Dissertation zur Erlangung des Doktorgrades
der Fakultät für Chemie und Pharmazie
der Ludwig-Maximilians-Universität München

T Cell Targeted Nanoparticles for Pulmonary siRNA Delivery as Novel Asthma Therapy

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aus

Attendorn, Deutschland

2019

Erklärung

Diese Dissertation wurde im Sinne von § 7 der Promotionsordnung vom 28. November 2011 von Frau Prof. Dr. Olivia M. Merkel betreut.

Eidesstattliche Versicherung

Diese Dissertation wurde eigenständig und ohne unerlaubte Hilfe erarbeitet.

Olpe, den 10.09.2019

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(Rima Kandil)

Dissertation eingereicht am:	16.09.2019
1. Gutachterin:	Prof. Dr. Olivia M. Merkel
2. Gutachter:	Prof. Dr. Ernst Wagner
Mündliche Prüfung am:	07.11.2019

Für meine Familie

Ihr seid alles.

ACKNOWLEDGEMENTS

The present thesis was prepared under the supervision of Prof. Dr. Olivia Monika Merkel at the Department of Pharmacy, Pharmaceutical Technology and Biopharmaceutics at the Ludwig-Maximilians-Universität München (LMU) in Munich, Germany.

This work could not have been achieved without the support and guidance of my supervisors, colleagues, collaboration partners, friends, and particularly my family. Within the next lines, I would therefore like to express my deepest appreciation and gratitude to them.

First and foremost, I would like to thank Prof. Dr. Olivia Monika Merkel for welcoming me in not only one but two of her research groups and giving us all the chance to work and collaborate interdisciplinary and even intercontinentally. Thank you for enabling 6 unforgettable months in Detroit never shying at any complications or the never-ending paperwork. Your scientific and personal guidance especially in these early stages of my career has been of inestimable value and is very much appreciated. Letting me join your lab in Munich, you gave me the chance to be part of building a new group with all its challenges and chances. Thank you for giving me the opportunity to work on such an interesting, innovative, and interdisciplinary project, your dedicated and enthusiastic supervision, scientific advice, creative ideas, trust, patience, and encouragement to work through difficult times. I am also deeply thankful for all the possibilities you offered me, including collaborations, additional workshops and certificates, research stays to learn new techniques, and the joining of several scientific conferences all over the world.

This work was in part further supervised by Dr. Aditi Mehta. Aimar, I would like to thank you for your constant advice for my work and life, patient teaching of your endless biological knowledge, diligent proof-reading of posters and manuscripts and most notably for stepping up for us when we needed it.

Furthermore I would like to thank Prof. Dr. Gerhard Winter and Prof. Dr. Wolfgang Frieß for providing such a great and welcoming working atmosphere, especially during our acceleration time in Munich. Thank you for encouraging your students and employees to

support the newest members of the chair and for giving us a platform for scientific collaboration and discussion, but also for numerous fun activities outside of work.

I would like to thank Prof. Dr. Ernst Wagner for influencing our work with his inspiring research and for warmly welcoming the new RNA people on campus. Thank you for kindly agreeing on being the co-referee of this thesis. I would like to extend my thanks and appreciation to Prof. Dr. Angelika Vollmar, Prof. Dr. Gerhard Winter, Prof. Dr. Franz Paintner, and Prof. Dr. Franz Bracher for kindly being members of my examining board.

Special thanks go to my former Wayne State colleagues for helping me lay my scientific groundwork and keeping on supporting me throughout my thesis. Thank you Dan, Steve, Sara, and Barani for your help and guidance together with fun times in and outside of the lab and especially thank you Tim and Vinny for providing me families abroad. My deepest gratitude has to be expressed here to Dr. Yuran Xie, the Tf-PEI expert of AK Merkel, who made this project even possible as she never stopped trying and trouble-shooting. Thank you for helping my getting started and your constant advice!

I highly appreciate the help and scientific input of several colleagues and collaboration partners, such as Prof. Ralf Heermann for spending numerous hours at the Biacore with and for me, Dr. David Bassett who came all the way to build up our animal equipment and enlighten us with his stories, Dorothee Dormann who always provided us with mice and a smile, Dr. Ayse Kilic and Julia Milleck for expert T cell advice, and everyone else who kindly offered help, cells or other materials.

I would moreover like to thank all students who gave me the chance to guide and teach them, especially my bachelor student Katharina Heigl and my special intern Barbara Cerullo, you did great work and I wish you all the best for your future careers!

Many thanks and the kindest regards go to my friends and former colleagues from the LMU, with a special emphasis on my AKM team: thank you Aditi, Gabriella, Rike, Tobi, Tasch, Bettina, Lorenz, Domizia, and Christoph for a lot of fun, but also deep talks and discussions and for always offering a helping hand. In particular, thank you Gabi for being the heart of the group and my second mum away from home in this foreign country. Thank you Domizia for eager learning and letting me be sure that my project will be in great hands! Special thanks also go to Lolo, my favorite lab mate, for always being there and providing me with enough fresh air, water and caffeine. Thank you

Bettina, for sticking together even in the hardest times and getting out stronger than ever, for countless unforgettable moments, and for the 32 reasons.

Of course I would also like to thank all members of AK Winter and AK Frieß for their support, fun parties and outdoor activities and great lunch and coffee breaks. Thank you Sabine Kohler for being the good soul of the chair and helping with every big or small problem in daily office life. Thank you Olli for sharing your knowledge about coffee, meditation, and life and for becoming a friend! Last but definitely not least thank you Kat, for your infinite wisdom, for always trusting in me and our friendship and for just being you!

Kristina, you deserve a special line here. Thank you so much for showing me that the perfect friend does exist.

Above all, I would like to express my deepest gratitude and appreciation to my friends and family, who always believed in me and supported me in any imaginable way. Thank you Steffi for 16 years of friendship and many more to come! Thank you Britta for being much more than a sister, but my best friend, life saver and soulmate. Thank you Kinan for being the best little brother I could think of. Thank you Norbert for being the older brother I never had and always having my back. A more than special thank you also goes to the greatest parents a child could ever ask for. Thank you with all my heart for your unconditional and never ending love and support! And finally, as the newest member of the family, thank you Martin. For everything.

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General Introduction

CHAPTER I – General Introduction

1. Asthma and the Role of T Cells

Affecting 339 million people worldwide and accounting for 250.000 deaths per year, [1] asthma as a disease still displays a serious public health issue at the present day, held responsible for a tremendous drain on economic resources. Even though the number of deaths from asthma has decreased with the use of modern therapy forms, the overall prevalence even appears to be increasing. [2] Asthma symptoms can manifest in very heterogeneous ways with different degrees of severity; however, the disease is generally characterized by chronic inflammation of the airways, bronchoconstriction, enhanced mucus secretion and airway hyperresponsiveness. Ultimately, these pathologic abnormalities may even result in structural and functional changes within the diseased lung. [3] The immune-system of asthmatic patients inappropriately recognizes common environmental stimuli such as pollen or dust as antigens, inducing inflammatory responses [4] leading to the typical asthma symptoms including chest tightness, shortness of breath, coughing and wheezing.

Just as the symptoms and severity of different asthma cases vary, its pathogenesis is equally complex and differs between clinical endotypes, stemming from a diverse interplay of genetic, epigenetic, and environmental factors. [5] It is currently assumed that the disease is approximately 60-75 % heritable, [6] with several candidate genes identified in genome-wide association studies. Moreover, exposure to particle matters, smoking and obesity have been found to increase asthma prevalence. [7]

Although standard therapies applying corticosteroids as controller and beta-2-sympathomimetics as reliever treatments, as outlined in **Figure 1**, can sufficiently control symptoms in most asthma patients today, a substantial share of diseased individuals still experience unsatisfactorily controlled disease patterns. [8] These 5-10 % of the whole asthma population with severe or difficult to treat asthma suffer tremendously and have the highest levels of morbidity. [9] It is therefore crucial to develop novel therapies for this group of patients overcoming the shortcomings of current basic medications and focusing on specific pathophysiologic pathways to early-

on halt disease causing patterns rather than just intervening at the end of the disease cascades in order to palliate symptoms.

The allergic form of asthma comprises all levels of severity and plays a major role in disease development in 50-80 % of all patients suffering from severe asthma forms. [10] A considerable share of severe cases exhibit early onset allergic disease with a noticeable T helper 2 (Th2) cell component, resulting in high levels of eosinophils, mast cells, and IgE in the affected airways. Th2 pathways are also intensely involved in patients with severe adult onset asthma showing prominent eosinophilia in the absence of any other allergic disease. [5] Th2 cells specifically, and T cells in general, therefore display a popular target for novel therapy approaches tackling the unsolved problems of serious and difficult to treat asthma cases.

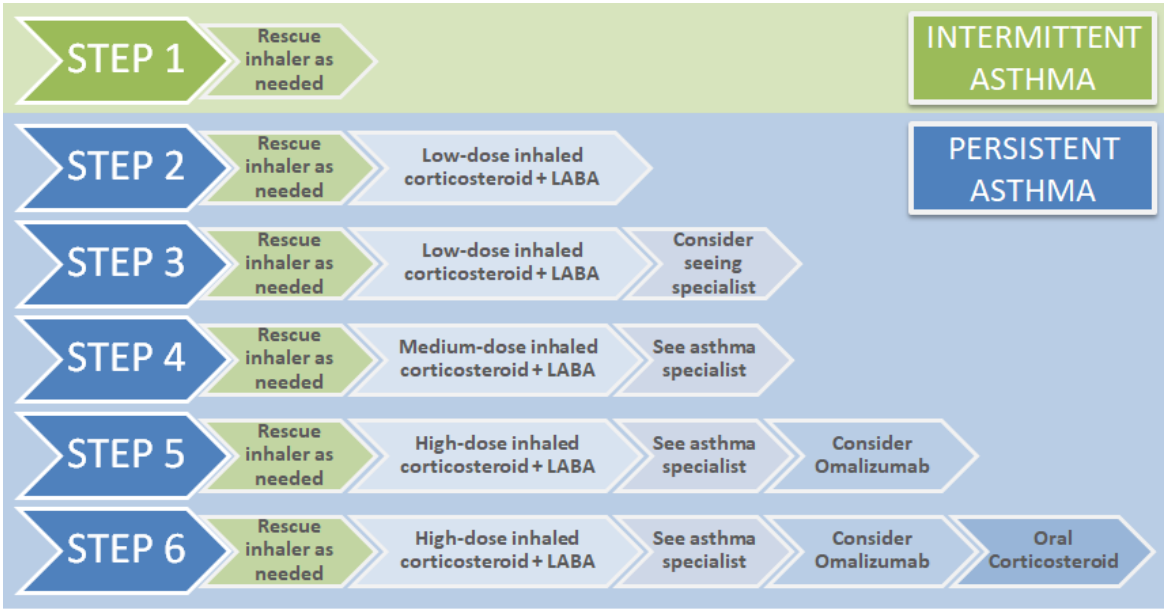


Figure 1: Standard treatment scheme for asthma management according to the guidelines of the National Heart Lung and Blood Institute. Adapted from the National Asthma Prevention and Education Programme. [11]

2. RNA interference (RNAi)

Since the discovery of RNA interference (RNAi) in 1998 [12] as a regulatory mechanism of eukaryotic cells to control gene expression, [13] it is extensively being studied as a method to specifically and therapeutically downregulate the activity of disease-related genes. Offering the potential to theoretically silence any chosen gene with a known sequence, [14] RNAi guides the way towards novel treatment options fulfilling unmet medical needs and enabling therapeutic intervention with diseases thus far regarded as untreatable. Within this process, small interfering RNAs (siRNA) are incorporated into RISC, the RNA-induced silencing complex, as depicted in **Figure 2**. [15] This enzymatic machinery subsequently targets sequence-complementary messenger RNA (mRNA) which is consequently degraded or inactivated. [16] After loading of double-stranded siRNA into RISC, so-called Argonaute (Ago) slicing proteins cleave and release the passenger RNA strand, leaving the now single-stranded guide RNA to activate and direct the target recognition with the respective mRNA via intramolecular base pairing. [17] To exploit this cellular machinery therapeutically, siRNA has to be delivered into the cytosol of appropriate target cells, where the endogenous RISC is located.

The key advantages of all nucleic-acid based antisense strategies for sequence-specific inhibition of gene expression are the precision of target discrimination controlled by Watson-Crick base pair interactions as well as the before-mentioned unrestricted target repertoire. [15] In contrast to other typical antisense strategies such as DNA oligonucleotides or ribozymes, siRNA, however, utilizes an existing cellular machinery, resulting in much more potent gene silencing effects. [18] This in turn enables the use of distinctly lower concentrations which is very relevant for therapeutic applications. Despite all its promising attributes, siRNA therapy still faces some challenges, eventuating in only one siRNA therapeutic currently approved for the market. [19] One of the biggest hurdles to overcome on the way from bench to bedside is successful and sufficient delivery of the RNA molecules. Unfortunately, siRNA features some unfavorable physiological properties, such as instability towards serum nucleases as well as its negative charge impeding efficient cellular uptake. [20] Besides cellular delivery of the nucleic acid cargo in general, it recently came to attention that oftentimes also the cytosolic delivery in particular is a crucial step and can considerably influence the treatment success of siRNA therapies. [21]

To achieve these steps allowing for successful delivery to the site of action, it is crucial to employ a suitable carrier system. In short, the ideal delivery system should be biocompatible and -degradable, effectively protect the sensitive RNA payload from degradation, enable specific targeting of and uptake into target cell types, facilitate liberation from cellular compartments into the cytoplasm and preferentially release the active nucleic acids in a controlled way at the target site. Numerous carrier systems for siRNA delivery are currently being investigated to fulfill these demands, with a focus on polymeric and lipid vectors.

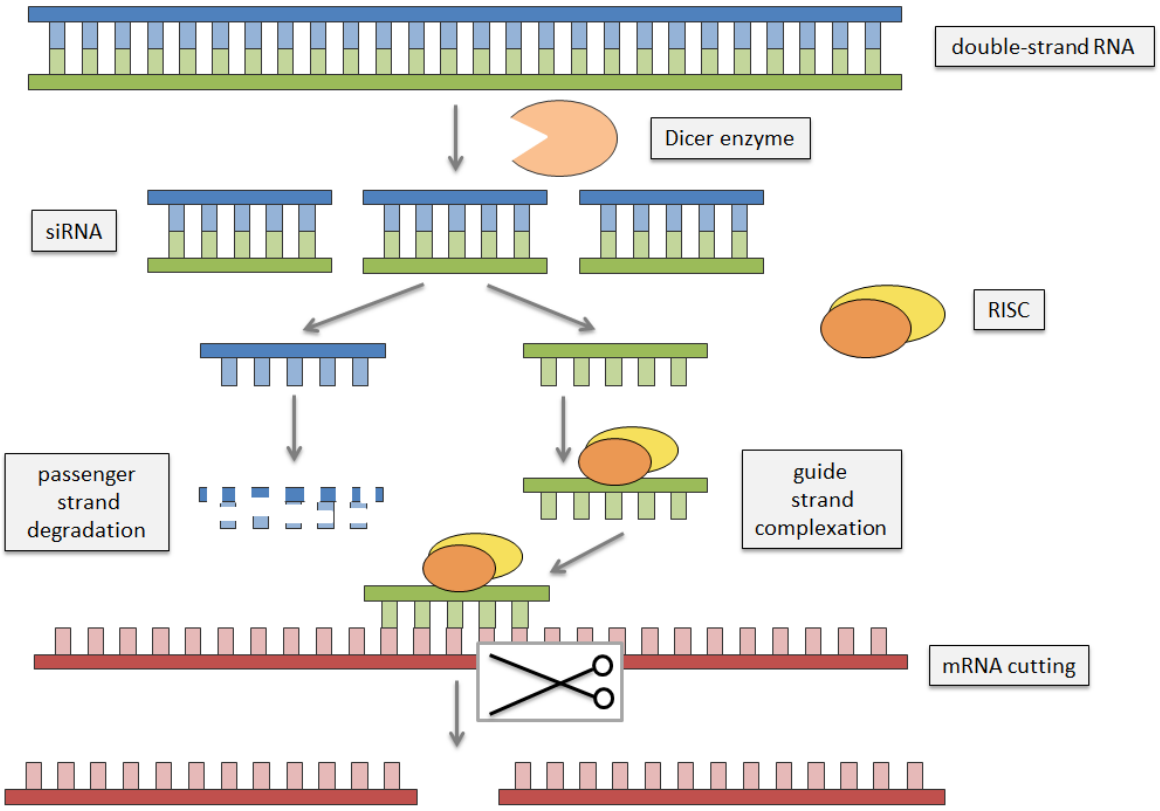


Figure 2. Mechanism of RNA interference (RNAi) as initiated by small interfering RNA (siRNA). Adapted from Matzke et al. [22]

3. GATA3 Silencing

Activated T cells play a key role in numerous immune responses including inflammation processes in asthma, and are therefore targeted in several preclinical studies. [23] Within the inflammatory cascade, antigen presenting cells (APCs) process antigenic molecules, such as allergens, and present them to naïve T helper (Th0) cells. Subsequent activation of allergen-specific Th2 cells results in secretion of interleukins (IL) IL-4, IL-5, and IL-13, which in turn initiate and enforce further pathways via recruiting of other immune cells. [24]

As illustrated in **Figure 3**, IL-4 enhances Th2 cell activation and thereby cytokine secretion, and leads to the activation of B cells, which then produce IgE that is presented on the surface of mast cells and can interact with the antigen directly. IL-5 recruits and activates eosinophils, while IL-13 stimulates goblet cells to enhance their mucus production. Consequently, eosinophils and mast cells secrete second messengers, such as cytokines, leukotrienes, and histamine. These trigger smooth muscle cell constriction as well as epithelial cell damage, resulting in inflammation, hyperresponsiveness and ultimately remodeling of the airways. [4, 24]

Although attempts have been made to prevent these processes by silencing inflammatory cytokines such as IL-5 [25] or IL-13 [26] with siRNA, interference with a single interleukin modulates only certain parts of the process, while the other pathways still proceed. A more promising way to adjust the entire Th2 driven inflammatory cascade is to downregulate GATA3, the central transcription factor regulating the expression of all these Th2 cytokines, [27] thereby silencing the expression of all of them simultaneously. In this way, the underlying inflammation process in asthmatic lungs could be prevented early-on in contrast to most current therapies that only palliate symptoms. GATA3 silencing was already proven successful in a murine asthma model [27] and even in a human phase IIa clinical trial using a GATA3 DNAzyme. [28] Studies concerning siRNA, however, either applied naked nucleic acids which are not expected to be taken up to the cytoplasm efficiently, holding the full knockdown potential back, or viral vectors were used which are associated with a high risk of undesired immune-responses [29]. Therefore, a non-viral delivery system for siRNA specifically targeting GATA3 in activated T cells would be preferable.

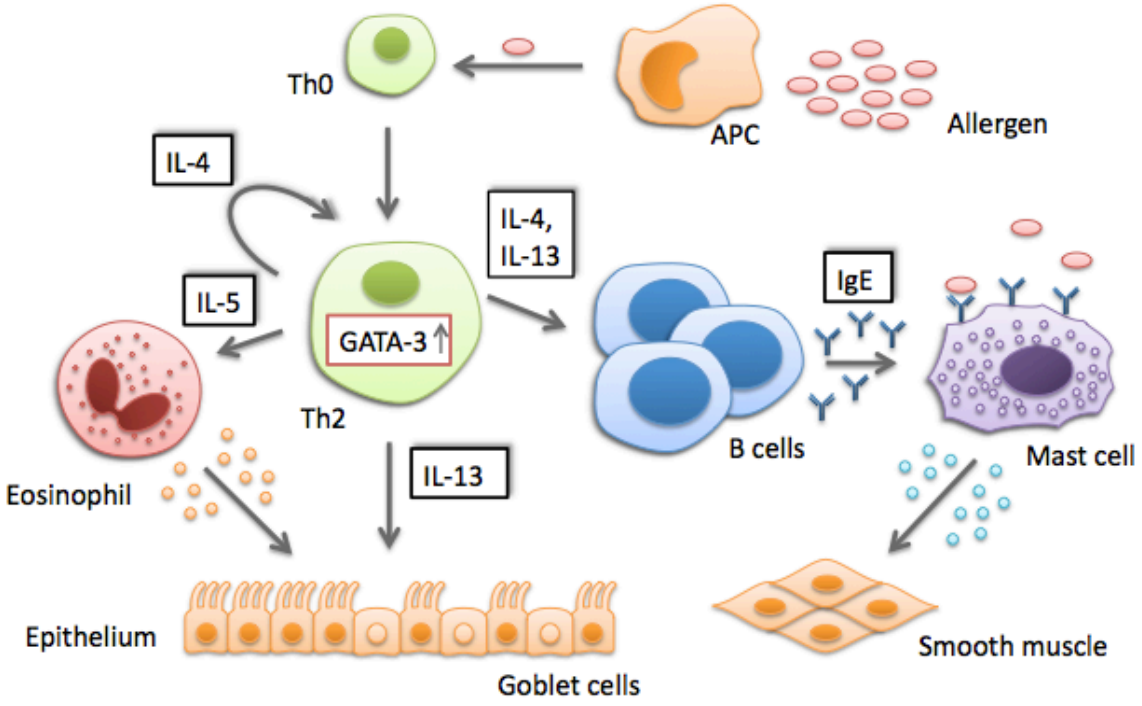


Figure 3. Th2 driven inflammatory pathway as part of the pathobiology of asthma. Adapted from Xie et al. [4]

4. Pulmonary Delivery of siRNA

A promising approach to facilitate delivery to target cells in the lung and circumvent problems of systemic delivery is to use local administration routes for the application of siRNA as asthma therapy. Although siRNA is prone to be degraded by nuclease digestion and rapidly excreted via the kidneys after intravenous (i.v.) injection, [30] a large number of studies still focuses on systemic application routes. Local administration such as pulmonary delivery, however, not only avoids first pass metabolism of applied drugs, but also enables significant reduction of doses, subsequently decreasing respective side effects. In case of lung diseases, the therapeutic molecules are, moreover, directly available in the airways and can bring about their effect at the site of disease within a prolonged lung retention period. [31] Despite the absence of serum proteins and comparably low nuclease activity [32], the lung also offers several other beneficial characteristics as a target organ. With its large and highly vascularized

alveolar surface area and thin epithelium, it provides ideal conditions for exhaustive drug absorption if systemic effects are desired. [33]

However, certain requirements have to be fulfilled for successful drug application. Besides the status of the patient's pulmonary function and the influence of cough and mucociliary clearance, particle size of the siRNA formulation is a crucial parameter directing deposition in the airways. [31] While the movement of small particles with an aerodynamic diameter < 1 μm is determined by Brownian motion and they are likely to be exhaled during normal breathing, larger particles (aerodynamic diameter of > 6 μm) are mostly impacted on the airway wall instead of following the airstream due to their high momentum. [34] The ideal particle size for sufficient deposition at the lower respiratory tract is therefore assumed to be between 1-5 μm in aerodynamic diameter. It has, however, to be noted, that the particles should also have the correct size not to be recognized by alveolar macrophages in the alveoli rapidly phagocytosing foreign particulate matter as a defense mechanism. [35]

Furthermore, the highly branched airways are covered with mucus in their upper parts and surfactant in their lower regions, posing significant obstacles for efficient delivery. While surfactant can severely decrease transfection efficiency of lipid-based nucleic acid delivery systems, it was found that polymeric carriers are hardly impeded. [36] Major difficulties in translating laboratory results to actual conditions in patients comprise the oftentimes missing correlation between *in vitro* and *in vivo* studies and the distinct differences in respiratory tract anatomy between animals and humans. With all points taken into consideration and reasonable formulation adjustments, the pulmonary route, however, displays a very promising way of delivering therapeutic siRNA to asthmatic lungs. **Figure 4** summarizes the essential steps for successful gene knockdown via pulmonary siRNA delivery.

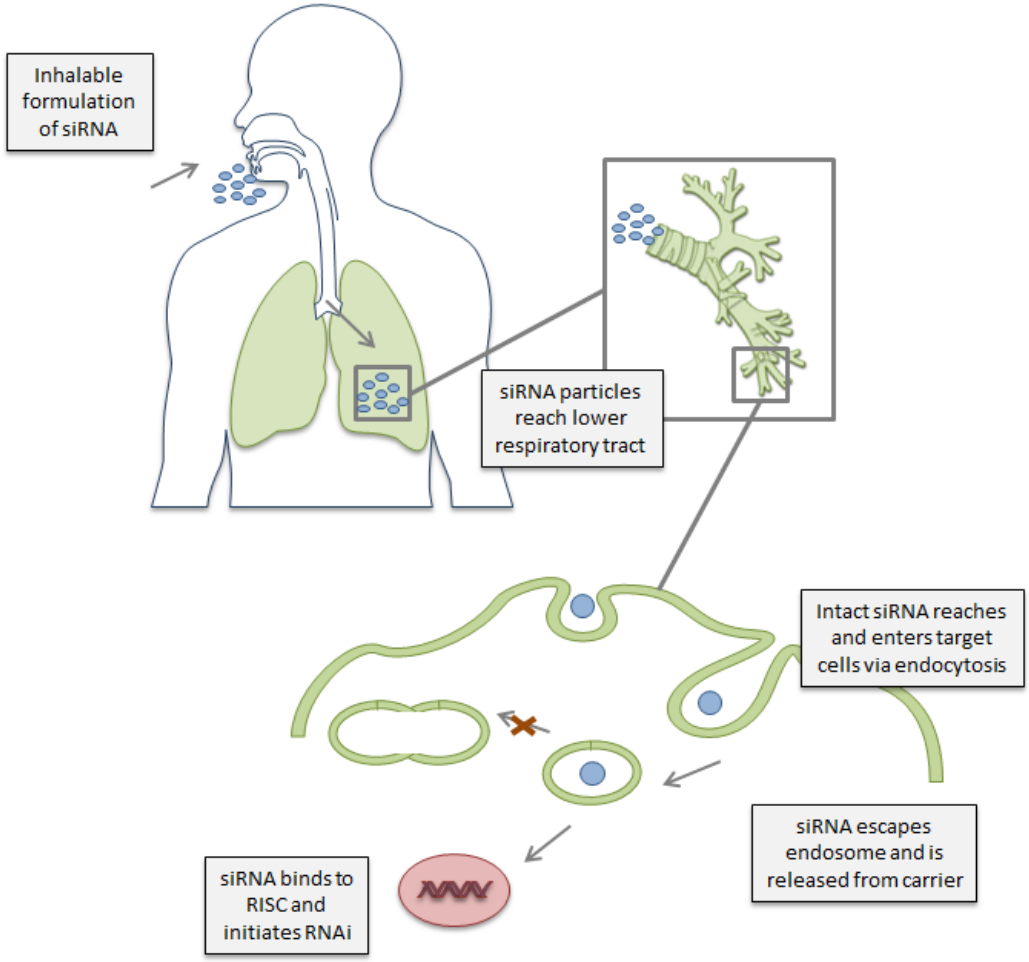


Figure 4. Important steps for pulmonary delivery of siRNA for gene silencing. Adapted from Lam et al. [34]

5. T Cell Targeting via Transferrin Receptor (TfR)

Activated T cells play a crucial role in orchestrating immune and inflammatory cascades which are the underlying cause of diseases such as asthma and therefore display a favorable target for respective nucleic acid based therapies. However, transfection of T cells was found to be an especially challenging quest, as they typically do not express the protein caveolin [37] which is essential for the formation of caveolae, the favored entry port of many conventional non-viral vector based transfection procedures. Transferrin, a native plasma glycoprotein responsible for iron transport in biological fluids, was discovered to present a solution for both tasks: specific targeting of activated

vs. naïve T cells and facilitating cellular transfection in general. Highly proliferating and differentiating cell types, such as activated T cells, overexpress their transferrin receptor (TfR) in order to meet their high iron requirements. [38] Naïve T cells, in contrast, only show negligible TfR expression, enabling specific targeting of the disease-related cells by utilizing transferrin as a targeting ligand. In this way, healthy cells are left untreated and a general immune suppression can be avoided, resulting in a very specific therapy.

Once iron-bound transferrin (holo-Tf) binds to its receptor on the cell surface, the transferrin-TfR complex is internalized via receptor mediated endocytosis, as outlined in **Figure 5.** Iron is released into the cell and the whole complex is recycled back to the surface, where iron-free transferrin (apo-Tf) is released and the receptor can take up even more transferrin, [39] or transferrin-loaded therapeutic particles, respectively. This process is presented in Figure 5. TfR targeting was confirmed successful in several *in vitro* and *in vivo* studies aiming for T cell transfection [40] or nucleic acid delivery to TfR overexpressing tumors [41-43] and even in a human phase I clinical trial. [44]

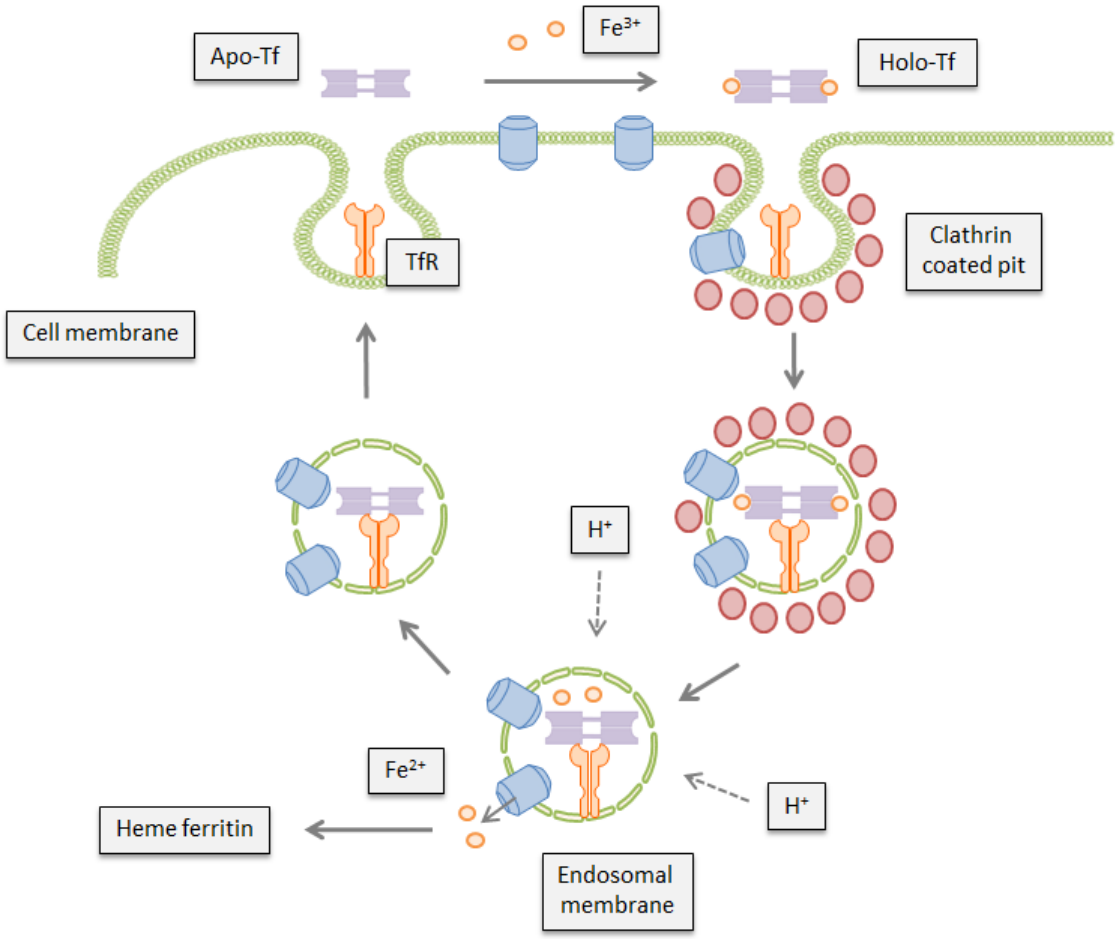


Figure 5. Iron transport via transferrin receptor mediated uptake and subsequent recycling of the receptor. Adapted from Qian et al. [45]

6. Endosomal Escape

A major advantage of siRNA therapeutics compared to DNA-based therapies is that the former do not have to be transported into the cell nucleus, but only to the cytoplasm of the cell, where the RNAi machinery is located. This cytoplasmic delivery, however, still poses several challenges, since most nanosized delivery systems are taken up into cells via endocytosis [46] and are subsequently often trapped in the endosomes. For sensitive payloads such as nucleic acids this is particularly disadvantageous, since the acidic pH activates reductive enzymes leading to degradation. [47]

Many cationic polymers, such as polyethylenimine (PEI), the most extensively studied polymer for gene delivery, are said to exhibit the so-called proton-sponge effect, meaning that they capture many of the protons during acidification of the endo/lysosome with their free amine groups. [48] This in turn leads to an influx of counter ions and water, resulting in endosomal swelling and ultimately rupture, releasing the trapped particles into the cytoplasm, as **Figure 6** illustrates. Recent studies, however, have questioned the actual effectiveness of this procedure [49] and suggest that endosomal release of delivered molecules is oftentimes still not sufficient, detaining their full therapeutic potential. Moreover, focusing on T cells in particular, it was recently found that endosomal acidification generally proceeds slower and less robustly in human T cells compared to other cell types, further querying the usefulness of this mechanism for sufficient release in this context. [50]

Endosomal escape was in fact determined to be the rate-determining step in the delivery of therapeutics, preventing entrapment and breakdown in lysosomal compartments. [51] It is therefore advisable to apply additional release mechanisms to ensure successful delivery of siRNA molecules to the cytosol as the site of action in an adequate quantity. Ways to improve vesicular escape comprise modulation of the carrier system with specific functional groups including positively charged or pH sensitive moieties, [52] utilizing viral or liposomal systems enabling fusion with the endosomal membrane, [53] and other mechanisms based on enhancing osmotic pressure, particle swelling, or membrane destabilization. [49] Endosomolytic agents, such as melittin, a bee venom peptide exhibiting inherent capacities for membrane disruption, were shown to be able to enhance transfection efficiencies of polymeric [54-56] as well as lipid-based [57] gene delivery systems.

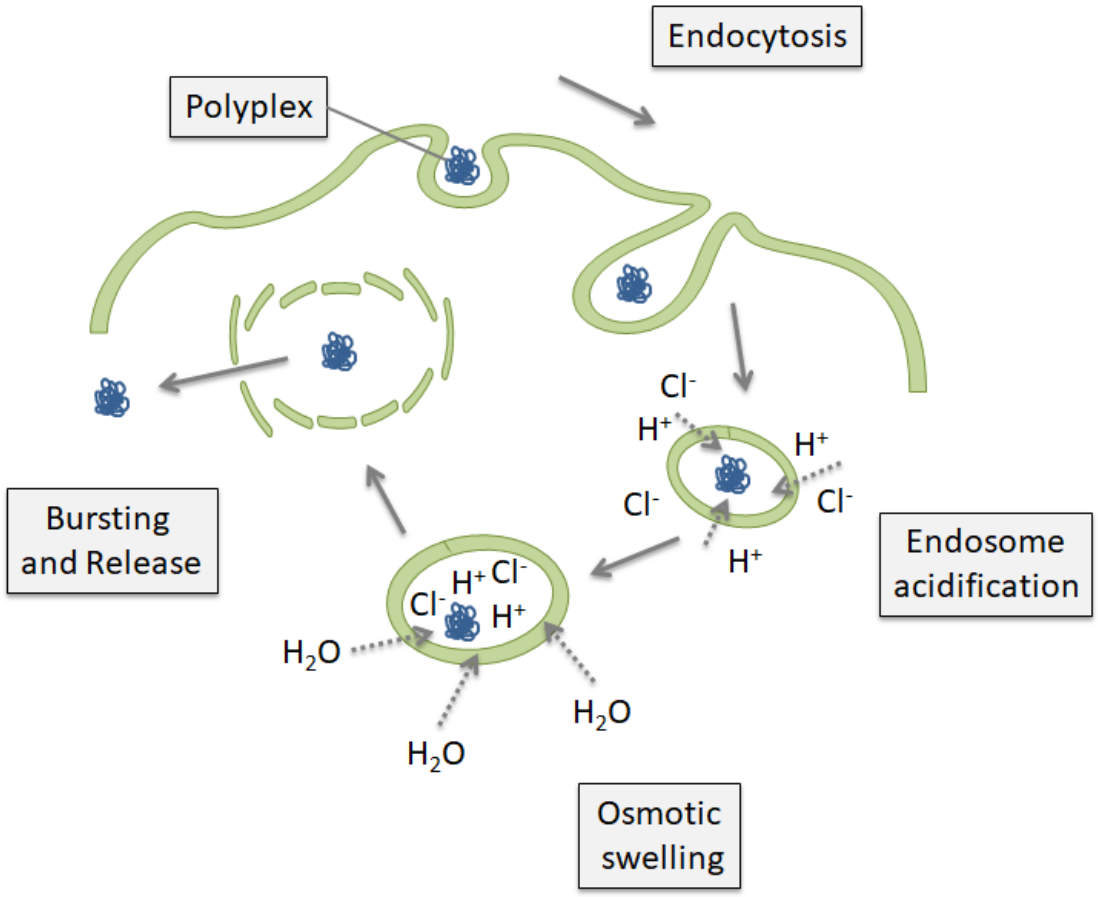


Figure 6. Endosomal release of polyplexes via acidification and osmotic swelling of the vesicles. Adapted from El-Sayed et al. [48]

7. Aim of the Thesis

The aim of this work was to develop and optimize a T cell targeted delivery system for pulmonary delivery of siRNA directed against GATA3, the central transcription factor of Th2 cytokines, as a novel therapy for asthma. Therefore, an existing carrier system on the basis of polycationic polymer polyethylenimine (PEI) and targeting ligand transferrin (Tf), resulting in the so-called Tf-PEI, was chosen and fully characterized concerning relevant siRNA polyplex characteristics such as size, zeta potential, siRNA encapsulation efficiency and gene silencing capability *in vitro* and *in vivo*. Subsequently, Tf-PEI was blended with a second conjugate, Tf-Mel, containing the lysosomal peptide melittin, in order to increase endosomal escape of the polyplexes. Resulting Tf-Mel-PEI blends were characterized and optimized to achieve siRNA polyplexes combining

specific targeting of activated T cells and efficient cytoplasmic siRNA release, resulting in successful gene knockdown. For GATA3 silencing, a suitable siRNA sequence combination was found and applied within the Tf-Mel-PEI blend polyplexes to investigate down-stream effects of the gene knockdown on cytokine levels. These were concludingly tested in an optimized model for activated T cells as a first step for evaluation of relevant therapeutic effects in an inflammatory environment.

Chapter 1 of this thesis provides a general introduction briefly covering all relevant topics affecting the content of this work.

Chapter 2 introduces the concept of using pulmonary siRNA delivery as a novel treatment strategy for lung diseases such as asthma. Besides relevant characteristics of the lung as a target organ, general advantages of and hurdles for pulmonary delivery of siRNA are discussed and current studies are summarized to conclude perspectives of the field.

Chapter 3 provides an overview of polymeric nanogels as a further form of delivery system for nucleic acids. In this chapter, important aspects of nanogel formulation and characterization are described and possible modifications as well as stimuli-responsive behavior of these systems are exemplified rounded off with information about active targeting, co-delivery and respective add-on treatments. Moreover, relevant *in vivo* studies are reviewed.

Chapter 4 evolves around the disease asthma, approaching its biology and pathogenesis as well as current treatment forms. In this context, epidemiology, etiology, pathophysiology and diagnosis of asthma are described in detail. Current disease management and patient care as well as hurdles for efficient treatment are discussed and new treatment forms are introduced as possible solutions.

Chapter 5 attempts to answer the question of how to effectively translate nanomedicines for asthma from basic research to standard clinical routine and thereby close the gap between bench and bedside. Therefore, the different groups of drugs available for asthma treatment are described with a special focus on nanomedicines. Subsequently, adequate study planning as well as factors having to be considered relating the drug approval process are critically discussed leading to an evaluation of the current situation and future of the field.

Chapter 6 explains the use of Tf-PEI as a method for targeted non-viral siRNA delivery to treat cancerous and inflammatory diseases. Conjugate synthesis and polyplex preparation as well as characterization are described in detail to offer a way to specifically target transferrin overexpressing cell types being involved in the development of tumors or inflammation.

Chapter 7 deals with evaluating respective treatment effects after using siRNA for gene silencing purposes *in vivo*. A particular emphasis is placed on antigen specific T cells being investigated via intracellular staining to evaluate regulation of relevant cytokine levels by RNAi.

Chapter 8 describes the blending of Tf-PEI as the conjugate described in the previous chapters with Mel-PEI yielding the Tf-Mel-PEI blend and characterization as well as optimization of resulting polyplexes. In the presented study, transferrin receptor binding, cellular uptake, transfection and gene silencing efficiency as well as endosomal release and tolerability of Tf-Mel-PEI/siRNA polyplexes are examined concluding in the superior suitability of the blend polyplexes made together with the melittin containing conjugate.

Chapter 9 addresses the specific knockdown of GATA3 using the Tf-Mel-PEI blend polyplexes including evaluation of respective treatment impact. For this purpose, T cell activation processes are optimized in continuous as well as primary T cells to generate a suitable model for evaluation of RNAi effects in disease-like conditions. Moreover, an

appropriate siRNA sequence combination is found for successful knockdown of GATA3 in human cells.

Chapter 10 provides a concluding summary of the gathered findings in this work and points out which parameters still need to be addressed in future investigations.

Chapter II
Pulmonary Delivery of siRNA

Please note that the following chapter has been published in *Future Science – Therapeutic Delivery:*

Rima Kandil, Olivia M. Merkel: Pulmonary Delivery of siRNA as Novel Treatment for Lung Diseases. Ther Deliv. 2019 Apr;10(4):203-206. doi: 10.4155/tde-2019-0009.

CHAPTER II - Pulmonary Delivery of siRNA

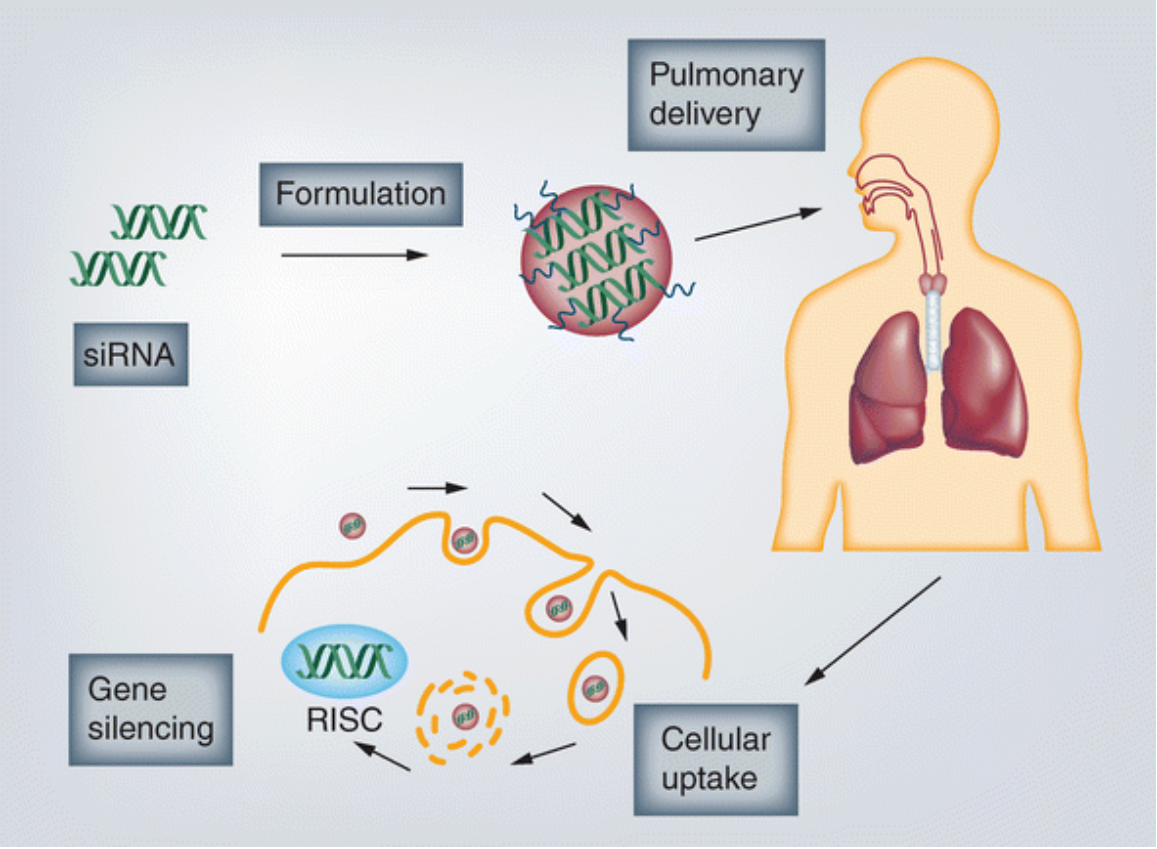
Pulmonary Delivery of siRNA as Novel Treatment for Lung Diseases

The authors of this editorial article include Olivia M. Merkel and me. I am the first author of the respective publication, performed the literature search and wrote the article.

Keywords

RNAi, siRNA, pulmonary delivery, inhalation, lung diseases

Graphical Abstract



The lung offers various beneficial characteristics as a target organ for therapeutic approaches. Besides the large (hundreds square meters) and very well perfused (5 L/min) surface area, extremely thin epithelium (0.1-0.2 μm) and high blood volume in pulmonary capillaries (0.25 L), it also captivates with a relatively low enzyme activity and slow surface clearance. [58] Owing to its location and function, the pulmonary region is susceptible to a number of specific diseases, being directly accessible to harmful substances. Lung-related disorders therefore contribute substantially to the global disease burden in regards to public health and economic resources. For a considerable number of those pathologies including asthma, idiopathic pulmonary fibrosis (IPF), respiratory syncytial virus (RSV) and, moreover, lung cancer as the leading cause of cancer death worldwide, the underlying cause is elevated transcription of certain genes. [59, 60] Therefore, the interference with gene expression via RNA interference (RNAi) in principle offers great potential to help address unmet medical needs in this regard.

Since small interfering RNA (siRNA) is the most extensively studied RNAi molecule for therapeutic application, accounting for half of all recent clinical studies focusing on this mechanism, [61] direct delivery of siRNA to diseased lung tissue presents a promising treatment option. SiRNA can induce post-transcriptional gene silencing by inhibition of respective mRNA, making it possible to treat even diseases for which currently no drugs are available. [20] Compared to other gene therapy approaches, siRNA provides several benefits such as robustness of expression, specificity of inhibitory effect, high efficiency even at low concentrations and reduced toxicity [58]. Furthermore, unlike DNA, siRNA does not need to be delivered to the cell nucleus, but only to the cytoplasm in order to bring about its therapeutic effect. Systemic injection of siRNA has proven to be difficult as it is easily degraded by ubiquitous nucleases and rapidly excreted via the kidneys, not being retained by glomerular filtration. [62] Local delivery, however, enables direct transport to the target site while circumventing the first pass effect resulting in lower doses and respective side effects.

The primary barrier for pulmonary siRNA delivery is the extensively branched structure of the airways with variable lengths and diameters, followed by the presence of lung fluids, such as mucus and surfactant [63], as well as mucociliary clearance mechanisms including alveolar macrophages. As the human respiratory tract occupies an efficient

system for gas exchange and protection against foreign particles, careful consideration of these natural defense mechanisms is necessary when formulating pulmonary therapeutics. [64] To improve siRNA stability and efficacy without using delivery systems, it can be chemically modified or conjugated to appropriate biomolecules. [65] Besides this, carrier vectors can of course help to overcome delivery challenges of naked nucleic acids. Due to improved toxicity profiles concerning immunogenicity and tumorigenicity as well as ease and reproducibility of manufacturing, non-viral vectors are mostly preferred over virus-based systems. [31] A great range of different materials is available, including polymers, lipids, peptides and inorganic material, which can furthermore be tailored with diverse surface modifications [66], such as targeting ligands or membrane-active substances.

Administration of siRNA to the lungs is usually performed via inhalation or intranasally, as both ways present easy and non-invasive options. For inhalation, siRNA can be formulated into liquid aerosols produced by inhalers or nebulizers or as a dry powder aerosol. [65] Currently available inhalation devices comprise pressurized dry powder inhalers (DPIs), nebulizers, soft mist inhalers (SMIs), and the most commonly used metered dose inhalers (pMDIs). For successful pulmonary application, particle deposition near the targeted lung cells is fundamental, arrangeable with optimal particle size, shape and density. The ideal aerodynamic diameter of droplets or powder particles would be between 1-5 μm, as smaller particles are very likely to be exhaled, while larger ones might easily deposit in the throat and upper airway walls. [63] By precisely controlling these particle characteristics and thereby deposition mechanics of drug formulations, specific regions in the pulmonary tract can be reached. [67] Even more distinct delivery is possible by targeting of certain cell types in the lung, for example by coupling ligands that bind to receptors expressed on the surface of target cells. The native glycoprotein transferrin, for instance, was shown to be an effective mediator to reach activated T cells as disease mediators in asthmatic lungs [68] or malignant lung cancer cells [69]. After successfully reaching the target cells, siRNA particles have to be efficiently taken up which is mostly achieved by endocytosis. It is then crucial for the nucleic acid to escape the endosomes to avoid degradation upon acidification and to achieve cytoplasmic delivery to the RNA-induced silencing complex (RISC), the target location of siRNA. In fact, recent studies identified escape of the

endosomal pathway as the rate-determining step in delivery of therapeutic molecules [51], justifying the various approaches to improve endosomal release. Among others, membrane fusion or disruption, pore formation, and osmotic lysis via buffering effects are currently investigated options. [70]

Almost all recent studies investigating the pulmonary delivery of therapeutic siRNA showed positive outcomes, proofing a working principle. Nevertheless, some reoccurring challenges were identified along the way, including distribution of siRNA in tissues other than the lung, safety issues of delivery vectors, unclear effects of long-term application, and a poor *in vitro-in vivo* correlation. [59] A crucial step for the transfer of pulmonary siRNA delivery from bench to bedside is to develop and characterize inhalable drug products. Appropriate ways to analyze aerosolized siRNA delivery vectors *in vitro* comprise dissolution tests, inertial cascade impaction, delivered dose uniformity assay, laser diffraction, and laser Doppler velocimetry, as well as the isolated lung perfusion model as a useful *ex vivo* option. Subsequently, formulations can be examined *in vivo* using methods such as gamma scintigraphy, 3D SPECT or MRI fluorescence imaging. [64]

The majority of *in vivo* studies are still carried out in mice, as they are small, rather economical in terms of husbandry and offer fast and efficient reproduction. However, rodent airways distinctly differ from those of humans in regards to anatomy, histology and physiology. [71] As different pharmacokinetic properties such as particle deposition and mucociliary clearance strongly affect the eventual active dose, it is essential to consider this fact before extrapolating these results to human application. Furthermore, animal models should ideally mimic the clinical conditions as accurately as possible to examine potential medications in a realistic disease environment and integrating the fact that respiratory function is compromised in patients with lung diseases. Due to the ease of experimental setup and control, the application route of choice is usually intratracheal or intranasal. Although intranasal delivery is very straightforward, since the formulation just has to be instilled in the nasal cavity and breathed in, humans are not obligate nose breathers and their nasal cavity filters out the vast amount of particles, resulting in a low lung deposition. [72] Intratracheal administration, on the other hand, does not perfectly translate inhalation outcomes, resulting in comparably less uniform particle deposition and neglecting of oropharynx

deposition, eventuating in a lower total drug loss. [64] Moreover, this method is not suitable for human clinical studies or even usage in the clinical routine due to its invasiveness, as inserting a tube within surgical incision is highly impracticable and uncomfortable for the patient. [34] Accordingly, focusing more on the testing of actual inhalable dosage forms would be rather expedient. Most studies also use liquid formulations, raising problems in regards to stability, as those are highly susceptible to nuclease contamination and not suitable for long-term storage. Therefore, dry powder formulations seem to be the more promising option and have, not surprisingly, gained more and more attention in recent work. [59]

Altogether, the therapeutic potential of RNAi in lung diseases was clearly demonstrated in several *in vitro* studies as well as clinical trials. With patisiran (ONPATTRO™) recently being approved in the US and EU as the very-first siRNA medication, [19] significant progress in the development of RNAi based therapeutics has been made, paving the way for more products to follow. As a large share of airway related diseases evolve around inflammatory processes for which long-term therapies are needed, it is inevitable to further investigate long-time stability of siRNA formulations in appropriate devices as well as effects of continuous repeated application. Here, both the nucleic acid itself, with particular regards to undesired immune responses and potential off-target effects, and the respective carrier system have to be closely examined. Preferably, efforts should be further focused on the development of biodegradable delivery vectors in order to achieve safe and compatible formulations. With all points considered, the pulmonary route presents a promising approach for siRNA based therapies presumable to be realizable in the near future.

