNA activity.[43–45]

Karikó et al.[43–45] demonstrated that substitution with modified bases reduced innate immune responses to transfected mRNAs. Based on this work, many first-generation mRNAs were modified with 5meC and Ψ.[46] They showed that chemical modification of mRNA limited TLR signaling,[43] decreased activation of 2′-5′-oligoadenylate synthetase,[45] and decreased binding to PKR.[44] Durbin et al.[47] showed that RNAs modified with m^6A bind RIG-I with reduced affinity, while Ψ, N1-methylpseudouridine (N1-me-Ψ), and 5meC RNAs bind RIG-I with high affinity yet fail to activate RIG-I signaling. Work by Peisley et al.[48] also reported reduced RIG-I filament formation triggered by Ψ, 2-thiouridine (2sU), or m^6A RNAs.

In addition to reducing innate immune responses, Karikó et al.[49,50] also showed that full substitution of mRNA with Ψ increased activity *in vivo*. Pardi et al.[51] showed that N1-me-Ψ mRNAs were efficiently expressed in mice when delivered by a variety of routes. Andries et al.[52] also reported that N1-me-Ψ mRNAs gave higher expression relative to Ψ-substituted mRNAs in mice.

Previously, we used partial substitution of mRNAs with 2sU and 5meC to express surfactant protein B (SP-B) to rescue SP-B-defective mice[53] and to reduce asthma by expression of Foxp3.[54] We have also used 2sU/5meC-modified mRNAs encoding zinc-finger nucleases and transcription activator-like effector nucleases (TALENs) in mouse lung, and we were able to demonstrate gene correction *in vivo* at the SP-B locus.[55] In this study, we screened modifications such as Ψ, 5meC, N1-me-Ψ, 2sU, and others (Table 1) to identify modifications that improved Cas9 activity.

Sequence Engineering

In addition to chemical modifications, studies have reported that sequence-engineered unmodified mRNAs may be superior to Ψ-modified RNAs *in vivo*.[56,57] Sequence engineering of mRNA utilizes the degeneracy of the genetic code to substitute specific nucleotides of an mRNA sequence or optimize codon utilization without altering the resulting amino acid composition. Several groups have reported that codon optimization could increase the activity of transfected mRNAs. Karikó et al.[50] saw an increase in erythropoietin (Epo) expression in human dendritic cells upon codon optimization of both unmodified and Ψ-modified mRNA. Thess et al.[56] sequence-engineered luciferase and Epo mRNAs by using only the most guanosine/cytosine (GC)-rich codons. They found that, while Ψ substitution improved the activity of non-sequence-engineered luciferase, Ψ modification decreased the expression of a sequence-engineered luciferase mRNA relative to an unmodified mRNA in HeLa cells. In mice, they found that sequence-engineered Epo performed better than non-sequence-engineered Epo mRNA and that Ψ modification of the optimized sequence decreased activity.

While designing our study, we found that, in the context of the luciferase open reading frame (ORF), depletion of uridines in the transcript using synonymous codons increased the luciferase activity for unmodified, Ψ, and 5-methoxyuridine (5moU)-modified RNAs (Figure S1). Based on these preliminary results and on reports by other groups that sequence engineering could improve mRNA activity,[56,57] we uridine-depleted the Cas9 ORF and synthesized 3 additional *Cas9* mRNAs containing WT bases, Ψ, or 5moU (Table 1). We selected these modifications for uridine depletion because work with other reporter mRNAs had shown improved activities with these modifications upon uridine depletion (data not shown). We compared the activity and immune response of these uridine depletion (UD) variants to the previously published ARCA Cap 0 Ψ/5meC mRNA.

HPLC

dsRNA is produced as an undesired side product during *in vitro* transcription with T7 RNA polymerase.[58,59] This dsRNA could activate innate immune sensors, including TLRs, PKR, or MDA5. Karikó et al.[60] reported that purification of mRNAs by HPLC reduced the levels of dsRNA impurities as assessed by a slot blot analysis with a dsRNA-specific antibody. They found that HPLC-purified Ψ-modified mRNAs had significantly higher activity *in vivo* than mRNAs that had not been HPLC purified.[60] In this study, we adapted this method to HPLC purify a portion of each mRNA listed in Table 1 in order to test if HPLC purification of *Cas9* mRNA would reduce innate immune stimulation or increase indel activity.

Thus, we performed an unbiased investigation of ability of these different methods to increase *Cas9* mRNA activity with minimal immune activation. We found the highest frequency of genome editing with uridine-depleted 5moU, and these transcripts showed minimal *in vitro* and *in vivo* activation of the innate immune response without the need for HPLC purification.

Uridine Depletion Improved Cas9 Activity by Indel Formation Assay

We conducted an initial indel formation screen of the mRNAs listed in Table 1 in primary $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) mobilized from peripheral blood. We used primary human cells for these assays as they are more sensitive to the immunostimulatory activity of delivered nucleic acids than cancer cell lines. Cells were co-transfected with 3 μg *Cas9* mRNA and 2 μg MS-single-guide RNA targeting the interleukin-2 receptor subunit gamma (IL2RG) locus. As a control, we also included Cas9 RNP using 6 μg Cas9 protein with 3.2 μg guide strand, as we have previously described.[6] Indels were quantitated using TIDE analysis.[61] Based on this initial screen, we narrowed our list to 11 candidate mRNAs (each with and without HPLC purification; Table 1, lead candidates). We tested the lead candidates on $CD34^+$ cells from five different donors.

The uridine-depleted mRNAs (WT UD, WT UD HPLC, 5moU UD, 5moU UD HPLC, Ψ UD, and Ψ UD HPLC) yielded the highest indel rates (~77%–87%) (Figure 2). This was a major improvement over our first-generation Cap 0 5meC/Ψ mRNA, which gave 61% indel. Indel frequencies with the Cas9 RNP complexed to guide were 67%. WT UD and 5moU UD HPLC showed a statistically significant improvement in indel frequency relative to their non-uridine-depleted counterparts ($p < 0.0001$). Among the non-uridine-depleted sequences, WT HPLC, Ψ, and Ψ HPLC gave 66%–69% indel formation. The chemical modifications in combination with UD did not improve *Cas9* mRNA activity. Even among the non-UD samples, the chemical modifications did not outperform WT HPLC-purified samples. Chemical modifications among non-UD samples were still relevant from the perspective of immune response, as discussed below.

To our surprise, in most cases, HPLC purification did not increase indel formation significantly. One notable exception was WT, where HPLC purification improved activity from 40% to 68% ($p = 0.028$). To assess the level of dsRNA contamination and depletion of dsRNA during HPLC, we adapted a previously described dsRNA

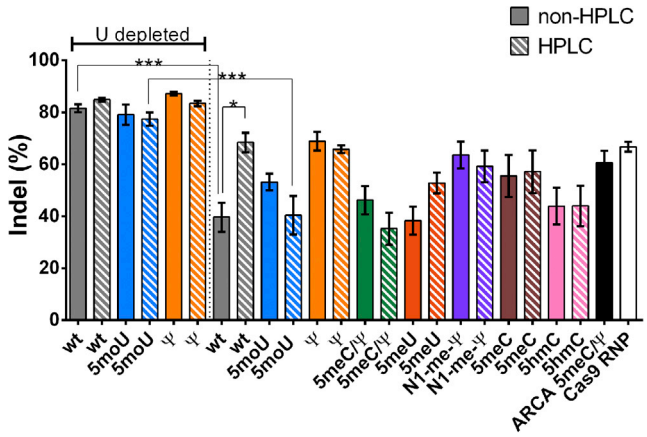

Figure 2. Indel Formation in CD34+ HSPCs Nucleofected with Modified *Cas9* mRNAs

CD34+ HSPCs were nucleofected with 3 μg of the indicated *Cas9* mRNA and 2 μg IL2RGlocus MS-sgRNA. 6 μg Cas9 RNP complexed to 3.2 μg IL2RGlocus MS-sgRNA was nucleofected for comparison. ARCA 5meC/Ψ is our previously published *Cas9* mRNA[6] and was also included for comparison. Cells were isolated after 4 days, and indel formation was assessed by TIDE analysis. Bars represent mean ± SEM of at least 5 independent transfections. White and gray bars indicate RNeasy and HPLC-purified mRNAs, respectively. sgRNA complexed to Cas9 RNP was included as a control. ***$p < 0.0005$ and *$p < 0.05$.

immunoblot with a dsRNA-specific antibody.[60] Based on this qualitative assay, we estimate that HPLC purification reduced dsRNA levels by 50%–80%, while uridine depletion reduced dsRNA levels by approximately 30% (Figure S2). While Karikó et al.[60] interpreted a decrease in blot signal to reflect removal of dsRNA, another alternative is that heating and denaturation of the mRNA during HPLC unfolded long double-stranded intramolecular structures within the purified mRNA, resulting in a decrease in slot blot signal.These experiments suggest that uridine depletion of *Cas9* mRNA can significantly improve CRISPR gene editing and may then obviate the need for HPLC purification in CD34+ cells.

Lastly, we tested the influence of the CleanCap, ARCA, and anti-reverse CleanCap cap structures on the performance of the 5moU UD mRNA. Notably, there was no significant difference in the indel activity between the 5moU UD ARCA CleanCap and 3′ O-methyl CleanCap *Cas9* mRNA (Figure S3).

IFN Responses in Differentiated THP-1 Cells Transfected with Unmodified and Modified mRNAs

We next tried to narrow down mRNA variants with improved activity that also induced low innate immune responses in the *ex vivo* setting. We used an IFN reporter cell line to assess IFN stimulation upon transfection of the various mRNAs into THP-1 Dual cells. THP-1 Dual cells are human monocyte stable transfectants, which, upon IFN stimulation, express a secreted coelenterazine luciferase (Lucia) driven by the ISG54 (IFN-stimulated gene) minimal promoter and five IFN response elements. The majority of modified mRNAs did not induce significant IFN responses above the negative control with or without HPLC purification (Figure 3). Notably, Ψ-modified mRNAs induced elevated IFN responses both with and without UD. Apart from Ψ-modified mRNA, WT UD and 5meU that had not been HPLC purified gave significantly elevated IFN signaling relative to the negative control. In each case, HPLC purification reduced IFN signaling to background levels. To our surprise, non-HPLC-purified WT *Cas9* did not induce significant IFN signaling. Among the UD variants, 5moU UD did not exhibit significant IFN response even without HPLC purification, and, thus, it appears to be an attractive choice for editing applications.

IL-12, TNF-α, and IL-6 Measurements in Whole Blood

In addition to *ex vivo* editing of isolated cells, the Cas9/gRNA system can be applied in whole organisms. The activation of the innate immune response *in vivo* has been a major barrier to gene therapy vectors, even inducing a patient death.[62] We next sought to assess immune responses in the more complex environment of whole human blood and identify mRNA variants that induced lower immune responses in whole blood. Whole blood obtained from healthy donors was transfected with mRNAs that were complexed with TransIT delivery reagent. At 6 and 24 hr, serum was isolated and interleukin (IL)-12 (Figure 4A), tumor necrosis factor alpha (TNF-α) (Figure 4B), and IL-6 (Figure 4C) were measured by ELISA.

Most *Cas9* mRNA variants did not induce IL-12 secretion. Among the UD samples, levels of IL-12 were significantly elevated relative to blood-only controls for WT UD, but not the other variants, at 6 and 24 hr. For the non-UD variants, levels of IL-12 were significantly elevated relative to 6-hr blood-only control for 5meC at 24 hr. While some other groups were slightly elevated, they did not reach statistical significance.

Many *Cas9* mRNA variants induced TNF-α secretion. Among the UD samples, TNF-α levels reached significance at 24 hr only for WT UD and WT UD purified by HPLC. Among the non-UD samples, TNF-α levels were significantly elevated at 6 and 24 hr regardless of HPLC purification for WT, 5moU, N1-me-Ψ, 5meC, 5meC/Ψ, and 5hmC. But HPLC purification abolished TNF-α induction only for 5meC/Ψ. Interestingly, 5moU UD did not increase TNF-α even though the non-UD 5moU increased TNF-α.

Most *Cas9* mRNA variants that induced TNF-α secretion also induced IL-6 secretion (except WT UD). For uridine-depleted sequences, IL-6 levels reached significance only for WT UD purified by HPLC at 24 hr. Among the non-UD samples, IL-6 levels were significantly elevated at 6 and 24 hr regardless of HPLC purification for WT, N1-me-Ψ, 5meC, and 5hmC. 5meU increased IL-6 at 6 and 24 hr without HPLC purification but only increased IL-6 after 24 hr when purified by HPLC. For 5meC/Ψ, IL-6 levels were significantly elevated at 24 hr, but HPLC purification abolished IL-6 induction. IFN-α was also tested, but it was not measurably induced by any of the tested mRNAs (data not shown).

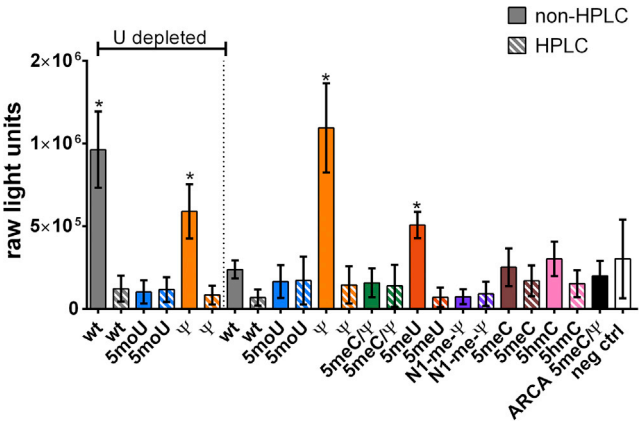

Figure 3. IFN Response Generated by THP-1 Dual Cells Transfected with Modified *Cas9* mRNAs

THP-1 dual cells were transfected in sextuplicate with 100 ng of the indicated mRNAs complexed with 1 μL transfection reagent mRNA-In. At 24 hr, Lucia expression in the media was assayed as a measure of IFN activity. Bars represent mean ± SEM of three independent assays comprising a total of 18 replicates. *$p < 0.05$.

IL-12, TNF-α, and IL-6 Measurements in Mice

To measure immune responses in mice *in vivo*, *Cas9* mRNAs were encapsulated in chitosan-coated poly-D,L-lactide-co-glycolide (PLGA) nanoparticles and injected into the tail vein of mice (n = 3). At 6 or 24 hr, mice were sacrificed and serum IL-12 (Figure 5A), TNF-α (Figure 5B), and IL-6 (Figure 5C) were measured by ELISA. None of the UD samples showed elevated serum IL-12, TNF-α, or IL-6. Only WT non-HPLC-purified IL-12 and IL-6 levels were significantly elevated relative to 6-hr blood-only control at 6 hr. Similarly, at 6 hr, TNF-α levels were elevated relative to control for both WT and WT HPLC, while no other significant changes were observed.

DISCUSSION

Improvement in *Cas9* mRNA Activity by UD

In our current study, we applied four design principles to improve the activity of our previously published ARCA Cap 0 Ψ/5meC *Cas9* mRNA.[6] These included exploring sequence engineering, screening different modified bases, examining the influence of HPLC purification, and using a Cap 1 structure. Among these strategies, a combination of UD with 5moU modification was able to achieve indel rates as high as 87% in primary $CD34^+$ HSPCs while avoiding immune responses even in the absence of HPLC purification (Figure 2).

UD was most effective in increasing *Cas9* mRNA activity, but further chemical modification was necessary to reduce immunogenicity. However, this increase in indels by UD variants only reached statistical significance for WT UD and 5moU UD HPLC relative to their non-uridine-depleted counterparts ($p < 0.05$). We also tested the off-target activity of 5 moU UD against ARCA 5meC/Ψ at a previously reported off-target site (chromosome [chr]1: 167730172–167730194).[6] The off-target indel activity was close to the limit of detection, and it was not significantly different between 5 moU UD (8% ± 1%) and ARCA 5meC/Ψ (5% ± 2%). Uridine depletion may improve indel levels by increasing protein expression, reducing immune responses, or a combination of the two effects. In our studies with luciferase (Figure S1), uridine depletion increased protein expression, but the percent increase was significantly different between 5moU and Ψ. Consistent with our results, studies have reported that codon optimization can influence both expression and mRNA stability.[63] More specifically, GC-enriched (adenosine/uridine [A/U]-depleted) genes have been reported to exhibit higher steady-state mRNA levels when expressed using plasmids.[64] The study further seemed to suggest that UD did not affect mRNA degradation rates, and it speculated that some mRNA-processing pathway(s) may influence mRNA levels.[64] It is also possible that uridine depletion could reduce recognition by TLRs. Interestingly, it has been reported that TLR7 recognizes uridine stretches.[20] Tanji et al.[65] also reported that uridine containing single-stranded RNA degradation products could be sensed by TLR8. Further studies with a variety of primary sequences may be necessary to understand the mechanism by which the combination of mRNA sequence and chemical modifications influences protein translation in different cell types.

Immunogenicity of UD and non-UD *Cas9* mRNA Variants

To test immune responses, we employed two complementary assays, a whole-blood assay and an *in vivo* mouse model. The whole-blood assay appears to be the most sensitive assay for monitoring these responses. The difference between the whole-blood and *in vivo* results may reflect difficulties in measuring systemic cytokines in mice in response to local delivery to a subset of cells. In these assays, uridine depletion also decreased most, but not all, immune responses elicited by WT, 5moU, and Ψ. Specifically, UD reduced TNF-α and IL-6 for 5moU (Figures 4B and 4C). For WT *Cas9* mRNA, UD significantly reduced IL-6 levels in whole-blood assay (Figure 4C) and reduced TNF- α, IL-6, and IL-12 in the mouse assay (Figure 5). However, UD was insufficient to reduce IFN responses completely for Ψ and increased IFN for WT (Figure 3).

The mechanism by which uridine depletion affects immune responses is unclear. It is possible that sequence engineering could influence binding to PRRs and thereby minimize the translational inhibitory effects that are activated through PRRs. It seems possible that PRRs designed to recognize aberrant RNAs might focus on uridine residues, as this is a major difference between DNA and RNA. For example, it was reported that triplets of sequential uridines could activate TLR-7 and cause dendritic cells to release IFN-α.[20] Likewise, RIG-I is reported to recognize uridine-rich sequences.[25–28] Further research will be required to define the precise mechanism by which uridine depletion improves the activity of some *Cas9* mRNA variants despite immune activation.

In the context of nucleofection of $CD34^+$ HSPCs, we show that *Cas9* WT UD can have similar indel formation rates as Ψ and

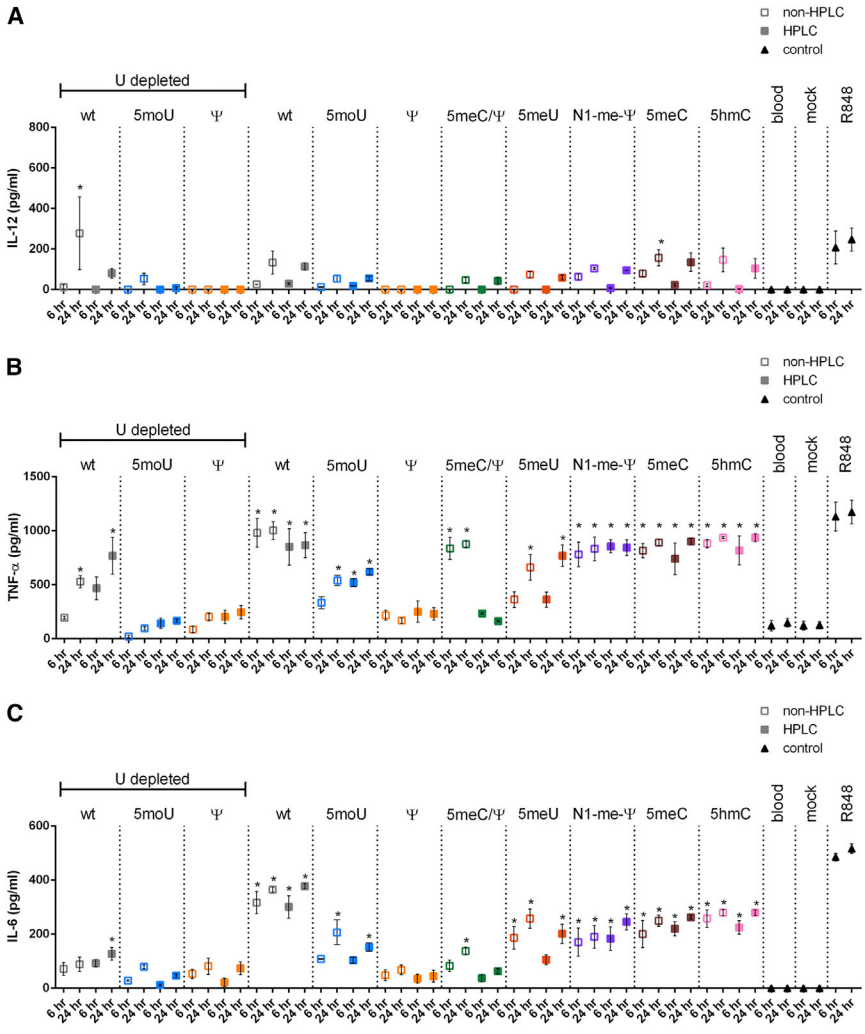

Figure 4. Amounts of IL-12, TNF-α, and IL-6 in Whole Human Blood Transfected with Modified *Cas9* mRNAs

To assess immune responses to transfected mRNAs, whole blood from healthy human volunteers (n = 3) was transfected with 10 μg mRNA complexed with 10 μL TransIT (https://www.mirusbio.com/). After 6 or 24 hr of incubation, sera were isolated and (A) IL-12, (B) TNF-α, or (C) IL-6 was measured by ELISA. Bars represent mean ± SEM. *p < 0.05 relative to 6-hr blood-only control.

5moU-modified RNAs (Figure 2). Thus, at least in CD34+ cells, uridine depletion may be sufficient and chemical modification may not be necessary. By way of contrast, reducing immune responses may be still important for *in vivo* applications, since the route of delivery may be an important determinant of efficacy and innate immune induction.[66] Since electroporation likely bypasses the endosomal compartment (where RNA-sensing TLRs reside), it remains to be determined if the same results would be observed with lipid or polymer transfection. A significant body of research supports the idea that chemical modification of mRNA can improve its activity by reducing innate immune stimulation in several instances.[49–55,60] Indeed, chemical modification of mRNA has been reported to decrease binding to or activation of TLRs,[43] 2′-5′-oligoadenylate synthetase,[45] PKR,[44] and RIG-I.[47,48] Chemical modifications are known to change the structure, base pairing, and codon/anti-codon pairing of mRNAs (reviewed in Harcourt et al.[67]), and they may thus make them poor substrates for PRRs. In contrast to the above reports, several groups have reported that sequence-engineered mRNAs may not require chemical modification. Thess et al.[56] found that, while chemical modification improved the activity of some sequences, when they used mostly GC-rich codons, WT mRNAs had the highest activity. Kaufmann et al.[57] found that when they formulated mRNAs in lipid nanoparticles and delivered them intravenously to mice, WT and Ψ mRNAs had similar activities and immune responses. Thus, the need for chemical modification may depend on the individual mRNA sequence as well as the route of administration. Since UD did not completely inhibit immune responses, we also explored the combination of UD with chemical modification.

In our studies, most chemical modifications reduced IFN responses except for Ψ and 5 meU. This suggests that the chemical modification present in many of the *Cas9* mRNAs tested may mask dsRNA recognition. It is also possible that these modifications are not recognized by the relevant PRRs. The observation that Ψ did not reduce IFN stimulation was somewhat surprising based on the literature.[49] Unfortunately, all chemically modified variants, except Ψ, still induced TNF-α and IL-6 in the absence of UD (Figures 4 and 5). Therefore, we tested the ability of HPLC to reduce the remaining immune responses.

Influence of HPLC Purification on Reducing Immunogenicity of *Cas9* mRNA Variants

HPLC was successful in reducing IFN responses when a combination of chemical modification and UD did not reduce IFN (WT UD, Ψ UD, Ψ, and 5meU) (Figure 3). Surprisingly, this reduction in immune response by HPLC purification only resulted in significant improvement in indel frequencies for non-uridine-depleted WT (Figure 2). This may reflect that the natural substrate for dsRNA-sensing PRRs is WT RNA. The reduction of IFN response after HPLC purification is consistent with literature (Karikó et al.[60]), especially for Ψ.[60] However, we also noticed that several chemical modifications (for 5moU UD, 5moU, 5meC/Ψ, N1-meΨ, 5meC, and 5hmC) showed no IFN response even without HPLC purification. Indeed, on further

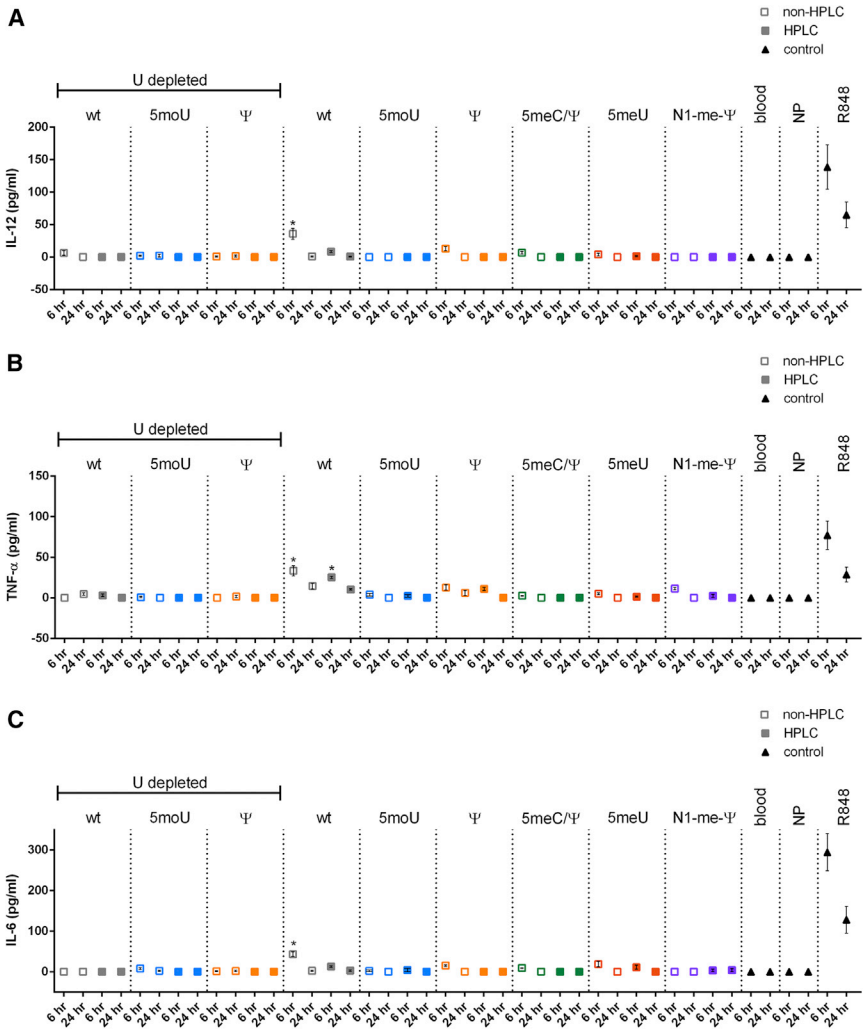

Figure 5. Amounts of IL-12, TNF-α, and IL-6 in the Sera of Mice after Intravenous Infusion of Modified *Cas9* mRNAs

To assess immune responses *in vivo*, 20 μg *Cas9* mRNA encapsulated in chitosan-coated PLGA nanoparticles was injected intravenously (n = 3) into the tail vein of mice. After 6 or 24 hr of incubation, sera were isolated and (A) IL-12, (B) TNF-α, or (C) IL-6 was measured by ELISA. Blood treated with R-848 serves as a positive control. Bars represent mean ± SEM. *p < 0.05 relative to 6-hr blood-only control.

comparison with Karikó et al.,[60] we noticed that the influence of chemical modification on IFN and TNF responses was protein dependent in their study. Thus, our data are broadly consistent with Karikó et al.[60] that HPLC purification reduces IFN responses if they are present.