Chapter III

Polymeric Nanogels for Gene Delivery

Please note that the following chapter was published in *Current Opinion in Colloid & Interface Science:*

Rima Kandil, Olivia M. Merkel: Recent Progress of Polymeric Nanogels as Nucleic Acid Delivery Systems. Curr Opin Colloid Interface Sci. 2019 Feb;11-23. doi: 10.1016/j.cocis.2019.01.005.

CHAPTER III - Polymeric Nanogels for Gene Delivery

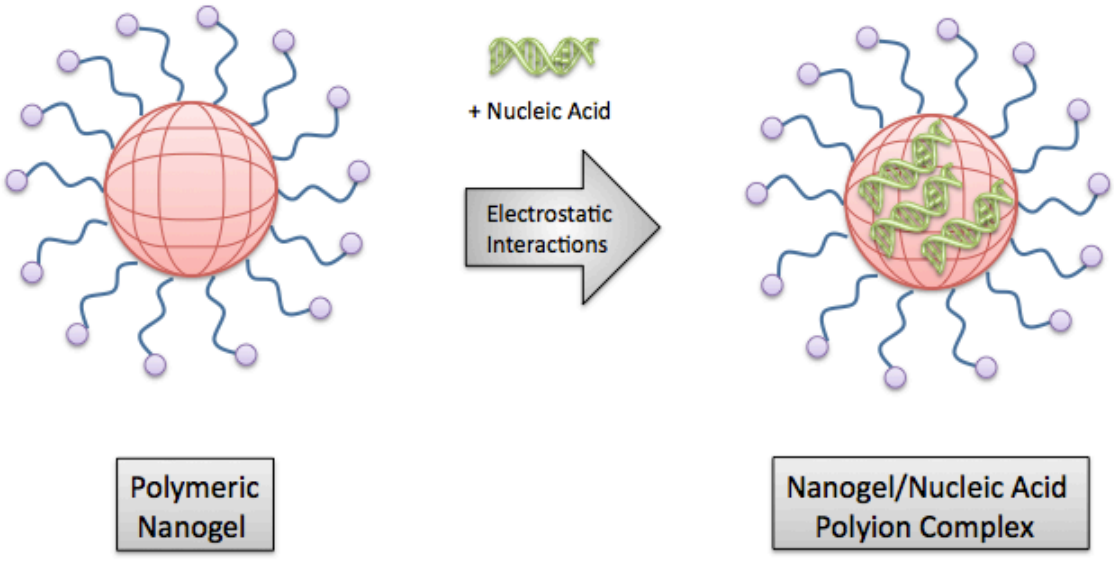
Recent Progress of Polymeric Nanogels as Nucleic Acid Delivery Systems

The authors of this review article include Olivia M. Merkel and me. I am the first author of the respective publication, performed the literature search and wrote the article.

Keywords

Nanogels, Polymeric Carriers, Gene Delivery, Nucleic Acids, siRNA, pDNA

Graphical Abstract



Abstract

With its nearly unrestricted possibilities, gene therapy attracts more and more significance in modern-day research. The only issue still seeming to hold back its clinical success is the actual effective delivery of genetic material. Nucleic acids are in general challenging to administer to their intracellular targets due to their unfavorable pharmaceutical characteristics. Polymeric nanogels present a promising delivery platform for oligonucleotide-based therapies, as the growing number of reports deliberated in this review represents. Within the scope of this article, recent progress in the employment of nanogels as gene delivery vectors is summarized and different examples of modified, stimuli-responsive, targeted and co-delivering nanogels are discussed in detail. Furthermore, major aspects of successful gene delivery are addressed and critically debated in regards to nanogels, giving insights into what progress has been made and which key issues still need to be further approached.

1. Introduction

Gene therapy describes the process of introducing foreign genomic material into specific host cells in order to gain a therapeutic benefit by correcting existing disfunctions or sustaining respective cells with new functions. [73] While at early stages, gene therapy mainly focused on rare genetic disorders, the concept of delivering nucleic acids, including plasmid DNAs, short interfering RNAs (siRNAs), as well as messenger RNAs (mRNAs), aiming to restore a specific gene function or to silence certain genes, is nowadays exploited for a great range of various diseases. Following the first human gene transfer in 1989 [74], the first gene therapy was applied in 1990. [75] Since the release of the human genome sequence in 2001 [76] and the discovery of the mechanism of RNA interference (RNAi) just a few years later [12], the opportunities of gene therapy vastly increased, as it became hypothetically possible to target and treat any chosen gene. Despite all progress, however, there are still several hurdles yet to overcome on the way to a successful translation of these findings into the clinical routine. It should be noted that after 20 years of research, only one RNAi-based drug has been approved by the FDA and EMA. [77]

The most challenging step towards effective gene delivery, in fact, appears to be the search for a suitable carrier system. As for most biotherapeutics, the transport of genetic material to their intracellular targets is demanding, due to their unfavorable biopharmaceutical properties. [78] Nucleic acids are not only heavily susceptible to enzymatic and chemical degradation and rapidly cleared upon systemic injection, but also generally hindered from crossing cellular membranes. It is therefore inevitable to package therapeutic DNA or RNA in appropriate delivery systems that protect their payload, facilitate cell internalization and guide its way towards the required intracellular target compartment: nucleus for DNA, or cytosol for siRNA and mRNA. Although viral vectors show high gene transfection efficiencies, their clinical utility remains very limited due to their potential immunogenicity and severe side effects. [79] Modern gene delivery approaches, therefore, mainly focus on nonviral vectors with a particular emphasis on polymeric carrier systems. Polymers can purposefully be designed for specific application needs regarding characteristics such as different molecular weights or charge densities and can be modified by coupling of targeting ligands or tailored to be reactive to certain physiological conditions. Furthermore, their production can rather easily be scaled-up to large quantities. [80]

One central aspect in successful drug delivery is the controlled release of the delivered therapeutic agent. The drug has to be available at the target region in a specific concentration within the therapeutic window in order to bring about its desired effect without causing any unwanted toxic reactions due to overdosing. Owing to their large surface area, accordingly designed nano-sized systems can offer finer temporal control over drug release rates than macro-sized vehicles. As opposed to bulky delivery systems, vehicles in the nano-scale can enter target cells with greater ease and are able to specifically attack diseases at their site of action as they can circulate in the body after injection. [81]

Polymeric nanogels are a special representative of nano-sized systems, consisting of nanoparticles composed of hydrogels which are in turn made of cross-linked polymer networks. Combining beneficial functions of dendritic systems with those of hydrogels such as large encapsulation cavities and the capability of swelling as well as responsiveness, these novel structures not only fill the size gap, but also present a functional link between common dendrimer or polymer scaffolds and macroscopic

hydrogels. [82] As opposed to larger hydrogel particles, nanogels can easily be administered intravenously and deliver their payload to various target regions and cells. Further advantages of these promising drug delivery platforms comprise simple and efficient drug loading, physical stability of both carrier and incorporated drug, and a versatile design. As they form complexes with biomacromolecules such as proteins in suitable size dimensions, they not only ease the way for their delivery, but also help to maintain their biological activity by keeping them in the correct confirmation, arousing special interest for biomedical applications. [81] Due to their characteristic properties such as softness and swelling behaviour, nanogels are predestined to achieve controlled as well as responsive release at the target location [83]. The possibility to trigger these soft delivery systems to alter their structure upon changes in parameters such as temperature, pH, or ionic environment facilitates both storage and administration of the therapeutic formulation compared to hard nanomedicines. Nanogels can therefore e.g. be applied in a low viscous form that transitions into a dense film or a high viscous depot form after administration. [84] The versatile architecture of nanogels enables the loading with various cargos holding different physical properties while maintaining their gel-like behaviour. Their high degree of porosity, owed to their weakly crosslinked polymer chains, even allows for efficient encapsulation of macromolecules, usually not realizable with conventional nanoparticles. [83]

Their stimuli-responsive nature makes nanogels particularly suitable for the treatment of cancer and inflammatory diseases, since those are commonly paired with acidic pH, generation of heat and ionic changes. [82] Nevertheless, nanogels are nowadays intensely investigated throughout a great variety of application fields, having the great benefit of being customizable for respective needs not just in terms of size and crosslinking density, but also surface modifications such as with specific targeting ligands. [78] Drug delivery representing the area with the greatest impact of nanogels, they have also emerged to be vastly applicable in other sections, particularly in the biomedical field, comprising imaging and diagnostic purposes [85], sensing [86], bioengineering [87] and the exploitation of responsive nanomaterials [82]. A tabular compilation of nanogels that have been formulated and investigated as gene delivery systems during the last five years can be found in **Table 1**.

Year of Publication	Carrier Material	Genetic Payload	Target Cells/Organism	Therapeutic Aim/Disease	Special Features	Ref.
2018	Epigallocatechin-gallate, protamine	siRNA	MDA-MB-231 cells and -tumor-bearing mice	Drug-resistant triple-negative breast cancer	Targeting ligands: hyaluronic acid, cell-penetrating peptide	[88]
2018	DNA-grafted polycaprolactone	siRNA	HeLa cells, U2OS cells, MDA-MB-231 tumor-bearing mice	Cancer		[89]
2018	PEI, R8	pDNA	HCT-116 cells, BALB/c mice	Abdominal metastatic colon carcinoma	Heparin modification	[90]
2018	Thiolated PEI, dextrin	siRNA	4T1-luc cells and -tumor-bearing mice	Cancer	Reduction-sensitive	[91]
2017	Dextran	siRNA	H1299 cells		Add-on treatments for lysosomal escape	[92]
2017	Dendritic polyglycerol, PEI	siRNA	HeLa cells		PH-sensitive	[93]
2016	Polyglycerol, varying amines	miRNA	U-87 cells, U-87 MG GBM-bearing SCID mice	Glioblastoma multiforme		[94]
2016	PGMA, lipoic acid	pDNA, siRNA	Hepatoma cells		PH-responsive	[95]
2016	PEI, heparinized pluronic 127	pDNA	Mesenchymal stem cells		Quantum dots complexes	[96]
2015	Methacrylates	siRNA	MC3T3 E1.4 cells, wild type mice			[97]
2015	Methacrylates	siRNA	Murine osteoblasts	Trauma-induced heterotopic ossification		[98]
2015	Dextran	siRNA	BALB/c mice	Inflammatory pulmonary disorders	Surfactant shell	[99]
2015	PEI	pDNA	SKOV3 cells, BALB/c mice	Ovarian cancer	Heparin modification	[100]
2015	Glycol chitosan	siRNA	HeLa cells		Folate receptor targeting	[101]
2015	PEI, Cellulose	pDNA	Various stem cells			[102]
2015	Dextran	siRNA	H1299 cells, A549 cells	Lung cancer	Folate receptor targeting, surfactant coating	[103]
2015	PNIPAM-g-PEI	pDNA	BALB/c mice	Gastric tumors	Thermo-responsive	[104]
2015	EGDE	pDNA	Human fibroblasts	Cancer	Photo-responsive, co-delivery	[105]
2015	Methacrylates	siRNA	Murine calcarial prosteoblasts	Heterotopic ossification		[106]
2014	PFPMA, MEO3MA, spermine	siRNA	HeLa cells			[107]
2014	Cycloamilose, spermine	siRNA	ACHN cells, 786-O cells, tumor-bearing mice	Cancer		[108]
2014	Chitosan, alginate	repRNA	Dendritic cells			[109]

Table 1. Nanogels as Gene Delivery Systems published during the last five years.

2. Synthesis and Characterization of Nanogels

The synthesis of nanogels is mostly achieved by two major strategies that are illustrated in **Figure 1**: the use of polymer precursors or the heterogeneous polymerization of monomers. As amphiphilic copolymers are prone to self-assemble into nanoscaled structures in an aqueous environment, the former can be stabilized by utilizing different cross-linking methods, based on amines or disulfides, click chemistry, or are photo- or physically induced. The fabrication of nanogel networks by polymerization of monomers can proceed in an emulsion or inverse emulsion process, depending on the continuous phase. By incorporation of bifunctional monomers and initiation of polymerization in these heterogeneous colloidal systems, nanogels can be manufactured. [81] As opposed to this approach, in the initially homogenous dispersion and precipitation polymerization, all components are soluble in the solvent, allowing a synthesis in a single batch process. More detailed information concerning the synthesis of nanogels can be found nicely summarized in a review article by Asadian-Birjand et al. [82] Due to the mostly rather harsh conditions and the oftentimes required use of catalysts during the synthesis of nanogels [93], it is usually preferred to add the sensitive nucleic acid payload in the aftermath. In order to use polymeric nanogels as effective gene delivery vectors, it is generally necessary for them to possess or get incorporated site-specific cationic entities [110]. In most cases, the siRNA, pDNA, or mRNA is then just added to the readily prepared nanogel at the desired N/P ratio (residual molar ratio of the amine groups of the nanogel to the phosphate groups of the nucleic acid) and mixed thoroughly. During a short incubation period, a polyion complex is now formed spontaneously via electrostatic interactions between the cationic nanogel and the negatively charged nucleic acid.It was recently shown that the size of nanogel particles is a crucial factor influencing the gene knockdown potential of siRNA loaded systems. Two well-defined types of cationic nanohydrogel particles were synthesized using amphiphilic reactive ester block copolymers of pentafluorophenyl methacrylate (PFPMA) and tri(ethylene glycol)methyl ether methacrylate (MEO3MA) with similar compositions, but different molecular weights, resulting in differently sized particles after crosslinking. Only those particles with an average diameter of 40 nm, but not with 100 nm, induced moderate gene knockdown. As the smaller-sized ones were revealed to especially avoid acidic compartments and hence endolysosomal uptake

pathways, it is suggested that these properties explain their greater knockdown potential. [107]

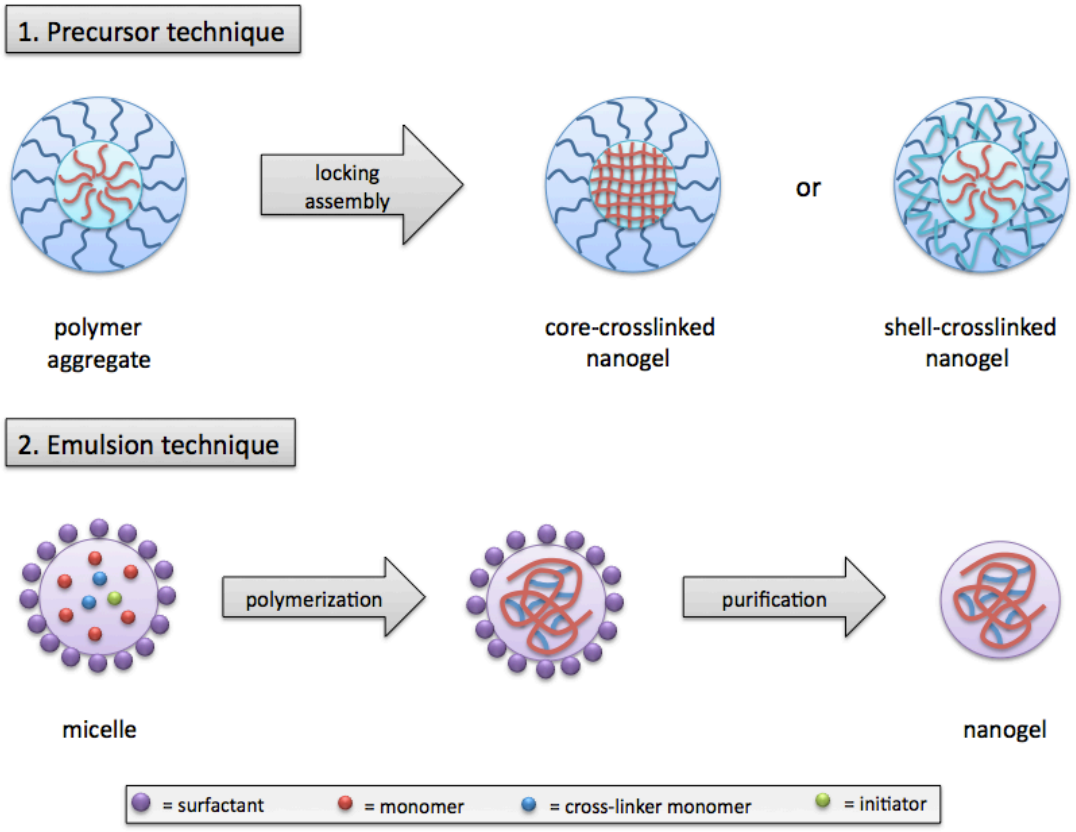


Figure 1. Typical nanogel synthesis techniques: The precursor method vs. the emulsion method. (Adapted from [81])

Moreover, the applied nanogel : nucleic acid ratio appears to play an important role. In a study aiming for gene knock-down in primary mouse osteoblasts, weight to weight ratios of nanogels : siRNA from 1:1 to 1:10 of quaternized dimethyl aminoethyl methacrylate (qDMAEMA) based nanogels were tested, revealing that two compositions (1:1 and 1:5) were particular favorable for the use of gene silencing. [98]

Shatsberg et al. prepared functionalized nanogels for microRNA (miRNA) delivery with a surfactant-free inverse nanoprecipitation method resulting in disulfide crosslinked redox-sensitive gels based on polyglycerol scaffolds that are degradable under intracellular reductive conditions. By attaching different amine-modified linkers to the

polyglycerol moieties in the nanogel structures, they were able to vary and thereby investigate the interactions between the nanogels and the miRNA in more detail. In this way, they synthesized and characterized six potential nanocarriers, depicted in **Figure 2**, giving new insights into some important features for the design of oligonucleotide delivery systems by comprehensive comparison of the varying nanogels. Nanogels 3 and 4 (NG3 and NG4) showed particularly high efficiencies to complex miR-34a, a miRNA that targets genes playing a key role in the regulation of apoptosis and cell cycle arrest as well as inducing the inhibition of cell proliferation and migration. Both cationic nanogels were able to neutralize the negatively charged miRNA in a dose-related manner and showed higher cellular uptake than the less positively charged NG2, confirming the widely accepted hypothesis that cationic surface charge of nanoparticles aids their internalization process. Complexes of miR-34a with NG3 and NG4 were successfully taken up by U-87 MG cells and significantly increased the miR-34a levels after transfection. NG3 complexes, however, showed superior knockdown abilities *in vitro* as well as *in vivo*, inhibiting the proliferation of U-87 MG cells and significantly arresting the tumor growth in mice bearing human U-87 MG glioblastoma multiforme, respectively. A polyanion competition assay revealed a distinct difference in the stability of the miRNA complexes, showing a lower affinity of NG3 towards miR-34a that resulted in a higher capability to release the miRNA. The authors, therefore, concluded that the stability of nanogels has to be carefully weighed with their ability to liberate the encapsulated cargo upon successful delivery. [94]

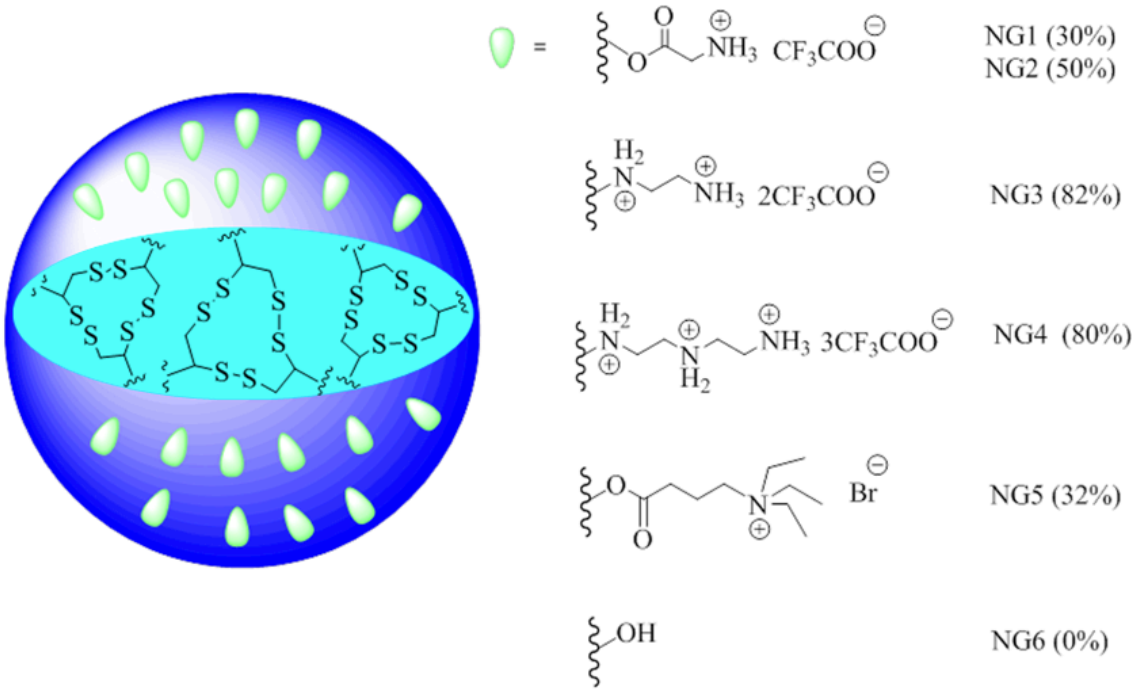


Figure 2. Schematic representation of the polyglycerol-based nanogels with different amine-bearing moieties. (Reproduced with permission from [94])

3. Modification with Natural Components

3.1. Heparin

In an attempt to overcome known drawbacks of using the non-biodegradable polymer polyethylenimine (PEI) as a gene carrier system, including its relatively high cytotoxicity and induction of aggregation of erythrocytes and hemolysis, the former was coupled to the natural polysaccharide heparin to form novel biodegradable cationic hydrogels. [111]

The transfection efficiency of heparin-PEI (HPEI) was found to be comparable to that of 25k PEI, while demonstrating improved blood and biocompatibility and decreased toxicity. While being stable *in vitro*, the nanogels were easily degradable through enzymolysis and hydrolysis into low molecular weight PEI and excreted through the urine *in vivo*. In several follow-up studies, the HPEI nanogels were tested for application in antitumor therapy. For instance, HSulf-1, a gene playing a key role in regulation of cell

proliferation, tumorigenesis and angiogenesis, which is down-regulated in most examined tumor types, was successfully transfected and expressed in SKOV3 ovarian cancer cells by HPEI nanogels. [112] The observed reduction in tumor weight, angiogenesis and cell proliferation as well as the induction of tumor cell apoptosis could even be extended in a pursuant combination of the HSulf-1 HPEI complexes with the anticancer drug cisplatin. [113]

Moreover, heparin-Pluronic supramolecular nanogels were synthesized by coupling aminated Pluronic to heparin through amide linkages. This conjugate was loaded with basic fibroblast growth factor (bFGF), an inducer of neovascularization, by a direct dissolution method. The highly negatively charged heparin was shown to stabilize genetic material and growth factors such as bFGF by forming high-affinity complexes. After coating these with PEI, polyplexes were prepared with pDNA encoding vascular endothelial growth factor $VEGF_{165}$ and delivered to endothelial progenitor cells (EPCs), where they promoted endothelial cell differentiation and neovascularization in an ischemic limb model system. [114]
The shielding effect of heparin was utilized to successfully diminish the toxicity of a PEI-based nanogel comprising the cell penetrating peptide R8. This peptide is in turn able to enhance the cellular uptake of the vehicle containing a therapeutic plasmid, making the PEI-R8-heparin nanogel a promising gene delivery system. [90]

3.2. Surfactant

Although RNAi has great potential for application in the treatment of pulmonary diseases, the lack of stable, biocompatible carriers to overcome the various intra- and extracellular barriers still impedes clinical translation. To enhance the colloidal stability of siRNA-encapsulating nanogels and to prevent siRNA release in the presence of competing polyanions abundantly present in respiratory biofluids, such as lung surfactant and mucus, novel bioinspired hybrid nanogels were manufactured. siRNA-loaded dextran nanogels were combined with a pulmonary surfactant coating to build up a core-shell nanoarchitecture. Despite the fact that the surfactant shell considerably reduced the uptake of the obtained nanogels in lung cancer cells, the resulting lower intracellular doses did not hamper the gene silencing effect, indicating that pulmonary

surfactant may play an important role in the processing of the nanogels inside the cells. To stimulate receptor-mediated endocytosis of the particles, folate was attached as a targeting ligand. Indeed, both uptake and gene knockdown were enhanced, eventuating in efficient silencing at nanomolar siRNA concentrations. [103]

Subsequently, surfactant-coated as well as uncoated nanogels were delivered to resident alveolar macrophages (rAM), critical contributors in lung inflammatory responses, via pharyngeal aspiration in BALB/c mice. While both achieved high levels of siRNA uptake in rAM, only the coated formulation significantly reduced gene expression on the protein level. Additionally, 70 % knockdown of target mRNA levels could be achieved with ~1 mg kg^{-1} siRNA doses, while only evoking mild acute pro-inflammatory cytokine and chemokine responses. [99]

3.3. Silica

An enhancement in stability and functionality of PEG-block-polycation/siRNA complexes was achieved by wrapping them with hydrated silica via polycondensation of soluble silicates onto their surface comprising a disulfide cross-linked core. This nanogelling process efficiently protected the polyplexes from counter polyanions under non-reducing conditions, while maintaining the environment-responsive disulfide cleavage leading to the release of the siRNA. Assumedly, a lower endosomal entrapment or lysosomal degradation of the siRNA resulted in an increased gene silencing effect in HeLa cells without arousing respective cytotoxicity. The authors hypothesized that deprotonated silanol groups and/or a modification of the intracellular trafficking could be responsible for a faster endosomal escape of the particles. [115]

4. Stimuli-responsive Nanogels

Several nanogel types have been generated to change their assembly or architecture in response to certain stimuli. Variations in the chemical design enable them to respond to a variety of environmental factors, such as temperature, pH, ionic strength, reduction, and light. [116]

4.1. Temperature

Thermally responsive nanogels for gene delivery have widely been investigated as drug delivery systems, especially in the research for tumor therapy and inflammatory diseases, since they are able to accumulate and release their payload at the desired target site via structural changes evoked by temperature changes. [117]

Cao et al. integrated the thermo-sensitive polymer Poly(N-isopropylacrylamide) (PNIPAM) in the side chain of low molecular weight PEI via the conventional radical graft copolymerization to form an amphiphilic graft copolymer at a reaction temperature of 80 °C and encapsulated TRP53 gene, a tumor suppressor gene playing a central role in cell cycle regulation and programmed cell death. The resulting cationic thermo-responsive non-cytotoxic nanogel with a well-defined core-shell structure showed considerably higher transfection efficiency compared to Lipofectamine 2000 or PEI alone. Additionally, distinctly higher *in vivo* tumor accumulation and inhibition were achieved after i.v. administration to Balb/c nude mice compared with PEI. [104]

In an approach to enable the challenging cutaneous application of proteins, thermo-responsive PNIPAM-polyglycerol (PNIPAM-dPG)-based nanogels were synthesized with a thermal trigger point at 35 °C, congenial to the native thermal gradient of human skin. The size of the ~200 nm protein loaded particles was instantly reduced by 20% at ≥ 35 °C, releasing 93% of the protein without any alterations in its structure or activity. Efficient intraepidermal protein delivery, especially in barrier deficient skin, was detected and transglutaminase 1 was successfully transported to respective knock-down models of human skin, restoring skin barrier function. [118]
Further thermo-sensitive hydrogels were constructed by PNIPAM-co-acrylic acid (PNIPAM-co-AAc) to generate self-assembled particles with a nanogel character. Binding of the carboxyl group on the outside of PNIPAM-co-AAc with the amine group of amine functional magnetic iron oxide nanoparticles was mediated by hydrophobic interactions. Fluorescent dye containing nanogels were then coated with cationic PEI and complexed with certain genes by the electrostatic formation of polyplexes. Efficient internalization of the nanogels by human mesenchymal stem cells (hMSCs) and expression of incorporated green fluorescent protein suggest a potential for respective gene delivery systems. [119]

4.2. pH

Similar to thermo-responsive carriers, pH-sensitive nanogels are well suited to deliver the incorporated payload to certain target areas with altered environmental conditions due to pathological processes taking place.

Based on two linear polymer precursors, disulfide-containing tetralysine (TetK) and oligoethylenimine (OEI), pH-dependent nanogel particles were formed by covalent *in situ* cross-linking with homobifunctional cross-linkers. The resulting nanogels were proven to increase in size as well as zeta-potential when encountering a decrease in environmental pH. Besides that, they were degraded in presence of glutathione at 10 mM, a concentration similar to the intracellular space. [116]

In another approach to generate both pH- and temperature-responsive delivery systems for the co-delivery of plasmid DNA and proteins, carbohydrate-based nanogels were synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization technique containing 2-lactobionamidoethyl methacrylamide (LAEMA) and methyl ethyl methacrylate (PEGMA). DNA-nanogel complexes were formed by the interaction of carbohydrate tails with DNA and stabilized with a linear cationic glycopolymer which further improved cellular uptake and gene expression *in vitro*. The acid-degradable profile of the nanogels enables a burst release of the encapsulated biomaterials in cell endosomes. [120]

Dimde et al. developed an elegant pH-sensitive nanogel on the basis of two macromolecular precursors, pH-reactive dendritic polyglycerol (dPG) and low molecular weight PEI-acrylamide, that were combined via thiol-Michael nanoprecipitation method. Owing to these mild conditions, lacking the need of any catalyst, the sensitive siRNA cargo could be encapsulated directly during the synthesis process minimizing siRNA loss or degradation. Resulting nanogels with pH-sensitive benzacetal-bonds containing GFP-siRNA demonstrated comparable gene silencing effects as unmodified PEI while showing significantly reduced cytotoxicity. [93]

4.3. Light

The swelling behavior of nanogels, caused by the difference in osmotic pressure in- and outside of the gel and alterable by charge or cross-linker density can be influenced by external stimuli to a great extent. Nanogels with unique photodegradation properties were developed using ethylene glycol diglycidyl ether (EGDE) as cross-linker, in conjugation with the polyamines spermine, protamine sulfate and PEI. These systems were then employed to condense different plasmids as well as anticancer drugs into nano-range particles encouraging their controlled release. Degradation of these nanogels upon UV light exposure occurs via the photo-oxidation of EGDE, leading to the removal of cross-links allowing the subsequent release of the constituent network polymer, resulting in changes in gel weight, mechanical properties, mesh size and porosity as well as swelling degree. The tunability of these systems harbors their potential for the controlled release of mono or dual delivery of biomolecules as well as for biosensing or –patterning technological purposes. [105]

5. Active Targeting

Under certain pathological circumstances, such as inflammation or hypoxia which are for example characteristic for tumors or infarcts, a phenomenon called 'EPR', enhanced permeability and retention, is hypothesized to take place at the disease area. The impairment of the protecting endothelial lining of the blood vessel walls by secreted factors such as kinin and vascular permeability factor leads to leaky vessels with larger cell gap sizes. This increased vascular permeability combined with vitiated lymphatic drainage can be exploited for a form of passive targeting, as it results in an increased accumulation of drug payloads in the desired regions while circumventing off-target sites, thereby minimizing side effects in healthy organs. [121] It is currently critically discussed if EPR has a viable impact on tumor targeting in patients, or if it is more relevant in artificially induced animal models of tumors. However, the pathological changes that cause experimental passive targeting are well described. [122]

As opposed to this passive targeting, it is also possible to attach specific ligands to the delivery systems in order to actively target certain diseased regions. This is often

achieved by aiming for cell surface receptors which are overexpressed in the respective disease in order to enhance accumulation of the delivered agents in the tissues of interest. Since folic acid is a vital nutrient crucial for cells to biosynthesize nucleotides and maintain important cellular pathways [123], many human malignancies, especially aggressively growing cancers, are associated with elevated expressions of the folate receptor, making it an attractive candidate for actively targeted drug and gene delivery. [121]

In a recent attempt to generate targetable nanogels for siRNA delivery, a glycol chitosan nanogel was synthesized by chemically grafting hydrophobic chains onto a polysaccharide, and the obtained macromolecular micelles were decorated with folate using a PEG linker. An extra amount of PEG was added to overcome the slight decrease of solubility caused by folate grafting and, additionally, to reduce the opsonin adsorption and subsequent scavenging by the mononuclear phagocyte system in order to lengthen the system's lifespan in blood. After incubating HeLa cells with both the targeted folate-decorated and non-targeted nanogels, the latter were detected on the cell surface, while targeted gels were localized in the cytoplasm, proving the support of internalization by receptor-mediated endocytosis via folate. [124] In a subsequent study aimed to further characterize the uptake mechanisms and intracellular fate of folate-functionalized nanogels, specific siRNA sequences were used to selectively inhibit uptake-mediating proteins such as clathrin or caveolin and thereby attenuate the respective endocytic pathways. Nanogel uptake was shown to occur mainly via flotillin-1 and Cdc42-dependent endocytosis and a shown impairment by free folate suggests a competitive inhibition and shared internalization mechanism. Cdc42- and Pak-1 involvement, furthermore, strongly hint to the need of actin reorganization for nanogel uptake. [101]

6. Co-Delivery and Add-on Treatments

Several of the mentioned studies employed co-delivery of different substances in one transport system in order to strengthen the therapeutic effect of the delivered genetic material. While a certain emphasis is put on the dual delivery of nucleic acids with anticancer drugs such as doxorubicin [125], paclitaxel [126], or cisplatin [113], there

are also approaches to co-deliver growth factors [114] or other proteins [120] alongside with DNA therapeutics.

Ding et al. recently published their findings on a self-assembling protamine-based nanogel co-delivering epigallocatechin-3-O-gallate (EGCG) with matching siRNA for sensitization to EGCG-involving chemotherapy in an approach to combine two promising strategies, co-delivery and selective tumor targeting. Their multicomponent carrier system was able to increase the cytotoxicity to a drug-resistant cell line by 15-fold compared to EGCG chemotherapy alone and demonstrated enhanced selectivity and tumor growth inhibition in respective xenograft tumor-bearing mice. [88]

A special type of co-delivery was used in an attempt to cope with one of the most challenging hurdles for polymer-based gene delivery: the issue of overcoming the endosomal entrapment of polyplexes. Therefore, a polysaccharide-based cationic nanogel composed of hexadecyl group-bearing cycloamylose was generated and complexed with plasmid DNA (pDNA) as well as the membrane phospholipid hydrolyzing enzyme phospholipase A_2 (PLA_2). Complexation with specific concentrations of PLA_2 were shown to enhance pDNA expression levels and resulted in similar hemolytic activity to that of native PLA_2, implicating a membrane disruption ability of the nanogel/PLA_2 complex when delivered into cells, triggering the subsequent release of pDNA from the endosome to the cytoplasm. [127]

Another approach to facilitate the cytosolic transport after delivery by nanogels was pursued by Joris et al. for siRNA. Here, it is thought to be of particular importance that the sensitive cargo is released to the cytoplasm prior to fusion of endosomes with lysosomes to prevent degradation. In an effort to circumvent this issue and even take advantage of the lysosomal accumulation, cells were treated with drugs provoking siRNA release from lysosomes to the cytosols, after having been transfected with nanogels. The group was able to show how a simple incubation of H1299 eGFP-expressing cells with the applied FDA-approved cationic amphiphilic drugs (CADs) after siRNA treatment could significantly increase the induced gene silencing effect. CADs cause lysosomal phospholipidosis in the cancer cells resulting in the permeabilization of lysosomal membranes, as illustrated in **Figure 3**, aiding siRNA release without affecting

cell viability. These findings might pave the way for intracellular depot forms allowing for controlled siRNA release via respective CAD treatments. [92]

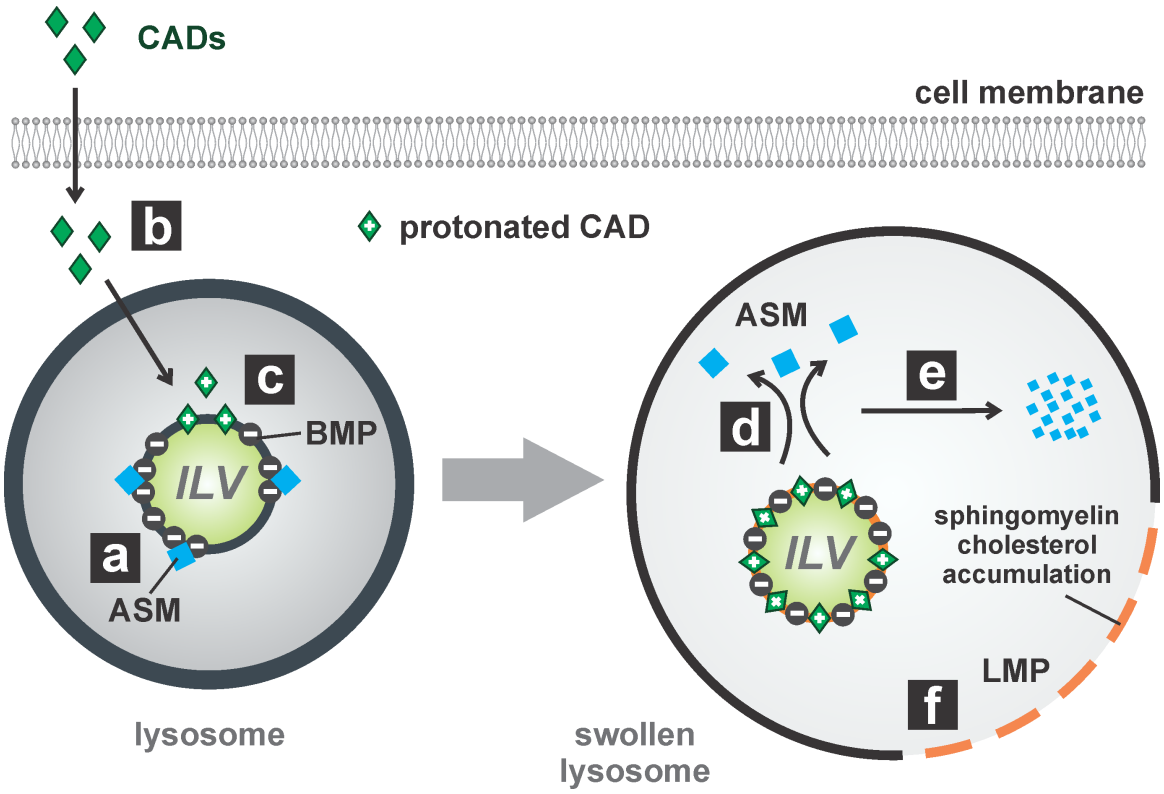


Figure 3. CAD-mediated inhibition of Acid sphingomyelinase causes lysosomal lipid accumulation and lysosome membrane permeabilization. (Reproduced with permission from [92])

7. *In vivo* Studies

Several of the nanogels currently under investigation for their use as gene delivery systems were already examined *in vivo* in respective animal models, the majority thereof were considering siRNA or other types of therapeutic RNA.

A recent report describes cationic nanogel polymers prepared by atom transfer radical polymerization (ATRP) in inverse miniemulsion and loaded with siRNA. The use of

quaternized dimethylaminoethyl methacrylate (Q-DMAEMA) as hydrophilic cationic moiety enabled siRNA binding without facing the problem of polymer aggregation. Moreover, the poly (ethylene oxide) (PEO) arms partially masked surface charges, enhancing biocompatibility and preventing enzymatic siRNA degradation through steric hindrance. 2'-O-methylation of siRNA was proven to maintain the polyplex integrity in presence of RNase A without compromising gene knockdown efficiency. After successful reduction of GAPDH enzyme activity by nanogel mediated siRNA treatment, significant inhibition of *in vivo* GFP expression via sequence-specific knockdown was confirmed in wild type mice. [97]

Intra-tumor delivery of siRNA was aimed for with self-assembled nanogels of cholesterol-bearing cycloamylose with a spermine group to deliver vascular endothelial growth factor (VEGF)-specific siRNA. The nanogel complexes were taken up by renal cell carcinoma (RCC) cells through endocytosis resulting in efficient knockdown, and intra-tumor injections were able to significantly suppress neovascularization and growth of RCC in mice. [108]

Another study tested the delivery of self-amplifying replicon RNA (RepRNA) in biodegradable, chitosan-based nanogel-alginate and demonstrated RepRNA delivery to dendritic cells. Accumulation in vesicular structures with patterns typifying cytosolic release promoted RepRNA translation *in vitro* as well as *in vivo* after vaccination of Balb/c mice and New Zealand white rabbits. [109]

A further nanogel for siRNA delivery was manufactured on the base of fully water-soluble DNA-grafted polycaprolactone brushes, that were used to further assemble a crosslinked nanogel via functional nucleic acid hybridization. After being intravenously administered to MDA-MB-231 tumor-bearing mice, the delivery system exhibited favorable physiological stability as well as a prolonged blood half-life and an increased accumulation at the tumor site compared to lipofectamine as positive control. siRNA specific for polo-like kinase 1 (PLK1), the chosen oncogenic target over-expressed in many tumor cells, resulted in the highest knockdown effect and, consequently, in the most effective tumor inhibition when delivered inside of the fabricated nanogel. [89]

Li et al. designed a reduction-sensitive nanogel by introducing thiolated low molecular weight PEI (1.8 kDa) into a biodegradable dextrin backbone resulting in a bioreduction-rupturing siRNA delivery system with a switch on/off controlled release. The dynamic

covalent bond crosslinked nanogel is susceptible to high cytosolic concentrations of glutathione, leading to rupture of the bioreducible crosslinks and degradation of the nanocarriers into its base materials PEI and dextrin, followed by a burst release of the incorporated siRNA. The nanogel exhibited equally high downregulation capabilities on the protein expression level as 25 kDA PEI *in vitro* and even superior tumor suppression rates in 4T1-luc tumor cell bearing BALB/C mice. At the same time, the developed system showed lower cytotoxicity and negligibly low hemotoxicity in healthy mice, evading recognition and clearance by the reticuloendothalial system. Biodistribution of the nanogel comprising Cy5-siRNA was, moreover, tested in tumor bearing mice after injection into the tail vein. The highest fluorescence intensity signal was obtained at the tumor site 12 h post-injection and the preferred accumulation in the tumor tissue was confirmed by examination of harvested single organs, as shown in **Figure 4**. The authors concluded that the longer blood circulation time, as opposed to naked siRNA that was rapidly cleared by liver and kidney, as well as the suitable size of the designed nanoparticles were decisive characteristics leading to the effective tumoral enrichment. [91]

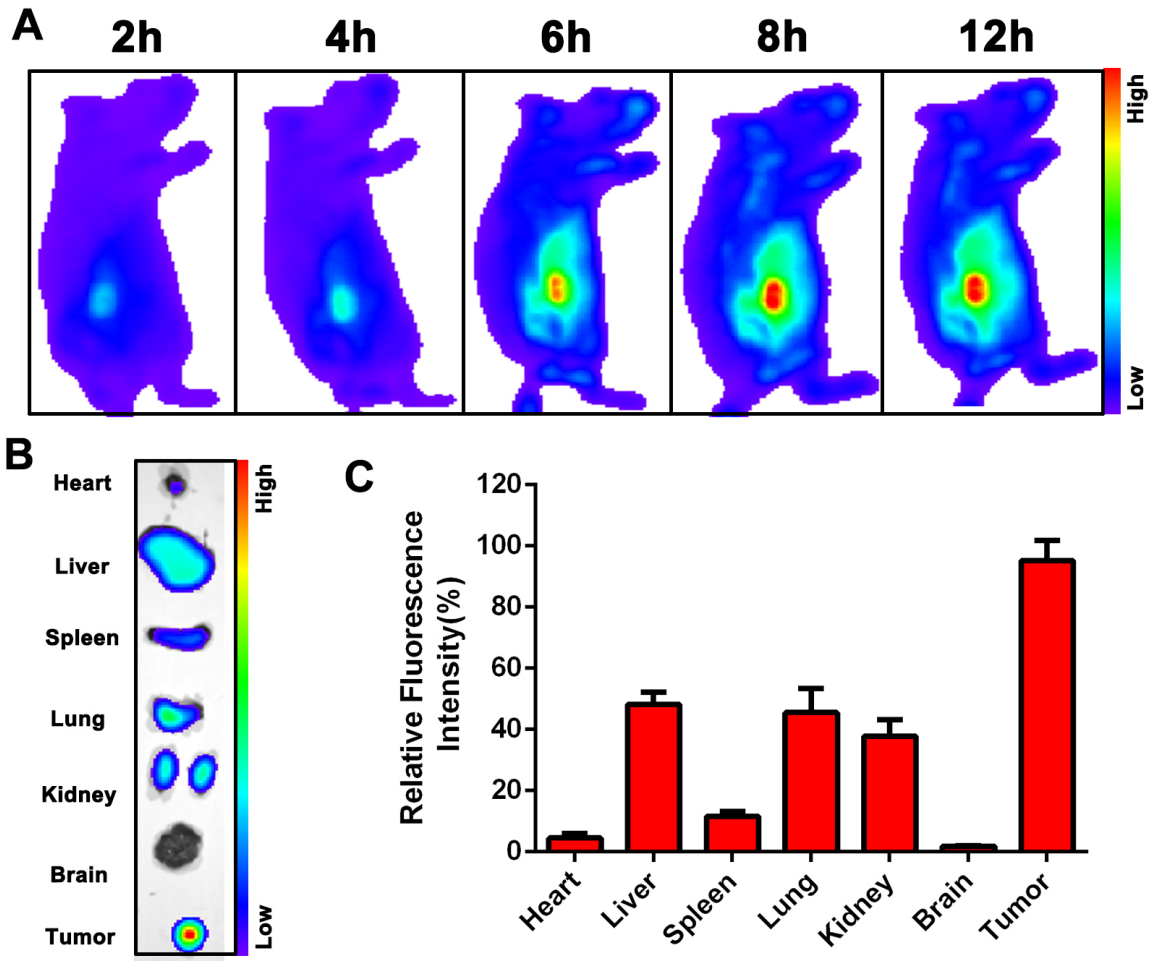


Figure 4. *In vivo* distribution of Cy5 after i.v. injection of the Cy5 siRNA loaded nanogel into 4T1-luc tumor-bearing mice. (Reproduced with permission from [91])

Albeit not explicitly falling into the scope of this review focusing on gene delivery, chimeric antigen receptor (CAR) T cell therapy as a kind of gene therapy with immense relevance is worth mentioning here as an example of the diverse application field of nanogels. In a recent approach, nanogels were used to selectively deliver large quantities of supporting protein drugs onto T cells and release their payload in a particular responsive way, namely upon T cell receptor (TCR) activation. Therefore, surface-conjugated nanogels were designed reacting to the increased redox activity of activated T cells in contrast to naïve ones and carrying an IL-15 super-agonist (IL-15Sa). In contrast to most conventional delivery systems, these nanogels operate as so-called

"backpacks" that are not expected to be internalized by the cells, but in fact to bind to the cell surface in order to sustain stimulation. In vitro, T cells treated with TCR-responsive nanogels expanded 16-fold more in tumors than those supported with systemic cytokine injections, and in vivo, nanogels backpacked CAR T cells eradicated tumors in four of five mice, while responses were only marginally improved with equivalent systemic doses of free IL-15Sa. [128]

8. Conclusion

Altogether, the ideal carrier system for successful gene delivery has to meet several specific requirements. First of all, the genetic material has to be efficiently encapsulated in a stable complex that at best endures circulation in the body. Nanogels certainly fulfill this demand, as their hydrophilicity contributes to high loading capacities for hydrophilic biotherapeutics and their tailorable size and crosslinking density allow for adjusting their pore sizes to various loaded molecules. Thus, nanogels can stably encapsulate their cargo during the synthesis. It is, however, essential to ensure that drug molecules are not chemically modified during this process. [129] Nucleic acids, as strongly charged biomolecules, can also be loaded post-synthesis into an oppositely charged nanogel via electrostatic interaction. Once loaded, nanogel networks generally protect their payload well from degradation, as enzymes are not able to penetrate into the particles. For special stability needs, the surface properties can easily be adjusted, for example via PEGylation.

The next step would be to transport the therapeutic molecule to the desired target region and, if applicable, to specific target cells. As discussed before, nanogel systems are both adjustable to benefit from passive targeting effects and to be customized by the linkage of certain targeting ligands to actively aid pointed drug delivery. By these means, high drug concentrations can be achieved in diseased areas, while healthy tissue is not affected, and side effects can significantly be reduced.

A critical point after reaching the target location is the exhaustive release of the carried drug so that it can bring about its effect. As important as a stable complex of delivery system and payload is for transporting intact therapeutics, it has to be stated that the

affinity can also be too strong, compromising release and, therefore, therapeutic effect. Nanogels just like nano-systems in general have the advantage of providing comparably fine control over release profiles [81] and feasibility for both sustained or burst release. Most common nanogels release their payload by hydrolytic degradation of their gel network, resulting in a sustained release leading to relatively low drug concentrations in- and outside of cells. As nucleic acids need to reach their site of action in the intracellular room, respective nanogel carriers should be taken up by cells and degrade upon the altered physiological conditions therein. Several bio- and stimuli-responsive nanogels have successfully been designed for the use as gene delivery systems as described in this review. Improvement of therapeutic efficacy as reflected in parameters such as enhanced transfection efficiency for reduction-sensitive nanogels as opposed to non-reducible representatives, implicates the intracellular cleavage of disulfide bonds. However, no direct evidence for this phenomenon has been found so far, leaving the exact intracellular fate of these nanogels uncertain. [78] The low pH of lysosomes is commonly used to reduce respective carrier systems releasing their payload in a controlled way. For nucleic acids, however, it is particularly important that they are able to escape from these acidic compartments in order not to be degraded. Some approaches to cope with this dilemma have been evaluated in this article, nevertheless, more efforts have to made to accurately comprehend and exploit these mechanisms to the fullest extent.

A further aspect not to be underappreciated is the biocompatibility of a drug delivery system, as this eventually determines whether it can be applied as a therapeutic. To achieve non-toxic carriers, they can either be straightaway manufactured from well-tolerated materials, or they have to be built upon substances that are transformed into non-toxic products via metabolization. Most modern nanogels currently designed for therapeutic purposes are already constructed from some of the many available biodegradable polymers. Even though sparsely relevant for the scientific level, but nonetheless crucial for ultimate drug approval and industrial production is the ease of synthesis, scale-up and purification of a drug transport system. Polymeric nanogels provide an attractive option in this matter as well, as their manufacturing is commonly straightforward and can easily be scaled up to larger quantities.

The highlighted favorable characteristics and discussed examples of promising nanogel formulations underline the great potential of this carrier system for drug delivery in general, and for the application as gene delivery vectors in particular. [130] Despite the considerable progress that has been made over the last years of intense research, there are, however, still some aspects that need closer assessment on the way towards clinical translation. Taking into account that toxicity and immunogenicity rank among the most important criteria for drug evaluation, one crucial factor for easing this translation especially in regards to nucleic acid delivery is a comprehensive investigation of the immune-compatibility of respective systems. Both nanotechnology-based formulations in general and biological products such as therapeutic nucleic acids in detail bear the risk of unintended immune-mediated adverse effects. [130] Although this area is substance of intensive research and several immunological targets such as cell surface or endosome Toll-like receptors and cytosolic sensors have already been identified [131], there is still an unmet need to further examine underlying molecular mechanisms in order to produce safe nanogel formulations. Guo et al. recently compared numerous different RNA nanoparticle formulations and demonstrated that their immune response is not only highly dependent on size and shape of the carrier system, but also the RNA sequence itself has influence on possible immunostimulations. [131] Their results suggest that immunogenicity of respective formulations is tunable and can be used to manufacture delivery systems with specifically designed immune responses as needed in order to achieve a minimal response for safe therapeutics or a strong response for cancer or vaccine adjuvants.

One of the main hurdles specifically related to nanogel-based delivery systems is that currently only 5-10 % of injected doses effectively reach the target location, while the largest share is still gathered by clearing organs such as kidney, liver and spleen. [132] The molecular weight of copolymeric nanogels lying above the renal threshold (~40 KDa), they are not excretable via the kidneys and might tend to accumulation, making it inevitable to accurately examine metabolism and elimination of the carrier system before planning for long-term clinical application. [83] Several parameters such as size, shape, composition and surface properties influence tissue distribution and clearance of nanogels and have to be carefully balanced in order to achieve successful delivery to the desired areas. PEGylation, or coating with polysarcosine as a newly arising alternative

[133], can be a way to prevent adsorption to plasma proteins and subsequent uptake by liver and spleen and at the same time provide shielding of undesired charges, as a rather neutral surface charge has been shown to prolong the circulation time of gel particles. [83] This improvement, however, has to be carefully weighed with maintaining the stimuli-responsiveness of the nanogel, oftentimes being reliant on charged groups. Another nanogel-related drawback is the continuing heterogeneity of respective formulations. Despite advances in nanoscale fabrication allowing for finer particle size distribution control, exact reproducibility of particle size and stoichiometry of nanogels remains difficult to achieve. [83]

Furthermore, some questions not satisfyingly acknowledged yet, such as the exact intracellular fate of both carrier material and nucleic acid cargo or the accurate balance between stability and release behavior of built complexes, still leave room for further improvements. More detailed investigations of the pharmacodynamics and -kinetics of nanogels as well as their interactions with their encapsulated payload would be an important step towards application in the clinical routine. Rationally designed nanogels taking all mentioned aspects into account then offer an auspicious base for a variety of biomedical applications, one of particular interest being the usage as a versatile gene delivery platform.