In contrast to IFN responses, HPLC was unsuccessful in reducing TNF-α and IL-6 secretion induced by all variants except 5meC/Ψ, as measured by the whole-blood assay. This result is inconsistent with Karikó et al.[60] Unlike Karikó et al.,[60] our results show that we were only able to deplete, but not completely rid, our mRNAs of dsRNA using HPLC purification (Figure S2). Thus, it is also possible that the remaining dsRNA in the HPLC-purified samples is sufficient to trigger immune responses that are equivalent to the non-HPLC-purified RNAs, rendering HPLC purification insufficient to provide a benefit within the context of $CD34^+$ HSPCs. These results suggest that a further reduction in dsRNA may be necessary to reduce IL-12, IL-6, and TNF- α and increase indels.

Influence of Capping Strategy *Cas9* mRNA Activity and Immunogenicity

Lastly, the capping strategy did not seem to impact indel formation (Figure S2). We had hypothesized that the presence of Cap 1 in our mRNAs may also decrease binding to PRRs and reduce the need for HPLC purification. It has been reported that MDA5 does not recognize Cap 1 mRNAs efficiently.[38] It remains to be tested if other dsRNA sensors such as RIG-I have decreased sensitivities to Cap 1 mRNA. We only compared the Cap 0 and Cap 1 modifications for the 5moU UD variant mRNAs, and we did not observe any difference in indel levels (Figure S3). However, there was a small but statistically significant increase in IL-12 production, but not TNF-α or IL-6, in the whole-blood assay in response to Cap 0 ARCA relative to the Cap 1 CleanCap analogs (Figure S4). There was no difference in cytokine production in the less sensitive mouse model system for the Cap 0 or Cap 1 mRNAs (Figure S5). It is possible a Cap 1 structure would have provided more benefit for 5moU-modified mRNA in the absence of UD.

Conclusions

In summary, we have used a variety of approaches in combination to identify several improved *Cas9* mRNAs. Among these strategies, uridine depletion resulted in the greatest increase in indel levels (Table 2), but WT UD samples still elicited innate immune responses. Ψ UD showed high indel levels and reduced both IL-6 and TNF-α, but not IFN. IFN induction by Ψ UD was resolved by using HPLC. By way of contrast, 5moU modification of WT UD *Cas9* mRNA maintained indel frequencies and reduced all immune responses, even without HPLC purification. Given that no benefit was seen upon HPLC purification, HPLC would not be recommended for this modification, because of the additional cost and the significant loss of yield upon HPLC purification. Taken together, 5moU UD would be the preferred candidate for *Cas9* mRNA given that it does not require HPLC purification (Table 2). Future studies may be necessary to

Table 2. Summary of Assay Results for Uridine-Depleted Sequences

	WT UD	WT UD HPLC	5moU UD	5moU UD HPLC	Ψ UD	Ψ UD HPLC
High indel	+	+	+	+	+	+
Lack of IFN in THP-1 Dual cells	–	+	+	+	–	+
Lack of IL-12 in whole blood	–	+	+	+	+	+
Lack of TNF-α in whole blood	–	–	+	+	+	+
Lack of IL-12 *in vivo*	+	+	+	+	+	+
Lack of TNF-α *in vivo*	+	+	+	+	+	+

investigate the influence of sequence engineering and chemical modification on the *in vivo* activity of 5moU *Cas9* mRNA.

MATERIALS AND METHODS

Transcription Templates and Sequence Optimization

The Cas9 ORF with C-terminal nucleoplasmin nuclear localization signal (NLS) and hemagglutinin epitope tag was provided by Feng Zhang. It was cloned into the mRNA expression vector pmRNA, which contains a T7 RNA polymerase promoter, an unstructured synthetic 5′ UTR, a multiple cloning site, and a 3′ UTR that was derived from the mouse α-globin 3′ gene. An N-terminal SV40 NLS was added to generate the vector pmRNA_NLS_Cas9_NLS. A transcription template was generated by PCR using mRNA forward primer 5′-TCGAGCTCGGTACCTAATACGACTCAC-3′ and mRNA reverse primer T(2′OMe)T(2′OMe)$(T)_{118}$CTTCCTACTCAG GCTTTATTCAAAGACCA-3′. The poly A tail was encoded in the template, and the resulting PCR product encoded a 120-nt poly A tail.The uridine-depleted plasmid pmRNA_UD_NLS_Cas9_NLS was created by codon optimization of the *Cas9* mRNA ORF contained within pmRNA_NLS_Cas9_NLS plasmid. UD of the *Cas9* mRNA sequence was performed with the "optimize codons" tool in Geneious version R8.1.8 (https://www.geneious.com).[68] A new sequence in Geneious was created for the Cas9 ORF; this sequence was selected, and under the tab "annotate and predict," the "optimize codons" function was chosen. Parameters were chosen as follows: source of genetic code, standard; target organism, *Homo sapiens*; target genetic code, standard; threshold to be rare = 1; and avoid restriction sites, No. Base content for our standard Cas9 ORF was as follows: 28.6% A, 27.8% C, 28.1% G, 15.5% U, and 55.8% GC. Base content for our uridine-depleted Cas9 ORF was as follows: 25.3% A, 30.6% C, 31.5% G, 12.6% U, and 62.1% GC.

In Vitro Transcription of Modified mRNAs

Chemically modified, co-transcriptionally capped Cap 1 Cas9 and firefly luciferase (FLuc) mRNAs were synthesized by T7 RNA polymerase *in vitro* transcription. All enzymes were purchased from New England Biolabs (Ipswich, MA). Transcriptions were done in 1× transcription buffer (40 mM Tris, 10 mM dithiothreitol, 2 mM spermidine, 0.002% Triton X-100, and 27 mM magnesium acetate) using final concentrations of 8 U/μL T7 RNA polymerase (M0251L); 0.002 U/μL inorganic pyrophosphatase (M2403L); 1 U/μL murine RNase inhibitor (M0314L); 0.025 μg/μL standard or uridine-depleted transcription template; 5 mM CleanCap Cap 1 AG trimer; and 5 mM each of ATP, cytidine triphosphate (CTP) (or CTP analog), GTP, and uridine triphosphate (UTP) (or UTP analog), as indicated in Table 1. Transcription reactions were incubated at 37°C for 2 hr and treated with final 0.4 U/μL DNase I (M0303L) in 1× DNase I buffer for 15 min at 37°C. We initially made anti-reverse CleanCap trimers with a 3′-O-methyl group on the sugar of the ^{m7}G to prevent incorporation in the opposite orientation, but we found this to be unnecessary, as the 3′-O-methyl version functioned equivalently to CleanCap with a 3′ OH. mRNAs were purified by RNeasy Maxi (QIAGEN, 75162), phosphatase treated for 1 hr with final 0.25 U/μg Antarctic phosphatase (M0289L) in 1× Antarctic phosphatase buffer, and then re-purified by RNeasy. A portion of each mRNA was purified by HPLC as described by Kariko et al.,[60] except that mRNA was recovered from HPLC fractions by RNeasy purification. Purification was carried out on a PRP-H1 column (Hamilton Company) at 65°C using a gradient of 100 mM triethylammonium acetate/acetonitrile. Transcription quality was measured by bioanalyzer analysis (Agilent 2100 Bioanalyzer). mRNA concentrations were measured by UV spectroscopy and corrected for predicted extinction coefficient.

dsRNA Slot Blot

Detection of dsRNA was performed by slot blot based on previously established methods[60] adapted for use with a 48-well slot blot vacuum manifold (GE Whatman, Pittsburgh, PA, 10447941) and SNAP i.d. 2.0 Protein Detection System (EMD Millipore, Burlington, MA, SNAP2MIDI). In brief, RNA samples (1,000 or 200 ng) were blotted on a super-charged nytran membrane (GE Amersham, Pittsburgh, PA, 10416230) pre-wetted in 1× TBST (50 mM Tris-HCL, 138 mM NaCl, 27 mM KCl, and 0.05% Tween-20 [pH 7.5]) by applying vacuum. The membrane was then transferred to a SNAP i.d. apparatus and blocked with 30 mL 0.5% w/v nonfat dried milk in 1× TBST for 1 min prior to the application of vacuum. Blocking buffer was incubated over the membrane for 1 min before the vacuum was applied. The membrane was probed with 15 mL 1:1,500 dsRNA-specific monoclonal antibody (mAb) J2 (English & Scientific Consulting, Hungary) in 0.5% milk for 20 min and washed 4 times with 30 mL 1× TBST. The membrane was incubated for 20 min with 15 mL 1:1,500 horseradish peroxidase (HRP)-conjugated donkey anti-mouse immunoglobulin G (IgG) (Jackson ImmunoResearch, West Grove, PA, 715-035-150) in 0.5% milk and washed 4 times with 1× TBST. The membrane was developed in the dark with 30 mL enhanced chemiluminescence (ECL) western blotting

detection reagent (GE Amersham, RPN2134) for 5 min before being imaged on G:BOX Chemi XRQ (Syngene, Frederick, MD) chemiluminescent imaging system with accompanying GeneSys (version [v.]1.5.6) software. Raw light units (RLUs) were measured and background corrected using densitometry software (GeneSys). For comparison of HPLC samples, RLU signals per mRNA were normalized to matched non-HPLC and reported as a percentage. Similarly, for comparison of UD samples, RLU signals per mRNA were normalized to non-UD and reported as a percentage. Percent reduction in dsRNA signal resulting from HPLC purification or UD was calculated using the following formula:

$$\%\ \text{reduction} = 100 - \left(\frac{\text{HPLC intensity}}{\text{non} - \text{HPLC intensity}}\right) * 100$$

$$\%\ \text{reduction} = 100 - \left(\frac{\text{uridine} - \text{depleted intensity}}{\text{WT intensity}}\right) * 100.$$

We calculated this for the 200- and 1,000-ng inputs and averaged these two values.

THP-1 and THP-1 Dual Cell Culture

THP-1 cells (ATCC, TIB-202) and THP-1 Dual cells (InvivoGen, San Diego, CA, thpd-nfis) were grown in RPMI-1640 medium (ATCC, 30-2001) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, 10437-028), 1 mM sodium pyruvate (Gibco, 11360-070), 1× MEM non-essential amino acids (Gibco, 11140-050), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, 15140-122), and 100 μg/mL Normocin (InvivoGen, ant-nr-1) at 37°C in an atmosphere of 5% CO_2. THP-1 Dual cells were grown in the presence of 100 μg/mL zeocin (InvivoGen, ant-zn-1) and 10 μg/mL blasticidin (InvivoGen, ant-bl-1) every other passage to maintain positive selection of reporters.

Luciferase Assay in Cultured THP-1 Cells

In preparation for THP-1 cell transfections, 2×10^5 cells per well were seeded in a 24-well plate format (Corning Costar, Tewksberry, MA, 3527) and allowed to differentiate in culture for 72 hr using 200 nM phorbol ester 12-O-Tetradecanoylphorbol-13-Acetate (Cell Signaling Technology, Danvers, MA, 4174). Cells were then transfected with 100 ng *in vitro*-transcribed, non-HPLC, and HPLC-purified unmodified or modified FLuc mRNAs complexed with 1 μL transfection reagent mRNA-In (MTI-GlobalStem, Gaithersburg, MA, 73741) and Opti-MEM (Gibco, 11058-021) in a total volume of 50 μL. Complexed mRNAs were briefly vortexed and incubated for 10 min at room temperature, then added drop-wise to each well. Modified FLuc mRNAs were transfected in sextuplicate. 24 hr post-transfection, media were aspirated from each transfected well, and adhered monolayers were lysed with the ONE-Glo Luciferase Assay System (Promega, Madison, WI, E6120) to assay for FLuc activity. Lysates were incubated in the dark for 10 min at room temperature with gentle rocking, then transferred to a white 96-well microtiter plate (Greiner Bio-One, Monroe, NC, 655073). Luciferase activity was measured using a GloMax Multi+ Detection System luminometer (Promega, E8032) with a 0.5-s integration per well.

IFN Response Assay in Cultured THP-1 Cells

THP-1 Dual cells were seeded, differentiated, and transfected as above except that cells were transfected with 100 ng *in vitro*-transcribed, non-HPLC, and HPLC-purified unmodified or modified *Cas9* mRNAs. Modified *Cas9* mRNAs were transfected in sextuplicate, and supernatants from each transfected well were assayed for Lucia activity 24 hr post-transfection. To assay Lucia activity as a measure of an IFN response, 50 μL media were mixed with 150 μL QUANTI-Luc coelenterazine luciferase substrate (InvivoGen, rep-qlc) in a white 96-well microplate (Greiner Bio-One, 655073), and luminescence was measured using a GloMax Multi+ Detection System luminometer (Promega, E8032) with a 10-s integration per well.

$CD34^+$ HSPC Tissue Culture

$CD34^+$ HSPCs derived from mobilized peripheral blood donated by male donors were purchased from AllCells (Alemeda, CA). Cells were thawed according to the manufacturer's instructions and cultured at a density of 250,000/mL in a 24-well plate. $CD34^+$ HSPCs were cultured in StemSpan SFEM II (STEMCELL Technologies, Vancouver, Canada) supplemented with stem cell factor (100 ng/mL), thrombopoietin (100 ng/mL), Flt3-Ligand (100 ng/mL), IL-6 (100 ng/mL), StemRegenin1 (0.75 mM), and UM171 (STEMCELL Technologies, 35 nM). Cells were cultured at 37°C, 5% CO_2, and 5% O_2.

Nucleofection of CD34+ HSPCs

Nucleofection was performed 48 hr after cells were thawed. Cell viability was confirmed to be >80% using trypan blue before nucleofection. Cells were resuspended in 1 M buffer (5 mM KCl, 15 mM $MgCl_2$, 120 mM Na_2HPO_4/NaH_2PO_4 [pH7.2], and 50 mM Manitol) at a density of 5 million cells/mL. As a control, we also included Cas9 RNP at a Cas9:sgRNA molar ratio of 1:2.5 as previously described by Hendel et al.[6] Briefly, 6 μg Cas9 protein was incubated with 3.2 μg IL2RG locus MS-sgRNA[6] (ACAACTTCGGTAG TAATGGT…) for 15 min prior to nucleofection. 3 μg *Cas9* mRNA and 2 μg MS-sgRNA were used for nucleofections.[6,69] *Cas9* mRNA and RNP were then mixed with 100,000 $CD34^+$ cells (20 μL of cell suspension) and transferred to a 16-well nucleofection strip (Lonza, MD, USA). Each treatment was performed in duplicate. Cells were nucleofected using DZ100 program in the Lonza 4D nucleofector. Cells were suspended in 200 μL $CD34^+$ media after nucleofection.

Measurement of Indels

Genomic DNA was obtained using QuickExtract DNA Extraction Solution (Epicenter, Madison, WI). The mixture was vortexed and incubated at 65°C for 6 min followed by 100°C for 10 min, a slight deviation from the manufacturer's recommendations for more optimal downstream applications. The target sequence in the IL2RG locus was amplified using PCR and sequenced. Indels were measured using TIDE software as previously described.[61] Briefly, the software uses quantitative sequence trace data from control cells and cells edited using Cas9 RNP or mRNA. The software decomposes the edited sequence trace into individual components using multivariate non-negative linear modeling, and it uses the control sequence as a template to model indels.

The following primers were used for PCR amplification of the IL2RG site: forward, 5′-TCACACAGCACATATTTGCCACACCCT-3′ and reverse, 5′-TGCCCACATGATTGTAATGGCCAGTGG-3′.

Whole-Blood Assay

Blood samples from three different healthy donors was taken and collected in EDTA collection tubes (Sarstedt, Germany). For each treatment group, 2 mL EDTA-blood was transferred into 12-well plates and treated accordingly. 10 μL 1 mg/mL (un-)modified mRNAs were complexed to 10 μL TransIT (Mirus Bio, Madison, WI). For a positive control group, blood was treated with the TLR 7 and 8 agonist R-848 (Resiquimod, Sigma-Aldrich, St. Louis, MO). Samples were incubated for 6 or 24 hr at 37°C in a humidified atmosphere containing 5% CO_2. At each time point, 1 mL whole blood was transferred into columns for serum separation (Sarstedt, 41.1378.005) and spun down at 10,000 × *g* for 5 min to obtain serum. Sera were stored at −20°C until further cytokine measurement analyses.

Animal Experiments

All animal experiments were approved by the local ethics committee and carried out according to the guidelines of the German Law for the Protection of Animals. BALB/cJ mice were purchased from Jackson Laboratory (Bar Harbor, ME) at an age of 6–8 weeks and were maintained under standardized specific-pathogen-free conditions on a 12-hr light-dark cycle. Nesting material was provided and food and water were provided *ad libitum*. Prior to injections, mice were anesthetized intraperitoneally with a mixture of medetomidine (0.5 mg/kg), midazolam (5 mg/kg), and fentanyl (50 μg/kg). BALB/cJ mice received 20 μg *Cas9* mRNA encapsulated in chitosan-coated PLGA nanoparticles (Chitosan, 83% deacetylated [Protasan UP CL 113] coated PLGA 75:25 [Resomer RG 752H] nanoparticles [NPs]) by intravenous injection (n = 3) into the tail vein. For both interventions, mRNA-NPs were administered in a total volume of 200 μL. To assess immune responses after 6 and 24 hr, mice were sacrificed and blood was collected to obtain serum.

Cytokine Measurement

Blood from mice and human donors was used to obtain serum and tested for IL-12, IL-6, and TNF-α (human and mice, respectively) production by ELISA, as directed in the manufacturer's instructions (BD Biosciences, San Jose, CA).

Statistics

Data were analyzed using Prism 6 Software (GraphPad, San Diego, CA) using a 95% confidence interval. For indel measurements, data were analyzed by one-way ANOVA with a Tukey's multiple comparisons test. For IFN, mouse cytokine, and whole-blood cytokine measurements, an ANOVA with a Dunnet's test for multiple comparisons was used.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.06.010.

AUTHOR CONTRIBUTIONS

S.V., K.T.A., A.K.M.A.H., J.M.H., A.H., S.S., J.S.A., R.I.H., M.S.D.K., M.H.P., and A.P.M. designed experiments. S.V., K.T.A., A.K.M.A.H., J.M.H., A.H., S.S., and J.S.A. conducted experiments. S.V., K.T.A., A.K.M.A.H., J.M.H., S.S., J.S.A., and A.P.M. wrote the manuscript.

CONFLICTS OF INTEREST

M.H.P. receives consulting fees from CRISPR Therapeutics for serving on their scientific advisory board.

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Molecular Therapy
Review

Recent Developments in mRNA-Based Protein Supplementation Therapy to Target Lung Diseases

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Protein supplementation therapy using *in vitro*-transcribed (IVT) mRNA for genetic diseases contains huge potential as a new class of therapy. From the early ages of synthetic mRNA discovery, a great number of studies showed the versatile use of IVT mRNA as a novel approach to supplement faulty or absent protein and also as a vaccine. Many modifications have been made to produce high expressions of mRNA causing less immunogenicity and more stability. Recent advancements in the *in vivo* lung delivery of mRNA complexed with various carriers encouraged the whole mRNA community to tackle various genetic lung diseases. This review gives a comprehensive overview of cells associated with various lung diseases and recent advancements in mRNA-based protein replacement therapy. This review also covers a brief summary of developments in mRNA modifications and nanocarriers toward clinical translation.

RNA, a fundamental molecule in the eukaryotic and prokaryotic cells and viruses, came recently into focus for therapeutic approaches:[1–3] tRNAs for nonsense mutation correction, RNA aptamers for binding to a specific target molecule, and RNAi and long noncoding RNAs (lncRNAs) for gene regulation.[4–8] mRNA gives rise to a new therapy for diseases associated with functional protein loss by supplementing the protein with a transcript encoding. The first study of *in vitro*-transcribed (IVT) mRNA in the late 1980s showed that this mRNA can be directly translated into a functional form immediately after transfection *in vitro* and *in vivo*.[9,10] This was shortly followed by a therapeutic application of mRNA in a temporary reversal of diabetes *insipidus*, unraveling its therapeutic potential.[11]

Therapeutic applications of mRNA are advantageous due to its unique properties. Cytoplasm being mRNA's functional site requires no transportation across the nuclear membrane. Furthermore, due to the transient nature and biodegradability of mRNA, permanent adverse effects can potentially be avoided.[12] This includes preventing the permanent manipulation of the genome, making mRNA a prevalent molecule for protein supplementation therapy.[13–15] Several aspects of mRNA have to be addressed in order to achieve therapeutic benefits: potential immunogenicity mediated by innate immune system reactions (pattern recognition receptor),[16–19] degradation of single-stranded mRNA by nucleases, and its negative charge that inhibits the passive crossing of the cell membrane. Concerning these obstacles, solutions such as chemical modifications of nucleosides to reduce immunogenicity and usage of nanocarriers to facilitate crossing the cell membrane are emerging. The recent advancements in the field of nanocarriers suggest the possibility to customize particles for target organs.[20,21]

Based on our research interest in lungs, this review focuses on protein replacement therapy of lung disease, especially monogenetic diseases, such as cystic fibrosis (CF) and surfactant protein B (SP-B) deficiency, as well as multifactorial diseases, such as chronic obstructive pulmonary disease (COPD) and asthma. To achieve targeted therapy options, lung structure and methods to reach specific lung cell populations are critical. Therefore, this review gives an overview of lung cell populations and diseases associated with them. Furthermore, a comprehensive summary of mRNA transcript improvements by chemical modification of nucleosides or capping and nanocarriers to target lung cell populations is also featured.

Lung Architecture and Disease Pathology

The lung has a very unique architecture to enable efficient transfer of oxygen and carbon dioxide required for oxidative metabolism. Inhaled gases travel through the airway tubes via trachea bronchi and bronchioles to the alveoli enriched with blood vessels, the primary site of gas exchange. Inflation and deflation of the lung is a prerequisite for gas exchange at the alveoli. This process requires multiple components like the extracellular matrix, smooth muscle cells, and cartilage for support and flexible collagen and the elastin fiber network for flexibility during inflation and deflation. Precisely regulated surface fluids, electrolytes, and mechanical activity of secretory

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and ciliated cells determine the mucociliary clearance, whereas on the other hand the epithelium maintains the barrier function.

The airway epithelium (tracheal and bronchiolar) consists of goblet cells, brush or tuft cells, ciliated cells, basal cells, neuroendocrine or neuroepithelial bodies, club cells, lineage-negative epithelial progenitor (LNEP) cells, and the newly identified ionocytes.[22,23] The ducts of submucosal glands consist of goblet cells, serous cells, and myoepithelial cells. The alveolar epithelium consists primarily of alveolar type I (ATI) cells (~95%), macrophages, and alveolar type II (ATII) cells, with close association to capillary endothelial cells of pulmonary microcirculation.[24] The terminal airway ducts and alveoli are supported by fibroblasts and myofibroblasts producing extensive elastin-collagen networks, which help with inflation and deflation.[25–27] Other important cell populations include stem cells and immune cells, which help in region-specific regeneration and protection against pathogens, respectively. Abnormalities of lung mechanics are observed in various diseases, including CF,[28,29] asthma,[30,31] idiopathic pulmonary fibrosis (IPF),[32,33] COPD,[34,35] and bronchiolitis *obliterans*.[34,36] These conditions are detrimental in nature and involve multiple factors, such as increased resistance of lung tissue due to fibrosis of the lung; collapsing tubes or thickening of airway walls due to mucus overproduction; and the loss of ciliary function, resulting in airflow obstruction, mucus plugging, chronic infection, and inflammatory damages. In that process, multiple cell populations undergo remodeling, directly contributing to clinical symptoms.

Broncho-Epithelial Cells and Associated Diseases

Goblet cells, present in the broncho-epithelia and submucosal glands, produce mucins (MUC5B and MUC5AC), and they are differentiated from basal and club cells upon various stimuli (toxic substances, pathogens, particles, and neural and innate immune signals). Goblet cells are not only helping to establish the innate immune system but also key players in pulmonary diseases, as described before.[37] These cells produce cytokines and chemokines that recruit and educate innate immune cells, including dendritic cells (DCs), innate lymphoid cells, and eosinophils. This contributes to the $CD4^+$ T helper cell 2 (Th2)-mediated immune response typical of asthma.[38,39] Hyperproduction of mucus and goblet cell metaplasia are characteristics of Th2-mediated and non-mediated inflammation (pathogen mediated), which leads to complications in CF, COPD, and IPF.[40,41] The fluids for hydration are regulated by serous cells that line the acinar region of submucosal glands and are important for the pathology of CF. The mucins are precisely balanced by fluid, and electrolyte transport enables rapid secretion and dispersal of mucus onto the airway surfaces and the movement of the mucus gel up the airway by ciliary activity. In CF, the secretion of chloride and bicarbonate is impaired, disrupting mucociliary clearance due to mutations in CF transmembrane conductance regulator gene (*CFTR*), which leads to recurrent airway infections, sinusitis, bronchiectasis, and pulmonary tissue remodeling.[42]

Brush cells, containing distinctive apical microvilli, are found in multiple organs, e.g., pancreas, intestine, nose, and trachea. Brush cells are known to play an important role in activating the innate immune system in the intestine[43] and nose (*Tas2R* receptors),[44] however, a similar phenomenon in airway trachea requires testing. Recent studies in the trachea have indicated their role as chemosensory for immune surveillance and as respiratory regulators.[45] These might be responsible in transducing signals regulating wheezing and coughing during episodes of asthma. However, further studies are required to understand their role.[46]

Ciliated cells are characterized by their multiple apical, motile cilia composed of structural proteins and motor proteins (dynein)[47–49] that regulate the coordinated bidirectional beating critical for particle and pathogen clearance.[50,51] Ciliated cells respond to both physical[52] and chemical[53] stimulation. Mucociliary clearance can be affected by ciliary dysfunction, impaired fluid secretion, disruption of epithelial cell lining, or lack of cough. This impairment can initiate an inflammatory response, damaging the airway epithelium. Disruption in ciliated cell function results in recurrent and persistent infections, morbidity, and mortality in chronic pulmonary disorders.[54] In COPD, direct evidence has been provided of suppressed ciliary beating in nasal epithelium[55] with normal mucus production. Cigarette smoking has been shown to have a detrimental effect on the number and size of cilia *in vitro*,[56] whereas *in vivo* a slight increase in ciliary beating initially followed by significant loss of cilia over prolonged time was observed.[57] Primary ciliary dyskinesia (PCD) resulting from ciliary dysfunction is caused by recessive mutations in one of multiple genes involved at different points in cilium structure, assembly, and function, which include *DNAI*,[58] *DNAH*(*5*,[59] *11*[60]), *ARMC4*,[61] *TXNDC3*,[62] *HEATR2*,[63] *HYDIN*,[64] *CDC* (*39*,[65] *40*,[66] *65*,[67] *103*,[68] *114*,[69] *151*[70]), *DNAAF*(*1*,[71] *2*,[72] *3*[73]), *RHSP*(*4A*, *9*),[74] DYX1C1,[75] LRRC6,[76] ZMYND10,[77] *CCNO*,[78] and recently identified *MCIDAS*.[79]

Club cells are columnar, secretory cells that express high levels of cytochrome P450-detoxifying enzyme CYP2F, surfactant proteins (SP-A, SP-B, and SP-D), and innate immune proteins, including defensins, lactoferrin, and secretaglobins (SCGB1A1 and SCGB3A1).[80] Upon stimulus or injury, these differentiate into alveolar cells,[81,82] goblet cells,[38,83,84] or ciliated cells.[85] However, in humans, these are only localized in terminal bronchioles, and they may play a role in the maintenance of distal bronchioles.[86] Club cells being progenitor cells also plays an important role in the repair mechanism of the airway epithelium, and, therefore, they are connected to damage responses in CF, COPD, and IPF.[80] Club cells also metabolize chemical toxins (e.g., naphthalene) to toxic compounds that selectively kill club cell subsets. SP-A and SP-D (C-type lectins) are responsible for host defense, enhancing the clearance of various microbial pathogens, whereas the secretory lipids and proteins (SP-B and SP-C) help in minimizing surface tension and collapsing forces caused by inhaled gases when in direct contact with alveolar structures, protecting peripheral saccules from atelectasis during ventilator cycles.[87]

Neuroendocrine cells represent less than 1% of the airway epithelium,[88] and they are found either isolated or in clusters known as neuroepithelial bodies located at precise airway branch points.[89] The

localization aids in their role as airway (environmental) sensors (acidosis, hypoxia, and hypercarbia),[90,91] and clustering is required for the appropriate innate immune responses.[92] The response to a stimulus is via the release of stored amines (serotonin) and peptides (calcitonin gene-related peptide [CGRP]).[92] Hyperplasia is associated with a wide range of congenital and infantile lung disorders. Though the underlying cause is unknown, NKX2-1 mutation has been associated with neuroendocrine hyperplasia of infancy (NEHI).[93]

Ionocytes (Foxi1^{+}) are the rare cell type of the airway epithelium recently characterized, in both mice and humans, by two independent research groups.[22,23] These reside at multiple levels of the airway tree, and they are needed to maintain airway surface physiology, including mucus viscosity.[23] Foxi1 is already known to regulate V-ATPase, which is important for transport and fluid pH in other cell types in skin.[94,95] Knockout in a mouse model reduced Cftr and Ascl3 expression, indicating the role of Foxi1 in CFTR regulation.[23] Montoro et al.[23] also performed pulse-sequence tracing that indicated the basal cell lineage, with an increased expression of *CFTR*.