Chapter IV

Biology and Treatment of Asthma

Please note that the following chapter was published in the book *Nanomedicine for Inflammatory Diseases (Taylor & Francis Ltd.):*

Rima Kandil, Jon R Felt, Prashant Mahajan, Olivia M. Merkel: The Biology and Clinical Treatment of Asthma. Nanomedicine for Inflammatory Diseases, 2017, CRC Press, pp.217-244.

CHAPTER IV - Biology and Treatment of Asthma

Nanomedicine for Inflammatory Diseases – The Biology and Clinical Treatment of Asthma

The authors of this book chapter include Jon R Felt, Prashant Mahajan, Olivia M. Merkel and me. Jon R Felt and I are the joined first authors of the respective publications, we performed the literature search and wrote the article.

1. Introduction

Asthma is among the most common chronic diseases globally, and represents one of the most common inflammatory conditions of both children and adults. Asthma is a chronic inflammatory state of the lungs and lower respiratory tract characterized by acute, intermittent episodes of increased inflammation and airflow obstruction. [134] Acute obstruction is usually reversible and therefore manageable, but the chronic inflammatory state results in physiologic changes within the lungs that can lead to more severe and in some instances life-threatening exacerbations, permanently decreased lung function, and poor quality of life. The high social and economic burden associated with the management of asthma and its sequelae have fostered a significant effort in researching the underlying mechanisms and effective management of this disease.

2. Epidemiology

Asthma affects an estimated 330 million people worldwide, including 15% of children and adolescents. [135] Asthma occurs in all parts of the world, but appears to have the highest prevalence in Australia, Northern and Western Europe, and Brazil. [136] In childhood, the disease is more common in males, but as the disease progresses into adulthood, slightly more women are affected. Though asthma can be diagnosed at any age, symptoms manifest in early childhood with 80% of asthmatics diagnosed by 6

years of age. [137] Many children with asthma improve while some achieve complete remission in adolescence, however some have persistent asthma and some demonstrate recurrence after apparent remission. Children who experience multiple wheezing episodes before 3 years of age and who continue to wheeze after age 6, experience a decline in their lung function and often have persistent symptoms through adolescence and into adulthood. [138-140] Other factors associated with persistence of asthma into adulthood are parental asthma, low birth weight or prematurity, male gender, atopic dermatitis (eczema), allergic rhinitis, and food allergies. [141]

The development and severity of asthma is most closely linked to atopic conditions such as eczema and allergic rhinitis. The overall prevalence in the developing world is less than that of the more metropolitan and affluent nations, but is increasing steadily with greater urbanization by approximately 50% per decade. [142, 143] In contrast, the prevalence in the developed world remains relatively unchanged. Death from asthma is fairly uncommon in the developed world (1.1 per 100,000 population in the United States). Independent risk factors for asthma related mortality include older age, presence of severe and persistent symptoms, and those living in the developing world. [144]

The economic and social burden of asthma is significant. It is a disease that disproportionately affects those of lower income and education, and those of minority status. In the Global Asthma Report on the burden of asthma on society, as measured by years of life lost due to premature death and years of life living with disability, is greatest in the young and the elderly and leads among chronic diseases in these categories. [145] In the United States, more than 50% of asthmatics report having an exacerbation each year, accounting for more than 14 million doctor's visits, 2 million emergency room trips, and close to half a million hospital admissions. [146] The economic cost in the United States alone approaches 50 billion US dollars in direct healthcare expenditure, and more than 6 billion dollars in lost productivity. [147]

3. Etiology

While the exact causes and mechanisms for the development of asthma remain unclear, it is understood to be greatly influenced by both genetics and environmental exposures. There is a high degree of concordance for asthma in identical twins, and those individuals with severe asthma are more likely to have children who develop asthma. More than 100 genetic loci have been linked to asthma in multiple different studies, but no single gene or family of genes has been identified that can explain the development or severity of the disease. [148] Most of these genes have been identified as related to other allergic/atopic conditions as well as pro-inflammatory states, suggesting that the development of asthma is polygenic and likely requires significant environmental interaction.

The high incidence of allergy/atopy in those with asthma, as well as the striking lack of asthma and atopy among those born and raised in rural farming communities with early exposure to domestic animals suggests the role of epigenetics, or environmental influence. [149, 150] This is also supported by the increasing prevalence of the disease in developing countries with the spread of urbanization. Additionally, recurrent episodes of wheezing in infants from acute viral respiratory infections due to influenza virus, respiratory syncytial virus, and human metapneumovirus, among others, have been shown to be directly related to later development of airway hyperreactivity and asthma. [151, 152] All of these highlight the significant role that environmental exposures in combination with genetic predilections have in the development and natural progression of asthma. Understanding these interactions and modifying environmental exposures have long been a focus for management of the disease. [153]

4. Pathophysiology

The underlying mechanisms behind the development of asthma in those who are genetically susceptible are not completely understood. It is known that asthmatics have both structurally and functionally different lower airways than non-asthmatics, though it is unclear whether these changes are what cause the disease or are simply a result of frequent exacerbations. Overall, asthmatics have a nearly constant level of underlying

inflammation of the lower respiratory tract and a hyperresponsiveness to triggers. The underlying reasons for this persistent inflammation are not fully understood. [154]

The pathophysiology of an acute asthma attack is mediated by a multitude of different inflammatory cells, structural cells, and inflammatory mediators in a complex interaction. [155-157] Generally, the underlying cause of airflow obstruction and the patient's distress is two-fold; smooth-muscle surrounding the airways contracts with resultant bronchospasm, and increased inflammation causes edema and mucous production, both resulting in sometimes severe airway narrowing. **Figure 1** compares the appearance of an asthmatic airway with normal.

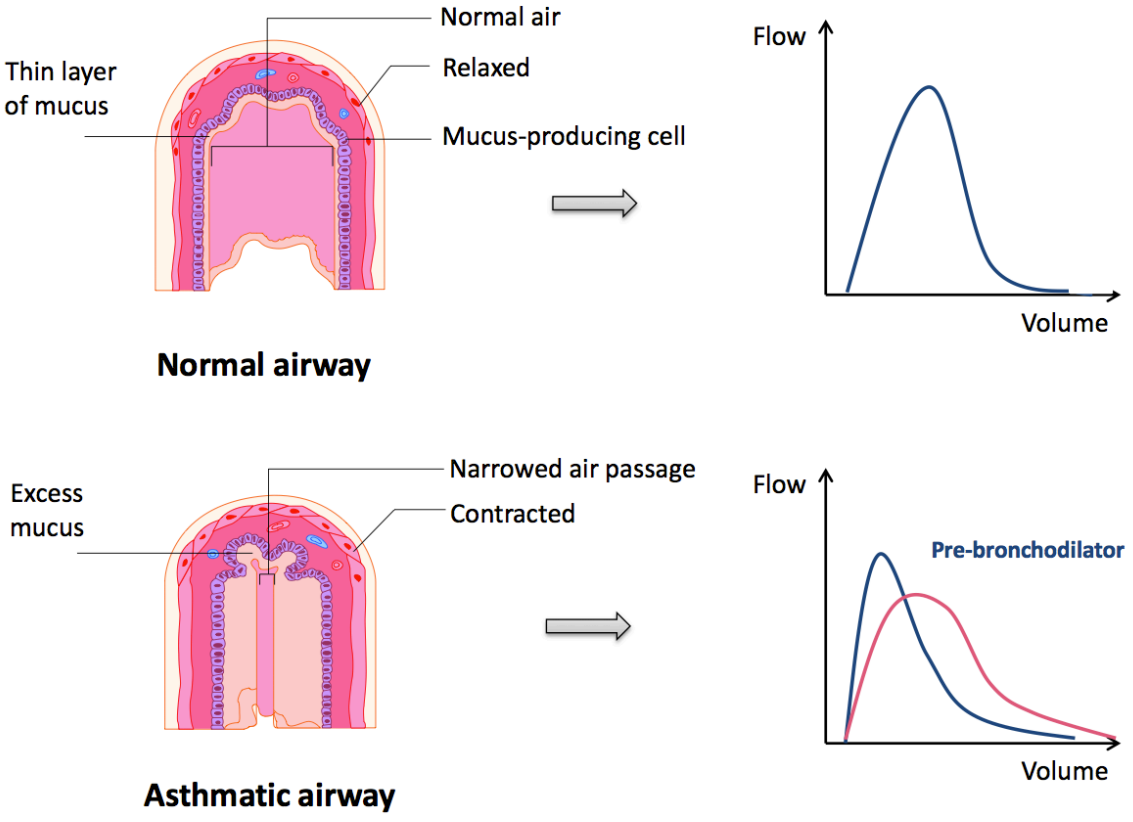


Figure 1. How obstruction of asthmatic airways affects the resulting flow diagram.

4.1. Bronchospasm

Bronchospasm of the airways is a reflex response designed to protect the lungs from harm. In healthy individuals, this is commonly seen as a response to extreme cold, heat, and environmental pollution (smoke, dust, heavy fog). In asthmatics, the lungs become hyperresponsive to triggers, and therefore the response is exaggerated and often persistent causing a significant narrowing of the airways and decreased airflow. [157] Multiple factors play a role in this mechanism and will be discussed here.

Physical disruption of the epithelium from trauma, invading pathogens, extremes in temperature, or changes in osmolality, expose sensory nerves which directly activate smooth muscle cells to contract. Indirectly, damage or loss of epithelial cells also causes decreases of various enzymes, inflammatory mediators, and relaxants that are secreted by the epithelial cells. Each of these either act directly to modify the reaction of smooth-muscle cells (epithelial-derived relaxant factor), or indirectly to degrade inflammatory mediators which also promote muscle contraction. [156]

Multiple inflammatory mediators (histamine, prostaglandin D_2, and cysteinyl-leukotriene) released by mast cells, act directly on smooth-muscle to induce bronchospasm. Histamine acts directly on peripheral nerves innervating smooth muscle, specifically causing bronchial smooth muscle to contract via H_1 receptors. Mast cells are found lining the epithelium as well as airway smooth muscle layer. While mast cells have important roles in wound healing and coagulation, they are best known for their release of histamine and its role in allergic reactions. [157]

They are activated by two mechanisms, namely binding of immunoglobulin E (IgE) and changes in osmolality. Mast cells express a high-affinity receptor which is highly specific to IgE, resulting in the cell being coated with IgE antibodies. When an allergen attaches itself across two matching IgE molecules (cross-linking), the high-affinity receptor is activated beginning a tyrosine-dependent cascade resulting in activation of the mast cell and release of its granule stores. Crosslinking by certain antigens, rather than full activation, causes the mast cell to become sensitized to environmental changes in the airway, most commonly changes in osmolality. [158] Changes in air temperature or humidity cause a shift in the osmolality of the thin fluid lining the mucosal layer of the

airways, which stimulates degranulation of the mast cells and subsequent bronchospasm. [157]

4.2. Airway Inflammation

Inflammation of the airways is characterized by mucosal edema and hypersecretion of mucus. In addition to mast cells and epithelial cells continuing to play a role in these events, dendritic cells, T-lymphocytes, and eosinophils also play a critical role. **Figure 2** illustrates the many cells involved in the pathophysiology of inflammation in the lungs and their interactions. Dendritic cells, specialized macrophages in the airways, are the major antigen presenting cells that activate the cell-mediated immune response. In healthy lungs, T-helper 1 cells predominate. However, for less understood reasons, in asthmatic lungs, the dendritic cells are mostly immature and promote differentiation and response of T-helper 2 (T_H2) cells instead. This is partially accomplished through the release of thymic stromal lymphopoeitin from epithelial cells, which causes the immature dendrites to release chemokines attracting T_H2 cells to the airways. [159]

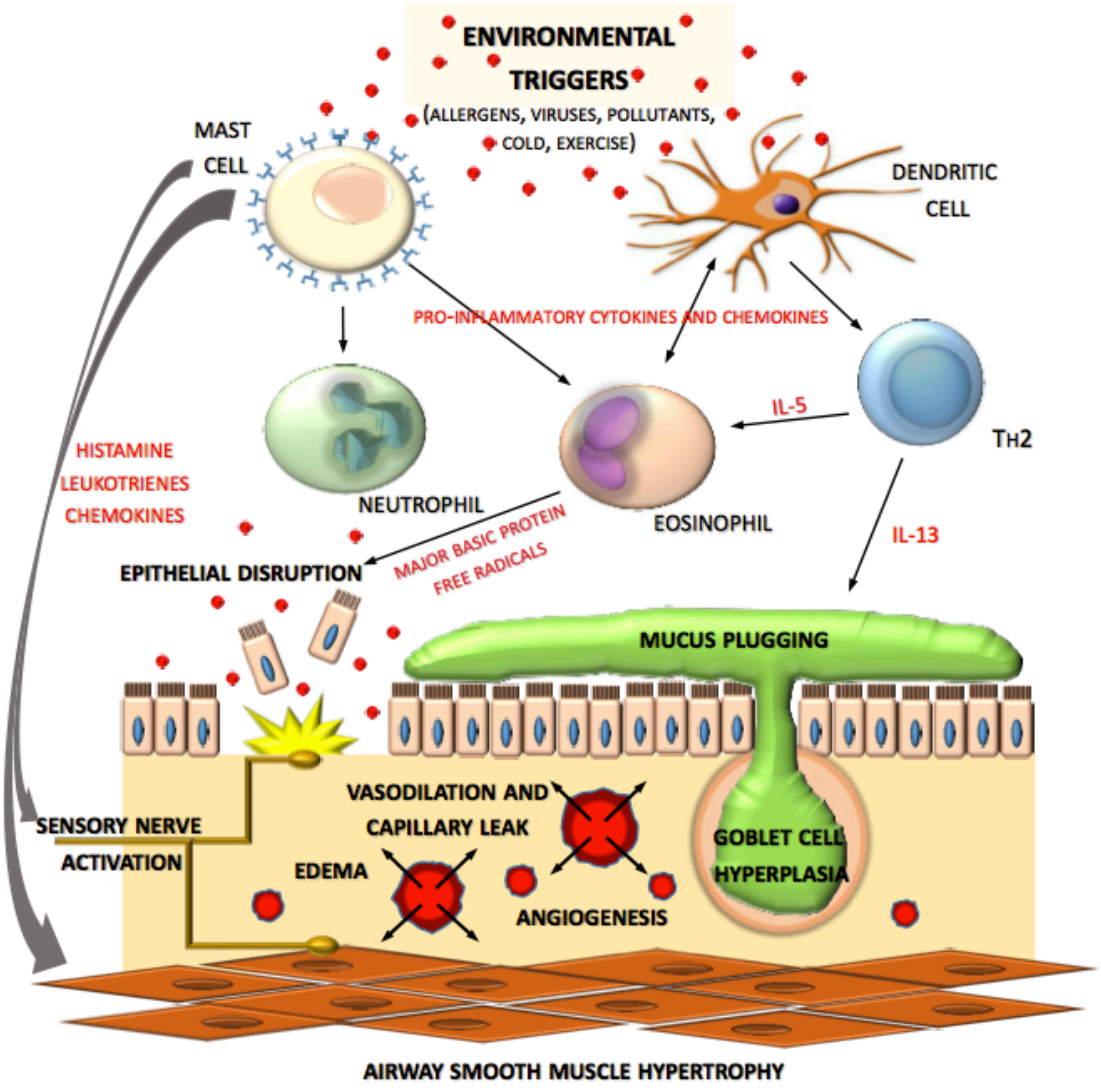


Figure 2. Cell-mediated and molecular pathogenesis of bronchospasm and airway inflammation during an acute asthma exacerbation.

T_H2 cells are critical to the coordination of the inflammatory response through the release of IL-4, IL-5, and IL-13. Release of these cytokines result in recruitment of eosinophils (IL-5), the maintenance and survival of both eosinophils and mast cells within the airways, and increased IgE formation (IL-4 and IL-13). IL-13 also induces hypersecretion of mucus from goblet cells lining the airways. Eosinophils increase airway hyperreactivity by releasing major basic protein and free radicals, which cause injury to the mucosa. They also release a small amount of histamine, though much less than mast cells. Overall, the importance of eosinophils in asthma is unclear as they

appear to play dual roles in both a dysregulated allergic response as well as tissue homeostasis and repair. [160] In some patients, particularly those with adult-onset asthma, neutrophils are attracted into the airways in larger numbers than eosinophils, but their role and how they affect the course of the disease is largely unknown. [161]

The disruption of the epithelium, already mentioned, also releases several additional inflammatory mediators, including vascular endothelial growth factor which promotes angiogenesis in the lungs. The significant increase in vasculature surrounding the airways results in more edema during periods of high inflammation, due to leakage of plasma into the interstitium. [162] Many additional inflammatory cytokines and chemokines, including TNF-α and IL-1β that amplify the overall inflammatory response, [163] are also involved but are beyond the scope of this manuscript.

Over time, due to constant inflammation, a physical remodeling of the lungs occurs. The mucosa of the lower airways is characterized by infiltration of a larger number of eosinophils, T-lymphocytes, and mast cells as compared to healthy lungs. There is a greater susceptibility for viral and bacterial infections and an exaggerated inflammatory response to such infections. [152] The basement membrane is thickened due to subepithelial collagen deposition from fibrogenic mediators released by the increased numbers of eosinophils. [160] And the smooth muscle surrounding the airways becomes hypertrophic, in part due to the increased vascularity, but also from growth factor stimulation and frequent episodes of bronchospasm. [156]

These changes are most often found in the bronchi, however, they can be present anywhere throughout the respiratory tract from the trachea to the terminal bronchioles in either a continuous or patchy distribution. [164] Unfortunately, the severity and/or extensiveness of these changes do not correlate with the severity of patient symptoms or disease.

5. Diagnosis

Chronic symptoms of asthma include frequent non-productive cough, often worse at night, occasional chest tightness and shortness of breath, and wheezing. Acute exacerbations lead to significant airflow obstruction and air-trapping. Patients present

with wheezing, dyspnea, and worsening cough. They often complain of significant chest tightness and pain. There is increased mucus production that is difficult to clear and contributes to air trapping (difficulty exhaling) and dyspnea. [163] If suffering from severe exacerbations, patients present with significant increase in their work of breathing with increased respiratory rate, accessory respiratory muscle use, and nasal flaring. Though many will have audible wheezing, auscultation of the lungs reveals an expiratory wheeze (sometimes also inspiratory wheezing in severe attacks) and a prolongation of the expiratory phase due to air trapping. There will often be asymmetry of breath sounds due to segmental atelectasis from mucus plugging. This mucus production and inflammatory exudate can also cause crackles to be heard. In severe exacerbations, airflow may be so limited that no wheezing may be heard and is an ominous finding. [134]

Diagnosis of asthma is based primarily on symptoms and response to therapy. Testing of lung function can confirm the diagnosis, but is difficult to perform on very young patients, and therefore has limited utility in the initial diagnosis of patients under the age of 6 years. While full pulmonary function testing can confirm the diagnosis, simple spirometry and a flow-volume loop is sufficient. A reduction in forced expiratory volume in 1 second (FEV_1), but no reduction in forced vital capacity (FVC) (reduced FEV_1/FVC ratio < 70% of predicted) is suggestive of the disease. [134] This combined with a reduction in peak expiratory flow (PEF) as seen by a convex shape on the flow-volume loop is diagnostic of asthma. The measurements obtained by lung function tests are depicted in **Figure 4**, which also demonstrates the characteristic convex shape of the flow-volume loop obtained by spirometry seen in asthmatic versus normal lungs. Often, spirometry is repeated after administration of a bronchodilator and a subsequent improvement of FEV_1 of greater than 10% confirms the diagnosis. [134]

Generally, a diagnosis of asthma can be made with observation of multiple episodes of airway obstruction and wheezing over time. However, not all wheezing is from asthma, and careful examination and review of the history may indicate a different etiology. **Table 1** lists the other causes of wheezing categorized by age when onset of symptoms is often observed. Overall, failure to correctly diagnose any of these other conditions will, at a minimum, result in improper use of medication with subsequent risk of side

effects. In the case of inhaled foreign bodies, delay in correct diagnosis and prompt removal can result in permanent, sometimes life-threatening, injury and scarring. [165]

Infant	Toddler	School-age	Adolescent	Adult
Bronchiolitis	Bronchiolitis	Foreign body	Vocal cord dysfunction	Interstitial lung disease
Bronchopulmonary dysplasia	Foreign body	Cardiomegaly	Cardiomegaly	Chronic obstructive pulmonary disease
Tracheo-bronchomalacia	Cystic Fibrosis	Pulmonary edema	Pulmonary edema	Cardiomegaly
Vascular ring	Bacterial tracheitis	Tumor	Tumor	Pulmonary edema
Tracheal stenosis	Cardiomegaly			Tumor
Laryngeal web	Pulmonary edema			
Cystic lesion/tumor	Tumor			
Cardiomegaly				
Pulmonary edema				

Table 1. Non-asthmatic causes of wheezing by age group.

Once an asthma diagnosis is made, the severity of the disease is classified to guide initial management. Severity is based on patient symptoms, functional impairment, and the patient's risk for future adverse events. Symptom severity can range from mild infrequent episodes of wheeze or cough without limitation to daily activities, to frequent episodes of severe exacerbation with daily symptoms causing considerable limitation in daily activities.

Asthma is classified as either intermittent or persistent based on the number of days with symptoms, frequency of nighttime symptoms, use of short acting rescue medications, interference with normal activities, objective measurements of lung function, and the frequency of exacerbations requiring systemic corticosteroids. [134] Patients with persistent symptoms (occurring at least biweekly with some limitation of

daily activity) are then classified as mild, moderate or severe. Care should be taken to provide equal consideration to both symptoms and objective data when classifying asthma. This is especially true in pediatric patients, as they may demonstrate normal lung function with objective testing but still suffer from severe or frequent symptoms. [166-168] In addition, it should be recognized that even those classified with mild or intermittent disease can still present with severe, life-threatening exacerbations. **Table 2** lists the criteria used to classify asthma severity and stratify risk.

Components of Severity			INTERMITTENT			PERSISTENT								
						MILD			MODERATE			SEVERE		
AGE (years)			0-4	5-11	>12	0-4	5-11	>12	0-4	5-11	>12	0-4	5-11	>12
IMPAIRMENT	Symptoms		≤2 days/week			≤2 days/week, but not daily			Daily			Throughout the day		
	Nocturnal Symptoms		0	≤2x/month		1-2x/ month	3-4x/month		3-4x/ month	≥1x/week		≥2x/ week	Often 7x/week	
	SABA use, excluding exercise-induced treatment		≤2 days/week			≥2 days/week			Daily			Several times/day		
	Limitation of normal activity		None			Minor			Some			Constant		
	Lung Function	FEV_1	-	>80%		-	80%		-	60-80%		-	<60%	
		FEV_1/ FVC	-	>85%	Normal	-	>80%	Normal	-	75-80%	Reduced >5%	-	<75%	Reduced >5%
RISK	Exacerbations requiring oral steroids		0-1x/year			≥2x/6 months OR >4x/year + risk factors	≥2x/6 months OR >2x/year + risk factors							

Table 2. Determination of asthma severity and risk.

Assessing risk of future adverse events helps clinicians identify those patients that require more careful monitoring and also helps to influence more aggressive management strategies to prevent complications. Patients are categorized as low, medium and high risk. Those factors that are predictive of increased exacerbations, severe exacerbations, or death include: Two or more emergency department visits or hospitalizations in the past year for asthma, previous intensive care unit admission, previous endotracheal intubation from asthma, tobacco use or second-hand exposure, severe and persistent airflow obstruction (seen with objective lung function testing), history of medication non-compliance, and low socio-economic status. [134, 136, 144]

6. Management

The goals of management are to reduce the frequency and severity of exacerbations and use of rescue medication, prevent chronic symptoms (cough, exercise intolerance, shortness of breath, etc.), maintain normal activity, prevent loss in pulmonary function, and reduce the side effects of medication. Although pharmacologic treatment is the mainstay in asthma management, it is only a part of successful treatment of the disease. Once the severity of asthma has been classified, successful management comes from identification and avoidance of triggers, identification and management of comorbid conditions that can worsen the disease, education and empowerment of the patient and family to develop skills in self-management, and frequent assessment and monitoring. [134]

Comorbidities that directly affect the ability to successfully manage asthma are important to identify and treat. Foremost are other allergic or inflammatory conditions, such as sinusitis, rhinitis, eczema, and gastroesophageal reflux disease, which add to the severity of inflammation in the body and can worsen asthma symptoms. Obesity and obstructive sleep apnea alter pulmonary mechanics and add to airflow obstruction, particularly at night. Additionally, obesity and metabolic syndrome have been linked to an increase in inflammatory markers, which would impact the chronic inflammatory state associated with asthma. [169] Finally, multiple psychosocial factors, including stress and depression have been shown to worsen asthma symptoms and outcome. While the exact cause of this correlation has yet to be identified, there is evidence to suggest that increased stress is associated with more pro-inflammatory cytokines released throughout the body. [170]

Patients with asthma should be monitored frequently to assess whether symptoms are being well-controlled. Clinicians are encouraged to use judgment in the frequency of clinical assessment, but according to the United States Department of Health and Human Services guidelines for management of asthma, the interval should not exceed 6 months in the well-controlled patient and should be much more frequent in the poorly controlled or those in the high risk category. [134] Assessments should include both clinical assessment via history and focused physical exam as well as patient self-assessment of their own symptoms and understanding of their disease control and

compliance. Objective measurements with spirometry should be obtained every 1-2 years.

Pharmacologic management of asthma is divided into acute and chronic therapies. Chronic management is aimed at reducing underlying lung inflammation and chronic symptoms while preventing exacerbations. Acute management consists of providing rapid relief of symptoms and airflow obstruction during exacerbation and returning inflammation to its baseline as quickly as possible. To minimize adverse effects, pharmacologic agents are preferably administered directly into the respiratory tree via inhalation, with systemic agents reserved for either acute exacerbations or to escalate chronic management in the poorly controlled or moderate to severe cases. [171]

6.1. Chronic Management

Pharmacologic agents designed for chronic management generally fall within two main categories, beta-receptor agonists and corticosteroids. Inhaled short-acting beta-$_2$ adrenergic agonists (SABA), albuterol and levalbuterol, act directly to relax the smooth muscles of the respiratory tree and decrease bronchospasm. Inhaled, long-acting beta-$_2$ adrenergic agonists (LABA), salmeterol and formeterol, also act directly to relax smooth muscle in the lungs but are more beta-$_2$ selective and therefore have less effect on heart rate. When used alone, they have been shown to increase the risk of asthma-related death and therefore are only recommended for use when combined with an inhaled corticosteroid and when a patient's symptoms are difficult to control. [172-174]

Corticosteroids work via direct immune suppression of leukocytes and inhibition of inflammatory products. They inhibit the genes that encode for a host of inflammatory cytokines that activate and direct the immune response of leukocytes. Specifically, the suppression of IL-2 prevents T lymphocyte activation and suppression of phospholipase A2 which is a critical factor in lymphocyte adhesion, emigration, and chemotaxis. The inhibition of prostaglandins and leukotrienes also helps to reduce mast cell activation, bronchial vasodilation, and smooth muscle contraction. [175] Inhaled formulations (budesonide, mometasone, and fluticasone) are preferred in order to decrease systemic side effects, but are ineffective in treating the increased inflammation during asthma

exacerbations. In these instances, the more potent systemic corticosteroids (prednisone, methylprednisolone and dexamethasone) are preferred. [176]

Additional agents are used that either target other steps in the inflammatory pathway, or work to decrease bronchospasm and hyperreactivity. Montelukast and zafirlukast are leukotriene receptor antagonists and zileuton is a 5-lipoxygenase inhibitor which blocks the formation of the leukotrienes. [177] Several anticholinergics, ipratropium and tiotropium, help to counter bronchospasm and are generally used in conjunction with a SABA. The cromoglycates, cromolyn and nedocromil (available only outside the United States) act as mast cell stabilizers, but have additional anti-inflammatory effects on eosinophils, monocytes, and T-lymphocytes. [178] Theophylline is a methylxanthine and acts to inhibit leukotriene production as well as relax bronchial smooth muscle. Historically it was the agent of choice in management of acute exacerbations, but due to its narrow therapeutic window and many drug interactions, it is only recommended as adjunctive treatment in severe asthma. [134] Finally, several monoclonal antibodies have been developed that specifically target IgE (omalizumab) and IL-5 (mepolizumab) and have been shown effective in reducing asthma symptoms in the poorly controlled. Although there both drugs have been shown effective in treatment younger children with severe asthma, neither drug is yet approved under age 12 in the United States. [179, 180]

The choice of which medication to use focuses on a step-wise approach, which escalates therapy (adding new medication or increasing doses) based on a patient's symptoms and control, and deescalates therapy as symptoms improve and control is maintained. The main goal is to use the least number and lowest doses of medications to achieve symptom control and thereby avoid unnecessary side effects. **Figure 3** illustrates the step-wise approach to pharmacologic treatment of asthma. Patients with intermittent asthma are generally treated with as needed use of a SABA. For those with exercise-induced symptoms, often pretreatment with a SABA is all that is needed to decrease exacerbations. As symptoms progress, a daily controller medication is added, generally a low dose inhaled corticosteroid (ICS). The next step up is to increase the dose of ICS or add an adjunct, such as a leukotriene modifier (LM), a cromoglycate, or theophylline (in adolescents or adults). If symptoms remain uncontrolled, a LABA (in adolescents and adults) or LM is added if it has not been done previously. If therapy is escalated, high

dose ICS is used plus introduction of a monoclonal antibody in adolescents or adults. The final step in the worst controlled asthmatics is the addition of a low-dose oral corticosteroid to the previous regimen of high-dose ICS, LABA, LM, and monoclonal antibody (in older patients). [134] Patients should be frequently assessed and stepped down in therapy if their symptoms have been controlled for at least 3 months.

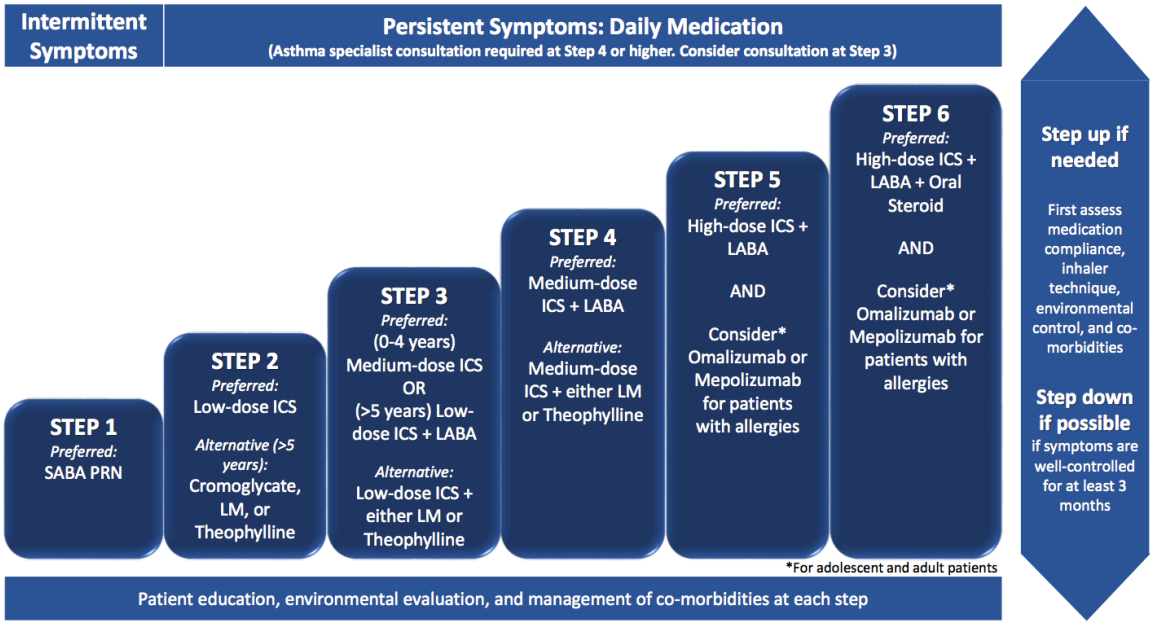


Figure 3. Treatment algorithm for the chronic management of asthma demonstrating a stepwise approach to pharmacotherapy.

6.2. Acute Management

The management of an acute asthma exacerbation should focus on relief of airflow obstruction, correction of hypoxemia if present, and reduction in acute inflammation. The majority of the medications used to control asthma previously mentioned have a longer onset of action, and therefore have no utility in the acute exacerbation. [134] For most exacerbations, multiple doses of a SABA and initiation of short course of systemic corticosteroids is sufficient to achieve improvement of symptoms and return to baseline. Many scoring systems have been developed and validated to determine the severity of an acute attack and may be useful for clinicians to guide management. In

general, a patient should be assessed for their work of breathing, subjective feeling of dyspnea, degree of air exchange, and presence of hypoxemia. [134]

Mild exacerbations are characterized by dyspnea with exertion, subjective feeling of tightness in the chest or shortness of breath, increased cough (particularly at night), and occasional expiratory wheeze or prolonged expiratory phase on auscultation. These patients can be managed at home with intermittent use of a SABA, generally with prompt relief. If symptoms have persisted for more than 48 hours or are worsening, consideration can be given to start a short (3-5 days) course of oral corticosteroids. [176] Moderate exacerbations are generally characterized by dyspnea interfering with normal activity, persistent expiratory wheeze, slight decrease in air exchange, and increased cough. These patients have no increased work of breathing or accessory muscle use. Those in moderate exacerbation should be evaluated promptly by a healthcare provider. Several doses of a SABA, either repeated every 20 minutes or given continuously via aerosolization should be administered and the patient started on a short course of oral corticosteroids. Often these patients will require scheduled use of their SABA (frequently every 4-6 hours) for the first 24-48 hours of treatment until peak effect of the steroids has been obtained. [134]

Severe exacerbations present with dyspnea at rest, difficulty speaking and increased work of breathing with accessory muscle use. Air exchange is usually diminished and wheezing may occur both during inspiration and expiration. Often, these patients are hypoxemic and require urgent attention. They should be helped into a position of comfort and provided supplemental oxygen if needed. A SABA should be given continuously over the first hour of evaluation and systemic corticosteroids initiated. The addition of ipratropium in combination with a SABA early in the course of treatment has been shown to reduce hospitalization and is recommended. [181, 182] Patients should be reassessed frequently and care escalated quickly if little or no improvement is observed. Being unable to speak at all, appearing panicked, little air movement, no wheezing, or altered mental status are all ominous signs and indicate the patient is rapidly progressing toward respiratory arrest and subsequent cardiovascular collapse. [134]

The use of epinephrine or terbutaline (both potent beta-$_2$ adrenergic agonists), given intramuscularly, can provide temporary bronchodilation allowing the inhaled particles of the SABA to penetrate deeper into the lungs, and should be considered very early in those presenting with severe or life threatening symptoms. Intravenous access should be established as quickly as possible and isotonic fluid administration begun to support cardiac function and correct dehydration if present. Magnesium sulfate, a potent bronchodilator, can be given intravenously in severe exacerbations not responding to SABAs, and can improve pulmonary function and possibly prevent hospitalization. [183, 184] Additional adjunctive therapies that have shown some benefit in severe asthma attacks include heliox (a helium and oxygen mixture), non-invasive positive pressure ventilation (NIPPV), intravenous terbutaline, and ketamine sedation.

Heliox (with the oxygen component not exceeding 40%) has been shown to increase laminar flow into and throughout the respiratory tree. It is felt that this improved flow allows better penetration of inhaled medications and improved ventilation. [185, 186] Intravenous beta-$_2$ agonists (terbutaline) can augment the action of inhaled SABAs when air exchange is poor, though care must be taken with these formulations due to their more prominent cardiac side effects. [187] Ketamine, an N-methyl-D-aspartate receptor antagonist and dissociative anesthetic, causes bronchodilation and can be used in refractory cases. It can also be useful in decreasing agitation and anxiety in those suffering severe exacerbations and is a preferred induction agent if endotracheal intubation is required. [188, 189] It is effective both in small titrated doses and as a continuous drip.

NIPPV can ease dyspnea, improve work of breathing, and in some cases prevent endotracheal intubation. Previously, the addition of positive pressure was contraindicated in asthmatics out of concern for worsening air trapping and increasing the risk of barotrauma to the lungs. [190] However, research now suggests that the positive pressure can actually reduce air trapping by stenting open the airways and allowing better ventilation. This has been shown in several small studies in adult patients, and has yet to be validated in the pediatric population, but provides an alternative to avoid endotracheal intubation. [190]

If necessary, endotracheal intubation of an asthmatic patient should not be delayed, however, extreme care should be taken to avoid complication. Manipulation of the airway can cause severe bronchospasm due to airway hyperreactivity often resulting in rapid decline and progression to cardiac arrest from loss of the airway and no ventilation. In addition, there is a significant increase in the risk of barotrauma from increased hyperinflation, air trapping, and airway pressure, all of which are already elevated within the lungs during exacerbation. [191] Indications for endotracheal intubation include persistent hypoxemia despite optimal delivery of supplemental oxygen or NIPPV, persistent respiratory distress and respiratory muscle fatigue, altered mental status, and respiratory or cardiac arrest. Endotracheal intubation should be performed by the most experienced provider to avoid repeated attempts. [192]

Once intubated, great care must be taken in choosing correct ventilator settings to avoid barotrauma or cardiac compromise from hyperinflation. Generally, in intubated patients, the goal of ventilation is to reduce hypercarbia but this is not always possible or advisable in those with asthma. Due to air trapping and inflammation, asthmatics often have prolonged expiration, which poses a significant problem with ventilator management. [191] If the expiratory time during the respiratory cycle is not long enough, there will be a steady increase in functional residual capacity (known as breath-stacking) which results in increasingly higher airway pressures which can lead to alveolar rupture, pneumothorax, and increased intrathoracic pressure that reduces pulmonary blood flow and venous return to the heart. This leads to both reduction in preload and outflow obstruction and results in eventual cardiovascular collapse. [191] To avoid these complications, often respiratory rate and tidal volumes must be decreased, and inspiratory to expiratory ratios adjusted. In most cases, this does not allow for adequate removal of carbon dioxide from the lungs and therefore a certain degree of hypercarbia must be allowed. Overall, patients who require endotracheal intubation generally suffer higher morbidity and mortality and longer hospital stays. [191, 192]

In intubated patients, a previously unavailable therapeutic option can be considered. Inhalational anesthetics (halothane, isoflurane, sevoflurane) are all potent bronchodilators and can be administered to improve airflow obstruction. [193] Other rescue medications should continue to be used, either intravenously or via inhalation

through the anesthesia circuit. The final option of rescue in the severe asthmatic, in whom all other therapies have failed, is extracorporeal membrane oxygenation, though this should be reserved as a last resort in the direst of circumstances. [194]

Overall, most patients in acute exacerbation will respond completely to repeated use of a SABA and initiation of a short course of oral steroids. Generally, SABAs act as a rescue medication to reverse acute airway obstruction and buy time until steroids can decrease the flare in inflammation and subsequent edema. In the most severe cases, steroids are still the mainstay of treatment and the only thing known to decrease the acute inflammation and eventually end the acute exacerbation. All other treatments mentioned here, including SABAs, are simply short-term rescue measures that provide relief from the symptoms of acute airflow obstruction and cannot treat the underlying inflammation. This highlights the importance of optimizing the long-term chronic management of asthma, with the most important goals being minimization of acute exacerbations and the degree of chronic inflammation in the patient's lungs.

7. Barriers and Hurdles for Efficient Treatment

Due to the detailed understanding of asthma and its underlying pathologic conditions as well as the great range of available treatment options, the disease and its symptoms should nowadays be expected to be controllable in a satisfactory way. A Canadian study, however, revealed that only in 47% of the patients' asthma symptoms are appropriately controlled according to symptom-based guideline criteria, while 53% are considered to have "poorly controlled" asthma. Nevertheless, 97% of the questioned patients would describe their asthma as "adequately controlled" themselves. [195]
According to the Global Asthma Report (GAR) 2014 [196], asthma is not only a cause of substantial burden to patients regarding their reduced quality of life due to physical, psychological and social effects, but also a tremendously high global economic burden. Thus, the indirect costs of the disease, with its negative impact on productivity leading the way, are at least as high as its direct costs, making it a problem of worldwide concern. Given the fact that there are essential asthma medicines with proven benefit available for most patients, a major global focus should be put on the improvement of access to care and adherence to these evidence-based treatments. Both in developing

and developed countries, this especially requires education of both health care providers and patients about the correct use and respective long-term benefits of medications. Additional barriers to effective management occur in developing countries, including lack of affordability of quality-assured medicines and poor infrastructure, indicating the need for political commitment for better asthma care. The key intention from the public health perspective should be the systemic implementation of the best standards of care in everyday practice in order to reduce both human suffering and the associated societal costs. Healthcare professionals and asthma experts are responsible to collaborate with national public health authorities and international organizations to develop national strategies and action plans. However, in 2013, approximately only 1 in 4 countries had national asthma strategies in place for children and/or adults. Therefore, the GAR's key recommendation to health authorities in all countries is to ensure the availability of nationally appropriate asthma management guidelines and provide access for everyone to the quality-assured, affordable essential medicines these guidelines suggest. Although suchlike guidelines first created in the 1980s were in a great measure commercially sponsored consensus statements, today they are most commonly evidence-based and independent of support from the pharmaceutical industry. They play a crucial role in both standardizing timely and correct assessment of asthma symptoms and severity and effective case management, hence lessening the overall burden of the disease. The World Health Organization (WHO) recently published respective asthma management guidelines in their report "Prevention and Control of Noncommunicable Diseases: Guidelines for primary health care in low resource settings". [197]

7.1. Detection/Diagnosis Problems

One hurdle in the very beginning on the way of treating asthma efficiently is the correct detection and diagnosis of the disease. Since the before-described symptoms are manifold and can vary strongly between patients, it can be challenging to detect and treat asthma early on without missing other possible disorders. Particular emphasis has to be put on a distinct and thorough differential diagnosis, as most symptoms are also observed in a number of other pulmonary and airway diseases which can, therefore,

mimic asthma clinically. Hence, it is necessary to maintain a rather broad differential diagnosis in order not to misdiagnose other lung diseases with airflow obstruction as asthma, especially for patients believed to have severe asthma that do not respond adequately to standard asthma therapy.

Although a clear and precise official definition is important to distinguish similar diseases, the guidelines concerning asthma have become rather ambiguous and vague over the last years. While former directives still highlighted eosinophils and mast cells as predominant cells in asthma development [198], the emphasis in the latest specifications by the Global Initiative for Asthma (GINA) is only put on variability in lung function and airflow limitation [199]. These symptoms, however, are also present in chronic obstructive pulmonary disease (COPD) and other related lung diseases. Particularly in atopic patients with a long smoking history, the differentiation between asthma and COPD is a demanding task, especially since it was noted that some COPD patients do in fact benefit from high doses of bronchodilators in terms of an improved FEV1 [200]. Before, it had been assumed that COPD-related airway obstruction can be regarded as irreversible in response to this treatment, a traditional way to demarcate it from asthmatic origins. Since it became obvious that a considerable group of asthma patients also have a clinically relevant history of smoking and, therefore, respective severe airway symptoms due to the two different kinds of airway inflammation, this condition is described as so-called Asthma-COPD-overlap-syndrome (ACOS). Although recent studies suggest that subgroups of asthma and COPD patients do have overlapping immune responses [201], critics question the benefit of this new affiliation of two diseases with different underlying pathological processes.

During viral infections caused e.g. by respiratory syncytial virus or adenoviruses, especially children can feature airflow obstruction, wheezing and other asthma-like symptoms [202] which can be easily misdiagnosed as childhood asthma. Furthermore, dysfunctional breathing disorders like the vocal cord dysfunction syndrome (VCD) can show clinical pictures very similar to asthma and often even coexist in patients with actual bronchial asthma. Although in many cases these patients receive high-dose anti-asthmatic therapy, treatment of the underlying disorders such as gastroesophageal reflux and postnasal drip would really be needed. Pulmonary sarcoidosis can, likewise, not satisfactorily be treated with bronchodilators or corticosteroids and has to be

distinguished from asthma by the lack of seasonal symptom variation and wheezing on auscultation.

Because of the described circumstances it is strongly suspected that quite some diagnosis of asthma has been made overhasty and haphazardly in the past. In order to prevent these misjudgments, the first measure for patients with alleged asthma should be a chest X-ray to preclude parenchymal disease, tumors, pneumothorax, and other thoracic conditions. A particular mistrust of asthma diagnosis is applicable in patients without eosinophilia and in those who do not respond to adequate anti-asthmatic, anti-inflammatory treatment with bronchodilators and inhaled corticosteroids with an expected normalization of lung function. Here, the differential diagnosis has to be broadened and the diagnostic accuracy has to be improved by considering ancillary examinations such as high-resolution CT, bronchoalveolar lavage and sputum cytology or even open lung biopsy. [200]

Besides the dissociation from other possible diseases, in modern asthma therapy, it is crucial to identify and differentiate clinical subphenotypes within patients, which is emphasized more precisely in the last segment of this chapter.

7.2. Corticosteroid Resistance

Another major hurdle in the effective treatment of asthma, especially in smokers and patients with more severe forms of the disease, is the reduced responsiveness to the anti-inflammatory effects of corticosteroids. Although the regular application of low doses of inhaled corticosteroids (ICS) can control most patients' symptoms appropriately nowadays, approximately 10% need maximal doses and in 1% even oral corticosteroids are required in order to maintain optimal control. In contrast to this so-called steroid-insensitive asthma, the complete resistance to corticosteroids is very rare, but still presents a serious problem in asthma care. The general definition of this resistance is no clinical improvement after high doses of an oral corticosteroid, meaning 40 mg/d prednisone or prednisolone for 2 weeks. As there is no well-defined procedure to quantify clinical steroid responsiveness, it remains a difficult task to measure the degree of resistance in individual patients. However, trying an oral application or a

single injection of a depot corticosteroid such as triamcinolone acetonide can be helpful to identify complete resistance. [203] Resistant patients were observed to clinically differ from responsive asthmatics by showing a longer duration of symptoms, a greater degree of airway hyperresponsiveness, and a more frequent family history of asthma [204]. The lower responsiveness in patients with severe asthma implies that the mechanisms of steroid resistance are possibly contributing to the grade of disease severity [205]. In contrast to general familial glucocorticoid resistance, steroid-resistant asthma patients are not cortisol deficient, nor do they have any abnormalities in sex hormones [206]. Furthermore, plasma cortisol and adrenal suppression in response to exogenous corticosteroids are normal and usually the typical side effects of systemic corticosteroids can be observed. Nevertheless, bronchial biopsies of resistant patients showed increased eosinophil and lymphocyte counts as well as a missing suppression of the T_H2 cytokines IL-4 and IL-5 compared to sensitive patients despite the treatment with high doses of corticosteroids [207].

As circulating cells from patients with steroid-resistance asthma also show reduced responses in vitro, it is feasible to investigate the underlying molecular mechanisms experimentally. Hence it could be shown that proliferation of peripheral blood mononuclear cells (PBMCs) from steroid resistant patients and complement receptors on monocytes from the latter were not inhibited by steroids, indicating that circulating T lymphocytes and monocytes are resistant in these patients. [208] Subsequently, it was observed that IL-2 and IFN-γ secretion are not inhibited [209] and secretion of cytokines and chemokines of peripheral monocytes and alveolar macrophages are less restricted than in patients with corticosteroid-sensitive asthma [210, 211].

Several mechanisms resulting in a reduced responsiveness to steroids have already been identified, indicating that individual therapeutic approaches may be needed to overcome this pitfall in asthma treatment. As this phenomenon is apparently more common within families, it can be expected that genetic factors play a crucial role in its occurrence. Eleven genes have been found to differ between patients with corticosteroid-resistant and -sensitive asthma in microarray studies of PBMCs [212], giving reason to assume that it might be possible to develop a genetic test for steroid resistance. A large proportion of patients with steroid-insensitive asthma shows a reduced nuclear translocation of glucocorticoid receptor (GR) α after binding of

corticosteroids which might be explained by modification of the GR via phosphorylation [213]. This can be the result of activation of several kinases, such as p38 mitogen-activated protein kinase α or γ, which in turn might be due to reduced activity and expression of phosphatases [203]. A further related mechanism is the increased expression of GR β which inhibits activated GR α. Besides that, increased secretion of macrophage migration inhibitory factor, competition with the transcription factor activator protein 1, and reduced expression of histone deacetylase 2 (HDAC 2) were proposed to be possible causes. The decreased activity of HDAC 2 in severe asthma patients is caused by oxidative stress via activation of phosphoinositide 3-kinase δ [214] and appears to interfere with the action of steroids to switch off activated inflammatory genes. Therefore, a novel approach to reverse steroid-resistance is the enhancement of HDAC 2 expression by theophylline or other phosphoinositide 3-kinase δ inhibitors. Respective long-term studies with low-dose theophylline combined with oral corticosteroids and ICSs are underway. [203] Besides that, common strategies to manage steroid-resistance include the use of alternative broad-spectrum anti-inflammatory drugs, such as phosphodiesterase-4 (PDE4) and p38MAPK inhibitors. Unfortunately, when given systematically, these treatments show side effects that limit the oral dose and efficacy, however, developing inhalable versions was proven to be difficult. Therefore, the well-tolerated ICSs would be the preferred anti-inflammatory therapy in combination with other drugs for increased responsiveness. This effect can for example be achieved by long-acting β_2-agonists via the reversion of GRα phosphorylation. [203]

7.3. Failure to Treat Vascular Problems

In a recent review, Harkness et al identified the failure of current asthma medication to regularize the vascular remodeling in affected airways as a further barrier in efficient treatment [215]. Numerous studies have shown the abnormal expansion and morphological dysregulation of the bronchial vascular network in asthmatic lungs by reporting an increased number, size and density of blood vessels, vascular leakage as well as plasma engorgement. A more intense blood flow to the airway tissue is

suggested to promote chronic influx of inflammatory mediators and pathological cell proliferation. [216]

Nevertheless, appropriate treatment attempts and respective trials are scarce. However, some novel findings give reason to further explore the potential of anti-angiogenic therapies as a new drug class for future asthma management. Re-purposing anti-angiogenic tumor therapy agents for asthma as a genomically and phenotypically more stable target is claimed to be relatively straightforward [215] and some of those agents have indeed already been tested successfully in asthma mouse models. As vascular endothelial growth factor A (VEGF-A) is a key driver of microvascular remodeling in asthma [217], several treatment approaches have tried to use it as a target. It was shown that the multi-kinase inhibitor sunitinib significantly inhibits eosinophilic airway inflammation and remodeling in chronic experimental asthma in response to either Ovalbumin (OVA) or toluene diisocyanate. [218, 219] Likewise, the VEGF neutralizing monoclonal antibody bevucizamab reduced epithelial, airway smooth muscle and basement membrane thickness compared to untreated OVA-challenged mice. [220] In another study, co-administration of the collagen XVIII fragment endostatin with OVA challenge in mice inhibited airway hyperresponsiveness as well as pulmonary inflammation and sub-epithelial angiogenesis was greatly reduced. [221] Further trials targeted circulating endothelial precursor cells, vasculogenesis or lymphangiogenesis. Nonetheless, the authors concede that there are still substantial hurdles to therapeutic implementation of anti-angiogenic medication in asthma treatment, most notably limiting side effects and the lack of good biomarkers to predict therapy responsiveness. [215]

8. Patient Care

8.1. Patient Monitoring and Assessment Tools

The National Institutes of Health and Expert Panels currently agree that achieving and maintaining asthma control are essential goals of therapy and the periodic assessment of this control should be part of patient monitoring once treatment is established. Therefore, asthma control is also increasingly being used as an outcome measure in

respective research studies. Due to the complexity and individuality of the disease, precise determination of the control level is difficult and commonly used assessment methods such as lung function tests have their limitations. As patients can show normal lung function between exacerbations but may still not have adequate control over their symptoms, the degree of control is often over-estimated by both clinicians and the patients themselves. In order to measure the level of asthma control in a quantitative and comparable way, several composite assessment tools have been developed.

In a recent work, studies were identified by a comprehensive literature search which are attempting to develop and/or test composite score instruments for asthma control. [222] Seventeen score instruments with published validation information where identified, which all have comparable content and assess nocturnal symptoms or interference with sleep. Symptom frequency, either of specific symptoms, such as cough, wheeze or dyspnea, or of any asthma symptom, is captured in all but 1 instrument and most of them also detect use of short-acting beta-2 agonists (SABA). All but 1 evaluate some form of activity limitation, such as interference with daily activities and school or work attendance, whereas only 2 (Asthma Control Questionnaire (ACQ) and Asthma Control Scoring System (ACSS)) include pulmonary function parameters and 1 (ACSS) assesses sputum eosinophilia. While more than half of the evaluated instruments assess exacerbations, only the Test for Respiratory and Asthma Control in Kids (TRACK) includes the "risk of exacerbations" domain, although this is recommended by the National Asthma Education and Prevention Program Expert Panel Report 3 guidelines [11]. In most cases, the score tools are designed to reflect the disease activity over a 1- to 4-week time period and are not validated to be used during asthma exacerbations.

The most widely used tool in patients older than 12 years is the Asthma Control Test (ACT) [223], that quantifies asthma control as a continuous variable and offers a numeric value to differentiate between controlled and uncontrolled asthma, similar to most available instruments. It is a multidimensional, standardized and validated patient-centered questionnaire that enquires the patient's experience of nocturnal and daytime asthma symptoms, the use of rescue medications, the effect of asthma on daily functioning, and the patient's perception of asthma control over the previous 4 weeks. [224] For children aged 4-11 years, the Childhood Asthma Control Test (cACT) was developed in 2006 [225], a self-administered tool that involves the child's as well as its

caregiver's perspectives. Another option is a parent completed version of the Asthma Therapy Assessment Questionnaire (ATAQ), designed to identify children and adolescents of 5-17 years with current problems in asthma control [226].

To ensure that the selected instrument will really measure the wanted outcome, profound knowledge of the psychometric properties of the tool is needed. Furthermore, it is essential to choose tools that have been evaluated with a comparable population and in a similar setting as their intended utilization. [227]

In order to facilitate implementation of asthma guidelines to primary care practices, the Asthma APGAR scheme was developed, documenting **A**ctivity limitation, **P**ersistence of symptoms, tri**GG**ers, **A**sthma medications and **R**esponse to therapy. This tool provides a patient survey to collect information based on control scores and contains a management algorithm to incorporate recommendations for education, therapy adherence, adequate inhaler techniques and follow-up visits. A study evaluating the benefits of implementing the Asthma APGAR in practice demonstrated that it resulted in both enhanced medical record documentation and significantly increased care processes. Involved patients and clinical staff described the tools to be easy to use and stated that they "made sense" and led to "improved care". [228]

8.2. Pharmaceutical Care and Patient Training

Beyond the care in medical practices, pharmacists play an equally important role in achieving optimal therapy and maximum improvement in the patient's quality of life (QoL). Although the scope of pharmaceutical care is often primarily focused on pharmacotherapy and drug-related outcomes, it is encroaching upon other areas of asthma patient care, too, beginning from the very early step of diagnosis. As a recent study reported, pharmacists are qualified to take part in this procedure by performing quality spirometry testing [229]. Spirometry is the most commonly used mean to detect and quantify the degree of airflow obstruction in patients and the lung function test of choice for both diagnosing asthma and assessing asthma control in response to treatment. This method is easy and safe to perform, however, for the patient it is physically demanding as it requires maximal effort and may cause breathlessness,

syncope and cough and can even induce bronchospasm in poorly controlled patients. Two important values obtained from the spirometry measurement are the FEV1, a criterion for airway caliber, and the FVC, the maximum volume of air that can be expired during the test. An exemplary spirometer curve with its components is depicted in **Figure 4**. As the spirometer measures timed expired and inspired volumes, it can be calculated from these how effectively and quickly the patient's lungs can be emptied or filled, making the spirogram a volume-time curve. Conventional volume-displacement spirometers provide a direct measurement of the respired volume from the displacement of a water sealed bell, a rolling sealed piston or bellows. New forms of advanced spirometers, however, utilize a sensor detecting flow as the primary signal, for example from the pressure drop across a resistance or the cooling of a heated wire. The sensed flow is then converted into volume by electronic or numerical integration of the signal. These portable devices are more suitable for personal use and generally easier to clean and disinfect. [230]

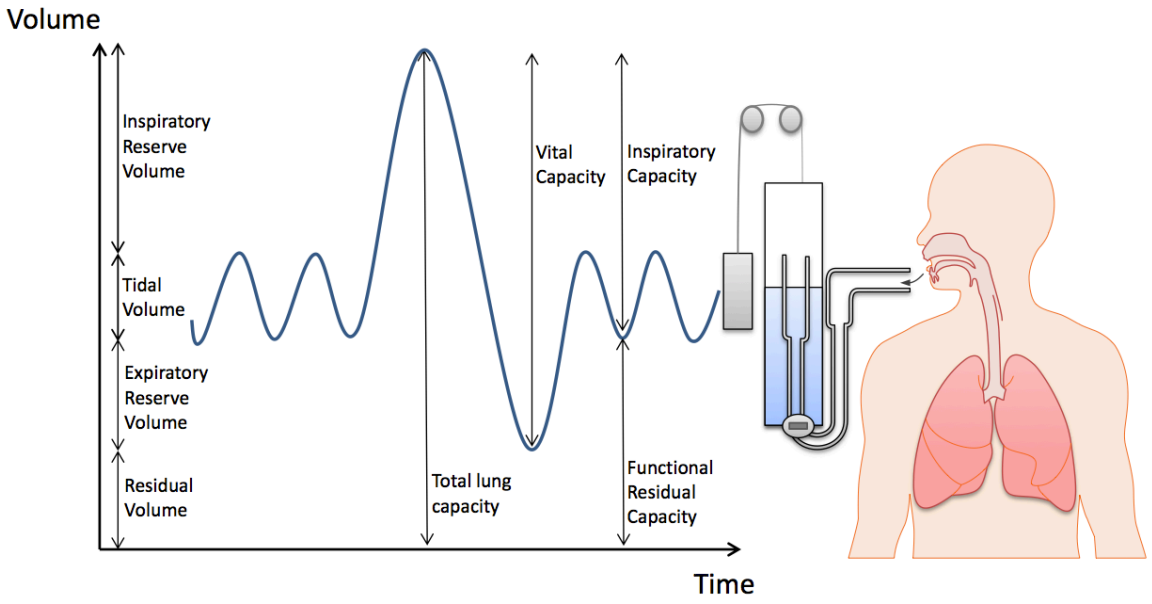


Figure 3.1. Exemplary spirometry curve with its component parts.

A further pulmonary function test rather used to detect and measure a patient's variability of lung capacity is the PEF measurement. It assesses the maximum expiratory flow occurring just after the start of a forced expiration from the point of maximum inspiration. In contrast to a spirometer, the peak flow meter has significant limitations, as its measurements are effort dependent and results vary considerably between different instruments. Furthermore, the range of values regarded as normal and healthy is rather wide. Nevertheless, PEF monitoring by patients can be useful when they suffer from intermittent symptoms and find it difficult to gauge asthma severity based on those. [231]

Besides lung function measurements, pharmacists are a crucial factor in the patients' education. As a successful treatment outcome in asthmatic patients depends on continuing compliance with their therapy, even in times they do not suffer from any obvious symptoms, the importance of a regular intake of medication has to be emphasized. In addition to a personalized drug regimen, every patient should get an individually tailored care plan in order to cope with variations in asthma severity. In this way, asthmatics are able to slightly adjust their therapy themselves without having to consult their physician every time. When they use PEF measurements to monitor their disease, as with inhaler devices, their technique should regularly be inspected and reinforced. In general, patients have to be familiarized with clinical symptoms of toxicity or undertreatment, such as shortness of breath, wheeze, tremor or change in O_2 saturation. Patients receiving corticosteroids in particular, should be educated and monitored concerning adverse effects of this drug class and respective measures they can take to avoid or reduce them. While local side effects of inhaled corticosteroids include oral thrush, hoarseness and dysphonia, patients on oral therapy should be monitored for hyperglycemia, gastrointestinal and neuropsychiatric effects, adrenal suppression, osteoporosis and infections. [232]

Oftentimes, medication adherence rates decline for children becoming teenagers. In a study assessing adolescent asthmatic needs and preferences regarding medication counseling and self-management, it was shown that effective adherence-enhancing interventions for this patient group are missing. Although lack of perceived need or beneficial effects were also mentioned as reasons for not taking the medication as prescribed, forgetting was identified being the major cause for non-adherence.

Participating adolescents revealed that their parents mainly still play a role in reminding and collecting refills and suggested smartphone applications with a reminder function and easy access to online information as favorable means for successful self-management. [233] It has been reported that inhaler reminders also offer an effective strategy to improve adherence in adult patients. A 6-month randomized trial compared 3 patient groups receiving asthma controller treatment: while one just obtained the usual care, personalized adherence discussions were performed with the second group. In the third group patients received twice-daily SmartTrack reminders for missed doses and automated e-mails about their daily inhaler use were sent to their practitioner. Results demonstrated that the electronic inhaler reminders including adherence feedback were able to improve the compliance even more than the patient-specific behavioral interventions. [234]

Besides poor adherence, inadequate inhaler techniques can also be a substantial cause for persisting asthma symptoms. The correct use of devices is central to effectively deliver the contained medication, however, especially for dry powder inhalers, serious technique errors are not uncommon. The failure to exhale before inhalation, insufficient breath-hold at the end of the inhalation process, and inhalation not forceful from the start are among the most frequent mistakes which are often connected to not only poor asthma control, but also severe consequences such as asthma-related hospitalizations. [235] This demonstrates the great need of comprehensive education and training measures, whose success has already been proven in numerous studies. Among others, Plaza et al. showed the effectiveness of repeated educational interventions including a written personalized action plan and training on inhaler techniques in improving control over asthma symptoms as well as future risk and quality of life [236]. Moreover, it was reported how an efficient symptom management training increases self-efficacy for children and adolescents [237]. Especially in younger children, particular attention has to be drawn to a thorough training of correct inhalation techniques involving encompassing education of the parents.

Although asthma was and is often regarded as being mainly a childhood disease, it is also a relevant origin of morbidity and mortality in the older generation leading to hospitalization, medical costs and most importantly a significant decrease in health-related quality of life, being an even greater burden for seniors. Recent findings suggest

that asthma in the elderly phenotypically differs from that in younger patients [238], showing the need for new diagnostic and therapeutic strategies in this population, especially considering the fact that life spans are rising and the proportion of individuals aged 65 and older is evermore increasing worldwide. The high occurrence of various comorbidities, aging-related lung and immune alterations as well as epigenetic factors can lead to complex interactions and a diverse pathophysiology. As most former studies were based on allergic or T_H2-mediated asthma, which is not a predominant characteristic of asthma in the elderly [239], and respective clinical studies often exclude older patients [240], the knowledge in this area is still incomplete. Examinations have to consider numerous age-related changes, e.g. decreasing respiratory mechanical properties, a shift in immune cells from naïve to memory lymphocytes, reduction of total serum immunoglobulin E (IgE), loss of lung volume and decline in FEV1 to name only a few. Regarding pharmacotherapy, special attention has to be paid to the higher sensitivity to side-effects of medications [241], the frequent polypharmacy and thereby increasing risk of drug interactions, as well as factors like misunderstanding of the disease and treatment regimen, poor compliance and memory problems [242, 243].

Furthermore, as aging is often associated with a sedentary everyday life and weight gain, recommendations for regular exercise and promotion of a healthy lifestyle are particularly advisable [238], however, this is not less true for all other age groups. A recent study evaluating the association of severity and control of asthma with factors like body mass index, insulin resistance, levels of adipokines and inflammatory markers in asthmatic children and adolescents found that asthma was associated with insulin resistance and a systemic inflammatory response possibly mediated by adipokines, with leptin levels standing out among the subjects with excess weight. [244]

Assessing the effect of exercise therapy on overweight women with chronic inflammatory diseases led to the observation that neutrophil counts can be reduced [245] and in asthma patients in particular, aerobic exercise training was proven to lower the number of eosinophils in induced sputum as well as the levels of the fraction of exhaled nitric oxide (FeNO) [246]. In children with persistent allergic asthma, a physical training program decreased their total and allergen-specific IgE levels [247]. The molecular background of this phenomenon was investigated in a study determining

the effect of aerobic exercise in an OVA asthma mouse model. It was shown that exercise is able to reverse the OVA-induced reduction of glucocorticoid receptor and consequently induce an increased expression of the anti-inflammatory cytokines IL-10 and IL-1ra, while inflammatory mediators like NF-κB and TGF-β as well as airway inflammation and remodeling were reduced [248]. Besides that, aerobic exercise was proven to show an anti-inflammatory effect in mice exposed to air pollution [249] and a single session of moderate aerobic exercise can down-regulate inflammatory mediators' genes expression and T_H2-derived cytokine production [250].

Nevertheless, it has to be minded that vigorous activity can also provoke asthma symptoms like cough, wheeze or dyspnoea. While sports with physical efforts of rather short durations and low ventilary levels have a small risk for the development of asthma symptoms, team sports in general can be regarded as medium-risk sports. Participating in swimming, endurance and winter sports, however, entails a higher risk for triggering exercise-induced asthma (EIA) and bronchoconstriction (EIB) due to the long duration of exertion and the low air temperature, respectively. Other factors influencing how beneficial or detrimental different sports can be for asthmatic patients are e.g. the humid air inhaled during swimming vs. chlorine-based irritants in the pool or the exposure to environmental pollutants and allergens like pollen and moulds in outdoor sports [251].

9. Recent Developments and New Treatment Options

Although in most patients asthma symptoms can nowadays effectively be managed by guideline-directed conventional medications, some disease forms are more severe and complex and therefore difficult to control. This may only apply to a relatively small group of the overall patient population, however, they account for more than 50% of asthma-related healthcare utilization [252] and are at increased risk of asthma-caused death [253], reasons enough to make them a top priority in respective research.

One approach to find new therapies is to modulate and improve currently successful drugs, for example by advancing delivery systems and prolonging their duration of action. Among others, indacaterol, a 24 hour ultra-long acting β agonist, was shown to

be safe and effective in clinical trials and dissociated corticosteroids, a new class of glucocorticoids still in preclinical development, are expected to maintain efficacy with reduced side effects [5]. However, there are also some completely new drug classes in development. Overall, the main interest currently concentrates on finding and targeting novel specific pathways in order to optimize treatments for individual patients.

9.1. Pharmacogenetics

More than 100 genes are considered to conduce to asthma manifestations, including primary disease conferring genes, asthma severity varying genes and treatment modifying genes. A well-investigated example that has been associated with a more severe form of asthma and a decreased response to beta agonists is the substitution of arginine with glycine at position 16 of the β2-adrenergic-receptor [254]. Recent genome-wide association studies combined with replication in additional cohorts and in vitro cell-based models identified novel pathway-related pharmacogenetic variations. As these have the potential to influence the efficacy of therapeutic measures, a more detailed understanding of the underlying genetic mechanisms may lead to the development of biomarkers to determine the most suitable therapy for individual patients. [255]

9.2. Asthma Phenotyping and Personalized Treatment Approaches

For a long time, asthma has been viewed as a single disease characterized by chronic airway inflammation and remodeling with anti-inflammatory therapy as the major approach to treatment. But more recently, the disease is recognized to be a multidimensional syndrome involving clinical, physiologic, and pathologic domains, which may coexist, but are not necessarily related. [253] As the understanding of the heterogeneity of asthma is increasing, more and more different phenotypes and endotypes are identified, incorporating observable clinical characteristics and specific biologic mechanisms in a more complex way. [254] A phenotype can be “defined as the composite observable characteristics or traits of an organism that result from genetic as well as environmental influences” [256]. These kinds of phenotypes including

dependence on high-dose corticosteroid treatment, severe airflow obstruction and recurrent exacerbations concomitant with an allergic background and late onset of disease have been revealed by analytical clustering methods, among others in the Severe Asthma Research Program (SARP) [257]. This investigation identified five clusters of patients differing in age, sex, age of onset, presence of atopy and/or obesity, degree of lung dysfunction, and reversibility of airflow obstruction. In another approach using sputum analysis, four phenotypes based on the predominant inflammatory cell type, such as eosinophilic or neutrophilic, were found [258]. In these groupings, the most evident differentiation was between patients with mild to moderate, early-onset asthma with eosinophilic or paucigranulocytic predominant sputum patterns and patients with more severe form of asthma and greater sputum neutrophilia. Furthermore, to comprise not only the clinical picture, but also find subtypes based on the underlying biologic mechanisms, different endotypes were defined, including biomarkers, lung physiology, genetics, histopathology, epidemiology and respective treatment response. For example, the aspirin-exacerbated respiratory disease endotype features leukotriene-related genetic polymorphisms leading to an upregulated leukotriene synthesis. As 5-lipoxygenase inhibitors block the synthesis pathway upstream, they are recognized as the superior treatment for this patient group. [259] The allergic bronchopulmonary mycosis endotype, in contrast, is associated with colonization of the airways by mold and might therefore benefit from antifungal agents. [254] Nevertheless, the major part of novel individual treatment approaches concentrates on biologic therapies.

9.3. Biologics

The most common grouping of asthma patients divides into allergic and non-allergic forms. While allergic asthma is present in all groups of asthma severity, with 50-80% it is of particular importance in patients with severe asthma [260], therefore finding new treatments for this population is an interesting area of research. Besides the well-known function of IgE, it is also assumed that several different cytokines and chemokines play a crucial role in the pathogenesis of the disease. A number of these have already been identified as suitable targets for therapy, demonstrating the potential

of biological drugs such as monoclonal antibodies and small-molecule inhibitors, especially as reasonable add-on treatments for those patients whose severe asthma forms do not respond to conventional therapies in a satisfactory way. While the anti-inflammatory effect of corticosteroids interferes with several pathways that are involved in asthma pathogenesis, cytokine-based therapy usually only targets a restricted cascade. As experimental anti-cytokine therapies have also been shown to induce variable responses in individual patients, the need to accurately characterize the patient's phenotypic pattern becomes even more evident. A certain biological drug addressing the particular molecular targets relevant for each subgroup has to be found in order to achieve the best-possible outcome for individual patients. [24]

As the first biologic in asthma treatment, omalizumab (Xolair®, Novartis) has been approved in the United States in 2003. The murine monoclonal antibody (mAb) is produced by the somatic cells hybridization method and contains a paratope able to bind to high and low affinity IgE receptors on basophils and mast cells, inhibiting both degranulation and activation of respective cellular mediators. Although several clinical trials have already proven the effectiveness of omalizumab with a significant decline in asthma exacerbations, improvement of quality of life and steroid-soaring effects, it still has some limitations. To overcome those, new mAbs are currently under investigation showing a greater avidity for IgE, such as RG7449 that targets B-lymphocytes before they are activated to produce IgE. [261]

After more than a decade without any new appearances on the market, the anti-interleukin 5 (IL-5) humanized mAb mepolizumab (Nucala®) by GlaxoSmithKline was approved in the end of 2015 as an add-on treatment for patients with severe asthma and eosinophilic inflammation. IL-5 was identified as a useful target as it promotes eosinophil growth and activation and two additional mAbs, reslizumab (Cinqair®) and benralizumab are already in the pipeline. While Nucala and Cinqair both bind IL-5 directly to hinder it from tacking to its receptors on eosinophils, benralizumab targets the receptor α subunit in order to mediate the death of eosinophils by enhanced antibody-dependent cell-mediated cytotoxicity. As IL-5 is not the only cytokine promoting eosinophil growth and survival, this active cell depletion approach can potentially be even more efficient. [262]

Besides that, several different biological targets are currently explored in asthma research. In addition to the IL-5 mAbs, atopic patients with T_H2-driven eosinophilic asthma could also benefit from inhibition of IL-4 and IL13. A blockade of IL-17 that also contributes to steroid resistance, instead, would most likely be useful for patients with severe neutrophilic asthma. Combinations of biologics targeting different types of cytokines could be applied for the mixed neutrophilic-eosinophilic phenotypes, a group represented quite frequently among the exacerbation-prone form of severe asthma. Another promising approach is the neutralization of the effects of innate cytokines IL-25, IL-33 and thymic stromal lymphopoeitin (TSLP), all of which play a crucial role in the initial priming of T_H2-mediated airway inflammation. This strategy could have the potential to disconnect the link between adaptive and innate immune responses which might be responsible for the development of severe subtypes of asthma that are difficult to treat. [24]

The two major hurdles yet to overcome on the way to more efficient and easily accessible biological therapies are the lack of reliable biomarkers to characterize specific phenotypes and predict medication responsiveness and the need to design reasonable clinical trials to evaluate the long-term safety of these immunomodulatory agents. Furthermore, the cost factor is not to be underestimated. As Nucala, for example, has a wholesale acquisition cost of 2500$ per single-use vial [262], it remains a demanding task to define which patients really need expensive new biologics and which are able to cope with their symptoms by using standard therapies.

Chapter V

Clinical Development of Nanomedicines for Asthma

Please note that the following chapter was published in the book *Nanomedicine for Inflammatory Diseases (Taylor & Francis Ltd.):*

Yuran Xie, Rima Kandil, Olivia M. Merkel: Bridging the Gap between the Bench and the Clinic:Asthma. Nanomedicine for Inflammatory Diseases, 2017, CRC Press, pp.255-286.

CHAPTER V - Clinical Development of Nanomedicines for Asthma

Nanomedicine for Inflammatory Disease - Bridging the Gap between the Bench and the Clinic: Asthma

The authors of this book chapter include Yuran Xie, Olivia M. Merkel and me. Yuran Xie and I are the joined first authors of the respective publications, we performed the literature search and wrote the article.

1. Background

Asthma is a disease characterized by the chronic inflammation of respiratory airways. Asthma remains a public health problem, particularly in developing countries [263]. A large global market for asthma drugs [264] fuels research and development by pharmaceutical companies to develop new therapies to treat asthma. A stepwise approach for controlling asthma symptoms is recommended by the Global Initiative for Asthma (GINA). For this stepwise approach inhaled rescue medications (e.g. short acting β_2-adrenoceptor agonist (SABA)) are prescribed and taken as needed to relieve asthma symptoms in all asthmatic patients, and inhaled corticosteroids (ICS) with or without long acting β_2-adrenoceptor agonist (LABA) are recommended for patients with moderate persistent asthma. Additionally oral corticosteroids (OCS) and anti-IgE (omalizumab) may be applied in patients who have severe asthma [265]. Although current treatment strategies can control asthma symptoms in most patients, there is still a need to develop alternative therapies for asthma primarily because of concerns of low patients compliance [266]. Patient compliance remains low for asthma therapies primarily because of the necessary frequent dosing (inhalation) and a fear for undesirable side effects. Nonadherence results in the overuse of rescue medications and

a high frequency of uncontrolled asthma symptoms. Moreover, 5-10 % of the patients whose asthma symptoms remain uncontrolled, despite the maintenance therapies, have a higher risk of exacerbation and hospitalization due to asthma [267].

Extensive research has been conducted to understand the detailed pathologic mechanisms of asthma and to develop new therapies for asthma. Numerous drugs have entered clinical trials but failed to demonstrate satisfactory therapeutic efficacy and safety. Nanomedicine may be a way to solve the poor safety and efficacy problems encountered by previously tested new drug candidates. Nanomedicine was defined by the Medical Standing Committee of the Europe Science Foundation on 2004 as "the science and technology of diagnosing, treating, and preventing disease and traumatic injury, of relieving pain, and of preserving and improving human health, using molecular tools and molecular knowledge of the human body" [268]. The advantages of nanomedicine include increased solubility, sustained release, and enhanced specificity of drug treatments. For example, a doxorubicin encapsulating liposome (Doxil®), the first nanodrug approved by FDA in 1995 to treat cancers, demonstrates increased circulation time, consequently enhanced amounts of drug in cancer cells and less side effects compared with free doxorubicin [269]. Nanomedicine is also suitable for the delivery of biopharmaceutics [270]. Despite the promise of nanomedicine for the development of new asthma treatments, no asthma treatments utilizing nanotechnology have undergone clinical trials or have been brought to market, so far. Here, we will first discuss several new asthma drugs (currently in clinical trials or on the market) and how the development process of these drugs may be utilized in developing a nanomedicine for asthma therapies. Then, we will elaborate on how studies need to be planned for inhaled nanomedicines, and what the regulatory hurdles are, before we will give an outlook (**Figure 1**).

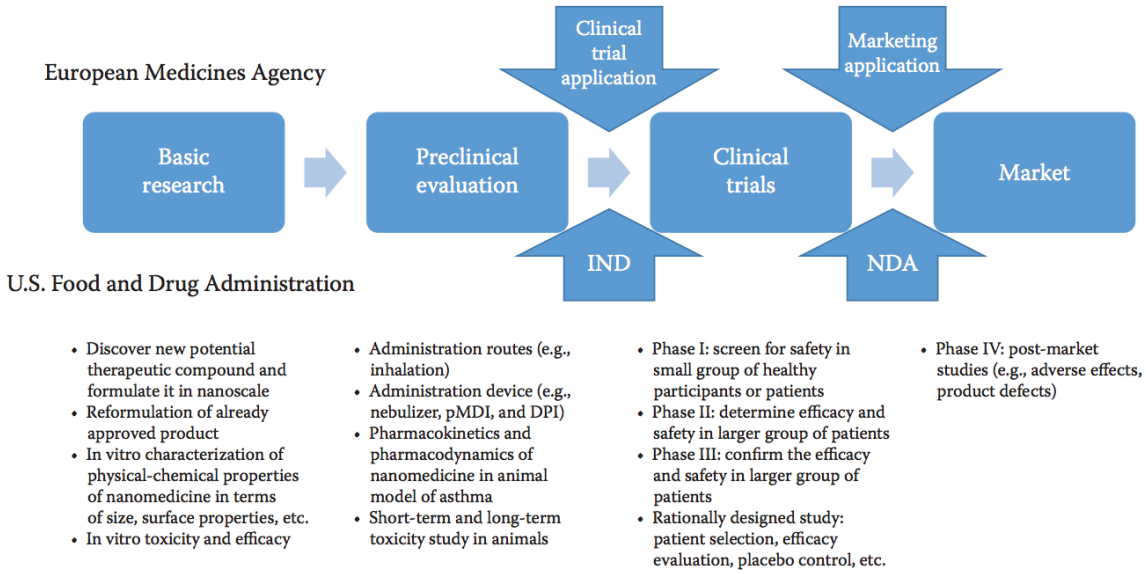


Figure 1. Process of developing new nanomedicine from laboratory to market.

2. Drugs for Asthma

Drugs available for asthma therapy can be categorized into small molecule drugs and biopharmaceutics. The majority of asthma drugs are small molecules formulated for either oral administration or inhaled administration. Theophylline, leukotriene receptor antagonists (e.g. montelukast), cromones, and corticosteroid are usually formulated for oral delivery. Alternatively, corticosteroids, SABA, LABA, anticholinergic and long-acting muscarinic antagonists (LAMA) are preferably formulated for pulmonary delivery. Currently, there are only two biopharmaceutical treatments for asthma on the market, both of which are monoclonal antibodies, formulated for subcutaneous (s.c.) administration. The first antibody, omalizumab, targets a high-affinity receptor binding site on IgE and is approved for the treatment of patients with severe persistent allergic asthma. The other antibody, mepolizumab, is raised against the antigen IL-5, and was approved by the FDA in 2015 to treat patients with severe asthma.

2.1. Small Molecules

Most small molecules developed for the treatment of asthma are formulated for either oral or pulmonary delivery. New drugs for anti-leukotriene therapies have demonstrated oral activity, such as a dual antagonist of cysteinyl-leukotriene receptor 1 ($CysLT_1$) and CysLT2 (Gemilukast) and a 5-lipoxygenase-activating protein inhibitor (GSK2190915). Furthermore, chemokine receptor antagonists (e.g. GW766944), phosphodiesterase (PDE) inhibitors (e.g. Revamilast), and mast cell inhibitors (e.g. Masitinib) are also administrated orally in clinical trials.

Another typical example of orally administrated drugs is the group of antagonists of chemoattractant receptor- homologous molecule expressed on T helper 2 (Th2) cells (CRTH2), also called D prostanoid receptor 2 (DP_2) [271]. Selective antagonists of CRTH2 alleviate allergic inflammation by inhibiting the activation and recruitment of pro-inflammatory cells such as Th2 cells, eosinophils, and basophils [272]. At least 13 different CRTH2 antagonists have been brought to clinical trials for the treatment of asthma, allergic rhinitis, and COPD [271]. Most of the new CRTH2 antagonists are delivered orally. OC000459 is a promising CRTH2 antagonist which is currently studied in clinical trials. In preclinical studies, the ability of OC000459 to displace prostaglandin D_2 (PGD_2) from human DP_2 was first evaluated using recombinant DP_2 expressed on the membrane of CHO cells and native DP_2 expressed on Th2 cells. Both of these studies demonstrated the high potency of OC000459 for DP_2 (K(i) = 0.013 μM and K(i) = 0.004 μM, respectively). OC000459 had minimal inhibitory activity when assessed using a library of 69 non-related receptors and 19 enzymes, suggesting OC000459 was highly specific for DP_2. OC000459 treatment inhibited PGD_2 induced chemotaxis of Th2 cells (IC_{50} = 0.028 μM) and IL-13 production of Th2 cells (IC_{50}= 0.019 μM) as well as competitively antagonized eosinophil shape change in response to PGD_2 (pK_B = 7.9). Pharmacokinetics of OC000459 oral administration has been investigated in rats, and the plasma concentration of OC000459 at $t_{1/2}$ was found to be much higher than its in vitro IC_{50} evaluated on cells. Additionally, the distribution volume (V_d) was estimated to be 0.5 l/kg, indicating high oral activity of OC000459. The anti-inflammatory effect of OC000459 was evaluated in rats, and oral administration of drug inhibited blood eosinophilia induced by systemic administration of 13, 14- dihydro-15-keto-PGD_2 (DK-

PGD_2) in a dose dependent manner. Furthermore, in guinea pigs, OC000459 treatment inhibited airway eosinophilia induced by inhalation of aerosolized DK-PGD_2 [273].

Promising preclinical results have led to the clinical investigation of OC000459 for the treatment of asthma. To determine the efficacy of OC000459, in a phase II clinical trial, patients with mild persistent asthma received 200 mg of OC000459 orally or placebo twice daily for 28 days and the lung function was assessed by measuring the forced expiratory volume in 1 second (FEV_1). Results revealed that treatment with OC000459, and not placebo, increased FEV_1 while also reducing serum IgE and sputum eosinophil count. Therefore, it was concluded that OC000459 is pharmacologically active in asthma patients [274]. To investigate if OC000459 can reduce lung inflammation in response to an allergic stimulus, another clinical trial was conducted in corticosteroid naïve asthmatic patients. It was reported that treatment with 200 mg OC000459 twice daily for 16 days reduced allergen induced later asthmatic response (LAR) determined by calculations of the area under the curve (AUC) of FEV_1. Furthermore, this treatment regimen inhibited the induction of sputum eosinophils 1 day following allergen challenge when compared with placebo. Together, OC000459 was shown to inhibit allergic asthma-related inflammation when administered orally to human patients [275]. To determine the optimal dose of OC000459 for the treatment of asthma, patients with mild to moderate persistent asthma were treated with three doses of OC000459 (25 mg or 200 mg once daily, or 100 mg twice daily) for 12 weeks. Results reflected that the lung function, as determined by change in FEV_1, was improved in all patients who received OC000459 when compared to placebo. Therefore, the lowest dose of 0C000459 tested (25 mg daily) was sufficient to provide therapeutic efficacy. To investigate whether sub-populations would respond to treatment differently, lung function data from asthmatic patients with eosinophilia (blood eosinophils count ≥ 250/µl) and without eosinophilia (blood eosinophils count < 250/µl) were compared. Patients with eosinophilia showed significant improvement of lung function with OC000459 treatment when compared to placebo. Conversely, lung function was not improved in patients without eosinophilia following OC000459 treatment, suggesting OC000459 was more effective for the treatment of eosinophilic asthma [276]. More clinical trials are ongoing to explore the application of OC000459 in severe eosinophilic asthma or other diseases such as eosinophilic esophagitis, atopic dermatitis, and allergic

rhinitis. The safety of OC000459 is also under investigation in healthy participants because of a potential interaction between OC000459 and cytochrome P450 3A4.

Numerous small molecule asthma drugs have been formulated for pulmonary delivery because inhalation of the drug has the advantage of rapid delivery directly to the effected tissue (i.e. the lung). Small molecule drugs formulated for inhalation in clinical trials include LABAs (e.g. GW642444), long-acting muscarinic antagonists (e.g. Seebri), non-steroidal selective glucocorticoid receptor agonists (e.g. AZD7594), PDE inhibitors (e.g. RPL554), and very late antigen (VLA)-4 inhibitors (e.g. GW559090X).

RPL554, a dual inhibitor of PDE3& PDE4, is administrated trough inhalation to avoid adverse gastrointestinal side effects reported for oral formulations of PDE4 inhibitors [277]. In preclinical studies, RPL554 was shown to potently inhibit the activity of isolated human PDE3 and PDE4 (IC_{50} = 0.4 nM and IC_{50} = 1479 nM, respectively). Inhibition of PDE3 was thought to mediate human airway smooth muscle relaxation, and both an in vitro model and an isolated guinea pig tracheal tissue were used to investigate the ability of RPL554 inhibit the activity of PDE3. RPL554 can relax the reproducible contractile of tracheal smooth muscle elicited by electrical field stimulation in a concentration dependent manner. The ability to inhibit stimulation of immune cells of RPL554 was evaluated in human primary cells. It was shown that RPL554 can inhibit the release of tumor necrosis factor alpha (TNFα) from lipopolysaccharide (LPS) activated human mononuclear cells (IC_{50} = 0.52 μM) and inhibit the proliferation of mononuclear cells stimulated by phytohemagglutinin (IC_{50} = 0.46 μM). In guinea pigs, inhalation of RPL554 dry powder (3-5 mg, blended with lactose) can reduce the bronchoconstriction and airway edema in response to i.v. administration of histamine as well as decrease the infiltration of eosinophils to the airway of ovalbumin-sensitized asthmatic animals, indicating the feasibility to deliver RPL554 through inhalation for anti-inflammatory therapies [278].

Based on the efficient activity of bronchodilation and anti-inflammation, RPL554 was evaluated in clinical trials for asthma therapy. The safety of a single administration of different doses of RPL554 (0.003, 0.009, 0.018 mg/kg) was evaluated in both healthy and asthmatic participants. Inhalation of RPL554 was well tolerated at all doses tested based on adverse event reports, vital signs, and electrocardiograph (ECG) data. RPL554

treatment can improve lung function of asthmatic patients, especially at high dose, as determined by more than 1.5 fold of the concentration of methacholine needed to induce a 20% decrease in FEV_1 compared with the original dose. To further evaluate the efficacy as a bronchodilator and the safety of RPL554, asthmatic patients received multiple administrations of inhaled RPL554 at a dose of 0.018 mg/kg, for 6 days. FEV_1 was determined for 6 h post-inhalation of RPL554 or placebo on days 1, 3, and 6. Results showed that RPL554 improved FEV_1 when compared to placebo. In another clinical trial, the anti-inflammatory effect of RPL554 was investigated in healthy volunteers challenged by inhaled LPS. Participants received a daily dose of RPL554 of 0.018mg/kg for 6 days before LPS challenge, and their sputum was collected 6 h and 24 h post- challenge. Results showed significantly less macrophages, lymphocytes, neutrophils and eosinophils in the sputum from participants treated with RPL554 when compared with the placebo-treated group. Therefore, inhalation of RPL554 was reported to inhibit the inflammatory response. [279]. Currently, RPL554 is investigated in a clinical trial to compare its efficacy to an active comparator, salbutamol, in asthmatic patients.

Small molecular drugs for asthma are rarely administrated intravenously. However, the novel β_2-adrenoceptor agonist, MN-221, was evaluated in clinical trials as an adjunct to standard therapy in patients experiencing an acute exacerbation of asthma. Phase I and II studies demonstrated that MN-221 (dose: 5.25- 1125 μg; rate: 0.35- 60 μg/min) was well tolerated in patients with mild-to-moderate or moderate-to-severe asthma, according to the reported adverse events, laboratory tests, vital signs, and ECG. Moreover, lung function was improved, as determined by FEV_1 measurements, in a dose dependent manner in patients treated with MN-221 [280]. Another clinical trial was conducted to evaluate the efficacy of MN-221 in patients with an acute exacerbation of asthma. In this trial, patients admitted to the emergency room received the standard treatment for acute asthma exacerbation, and those who did not response to standard therapy (FEV_1 was less than 50% of predicted) received an i.v. infusion of MN-221 (1200 μg) or placebo, followed by measurements of FEV_1 for 3 h. MN-221 treatment in addition to standard therapy failed to significantly improve the lung function determined by $AUC_{0\text{-}3h}$ of $\%FEV_1$ when compared to standard treatment [281]. Despite the apparent lack of improvement with MN-221 treatment, it is possible that these

patients were resistant to any asthma treatment, because these patients also did not respond well to standard treatment. To better determine the efficacy of MN-221, future clinical trials should include a more heterogeneous population, to avoid a disproportionate number of treatment resistant patients.
In conclusion, small molecules can be effective in several formulations and routes of administration.

2.2. Biopharmaceutics

Asthma is a complicated disease involving numerous inflammatory cells and cellular mediators. Biopharmaceutical drugs can be used to intervene specific events, for example, blockage of interaction between cells or between receptors and cytokines/chemokines and inhibition of expression of key pathological factors (e.g. cytokines, receptors, transcription factors). Numerous biopharmaceutical drugs are currently in preclinical and clinical development for the treatment of asthma. There are two major kinds of biopharmaceutical drugs which have been in clinical trials for the treatment of asthma: 1) protein based drugs, such as monoclonal antibodies and cytokines, and 2) nucleic acid based drugs including DNAzymes, anti-sense oligonucleotides and small interference RNA (siRNA).

Protein based drugs, specifically monoclonal antibodies, are particularly promising treatments for asthma. Monoclonal antibodies have been successfully brought to market for the treatment of various diseases. Monoclonal antibodies can be raised against specific antigens (i.e. drug targets) and administered through i.v. or s.c. injection. Monoclonal antibodies have the benefit of being highly specific for a particular drug target, and with little off-target binding. Currently, there is large number of monoclonal antibodies under clinical investigation. Their targets include 1) cytokines such as IL-4 (e.g. pascolizumab), IL-5 (e.g. mepolizumab), IL-9 (e.g., MEDI-528), IL-13 (e.g. QAX576, CAT-354), and IL-17A (e.g. secukinumab); 2) receptors such as IL-13 receptor α1 and α2 (e.g. GSK679586), IL-4 receptor α (e.g. AMG 317), and IL-5 receptor α (e.g. MEDI-563); and 3) other cellular elements such as IgE (e.g. MEDI4212) and OX40L (e.g. huMAb OX40L).

Mepolizumab is a humanized monoclonal antibody raised against IL-5. Mepolizumab can bind to IL-5 inhibiting the interaction between IL-5 and its receptor [282]. Because IL-5 regulates the activation and survival of eosinophils, a proinflammatory cell type involved in asthma, mepolizumab could serve as a potential therapy for asthma. Mepolizumab has been evaluated both pre-clinically and in clinical trials. Mepolizumab was determined to have high binding affinity for purified human IL-5 (K_d of 4.2 pM) as determined by surface plasmon resonance (Biacore) and titration microcalorimetry. In human TF-1.28 cells, a human erythroleukemia cell line, mepolizumab treatment can inhibit IL-5 dependent cellular proliferation with an IC_{50} value of < 150 pM. Mepolizumab binding is highly specific for the human IL-5 protein and does not bind to IL-5 derived from mouse, rat, guinea pig or dog [283]. Therefore, pharmacokinetic and pharmacodynamic studies of mepolizumab were conducted in healthy cynomolgus monkeys. The mean terminal half-life of mepolizumab after i.v. administration (13.0± 2.2 days) and s.c. administration (14.5± 3.8 days) were similar and relatively long, indicating mepolizumab was bioavailable following s.c. route. A single dose (1 mg/kg) or repeated doses (10 mg/ml) of s.c. administration of mepolizumab both can reduce the number of peripheral eosinophils in the monkeys and the IC_{50} of mepolizumab was only 1-2 μg/ml, suggesting it could be a valuable drug candidate for diseases associated with eosinophilia, such as asthma. [284]. To characterize the pharmacologic activity and long-term safety profile, mepolizumab was evaluated in a monkey allergy model or healthy monkeys. In monkeys naturally sensitized to *Ascaris suum* (*A. suum*), a single dose of mepolizumab (i.v., 10mg/kg) inhibited the infiltration of eosinophils to the airways and blood eosinophilia induced by inhaled *A. suum* antigen. Healthy monkeys were treated (i.v.) twice with mepolizumab at different doses (0.05, 0.5 or 50 mg/kg) on days 1 and 29. Mepolizumab mediated a dose-dependent reduction of peripheral blood eosinophils and inhibition of blood eosinophilia induced by s.c. injection of rhIL-2 on day 30. Repeated i.v. administration (10 or 100 mg/kg) or s.c. (10 mg/kg) of mepolizumab to monkeys monthly for 6 months was conducted to investigate long term toxicity and pharmacodynamics. All doses were well tolerated and no adverse side effects were observed. More importantly, all doses effectively decreased circulating eosinophils but not significantly affected immature or mature eosinophils in bone marrow. Therefore, mepolizumab treatment appears to be a safe and effective treatment for the inhibition of eosinophil-mediated inflammation [285].

Several clinical trials of mepolizumab have been conducted. The efficacy and safety of single i.v. administration of mepolizumab (2.5 mg/kg and 10 mg/kg) were tested in patients with mild allergic asthma. Mepolizumab treatment can inhibit the increased blood eosinophils and sputum eosinophils count induced by inhaled allergen. However, mepolizumab did not significantly change airway response to inhaled histamine as determined by histamine PC_{20} (mg/ml) nor did it attenuate early asthmatic response (EAR) and LAR. These results indicated that eosinophils may not play a significant role for airway hyper-responsiveness (AHR) and LAR but be more relevant to the pathogenesis of asthma. [286]. Larger scale clinical studies on the safety and efficacy of mepolizumab were conducted and provide more information about the role of eosinophils in asthma pathogenesis. In a placebo controlled clinical trial, asthma patients with persistent symptoms despite inhaled corticosteroid therapy received three i.v. infusions of mepolizumab (250 and 750 mg) at 4 weeks interval. Results showed that mepolizumab treatment was well tolerated and significantly reduced eosinophils count in sputum and blood. Similar to the previous clinical study, mepolizumab did not improve lung function as determined by changed morning peak expiratory flow (PEF) and FEV_1 when compared to placebo, indicating that mepolizumab treatment may not be effective to alleviate asthma symptoms and other clinical outcome measures. However, patients that received high dose mepolizumab treatment (750 mg) had a 50% lower exacerbation rate (although not statistically significant) when compared to placebo, suggesting a potential benefit of mepolizumab treatment in preventing exacerbation [287]. Because mepolizumab may be most effective in reducing the exacerbation rate of asthmatics, two clinical trials were conducted with patients displaying eosinophilia to determine the efficacy of mepolizumab to control exacerbation. In one of the clinical trial, patients, who had refractory eosinophilic asthma and a history of recurrent severe exacerbation, received i.v. infusions of 750 mg mepolizumab every month for 12 consecutive months [288]. In the other clinical trial, patients that had persistent sputum eosinophilia despite prednisone treatment received i.v. infusions of 750 mg mepolizumab every four weeks for 24 weeks [289]. Both studies confirmed that mepolizumab treatment significantly reduced the exacerbation rate in asthmatic patients with eosinophilia when compared to placebo, without significantly improving lung function. To optimize the administration routes and determine an efficient dose of mepolizumab, a phase III, a

placebo controlled clinical trial studied mepolizumab as adjunctive therapy in patients with severe asthma was conducted. The efficacy of s.c. administration of 100 mg mepolizumab was compared with i.v. administration of 75 mg mepolizumab for 32 weeks at a 4-week interval. Results showed that mepolizumab treatment reduced the annualized frequency of clinically significant exacerbations when compared to placebo. More importantly, s.c. administration achieved slightly better efficacy compared with i.v. administration. The mepolizumab treatment group showed a trend of increased lung function, as determined by FEV_1 (although not statistically significant) and an increased quality of life according to the score of Asthma Control Questionnaire (ACQ) and St. George's Respiratory Questionnaire (SGRQ) [290]. Based on the outcome from these clinical trials, in 2015, the s.c. administration of 100 mg mepolizumab was approved by the FDA for the treatment of severe asthma.

Another class of biopharmaceutical drugs in clinical development are nucleic acids [291]. Specific nucleic acid based therapies include DNA vaccines to induce immune tolerance [292], antisense oligonucleotides [293], small interfering RNA (siRNA) [293, 294] and deoxyribozymes (DNAzyme) for the selective silencing of asthma related genes. For example, SB010 is a 10-23 DNAzyme that targets (i.e. cleaves) GATA-3, possessing a catalytic domain and two GATA-3 mRNA specific binding sites. GATA-3 is a key factor in asthma pathogenesis and it is important for Th2 cells differentiation, the secretion of Th2 cytokines, and the production of IgE and airway recruitment of eosinophils. In preclinical evaluation, in a mouse model of acute asthma, intranasally (i.n.) applied 200 μg SB010 (hgd40), which is more active in humans, significantly reduced infiltration of leukocytes while the scrambled DNAzyme was inactive. In the same study, administration (i.n.) of another GATA-3 DNAzyme Gd21, which is more active in mice, to asthmatic mice inhibited the secretion of IL-5 and infiltration of leukocytes and alleviated AHR and airway remodeling (e.g. goblet cells hyperplasia). These results indicate the therapeutic potential of GATA-3 DNAzymes in asthma [295]. Potential off-target effects of SB010 in immune cells were investigated in TLR-9-transfected HEK293 cells, macrophage cell lines, and primary innate immune cells. Results showed that SB010 did not induce non-specific innate cell stimulation, such as activation of neutrophils and degranulation of mast cells and basophils, suggesting that SB010 could be used as a safe treatment for the treatment of allergic asthma [296]. In

animal studies, potential toxicity of SB010 inhalation was evaluated in healthy rats and dogs because both rats and dogs share the same mRNA sequence to human GATA-3 which SB010 binds to. Results showed that prolonged exposure (28 days) to inhaled SB010 in dogs (14.6, 40.8 and 115.5 mg/m^3 and the corresponding theoretical doses were 103.3, 250.4 and 739 μg SB010/kg body weight (BW)) and in wistar rats at different doses (14, 38 and 113 mg/m^3 and the corresponding theoretical doses were 112 and 302 μg SB010/kg BW) did not produce signs of systemic toxicity as measured by clinical chemistry, urinalysis, as well as gross pathology and histological analysis of the lung. Although minimal histopathological changes, including interstitial leukocytes infiltration, bronchus-associated lymphoid tissue hyperplasia, and compound related lesions in the lung, were observed at high dose of SB010 (888.8 μg SB010/kg BW) in rats, these changes only occurred at the highest dose and were completely recovered after withdrawing SB10. Furthermore, long term exposure in rats didn't change the splenic cell population (e.g. T helper cells, NK cells) or serum levels of IgG, IgA and IgE antibodies and there was no abnormal expression of cytokines in the BALF except a small increase of IL-10 and IFN-. Dogs received i.v. infusion of SB10 (10 μg/kg) for 10 min and ECG and blood pressure were monitor. The results suggested that SB10 treatment didn't cause cardiac abnormalities. Together, these toxicity results suggested that SB10 treatment was well tolerated in dogs and rats and did not cause significant histopathological changes (e.g. respiratory, cardiac) [297]. Biodistribution studies of inhaled SB010 were conducted in mice with OVA induced airway inflammation, healthy rats and dogs. Fluorescently labeled 200 μg SB010 was retained in the lungs of asthmatic mice up to 24 h after administration (i.n.) as observed by confocal microscopy. Long term pharmacokinetics of intratracheal (i.t.) instillation of ^{111}Indium labeled SB010 was monitored by a single-photon emission computerized tomography (SPECT) in asthmatic and healthy mice. Results showed that ^{111}Indium labeled SB010 rapidly accumulated in the lung and bladder and was detectable in the lungs of asthmatic mice for up to 150 h. In comparison, less ^{111}Indium labeled SB010 accumulation was observed in the lungs of healthy mice. Pharmacokinetics of inhaled different doses of SB010 were evaluated in rats (38.3 and 113.2 mg/m^3 and the corresponding theoretical doses were 303 and 889 μg SB010/kg BW) and dogs (40 and 115 mg/m^3 and the corresponding theoretical doses were 250 and 750 μg SB010/kg BW). The SB10 exposure lasted for 28 days, serum samples were collected on different

post-exposure time points, and the concentration of SB10 was measured by ELISA assay. SB010 was detectable shortly after application in both species indicating high systemic availability, while no plasma accumulation observed after multiple administration. These favorable pharmacokinetics characteristics support that inhalation of SB10 could be a safe and efficient administration route and also provides guidance for future administration dose and regimen [298]. In human clinical trials, the safety, tolerability and pharmacokinetics of inhaled SB010 was firstly evaluated in healthy participants. Inhalation of a single dose of SB10 (0.4, 2, 5, 10, 20 and 40 mg) and multiple-doses (5, 10 and 20 mg) for 12 days didn't cause serious adverse events. In the single dose treatment, the drug plasma concentration was below the detection limit in patients receiving doses in the range from 0.4 to 5 mg. In contrast, SB10 was detected in plasma after inhalation of 20 and 40 mg SB10 and reach the maximal concentration (C_{max}) at 0.5-2 h after administration. Next, the safety and pharmacokinetics of single dose of inhalation of SB10 (5, 10 and 20 mg) was investigated in asthmatic patients. The asthmatic patients overall had higher AUC and C_{max} at given doses compared with healthy participants since their airway epithelium may be damaged [299]. Since SB10 treatment was safe and well tolerated, its pharmacologic activity was further evaluated in asthmatic patients. Patients with mild asthma received 10 mg SB10 or placebo via inhalation for 28 days and an allergen challenge after the 28 days treatment. Results showed that SB010 treatment improved the lung function and attenuated allergen induced EAR and LAR as measured by FEV_1. SB010 treatment also resulted in reduced eosinophils in the sputum and lower IL-5 plasma levels when compared to controls, indicating that SB010 alleviated Th2-driven inflammatory responses and may consequently improve lung function [300]. Despite promising results, clinical trials assessing the long-term safety and efficacy of SB010 still need to be conducted in a larger number of asthmatic patients to determine the usefulness of S010 for the treatment of asthma.

3. Nanomedicine for Asthma

As described above, numerous drugs for the treatment of asthma are currently available or in clinic development, however, none of them can be considered a nanomedicine. The use of nanomedicine for the treatment of asthma is the subject of numerous pre-clinical

studies. Nanomedicine holds the promise of providing a superior drug formulation that may enhance drug delivery. Specifically, for asthma therapy, nanomedicine should focus on refining the formulation of potential small molecules and biopharmaceutical drugs for oral administration, inhalation, and subcutaneous injection.

Oral delivery of a drug is most desirable as it can greatly increase patient compliance, especially in chronic diseases which require frequent administration, such as asthma. Nanomedicine allows for small molecule drugs to be encapsulated in a polymer enhancing the drugs solubility and stability in the GI tract, ultimately increasing bioavailability [301]. Only a few orally administered anti-inflammatory drugs are being developed using nanomedicine. For example, a nanoemulsion of vitamin D demonstrated increased bioavailability and attenuated inflammatory response in OVA-induced asthmatic mice when administered orally [302]. A nanoemulsion of curcurmin was evaluated in transgenic mice which a firefly luciferase reporter gene driven by NFκB responsive elements, and may serve as a potential therapy for inflammatory diseases through suppression of NFκB signaling pathway and macrophage migration [303]. However, results reported in these two studies such as decreased production of IL-1β and TNF-α [302] and the reduction of peripheral macrophages [303] are insufficient to prove their clinical potential for asthma therapy. Additional investigations are needed to assess therapeutic effects in asthmatic animal models such as the inhibition of the production of asthma related cytokines (e.g. IL-13, IL-5) in plasma or BALF, improved lung function, and reduced infiltration of leukocytes in BALF. Furthermore, the stability of the loaded drug and nanocarrier in the GI tract and potential systemic toxicity induced by drugs (e.g. β_2-adrenoceptor agonists and glucocorticoids) or nanocarriers also need to be considered. Regarding the peroral delivery of biologics in nanomedicines, we can learn from other examples where efforts have been made to develop an oral formulation. Polyester (poly(-ε-caprolactone) and a polycationic non-biodegradable acrylic polymer were used to encapsulate insulin for oral administrated to diabetic rats. Decreased glycemia was achieved by insulin nanoparticles indicating the potential of using polymer as oral administration carriers for proteins [304]. Further evaluation need to be performed to apply biopharmaceutical drugs orally for asthma therapy. Particularly, the bioavailability of drugs and the efficacy when compared with traditional administration such as s.c. injections need to

be evaluated. In conclusion, oral formulation of nanomedicine still requires several steps of development to reach the clinic.