Basal cells are progenitor cells[96–98] that are regulated by NOTCH signaling to give rise to ciliated cells (NOTCH^{-}), goblet cells (NOTCH^{+}), and club cells (NOTCH^{2+}).[99–102] Due to their basal proximity, these cells interact with the columnar epithelium; underlying mesenchymal cells; basal membrane; neurons; and also lymphocytes, inflammatory cells, and DCs.[103] A loss in tight regulation of basal cell differentiation can result in inappropriate cell fate determination, leading to pathological airway remodeling. This includes epithelial hypoplasia (proliferation failure), basal cell hyperplasia (excessive proliferation with no differentiation), goblet cell metaplasia or hyperplasia instead of ciliated cell generation, and squamous metaplasia (suprabasal cells) instead of luminal cells. Pathological airway remodeling occurs frequently in association with CF, COPD, and chronic asthma.[103] Araya et al.[104] in their study showed that hyper-proliferating basal cells secrete cytokines (interleukin-1β [IL-1β]) that promote airway wall fibrosis via transforming growth factor β (TGF-β) signaling in COPD.

Alveolar Cells and Associated Diseases

Alveolar type I cells (ATI) are squamous cells lining the alveolar compartment involved in gas exchange. These are terminally differentiated cells (lifespan ~120 days) that form a barrier to sense microbial products and generate inflammatory responses.[105,106] These cells undergo excessive physical and chemical stresses due to their higher exposure, and they require constant regulated repair.[107,108] A defect in repair directly and indirectly contributes toward injurious manifestations of the lung, leading to diseases like acute respiratory distress syndrome (ARDS) and IPF.[109,110] ATII (progenitor of ATI cells) are cuboidal cells and cover about 7% of the total alveolar surface. These produce surfactant lipids (phosphatidylcholine) and surfactant proteins (SP-A to -D). Congenital SP-B deficiency leads to death[87,111] soon after birth; however, targeted disruption of SP-C,[112] SP-A,[113] and SP-D[114,115] gene loci does not show detrimental effects. In cases with acute respiratory distress syndrome, a decrease in the expression of SP-B is also observed.[116,117]

Stem Cells of the Airways

To maintain the constant dynamic function of the lung, it is very crucial that the respiratory epithelium is equipped with fast and extensive regenerative ability following injury. Airway basal cells and ATII cells have been known for their role in repair of the airway epithelium. Recently, studies have identified distinct niches throughout the lung that can mediate graded and region-specific responses.[118,119] Myoepithelial cells (MECs) and bronchoalveolar stem cells (BASCs) are a couple of the established stem cells in lungs. It was well established that the innervated MECs encircle the submucosal glands and mediate mucus secretion in response to neural inputs, which can activate massive secretory responses after stimulation by irritants and toxins (also reviewed in Boers et al.[86] and Yei et al.[120]).[37,42,121] However, recent work by Lynch et al.[122] and Tata et al.[123] have explained the potency of MECs in generating seven cell types of surface airway epithelium and the submucosal gland following airway injury. These can be activated via Sox9 or Lef-1 transcriptional signaling, and they can be exploited for regenerative medicine. BASCs have been identified as stem cells that co-express both club cell and ATII cell markers.[124] BASCs sorted by flow cytometry and cultured *in vitro* showed differentiation, self-renewability, and response to injury.[124–126] Lineage-tracing studies have revealed the BASC's ability to give rise to alveolar epithelial cells *in vivo*[127,128] and its contribution to homeostasis and repair, along with club and ATII cells.[127–130]

Respiratory Diseases Targeted by Protein Replacement Therapy

SP-B Deficiency

SP-B deficiency is a rare genetic disease leading to neonatal lethality, including interstitial lung disease (ILD) and ARDS.[131–135] SP-B is crucial for breathing transition of neonates at birth, and it helps in reducing surface tension of the alveolus. Dipalmitoylphosphatidylcholine (DPPC) is the principal surface tension-reducing component that combines with hydrophobic SP-B or SP-C peptides to form stable surfactant film.[136] Surfactant supports rapid adsorption and insertion of phospholipids, reduction in surface tension upon compression, and rapid re-spreading during expansion. Changes in surfactant lead to alveolocapillary leakage, alveolar instability, compromised gas exchange, and respiratory failure. Both SP-B and SP-C peptides are processed from their pro-peptide forms to their functional form. Absence of proSP-C processing leads to the accumulation of misprocessed SP-C, consisting of the mature peptide with an N-terminal extension (relative molecular weight [M_r] ~6,000), and to a significant decrease in mature SP-C peptide in alveolar surfactant. ProSP-C processing is also closely related to SP-B expression.[87,111,137] The combinatorial effect exacerbates lung function at birth, and *in vivo* studies showed respiratory failure in selective loss of SP-B in adult.[138]

Natural surfactant replacement, such as Survanta beractant (modified bovine surfactant 8 mg SP-B/mL) by Abbott Laboratories, Curosurf

(porcine surfactant 80 mg/mL) by Chiesi, and Infasurf (calf surfactant), are a few of the FDA-approved preventive medications for infants with ARDS or premature babies at the risk of developing RDS. Accompanied by physical measures, corticosteroids, or immunosuppressants and repeated lung lavage, surfactant replacements have shown improvement in the disease condition until lung transplantation.[139–144] The potential risks with animal-derived protein include immunological reactions and transmission of animal-derived diseases, justifying the need for standardized human-like alternatives.[145–147] One alternative involves synthetic mimics that have shown superior surfactant properties.[144,148]

Since SP-B deficiency is a monogenetic disorder, it acts as a perfect model for gene therapy. Both DNA- (virus[120] and plasmid[149]) and mRNA-[150] based gene supplementation have been tested in a conditional SP-B-knockout mouse model, which indicated improvements in lung function and SP-B expression and a significant increase in survival. Kormann et al.[150] showed for the first time that intratracheal (i.t.) instillation of modified *SP-B* mRNA to the lung can restore up to 71% of the wild-type SP-B expression, and the treated conditional SP-B-knockout mouse model survived until the predetermined end of the study of 28 days. Presently, the Rudolph team from Ethris holds a patent for pulmonary delivery of mRNA with polyethylenimine (PEI) (US patent application 20150126589), and their teaming up with AstraZeneca and MedImmune could bring the therapy closer to the reach of patients.[151]

Asthma

Asthma is a multifactorial disease and can be characterized by airway obstruction, chest tightness, wheezing, cough, and breathlessness, followed by recurrent pneumonia or bronchitis. The initiating event in asthmatic airway diseases revolves around interactions between DCs and T cells. DC and T cell interactions favor the generation of Th2, leading to eosinophilia, mucus hypersecretion, and chronic airway inflammation.[152,153] The overactive Th2 response induces the production of cytokines and chemokines, followed by a cascade of immune-activating events, leading to changes in airway smooth muscle contractility,[154] a characteristic of asthma. Studies by Hellings et al.[155] and Wilson et al.[156] showed that Th17 cells orchestrate airway inflammation by enhancing neutrophil recruitment to the lung. The Th2-mediated immune response can be contained via Th1-type cytokines (by Th1 cells), IL-10, and TGF-β (by T regulatory cells [Tregs]), but the roles of IL-17 and IL-22 (by Th17 cells) are debated.[155,157,158] Both circulatory and airway fluids of asthmatic patients indicate increased IL-17 levels[155] and decreased airway Tregs,[159] indicating an imbalance in Th2 regulation.

Corticosteroid treatment is found to suppress the Th2 immune response via increased Foxp3$^+$ Tregs in asthmatic patients.[160] Similar results were found with exposure to microbes[161,162] influencing Treg expression, modulation of IL-6,[163] prostanoids,[164] and tumor necrosis factor (TNF) pathway enhancement.[165] Mays et al.[166] has successfully demonstrated the protective role of Foxp3 by delivering chemically modified *Foxp3* mRNA into the lung of an asthma mouse model. Site-specific instillation of chemically modified *Foxp3* mRNA can modulate both Th2 and Th17 responses in an IL-10-dependent manner.[166] Local administration of *Foxp3* mRNA can influence the balance among Treg, Th2, and Th17 responses, and it can reduce side effects in terms of the anti-tumoral and anti-infective[167,168] effects of Tregs in comparison to systemic delivery.[165] Kormann et al.[169] produced a unique insight into Toll-like receptors (TLRs), as polymorphisms in TLRs 1, 6, and 10 have shown protective effects on atopic asthma in humans by forming heterodimers with TLR2. A subsequent study by Zeyer et al.[170] demonstrated that *Tlr1/2* and *Tlr2/6* mRNA instillation in the lungs of a house dust mite-induced mouse model of asthma reduced airway resistance by 40%.

CF

CF, caused by mutations in the CFTR gene, is the most common life-limiting autosomal-recessive disease in the Caucasian population, and it affects more than 80,000 people worldwide. Around 2,000 mutations have been identified and are categorized into 6 classes, ranging in severity from no production of functional protein to decreased synthesis, stability, or function of CFTR protein. CFTR protein acts as a small conductance ATP and cyclic AMP (cAMP)-dependent chloride channel, found at the apical side of epithelial cell lining of most exocrine glands. In the lung epithelium (ionocytes and ciliated and goblet cells), CFTR ensures the secretion of chloride ions, resulting in more hydration and regulated mucus clearance in the airway. A lack of functional CFTR leads to decreased chloride secretion and increased sodium absorption, resulting in dry and rigid mucus production by goblet cells.[171] An increase in inflammatory response is also observed, possibly due to the breakdown of elastin fibers by neutrophil elastase and reduced IL-10.[172] The defective mucus clearance enables further pathogen (Pseudomonas aeruginosa and Staphylococcus aureus) colonization, repetitive inflammatory responses that result in irreparable lung damage, and ultimately cardiac arrest. Defects of the CFTR channel lead to a failure in LPS recognition, endocytosis of pathogens, and changes in airway fluid composition. This inactivates beta defensins, causing detrimental effects on the primary defense in the lung.

A readthrough agent for CFTR class I mutation (Ataluren, PTC Therapeutics) showed potential benefits *in vivo* by increasing CFTR production and function;[173] however, it failed phase III clinical trial due to a lack of efficacy.[174] Channel modulators categorized into potentiators, correctors, and amplifiers have been used for CF treatment. Potentiators (ivacaftor, Kalydeco) can help in gating and conduction mutations by increasing the open probability of the CFTR channels. Correctors (tezacaftor and lumacaftor) improve CFTR trafficking by facilitating the formation of correct 3D protein structure. Combinations of potentiator and corrector (Orkambi and Symdeko, Vertex Pharmaceuticals) are commercially available only for patients with F508del mutation, expanding the modulators also for application on class II mutations.[175,176] A triple combination of two correctors (VX-659, tezacaftor) and one potentiator (ivacaftor) has also shown greater potency in pre-clinical trials. PTI-428 (Proteostasis Therapeutics), an amplifier to increase the amount of CFTR protein,

Table 1. Current Clinical Trials Involving mRNA Delivery

Name	Disease	Genetic/Protein Target	Administration Route	Administration Vehicle	ClinicalTrials.gov Identifier	Phase
Lipo-MERIT	melanoma	tumor-associated antigens	intravenous infusion	mRNA-Lipoplex	NCT02410733	1
TNBC-MERIT	triple-negative breast cancer	tumor-associated antigens	intravenous infusion	mRNA-Lipoplex	NCT02316457	1
IVAC mutanome /warehouse	triple-negative breast cancer	patient-specific antigens	intra-nodal	naked	NCT02035956	1
mRNA-1851	influenza A	Hemagglutinin 7 (H7) protein	intramascular injection	not disclosed	Not disclosed	1
mRNA 1440	influenza A	Hemagglutinin 7 (H7) protein	intramuscular injection	not disclosed	not disclosed	1
CV7201	rabies	rabies virus glycoprotein	intramuscular injection	naked	NCT02241135	1
CV8102	HIV, rabies, RSV	RNA-based adjuvant	intramuscular injection	naked	NCT02238756	1
mRNA MRK-1777	not disclosed	vaccine	intradermal	not disclosed	not disclosed	1
mRNA AZD-8601	cardiovascular diseases	VEGF-A	intramuscular injection	naked	NCT02935712	1
mRNA-1325	Zika	viral antigenic protein	intramuscular injection	lipid nanoparticle	NCT03014089	1/2
CV9103	prostate cancer	tumor-Specific antigen	autologous dendritic cell therapy	naked	NCT01197625 NCT00831467	1/2
MRT5005	cystic fibrosis	CFTR	nebulization to the respiratory tract	lipid nanoparticle	NCT03375047	1/2
AGS-004	HIV	vaccine	autologous dendritic cell therapy	naked	NCT01069809, NCT02707900	1/2
AGS-003-LNG	non-small-cell lung cancer	tumor-specific antigen	autologous dendritic cell therapy	naked	NCT02662634	2
iHIVARNA-01	HIV	HIV-target antigen	intranodal route	naked	NCT02888756	2
AGS-003	renal cell carcinoma	tumor-specific antigen	autologous dendritic cell therapy	naked	NCT01482949 NCT00678119 NCT01582672	2/3