Subcutaneous injections or intravenous infusions are the preferred means of administration of monoclonal antibodies in asthma therapy because they are degraded in the GI and generally do not readily cross biological barriers such as cell membranes. Compared with i.v. infusion, s.c. injection is a preferred route of administration for monoclonal antibodies because it is less invasive, less time consuming, more convenient, and could be self-administrated at home [305]. Currently, there are several nanomedicines under development for s.c. administration, however there are none for the treatment of asthma. Poly (lactic-co-glycolic acid) (PLGA) [306], cyclodextrins [307] and a liposomal polymeric gel [308] have been used to encapsulate proteins for s.c. or i.v. administration. To evaluate the potential of these formulations for applications in asthma therapy, more studies have to be undertaken including the assessment of efficacy in asthmatic animal model and pharmacokinetics compared with drug only. Since there is only a limited amount of s.c. formulations which are in development, nanomedicines delivered through the s.c. route will most probably not enter the clinic any time soon.

The most common route of administration for nanomedicines in pre-clinical development for asthma therapy is inhalation. Inhalation is a non-invasive route and therefore is thought to have better patient compliance. Additionally, pulmonary inhalation has the advantage of delivering the drug directly to the diseased organ, of delivering the drug to a large absorption surface area, and the advantage of generally lower dose requirements. So this route can consequently increase bioavailability and reduce the possibility of systemic toxicity. Small molecule drugs can be encapsulated in polymers [309-311] or liposomes [312] to achieve increased solubility and sustained release after pulmonary administration. For example, a commercially available corticosteroid, budesonide has been encapsulated in porous poly(lactic-co-glycolic acid) (PLGA), a biodegradable polymer approved by the FDA for its use in the clinic, and was evaluated in a murine asthma model. Encapsulated budesonide provided sustained release for 24 h as observed in vitro. In the murine model, an aerosol of encapsulated budesonide significantly decreased the infiltration of inflammatory cells in BALF, reduced airway thickness, and improved lung function when compared to an aerosol of

free budesonide [309]. To achieve a long lasting effect, a β_2- adrenoceptor agonist, salbutamol sulfate, has also been formulated using nanomedicine. Specifically the drug was incorporated into artificial lipid vesicles, termed liposomes. When administered (i.t.) to healthy rats, the aerosol of salbutamol sulfate loaded liposomes increased the half-life of salbutamol when compared to free drug. Additionally, larger AUC of salbutamol sulfate in the lung and plasma were observed compared with free salbutamol sulfate, indicating that liposome encapsulation achieved sustained release of the drug. Salbutamol sulfate loaded liposomes increased the time of bronchodilation in an asthmatic guinea pig model when compared to free drug, confirming that the liposome suspension could provide a longer lasting therapeutic effect [312].

Currently, a limited number of biopharmaceutical drugs have been formulated using nanomedicine for the treatment of asthma. One example is IFN-γ-plasmid DNA which was encapsulated in chitosan and administered (i.n.) to asthmatic mice. Chitosan encapsulated IFN-γ-plasmid DNA attenuated AHR, and reduced the infiltration of eosinophils. Additionally, the levels of IL-4 and IL-5 in BALF as well as the infiltration of leukocytes in the airway were decreased [313]. However, pulmonary delivery of biopharmaceutical drugs such as siRNA and proteins has been extensively studied for diseases other than asthma. For example, inhalation of nebulized insulin loaded-liposomes can continuously reduce blood glucose up to 6 h, while inhalation of nebulized insulin with empty liposomes decreased blood glucose temporarily which, however, bumped back 2 h post inhalation [314]. Polyethylenimine (PEI) [315] and chitosan [316] are most commonly used for pulmonary delivery of siRNA.

Pulmonary delivery requires that inhalation devices are used including 1) pressurized metered dose inhalers (pMDIs) 2) nebulizers 3) and dry powder inhalers (DPIs) [317]. A pMDI is a widely used and inexpensive device, however, many therapeutics do not readily dissolve in the hydrofluoroalkane (HFA) propellants compatible with pMDIs. To administer nanomedicines using a pMDI, the properties of the nanomedicine including physical stability in the pMDI formulation (e.g. aggregation) need to be carefully evaluated. As an example, Conti et al. reported in 2014 that poly amidoamine dendrimer (PAMAM)/siRNA nanoparticles efficiently knocked down enhanced green fluorescent protein (eGFP) in eGFP expressing A549 cells. Similar knockdown efficiency was achieved even following a 2-month incubation of PAMAM/siRNA in HFA, indicating that

the siRNA nanoparticles were sufficiently stable in HFA. For this formulation, the spray dried nanoparticles were encapsulated in mannitol or chitosan-lactic-acid, dispersed in HFA, and no large/irreversible aggregation was observed within 5 h, suggesting the nanoparticles were stable in this pMDI formulation. The pMDI formulation was characterized by an eight-stage Andersen Cascade Impactor. Results showed that the aerosol contained a respirable fraction and fine particle fraction of approximately 77% and 50%, respectively, similar to commercially available pMDI's. Taken together, the authors concluded that the nanoparticles demonstrated high potential to be used in a clinical setting as a pMDI [318].

A nebulizer is an inhalation device that requires less coordination of the patient, compared with pMDIs and DPIs, and therefore it is suitable for pediatric, elderly, and unconscious patients. Nebulization is suitable for nanomedicines that are water soluble. However, special consideration needs to be taken such as the stability of the drugs during the production of the aerosol. There are three different nebulizers available on the market including jet, ultrasonic, and mesh nebulizers. If the nanomedicine payload is temperature sensitive, as in case of proteins and DNA, nebulizers need to be chosen carefully because of heat generated inside the medication reservoir of vibrating mesh [319] or ultrasonic nebulizers [317]. For example, plasmid DNA (15-20 kb) is easily degraded during aerosol production because of the shear effects [320]. The degradation of plasmid DNA can be prevented by both PEI [321-323] and liposomes [324]. Another consideration when formulating a nanomedicine for nebulizers is the large air-liquid interface created during nebulization, which may induce protein unfolding and aggregation [325]. Many nanomedicine formulations tend to aggregate during nebulization [326, 327] due to the concentration of nanoparticles in jet and ultrasonic nebulizers [327]. For example, Ewe et al. reported in 2014 that lipopolyplexes formulated from liposome-PEI loaded with pDNA or siRNA can mediate efficient transfection in SKOV-3 and in a luciferase expressing SKOV-3 cell line (SKOV-3-LUC). The size of pDNA and siRNA lipopolyplexes after nebulization was generally larger than prior to nebulization, suggesting that aggregation occurred. However, the apparent aggregation did not decrease the transfection efficiency of lipopolyplexes. In fact, the transfection efficiency of pDNA lipopolyplexes right after nebulization or when stored at 4°C for 1 day after nebulization was enhanced compared with to the corresponding

pDNA lipopolyplexes which were not nebulized. This effect can be understood as the result of accelerated sedimentation of larger particles in cell culture. In vivo, however, the transfection efficacy is yet to be assessed. The transfection efficiency of siRNA lipopolyplexes was also retained after nebulization or following storage suggesting that pDNA and siRNA were protected in the formulation during nebulization [328].

DPIs are the most popular inhalation devices since they are portable, and no propellant is used. The dry powder formulation demonstrates better chemical stability than the liquid formulation [317]. Several key considerations should be made when formulating nanomedicines for DPIs including the stability of the drug and nanocarrier during the production of the dry powder. Spray drying is a common method used to produce inhalable dry powders of drugs formulated with polymers (e.g. PLGA [329, 330], chitosan [331, 332], and polymer-lipid [333]). However, during the spray drying process, droplets of the formulation are rapidly dried by a hot gas, and therefore the use of thermolabile drugs or thermo-sensitive polymers is limited. Spray freeze drying [334] or freeze drying followed by milling [334] may be a better choice for heat sensitive drugs, particularly proteins. Aqueous solubility, yield, and size change of reconstituted nanoparticles must also be carefully assessed. Specifically for the lung, deposition of inhaled dry powder highly depends on its size with particles of 1-5 μm needed to achieve deep lung deposition. It is also important to assess the nanomedicine particle size once it is reconstituted in an aqueous environment (e.g. lung tissue). Once delivered to the aqueous environment of the target tissue, the nanomedicine must maintain small size to penetrate through the mucus layer and be taken up by cells. Spray dried and spray freeze dried formulations of drugs (e.g. levofloxacin) loaded into polymer (e.g. PLGA) or lipid-polymer (lecithin-PLGA) have been characterized for pulmonary drug delivery. Spray freeze dried formulations of nanomedicines achieve better aerosol properties including approximately 26% fine particle fraction and 5.8 μm mass median aerodynamic diameters compared with spray dried formulations. To ensure the desired particle size in the aerosol requires optimization of the formulation methodology by carefully controlling the ratio of lipid, polymer, and excipients. However, following optimization, the production yield was relatively low, 33% (w/w) making further optimization necessary. In addition, reconstitution of nanoparticles

from both formulations in an aqueous environment showed increased particle sizes, suggesting aggregation occurred [335].

4. Animal Models of Asthma

Animal models are required to determine the pharmacokinetics, therapeutic effect, and biocompatibility of new asthma therapies. Specifically, asthma is a heterogeneous disease involving numerous types of immune cells and cellular mediators, rendering in vitro models of limited use. The European federation of pharmaceutical industries and associations (EFPIA) [336] and the US Food and Drug Administration (FDA) [337] agree that the combination of both new technologies and animal models is required for drug discovery. Several animal models of asthma are currently used including mice, rats, guinea pigs, dogs and sheep. Below we will review the applicability of these animal models for the development of treatments of asthma.

4.1. Rodent Asthma Models

4.1.1. Mouse

Mouse asthma models are the most commonly used to study potential asthma therapies. Mouse models of asthma have several advantages including cost effectiveness, availability of several transgenic strains, and a large number of commercially available mouse specific probes and tools. Because mice do not naturally develop asthma, allergens including ovalbumin (OVA) and house dust mite (HDM) extract have been used to artificially induce airway inflammation. In both models, mice are first sensitized by several injections (i.p.) of an allergen with or without adjuvant. Following sensitization (2-5 weeks later), mice are exposed to the inhaled allergen which produces an inflammation response and AHR. For example, to test the therapeutic effects of GATA-3 specific DNAzyme, SB010, of which phase II clinical trials have been completed [300], Sel et al. established a murine asthma model with the allergen OVA and adjuvant $Al(OH)_3$ which can preferentially induce a Th2 biased response. Female BALB/c mice were sensitized by i.p. injection of 10 μg OVA/1.5 mg $Al(OH)_3$ suspended in 200 μl PBS solution on day 0 and day 14. The first challenge of 3 consecutive days

starting on day 24, sensitized mice which inhaled aerosolized 1% (wt/v) OVA dissolved in PBS for 20 min daily. Before and during the second 3-day challenge, starting on day 36, animals received a 4 days i.n. treatment of GATA-3 DNAzyme (Days 35-38). The efficacy of DNAzyme was assessed on days 39 and 40. Treatment with GATA-3 DNAzyme relieved AHR, decreased mucus hypersecretion, and reduced the influx of eosinophils [295]. This short term (4-6 weeks) mouse model has been widely used and demonstrates high reproducibility. However, asthma in humans is a chronic airway inflammatory disease, and many patients show airway remodeling such as goblet cells hyperplasia, fibroblasts of smooth muscles, and long term AHR which is absent in this acute allergic inflammation model. To address this, a model of chronic airway inflammation has been developed in mice. This chronic model is similar to the acute mouse asthma model with the exception that the challenge phase is extended. In the same study, Sel et al. established a chronic murine asthma model to further investigate the efficacy of GATA-3 DNAzyme. Mice were sensitized by three i.p injection of OVA/$Al(OH)_3$ on day 0, day 14 and day 21. Sensitized mice were challenged on 2 consecutive days each week for a total 14 weeks (5^{th}- 15^{th} week and 19^{th}- 21^{st} week). This chronic asthma model more closely approximated human asthma indicated by eosinophil influx, AHR, goblet cells hyperplasia, mucus hypersecretion and subepithelial collagen deposition [295]. However, there are still some limitations to this chronic mouse asthma model. The inhalation of allergen causes an early-asthmatic response (EAR) in patients due to constriction of smooth airway muscles [338]. In some patients, 3-4 h after EAR, a second phase of decreased lung function, called LAR, often associated with AHR, may occur due to released cytokines and recruited eosinophils in EAR [338]. Mice can develop AHR to non-specific bronchoconstriction agents such as methacholine like humans, but there is a lack of evidence to prove EAR or LAR in mice after inhalation of the allergen.

4.1.2. Rat

Rat asthma models are also commonly used in asthma studies. The rat models of asthma, like the mouse models, are cheap and have many commercially available biological probes. In the rat asthma models, AHR and LAR can be induced by controlled exposure to allergens. Therefore, the rat models are better than the mouse models to

study the effects of new therapies on EAR and LAR. Rats can also be sensitized by OVA [339-341] and HDM extracts [342]. To establish the asthma models, rats are often sensitized by systemic injection of allergen with one ($Al(OH)_3$) or two adjuvants ($Al(OH)_3$ & heat-killed *Bordetella pertussis* bacilli) followed by challenge of inhaled allergen aerosol or intratracheal (i.t) instillation of allergen. A rat asthma model was established for testing the preclinical profile of ciclesonide. To this extent, Brown Norway (BN) rats, a strain of rat commonly used for allergic models, were sensitized by i.p. injection of suspension of 100 μg OVA/100 mg $Al(OH)_3$ on days 0, 12 and 21 and challenged with inhalation of 1% OVA for 30 min daily for 4 consecutive days on days 27-30. Meanwhile, ciclesonide and control drug fluticasone were administrated via i.t. instillation 1 h and 24 h before each challenge. Rats were sacrificed 24 h after final challenge and the efficacy of ciclesonide to inhibit eosinophilia in bronchoalveolar lavage fluid (BALF) and lung tissue, a sign of airway inflammation, was assessed [339]. A rat chronic asthma model has been reported, in which BN rats were sensitized by i.p. injection of 1 mg OVA/200 μg $Al(OH)_3$ on day 0. Sensitized rats were challenged with 1% OVA aerosol three times each week for 12 weeks starting from day 14. Clear airway structure changes were observed including goblet cell hyperplasia, sub-epithelial deposition of collagen and fibronectin, however, pro-longed exposure of allergen lead to reduced airway wall thickness and loss of AHR [343]. Therefore, further optimization of rat chronic asthma models is necessary.

4.1.3. Guinea Pig

As an asthma model, guinea pigs are less popular than mice and rats. However, guinea pig asthma models demonstrated well defined EAR and LAR after allergen challenge [344]. The pharmacology and anatomy in the guinea pig model is more similar to the conditions in humans as compared to rats and mice and can be used as a model for certain subtypes of asthma such as the cough variant asthma [345]. An acute guinea pig model of asthma has been reported where Dunkin-Hartley guinea-pigs were sensitized by i.p. injection of 100 μg OVA/100 mg $Al(OH)_3$ on days 1 and 5 and challenged by inhalation of 0.01% OVA for 1 h on day 15. In the same study, a chronic asthma guinea pig model was established, where the sensitization and first challenge were the same as in the acute model, but the animals received 8 additional challenges with inhalation of

0.1% OVA aerosol once every two days. To prevent fatal anaphylaxis, all additional challenges but the last one was under mepyramine cover (30 mg/kg, i.p). Fluticasone propionate, roflumilast and GW274150, an inducible nitric oxide synthase (iNOS) inhibitor have been tested in clinical trials for patients with mild asthma, and were pre-clinically tested in both aforementioned guinea pig models. The drugs were administrated three times at 24 h and 30 min before and 6 h after the final challenge either by aerosol (Fluticasone, 0.51 mg/ml) or orally (roflumilast, 1 mg/kg; GW274150, 5 mg/kg as the phosphate). EAR, LAR, AHR, and influx of inflammatory cells were observed in both models. There was no airway remodeling observed in the acute model compared with the saline challenged group, but in the chronic model such airway remodeling was observed including increased thickness of airway walls, bronchiolar collagen or hyperplasia of goblet cells [346]. GW274150 was effective in the acute model, however, not in the chronic model which agreed with the results of its phase I clinical trial [347]. Therefore, the authors proposed that this chronic model could be a better animal model for asthma to predict the results in humans.

4.2. Larger Animal Models

Lager animal asthma models have been developed in dogs, sheep and monkeys. They are not as popular as rodent models because the costs are much higher. However, it is necessary to test therapeutic reagents in larger animal models since they are more physiologically relevant for human.

4.2.1. Dog

Dog asthma models have been used for decades to study physiological and pathological mechanism of asthma as well as the pharmacological response of new therapeutic reagents. There are three ways to establish dog asthma models: allergen challenge, hyperventilation-induced and ozone-induced asthma. Dogs naturally sensitized to *Ascaris suum* [348] can be challenged by inhalation of an A. *suum* extract aerosol. Asthmatic response can be induced in dogs that are neonatally sensitized to ragweed by the inhaled antigen. Becker et al. described a ragweed sensitized dog model of asthma where new-born Basenji- Greyhound dogs were immunized by i.p. injection of a

suspension of 500 μg short ragweed and 30 mg $Al(OH)_3$ within 24 h of birth. Injection was repeated weekly for 8 weeks and biweekly for another 8 weeks. In this model, after inhalation of short ragweed (antigen E content 120 U/ml), all sensitized dogs demonstrated EAR and some individual developed LAR. Furthermore, AHR in sensitized dogs was determined by non-allergic airway response to inhaled acetylcholine [349]. The efficacy of GS-5759, a phosphodiesterase 4 (PDE4) inhibitor with long-acting β_2-adrenoceptor activity, was compared to indacaterol in the ragweed sensitized dog asthma model. Different doses of micronized powder of GS-5759 and indacaterol were insufflated into the lung of dogs one hour before ragweed challenge and the change of lung resistance was monitored for the next 30 min. GS-5759 demonstrated better ability to decrease the allergen induced pulmonary resistance compared with indacaterol, which is in line with the results in different animal asthma models including guinea pig, monkey and rat [350]. Ozone induced bronchoconstriction, airway inflammation and AHR in dogs was used to study the therapeutic effect of MK-0591, an antagonist of 5-lipoxygenase-activating protein (FLAP), namely the inhibitor of biosynthesis of leukotrienes (LT). MK-0591 (2mg/kg) was administrated intravenously to dogs and drug blood concentration was maintained by infusion of 8 μg/kg*min of MK-0591 throughout the whole experiment. Ozone was delivered through the endotracheal tube at a concentration of 3 ppm for 30 min to establish airway inflammation. MK-0591 treatment remarkably inhibited the production of LTB_4 in blood and in BALF cells as well as the production of LTE_4 in urine. However, there was no effect on ozone induced AHR, bronchoconstriction and influx of neutrophils in the airway [351]. This result in the dog asthma model in fact predicts the result in a preclinical evaluation of MK-0591 in patients with mild asthma. Orally administration of MK-0591 for three times in asthmatic patients before allergen challenge can inhibit LTB_4 biosynthesis in blood and urinary LTE_4 excretion. Furthermore, EAR and LAR determined by change of FEV_1 were alleviated by MK-0591 treatment but not AHR measured by inhalation of histamine [352]. Reversible bronchoconstriction in patients with asthma can be induced by exercise and inhaled cold air, and this process can be mimicked by the hyperventilation induced dog asthma model. Anesthetized dogs were intubated endotracheally and ventilated mechanically with room temperature dry air with 5% CO_2 at 200 ml/min. Flow rate was increased to 2000 ml/min for 2 min and bronchoconstriction, AHR and late phase airway obstruction can be observed [353, 354].

4.2.2. Sheep

Sheep are cheaper to purchase, maintain and handle compared with dogs and non-human primates. Furthermore, there are many similarities between the lung of sheep and humans, and sheep can be naturally sensitized by *Ascaris suum*. These characteristics make sheep asthma models the most popular large animal models for asthma. As an example, Abraham et al. selected sheep that were naturally sensitized to *A. suum* and can develop EAR and LAR upon inhalation of *A. suum* to establish experimental asthma. Sheep were challenged by inhaled *A. suum* extract aerosol (82,000 protein nitrogen unit/ ml) at 20 breaths/ min for 20 min. EAR and LAR were determined by specific lung resistance (SR_L), AHR existed for 9 days after antigen-challenge and an increased influx of inflammatory cells was observed in BALF and bronchial biopsies. The anti-inflammation effect of multiple doses, a single dose and different concentrations and formulations of BIO-1211, a small molecule inhibitor of integrin $\alpha_4\beta_1$ also known as very late antigen-4 (VLA-4), were tested in this model [355]. An experimental sheep asthma model where the animals were sensitized and challenged by HDM, a relevant human allergen, was developed by Bischof et al. [356]. Sheep were immunized subcutaneously with a 1 ml suspension of 50 μg HDM mixed with 50 μg $Al(OH)_3$ on days 0, 14 and 28. On day 42, tracheal instillation of 5 ml HDM solution (0.2 mg/ ml) was guided by a flexible fiber-optic bronchoscope deep into the left caudal lung lobe. For comparison, saline was instilled into right caudal lung. HDM specific antibodies IgE, IgG_1 and IgG_2 were increased on 7 days post-third immunization and increased eosinophil cell counts were observed in peripheral blood and BALF 48 h after HDM challenge in allergic sheep. In addition, infiltration of lymphocytes and eosinophil into the peribronchial region was shown in haematoxylin and eosin (H&E) stained lung tissue [356]. A sheep model of chronic asthma was developed based on this protocol by the same group in which sheep were challenged twice a week for the first 3 months and once weekly for another 3 months. Airway remodeling, including hyperplasia of goblet cells and increased collagen deposition and thickness of smooth muscle, were observed, however, the total time frame of approximately 7 month to establish this model is very long and time consuming [357]. Van der Velden et al. investigated the effect of senicapoc (ICA-17073), an inhibitor of the $K_{Ca}3.1$ ion-channel expressed widely in various cells involved in asthma, in a modified sheep model of

chronic asthma. Sheep were sensitized with HDM as described by Bischof et al. [356]. Two weeks after the final immunization, sheep were challenged with nebulized HDM solution (1 mg/ml) at 20 breaths/min for 10 min once every two weeks for 14 weeks. Allergic sheep received orally 30 mg/kg senicapoc twice daily from 7 days before the first challenge and throughout the 14-weeks challenge phase. The lung resistance R_L in allergic sheep was increased, EAR was observed but not LAR. However, increased collagen production and airway smooth muscles remodeling were not shown in this model, but hyperplasia of goblet cells and increased density of blood vessels in the airway wall were observed. Senicapoc treatment maintained normal R_L, reduced the EAR in allergic sheep, and inhibited the increased density of blood vessels. However, there was no significant difference in the influx of eosinophils or hyperplasia of goblet cells between the senicapoc and vehicle treated groups [358]. This result is in line with the result of a phase II clinical trial for allergic asthma. Senicapoc treatment reduced the allergen induced increased airway resistance and exhaled nitric oxide, an inflammatory marker, however, it did not achieve improved lung function [359]. An HDM challenge protocol to establish a sheep asthma model is more standardized compared with the *A. suum* challenged protocol. The HDM challenge sheep model of chronic asthma could be a relatively accurate model to predict clinical results, but further optimization is still needed to balance the time and development of physiological asthma hallmarks such as airway remodeling.

5. Study Planning

5.1. Nanomedicine Specific Considerations

A constitutive sector to examine when developing a new nanotherapy is the influence of the medication on the condition of the human body. Besides possible side effects that nanomedicines can induce, it is also important to monitor any changes they can provoke, e.g. in pH or temperature, as those might decrease the valuable therapeutic effects by altering the drug characteristics. As the success of nanotherapy in asthmatic lungs hinges on several factors, such as administration route and characteristics of the particles, but also physiological aspects of the diseased organ, it is crucial to have an encompassing understanding of the respective anatomy, molecular biology and cell

physiology. In order to observe the distribution of nanomedicines within the intended target organ as well as other parts of the body, different imaging techniques can be applied. Previous investigations focusing on nanoparticles as contrast enhancers [360], intracellular trafficking of nanocarriers [361], real-time monitoring of pharmacokinetics and biodistribution [76], and high throughput pharmacokinetics screening [362] have pointed out capabilities for high quality and precise data collection. [363]

5.1.1 Toxicity

Although the utilization of nano-scaled medications entails numerous advantages, including new material properties, an increased surface capability due to enlarged surface-volume ratio, a shorter transport time, the potential of selective targeting and the minimized exposure of healthy tissue to the incorporated drug [364], the miniaturization of systems always involves the danger of arousing toxicity. To determine a safe dose within the therapeutic window as well as the lethal dose of drug-loaded nanoparticles, it is essential to perform toxicological testing in vitro, ex vivo and in vivo in cell lines, tissue and animal models before starting clinical trials with humans. Although it is not possible to convey the data gained from those experiments directly to the conditions of an actual patient, it is crucial to reduce the risk of toxic effects and possible adverse reaction as much as possible beforehand.

Several assays to ascertain the toxicity of nano-based drug formulations have already been established and can employ different cellular targets, like mitochondria, lysosomal activity, cell membrane integrity or DNA ladder assays to determine cell death mechanisms. The standard testing method for cell viability after treatment with nanomaterials in a great range of cell lines is the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Other tests resting upon the principle of the metabolic activity of mitochondria involve tetrazolium salts or resazurin. To improve statistical validity and minimize the error chance, it is recommended to combine multiple experiments. For example, the simultaneous performance of MTT and resazurin assay can be rational, as they both utilize a similar principle of testing. [365] Studies revolving around the specific disease instances of asthmatic lungs should take into consideration the correspondent conditions, pre-

eminently airway hyperresponsiveness, mucus hypersecretion and the influx of inflammatory cells as well as their cytokines. [294] In order to detect possible immune-related and inflammatory responses, variations in activation levels of cytokines, e.g. TNF-α, interleukins and prostaglandin should be monitored.

Particular materials have to be chosen to reduce toxic effects contingent upon the desired target region. While polyamidoamine (PAMAM), for example, indeed exhibited favorable characteristics as nanocarriers in several studies, it was shown to foster acute lung injury by inducing autophagic cell death via the Akt-TSC2-mTOR signaling pathway [366]. Card et al. reviewed different imaging, diagnostic and therapeutic applications of engineered nanoparticles in the lung and identified whole groups of nanomaterials that can have negative repercussions on the pulmonary structure and function. According to their findings, nonbiodegradable substances such as carbon nanotubes, carbon black, fullerenes, silica, metals and metal oxides can generate inflammation and/or fibrosis in the lung after inhalation, intranasal or oropharyngeal aspiration as well as systemic administration [367]. Biodegradable nanoparticles, e.g. made of PEG-PLA (polyethylene glycol-polylactide) [363] or PLGA (poly(lactic-co-glycolic acid)) [368], on the contrary, have been proven to be useful pulmonary drug carriers.

Different studies in humans have been performed to investigate the deposition of inhaled nanoparticles in healthy and diseased lungs [369, 370]. An increased pulmonary deposition and retention in constricted airways was predicted by computational models [371] and demonstrated in obstructive lung disease [372] and asthma [373] patients. Regarding the latter, the exposure of subjects with mild to moderate asthma with ultrafine carbon particles during spontaneous breathing led to an increased fraction of deposited particles compared to healthy individuals. Pietropaoli et al., nevertheless, did not ascertain any differences in respiratory parameters between healthy and asthmatic subjects after inhalation of respective particles. No airway inflammation was observed in either group, but an exposure of healthy individuals to a higher concentration of particles resulted in a decreased midexpiratory flow rate and carbon monoxide diffusing capacity, indicating that nanoparticles may influence respiratory function and gas exchange [374]. Moreover, several investigations have been conducted to test the translocation of nanoparticles from the lung to the systemic circulation after inhalation, as cardiovascular effects similar to the impact of urban air pollution were apprehended.

Most findings indicate that the tested 99mtechneticum-labeled carbon nanoparticles are not detected outside the lungs in appreciable concentrations [370, 375, 376]. Nevertheless, it remains uncertain whether other nanoparticles behave similarly, and the possibility of particles influencing the vasculature is still not excluded. Besides that, all studies used single inhalation exposure protocols so that further investigations on the repercussions of repeated exposure, stronger pulmonary accumulation and, therefore, translocation of greater particle quantities are urgently needed. [367]

In a recent study, it was examined whether intratracheal instillation studies can be used for evaluating any harmful effects of inhaled nanoparticles. Therefore, rats were exposed to nanoparticles composed of Nickel oxide and titanium dioxide as high and low toxicity examples. Among others, increases in neutrophils in BALF (bronchoalveolar lavage fluid) and concentration of cytokine-induced neutrophil chemoattractants were compared after single intratracheal installations and inhalations over 4 weeks, and results suggest that intratracheal studies can be a useful tool in ranking adverse influences of nanoparticles. [377]

5.1.2. In vivo Pharmacokinetics, Administration and Metabolism

The lung and its large surface area with a high vascularization as well as a thin air-blood-barrier, on the one hand, displays an ideal location for the absorption of agents [31]. On the other hand, several physicochemical and biological barriers await the nanotherapeutics in the pulmonary system, making it essential to thoroughly track their routes and deposition in the body. **Figure 2** illustrates the different defense mechanisms nanoscale particles have to encounter in the lung.

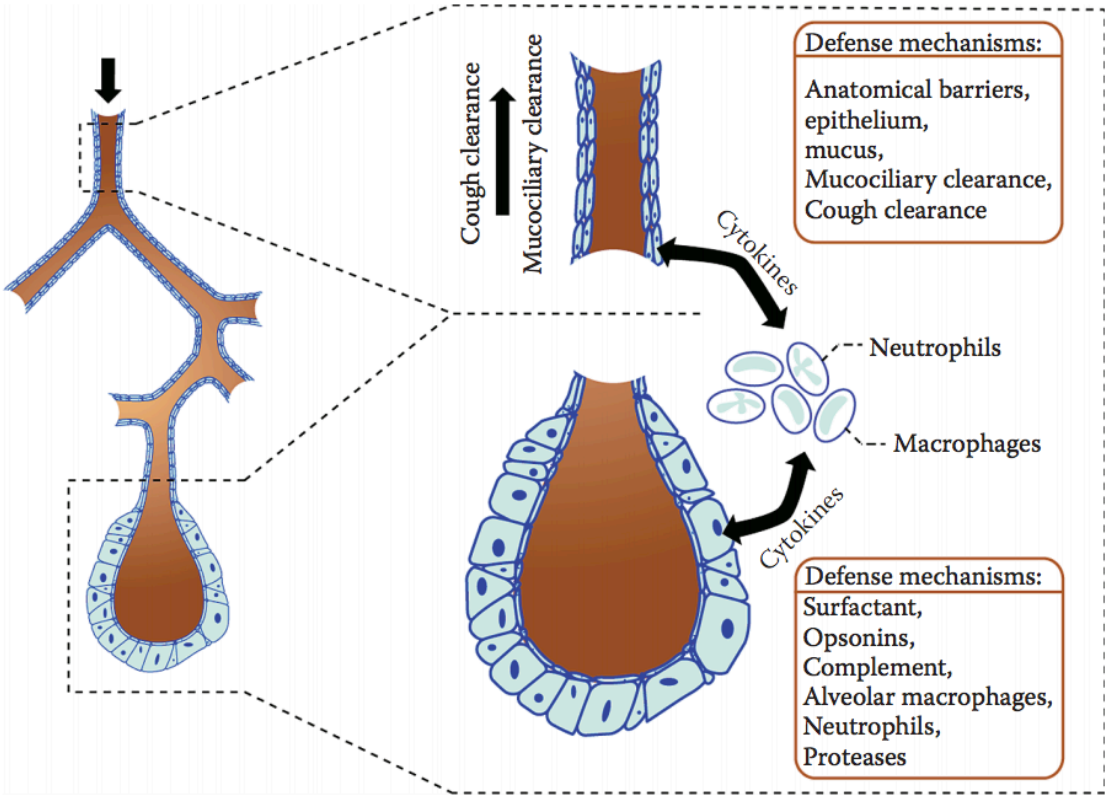


Figure 2. Lung-intrinsic barriers to efficient pulmonary siRNA delivery. Reprinted with permission from [20].

The most important parameter influencing the deposition of particles in the different areas of the lung is their size. Depending on that, three different mechanisms of allocation are possible: impaction, sedimentation and Brownian diffusion. Particles with a mass median aerodynamic diameter (MMAD) greater than 5 μm pass through the oropharynx and upper respiratory passage with a higher pace, collide with the respiratory wall due to the centrifugal force, and are deposited in the mouth and pharyngeal regions [20]. This so-called impaction usually occurs with dry powder inhalation (DPI) and metered dose inhalators (MDI). Deposition of drugs formulated as DPIs is especially dependent on the inspiratory effort of the patient: an insufficient force of inhalation leads to aggradations of the particles in the upper airways. Nevertheless, large and aggregated particles can also become subject to this process when MDIs are used, despite the higher speed of the generated aerosol. As gravitational forces

preponderantly condition the sedimentation process, particles with an MMAD between 1-5 μm are slowly deposited in the smaller airways and bronchioles, whereas Brownian motion is the prevalent mechanism in the lower alveolar sections. The molecules surrounding the aqueous lung surfactant underlie the Brownian motion itself and induce a random moving of the particles. The dissolution of the therapeutic agents in the lung surfactant, depending on the concentration gradient, influences this process as well. Particles with a size smaller than 1 μm deposit in the alveolar region or can be exhaled. Therefore, sedimentation is the preferably achieved process for therapeutic nanosystems in order for them to stay in the bronchiolar area for a long time and to result in the desired effects. In addition to these particle and target surface depending characteristics, breathing patterns, the holding of breaths and tidal volume, but also air velocity and humidity are factors influencing the deposition and hence need to be considered. [378]

The inside of the upper airways is covered with a film of mucus that is responsible for trapping and purging invading particles. Before those can reach lower sections of the lung through coughing or swallowing, they are often cleared by mucociliary movements. Consequentially, nano-sized drugs should be able to cross the mucus layer and reach the sol that covers the stratum below the gel coat. [379] As PEG nanoparticles were repeatedly shown to be capable of permeating across mucus [380-382], PEGylation is a possible approach to avoid bronchial clearance of nanomedicines. In the alveolar regions, the alveolar lining consisting of various proteins and lipids. Additionally, the existing tight junctions hamper the transport of molecules. According to the structure of the nanomedicine, active transport or passive diffusion through those transporter proteins are possible. Larger particles are, furthermore, prone to be cleared through phagocytosis by alveolar macrophages. [383]

Once arrived inside the peripheral lung, the particles have to dissolve, and the incorporated drug has to diffuse through the epithelial barrier in order to reach the blood. There are still some deficiencies in the exact understanding of the process of cell-uptake and how the particles are transported and reach the systemic circulation. Despite the existence of in vitro models for studying the uptake and permeation, the precise behavior of the cells under disease conditions has yet to be examined further. [365]

5.2. Disease-Specific Considerations: Asthma

Since asthma is a disease presenting itself in a very heterogeneous range of manifestations, it is of particular importance to define criteria both for categorizing the severity of the existing condition as well as for evaluating the success of any treatments. According to the current GINA (Global Initiative for Asthma) Report, 'asthma severity' should be "assessed retrospectively from the level of treatment required to control symptoms and exacerbations" [265]. This severity is not necessarily a constant feature, but can change over time, so that it can only be evaluated after a patient has already been experiencing a controlling treatment for several months. Subsequently, the patient's status can be rated as mild, moderate or severe asthma. The latter is thereby important to be distinguished from uncontrolled asthma due to incorrect inhaler technique or poor adherence. [384]

Corresponding to this prior definition, the main goal in asthma treatment is to maintain 'asthma control', which is described as "the extent to which the various manifestations of asthma have been reduced or removed by treatment" [265]. Among the current clinical situation of the patient, and therefore factors such as symptoms, lung function, the use of reliever medication and night awakenings, this concept additionally comprises the patient's future risk of exacerbations and decline in lung function as well as treatment related side effects. According to the GINA Guidelines, asthma can be regarded as controlled when daytime symptoms occur only twice per week or less, daily activities are not limited, the lung function is normal or near normal, there are no exacerbations and no symptoms at night-time and the reliever medications only needs to be used twice per week or less. The obtained control level through therapy determines the necessity to go up or down to the adjacent treatment level in the stepped management approach for asthma therapy that both the respective US and European guidelines recommend. Patients whose disease symptoms can not be controlled in an adequate way by the highest stage of treatment, which means a reliever medication and two or more controller treatments, are considered to suffer from *difficult-to-treat* asthma. [384]

5.2.1. Patient Selection

When choosing patients for clinical studies, it is important to use uniform clinical guidelines to diagnose and pre-define the disease status based on clinical symptoms and disease history of the patient as well as on assessment of airflow limitation. To examine this factor, the preferred approach is to perform spirometry under standardized conditions. In this way, both the FEV1 (forced expiratory volume in one second) and the FCV (forced vital capacity) can be measured to conclude on the patient's existing airflow limitation and its variability. In case this data is difficult to generate, e.g. because the patient is treated with a controller medication, the PEF (Peak expiratory flow) can also be ascertained. A determination of airway hyperresponsiveness can be of use for patients who show clinical symptoms, but possess a normal lung function. [384]

For patients who already receive a treatment against asthma, it is especially important to assess and establish the minimum level of treatment necessary to maintain control over their symptoms and standardize this treatment as much as possible in order to ensure an appropriate baseline to interpret subsequent results. An accurate and detailed profile of every patient has to be compiled, including characterization of attributes such as lung function, day- or night-time symptoms, and previous history of exacerbations, as well as a documentation of all co-morbidities and concomitant treatments. Concerning the treatment of asthma in co-morbid patients, particular attention has to be drawn to Chronic obstructive pulmonary disease (COPD), as these two conditions indeed have different pathways of causation, but can still coexist in one patient. Therefore, patients who suffer from COPD can distort respective results and should not be included in asthma studies. As opposed to this, smokers are able to take part in these, although their smoking history has to be accurately recorded and it is advisable to conduct a subgroup analysis to discover any influence the smoking could have on study results. Other possible classifications for relevant sub-populations would be frequency of exacerbations, sensitivity to NSAID, eosinophilia or co-sensitivity to other allergens. When investigating a specific immunotherapy, a documentation and characterization of the patients' allergy history and inflammatory airway profile is required. Moreover, a crucial point to consider before starting any clinical asthma study is the equalization of clinical procedures. All patients have to be trained in an adequate way to use their drug administration devices such as inhalers as well as equipment for

lung function testing and, where applicable, diary cards or other relevant evaluation tools. [384]

The high incidence of asthma in children leads to them being a target group of special interest in the respective research. For them, as well as for the older generation, particular attention has to be drawn to the adequate utilization of inhalation devices. Studies in children should be conducted in the early development process, when the therapy holds promise to be a major advance for the pediatric population, of course implying that appropriate safety data are available. More detailed advice regarding studies for these special patient groups can be found in the respective ICH (International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use) Notes for Guidance (Geriatrics: ICH Topic E7 [385], Pediatrics: CPMP/ICH/2711/99 [386]). [384]

5.2.2. Efficacy Evaluation

To appraise the success of the examined treatment forms, there are several measurable parameters available for asthma studies.

For characterizing the effect of therapies on the **lung function**, the previously mentioned FEV1 and PEF are suitable to detect and evaluate any airway obstruction. The most accurate factor represents the pre-bronchodilator FEV1, being influenced even by short-term fluctuations. Nevertheless, it rather poorly correlates with the symptoms the patient experiences. To achieve a more relevant portraiture of the actual experienced lung function, the measurement of PEF would be a more appropriate approach. Further spirometric recordings such as VC (vital capacity), the flow rates at 25% or 75% of VC above RV (residual volume) or post-bronchodilator FEV1 and FVC are possible supplementary study endpoints.

In achieving asthma control, the avoidance of **exacerbations** is a crucial factor, which makes it a relevant endpoint to evaluate controller treatments in patients. According to the EMA (European medicines agency) notes for guidance on clinical investigation of medicinal products for treatment of asthma, severe exacerbations can be described as “a requirement for systemic corticosteroids or an increase from the maintenance dose

of corticosteroids for at least three days and/or a need for an emergency visit, or hospitalization due to asthma" [384]. Moderate exacerbations, however, arouse the need for a change in treatment in order to prevent a deterioration of asthma symptoms and, therefore, the occurrence of severe exacerbations. In order to really capture any changes in the number of exacerbation events in an appropriate way, the EMA guidelines advice a study length of at least 12 month with a subsequent equally long follow-up period and the documentation of the respective seasons.

When studying specific immunotherapies, **challenge testing** with an applicable antigen can be of use to grade the tendency of airways to narrow after being stimulated in a way that has hardly any influence on healthy persons. Therefore, direct factors such as histamine or indirect ones such as mannitol or hypertonic saline can be applied. Although the hyperresponsiveness shows only a weak correlation with symptoms, a respective increase can be a predictor of losing asthma control. [384]

Symptom scores are another possible evaluation tool. Here, day- as well as night-time symptoms have to be gathered and pre-defined variables such as symptom-free days or number of night-awakening should be utilized. Besides that, there are special **composite scores** with categorical or numerical variables available for the measurement of asthma control, e.g. the Asthma Therapy Assessment Questionnaire (ATAQ), the Asthma Control Test (ACT) or the Asthma Control Scoring System (ACSS) [222].

A different approach to capture the patients' symptoms, is to take a look at the need of **reliever use** as this correlates with the frequency and intensity of the symptoms and their tolerance, when being separated from the prophylactic use. Likewise, the **reduction of controller medication** can provide information about the therapy's influence on the patient.

The underlying **airway inflammation** can be determined by measuring certain biomarkers, such as eosinophil counts and fractional concentration of exhaled nitric oxide (FE_{NO}).

Since the patients' sensation of the disease status might be different from the way clinicians would evaluate it, it is also expedient to utilize **Health related quality of life**

(HRQoL) Questionnaires to integrate their point of view. One asthma-specific kind of these questionnaires is e.g. provided by the American Thoracic Society [387]. [384]

5.2.3. Design of the Study

Before starting the clinical study, answers for the following questions need be found: How does the new therapy relate to current treatments? Is it a primary or add on therapy? Is it a reliever or controller treatment? What is the intended mechanism of action? Specific immunotherapies don't belong to neither reliever nor controller medications and therefore have to be evaluated in an individual way.

The guideline ICH E-4 Dose Response Information to Support Drug Registration recommends to examine the dose related benefit and adverse effects of new pharmaceutical products in randomized, double blind, placebo controlled studies [339]. While cumulative dose response studies with FEV1 or PEF as pharmacodynamic endpoints are applicable for β2 adrenergic agonists, anti-inflammatory therapies are advised to be tested in group comparative studies parallel to a control group. Both types of treatment can also be investigated with the bronchoprotection/bronchial reactivity model. More detailed information on this matter can be found in the CHMP (Committee for Medicinal Products for Human Use) guideline for orally inhaled products [388].

The duration of the trial is contingent on both the mechanism of action of the tested drug and a defined efficacy measurement. 6-12 week studies are recommended by the EMA for long-acting bronchodilators. Short-acting ones can, on the other hand, also be acceptably examined in smaller periods of time. The exploration of an effect on inflammation or exacerbations, however, asks for a longer trial duration. Reliever medications are advised to be investigated in parallel group studies, where efficacy tests can be performed in short-term trials of four weeks that should show the maintenance of efficacy without any tolerance. The effects on controller medication, in contrast, can only be examined in an encompassing way in studies of at least six months, according to the selected endpoint, but possibly for even longer periods of time. Products for specific immunotherapy should usually be evaluated as an add on treatment to required controller and/or reliever medication during a time span that comprises the period of high allergen exposure, e.g. pollen season. [384]

Regarding comparator products, the preferential approach for reliever medication would be a three-arm study in which the new product is compared to effects of both placebo and a short-acting β2 agonist. Medications that are expected to be utilized as a first-line controller therapy should be investigated in an active comparator trial comparing it with a standard treatment for a specific step in the treatment scheme. Typically, an inhalable corticosteroid is included in all steps, which is supposed to be administered in appropriate doses and durations. To guarantee assay sensitivity, it is generally advisable to perform a three-arm study in patients with milder asthma with a placebo adjustment in at least one fundamental clinical trial. When inhaled corticosteroids are not planned to substitute the new medication, add-on study designs are necessary, in which the drug being tested is compared to placebo and as the circumstances require also a standard comparator of the next higher treatment scheme step.

Since asthma is a disease that can lead to severe medical conditions, it is crucial to secure the patients' safety and well-being in each instance, meaning that they receive appropriate treatment according to their severity level and that concomitant rescue therapy always has to be available. However, this proceeding ought to be standardized and facilitated as much as possible to ensure that the interpretation of generated results is not distorted. Every concomitant treatment with bronchodilators, corticosteroids, antibiotics or mucolytic antioxidants has to be thoroughly documented and also balanced between different trial groups in order to establish a common base. [384]

If applicable, it is advised to always carry out clinical trials for asthma in a double-blinded way. When this can not be achieved, e.g. for some inhalable products, a three-arm study with a blinded comparison with placebo and an unblinded comparison with an active comparator is possible. Here, the respective personnel measuring and documenting data should leastwise be left unaware of the received treatment option. It is, anyhow, always preferable that the most important results concerning safety and efficacy are collected by an independent adjusting committee.

When selecting suitable endpoints for asthma studies, it is essential to consider the diversity of this disease. Since it shows multiple manifestations that might not all correlate with each other, it is highly recommended to use a range of evaluation

measures. The choice of the most convenient primary endpoint hinges on the type of the new medication (reliever or controller), its particular way of action and the asthma severity level. Whereas the examination should be concentrated on the airway construction and therefore FEV1 assessments are suitable for reliever treatments, controller therapies are expected to control the asthma and diminish exacerbations. Hence, studying the latter has to include both lung function and symptom improvement as primary endpoints. For anti-inflammatory drugs, the focus particularly lies on the occurrence of exacerbations, however, in patients with milder asthma this might not be adequate enough and other symptom-based endpoints can be more suitable. Since specific immunotherapy is aimed to regulate immunological mechanisms which needs some onset-time, their exploration should begin as an add-on treatment and can e.g. be studied during a stepwise reduction of controller therapy. Possible primary endpoints in this approach next to lung function and exacerbation number are composite scores or the reduction in need for controller medication. Irrespective of the chosen primary endpoint, it is crucial to determine the minimally important difference a priori according to the disease characteristics and severity, the control group, duration of the study as well as the tested conjecture. As a secondary endpoint, if not already chosen for primary, the lung function is recommended to be investigated in any case. Furthermore, variables such as symptom scores, hyperresponsiveness, biomarkers or the need for rescue medication can be considered. [384]

5.2.4. Safety

As asthma therapy is often started at a young age and has to be received over a great period of time, the safety of respective medications over a longer time is a crucial factor that needs to be addressed with appropriate attentiveness. Therefore, long-term safety studies of at least one year should be carried out, whereat the asthma severity determines the exact time span and intensity of treatment. Special attention has to be paid when investigating immunosuppressive therapies to detect any malignancy evoking effects. Concerning inhalable products, specific security issues such as oral infections, vocal cord myopathy or cataract formation might be necessary to address. Although the systemic exposition is lower here, its extent is advised to be evaluated regardless. Regarding inhaled corticosteroids, for example, the impacts on hypothalamic

pituitary adrenocortical (HPA) axis function, bone mineral density and the eyes should be monitored. In all cases, both pharmacokinetic and pharmacodynamic studies have to be used to examine the overall systemic safety. [384]

6. Regulatory and Ethical Hurdles – The Drug Approval Process

Before a new drug can be introduced into the market, by law it first has to be shown to be safe and effective before it can be approved by the appropriate regulatory agency governing the respective market. Apart from Japan, the United States of America and Europe are home to the two main regulatory agencies in the world. The United States Food and Drug Administration (USFDA) and the European Medicines Agency (EMA) set the most demanding and stringent standards for approving new medicinal products, bringing out legislations and guidelines for developing, testing, trialing and manufacturing drugs [389]. Overall, both departments base their work on similar key principles to warrant public safety and health, however, the exact courses of actions display some minor differences.

The USFDA (or in short, FDA) drug approval process generally consists of submitting two essential applications, an Investigational New Drug Application (IND) followed by a New Drug Application (NDA). As soon as a new drug is considered safe in preclinical trials, a firm or institution as a so-called sponsor is responsible for submitting the IND to the FDA in order to start clinical trials in humans. In a pre-IND meeting, issues such as design of supporting animal research, clinical trial protocols and the manufacturing and control of the novel drug can be deliberated with the FDA. Subsequently, the Center for Drug Evaluation and Research (CDER) evaluates medicinal, chemical, pharmacological, toxicological, statistical and safety aspects in a review. When the product is confirmed to be effective and not causing unreasonable risks in clinical trials, the manufacturer can next request to manufacture and sell the drug in the US by submitting the NDA. [389] In the following process, the FDA reviewers decide whether the new drug is safe and effective in its proposed use and the benefits outweigh the risks, whether its labeling is appropriate and whether manufacturing and control methods are adequate to preserve the drug's identity, strength, quality and purity. [390]

Comparably, the approval procedure in the EU likewise involves two main steps, the clinical trial application and the marketing authorization application. While the former is approved at the level of one of the currently 28 member states, marketing authorizations can be administered at both member state or centralized levels. Altogether it can be distinguished between four different processes, namely the Centralized, Mutual Recognition, Nationalized and Decentralized Procedures. The Centralized Procedure, which is compulsory for certain medicines, e.g. deriving from biotechnological processes, intended for cancer, HIV/Aids or diabetes and so-called orphan drugs is mandatory in order to be allowed to obtain a marketing authorization valid throughout the EU. The respective application gets reviewed by an assigned rapporteur and submitted to the European Commission for final approval. To receive a marketing authorization in a member state (Concerned Member State) other than the one where the drug was originally approved (Reference Member State), the Mutual Recognition Procedure is appropriate. The applicant submits dossiers to all desired states, from which one decides to take command and evaluates the drug and afterwards reports its findings to the other states. As opposed to this, the Nationalized Procedure only accomplishes an authorization for one member state and involves just the respective authority. Medicinal products that have not yet been approved in any EU country and do not come within the Centralized Process, can undertake the Decentralized Procedure and be authorized simultaneously in several member states. [389]

Focusing on nanoscience in particular, the FDA implemented the 2013 Nanotechnology Regulatory Science Research Plan with the aim to lay out a framework and implementation plan to provide coordinated leadership and foster the addressing of key scientific gaps in knowledge, methods, or tools needed to make regulatory assessments of FDA-regulated products that either contain nanomaterial or otherwise involve the application of nanotechnology. Led by the FDA Nanotechnology Task Force formed in 2006, issues such as the development of measurement tools for determination of physico-chemical properties of engineered nanomaterials and the development of appropriate in vitro and in vivo assays and models to predict human responses are scheduled to be addressed in partnership with other government agencies, academia and industry. [391]

The EMA equally takes action to ensure that nanomedicines safely and timely enter the clinic and the European Commission has developed several initiatives to stimulate research and facilitate commercialization of the technologies, including a consultation on nanotoxicology and nanoecotoxicology and a round table promoted by the European Group of Ethics in Science and New Technologies. [392] In 2009, the EMA's Committee for Medicinal Products for Human Use (CHMP) established an ad hoc expert group on nanomedicines that comprises selected experts from academia and the European regulatory network to provide specialist input and assist with the review of respective guidelines. Furthermore, the CHMP began to develop a series of four reflection papers on nanotechnology in 2011 to offer guidance to sponsors, covering the development of both new nanomedicines and nanosimilars [384]. [388]

7. Current Situation and Future of the Field

In recent studies, the potential of nanomedicines to treat asthma could clearly be demonstrated. It was shown that the effects of steroids against airway inflammation are prolonged and enhanced when compacting them with nanoparticles [393] and that particles with smaller sizes induce bronchodilation more effectively [394]. The advantages of nano-sizing respective substances were revealed by incorporating several drugs routinely used in asthma treatment in nanosystems. Salbutamol nanoparticles were proven to interact more intensively with the lung membrane due to augmented peripheral deposition and mucociliary movement to the tracheo-bronchial region, resulting in a higher and sustained drug concentration in the desired region [366], [363]. Budesonide showed preferable properties in nanocluster formulations for efficient drug delivery [395] and was successfully manufactured in nanosuspensions by microfluidizer method [396] as well as in freeze-dried soy phophatidylcholine-cholesterol liposome vehicles in combination with salbutamol [397], all approaches aiming for pulmonary delivery. Solid lipid nanoparticles and nanostructured lipid carriers containing Beclomethasone were produced by high-shear homogenization and efficiently nebulized to aerosols with a suitable particle size for deep lung delivery [398]. Furthermore, Indomethacin was incorporated in nanostructured lipid carriers showing a controlled drug release [399]. Curcumin, an anti-inflammatory substance

with favorable pharmacological effects but poor bioavailability and rapid metabolization, was formulated in solid lipid nanoparticles and tested in an ovalbumin-induced allergic asthma model in rats. The obtained release profile showed an initial burst followed by sustained release which resulted in significantly higher plasma concentrations than those after applying curcumin alone. Besides that, the particles were able to effectively diminish airway hyperresponsiveness and inflammatory cell influx as well as the expression of T-helper-2-type cytokines in BALF [307].