Modified from Kaczmarek et al.[321]

is in phase II trial. Eluforsen (QR-010) can bind to defective CFTR RNA, and it has shown increased CFTR function by nasal potential difference test in compound heterozygous or homozygous F508del CF patients.[177]

Protein replacement therapy with DNA, protein, or mRNA holds a great potential as a universal therapy for curing the underlying defect of CF. Initial attempts of *in vivo* protein transfer via phospholipid liposomes into the apical membrane of nasal epithelia of CFTR-knockout mice showed limited membrane incorporation by electron microscopy but improvement of nasal potential difference (NPD) measurement.[178] Similarly, DNA-based vectors (viral and plasmid) were tested by Alton's group (pGM169/GL67A), reaching phase II clinical trials with modest improvement in FEV_1 after repeated administration but no improvement in patient's quality of life.[179,180] Haque et al.[20] have observed a significant improvement in CFTR protein translation, expression, and function *in vitro* (CFBE41o- and 16HBE14o-) and *in vivo* (CFTR-knockout mice) by administering chemically modified human CFTR (hCFTR) mRNAs complexed with chitosan-coated poly(lactic-co-glycolic acid) (PLGA) nanoparticles. The study also showed a substantial improvement in $FEV_{0.1}$ up to 89% of the level of a healthy control group. Airway compliance and resistance are also significantly improved by the treatment with chemically modified hCFTR mRNAs. A significant decrease in chloride concentration (around 50%) was also observed, indicating a restoration of CFTR in the duct compartment of submucosal glands and thus leading to improved chloride absorption.[20] A separate study from Robinson et al.[181] confirmed nasal application of chemically modified CFTR mRNA can recover up to 55% of the net chloride efflux characteristic of healthy mice. Bangel-Ruland et al.[182] demonstrate restoration of cAMP-induced CFTR current following transfection of CFBE41o- cells with wild-type CFTR-mRNA similar to the values seen in 16HBE14o- control cells. Translate Bio is also working on CFTR-encoding mRNA (MRT5005) and has entered phase I/II clinical trial (Table 1). All these studies prove the potential of mRNA as a promising therapeutic in CF patients, irrespective of their CFTR mutation status.

COPD and COPD-like Symptoms in α1-Antitrypsin Deficiency

COPD is a progressive and largely irreversible smoking-related disease characterized by small airway obstruction, emphysema, and chronic bronchitis. It is mainly attributed to long-term exposure to tobacco, toxic gases, and particles, activating both innate and adaptive immune responses. The innate immune defense includes tight junctions, TLRs of epithelial barrier, macrophages, and alveolar fluid

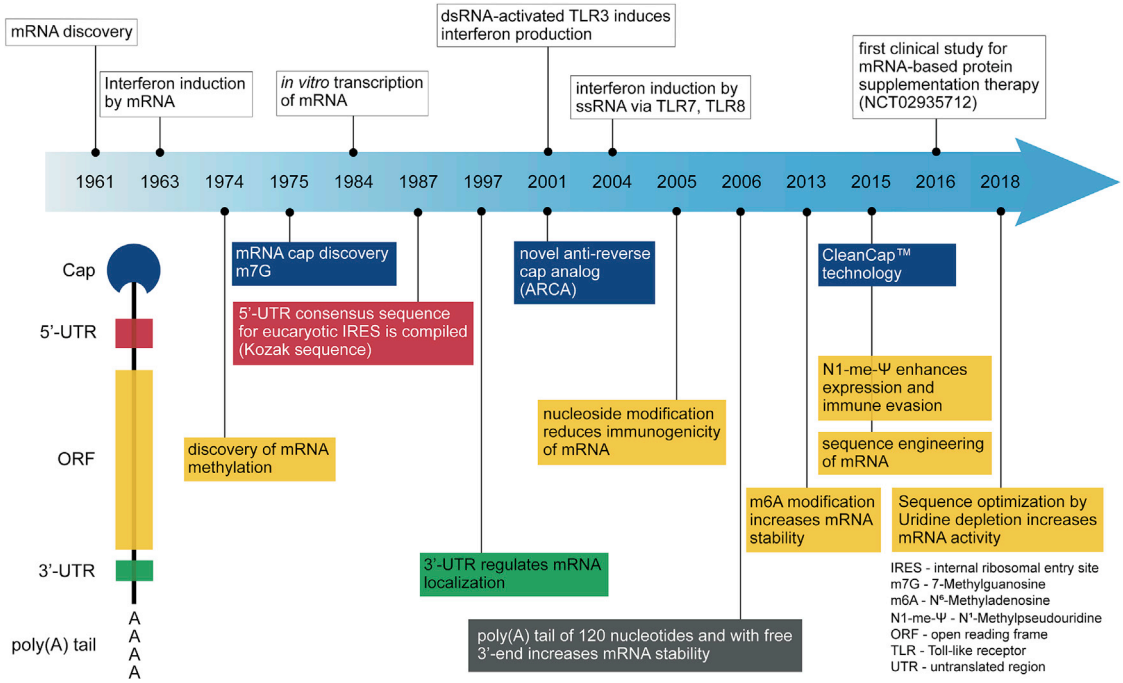

Figure 1. mRNA IVT, Modifications, and Function and Timeline

Overview of milestones in protein supplementation therapy, *in vitro* transcription, and mRNA modification. White boxes, important milestones for the development of mRNA therapy;[250] blue boxes, evolution of different cap structures;[215,220,322] red, green, and gray boxes, 5′ UTR, 3′ UTR, and poly(A) tail, respectively, the addition of regulatory elements in the modification of mRNA;[14,312,313] yellow boxes, nucleoside modifications and sequence optimizations in the development for mRNA therapy.[215,230,238,240,314,317]

(secreted by ATII cells of lung). A second line of defense includes exudation of plasma and circulating effector cells into damaged tissue, regulated by IL-1, IL-8, and TNF-α. The infiltration of both bronchial and alveolar tissue with macrophages, B and T lymphocytes, and eosinophils has been associated with emphysematous destruction. Both the responses are linked to tissue repair and remodeling that increase mucus content of airway lumen and metaplasia of mucous and squamous cells. This leads to a thickened wall and narrowed lumen of conducting airways. Second, emphysema limits air flow by reducing elastic recoil pressure for exhaling air during forced expiration.[183]

α_1-antitrypsin deficiency (AATD) can cause COPD-associated symptoms like emphysematous destruction along with innate inflammation in lung due to an imbalance in protease and antiprotease homeostasis.[184] In lungs, AAT has the major physiological function of protecting the healthy but fragile alveolar tissue from proteolytic damage of neutrophil elastases.[185] The AATD is largely associated with mutations within *SERPINA1*, resulting in abnormal protein folding, intracellular retention, and consequently low serum levels.[186] However, multiple other factors also contribute to disease severity, and much research is being done to obtain a comprehensive picture for enabling better diagnosis.[187–196] Danozol was found to significantly improve AAT circulating levels[197] without eliciting side effects. Another approach is to inhibit polymerization of AAT by small molecules,[198] peptides,[199] autophagy-enhancing drugs[200,201] (ClinicalTrials.gov: NCT01379469 Tregretol phase II clinical trial, rapamycin[202]), and RNA silencing of mutant AAT in liver hepatocytes.[203–205] Intravenous (i.v.) augmentation of plasma-derived AAT (Bayer Biologicals, ZLB Behring, Baxter Healthcare)[206,207] is an established method to raise circulating levels of AAT in blood and bronchoalveolar lavage fluid (BALF), slowing the progression of lung destruction.[208]

Various routes of administration and vectors have been tested for gene delivery of AAT in various animal models (rat, mouse, and dog), with varying expression efficiency in terms of time and localization (reviewed in detail[209]). However, only AAVrh.10hAAT (Adverum Biotechnologies, ClinicalTrials.gov: NCT02168686) has entered the phase I/II clinical trial to assess the safety and therapeutic level expression of M1-type AAT in the serum and alveolar epithelial lining fluid.[210] Connolly et al.[21] have shown successful expression of AAT with liposome-encapsulated *SerpinA1* mRNA *in vitro* and *in vivo* after transfection. In an independent study, Michel et al.[211] observed significant expression of AAT *in vitro* and *ex vivo* along with a significant reduction in elastase activity.

Steps toward Pharmacologically Auspicious mRNA

In recent years substantial efforts have been made for engineering mRNA with diverse pharmacokinetic properties (Figure 1). Modifications of structural elements such as 5′ Cap, 5′ and 3′ UTRs, poly(A) tail, and the coding region were the main focus.[14,212–215]

5′-Capping for Stability and Immune Evasion

5′-capping is vital for the robust translation of mRNA as the natural 7-methylguanosine (m^7G/Cap0), and it is connected by a 5′-to-5′ triphosphate bridge to the first nucleotide. Translation is initiated by binding to eukaryotic translation initiation factors (eIF4E and eIF4G), and mRNA deterioration is controlled by binding with Dcp1, Dcp2, and DcpS (mRNA-decaying enzymes).[57,216–218] In *in vitro* transcription, m^7G possesses the risk of constructing uncapped or inactive IVT mRNA, as the m^7G and GTP compete for incorporation. m^7GpppG cap was the first step to circumvent the restriction of m^7G, however, substantial proportions of m^7GpppG analog were incorporated in the reverse direction and thus yielded substandard translational activity.[9,219] Anti-reverse cap analog (ARCA; $m_2^{7,3'\text{-O}}GpppG$) can counteract the reverse integration and skip degeneration by Dcp2, and, thus, it results in superior translational efficiency and extended half-life.[13,150,220] Study on viral capping systems reveals that the 2′ ribose position of the first cap-proximal nucleotide is 2′O-methylated to form a Cap 1 structure ($^{m7}GpppN_{2'Om}$ N), and, in ~50% of transcripts, the second cap-proximal nucleotide is 2′O-methylated to form a Cap 2 ($^{m7}GpppN_{2'Om}N_{2'Om}$) structure.[221] Cap 1 2′O-methylation has been described to reduce recognition by pattern recognition receptors (e.g., interferon [IFN]-induced protein with tetratricopeptide repeats-1 and 5 [IFIT1 and IFIT5] and retinoic acid-inducible gene I [RIG-I]) in comparison to Cap 0.[222–224]

Modification in UTRs

The poly(A) tail plays an important role in regulating the stability and translational efficiency (half-life) of mRNA by preventing deadenylation by poly-specific nucleases.[225] Integration of the poly(A) tail during IVT mRNA synthesis can be conducted by encoding the poly(A) stretch in the template or by a two-step enzymatic reaction using recombinant poly(A) polymerase.[226] The ideal length of the poly(A) tail is between 120 and 150 nt, and the 3′ end of the poly(A) tail should not be concealed by additional bases.[14,213] 5′ and 3′ UTRs also play a vital role in the stability and expression of IVT mRNA by harboring several sequence elements. For example, mRNAs with adenosine in 5′ UTR can form a complex with Lsm1-7 at both the 5′ and 3′ ends and circularize the transcript to inhibit degradation by exosome and Dcp1/2.[227] Adenine uracil (AU)-rich elements in the 3′ UTR can destabilize the mRNA, and they might provide a mechanism to limit the duration of protein production.[228]

Post-transcriptional Modifications

Post-transcriptional chemical modifications of RNA are not uncommon, and over 100 modifications are listed by different studies.[229] In mRNA, only a small subset of these naturally occurring modifications is reported to be essential for reducing innate immune response and improving mRNA expression and stability.[215,230,231] N6-methyladenosine (m6A) is one of the most frequent modifications in eukaryotic mRNA. Insulin-like growth factor 2 (IGF2) mRNA-binding proteins 1, 2, and 3 (IGF2BP1/2/3) preferentially recognize m^6A mRNA and guard the modified mRNA against decay.[232] Based on the studies of Kormann and Warren et al.,[233] the first generation of modified mRNAs containing 5-methylcytidine (m5C) or pseudouridine (Ψ-UTP) reduces innate immune responses and enhances translation. TLR3, TLR7, TLR8, and RIG-I activations were significantly reduced when mRNA contained modified nucleosides such as m5C, m6A, 5-methyluridine (m5U), Ψ-UTP, and 2-thiouridine (s2U).[20,150,230,234] RNA-dependent protein kinase (PKR) arbitrated the immune response, and translation inhibition (by phosphorylating the alpha subunit of translation initiation factor 2 [eIF-2α]) can be escaped using Ψ-UTP- or m5C-modified nucleosides.[235] m5C is explicitly recognized by the mRNA export adaptor ALYREF, and it increases mRNA-binding affinity and associated mRNA export.[236] Activation of two important components of the innate immune response against unmodified mRNA, the interferon-induced enzymes 2′-5′-oligoadenylate synthetase (OAS) and RNase L, can be limited by Ψ-UTP.[237] N1-methylpseudouridine (N1-mΨ-UTP) is the most used chemical modification in recent studies, and it showed remarkable expression compared to Ψ-UTP-substituted mRNA, even when delivered by different routes *in vivo*.[238] N1-mΨ-UTP induces a tight binding to RIG-I but failed to activate RIG-I signaling (Figure 2).[234,239]

Codon Optimization

Codon optimization of mRNA uses the degeneracy of the genetic code to substitute specific nucleosides of a mRNA sequence without altering the resulting amino acid composition. Several recent studies have reported a high expression by codon optimization of unmodified and Ψ-UTP-modified mRNA through enriching guanosine and/or cytosine (GC).[231,240] Cas9 activity has been reported to produce significantly higher insertion or deletion (indel) and to be less immunogenic when uridine depletion has been used with 5-methoxyuridine (5moU) modification compared to unmodified and Ψ-UTP-modified Cas9 mRNA.[215] Pharmacologically favorable mRNA that has undergone modification and sequence optimization still needs to be transferred in lung using carriers, to circumvent the naturally occurring barriers the lung possesses.