A different approach was developed by the 2006 founded biotechnology company Revalesio. Their lead product RNS60 is based on charge-stabilized nanostructure technology, created by a patented device that produces stable nanostructures in an aqueous suspension by generating rotational forces, cavitation, and high-energy fluid dynamics. The obtained particles are smaller than 100 nanometers and consist of a stabilized oxygen bubble core. Other than traditional therapies, RNS60 does not target single cellular proteins, but aims to alter cellular inflammatory signaling in order to prevent cell and tissue damage by modulating the PI3K-Akt pathway, among others responsible for cellular protection from apoptosis and reduction of inflammation. In cell-based in vitro assays it was shown to change the responsiveness of epithelial, smooth muscle and immune cells to inflammatory stimuli that can trigger asthma attacks. Moreover, in vivo in an ovalbumin challenge model in rats it achieved a significant improvement in tidal volume as well as a downregulation of inflammatory cytokines and chemokines in blood and BALF. Two clinical safety studies of RNS60 administered via nebulizer have already been completed, showing no concerns regarding safety or tolerability in neither healthy volunteers nor asthma patients, but significant improvements in PEF and QoL in diseased individuals. A clinical trial studying the effects of RNS60 on the late phase asthmatic response to allergen challenge was just completed and final data collection is being conducted. A further study determining the impact on regional inflammation and allergen-induced bronchoconstriction is soon to be opened for participant recruitment. [381], [400]

Additionally, despite of promising potential, most of the new asthma therapies in pre-clinical investigations fail to show convincing effects in clinical studies thus far. Besides the challenge that asthma is a very patient-specific disease, a crucial factor leading to

this discrepancy is the absence of relevant models that are really able to accurately mimic the actual conditions in human asthmatic airway tissue.

One auspicious progression that could help reduce this lack is the recent development of a novel human airway musculature on a chip which is able to simulate the contraction of smooth muscles and hence imitate bronchoconstriction and bronchodilation in vitro under asthmatic as well as healthy conditions. In this model, asthmatic inflammation was imitated by exposition of the muscle tissue on a thin elastomeric film to interleukin-13 (IL-13), a native protein that often is hypersecreted in asthmatic airways. When consecutively subjecting it to acetylcholine, the airway muscle responds with hypercontractility, just as it is observed in both asthmatic patients and animal tissue studies. The reverse reaction, a relaxation, can be achieved by using a muscarinic antagonist and a β-agonist that are both utilized against constricted airways in the clinic. On the cellular level, the chip's reaction coincides with the in vivo conditions, too: the known phenomena of atrophing smooth muscle cells in the presence of IL-13 as well as the increased alignment of actin fibers in asthmatic airways were also observed in vitro. As a first example, HA1077, a RhoA targeting inhibitor, was tested. It resulted in decreased basal tone which prevented hypercontraction and improved relaxation and, therefore, made the muscle tissue less sensitive to the asthma triggering IL-13. Consequently, this tool potentially enables an innovative approach to evaluate the efficacy and safety of novel asthma treatments. [401]

Another hurdle to overcome on the way to clinical translation is to find the ideal biocompatible nanocarrier with respective characteristics. Although several studies have been conducted on the safety of nano-based systems and materials, extensive biocompatibility investigations are rather scarce, yet it is essential to scrutinize all possible levels of toxicity in a responsible way. Equally important is the securing of in vivo stability concerning both the nanovector and the active compound. Biodegradable nanoparticles indeed can be applied for sustained release effects, however, the exact drug release mechanisms still have to be clarified. Degradation rates of the polymer as well as release profiles and bioavailability of the incorporated drug demand comprising investigations. To successfully commercialize inhalable nanoparticles for asthma treatment, moreover, the development of suitable inhalation devices is of great importance. Apart from the appropriate device type, and, if applicable, respective

excipients and attached equipment, inhaler performance factors such as flow rate, administered volume and dose reproducibility need to be addressed.

Concerning the lung as a target organ, the largest gap of knowledge is the uptake and clearance of particles in the cells, as the exact transport mechanisms across the pulmonary epithelium, especially under realistic disease specific conditions, are still not understood in a satisfactory way [365].

Chapter VI

Targeted Non-Viral siRNA Delivery

Please note that the following chapter was published in the book *Methods in Molecular Biology – Drug Delivery Systems (Springer Link):*

Rima Kandil, Yuran Xie, Aditi Mehta, Olivia M. Merkel: A method for targeted non-viral siRNA delivery in cancer and inflammatory diseases. Methods Mol Biol. 2020;2059:155-166. doi: 10.1007/978-1-4939-9798-5_7.

CHAPTER VI - Targeted Non-Viral siRNA Delivery

A method for targeted non-viral siRNA delivery in cancer and inflammatory diseases

The authors of this book chapter include Yuran Xie, Aditi Mehta, Olivia M. Merkel and me. I am the first author of the respective publication, performed all experiments and wrote the article.

Key Words

siRNA, polyplexes, gene delivery, T cell targeting, transferrin, receptor expression, knockdown, inflammation, asthma, cancer

Abstract

Small interfering RNA (siRNA) based therapy has been subject of intense research since the discovery of RNA interference (RNAi), providing a tool to potentially silence any chosen gene. Nevertheless, efficient delivery still presents a major hurdle to translating this promising technology into medical practice. Here, we describe a straightforward method to prepare and characterize an effective delivery system consisting of low molecular weight polyethylenimine (PEI) and transferrin (Tf). Tf-PEI polyplexes are not only able to successfully transport and protect the sensitive nucleic acid payload from degradation, but also to selectively deliver the siRNA to transferrin receptor (TfR) overexpressing cells, playing key roles in the pathology of numerous cancer types as well as inflammatory diseases.

1. Introduction

RNA interference (RNAi) offers the potential to selectively target and functionally inhibit disease-related genes [14], paving the way for developing novel treatment options to solve unmet medical needs. Small interfering RNA (siRNA), as the most intensively studied RNAi mediator for therapeutic application in recent studies, [61] arouses significant interest in modern research. Despite numerous studies aiming to translate this revolutionary technology into an applicable medication, its way into clinical routine is still hampered, and so far only one siRNA drug product has been clinically approved. [19] Naked siRNA displays several unfavorable characteristics such as its strong charge and susceptibility to nuclease degradation. [402] In order to avoid rapid clearance and facilitate transport across extra- and intracellular barriers it is therefore imperative to employ a suitable delivery system for the delicate payload. Although viral vectors may offer high transfection efficiencies, due to their debatable safety profiles regarding immunogenicity and tumorigenicity, non-viral vectors are usually the preferred option. [403]

Cancerous and inflammatory tissue share several common features, both exhibiting enhanced vascular permeability orchestrated by mediators such as nitric oxide (NO), bradykinin, and prostaglandins. [404] The vascular anatomy of tumors is, moreover, characterized by reduced lymphatic drainage. Nanosized siRNA systems are, therefore, not only preferentially taken up into tumor tissue via the leaky vasculature, but subsequently also captured due to poor clearance effects. [405] Although this phenomenon, known as the enhanced permeability and retention (EPR) effect, is expected to improve delivery of nanoparticles into tumors, it actually only results in a rather modest 2-fold increase compared to critical normal organs in most tumor models but often is not reflected in the clinical picture. [406] The main difference between tumor and inflammatory tissue is the latter having a functional lymphatic system in place. It does, however, release various mediators inducing the EPR effect and additionally allows enhanced uptake of nanomedicines by dilation of vessels and contraction of endothelial cells. [407]

Despite the preferential passive accumulation of siRNA in pathological tissues, delivery can be further improved by precise and active targeting of desired tissues and cell types.

Targeted nanoparticles are generally designed to interact with specific cell subtypes via certain ligands in order to pointedly deliver their payload as well as increase retention in target cells. [408] This strategy is often achieved by aiming at receptors overexpressed on disease-related cells and eventuates in improved efficacy and reduced toxicity, resulting in an overall improved therapeutic outcome. Therefore, nanoparticles can be functionalized with targeting moieties that are recognized by specific cell types including peptides, oligosaccharides, antibodies, and proteins, of which transferrin is a promising example. Transferrin is a native circulating glycoprotein, responsible for controlling iron levels in biological fluids. Transferrin receptors (TfR) are transmembrane proteins which bind plasma iron complexes with transferrin, enabling iron internalization into cells via receptor-mediated endocytosis. [409] While TfR 1 is ubiquitously expressed on almost all generic cell surfaces at low levels, its expression is upregulated in highly proliferating cells such as in various cancer types and inflammatory tissues. [38] Enhanced TfR 1 expression has been reported in a wide range of different cancers, including breast, liver, colon, ovarian, and lung cancer, as well as leukemia and even glioblastoma. [410] This expression profile can not only be exploited to target cancer specific cells, but furthermore can facilitate cerebral delivery of siRNA by overcoming the otherwise hard to conquer blood brain barrier, since brain endothelial cells also express TfR. [411] In a similar approach, siRNA can successfully be delivered to activated T cells via transferrin targeting, presenting a way to precisely reach disease-related cells in inflammatory diseases such as asthma. [40] Transfection of T cells is a challenging task in general, as they are devoid of caveolae, and therefore do not actively endocytose nanoparticles. [37] Hence, the employment of transferrin as a targeting ligand again fulfills two purposes by offering a gate to enter the difficult to reach T cells and selectively targeting activated TfR overexpressing T cells while leaving naïve ones unaffected. This chapter describes a novel approach to generate a versatile siRNA delivery system composed of low molecular weight polyethylenimine (PEI) and transferrin (Tf) suitable for targeting of disease-related cells in cancer [42] as well as inflammatory diseases [40]. It is moreover depicted how the Tf-PEI conjugate and resulting siRNA polyplexes can be characterized and how respective cell lines are tested for sufficient TfR expression. Finally, a transferrin competition assay is presented as a method to verify ligand-mediated uptake of the targeted polyplexes *in vitro*.

2. Materials

2.1. Conjugate Synthesis

1. Buffers: (*see* **Note 1**)
   Buffer 1 (B1): 20 mM HEPES, 150 mM NaCl, pH 7.5.
   Buffer 2 (B2): 20 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.1.
   Buffer 3 (B3): 20 mM HEPES, 150 mM NaCl, 20 mM EDTA, pH 7.1.
   Buffer A: 20 mM HEPES, 0.5 M NaCl .
   Buffer B: 20 mM HEPES, 3 M NaCl.
2. 5 kDa PEI (Lupasol® G100, BASF, Ludwigshafen, Germany).
3. Human holo-transferrin (Sigma-Aldrich, St. Louis, USA).
4. Succinimidyl 3-(2-pyridyldithio) propionate (SPDP, ThermoFisher Scientific, Waltham, USA).
5. Dithiothreitol (DTT, ThermoFisher Scientific, Waltham, USA).
6. Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, USA).
7. Centrifugal filters:
   MWCO 3.000: Amicon® Ultra-4 (Merck Millipore, Burlington, USA)
   MWCO 10.000: VIVASPIN 6 (Sartorius, Göttingen, Germany)
8. HiTrap™ SP HP columns, 1ml (GE Healthcare, Chicago, USA).
9. Centrifugal tubes, 1.5 ml.
10. Glass vials, 4 ml.

2.2. TfR1 Expression

1. Phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, USA).
2. Human FcR binding inhibitor (eBioscience, Frankfurt, Germany).
3. Anti-human CD71PE (eBioscience, Frankfurt, Germany).
4. Mouse anti-human IgG1-PE (ThermoFisher Scientific, Waltham, USA).
5. Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, St. Louis, USA).

2.3. Polyplex Preparation and Particle Characterization

1. HEPES buffered saline (HBS, 20 mM HEPES, 150 mM NaCl, pH 7.5).
2. Disposable micro-cuvette (Malvern Instruments, Malvern, UK).
3. Folded capillary cells (Malvern Instruments, Malvern, UK).

2.4. siRNA Encapsulation

1. 5 % glucose solution.
2. FluoroNunc 96-well white plate (Thermo Fisher Scientific).
3. 4x SYBR® Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific).

2.5. Transferrin Competition Assay

1. Fluorescently labeled siRNA (here: amine modified siRNA (Integrated DNA Technologies, Coralville, USA) was labeled with succinimidyl ester (NHS) modified AF 488 (Life Technologies, Karlsbad, USA) according to the manufacturer's protocol).
2. 96-well plates (Thermo Fisher Scientific).
3. Human holo-transferrin (Sigma-Aldrich, St. Louis, USA).
4. Phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, USA).
5. Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, St. Louis, USA).

3. Methods

3.1. Conjugate Synthesis

1. Add 940 µl of a 5 kDa PEI solution (1 mg/mL in B1) in a 4 ml glass vial and drop-wise add 94 µl of SPDP solution (20 mM in DMSO) (*see* **Note 2**) while stirring, resulting in PEI-SPDP.
2. Wrap vial with parafilm and stir overnight at room temperature.

3. Add 10 mg of transferrin (*see* **Note 3**) to 1 ml B1 in a 4 ml glass vial and let stir until completely dissolved. Slowly add 31 µl of SPDP solution (20 mM in DMSO), wrap with parafilm and stir for 2 h at room temperature, resulting in Tf-SPDP.
4. In the mean-time, purify PEI-SPDP with a centrifugal filter (MWCO 3.000). Put 1 ml B2 in the column and add PEI-SPDP. Wash the used glass vial with more B2 and add to the column up to a total volume of 4 ml. Centrifuge at 4.000 g for 40 min at 4 °C. Discard the flow-through and mix the remaining solution well by pipetting up and down. Add B2 up to a column volume of 4 ml and centrifuge again at 4.000 g for 45 min at 4 °C. Transfer the remaining volume (*see* **Note 4**) to a 1.5 ml tube and wash the column 2 x with 100 µl B2. Store in the refrigerator (2-8 °C) for later use.
5. Purify Tf-SPDP with a centrifugal filter (MWCO 10.000). Put 1 ml B2 in the column and add Tf-SPDP. Wash the used glass vial with more B2 and add to the column up to a volume of 6 ml. Centrifuge at 4.000 g for 20 min at 4 °C. Discard the flow-through and mix the remaining solution well. Add B2 up to a column volume of 6 ml and centrifuge again at 4.000 g for 25 min. (*see* **Note 5**) Transfer to a 1.5 ml tube and wash column with 100 µl B2. Store in the refrigerator (2-8 °C) for later use.
6. Measure the SPDP amount in purified PEI-SPDP (from step 4) by Pyridine-2-thione assay. (*see* **Note 6**) Prepare a 50 x dilution of PEI-SPDP in B1 (8 µl PEI-SPDP + 394 µl of B1). Add triplicates of 100 µl each in a 96 well plate and measure absorbance at 342 nm against B1 as blank. Subsequently, add 1 µl of 150 mM DTT solution to each well, mix and incubate for 15 min at room temperature. Measure absorbance again and calculate moles of SPDP per mole of PEI with the following equation:

$$\frac{\Delta A}{8080} \times \frac{\text{MW of Protein}}{\text{mg/mL of Protein}} = \text{moles of SPDP per mole of Protein}$$

7. Based on the measured amount of SPDP in PEI-SPDP, add a 10 molar excess of DTT solution and let react under an inert atmosphere of nitrogen for 2 h at room temperature.
8. Purify activated PEI-SPDP with a centrifugal filter (MWCO 3.000). Put 1 ml of B3 in the column, add PEI-SPDP and wash used vial with B3 until a column volume of 4 ml. Centrifuge at 4.000 g for 40 min at 4 °C.

9. Mix activated PEI-SPDP with purified Tf-SPDP in a 4 ml glass vial and add 10 µl of DMSO to aid formation of the disulfide bonds. Wrap with parafilm and stir at 4 °C overnight.
10. Filter the Tf-PEI conjugate with a 13 mm, 0.22 µm syringe filter and purify with 2 connected HiTrap SP HP 1 ml columns using an FPLC system, such as the ÄKTA pure protein purification system. (*see* **Note 7**) After removing the storage solutions from the columns with buffer A, slowly (1 ml/min) let the conjugate run through and collect 0.5 ml fractions. Wash the vial used for the conjugate with 3 ml buffer A, then switch to buffer B and rinse with ~ 9 ml, collecting 0.5 ml fractions. (*see* **Note 8**)
11. To check which buffer B fractions contain the purified conjugate, measure absorbance at 280 nm with buffer B as blank.
12. Combine those fractions with the highest absorbance values and purify with centrifugal filters (MWCO 10.000) (*see* **Note 9**). Put 1 ml B1 in the column and add the conjugate fractions, fill with B1 up to a volume of 6 ml. Centrifuge at 4.000 g for 35 min at 4 °C. (*see* **Note 10**)
13. Quantify the final conjugate via 2,4,6-trinitrobenzenesulfonic acid (TNBS) (*see* **Note 11**) assay. It is advisable to 20 x dilute the conjugate for this step. First, determine the transferrin concentration by absorption measurement at 280 nm with the help of a standard curve starting with 1 mg/ml transferrin in B1, serially diluted to 0.03125 mg/ml. (*see* **Note 12**) Based on the standard curve, calculate the transferrin concentration in the conjugate.

    Dilute the TNBS stock solution to obtain a 3 mM working solution in B1. Prepare a PEI standard curve starting with 400 µg/ml PEI in B1, serially diluted to 3.125 µg/ml. (*see* **Note 13**) Based on the result of the transferrin concentration, prepare a solution with 2 x the calculated transferrin concentration in the diluted conjugate. Dilute each point of the standard curve 1:1 with this transferrin solution in order to account for the transferrin amount in the conjugate. Distribute 100 µl of each point of the standard curve in a 96 well plate and fill 3 wells with the 20 x diluted conjugate as well as 3 wells with only B1 as blank samples. Add 30 µl TNBS per well and cover with aluminum foil, incubate 5 min at room temperature and measure absorbance at 405 nm. Based on the PEI standard curve, calculate the PEI concentration in the conjugate.

14. If appropriate, filter the final conjugate with a syringe filter to make it sterile and store at 4 °C. The coupling reaction is shown in **Figure 1**.

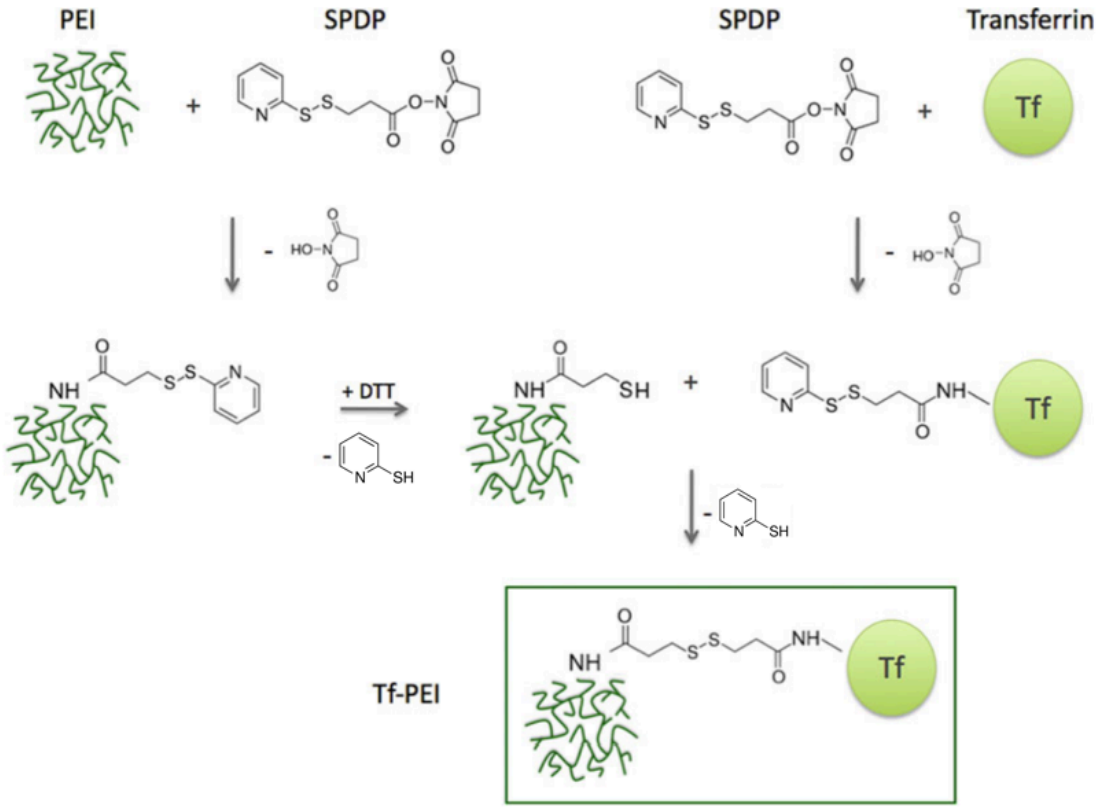


Figure 1. Schematic representation of the conjugation process of transferrin (Tf) and polyethylenimine (PEI), resulting in the Tf-PEI conjugate. Both components are first activated with succinimidyl 3-(2-pyridyldithio) propionate (SPDP). PEI-SPDP is subsequently reduced with dithiothreitol (DTT), before reacting with Tf-SPDP to the final conjugate. Adapted from [40].

3.2. TfR1 Expression

To validate whether your cell line is suitable for transfection with the Tf-PEI conjugate, it is advised to determine endogenous TfR1 expression levels.

1. Harvest cells following trypsinization (or other appropriate methods). Count viable cells and resuspend in PBS at 10 x 10^6 cells/ml and distribute 100 µl/tube of cell suspension (100.000 cells/tube) in 1.5 ml tubes in triplicates each for TfR1 (also

known as CD71), isotype control and unstained blank. If T cells are present, add 10 µl of diluted FcR binding inhibitor (1:10 in PBS) to each sample, mix and incubate for 5 min in the refrigerator (2-8 °C).

2. Add 1 µl undiluted antibody to appropriate samples, triplicates for CD71 and isotype control. Leave 3 samples unstained. Vortex and incubate 30 min in the refrigerator (2-8 °C).
3. Centrifuge at 350 g for 5 min, discard supernatant and add 500 µl of PBS/2 mM EDTA. Centrifuge again and discard supernatant, repeat this washing step 2 times. Resuspend cells in 400 µl PBS/2 mM EDTA.
4. Adjust the laser power at the flow cytometer using the unstained blank samples.
5. Gate the cells to exclude debris and cell clumps and count at least 10.000 events per sample.
6. Determine the % of positive cells in the PE channel for each sample as depicted in **Figure 2**.

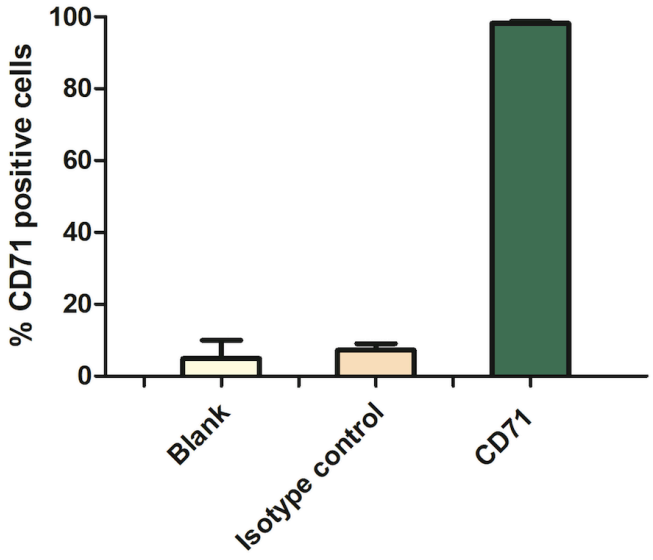


Figure 2. Levels of transferrin receptor 1 (TfR1, also known as CD71) on Jurkat cells as quantified by CD71-PE antibody staining and determination of median fluorescence intensity (MFI) by flow cytometry. Results are presented as means of triplicates ± SD. Blank samples were left unstained, while isotype controls were stained with an IgG antibody to exclude unspecific binding. The high TfR1 expression makes Jurkat cells a suitable model cell line for Tf-PEI experiments.

3.3. Polyplex Preparation and Particle Characterization

1. For polyplex preparation, dilute appropriate amounts of Tf-PEI in HEPES buffered saline (HBS) or 5 % glucose. Add defined amounts of siRNA solution and mix well by pipetting. (*see* **Note 14**)
   Allow the polyplexes to form via electrostatic interactions during 20 min incubation at room temperature.

2. To measure hydrodynamic diameter, PDI and zeta potential of prepared polyplexes, prepare 100 µl of Tf-PEI/siRNA formulations with different N/P ratios in HBS and add into disposable micro-cuvettes. Determine sizes with a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at 173° backscatter angle with 3 times 15 runs per sample. Use 0.88 mPa*s for viscosity and 1.33 for refractive index for data analysis with the Zetasizer software. Subsequently, dilute all samples with 900 µl of nanopure water and transfer to a folded capillary cell. Perform three zeta potential measurements per sample using the same device. Typical results compared to unmodified PEI polyplexes are shown in **Figure 3**.

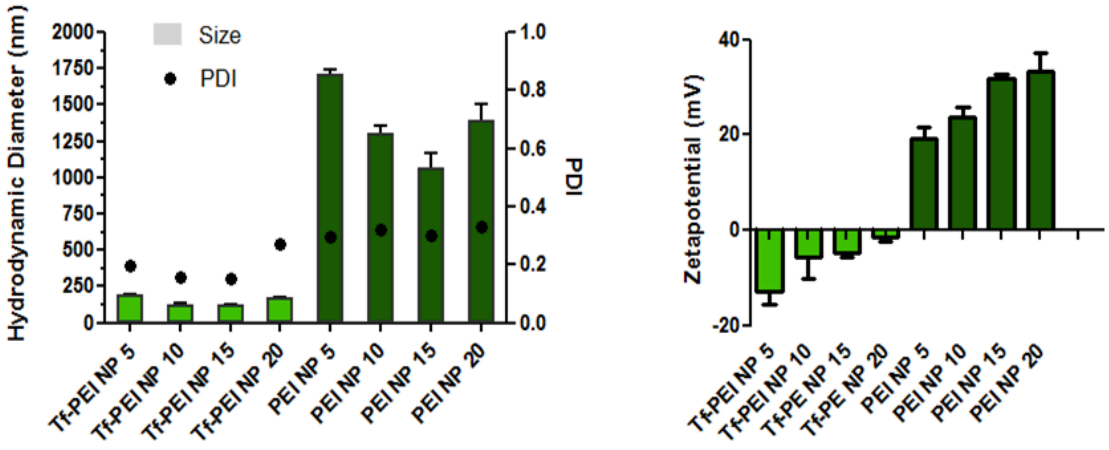


Figure 3. Hydrodynamic diameters, PDI, and zeta potentials of PEI and Tf-PEI polyplexes at different N/P ratios as measured by dynamic light scattering (DLS) and laser Doppler anemometry (LDA), respectively. Data points indicate means ± SD. Due to the shielding effect of the negatively charged transferrin, Tf-PEI polyplexes are distinctly smaller than those made of unmodified PEI and hold slightly negative zeta potentials, exhibiting improved characteristics for siRNA delivery.

3.4. siRNA Encapsulation

1. Determine siRNA condensation efficiencies via SYBR gold assay. Therefore, prepare polyplexes with 50 pmol siRNA at rising N/P ratios in 5 % glucose, distribute in a FluoroNunc 96-well white plate and incubate for 20 min. Use controls containing only siRNA (N/P=0) to account for 100 % free siRNA.
2. Subsequently, add 30 µl of 4x SYBR gold nucleic acid gel stain and incubate for another 10 min in the dark. (*see* **Note 15**)
3. Conduct fluorescence measurements at excitation wavelength of 485/20 nm and emission wavelength of 520/20 nm as illustrated in **Figure 4.**

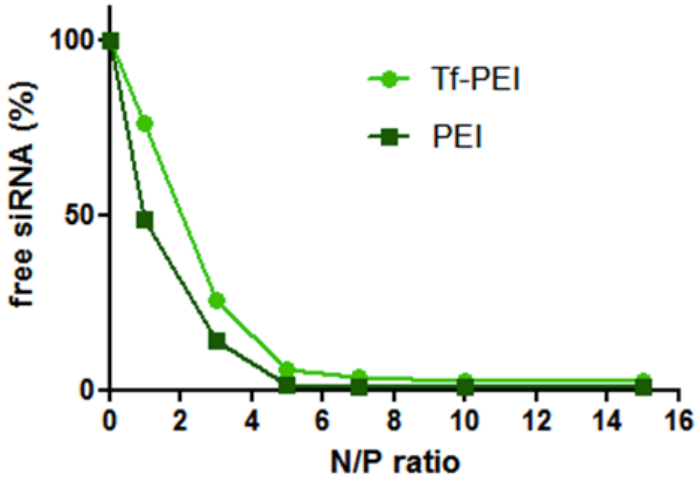


Figure 4. SiRNA encapsulation efficiencies of Tf-PEI and PEI polyplexes at increasing N/P ratios determined by SYBR gold assay. Tf-PEI polyplexes only show slightly enhanced amounts of non-encapsulated siRNA compared to regular PEI, potentially caused by steric hindrance of transferrin interfering with the interaction between PEI and siRNA.

3.5. Transferrin Competition Assay

1. Seed desired cells in an appropriate culture plate (here: 400.000 Jurkat cells per well in a 96 well plate) either in regular culture medium, or in medium containing defined concentrations of free transferrin (here: 0.1 and 2 mg/ml).
2. Prepare polyplexes with 50 pmol of fluorescently labeled siRNA (here: AF 488 labeled siRNA) in 5 % glucose and add to triplicates of appropriate wells. Leave 3 wells of cells untreated as blank controls.
3. Incubate the transfected cells for 24 h at a humidified atmosphere with 5 % CO_2 at 37 °C.
4. Harvest the cells and centrifuge at 350 g for 5 min. Discard the supernatant, wash the cells with PBS/2 mM EDTA, centrifuge again and discard the supernatant. Repeat this washing step 2 times.
5. Resuspend the cells in 400 µl PBS/2 mM EDTA per sample.
6. Adjust the laser power at the flow cytometer using the unstained blank samples.
7. Gate the cells to exclude debris and cell clumps and count at least 10.000 events per sample.
8. Determine the median fluorescence intensity in the respective channel for each sample as depicted in **Figure 5**.

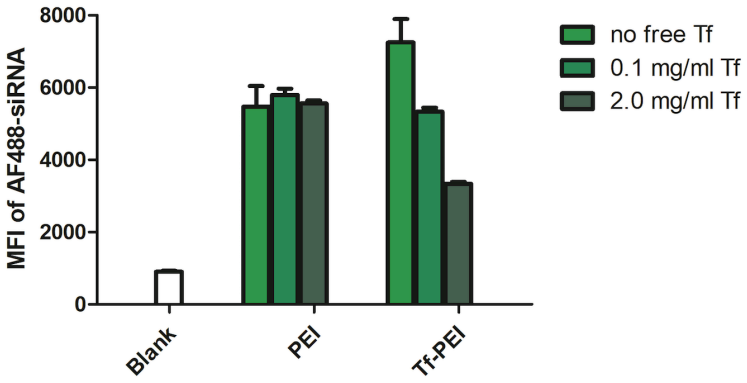


Figure 5. Cellular uptake of PEI and Tf-PEI polyplexes containing fluorescently labeled (AF488) siRNA in Jurkat cells with or without the presence of free transferrin in the culture medium. Results are depicted as means of triplicates ± SD, blank samples represent non-treated cells. While the concentration of free transferrin does not affect uptake of PEI polyplexes, uptake of Tf-PEI is clearly diminished by the addition of free transferrin in a concentration-dependent manner, indicating a transferrin mediated uptake mechanism.

4. Notes

1. Prepare all buffers in purified water and ideally filter them with a 0.33 µm bottle top filter to get rid of any airborne particles, they do not have to be sterile.
2. The SPDP solution should always be prepared freshly. Yellow coloring is a sign for oxidation, indicating the solution should not be used any longer.
3. Ideally, remove the transferrin from the refrigerator and allow adjusting to room temperature before use.
4. The remaining volume should be ~ 300 µl.
5. The remaining volume should be ~ 200 µl.
6. DTT dissolves the disulfide bonds, resulting in pyridine-2-thione as a free moiety absorbing at 343 nm. Since this is emerging in a 1:1 ratio, it can be accounted for the amount of SPDP in PEI-SPDP. In the next step of the conjugation, the whole amount of SPDP can therefore be reduced with DTT.
7. If no FPLC system is available, the purification can also be performed manually with a syringe. Therefore, fill a 6 ml luer lock syringe with the appropriate buffers or the conjugate sample, respectively. Connect the syringe to the HiTrap column and slowly (max. 1 ml/min) push the solutions through. It is advised to always add a bit of fluid to the column before connecting the syringe to avoid formation of air bubbles.
8. Rinsing with buffer A, containing a low amount of salt, results in removing of free unconjugated transferrin, while Tf-PEI and free PEI bind to the negatively charged groups of the HiTrap column. Rinsing with buffer A as a high salt buffer, consequently leads to replacement of PEI from the binding sites with Na^+, eluding purified Tf-PEI as well as free PEI.
9. This purification step is performed to remove any unconjugated free PEI polymer.
10. The remaining volume should be ~ 500 µl.
11. The TNBS assay measures the primary amine groups of PEI by reacting to a chromogenic product.
12. It is necessary to determine this concentration, as the transferrin in the conjugate might interfere with the PEI concentration measurement. Therefore, a transferrin solution with a respective concentration has to be added to the standard curve in order to account for any effects influencing the measurement of the conjugate.

13. Please be aware that each point is diluted 1:1 with the transferrin solution in the next step, meaning the 400 µg/ml will only account for 200 µg/ml PEI in the final assay analysis.
14. Calculate the mass of polymer required for 50 pmol siRNA to yield a certain amine to phosphate ratio (N/P ratio) according to the following equation: m (PEI in pg) = 50 pmol x 43.1 g/mol x N/P x 52 (protonable unit of PEI = 43.1 g/mol, number of nucleotides of 25/27mer siRNA = 52).
15. SYBR gold is a nucleic acid staining dye only fluorescing when intercalating with free siRNA. If no fluorescent signal can be detected, it can be expected that the siRNA is fully protected inside of coherent polyplexes and not accessible to the dye.

Chapter VII

Evaluation of siRNA Treatment Effects

Please note that the following chapter was published in the book *Methods in Molecular Biology – Nanotechnology for Nucleic Acid Delivery (Springer Link):*

Rima Kandil, Daniel Feldmann, Yuran Xie, Olivia M. Merkel: Evaluating the regulation of cytokine levels after siRNA treatment in antigen-specific target cell populations via intracellular staining. Methods Mol Biol. 2019;1943:323-331. doi: 10.1007/978-1-4939-9092-4_21.

CHAPTER VII - Evaluation of siRNA Treatment Effects

Evaluating the regulation of cytokine levels after siRNA treatment in antigen-specific target cell populations via intracellular staining

The authors of this book chapter include Daniel Feldmann, Yuran Xie and Olivia M. Merkel. I am the first author of the respective publication, performed the experiments (in part in collaboration with Daniel Feldmann) and wrote the article.

Key Words

siRNA delivery, knockdown, lung, inflammation, GATA-3, OVA challenged mice, flow cytometry, cytokines, T cell isolation, intracellular staining

Abstract

RNA interference (RNAi) offers a promising base for therapeutic knockdown of clinically relevant genes. Local delivery routes as well as targeted delivery to specific cell populations have been shown to circumvent several hurdles of successful siRNA delivery in vivo. To evaluate and quantify the treatment effect in a precise way, next to measuring the downregulation on gene and protein levels, it is equally essential to investigate the influence on down-stream factors such as generated cytokines. Here, we describe an expressive method to specifically isolate the desired target cells and determine their levels of intracellular cytokines by flow cytometry using the example of murine lungs after pulmonary in vivo transfection with siRNA.

Therefore, the lungs of treated mice are harvested and processed into single cell suspensions, in which CD4 positive T cells are marked by antibody-coupled magnetic beads and isolated via magnetic separation. These purified target cells are then fixed and permeabilized, making their intracellular interleukins accessible for staining with

fluorescently labelled antibodies. In this way, the cytokine levels and hence the precise influence of the siRNA treatment on intracellular conditions can be measured.

1. Introduction

Small interfering RNAs (siRNA) offer the theoretic potential to silence the expression of any chosen gene with a known sequence [14]. Over the last two decades, the regulatory mechanism of RNA interference (RNAi) has aroused great interest for therapeutic purposes and found its way into several human clinical trials [15]. Despite all progress, however, there are still hurdles that impede translation into the clinical routine. Since siRNA, due to its poor pharmacokinetics, was recognized at an early stage to be extremely problematic for systemic administration, a majority of the recent studies focuses on local delivery routes [31]. Pulmonary application, as such a way, not only circumvents several of the barriers that need to be overcome by nucleic acids and protects the sensitive payload from degradation, e.g. by serum nucleases, but obviously also enables reductions of doses, and therefore, side effects [365]. Furthermore, the therapeutics are instantly available at the target region where they are supposed to bring about their effect [34].

For immune-related diseases, this target is oftentimes displayed by the cellular contingent of the immune system. Activated T cells, as the most important cellular mediators in immune responses, are anticipated to be targeted and treated in several related studies [23]. One of the numerous diseases, in whose pathophysiology T cells play a crucial role, is asthma, a chronic inflammatory disease of the airways characterized by infiltration of immune cells including T helper 2 cells (T_H2 cells), a type of activated T cells (ATC), in the lung [412]. These $CD4^+$ T cells orchestrate various of the cytokine-based inflammatory cascades by secretion of interleukins such as IL-13, which in turn are produced upon activation of the key transcription factor GATA-3 [413]. There are attempts to capture the cells and their receptors directly [414, 415] as well as indirect methods that aim to modulate the cytokine environment [416]. Beyond targeting these single cytokines in particular, for example with respective antibodies [24], the down-regulation of GATA-3 is a promising approach to early-on undermine

pathologic pathways [27] and was recently even proven successful in a human phase II clinical trial employing DNAzymes [28].

To further characterize and optimize similar approaches in vivo, suitable animal models and appropriate read-out parameters to quantify the treatment success are imperative. A well-established and extensively studied method to examine inflammatory and immune responses is the Ovalbumin (OVA)-sensitization mouse model [28] which is well suited, but by far not limited, for the use in asthma research [40]. In these experimentally challenged animals, it is possible to check the distinct influence of siRNA treatment on enhanced cytokine levels in specific T cell subsets. To achieve this, an essential step is to isolate the desired cell type, which can be accomplished by antibody-based magnetic cell separation as a straightforward technique [417]. In order to access intracellular cytokines, the membrane of the obtained cells then has to be permeabilized, which is commonly approached with organic solvents or detergents such as saponin [418]. The respective cytokines are now attainable for fluorescently-labeled antibodies and can easily be stained and detected by flow cytometry as described in this chapter.

2. Materials

2.1. $CD4^+$ T Cell isolation

1. Optional. 0.7 % NaCl solution for cell counting.
2. Isolation buffer: phosphate-buffered saline (PBS), pH 7.2, 0.5 % bovine serum albumin (BSA), 2 mM EDTA, filtrated and degassed.
3. $CD4^+$ T cell Isolation Kit, mouse (Miltenyi Biotec, Bergisch Gladbach, Germany)
   Components:
   $CD4^+$ T cell biotin-antibody cocktail, mouse
   Anti-biotin microbeads
4. LS Separation Columns (Miltenyi Biotec, Bergisch Gladbach, Germany).
5. MidiMACS Separator and appropriate rack (Miltenyi Biotec, Bergisch Gladbach, Germany).
6. Centrifuge cups, 1.5 ml.

2.2. Cell Fixation and Permeabilization

1. Paraformaldehyde: 1% v/v in PBS buffer, freshly prepared.
2. Wash buffer: PBS supplemented with 2 mM EDTA and 0.5% v/v fetal calf serum.
3. Saponin buffer: 0.3% w/v saponin (Carl Roth, Karlsruhe, Germany) in PBS buffer.
4. FACS tubes.
5. Antibodies:

   Rat anti-mouse CD16/CD32 (Fc-Block; BD Biosciences, Heidelberg, Germany), 1:100 dilution in 0.3% saponin buffer.

   Anti-mouse CD4-PE, clone REA604 (Miltenyi Biotec, Bergisch Gladbach, Germany).

   Isotype control: Rat anti-mouse IgG1-PE, clone M1-14D12 (eBioscience, Frankfurt, Germany).

   Rat anti-mouse IL-13-PE, clone eBio13A (eBioscience, Frankfurt, Germany), 1:10 diluted in 0.3% saponin buffer.

   Rat anti-mouse IL-17A-eFluor 450®, clone eBio17B7 (eBioscience, Frankfurt, Germany), 1:10 diluted in 0.3% saponin buffer.
6. Attune NxT acoustic focusing cytometer (Life technologies, Carlsbad, USA) or similar flow cytometer equipped with the following lasers and filter settings:

   PE: excitation: 488 nm, emission filter: 574/26; eFluor 450®: excitation: 405 nm, emission filter: 440/50.

3. Methods

For in vivo transfection, harvesting of the lungs and preparation of single cell suspensions, please refer to Chapter 18 of *Methods in Molecular Biology – Nanotechnology for Nucleic Acid Delivery* (Springer Link). With these single cell suspension, obtained from lungs or other desired organs, respectively, proceed with the following steps.

3.1. Isolation of $CD4^+$ T cells (*see* **Note 1**)

1. Dilute the obtained single cell suspension in 10 ml of a 0.7 % NaCl solution and count the cells using a cell counting device, such as the Z2 Coulter Particle Count and Size Analyzer (Beckman Coulter, California, USA) (*see* **Note 2**).
2. Centrifuge the cell suspension for 10 min at 350 g.
3. Discard the supernatant and resuspend the cell pellet in 40 µl of isolation buffer per 10^7 total cells (*see* **Note 3**).
4. Optional: Take an aliquot of the original untreated cell population aside for later comparison.
5. Add 10 µl of biotin-antibody cocktail per 10^7 total cells.
6. Mix well and incubate for 5 minutes in the refrigerator (2-8 °C).
7. Add 30 µl of isolation buffer and 20 µl of anti-biotin microbeads per 10^7 total cells.
8. Mix well and incubate for 10 minutes in the refrigerator (2-8 °C).
9. For cell separation, place the LS column in the magnetic field of a MidiMACS Separator.
10. Rinse the column with 3 ml of isolation buffer (*see* **Note 4**).
11. Apply the cell suspension onto the column and collect the flow-through containing the unlabeled, enriched $CD4^+$ T cells (*see* **Note 5**).
12. Wash the column with 3 ml of isolation buffer, collect the flow-through and combine with the effluent from step 10 (*see* **Note 6**).
13. Optional: To obtain the labeled non-$CD4^+$ cells, remove the column from the separator, place it on a collection tube and add 5 ml of isolation buffer. Immediately flush out the remaining cells by firmly pushing the plunger into the column, collecting the flow-through containing the non-$CD4^+$ cells.

3.2. Validation of Successful Isolation

1. To determine the fraction of $CD4^+$ T cells in the enriched sample, count all cells and transfer triplicates of about 300.000 cells each of the original untreated cell sample, the $CD4^+$ fraction and the non-$CD4^+$ fraction into FACS tubes. (*see* **Note 7**).
2. Prepare additional samples as blank and isotype controls.
3. Centrifuge the aliquots of 300.000 cells per tube for 10 min at 350 g.

4. Discard the supernatant and resuspend the cell pellets in 35 µl PBS buffer.
5. Add 10 µl of 1:10 diluted Fc-block and 5 µl of the antibody against CD4 (or the appropriate isotype control), vortex and incubate for 10 min at 4 °C in the dark.
6. Centrifuge the stained cells for 5 min at 350 g.
7. Discard the supernatant, wash with 500 µl PBS buffer three times, and centrifuge the cells a fourth time for 5 min at 350 g.
8. Discard the supernatant and resuspend the cell pellet in 400 µl PBS buffer.
9. Adjust the laser power at the flow cytometer using the isotype controls and unstained samples.
10. Gate the cells to exclude debris and cell clumps. Count at least 30.000 events.
11. Determine the fraction of $CD4^+$ cells in the PE channel as shown in **Figure 1**. (*see* **Note 8**)

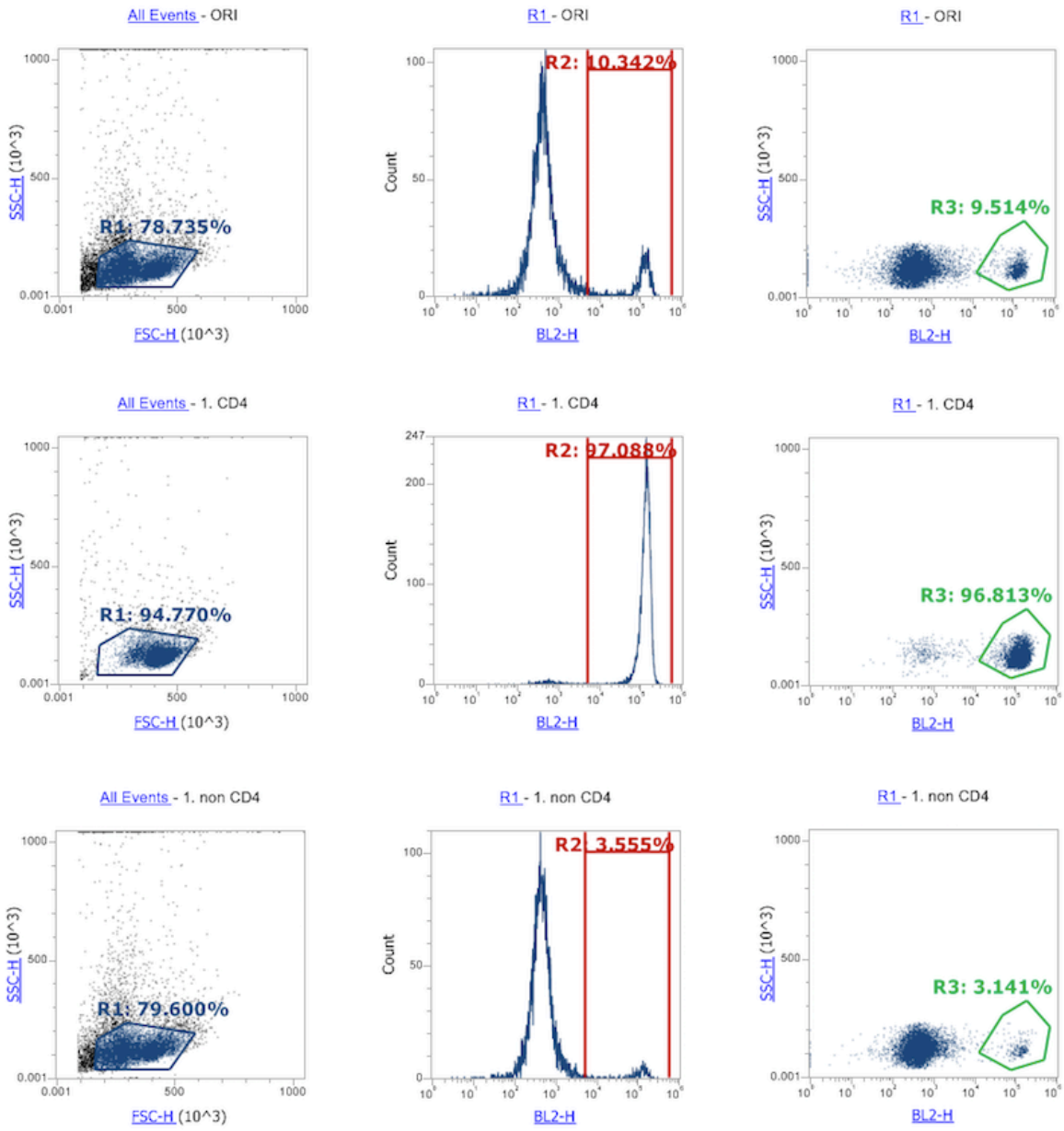


Figure 1. Dot and histogram plots obtained after measuring the mean fluorescence intensity (MFI) in the PE channel after staining with an anti-CD4-PE antibody. To exclude debris, cells are gated in the FSC-SSC dot plots on the left. $CD4^+$ cells are gated in the respective middle and right plot. From top to bottom the following samples can be seen: original cells before separation, isolated $CD4^+$ fraction, non-$CD4^+$ fraction.

3.3. Quantification of Intracellular Cytokine Levels

1. Centrifuge the isolated $CD4^+$ T cells for 10 min at 350 g.
2. Discard the supernatant and resuspend the pellet in 4 ml fresh 1% paraformaldehyde solution and incubate for 15 min on ice.
3. Centrifuge the fixed cells for 10 min at 350 g.
4. Discard the supernatant and resuspend in 5 ml wash buffer.
5. Centrifuge cells for 10 min at 350 g.
6. Discard the supernatant and resuspend the pellet in 4 ml wash buffer.
7. Count the cells and transfer about 300.000 cells per sample into FACS tubes.
8. Prepare additional samples as blank and isotype controls.
9. Centrifuge the aliquots for 10 min at 350 g.
10. Discard the supernatant, add 100 µl 0.3% saponin buffer per sample for permeabilzation, mix and incubate for 15 min at 4 °C.
11. Centrifuge FACS tubes for 5 min at 350 g.
12. Discard the supernatant, add 10 µl diluted Fc-block, add 10 µl of the appropriate dilutions of the antibodies against IL-13 and IL-17A (or isotype controls), vortex and incubate for 25 min at 4 °C in the dark.
13. Centrifuge the stained cells for 5 min at 350 g.
14. Discard the supernatant, wash with 100 µl 0.3% saponin buffer twice, and centrifuge the cells a third time for 5 min at 350 g.
15. Discard the supernatant, wash with 100 µl wash buffer once, centrifuge the cells again for 5 min at 350 g, and resuspend them in 200 µl wash buffer.
16. Adjust the laser power at the flow cytometer using the isotype controls and unstained samples.
17. Gate the cells to exclude debris and cell clumps. Count at least 30.000 events.
18. Determine the mean fluorescence in the PE channel (representing IL-13) as well as in the eFluor 450® channel (representing IL-17A) as shown in **Figures 2 and 3**.

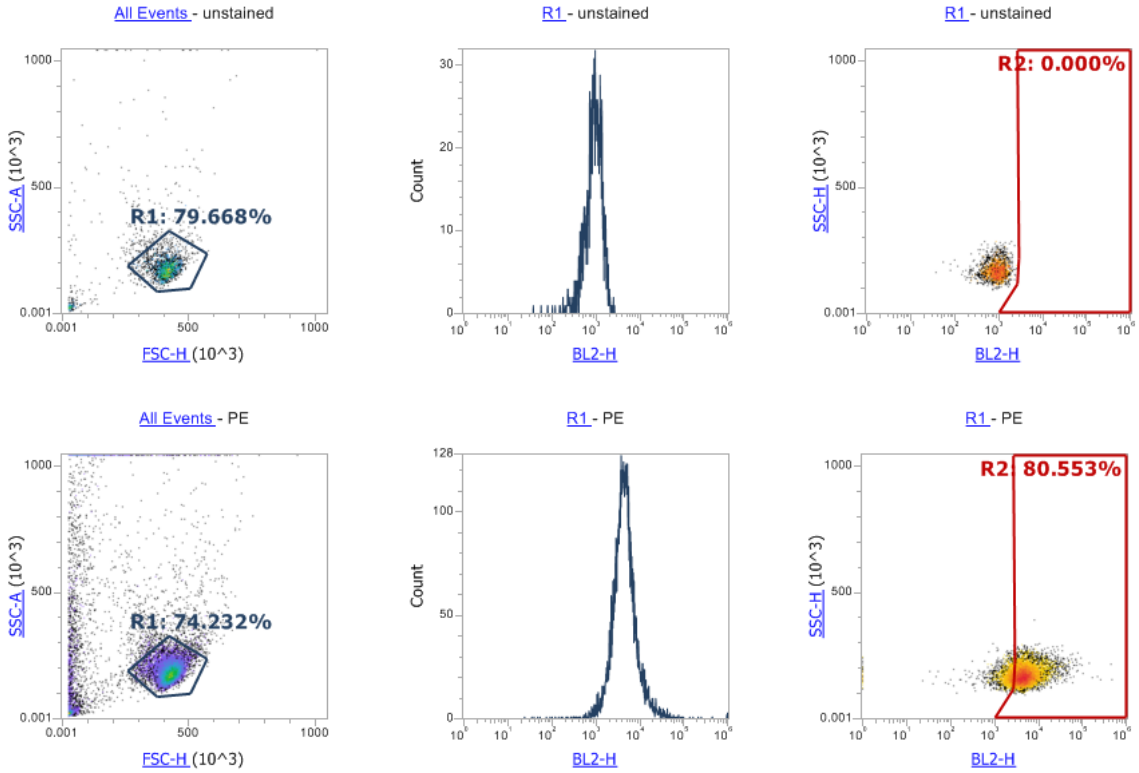


Figure 2. For illustration, an untreated blank sample and a stained sample of CD4+ T cells are shown after measuring the MFI in the PE channel. The respective peak and dot cloud of the positive stained sample distinctly shift to higher fluorescence values, indicating high levels of the respective cytokine as well as a high number of positive cells.