Reaching the Lung

In vivo delivery of mRNA therapeutics remains one of the biggest hurdles for mRNA-based therapies in general. Apart from the fragility of the mRNA molecules and the ubiquitous existence of RNases, there are two main obstacles in the delivery of mRNA *in vivo*: targeting specific lung cell populations and crossing the cellular membrane. For solving the latter part, nanocarriers as a delivery system have gained increased attention. Therefore, this review focuses on the benefits and obstacles in the use of mRNA-nanocarrier complexes.

In terms of transport of mRNA to a specific tissue, the route of administration plays an important role when discussing the hurdles of organ-specific mRNA therapy. Focusing on the lung, two main routes of administration were investigated in the past: i.v. application of mRNA or mRNA-complexes to reach the lung from the vascular structures and i.t. delivery of mRNA therapeutics via dry powder insufflation or high-pressure liquid suspensions using a microsprayer.[150,166,241–246]

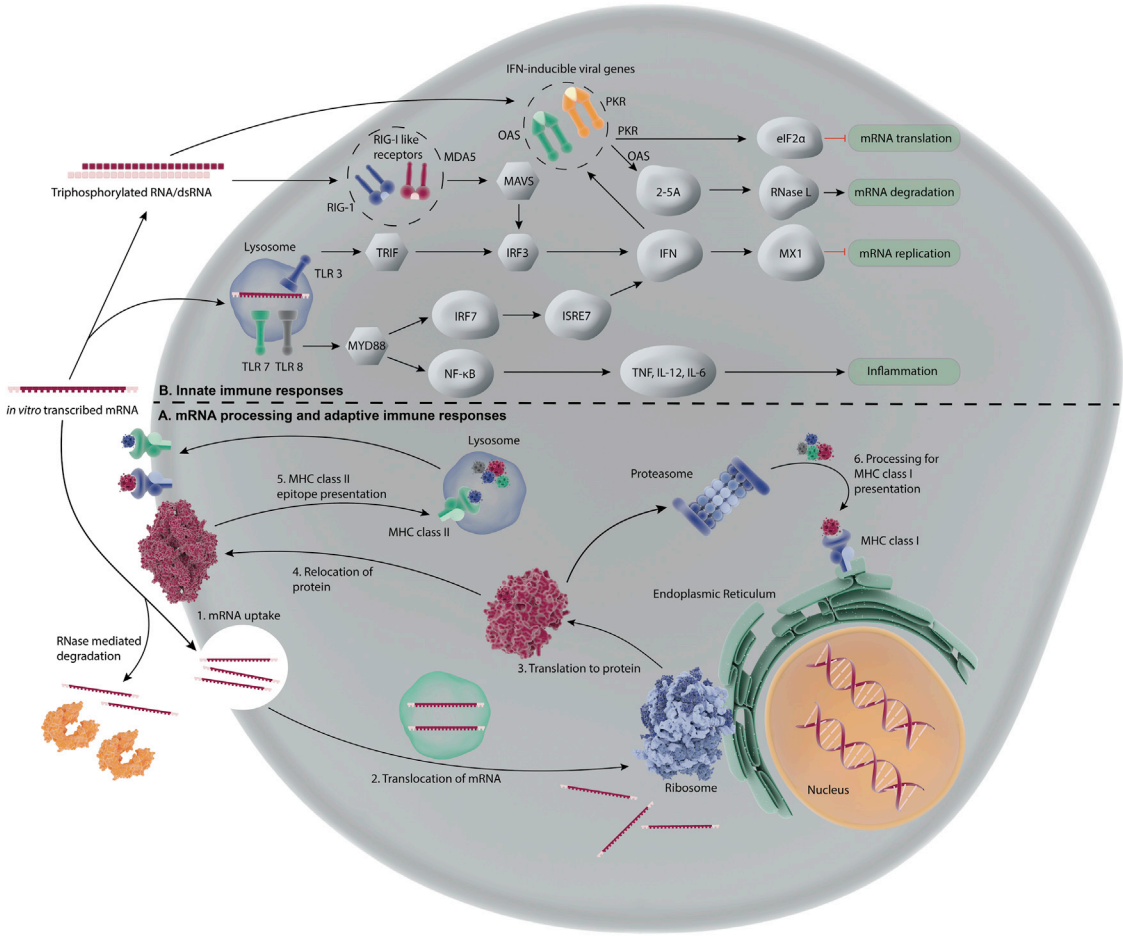

Figure 2. Processing of IVT mRNA in a Cell

(A) *In vitro*-transcribed (IVT) mRNA from linearized DNA or PCR-amplified fragment is used to transfect the cell of interest. Step 1: mRNA protection from RNase degradation and mRNA uptake are facilitated by various carriers. Step 2 of mRNA transport and release inside the cell is still unclear. Different capping modification can increase translation in step 3 and also protect from degradation. In steps 3 and 4, the translated protein from delivered mRNA gets transferred to various parts of the cell system based on post-translational modification. For an immunotherapeutic approach, the translated protein needs to get degraded by proteasome to antigen epitopes and delivered to MHC (major histocompatibility complex) class I located in the endoplasmic reticulum. MHC class I mediates surface presentation of the presented epitope to CD8+ cytotoxic T cells.[320] The T cell further initiates the immune response by relocating the antigen and presenting in to MHC II. (B) IVT mRNA cause inflammatory responses and inhibition of mRNA replication as triphosphorylated mRNA or double-stranded RNA (dsRNA) can be recognized by Toll-like receptors 3, 7, and 8 (endosomal innate immune receptors), which can initiate inflammation associated with type 1 interferon (IFN), interleukin-6 and -12, and tumor necrosis factor (TNF).[230] Cytoplasmic receptors, protein kinase R (PKR), retinoic acid-inducible gene I protein (RIG-I), melanoma differentiation-associated protein (MDA5), and 2′-5′-oligoadenylate synthase (OAS) can detect triphosphorylated mRNA or dsRNA and stalled translation through eIF2α, RNA degradation by ribonuclease L (RNase L), and inhibition of mRNA replication by IFN.[323,324]

Intravenous application of RNAs leads to a systemic distribution of the therapeutic throughout the whole organism.[247] This can be beneficial for certain pulmonary diseases like CF affecting other organs; on the contrary, only a small percentage of an active agent reaches the desired location.[239] A substantial amount of mRNA is removed from the bloodstream, especially in the liver and spleen.[248] This increases the amount of mRNA needed for reaching effective dose levels in the lung.[20] Another hurdle emerges in the lung itself. The lung has a capillary system that consists of mostly small and non-fenestrated capillaries.[249] This is very efficient for gas exchange at the alveoli, but it does not permit a free exchange of larger molecules out of the blood into the tissue. This phenomenon affects mRNA therapeutics by reaching cells in close proximity, while cells farther away from capillaries are harder to reach by i.v. administration.[250] In general, i.v. application of mRNA has the advantage of circumventing some initial innate defense systems and lung barriers while fighting with the problems of systemic application of dispersed distribution and losing the targeted administration toward the lung.

In contrast, i.t. administration of therapeutic substances gives the advantage of local application of mRNA in the lung and airways.

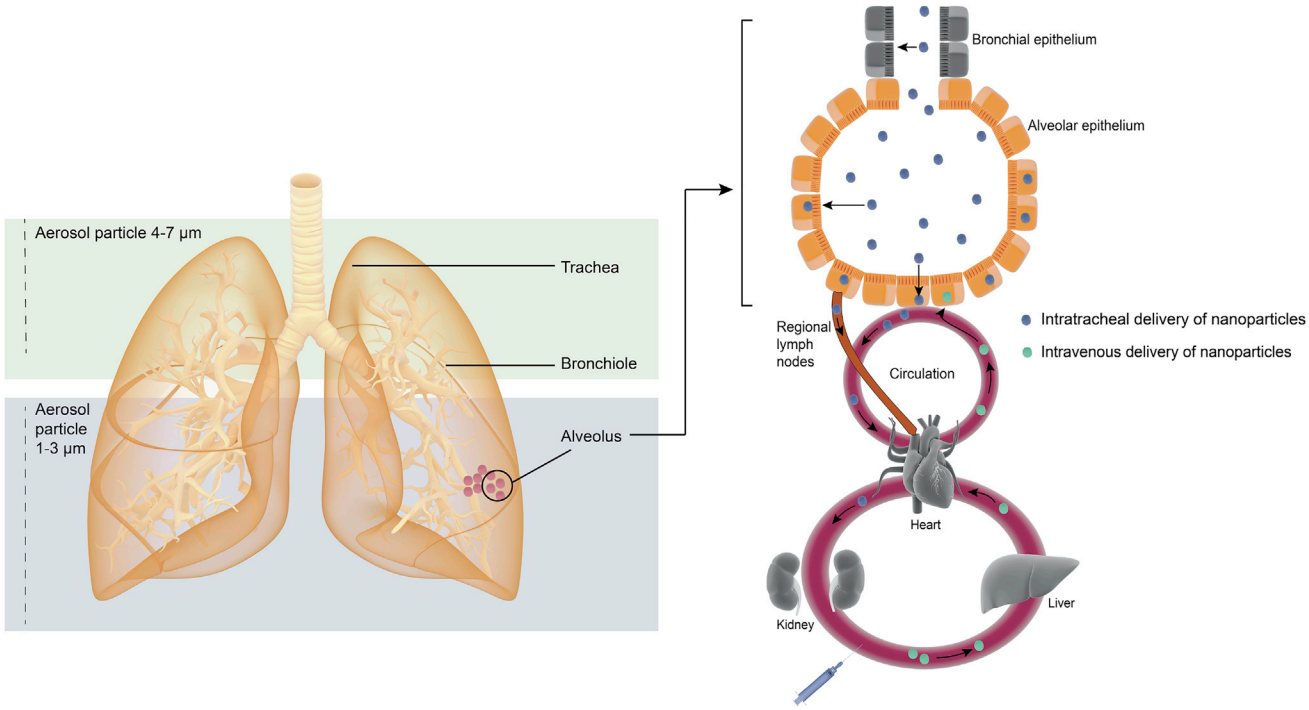

Figure 3. Deposition of Nanoparticles for Delivery in the Lung after Intratracheal or Intravenous Administration

Left: intratracheal instillation requires a particle size of 1–3 μm to reach the alveoli efficiently; particles from 4 to 7 μm are mainly distributed to the upper airways and main bronchioles, and particles smaller than 1 μm are exhaled again.[252,318] Right: inhaled nanoparticles can enter bronchial as well as alveolar epithelium; nanoparticles can enter lymph and blood circulation to be delivered to secondary organs.[319] Intravenous injection can systemically deliver nanoparticles to a limited part of the alveolar epithelium due to small and non-fenestrated endothelial cells in the capillaries in the lung.[250]

Moreover, the alveolar surface area is large and suitable for drug absorption, and the epithelial barrier is thin,[242] which could facilitate the delivery of mRNA to lung cells. While endothelial cells and lung stem cells are difficult to access, alveolar cells, epithelial cells, and macrophages can be targeted by i.t. administration.[251] A potential therapeutic has to be appropriately formulated to be able to reach deep lung surfaces. This includes a particle size of 4–7 μm in diameter[252] for targeting the tracheobronchial region and 1–3 μm for targeting the alveolar region, when preparing a powder for insufflation or nebulizing a liquid (Figure 3).[253]

To reach deep lung structures and alveoli, mRNA therapeutics still has to pass the respiratory mucus. In a non-pathological condition, the thickness of the mucus is between 2 and 5 μm in the bronchi and 10 and 30 μm in the trachea,[254] while in CF asthma and COPD the mucus layer is reported to be much thicker.[255] The gel-on-brush model of Button et al.[256] states that ciliary movement transports all material out of the lung at a rate of 3.6 mm/min,[257] while a layer of mucins and glycoproteins form a fine mesh preventing large particles from reaching the periciliary layer and epithelial cells in the lung.[256] The mucus layer, lining epithelial cells from the nose to the terminal bronchioles, also affects nanocarriers by sterical obstruction or direct interaction from diffusion to the target cells.[251] Different independent studies showed a correlation between nanocarrier size and mobility in respiratory mucus. Sanders et al.[258] and Dawson et al.[259] reported that nanospheres at the size of ~100 nm were able to pass more or less freely through CF sputum compared to nanospheres larger than 500 nm. Broughton-Head et al.[260] detected that CF sputa of three CF patients contained a mesh with a mean size of 300 ± 106 nm, 578 ± 191 nm, and 711 ± 328 nm, respectively, providing evidence for the distinct transport parameters of different-sized nanospheres. Studies by Stern et al.,[261] Kitson et al., [262] and Ferrari et al.[263] suggested that not only the retention by the mesh structure in the CF sputum but also the direct interaction with free DNA present in CF sputum can reduce the gene transfer of 3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl] (DC)-cholesterol (Chol)/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)-based lipoplexes up to 20-fold.

Respiratory mucus is not the only fluid presenting a barrier for the nanocarrier delivery of mRNA. Also, the alveolar fluid is known to inhibit cationic lipid nanocarriers, presumably by disintegration of the lipoplexes by negatively charged lipids in the surfactant.[251] In contrast, PEI and dendrimer polyamidoamine (PAMAM)-based gene delivery was observed to be resistant to the effects of pulmonary surfactant *in vitro* and *in vivo*.[264,265] Moreover, Exosurf (a synthetic surfactant) has been reported to increase the efficiency of PAMAM-pDNA complexes *in vitro*.[265]

To overcome the obstacles presented by both respiratory mucus and pulmonary surfactant, various approaches have been tested. Mucolytic agents, which degrade the biopolymer network built up out of DNA, actin, and mucins, are a focus of many research groups.[258–260,263] Recombinant human DNase (rhDNase) liquifies CF mucus by cleaving DNA chains in the biopolymer, and it has a direct effect on the mobility of nanocarriers of CF sputum. It increased the mobility of 270-nm nanospheres and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP)/DOPE-based lipoplexes 2.5-fold and 1.4-fold, respectively.[258,266] N-Acetylcysteine (NAC), a clinically used mucolytic agent for asthma, COPD, and CF patients, reduces the disulfate bonds between mucins and lowers the viscosity and elasticity of respiratory mucus.[267,268] NAC-mediated mucus clearance of an *ex vivo* sheep trachea model increased gene transfer via p-ethyl-dimyristoylphosphadityl choline (EDMPC)-Chol lipoplexes and PEI-based polyplexes 20-fold and 10-fold, respectively. If the nasal epithelium of mice is treated with NAC 30 min before the administration of EDMPC-Chol lipoplexes, the gene expression can be increased up to 100-fold.[263]

An alternative strategy is to coat nanocarriers with biocompatible hydrophilic but biologically inert polymers[251] to shield the nanocarriers from respiratory fluids and surfactant. GL67 (genzyme lipid 67)/DOPE lipoplexes can be coated with polyethylene glycol (PEG), and they have been reported to circumvent the adverse effects of CF mucus during gene transfection *in vivo*.[269] Maisel et al.[270] reported that 10–40 kDa PEG-coated nanocarriers can diffuse through the mucus as a mucoinert particle.

Nanotransporters to Target the Lung

The labile nature of mRNA and immunogenicity are the biggest hurdles of mRNA therapeutics. As discussed above, the immunogenicity has been overcome by chemical modifications; however, the instability of mRNA under physiological conditions requires additional action. Electroporation,[271] gene gun,[272] microinjection,[273] and sonoporation[274] have been investigated for mRNA delivery; however, these are restricted to *ex vivo* manipulation and ill suited for systemic delivery. Therefore, suitable mRNA carriers should exhibit the following functions: protection from RNase degradation, evasion of direct renal clearance, avoidance of nonspecific interaction, facilitation of mRNA stability, and sufficient mRNA loading and release.[275–277] The physiochemical properties, such as hydrodynamic diameter, shape, size, surface charge, solubility, flexibility, stability, formulation, and body composition with regard to route of administration, decide the target binding bio-distribution as well as the clearance of the nanocarriers. As many materials used to construct nanoparticles are toxic or potentially toxic, biocompatibility and biodegradability become key factors. Since our focus is delivering mRNA therapeutics to the lung, we cover biomaterials such as lipids, polymers, and combined formulations that are developed for delivery to the lung.

Lipid-based nanoparticles (LNPs) or lipoplexes have gained popularity since the beginning of drug and nucleic acid delivery.[9,231,240,278,279] These have the significant advantages of easy synthesis, scalability, low batch variability, and biocompatibility.[280–282] Commercially available lipoplexes, such as RNAiMAX, Stemfect, and Megafectin, have been successfully used in transfecting mRNA *in vivo*.[279,283–285] Cationic lipids, such as 1,2-di-O-octadecenyl-3-trimethylammonium-propane (DOTMA), DOTAP, and zwitterionic DOPE, have been used alone or in combination, as these readily form complexes with mRNA. Variants to reduce toxicity and immunogenicity associated with cationic lipids and with improved efficacy have been developed. Additionally, the ratio of components substantially affects LNP efficacy. These include ionizable lipidic systems that can reduce toxicity by possessing a neutral charge at physiological pH[286] and ionizable lipid nanoparticles consisting of ionizable lipid, cholesterol (hydrophobic), helper lipid (DOPE or 1,2-distearoyl-sn-glycero-3-phosphocholine [DSPC]), and PEG lipids. DOPE enhances efficacy by promoting membrane fusion (cell and endosomal), and PEG lipids prevent reticuloendothelial clearance and reduce opsonization by serum proteins.

LNP-mediated mRNA delivery has been extensively used in protein replacement therapies, vaccines, and cancer immunotherapies.[276] Earlier work by Litzinger et al.[287] showed that cationic liposomes of size 2.0 μm are transiently taken up by the lung, followed by rapid distribution to the liver. Similarly, efficient pulmonary endothelial delivery of plasmid was achieved with a lipid vector consisting of DOTAP liposomes, protamine, and oligo deoxynucleotides,[288] with minimum cytotoxicity and release of proinflammatory cytokines. The landmark clinical trial of CFTR gene therapy with pGM169[179] and multiple other clinical trials with nasal delivery tested lipid nanoparticle cholest-5-en-3-ol(3β)-,3-[(3-aminopropyl)[4-[(3-aminopropyl)amino] butyl] carbamate] (GL67A) due to its desirable stability during aerosolization,[289] gene transfer potency,[290] and well-characterized safety parameters.[291] The Wendel group[292] has shown DOPE liposomes as potential transfection agents for AAT mRNA, resulting in prolonged protein production of AAT *in vitro* with improved stability of mRNA in liposomes for up to 80 days, without the loss of transfection efficacy. Alexion Pharmaceuticals has shown human AAT expression in both mouse liver and lung upon i.v. injection of mRNA-ionizable LNP complex after 24 h.[21] Both approaches require further testing on knockout mouse models[293] to check its efficiency as a therapy for AATD.

Lipid-enabled and unlocked nucleomonomer agent-modified RNA (LUNAR) technology of Arcturus Therapeutics employs biodegradable ionizable lipids (ATX, Arcturus Therapeutics's proprietary lipid) that have shown no adverse events, hepatotoxicity, weight loss, or innate or adaptive immune reactions in response to treatment with repeated dosing of up to 4 months.[294] Ramaswamy et al.[294] observed faster translation (within 6 h) and major deposition of LUNAR-encapsulated mRNA in mouse liver with i.v. injection. Arcturus holds multiple patents on nanoparticles for RNA delivery with the potential of lung epithelial delivery via nebulization. Translate Bio holds a patent on multiple lipid nanoparticles with its collaborator at Massachusetts Institute of Technology (MIT) and Imperial College London, and it is the first one to enter clinical trials with

LNPs (ClinicalTrials.gov: NCT03375047) for mRNA-based CF therapy. Valera (by Moderna) has reported efficacy of modified hemagglutinin mRNA-LNP-formulated vaccines against H7N9 and H10N8 influenza virus (presently at clinical phase I), when immunized intradermally or intramuscularly in mice, ferrets, and non-human primates[293] (one must note that localization in lungs is not required for immunization).

Though not as equally advanced as LNPs, polymer-based nanoparticles have shown considerable potential in aiding therapeutics. Cationic polymers (linear or branched) can enable nucleic acid shuttling across membranes by compactly packing them into nanoplexes, and they can help in cellular uptake via endocytosis.[295] PEI is the vastly studied polymer for gene or oligonucleotide delivery,[296,297] however, toxicity due to nondegradability, high molecular weight (>20 kDa), and its highly branched formulations has limited its clinical applications.[298] The positive charge attributes to interaction with serum proteins (negatively charged), resulting in their aggregation and increase in size that causes toxicity, similar to that of cationic liposomes. Therefore, different groups have tried to modify PEI and achieve higher transfection with lower toxicity, which includes reducing size[299] (mRNA release), reducing molecular weight,[300] or using additives.[301]

Poly (L-lysine) (PLL), poly(2-(dimethylamino)ethyl methacrylate) (p[DMAEMA]), and PLGA are well known polymers, and diblock[302] and triblock polymers[303] have shown encouraging results for nucleic acid transfection. As already discussed, mucus acts as a strong barrier, and mucoadhesive particles can increase the residence time while bulking up the nanoparticle. PEGylated NPs (diblock copolymer composed of PLGA and PEG [PLGA-PEG], namely, PLGA-PEG mucous penetrating particle [MPP]) of >200-nm size are known to penetrate mucus and CF sputum.[304,305] Based on this, Schneider et al.[305] and others have shown that MPP ($\leq$300 nm in diameter) exhibits improved particle distribution and lung retention.[150] Chitosan (mucoadhesive) coating[20,306] of PLGA nanoparticles has shown successful delivery of *SP-B* mRNA and *hCFTR* mRNA in mouse models, significantly improving survival[307] and lung function,[308] respectively. It is possible that these enable deeper lung delivery instead of mere epithelial delivery, due to which we could observe the survival of an SP-B-deficient mouse model[309] when corrected with zinc-finger nuclease (ZFN) mRNA and donor template. In an attempt to develop nanoparticles for pulmonary delivery, Ethris used mRNA complexed with a polymer scaffold of poly (acrylic acid) of 20 kDa grafted with oligoalkylamines, which showed delivery in cranial parts of pig lung upon nebulization.[54] A study about the exact localization of developed nanoparticles in different lung cell populations would benefit the scientific society in moving toward disease-specific targeted therapy for different lung diseases.

Further, combinations of lipids and polymers have been tested as nanocarriers. These include self-assembling nano-micelles formed by copolymer consisting of polyamino acid block and PEG with mRNA at core. Commonly used polyamino blocks include poly(*N*′-(*N*-(2-aminoethyl)-2-aminoethyl) aspartamide (PAsp[DET]), which has shown protein expression in nasal neurons with mRNA coding for brain-derived neurotrophic factors (BDNFs).[310] The complex also reduced apoptosis when injected with anti-apoptotic protein B cell lymphoma (Bcl-2) mRNA in a fulminant hepatitis mouse model.[311] The group of Daniel G. Anderson has developed various nanoparticles to be used in therapeutics,[14,312] among which the polymer lipid combination of poly (β-amino esters) (PBAEs) and PEG has shown greater potential in delivering mRNA to lung via i.v. injection,[313] aiding successful systemic delivery. Desrosiers et al.[314] have developed amine-modified polyester-based nanocarriers in combination with triblock copolymers, with specific mRNA delivery to lung. Though degradable and optimized for serum stability and reduced toxicity, these need to be further tested for inflammatory reactions before clinical translation. Recent publication by Patel et al.[315] on hyperbranched PBAEs has shown ease in nebulization and uniform distribution of mRNA in all 5 lobes of lung, with no measured local or systemic toxicity.

To further increase the specificity of nanocarrier-based delivery, receptor-based technologies have been tested. Arrowhead Pharmaceuticals developed an asialoglycoprotein receptor-targeting nanocarrier to specifically administer an RNAi molecule (targeted RNAi molecule [TRiM]) to reduce the accumulation of AAT protein[230] for AAT-related liver diseases. If combined with pulmonary delivery of AAT mRNA, it can act as a complete therapy for diseases like AATD. A receptor-based method can also be developed for the lung epithelium, but specific markers of lung epithelial cells have to be identified to avoid cross-reactivity with other epithelial cell linings. In another approach for enhancing translation and reducing the degradation of mRNA upon entry, a delivery system has been tested that employs translation initiation factor eIF4E with cationic polyamine. A study showed that these nanoparticles induced mRNA expression in mouse lung upon systemic delivery.[316] Other nanoparticles developed for lungs include gelatin nanocarriers crosslinked with genipin, monomethoxypoly(ethylene glycol)-poly(lactic-co-glycolic acid)-poly-l-lysine (mPEG-PLGA-PLL) triblock copolymers, MUC-1 aptamer-functionalized hybrid nanoparticles, drug-loaded liposomes, anionic PAMAM dendrimers, and a recently developed virus-inspired polymer for endosomal release (VIPER).[215,230,238,240,250,252,317–320] However, as discussed before, these must be extensively tested for compatibility with mRNA.

Conclusions

This paper focused on respiratory diseases and associated cell populations of the lung. To understand the disease pathology and possible countermeasures, the physiological aspects of various lung cells have to be determined. This includes the awareness of connections of epithelial cells with alveolar cells, serous cells (goblet cells), and also stem and progenitor cells, as lung diseases may originate from a single defect but can affect the whole lung. Recent developments already produced major advancements in therapies such as chemical modulators for CF, but they also produced therapy resistance or were only effective for certain variants of a disease. This makes mRNA-based

protein supplementation therapy a viable alternative for diseases such as CF, SP-B deficiency, asthma, IPF, and COPD while offering a treatment independent of the underlying mutational status. Furthermore, stem cells of the lung can also be a target for mRNA-based CRISPR/Cas therapies that hold the potential for permanent cures for monogenetic lung diseases, such as CF, SP-B deficiency, and AATD.

The therapeutic potential of mRNA for protein supplementation therapy was widely unrecognized due to its instability for a long time. Over the last decades, an appreciation of mRNA as the molecule connecting the world of proteins and DNA is renewing the focus of research on mRNA.[9,20,215] The research focuses on the properties of RNA to increase or modulate stability and evade immune recognition as well as delivery of mRNA specifically to the lung and other organs. The use of naturally occurring nucleoside modifications has diminished the recognition of mRNA by the innate immune system.[230,235] These modifications also help in improving the stability and expression of mRNA. This is further promoted by modifications like 5′-capping and the addition of a poly(A) tail as well as modifications in the 3′ and 5′ UTRs. Sequence optimization increases expression and lowers immunogenicity of mRNA therapeutics. The most optimized RNA still needs to reach the target cell to be expressed into a protein.

The problem of delivery consists of three main parts: (1) how to find a suitable route of administration; (2) as mRNA for protein supplementation therapy usually are of substantial size and negatively charged, they will not cross cellular membranes unfacilitated; and (3) the composition of mRNA and nanocarriers to maintain the optimal stability of mRNA nanocarrier complexes. Looking at protein supplementation therapies for the lungs, i.t. delivery gives the ability to apply mRNA therapeutics locally and concentrated in the lungs. To overcome defense barriers like the respiratory mucus and alveolar fluids as well as the cellular membranes, nanocarriers developed into a favorable type of vehicle for mRNA. To date, research indicates that small nanocarriers (~100 nm) together with a polymer coating and the potential application of a mucolytic agent can improve the stability of the mRNA nanocarrier complex and mRNA uptake into the target cell. Nonetheless, the mechanism of how a nanocarrier facilitates the cellular uptake of mRNA is still not fully understood.

All in all, the pieces needed for an effective protein supplementation therapy in the lung are available in various preclinical and clinical stages. The next task is to find a formulation to bring all of these components together—route of administration, carrier, mRNA sequence and modification—to form a working therapy for patients. The first clinical trials in the slowly evolving field of protein supplementation therapy show that the concept is viable (ClinicalTrials.gov: NCT02935712 and NCT03375047; see also Table 1).

AUTHOR CONTRIBUTIONS

I.S., A.K.M.A.H., and B.W. wrote the main parts of the article and produced graphics. P.W. helped in the meticulous preparation of the manuscript. M.S.D.K. drafted the final version of the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

M.S.D.K. holds a patent on RNA modification (EP2459231B1). M.S.D.K. and A.K.M.A.H. hold a European patent on delivery of h*CFTR* cmRNA complexed with nanoparticles (17169561.2-1401).

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