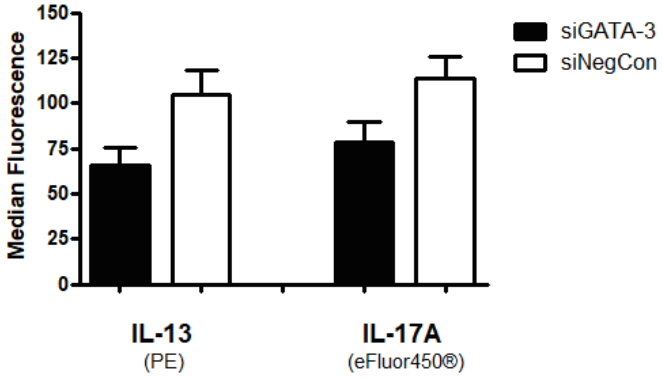


Figure 3. Mean fluorescence values from the respective channels can be depicted in bar graphs. Here, the cytokine levels after pulmonary treatment of OVA challenged mice with GATA-3 siRNA compared to a scrambled control sequence (siNegCon) are shown after assessment via intracellular staining of the isolated CD4+ T cells from the lungs.

4. Notes

1. Throughout the whole isolation process, it is advised to work fast, always keep the cells cold and use pre-cooled solutions (2-8 °C). Ideally, also pre-cool the columns in the fridge, so that they have a similar temperature as the isolation buffer.
2. Alternatively use a haemocytometer counting chamber.
3. For cell numbers lower than 10^7, use the same volumes as indicated. For higher cell numbers, scale up all reagent volumes accordingly.
4. Always wait until the column reservoir is completely empty, before proceeding with the next step.
5. A volume of not less than 500 µl is required for successful separation, smaller volumes should be filled up with buffer at this point.
6. If some of the T cells are desired to be kept in culture for further experiments, it is strongly recommended to stimulate them, e.g. with antibodies against CD3e (Hamster anti-mouse CD3e, clone 145-2C11 (BD Biosciences, Heidelberg, Germany)) and CD28 (Hamster anti-mouse CD28, clone 37.51 RUO (BD Biosciences, Heidelberg, Germany)). Therefore, coat the appropriate number of wells of a 96-well plate with 1 µl of CD3 antibody diluted in 200 µl of PBS buffer per well. Incubate overnight at 4 °C or, alternatively, at 37 °C for 3 h. Wash three times with rising volumes of PBS buffer and dispense 1-2 x 10^6 cells per well in 100 µl RPMI-1640 medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10 % fetal bovine serum (Sigma-Aldrich, St. Louis, USA). Add 0.2 µl of CD28 antibody diluted in 100 µl of RPMI-1640 medium per well and incubate the plate at a humidified atmosphere with 5 % CO_2 at 37 °C.
7. To check the cell viability after isolation, the samples can, additionally, be incubated with dead cell stains, such as DAPI (Biolegend, San Diego, USA) and distinguished by flow cytometry.
8. A purity value of at least 90 % is expectable.

Chapter VIII

Blending of Receptor Targeted Delivery and Endosomal Escape

Please note that the following chapter was published in *Advanced Therapeutics*:

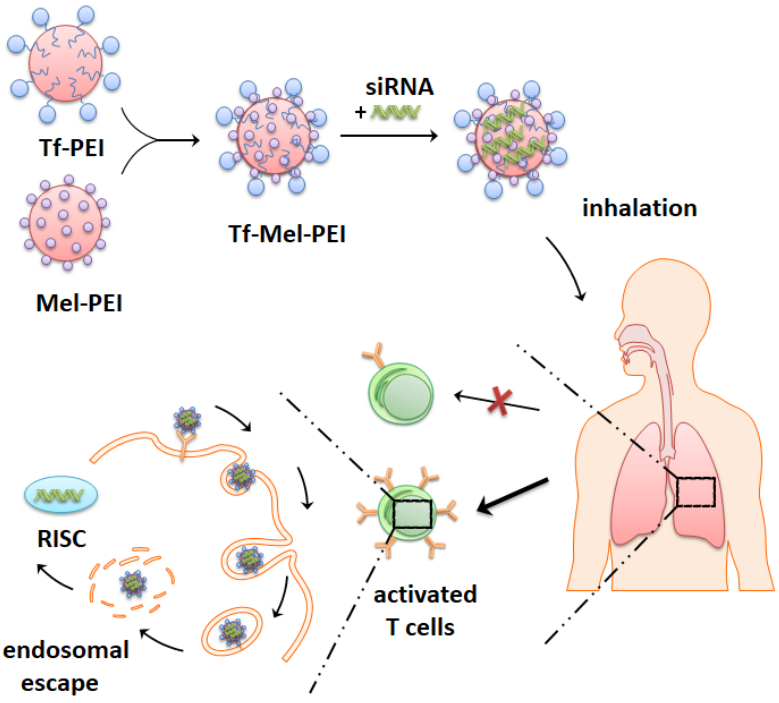
Rima Kandil, Yuran Xie, Ralf Heermann, Lorenz Isert, Kirsten Jung, Aditi Mehta, and Olivia M. Merkel. Coming in and Finding out: Blending Receptor Targeted Delivery and Efficient Endosomal Escape in a Novel Bio-Responsive siRNA Delivery System for Gene Knockdown in Pulmonary T Cells. Adv Ther (Weinh). 2019 Jul;2(7). pii: 1900047. doi: 10.1002/adtp.201900047.

CHAPTER VIII - Blending of Receptor Targeted Delivery and Endosomal Escape

Coming in and Finding out: Blending Receptor Targeted Delivery and Efficient Endosomal Escape in a Novel Bio-Responsive siRNA Delivery System for Gene Knockdown in Pulmonary T Cells

The authors of this chapter include Yuran Xie, Ralf Hermann, Lorenz Isert, Kirsten Jung, Aditi Mehta and Olivia M. Merkel. I am the first author of the respective publication, performed most of the experiments independently and wrote the article. Measurements of the SPR samples were performed by Ralf Heermann and confocal pictures were taken with the help of Lorenz Isert.

Graphical Abstract



Abstract

RNA interference (RNAi) offers the potential to selectively silence disease-related genes in defined cell subsets. Translation into the clinical routine is, however, still hampered by the lack of efficient carrier systems for therapeutic siRNA, endosomal entrapment presenting a major hurdle. We previously developed a promising siRNA delivery system on the base of polyethylenimine (PEI) and the targeting ligand transferrin (Tf) to specifically reach activated T cells in the lung. In the present work, we focused on optimizing Tf-PEI polyplexes for gene knockdown in primary activated T cells by improving their endosomal escape properties. Blending of the conjugate with membrane lytic melittin significantly enhanced endosomal release and thereby cytoplasmic delivery, while maintaining selective T cell targeting abilities and overall cell tolerability. The gathered data furthermore demonstrate that melittin addition also distinctly improved several other essential particle characteristics, such as siRNA encapsulation efficiency and stability in lung lining fluids. In conclusion, this results in a novel upgraded siRNA delivery system that is not only able to specifically deliver its payload to the desired target cells via receptor-mediated endocytosis, but also shows enhanced release from endosomal vesicles in order to initiate RNAi in the cytoplasm.

1. Introduction

Small interfering RNA (siRNA) has the capability to target and functionally inhibit any chosen gene via RNA interference (RNAi).[14] This specific altering of gene expression is not only of great interest for functional analysis, but can moreover be exploited therapeutically to precisely influence pathological processes. While siRNA has already emerged to be a standard research tool, its translation into therapeutic usage in the clinical routine has been slow, with overcoming extra- and intracellular barriers being a major impediment. Due to unfavorable characteristics such as relatively high molecular weight and strong charge, naked siRNA is hardly able to cross hydrophobic cell membranes and is furthermore very susceptible to degradation.[402] Therefore, it is necessary to employ a suitable delivery system that efficiently protects the siRNA, facilitates cellular delivery and aids cytoplasmic transport to achieve passage to the target location. Cationic lipids and polymers can spontaneously condense siRNA and

form nanoscale complexes, showing high gene delivery efficiencies and eluding immune responses often occurring with viral vectors.[31] However, these carrier systems usually have two major drawbacks: high toxicity due to their positive charge and abundant endosomal entrapment of their payload. Endosomal escape is generally important to avoid degradation of sensitive material in the acidic and enzymatic conditions of the endolysosomal compartment.[419] For siRNA, it is yet particularly relevant, as it has to reach the RNA-induced silencing complex (RISC), the RNAi machinery located in the cytoplasm, in order to bring about its effect.[79]

A promising approach to facilitate efficient delivery of siRNA to the desired target region is to use local administration routes, circumventing systemic degradation and first pass metabolism of the sensitive cargo while at the same time reducing required doses and minimizing side effects. By combining this strategy with specific targeting of diseased cells it is feasible to reduce the dose even further as delivery becomes as exact and efficient as possible. In various inflammatory and immune-related diseases, T cells play a key role as they mediate a majority of cellular immune responses. Via production of Th_2 cytokines, activated T cells orchestrate several pathologic cascades underlying chronic inflammatory diseases, such as asthma.[24] Here, blockage of these cytokines or preferably even their transcription factors can early-on undermine disease triggering processes and bears the potential to treat even severe forms in patients whose symptoms are currently not adequately controlled. Unfortunately, T cells have proven to be difficult to transfect as they are resistant to common non-viral vector based transfection methods. In contrast to most other cell types, T cells are devoid of caveolae as they do not express caveolin,[420] meaning that their active endocytosis of nanoparticles is limited. While other techniques such as viral delivery systems or electroporation as a physical method may be effective *in vitro*, but are not well suited for therapeutic *in vivo* application,[210] a more sophisticated way has to be found to reach the T cells.

Previously, our group developed a conjugate consisting of transferrin (Tf) and polyethylenimine (PEI), namely Tf-PEI, that can efficiently and selectively deliver siRNA to activated T cells in the lung,[40] exploiting the fact that Tf receptor (TfR) expression is upregulated in activated T cells, while naïve T cells only show negligible expression.[23] The differential expression of this activation marker concomitantly

being an internalizing receptor allows the targeting of specific cell subsets while leaving naïve T cells unaffected, thereby avoiding influence on the general immune system. Besides exhibiting optimal physicochemical properties, Tf-PEI showed significant gene silencing efficiencies *in vitro* and preferentially delivered siRNA to activated T cells in *vivo.*[40] However, despite promising results, one aspect that still left room for optimization and potentially prevented the conjugate from achieving therapeutically relevant *in vivo* knockdown rates was sufficient endosomal release of delivered siRNA. Recent studies have identified escape of the endosomal pathway as the rate-determining step in delivering therapeutic agents, being the only way to circumvent lysosomal entrapment and subsequent degradation.[51] We therefore hypothesized that the achieved gene silencing effect could possibly be enhanced by including an endosomolytic component. The peptide melittin (Mel) exhibits an inherent capacity for membrane disruption and was shown to be able to lyse red blood cells and model membranes. [421, 422] As this effect is not impaired by acidic conditions, melittin exhibits optimal features as an endosomolytic agent and was proven to be a useful tool to release delivered therapeutics from the endosomal compartment. [423] Besides enhancing transfection efficiencies of both polymeric [54-56]and lipid-based [57] gene delivery vectors, it can also be used directly as an oligonucleotide transfection agent. [424] The evoked pore-formation in membranes can, however, cause general toxicity if this effect is not limited to the endosomes.[423] For this reason, a pH-sensitive shielding of melittin was applied, yielding a responsive conjugate with minimized membrane-effects at extracellular neutral conditions, but precise lytic activity upon endolysosomal acidification.

The aim of this study was therefore to refine and optimize our Tf-PEI conjugate by incorporating a bioresponsive endosomolytic domain while maintaining the specific T cell targeting ability in order to develop a versatile siRNA delivery system combining the two essential features for successful directed delivery. The resulting Tf-Mel-PEI blend was fully characterized and compared to its single components Tf-PEI and Mel-PEI as well as unmodified PEI polymer and demonstrated superior competences in terms of siRNA encapsulation and protection, Tf related cellular uptake and transfection efficiency, sequence specific gene and protein knockdown and, moreover, endosomal release properties in various cell models, including primary human T cells. Based on the findings presented here, we therefore conclude that the blending of Tf-PEI and Mel-PEI

results in an efficient siRNA delivery system with optimal properties to preferentially reach and treat activated T cells in the lung.

2. Results

2.1. Conjugate Synthesis

Tf-PEI conjugates were prepared as described before [40], resulting in a molecular ratio of Tf to PEI of approximately 1.5:1. Low molecular weight (LMW, 5k) PEI was successfully coupled to 2,3-Dimethyl-maleic anhydride (DMMAn) modified melittin (Mel) to yield the Mel-PEI conjugate with pH-responsive lytic ability. The reversible masking of lysine residues and N-terminal amino groups with DMMAn resulted in bioresponsive membrane-disruptive properties only emerging in acidic environments, leading to a distinct reduction of toxic side effects in the extracellular area. As a first step, PEI was coupled to the SPDP-PEG4 linker and melittin was reacted with DMMAn after activation with dithiothreitol (DTT). DMMAn-Mel was then conjugated with PDP-modified PEI via formation of a disulfide bond in the presence of 1 M guanidine hydrochloride to prevent aggregation of positively charged modified PEI and negatively charged modified melittin. Acidic cleavage of DMMAn was in turn avoided by conducting the proceeding purification at pH 8.

2.2. Particle Characterization

Hydrodynamic diameter, polydispersity index (PDI) and zeta potential of prepared polyplexes were determined by dynamic light scattering and laser Doppler anemometry, respectively. As **Figure 1A** shows, PEI/siRNA polyplexes demonstrated agglomeration with sizes above 1000 nm in physiologic salt conditions for all tested N/P ratios (nitrogen to phosphate ratio, see experimental section) with an average PDI of 0.29, while Tf-PEI formulations resulted in significantly smaller particle sizes ranging from 37 to 106 nm and an average PDI of 0.26. Polyplexes prepared of Mel-PEI showed the most heterogeneous size distributions ranging from 154 to 750 nm and the largest PDI with up to 0.523. Tf-Mel-PEI blends, however, eventuated in polyplexes with

consistent hydrodynamic diameters below 200 nm exhibiting the smallest average PDI of all formulations (0.16), indicating the most homogeneous particle morphology.

Zeta potentials of PEI polyplexes increased with rising N/P ratio (Figure 1B), showing positive charges in the range of 2.0 to 35.64 mV, while Tf-PEI particles were negatively charged with an average of -4 mV. Positive zeta potentials of Mel-PEI formulations ranged from 0.133 to 10.23 mV and Tf-Mel-PEI polyplexes displayed slightly negative charges for low N/P ratios (3, 5 and 7), but positive zeta potentials starting at N/P 10.

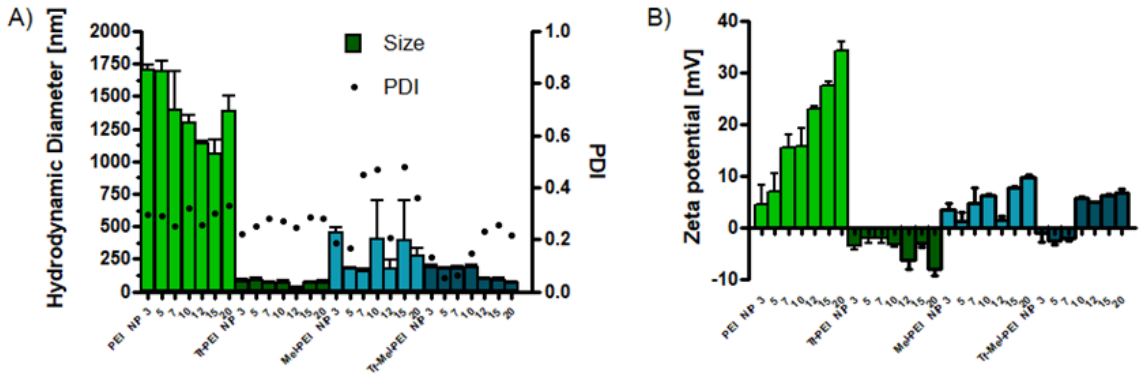


Figure 1. Dynamic light scattering and laser Doppler anemometry measurements of polyplexes. A) Hydrodynamic diameters (left y-axis) and polydispersity indices (PDI, right y-axis) and B) zeta potentials at different N/P ratios: 3, 5, 7, 10, 12, 15, 20. (Data points indicate mean ± SD, n = 3).

2.3. siRNA Encapsulation and Stability

Figure 2 depicts siRNA encapsulation efficiencies of the different polyplex formulations as investigated by regular (Figure 2A) and modified (Figure 2B) SYBR® Gold assays. While PEI and Tf-PEI polyplexes showed comparable siRNA encapsulation starting with approximately 85 % of free siRNA at N/P 1, the Tf-Mel-PEI blend left only 70 % of siRNA uncondensed at N/P 1 and showed full condensation beginning at an N/P ratio as low as 3, compared to maximum condensation at N/P 5 for PEI and Tf-PEI.

Analogous results were obtained when testing stability in the presence of Alveofact® or mucin (Figure 2B) mimicking the lung environment. In all cases, the amount of released siRNA increased with rising concentrations of simulated lung fluids, PEI and Tf-PEI

showing very similar release profiles. Tf-Mel-PEI blends, however, protected the siRNA payload better at all tested conditions, releasing only negligible siRNA concentrations, up to a concentration of 0.05 % for both Alveofact® and mucin.

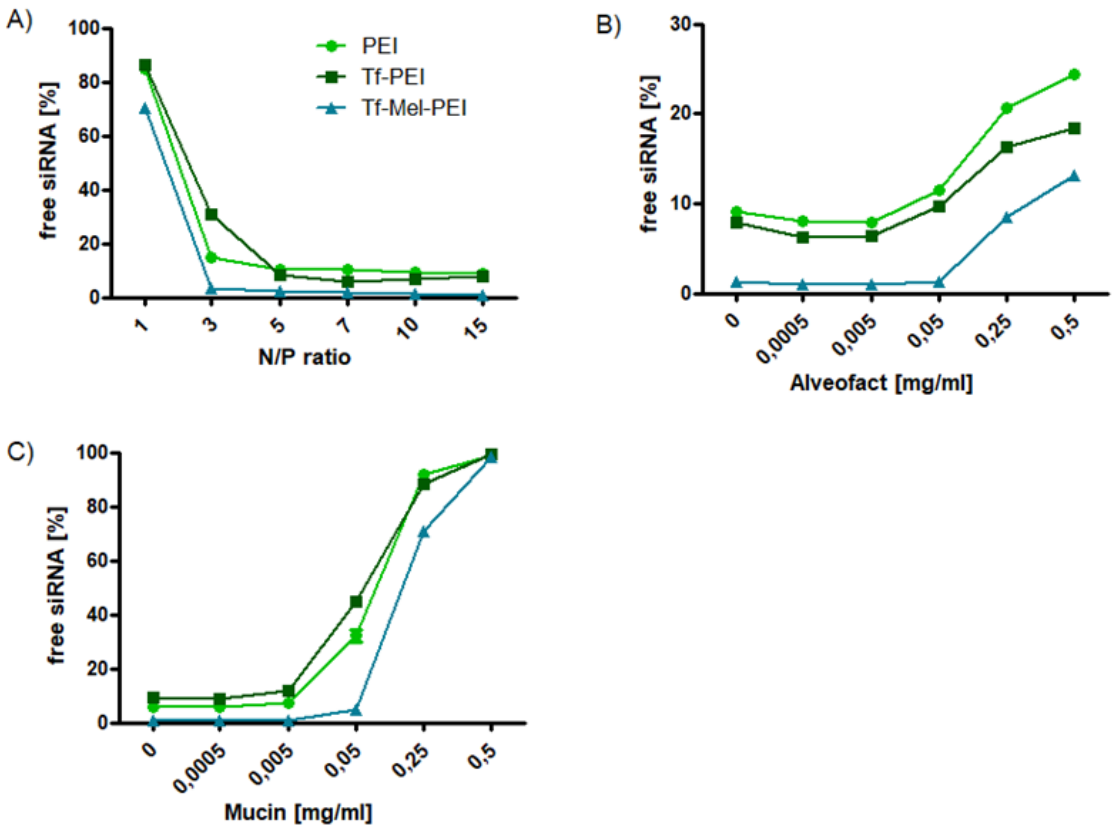


Figure 2. siRNA condensation efficiencies of polyplexes as measured by SYBR gold assay. A) Unmodified assay at different N/P ratios: 1, 3, 5, 7, 10, 15 performed in 5 % glucose. Fluorescence emitted by free siRNA (N/P = 0) represents 100 %. B) Polyplex stability at N/P 10 in the presence of Alceofact® and C) mucin. (Data points indicate mean ± SD, n = 3).

2.4. Transferrin Receptor Binding

We used Surface Plasmon Resonance (SPR) technique to determine binding kinetics of the interaction between the Tf-PEI and Tf-Mel-PEI conjugates, respectively, to the transferrin receptor (TfR). For that purpose, we used a capturing approach in which His-tagged TfR was captured onto a sensor chip that had been preloaded with anti-His antibodies. Then, increasing concentrations (1 nM-1000 nM) of Tf-PEI and Tf-Mel-PEI polyplexes were injected. After each cycle, TfR-His was regenerated from the chip and recaptured. As positive control, we injected free transferrin in similar concentrations over the chip surface. We clearly detected high affinity binding of transferrin to the Tf-receptor with an overall affinity (K_D) of 10 nM (**Figure 3A**). Binding was characterized by an association rate of k_a=3.1 x 10^5/M*s and showed a dissociation rate of k_d=3.2 x 10^{-3}/s, assuming a 1:1 interaction model. However, the dissociation rate emerged to be bi-phasic: a high dissociation rate was followed by a low dissociation rate, implying stable binding. High affinity binding could also be observed for the Tf-PEI conjugate to the Tf-receptor, though the overall affinity decreased nearly 6-fold (K_D=59 nM) compared to free transferrin, which was due to a lower association rate of Tf-PEI (k_a=5.3 x 10^{-3}/M*s) (Figure 3B). Binding of Tf-PEI to the TfR was more stable compared to free transferrin since no first high dissociation phase was observed. Assuming a 1:1 binding stoichiometry the overall dissociation rate of Tf-PEI to the Tf-receptor was comparable to that of free transferrin (k_d=3.1 x 10^{-3}/s). Free PEI showed no interaction with the chip surface, however, the Tf-Mel-PEI blend bound the Tf-receptor with higher affinity compared to Tf-PEI, showing an overall affinity of 14 nM with an association rate k_a of 6.6 x 10^4/M*s and an overall dissociation rate k_d of 9.2 x 10^{-4}/s (Figure 3C). In summary, for all Tf-conjugates a stable binding to the Tf-receptor could be demonstrated, whereby the binding of the Tf-Mel-PEI blend was the most stable one.

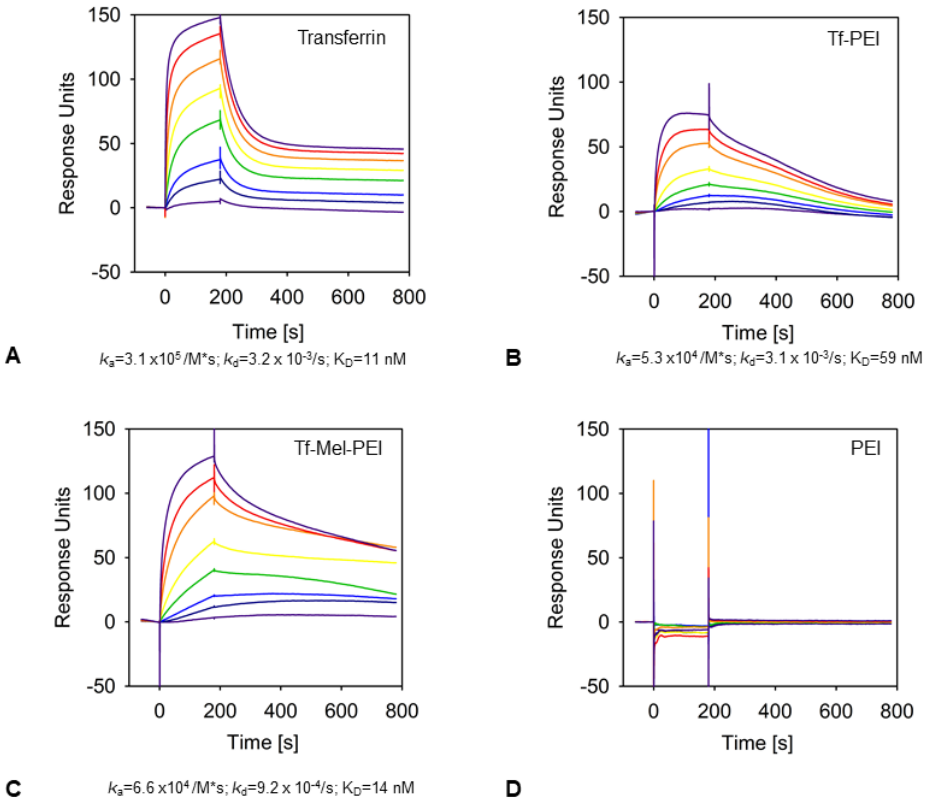


Figure 3. Binding of Tf-PEI and Tf-Mel-PEI was analyzed by Surface Plasmon Resonance (SPR) spectroscopy. The transferrin receptor (TfR) was captured via its His-tag onto a CM5 sensor chip coated with anti-His antibody, and solutions of 1 nM (light purple), 10 nM (dark blue), 25 nM (blue), 50 nM (green), 100 nM (yellow), 250 nM (orange), 500 nM (red), 1000 nM (dark purple) of free transferrin (A), TF-PEI conjugate (B), Tf-Mel-PEI (C) conjugate, and unmodified PEI polyplexes (D), respectively, were passed over the chip. The plots are representatives of four independently performed experiments.

2.5. Cellular Uptake of Polyplexes

Cellular uptake of Alexa Fluor 488-labeled siRNA (AF488-siRNA) was evaluated by flow cytometry. **Figure 4A** shows the Median Fluorescence Intensity (MFI) of Jurkat cells transfected with polyplexes composed of AF488-siRNA and different polymer formulations compared to untreated cells and free siRNA as negative controls, and lipofectamine (LF) as positive control. As previously observed, [9] coupling of Tf to PEI improved uptake efficiencies for N/P 5 and 10, leading to comparable results with LF.

Addition of melittin to PEI, however, tremendously increased the obtained MFI for all tested N/P ratios. Finally, blending of Tf-PEI and Mel-PEI in a 1:1 ratio (="Tf-Mel-PEI"), resulted in the most effective uptake for N/P ratios 10 and 15. Enhancing the Tf-PEI portion in the blend to 75 % did not further improve uptake, but rather lowered obtained MFI. Trypan blue quenching, to diminish extra-cellular fluorescent signals resulting from siRNA bound but not internalized by cells, eventuated in overall lower MFI values, nonetheless proving the basic correlations true and leaving Tf-Mel-PEI with a more than 5x higher uptake signal than the positive control LF.

Figure 4B illustrates cellular uptake of polyplexes in human primary $CD4^+$ T cells. While in naïve T cells with negligible TfR expression, Tf-PEI and Tf-Mel-PEI polyplexes were already more efficiently taken up than their Tf-free counterparts, after activation of the cells and thereby upregulation of their TfR expression, this enhancement was even more pronounced, implying Tf related uptake mechanisms. Furthermore, blending of Tf-PEI with Mel-PEI additionally increased the MFI 2.8 fold compared to Tf-PEI alone. As observed for Jurkat cells, trypan blue quenching slightly decreased measured signals, however, did not affect the captured correlations between the formulations (data not shown).

To further manifest Tf related uptake of Tf containing polyplexes, a Tf competition assay was performed in Jurkat cells as depicted in Figure 4C. MFI values, and therefore uptake, of both Tf-PEI and Tf-Mel-PEI polyplexes were distinctly and gradually reduced by increasing concentrations of free Tf in the culture medium during transfection, indicating a Tf dependent uptake mechanism. Cellular uptake of non Tf-containing polyplexes composed of PEI and Mel-PEI, however, was not influenced by free Tf concentration.

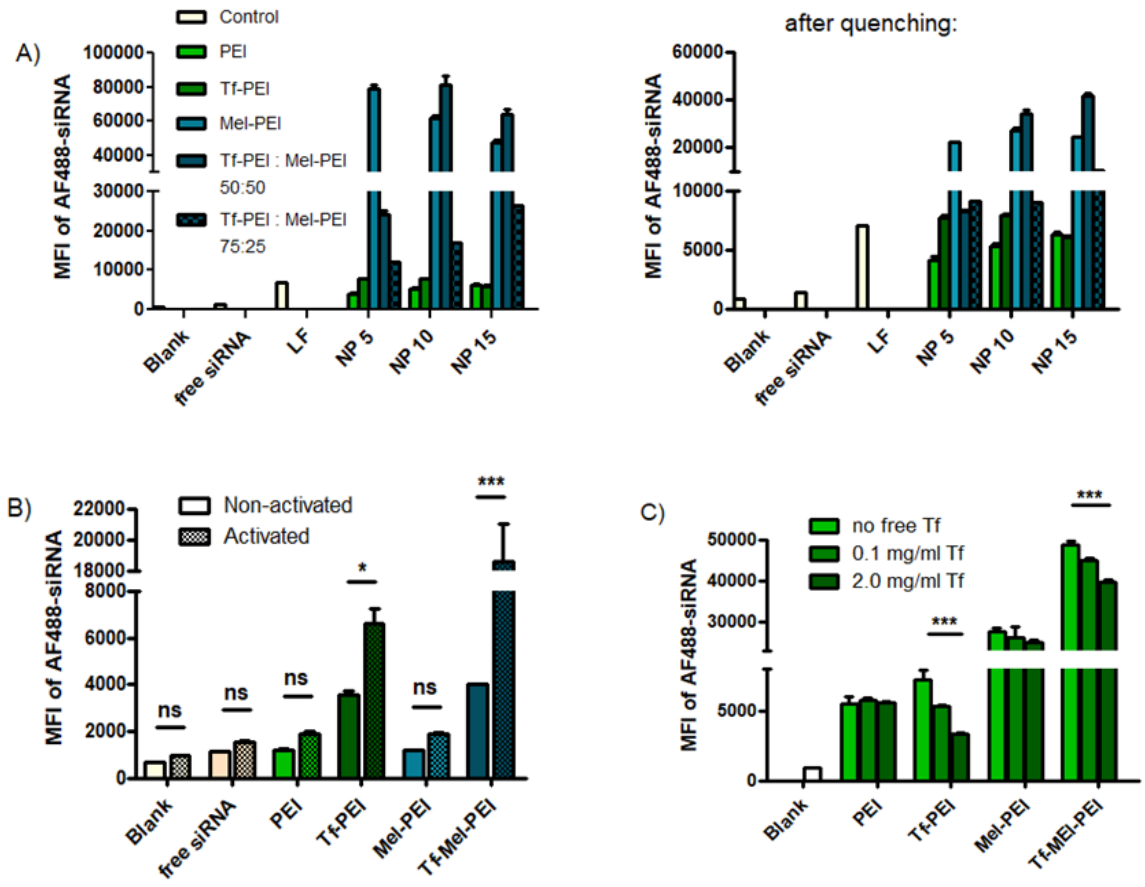


Figure 4. Cellular uptake of polyplexes as measured by flow cytometry and presented as median fluorescence intensity (MFI). A) Uptake in Jurkat cells before (left panel) and after (right panel) trypan blue quenching. B) Uptake in primary $CD4^+$ human T cells. "Blank" represents untreated control cells, "free siRNA" represents cells treated only with free siRNA, "LF" represents cells treated with lipofectamine lipoplexes. (Data points indicate mean ± SD, n = 3; Two-way ANOVA, *, $p < 0.05$; ***, $p < 0.005$). C) Uptake of polyplexes in Jurkat cells with or without increasing concentrations of free Tf in the culture medium. (Data points indicate mean ± SD, n = 3; One-way ANOVA, ***, $p < 0.005$).

2.6. GFP Transfection and Knockdown

To evaluate transfection efficiencies, Jurkat cells were transfected with polyplexes containing a plasmid expressing green fluorescent protein (GFP) under an active constitutive promoter and resulting fluorescence levels were determined by flow cytometry. As seen in **Figure 5A**, cells treated with PEI/plasmid polyplexes showed MFI values similar to untreated cells or cells only incubated with free plasmid, indicating no sufficient transfection. Tf-PEI and Mel-PEI, however, demonstrated comparable transfection efficiencies as LF, while the Tf-Mel-PEI blend even surpassed the positive control by approximately 40 %.

Next, GFP protein knockdown was tested in H1299 cells stably expressing EGFP (H1299-mEGFP) using polyplexes encapsulating GFP targeting siRNA (GFP-siRNA) (either with or without simultaneous treatment with the lysosomotropic agent chloroquine) compared to respective particles carrying NC-siRNA as negative controls. Figure 5B depicts that neither PEI nor Tf-PEI polyplexes achieved a silencing effect, while Mel-PEI and Tf-Mel-PEI resulted in significant knockdown. Tf-Mel-PEI was moreover the only tested treatment that could not be further enhanced with chloroquine, implying sufficient endogenous endosomal escape capacity.

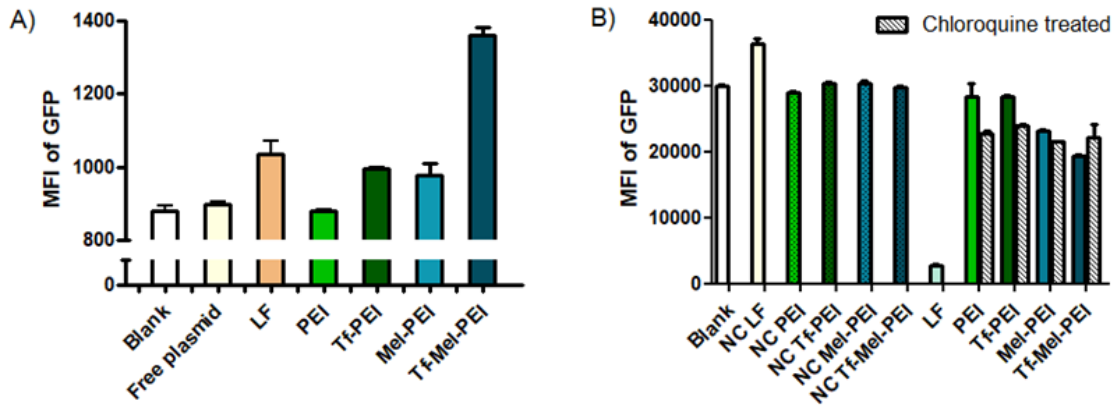


Figure 5. A) Transfection of Jurkat cells with a GFP expressing plasmid measured by flow cytometry as median fluorescence intensity (MFI) of GFP. B) GFP knockdown in H1299-mEGFP cells depicted as MFI of GFP after treatment with GFP siRNA or scrambled control siRNA (NC) with or without additional chloroquine treatment. "LF" represents cells treated with lipofectamine lipoplexes. (Data points indicate mean ± SD, n = 3).

2.7. GAPDH Knockdown

We then investigated whether the high cellular uptake also correlates with corresponding gene silencing. Downregulation was examined using siRNA specifically targeting the house-keeping gene *GAPDH* (GAPDH-siRNA) followed by quantification of respective mRNA levels via real time PCR (RT-PCR). All tested formulations were also applied containing scrambled non-targeting siRNA (NC-siRNA) as a negative control. **Figure 6A** shows *GAPDH* expression normalized to β-actin after treatment of Jurkat cells. While Tf-PEI and Tf-Mel-PEI/GAPDH-siRNA polyplexes were able to significantly silence the gene expression compared to respective NC-siRNA and untreated control groups, no significant difference was obtained between the groups of PEI/GAPDH-siRNA, PEI/NC-siRNA and untreated blank samples. The blending with Mel-PEI further enhanced the silencing effect of Tf-PEI, achieving a knockdown of 70 % compared to the negative control. Similar treatment effects were observed in activated human primary $CD4^+$ T cells (Figure 6B). Again, unmodified PEI polyplexes did not result in efficient gene silencing, while Tf-PEI and, particularly, Tf-Mel-PEI achieved considerable knockdown efficiencies of 43 and 76 %, compared to negative controls, respectively.

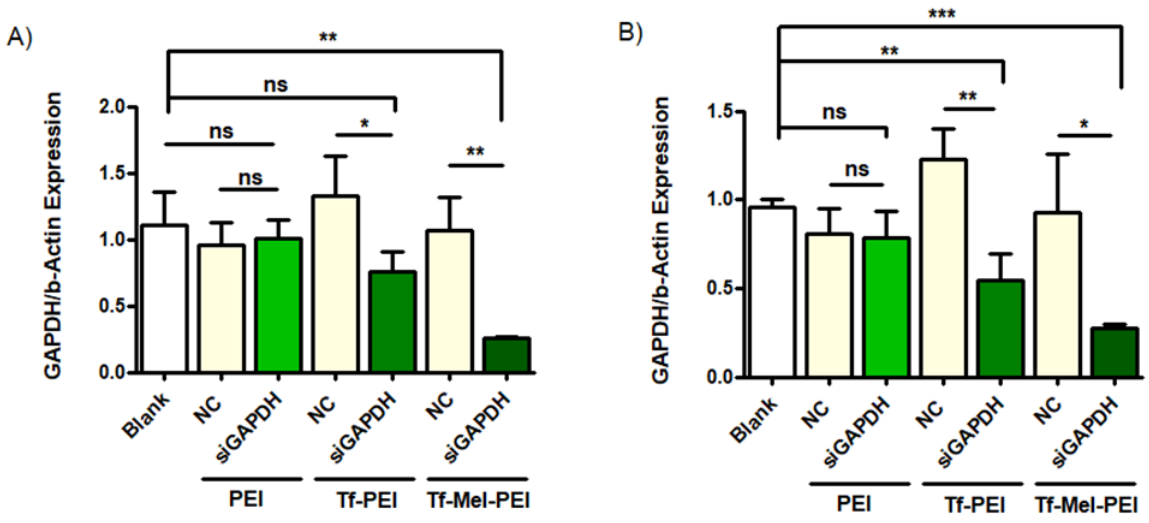


Figure 6. *GAPDH* knockdown in A) Jurkat cells and B) primary $CD4^+$ human T cells after treatment with *GAPDH* siRNA (siGAPDH; green bars, indicated by polyplex type used for transfection) or scrambled siRNA as negative control (NC). (Data points indicate mean ± SD, n = 3; One-way ANOVA, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$).

2.8. Endosomal Release

Endosomal disruption in living cells was examined by acridine orange staining. Adherent A549 cells were stained with the cell-permeant nucleic acid binding dye that emits red fluorescence in the endosome, but green fluorescence at cytoplasmic pH[425], and incubated with polyplexes. A positive control sample was exposed to chloroquine prior to imaging. **Figure 7A** summarizes the degree of lysosomal disruption as visualized by an increase in green fluorescence captured by fluorescence microscopy. Both melittin containing conjugates Mel-PEI (7A5) and Tf-Mel-PEI (7A6) induced acridine orange release from cytoplasmic endosomal vesicles similarly to chloroquine (7A2), proving efficient endosomal disruption in the treated cells. Polyplexes composed of unmodified PEI (7A3), however, did not visibly influence endosomal integrity, eventuating in red fluorescing cells comparable to the blank sample (7A1). Tf-PEI particles (7A4) only resulted in a vague green signal, suggesting insignificant dye release.

To portray endosomal release of polyplexes taken up into cells in a more accurate way, Jurkat cells were transfected with AF488-siRNA and stained with LysoTracker Red DND-99, a fluorescent probe that accumulates in acidic vesicles, as well as DAPI before analyzing them by confocal microscopy. **Figure 7B** illustrates the different polyplex treatments and resulting effects on cellular uptake and endosomal release of fluorescently labeled siRNA. Blue areas depict the cell nuclei colored with DAPI, while red staining reflects lysosomes, and green staining is accounted to the incorporated siRNA. As Figure 7B2 shows, no green signal can be detected in the free siRNA sample, meaning that no successful uptake can take place without an appropriate delivery system. Uptake with PEI (7B3) and Tf-PEI (7B4), however, results in a punctate distribution of fluorescent siRNA with an appearance similar to the red stained lysosomes, suggesting endosomal entrapment of the delivered cargo. In contrast to this, Mel-PEI treated cells (7B5) show rather cloudy green signals evenly spread over the cells, implying that the siRNA was not only successfully delivered inside the cells, but also able to escape the endosomes and consistently distribute in the cytoplasm. For cells transfected with Tf-Mel-PEI polyplexes (7B6), both the point-shaped and the homogeneously spread signal can be observed, entailing that while parts of the

delivered siRNA were still entrapped in the endosomes, a significant proportion was also able to escape and spread over the cytoplasm.

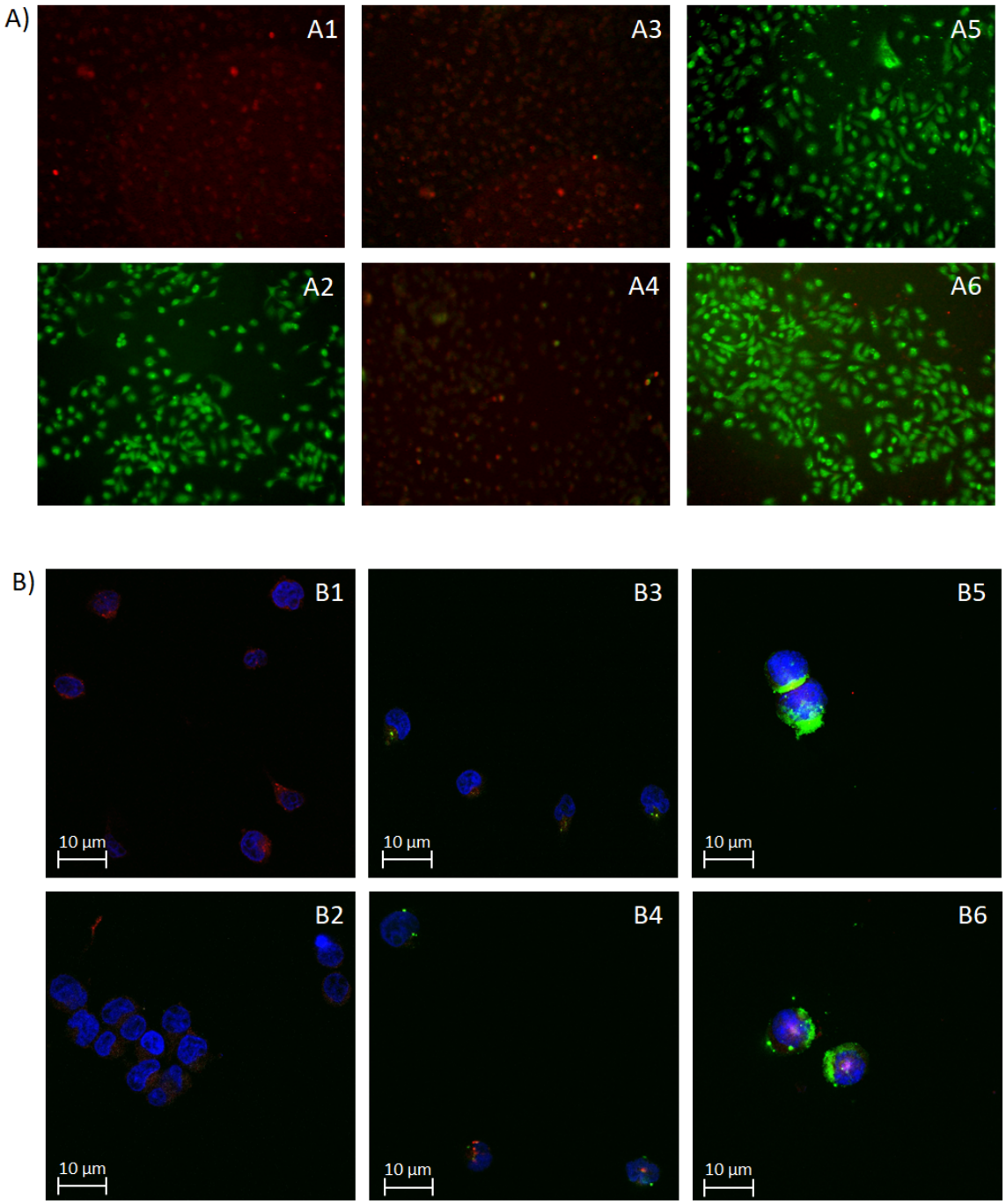


Figure 7. A) Fluorescence microscopy images of A549 cells after staining with acridine orange and treatment with chloroquine (A2), PEI polyplexes (A3), Tf-PEI polyplexes (A4), Mel-PEI polyplexes (A5) and Tf-Mel-PEI polyplexes (A6). A1 represents untreated cells as blank. B) Confocal images after transfection of Jurkat cells and staining with DAPI (blue, depicting the cell nuclei) and LysoTracker Red DND-99 (red, representing the lysosomes). B1 shows the DAPI and Lysotracker only control, B2-B6 show cells transfected with free siRNA, PEI, Tf-PEI, Mel-PEI and Tf-Mel-PEI polyplexes, respectively.

2.9. Toxicity

To confirm compatibility with living cells, polyplex cytotoxicity was evaluated with an MTS assay performed in A549 cells that had been incubated with the different formulations at increasing N/P ratios. As exemplified in **Figure 8A**, Mel-PEI demonstrated the greatest negative influence on cell viability, while unmodified PEI was slightly better tolerated. No significant differences were found for the effects of Tf-PEI and Tf-Mel-PEI for any tested condition, indicating that blending with Mel-PEI has no negative influence on cell tolerability. Notably, at treatment relevant N/P ratios of 10 and 15, both Tf-PEI and Tf-Mel-PEI showed average cell viability rates of around 90 %.
For further examination of cell tolerability focusing on membrane integrity, an LDH Assay was performed in A549 cells as presented in Figure 8B. Analogous to the MTS Assay, cells were incubated with polyplexes at different N/P ratios and the resulting LDH release was measured in comparison to untreated blank samples. Again, no significant difference could be found comparing Tf-PEI and Tf-Mel-PEI for any tested N/P ratios. Moreover, no significant increase in LDH levels was observed for any applied treatment compared to untreated cells, suggesting that neither formulation would have any noticeable effect on membrane stability.

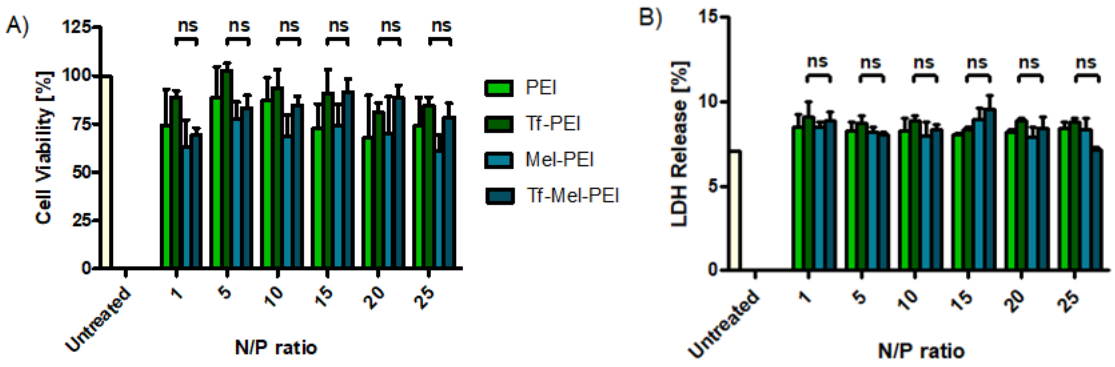


Figure 8. A) Cell viability as measured by MTS assay for polyplexes with different N/P ratios: 1, 5, 10, 15, 20, 25. Untreated cells represent 100 % viability. B) Cellular membrane integrity as measured by LDH assay. Cells treated with lysis buffer represent 100 % LDH release. (Data points indicate mean ± SD, n = 3; One-way ANOVA, ns, not significant).

3. Discussion and Conclusion

PEI is the most intensively studied cationic polymer in terms of non-viral gene delivery, being widely investigated for potential *in vivo* applications.[426] Its high density of positive charges mediates the arrangement of siRNA in non-covalent polyplexes via electrostatic interactions.[427] In our previous work, low molecular weight PEI (5 kDa) was used to minimize its toxic effects and coupled to Tf in order to create trojan horse like particles that specifically deliver siRNA to disease-related T cells in the lung.[40] As these activated T cells exhibit a high metabolic activity and hence high iron consumption, Tf receptors are overexpressed in order to enhance the uptake of Tf-coupled iron.[23] This can be exploited as an entry gate for Tf-targeted polyplexes reaching the, otherwise hard to transfect, primary T cells. Despite the critically-discussed hypothesis that positively charged polymers such as PEI demonstrate the proton sponge effect upon acidification of endosomal compartments,[428] leading to disruption and release of delivered payload, PEI generally exhibits rather low endosomolytic properties, which is especially true for LMW-PEI. Since it is crucial that sufficient siRNA molecules reach the cytoplasm to be therapeutically relevant, it was aimed to apply an additional endosomal escape mechanism in this work by incorporating melittin into the Tf-PEI delivery system. Melittin is a 26 amino acid peptide that was first purified from the venom of the European honeybee in 1958 and shows a particularly strong lytic activity due to its high affinity to lipid membranes.[423] Although the exact mechanisms of this properties have not yet been fully identified, it is currently assumed that the membrane destabilization is taking place in a two-step mechanism that is comparable to the effect of detergents:[422, 423] melittin first reacts with negatively charged lipid head groups via electrostatic interactions and binds parallel to the membrane. When a critical peptide concentration is reached, melittin molecules rearrange perpendicular to the membrane surface and form pores, thereby interrupting and destabilizing the membrane bilayer. It was successfully shown that despite of these lytic properties, *in vivo* toxicity of naked melittin can distinctly be attenuated by respective modifications, including sequestration of melittin in particles[429] or reversibly masking its hydrophobic residues.[430]

In this work, melittin was modified with dimethylmaleic anhydride (DMMAn) masking its lytic activity and hence minimizing acute toxicity at neutral pH. Once the polyplexes are taken up into the endosome, the acidic environment triggers cleavage of these protecting groups and recovery of melittin's membrane destabilizing effects. Initial studies showed diminished lytic activity by irreversibly masking lysines and the terminal amino function.[431] Rozema et al. then reversibly acylated melittin with a dimethylmaleic anhydride [432], following the role model of viruses such as adenovirus which presents its endosomolytic residues only upon endosomal acidification[433]. Meyer et al. further developed this approach by covalently linking this reversibly masked melittin to polycations delivering DNA.[54] The reversible reaction of maleic anhydrides with the amines of melittin is reverted at endosomal pH, restoring lytic activity[434] to aid subsequent cytoplasmic release of the delivered cargo. We ultimately combined the benefits of coupling both transferrin and melittin to PEI as a gene delivery system by blending the two conjugates Tf-PEI and Mel-PEI yielding one bioresponsive siRNA carrier capable of both T cell targeted and efficient cytoplasmic delivery.

DLS measurements of resulting polyplexes confirmed our previous observations that modification of PEI with the negatively charged hydrophilic glycoprotein Tf eventuates in a considerable reduction of particle sizes.[40] Coupling of PEI with melittin likewise resulted in smaller polyplexes, while the blending of Tf-PEI with Mel-PEI yielded particles with even smaller and more homogenous hydrodynamic diameters below 200 nm, presenting optimal characteristics for efficient pulmonary transfection. The assumed decoration with the soluble negatively charged Tf on the outside of polyplexes is supported by a decrease of positive surface charges for TF-PEI vs. PEI as well as for Tf-Mel-PEI vs. Mel-PEI. While all tested N/P ratios of Tf-PEI polyplexes held negative zeta potentials, Tf-Mel-PEI blend particles were slightly positively charged for N/P ratios higher than or equal 10. Thus, the shielding of positive charges from PEI by the negatively charged Tf appears to be influenced by the proportion of polymer to siRNA. At lower N/P ratios, more negative charges from transferrin as well as from negatively charged masked melittin seem to be present at the outer regions of the polyplexes resulting in an overall slightly negative zeta potential. At higher N/P ratios with excess polymer, the particle composition changes to more PEI being present at the surface,

raising the overall charge to positive values. Interestingly, viruses as well as virus-mimicking particles coated with both positively and negatively charged groups were found to avoid electrostatic mucus adhesion, eventuating in minimized entrapment. [435] The modified surface properties may hence facilitate particle mobility in lung fluids and thereby improve respective *in vivo* behavior of Tf-Mel-PEI polyplexes.

To investigate the influence of melittin unmasking in acidic environments on polyplex characteristics, DLS and LDA measurements were also conducted at pH 5. As shown in **Figure S1A**, zeta potentials of Mel-PEI as well as Tf-Mel-PEI polyplexes overall increase leading to invariably positively charged particles which can be explained by the unmasked amine groups of melittin. Concerning the hydrodynamic diameters, Mel-PEI polyplexes show smaller and more homogeneously distributed sizes at pH 5 (Figure S1B) compared to pH 7.5. Since the amines of melittin are now unprotected and free for interaction with the negatively charged siRNA, it can be expected that this results in an overall enhanced interaction of polymer and siRNA leading to an even tighter packing. In contrast, hydrodynamic diameters of Tf-Mel-PEI blend particles consistently increase at all tested N/P ratios. As they do no longer display negative charges at any of the tested N/P ratios, it can be expected that the assumed exterior decoration with transferrin leading to shielding and tight condensation does not convey to the acidic environment. It is even conceivable that negative charges of transferrin and positive charges of unmasked melittin now result in aggregation forming distinctly larger particles.

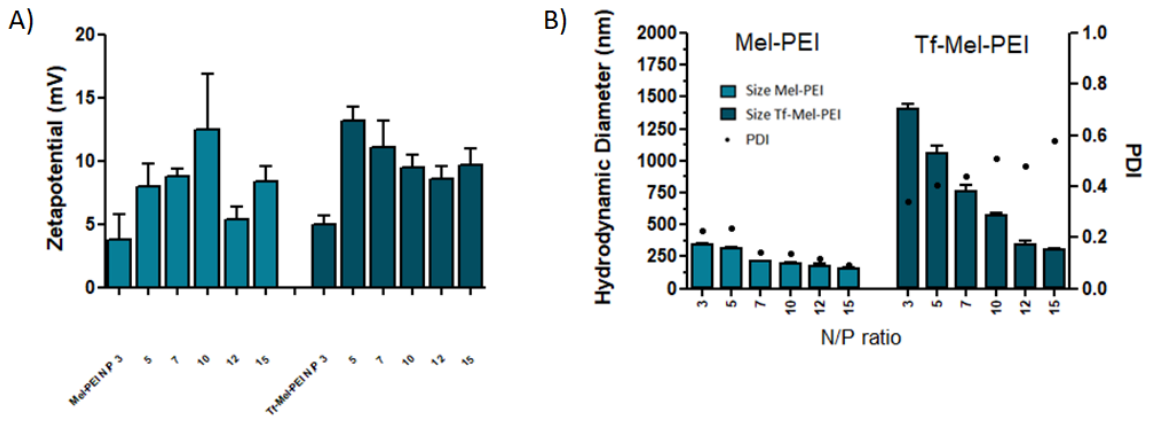


Figure S1. Laser Doppler anemometry and dynamic light scattering measurements of Mel-PEI and Tf-Mel-PEI polyplexes in HBS at pH 5. A) Zeta potentials and B) hydrodynamic diameters (left y-axis) and polydispersity indices (PDI, right y-axis) at different N/P ratios: 3, 5, 7, 10, 12, 15. (Data points indicate mean ± SD, n = 3).

Since siRNA is easily degraded and rapidly excreted upon systemic injection, local application is a promising concept to facilitate efficient delivery. The lung offers advantageous physiological characteristics as a drug target organ with its large surface area, thin and highly vascularized epithelium and the absence of serum proteins [436] Obstacles on this delivery route, however, can be respiratory mucus and airway surface liquid (surfactant). Cationic carrier systems, in particular, can nonspecifically interact with the negatively charged lung lining fluids, be entrapped, destabilized and their mobility is decreased.[437] It was shown that modifying positive PEI polyplexes with the negatively charged hydrophilic glycoprotein Tf results in reduced interactions with polyanions and thus enhanced stability.[43] This was also demonstrated in our previous work, wherein Tf-PEI polyplexes exhibited increased stability in artificial lung lining fluids[40]. Here, we examined the stability of all conjugates again, including the Tf-Mel-PEI blend. As shown by the SYBR gold assay, blending with Mel-PEI improved siRNA encapsulation efficiencies for all tested N/P ratios. The partly constrained encapsulation efficiencies of Tf-PEI compared to unmodified PEI polyplexes may be explained by steric hindrance of the relatively large Tf molecules which not only influence the interaction of PEI and siRNA in general, but also capture some of the primary amines in the polymer as potential binding sites for siRNA. By decreasing the Tf portion in the blend in favor of

adding melittin molecules differing both in size and surface charge, these destabilizing effects of Tf might be attenuated.

Adjusted experiments in Alveofact® and Mucin identified that the Tf-Mel-PEI blend also showed superior stability within the influence of surface active phospholipids and mucus glycoproteins, respectively, implying that addition of melittin has a further stabilizing effect on the polyplexes for successful pulmonary application, potentially due to additional positive charges present in melittin.

SPR measurements to quantitatively analyze binding affinities of the polyplexes to TfR confirmed the specific binding of transferrin containing polyplexes, while no binding was observed for unmodified PEI particles. Comparison of binding affinities revealed that Tf-PEI particles bind even more stable than free transferrin, showing an overall comparable dissociation rate. As we have previously demonstrated, multivalent targeted nanoparticles can in general display stronger receptor binding than respective monovalent ligands may be able to achieve under *in vitro* conditions. [438] The lowered association rate observed here for polyplexes may be caused by steric hindrance of PEI resulting in a reduced accessibility of Tf in the particles compared to the free ligand. Lack of the early high dissociation rate for Tf-PEI compared to free Tf, however, eventuates in an overall enhanced binding stability. Considering the fact that only an adequately stable and sustained receptor binding can result in sufficient downstream signaling and endocytosis, this parameter is extremely relevant for evaluating *in vivo* performance. Strikingly, Tf-Mel-PEI blend polyplexes presented an even increased binding affinity compared to the original conjugate. It was recently proposed that melittin itself also interacts with TfR[439], which taken together with evidently improved surface properties in the altered composition of the blend particles could explain this improvement in receptor binding. In any case, it can be expected that the addition of melittin to the conjugate would not interfere with specific binding on TfR of targeted cells.

Cationic materials such as PEI and melittin can non-specifically interact with negatively charged cell membranes triggering adsorptive endocytosis. This non-targeted uptake could potentially interfere with the desired uptake exclusively in activated T cells. In Jurkat cells, an immortalized cell line representative of T cells, the cellular uptake of Tf-

Mel-PEI blend particles was remarkably higher compared to all other polyplex types at treatment relevant N/P ratios 10 and 15. Increasing the Tf-PEI portion in the blend to 75 % could, however, not improve uptake further, suggesting that the 50:50 blend Tf-Mel-PEI already contains enough Tf for efficient uptake. Addition of positively charged melittin even seems to be beneficial for more efficient siRNA condensation, resulting in distinctly enhanced uptake. As continuous cell lines significantly differentiate from primary cells in their membrane composition and endocytosis profiles, we decided to validate these data in primary T cells. In TfR overexpressing activated CD4+ T cells, the distinctly higher uptake of Tf-targeted polyplexes compared to unmodified PEI and Mel-PEI suggests efficient Tf-mediated endocytosis of Tf-PEI and Tf-Mel-PEI. The preferential uptake of Tf-PEI in activated as opposed to naïve T cells with a negligible receptor expression, was even more pronounced after the addition of melittin. The improved binding characteristics of the Tf-Mel-PEI blend demonstrated in the SPR experiments are also reflected in the uptake behavior of the polyplexes. Tf-Mel-PEI blends, therefore, seem to exhibit ideal characteristics for most efficient internalization without losing any of the targeting potential of Tf-PEI. Increasing the Tf-PEI fraction to greater than 50% did not provide any additional targeting benefit and increasing the melittin containing fraction above 50% would presumably decrease the specific binding affinity too much. Therefore, we decided to further pursue the 50:50 blend of Tf-Mel-PEI as the most promising candidate for therapeutic application. Although N/P 15 showed slightly superior uptake efficiencies than N/P 10, an increased polymer excess usually correlates with a higher toxicity, so that N/P 10 was chosen for subsequent knockdown studies.

The specificity of uptake of the Tf-containing polyplexes was confirmed by a Tf competition assay. While uptake of those polyplexes without the targeting ligand, as expected, was not influenced by the presence of free transferrin, the Tf-targeted polyplexes exhibited clearly compromised uptake efficiencies, although the uptake was not blocked completely. We recently described how monovalent ligands cannot fully outcompete binding of multivalent ligands.[438] This concentration-dependent influence of additional free Tf molecules competing for the binding sites at the TfR supports the anticipated hypothesis that both Tf-PEI and Tf-Mel-PEI polyplexes are taken up via TfR mediated endocytosis. Most notably, these results show that the

addition of melittin to the original Tf-PEI conjugate does not affect its target specificity. Thus, even the reduced Tf amount of 50 % in the blend is apparently sufficient for specific receptor binding, and it can be expected that enough Tf is still located towards the surface of the blend polyplexes, being able to initiate TfR binding.

Although uptake experiments confirmed efficient internalization of siRNA, its subsequent fate and functionality was still unknown. We therefore evaluated whether the polyplexes are also able to deliver a plasmid that would subsequently be expressed by the cells. As expected, Tf-Mel-PEI showed the highest transfection efficiencies, mirroring the uptake results. This improved transfection did also result in enhanced knockdown effects on GFP protein levels, which could not be further amplified by chloroquine treatment for Tf-Mel-PEI, but for all other tested polymers. Thus, the blend polyplexes seem to be the only delivery system being able to induce sufficient uptake and endosomal release of the delivered cargo on its own. However, it has to be noted that the used cell line stably expresses GFP which has a reported half-life of 26 h [440] and in unsynchronized cultures protein turnover rates are varied, which might potentially mask the overall efficiency of the knockdown. Since siRNA effects directly influence mRNA levels, these were then evaluated by qRT-PCR after treatment with the different polyplexes in the Jurkat cell line and in human primary CD4+ T cells. The improved uptake and endosomal escape properties of the Tf-Mel-PEI blend did indeed further enhance gene silencing. This confirms our hypothesis that insufficient endosomal release had in fact been a major hurdle limiting the knockdown potential of Tf-PEI and gives reason to assume that Tf-Mel-PEI can achieve significant gene silencing *in vivo*.

Based on these findings, it could already be postulated that adding melittin to the polyplexes does in fact improve endosomal escape of delivered siRNA. To verify that melittin-containing polyplexes are actually inducing endosomal disruption, acridine orange staining was performed in living cells. Acridine orange is a cell permeant, lysosomotropic, metachromatic dye with a luminescence wavelength strongly dependent on its concentration and extent of polymerization.[441] Under acidic conditions, such as in endosomes, it becomes protonated, entrapped, and accumulates as dimers, trimers or oligomers emitting red fluorescence (640 nm). On the other hand, when the endosomes are disrupted, the dye is homogenously distributed as a monomer

all over the cytoplasm emitting a green signal (525 nm), meaning that endosomal leakage can be visualized by an increase in green fluorescence. As expected, neither PEI nor Tf-PEI polyplexes resulted in significant dye release, while both melittin-containing polyplex types were able to visually induce acridine orange and therefore siRNA release from endosomal vesicles.

Endosomal release was further verified in Jurkat cells after transfection with fluorescently labeled siRNA via the different polyplexes. Following uptake with PEI or Tf-PEI, siRNAs demonstrated a punctate distribution which correlated and partly colocalized with lysosomal staining, confirming endosomal entrapment. Mel-PEI, on the other hand, showed a homogenous distribution of the siRNA throughout the cytoplasm. Remarkably, Tf-Mel-PEI treated cells depicted both punctate and diffused siRNA distribution, indicating that while a fraction of the siRNA remains within the endosomes, a significant proportion was able to escape, marking a definite improvement to the original Tf-PEI. These results confirmed the favorable effect of melittin on endosomal release of delivered siRNA, resulting in a distinct improvement in cytosolic accumulation and eventual gene silencing. As most endocytic vesicles are rapidly trafficked to the early endosome, as long as the escape does not happen before this fusion, the influence of internalization mechanisms on endosomal escape is not clarified.[51] It is therefore conceivable that besides the mere lytic activity of melittin, also the altered uptake of the Tf-Mel-PEI blends plays a role in this improvement. Beyond confirmation of the endosomolytic properties of Mel-PEI, these results also demonstrate that this lytic activity can be conveyed to the Tf-Mel-PEI blends, while still maintaining efficient Tf targeting abilities. Albeit in our previous work we showed that the reducible crosslinker SPDP improved the knockdown efficiency of Tf-PEI,[68] only the combination with sufficient endosomolysis really resulted in optimal gene silencing, underlining that both disassembly of the nanocomplex and cytoplasmic delivery are crucial factors for successful cytoplasmic delivery and subsequent interaction with the RNAi machinery.

As destabilization of cellular membranes naturally affects cell viability which can arouse acute toxicity, it is crucial not to generate this effect until the delivery system is undergoing the endo/lysosomal pathway. In the Tf-Mel-PEI blend this is achieved by a reversible pH-responsive shielding of the reactive groups of melittin, whose lytic

activity is hence restored once the polyplexes enter acidic compartments. Despite this protection, potential detrimental effects of melittin addition on cell tolerability were examined by MTS and LDH assay, investigating metabolic activity and membrane integrity of treated cells, respectively. Cationic polymers and peptides such as PEI and melittin in general exhibit cell toxicity due to their high density of positive charges which can be shielded by negatively charged Tf. This is portrayed by a distinctly higher metabolic activity of Tf-PEI vs. PEI and Tf-Mel-PEI vs. Mel-PEI treated cells for all tested N/P ratios. Interestingly, despite the slight positive charges of Tf-Mel-PEI blends at N/P equal and higher than 10, no significant increase in cell toxicity was observed compared to regular Tf-PEI. Regarding membrane integrity, none of the tested polyplexes induced significant LDH release compared to untreated cells, indicating that all polyplexes are well tolerated. These results confirm that masking of melittin efficiently shields its membrane lytic properties where needed, namely in the extracellular space and outside of the endosomes. Altogether these results indicate that the blending of Tf-PEI with Mel-PEI has no negative influences on cell tolerability *in vitro*. Since *in vivo* compatibility of Tf-PEI polyplexes was already confirmed in our previous study,[40] it is therefore to be expected that Tf-Mel-PEI blend particles would be equally well tolerated.

In conclusion, the aim of this work was to develop an improved siRNA delivery system to selectively target activated T cells in the lung and mediate efficient endosomal release of the delivered cargo. By adding an endosomolytic agent to our original Tf-PEI conjugate, we did not only specifically improve the cytoplasmic delivery of siRNA, but furthermore distinctly advanced several other important properties. The pH-responsive shielding exploiting endosomal acidification triggers lytic activity only in lysosomes, avoiding general toxicity. Blending of Tf-PEI and Mel-PEI therefore yields a novel versatile delivery system combining the advantageous features of both singular conjugates resulting in efficient and very specific gene silencing in primary activated T cells. Hence, Tf-Mel-PEI offers an auspicious platform for pulmonary siRNA delivery to selectively alter gene expression in disease-related cell subsets in the lung.

4. Experimental Section

4.1. Synthesis of Conjugates and Preparation of Polyplexes

Tf-PEI conjugates were prepared as recently described[40] and Mel-PEI conjugation was performed based on a previously described protocol with respective modifications.[54] In detail, 5k PEI (Lupasol® G100, BASF, Ludwigshafen, Germany) was dissolved in HEPES buffered saline (HBS) (20 mM HEPES, 150 mM NaCl, pH = 7.4) at a concentration of 5 mg/ml and mixed with an excess of (4-polyethylene glycol-N-succinimidyl 3-(2-pyridyldithio) propionate (PEG4-SPDP, 20 mM) (Thermo Fisher Scientific, Waltham, USA) in dry DMSO and stirred overnight. Cysteine modified melittin (Pepmic, Suzhou, China) was dissolved in HBS (3 mg/mL), a 150 mM solution of dithiothreitol (DTT) was added, and the mixture was shaken for 2 h. Reduced melittin was purified via 2000 MWCO centrifugal filters (Sartorius, Göttingen, Germany) with 100 mM HEPES, 125 mM NaOH and mixed with 1 ml of 2,3-Dimethyl-maleic anhydride solution (DMMAn, 1.5 mg/mL in EtOH) (Sigma-Aldrich, St. Louis, USA), stirring for 1h. Mel-DMMAn and PEI-SPDP were purified in separate runs with 2000 MWCO centrifugal filters and 20 mM HEPES, 0.5 M NaCl, 1 M Guanidine hydrochloride (pH = 8) or 3000 MWCO centrifugal filters and HBS, respectively. For the coupling reaction, both precursors were mixed and stirred overnight. Purification and desalination of the final Mel-PEI conjugate was achieved with 10000 MWCO centrifugal filters and HBS and the concentration of PEI was determined spectrophotometrically at a wavelength of 405 nm via TNBS assay. [442]

For polyplex preparation, PEI, Tf-PEI, Mel-PEI, or blends of both Tf-PEI and Mel-PEI (the term “Tf-Mel-PEI” is used for the 50:50 mixture throughout this article if not stated otherwise) were diluted in either HBS or 5 % glucose and defined amounts of siRNA were added in order to obtain specific amine to phosphate (N/P) ratios. The mass of polymer required for 50 pmol siRNA to yield a certain N/P ratio was calculated according to the following equation: m (PEI in pg) = 50 pmol x 43.1 g/mol x N/P x 52 (protonable unit of PEI = 43.1 g/mol, number of nucleotides of 25/27mer siRNA = 52). The formulations were mixed by pipetting and incubated for 20 min before further experiments. Lipofectamine 2000 (LF, Thermo Fisher Scientific) lipoplexes were prepared according to the manufacturer’s protocol.

4.2. Particle Characterization

To measure hydrodynamic diameter, PDI and zeta potential, 100 µl of polyplexes with different N/P ratios were prepared in HBS and added into a disposable micro-cuvette (Malvern Instruments, Malvern, UK). As an example, for N/P 10, 1.1 µl of a 1 mg/ml polymer solution were added to 48.9 µl HBS and then mixed with 50 µl of a solution containing 50 pmol siRNA in HBS via pipetting. Sizes were determined with a Zetasizer Nano ZS (Malvern Instruments) at 173° backscatter angle running 15 runs three times per sample. Viscosity of 0.88 mPa*s and 1.33 for refractive index were entered for data analysis with the Zetasizer software. Polyplexes were then diluted with 900 µl of nanopure water and transferred to a folded capillary cell (Malvern) to perform three zeta potential measurements for each sample using the same device.

4.3. siRNA Encapsulation and Stability

To determine siRNA condensation efficiencies, polyplexes with 50 pmol siRNA were prepared at different N/P ratios in 5 % glucose, distributed in a FluoroNunc 96-well white plate (Thermo Fisher Scientific) and incubated for 20 min. Subsequently, 30 µl of 4x SYBR® Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific) was added and incubated for 10 min in the dark. SYBR® Gold is a fluorescent nucleic acid staining dye that fluoresces only when intercalating with free siRNA. When the siRNA is protected in a coherent polyplex and not accessible to the dye, no fluorescence signal is detected. Measurements were conducted on a FLUOstar Omega (BMG Labtech, Ortenberg, Germany) at an excitation wavelength of 485/20 nm and an emission wavelength of 520/20 nm. Samples with only siRNA were prepared and treated and measured in the same way as the polyplex samples. Fluorescence of free siRNA (N/P =0) was used as a control for 100 % free siRNA.

For evaluating polyplex stability in lung fluids, modified SYBR® Gold Assays were performed [36] in the presence of mucin (Sigma-Aldrich) or lung surfactant Alveofact® (Lyomark Pharma, Oberhaching, Germany). Therefore, the described protocol was followed with polyplexes at N/P ratio 10, but SYBR® Gold was already added after 15 min and incubated for 10 min. Subsequently, 50 µl of a serial dilution of mucin or Alveofact® were added to obtain final concentrations of 0, 0.0005, 0.005, 0.05, 0.5, and 0.25 mg/ml and incubated for another 20 min before fluorescence measurements.

Autofluorescence of mucin was accounted for by measuring free siRNA samples with corresponding concentrations of mucin.

4.4. Transferrin Receptor Binding

The binding affinities of prepared polyplexes to the transferrin receptor (TfR-His) were investigated by Surface Plasmon Resonance (SPR) spectroscopy. To eliminate initially observed unspecific interactions of sticky PEI polymer with the sensor chip surface, gradient addition of NaCl to the sample buffers was tested, revealing that an extra 625 mM resulted in minimized interactions. After ensuring that the presence of additional salt does not influence the receptor binding and confirming hydrodynamic diameters in the high salt conditions (data not shown), measurements of all samples were conducted in HBS-N buffer (10 mM HEPES, pH 7.4, 150 mM NaCl) + 625 mM NaCl. Polyplexes were prepared at N/P ratio of 10 in HBS-N + 625 mM NaCl with equivalent concentrations of Tf and/or PEI, respectively. For comparison, free human holo-transferrin (Sigma Aldrich) was dissolved in the same buffer in which the polyplex samples were dispersed, and recombinant human transferrin receptor protein (Abcam, UK) was diluted in HBS-N without additional NaCl.

SPR assays were performed in a Biacore T200 device using Biacore CM5 Series S carboxymethyl dextran sensor chips (GE Healthcare, Freiburg, Germany) that were coated with His-antibodies from the Biacore His-capture kit (GE Healthcare). First, the chips were equilibrated with HBS-EP buffer (10 mM HEPES pH 7.4; 150 mM NaCl; 3 mM EDTA; 0.005 % (v/v) detergent P20) until the dextran matrix was swollen. Then, two of the four flow cells of the sensor chips were activated by injecting a 1:1 mixture of N-ethyl-N-(3-dimethylaminopropyl)carbodiimide hydrochloride and N-hydroxysuccinimide using the standard amine-coupling protocol. Both flow cells were loaded with a final concentration of 50 µg/ml of anti-histidine antibody in 10 mM acetate pH 4.5 using a contact time of 420 s, so that the surfaces contained densities of approximately 10,000 resonance units (RU). Free binding sites of the flow cells were saturated by injection of 1 M ethanolamine/HCl pH 8.0. Preparation of chip surfaces was carried out at a flow rate of 10 µl/min. Interaction analyses between the prepared polyplexes and TfR-His were then performed in HBS-N buffer + 625 mM NaCl. First, TfR-His (30 nM) was captured onto the second flow cell using a contact time of 60 s at a

constant flow rate of 10 µl/min, followed by a stabilization time of 20 s so that approximately 300-400 RU of TfR-His were captured. Increasing concentrations (1 nM, 10 nM, 25, nM, 50 nM, 100 nM, 2 x 250 nM, 500 nM, and 1000 nM) of the polyplexes were then injected onto both flow cells using a contact time of 180 s each and a final dissociation of 600 s using a flow rate of 30 µl/min. As control, similar concentrations of PEI were injected onto the chip. After each cycle the chip was regenerated by injection of 10 mM glycine pH 1.5 for 60 s at a flow rate of 30 µl/min over both flow cells, which completely removed TfR-His from the surface. All experiments were performed at 25°C. Sensorgrams were recorded using the Biacore T200 Control software 2.0 and analyzed with the Biacore T200 Evaluation software 2.0. The surface of flow cell 1 was used to obtain blank sensorgrams for subtraction of bulk refractive index background. The referenced sensorgrams were then normalized to a baseline of 0. Peaks in the sensorgrams at the beginning and the end of the injections emerged from the runtime difference between the flow cells of each chip.

4.5. Cell Culture

Jurkat cells, a human T lymphocyte cell line, were a kind gift from Prof. Dr. Heissmeyer (Institute for Immunology, Biomedical Center Munich) and were cultured in RPMI-1640 cell culture medium (Sigma-Aldrich) supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/l glucose, 10 % (v/v) heat inactivated fetal bovine serum (FBS, Sigma-Aldrich) and 1 x penicillin/streptomycin (Pen/Strep, Sigma-Aldrich). $CD4^+$ human primary T cells were isolated from freshly obtained buffy coats (DRK, Berlin, Germany) via magnetic bead separation with a $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as described before.[443] Cells were subsequently cultured in the same media as Jurkat cells and, where described, activated with anti-CD3 and anti-CD28 monoclonal antibodies (BS Biosciences, Franklin Lake, USA) at a final concentration of 5 µg/ml or 1 µg/ml, respectively. A549 cells, an adenocarcinomic human alveolar basal epithelial cell line, were grown in RPMI-1640 medium supplemented with 10 % FBS and 1 x Pen/Strep. H1299-mEGFP cells (ATCC, Manassas, USA) were cultured in RPMI-1640 medium supplemented with 2 mM L-Glutamine, 10 % FBS, 1 x Pen/Strep and 0.2 mg/ml geneticin (G418, Sigma-Aldrich). All cells were grown at a humidified atmosphere with 5 % CO_2 at 37 °C.

4.6. Cellular Uptake of Polyplexes

For uptake experiments, amine modified siRNA (Integrated DNA Technologies, Coralville, USA) was labeled with succinimidyl ester (NHS) modified AF 488 (Life Technologies, Carlsbad, USA) according to the manufacturer's protocol resulting in siRNA-AF488, and purified via ethanol precipitation and spin column binding as described before. [444] Polyplexes were prepared with 50 pmol siRNA-488 at different N/P ratios and lipoplexes were prepared with LF as positive control. Per well, 400.000 Jurkat cells or human primary $CD4^+$ T cells were seeded in 96-well plates (Thermo Fisher Scientific) at a concentration of $2x10^6$ cells/ml, transfected and incubated for 24 h. Primary human T cells were transfected directly after isolation from fresh buffy coats for non-activated cells or after 48 h of activation with CD3/CD28 antibodies for activated cells as described above. As negative controls, blank samples were seeded and left untreated and free siRNA samples were transfected with 50 pmol of siRNA diluted in 5 % glucose. Cells were harvested and washed three times before resuspension in 400 µl PBS/2 mM EDTA (Sigma-Aldrich). Samples were analyzed using an Attune® NxT flow cytometer (Thermo Fisher Scientific) with 488 nm excitation and 530/30 emission filter. All cells were gated according to morphology based on forward/sideward scattering, and 10.000 events were evaluated per sample. For trypan blue quenching, one half of each sample was washed with 0.4 % trypan blue to mask any signal originating from extracellular fluorescence.
For Tf competition assays, Jurkat cells were seeded in medium either without free Tf or containing 0.1 mg/ml or 2 mg/ml free Tf and transfected and analyzed as described above.

4.7. GAPDH Gene Knockdown

To test gene silencing abilities of the polyplexes, 500.000 Jurkat cells per sample were seeded in a 96-well plate or 650.000 $CD4^+$ T cells were seeded in a 48-well plate (Thermo Fisher Scientific) and activated for 48 h as described above. Cells were transfected with polyplexes containing 100 pmol siRNA either directed against *GAPDH* (siGAPDH) or scrambled negative control siRNA (siNC), respectively, at an N/P ratio of 10. After 24 h incubation, cells were harvested, and total RNA was isolated with the PureLink RNA mini kit according to the manufacturer's protocol with additional DNAse I

digestion (Thermo Fisher Scientific). cDNA was synthesized from RNA and amplified with Brilliant III ultra-fast SYBR® green QRT-PCR master mix kit (Agilent Technologies, Santa Clara, USA) and QuantiTect® primer assays Hs_*GAPDH*_1_SG and Hs_*ACTB*_2_SG (Qiagen, Venlo, Netherlands) using a qTOWER real-time PCR thermal cycler (Analytik Jena, Jena, Germany). Cycle threshold (Ct) values were obtained with the qPCRsoft software (Analytik Jena). An untreated blank sample was used to prepare a 1:5 serial dilution with 5 points as a standard curve and respective Ct values were plotted against the assigned concentration of each point: 1, 0.2, 0.004, 0.0008, and 0.00016. GAPDH gene expression was normalized by corresponding β-Actin expression for each sample.

4.8. GFP Transfection and Protein Knockdown

To evaluate transfection efficiencies, 100.000 Jurkat cells were seeded in a 96-well plate and transfected with polyplexes or LF lipoplexes containing 0.75 µg of *pCMV-GFP* plasmid (PlasmidFactory, Bielefeld, Germany) at N/P 10. As controls, some samples were left untreated as blank and others were only transfected with the free plasmid. All cells were incubated for 48 h before washing and analyzing for the MFI of GFP protein expression with an Attune® NxT flow cytometer (Thermo Fisher Scientific) using 488 nm excitation and 530/30 nm bandpass emission filter set. All samples were gated based on morphology with forward/sideward scattering, analyzing a minimum of 10.000 viable cells.

For GFP protein knockdown, 20.000 H1299-mEGFP cells were seeded in a 24-well plate (Thermo Fisher Scientific) 24 h prior to the experiment. Cells were then transfected using polyplexes containing 100 pmol of either siRNA targeting GFP (siGFP) or scrambled negative control siRNA (siNC) and incubated in cell culture medium with or without 100 µM chloroquine (Sigma-Aldrich). An additional volume of 500 µl of fresh medium was added 4 h after transfection, and cells were further incubated for a total of 72 h. Subsequently, all samples were trypsinized, washed and analyzed by flow cytometry as described above.

4.9. Endosomal Release

For endosomal escape studies, 15.000 A549 cells were seeded per well in 8-well chamber slides (Ibidi, Martinsried, Germany) 24 h prior to the experiment. All wells were then stained with acridine orange (Sigma-Aldrich) (0.1 µM in PBS) for 15 min and washed three times with PBS. Cells were resuspended in culture medium, transfected with polyplexes containing siGAPDH at N/P 10 to obtain a final siRNA concentration of 100 nM and incubated for 24 h. Appropriate wells were treated with 100 µM chloroquine for 15 min as positive controls and, subsequently, all wells were washed and resuspended in 300 µl PBS before analysis with a BZ-8100 (Biozero) fluorescence microscope (Keyence, Osaka, Japan).

For confocal images, 300.000 Jurkat cells were seeded in a 96-well plate and transfected with polyplexes containing AF488-siRNA at N/P 10. After 24 h incubation, cells were harvested, washed with PBS and attached to shi-fix coverslips (Everest Biotech, Oxfordshire, UK) according to the manufacturer's protocol in a 24-well plate. Appropriate wells were stained with 100 µM LysoTracker Red DND-99 (Invitrogen) in pre-warmed cell culture medium for 1 h at 37 °C, 5 % CO_2. After washing, cells were fixed with 4 % paraformaldehyde (PFA) in PBS for 15 min and washed again. DAPI was added to appropriate wells at a final concentration of 1 µg/ml in PBS and incubated for 20 min. All cells were washed again and mounted using FluorSave reagent (Merck Millipore, Billerica, USA) prior to analysis with a SP8 Inverted scanning confocal microscope (Leica Camera, Wetzlar, Germany).

4.10. Toxicity

For cell viability assessment via MTS assay, 5.000 A549 cells were seeded per well in phenol red free RPMI-1640 medium (Sigma-Aldrich) in a 96-well plate 24 h prior to the experiment. Cells were transfected with polyplexes containing 5 pmol siNC at different N/P ratios ranging from 1-25 and incubated for 24 h. Untreated cells were analogously incubated as blank controls. Subsequently, 20 µl of CellTiter 96® AQ_{ueous} One Solution (Promega, Madison, USA) was added to each well and incubated for 4 h at 37 °C, 5 % CO_2 before absorption measurement at 490 nm using a FLUOstar Omega (BMG Labtech, Ortenberg, Germany).

LDH release of treated cells was determined using the CytoTox-ONE Homogeneous Membrane Integrity Assay (Promega) according to the manufacturer's protocol. For this assay, 8.000 A549 cells were seeded in a 96-well plate 24 h prior to transfection. Cells were treated with polyplexes containing 5 pmol siNC at N/P ratios ranging from 1-25 and incubated for another 24 h. No cell controls were used to represent 0 % LDH release, while cells treated with lysis buffer represent 100 % LDH release. Untreated cells were cultivated as blank controls. Afterwards, 100 µl of Cyto-Tox-ONE Reagent was added to each well and the plate was shaken for 30 sec before 10 min of incubation. Subsequently, 50 µl of stop solution was added to each well, the plate was shaken again for 10 sec and fluorescence was recorded at excitation wavelength of 560 nm and emission at 590 nm using a FLUOstar Omega (BMG Labtech).

4.11. Statistical Analysis

All results are given as mean value ± standard deviation (SD). One-way ANOVA and two-way ANOVA with Bonferroni posthoc post-test were performed in GraphPad Prism (GraphPad Software, La Jolla, USA).

Chapter IX

Therapeutic Knockdown of GATA3

CHAPTER IX - Therapeutic Knockdown of GATA3

Targeted GATA3 knockdown in activated T Cells via pulmonary siRNA delivery as novel therapy for allergic asthma

The authors of this manuscript include Yuran Xie, Aditi Mehta, Olivia M. Merkel and me. I performed all experiments and wrote the chapter.

Abstract

GATA3 gene silencing in activated T cells displays a promising option to early-on prevent the activation of pathological pathways in the disease formation of allergic asthma. The central transcription factor of T helper 2 (Th2) cell cytokines IL-4, IL-5, and IL-13 plays a major role in immune and inflammatory cascades underlying asthmatic processes in the airways. Pulmonary delivery of small interfering RNAs (siRNA) to induce GATA3 knockdown within disease related T cells in asthmatic lungs via RNA interference (RNAi) presents an auspicious basis to realize this strategy, however, this approach still faces some important hurdles. Main obstacles for successful siRNA delivery in general comprise stability and targeting concerns, while in addition, the transfection of T cells presents a particularly challenging task by itself. In previous studies, we have developed and advanced an eligible siRNA delivery system composed of polyethylenimine (PEI) as polycationic carrier, transferrin (Tf) as targeting ligand and melittin (Mel) as endosomolytic agent. Resulting Tf-Mel-PEI polyplexes exhibited ideal characteristics for targeted siRNA delivery to activated T cells and achieved efficient and sequence-specific gene knockdown of surrogate genes in primary T cells *ex vivo*. In this work, the therapeutic potential of this carrier system was evaluated in a cellular model displaying the activated status of T cells as seen in asthmatic lungs. Therefore, a protocol was optimized for antibody-based activation of both primary human T cells as well as Jurkat cells, a human T lymphocyte cell line. Activation status was evaluated via determination of Tf receptor expression, GATA3 gene expression, and respective cytokine levels and resulted in a distinct increase of all three parameters.

Moreover, a suitable siRNA sequence combination was found for effective and therapeutically relevant GATA3 gene silencing in human cells. The findings described here support the suitability of Tf-Mel-PEI as siRNA delivery system for targeted gene knockdown in activated T cells in general and show 75 % GATA3 gene silencing resulting in 30-100% reduction of Th2 cytokine levels which lays the groundwork for a potential novel allergic asthma therapy.

1. Introduction

Asthma is a major public health problem affecting 339 million people in all regions of the world and accounting for 1000 deaths per day. [1] The disease not only causes a high disability and death burden, but also raises tremendous socioeconomic issues. Although currently asthma symptoms can be controlled in the majority of patients using standard therapies mainly based on corticosteroids as controller and beta-2-sympathomimetics as reliever medications, in a distinct portion of patients (5-10 %) symptoms can still not be adequately commanded. [8] This is particularly dangerous, as these patients experience worse asthma symptoms, more concomitant comorbidities and, moreover, a specifically high risk of mortality. It is therefore inevitable to find novel, more precise treatment options for this group of patients. Asthma is a reversible disease of the airways characterized by inflammation resulting in bronchoconstriction, enhanced mucus production, hyperresponsiveness and ultimately remodeling of the airways. [3] The fact that current therapies only show limited benefits in certain subgroups of patients may be due to the heterogenicity of the disease resulting in several different phenotypes with distinct clinical, functional and pathological patterns. [445] The underlying pathological base is distinguished by different patterns of cytokine-based airway inflammation involving immune and inflammatory cell types such as T and B lymphocytes. These cytokines therefore display promising targets of novel therapies tailored for patients suffering from severe asthma who do not fully respond to conventional treatments. [24]

Allergic asthma plays a major role in this context, comprising all levels of disease severity and being involved in the disease pattern of 50-80 % of all severe asthma patients. [10] This asthma phenotype class is understood to be actuated by an immune-

inflammatory response driven by type 2 T helper (Th2) cells orchestrating a complex interplay between the innate and adaptive immune system. [205] Prevalently, these processes already start in the childhood via sensitization to common inhaled allergens such as house dust mites or pollens. These allergens are taken up by antigen-presenting cells (APCs), for example dendritic cells (DCs), processing and presenting antigenic molecules to naïve T helper cells. Consequently, allergen-specific Th2 cells are activated and produce and secrete the major Th2 cytokines interleukin (IL)-4, IL-5, and IL-13, playing an essential role in the development of asthmatic inflammation. [446] Besides individually interfering with these single cytokines, silencing of their common activating transcription factor GATA3 [27] provides the option of interfering with the production of all of them at once and therein early-on prevent the inflammatory cascade from commencing. However, transfection of T cells displays an especially challenging endeavor, since caveolae-mediated uptake, the commonly used strategy for non-viral vector-based transfection methods, cannot be applied to the non-caveolin expressing cells. [420]

RNA interference (RNAi) offers a promising option to even treat diseases that have so far thought to be "undruggable", enabling the potential targeting of any chosen gene with an established sequence. [14] Small interfering RNAs (siRNA) have therefore been subject to extensive research leading to the recent approval of the first siRNA-mediated therapy on the market. [19] The main drawbacks, however, still holding back the potential of these novel treatment forms, are the poor pharmacological properties of nucleic acids which are easily degraded by nucleases ubiquitously found in the bloodstream and rapidly excreted upon systemic injection. [20] In recent years, however, key improvements have been made in stable packaging of siRNA within nanoparticles mostly composed of polymers and/or lipid formulations. A further way to improve siRNA delivery and circumvent i.v. stability concerns, thereby also bypassing first pass metabolism, is to use local administration routes. In case of asthma, pulmonary delivery obviously offers a suitable approach to effectively target disease related cells in asthmatic lungs. [447]

In our previous work, we have developed and optimized an siRNA carrier system based on low molecular weight (LMW) polyethylenimine (PEI), [40] the most extensively studied polymer for gene delivery. [426] The polycationic carrier was coupled to

transferrin, a glycoprotein responsible for iron transport in biological fluids. Its receptor is therefore overexpressed on highly proliferating cell types such as activated T cells, [23] providing both an entry gate via receptor mediated endocytosis and the potential to specifically target such activated T cells. Since naïve T cells only show a negligible expression of the transferrin receptor (TfR), they are not considerably transfected and a general immune suppression by the treatment can be avoided. While administering Tf-PEI polyplexes containing fluorescently labeled siRNA to the lungs of ovalbumin-sensitized mice showed promising results in terms of T cell targeting in the diseased state, [40] administration of such polyplexes containing siRNA against GATA3 to the same experimental asthma model resulted only in non-significant gene silencing and intracellular cytokine levels. [448] In a follow-up study, [449] the Tf-PEI polyplexes were therefore further advanced by blending them with an LMW-PEI conjugate with pH-resonsively protected melittin, a cationic peptide with lysosomal properties. The resulting Tf-Mel-PEI blend formed polyplexes which were able to efficiently be released from the endosomes after being taken up reflected in more efficient *ex vivo* gene silencing of reporter and housekeeping genes, such that an optimized *in vivo* effect of therapeutic siRNA is expected.

The aim of this work was therefore to evaluate the therapeutic potential of these Tf-Mel-PEI/siRNA polyplexes in the context of targeted GATA3 knockdown in activated T cells in the lung for early interference with the inflammatory cascade in asthmatic patients. For this purpose, primary as well as immortal T cells were used to generate and optimize an appropriate model for the activated T cell status in inflamed lungs. A suitable GATA3 sequence combination was found for distinct gene silencing and downstream effects of this knockdown were investigated. Overall, the results gathered in this study give reason to assume that Tf-Mel-PEI polyplexes present a feasible approach for precise delivery of siRNA for specific gene knockdown in disease-related T cells as a novel therapy for allergic asthma.

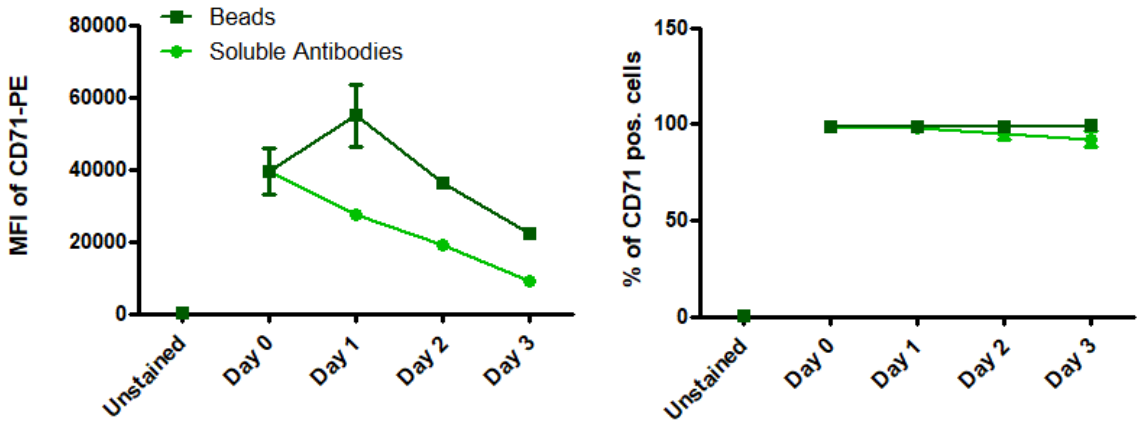
2. Results

2.1. Optimization of T Cell Activation

To optimize the T cell activation process, two different activation strategies were tested, and the standard protocol of using soluble antibodies directed against CD3 and CD28 was compared with so-called Dynabeads®, beads covalently coupled to anti-CD3 and anti-CD28 antibodies. Both strategies were applied to Jurkat cells, a human T lymphocyte cell line, as well as primary $CD4^+$ T cells freshly isolated from buffy coats via magnetic bead separation. To evaluate the activation status of the cells, upregulation of TfR, gene expression of GATA3, and secretion of interleukins IL-4, IL-5, and IL-13, were analyzed.

Figure 1 illustrates TfR (equivalent to CD71) expression in Jurkat cells (Figure 1A) and primary $CD4^+$ T cells (Figure 1B) as measured at different time points post activation. Since Jurkat cells express TfR constitutively, the percentage of TfR expressing cells could not be enhanced any further, but was steadily maintained for 3 days. The corresponding median fluorescence intensity (MFI) of CD71-PE labeled cells was, however, increased via bead activation at day 1, while treatment with soluble antibodies resulted in a consistent decrease of receptor expression. In contrast to this, naïve primary T cells only show a negligible TfR expression without activation stimuli and were therefore successfully activated, eventuating in a distinct increase in both CD71 positive cells and MFI of CD71-PE. Dynabead activation outperformed soluble antibodies, resulting in 45 % positive cells already after 24 h of activation which was maintained for an additional 2 days, reflected in an increase of MFI from 1500 up to approximately 3800.

A)



B)

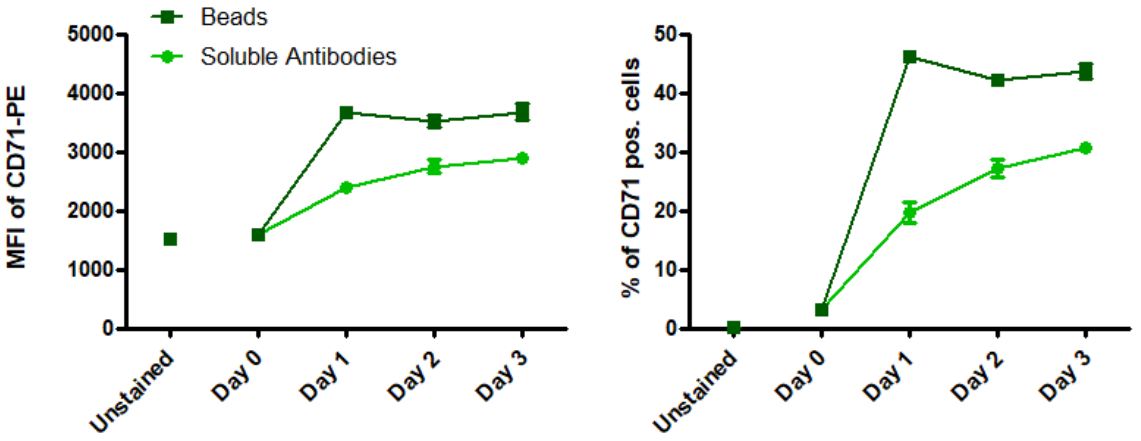


Figure 1. Transferrin receptor (TfR, equivalent to CD71) expression as measured by flow cytometry and presented as median fluorescence intensity (MFI) or % of CD71 positive cells, respectively. A) TfR expression in Jurkat cells, B) TfR expression in human primary $CD4^+$ T cells after 0, 1, 2, or 3 days of activation via beads (dark green, squares) or soluble antibodies (light green, circles) and staining with CD71-PE antibody. "Unstained" represents untreated control cells.

In **Figure 2**, GATA3 expression of Jurkat cells (Figure 2A) and primary CD4+ T cells (Figure 2B) normalized to β-Actin is depicted after the different activation procedures as quantified by qRT-PCR. In both cell types, GATA3 expression was distinctly upregulated via CD3/CD28 based activation compared to untreated blank cells. In Jurkat cells, beads and soluble antibodies worked comparably well, while in the primary T cells, the bead activation was also more effective in regards to GATA3 activation, resulting in a twice as high expression after three days of activation compared to soluble antibodies and a three times higher expression compared to non-activated cells.

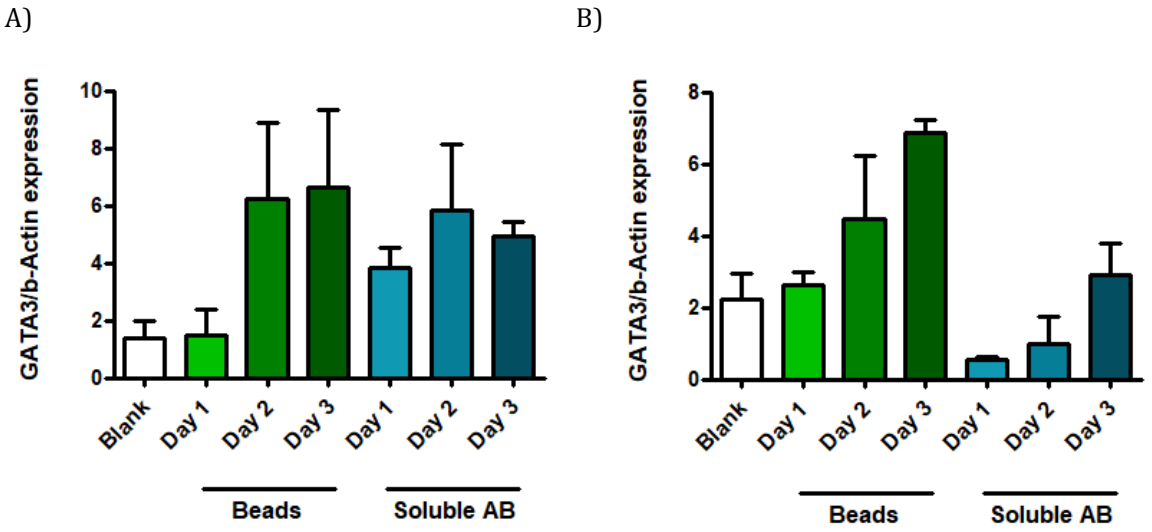
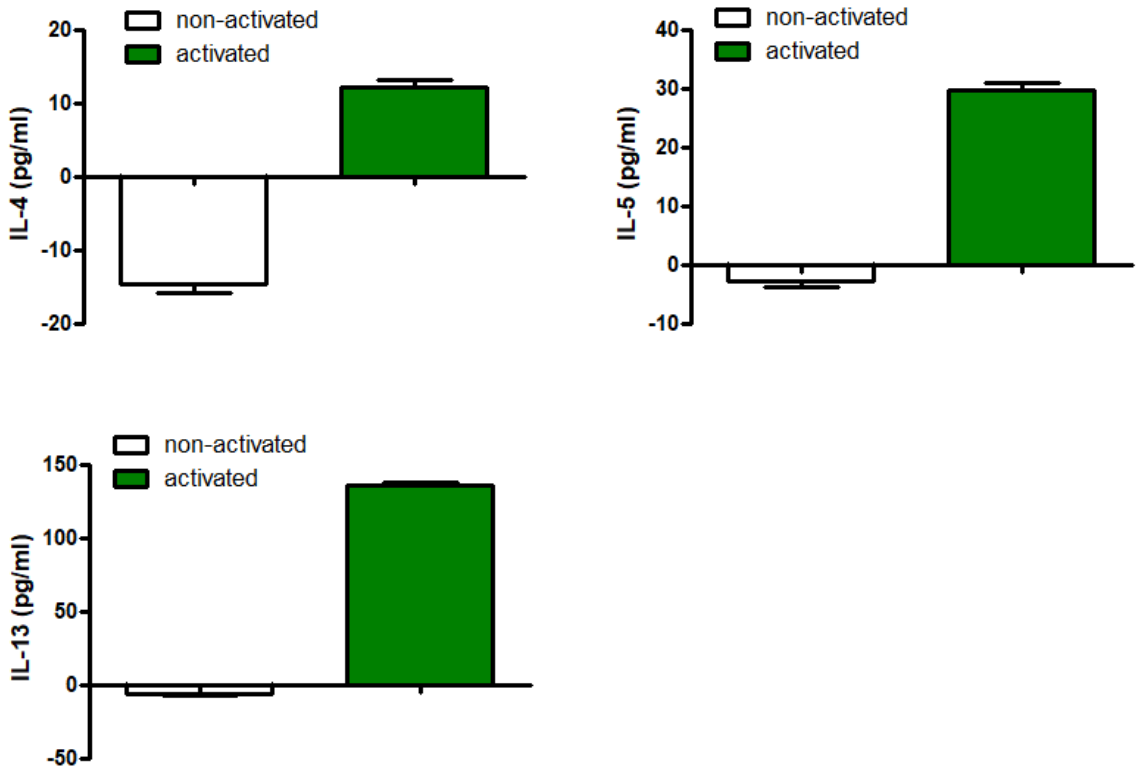


Figure 2. *GATA3* expression as measured by qRT-PCR in A) Jurkat cells and B) human primary CD4+ T cells after 1, 2, or 3 days of activation with beads (green) or soluble antibodies (blue), normalized to *β-Actin* expression. "Blank" represents untreated control cells.

As a third activation parameter, the secretion of GATA3 related interleukins IL-4, 5, and 13, was chosen to be analyzed. Results of sandwich ELISA assays from primary $CD4^+$ T cells are shown in **Figure 3,** 2 days after activation with beads (Figure 3A) or soluble antibodies (Figure 3B) compared to non-stimulated naïve T cells. In general, all 3 interleukins were only secreted at a non-detectable level without activation. In marked contrast to this, secretion of all tested cytokines was considerably upregulated by both activation processes, with IL-13 showing the strongest increase in each case. Bead based activation enhanced IL-13 concentration approximately 10-fold, from 20 to 200 pg/ml. In general, both beads and soluble antibodies show a comparable trend in the activation of T cells. However, bead based activation showed more reproducible and reliable results reflected in smaller deviations between replicates as compared to soluble antibodies. Unfortunately, interleukin measurements in activated Jurkat cells revealed strongly varying results (data not shown).

A)



B)

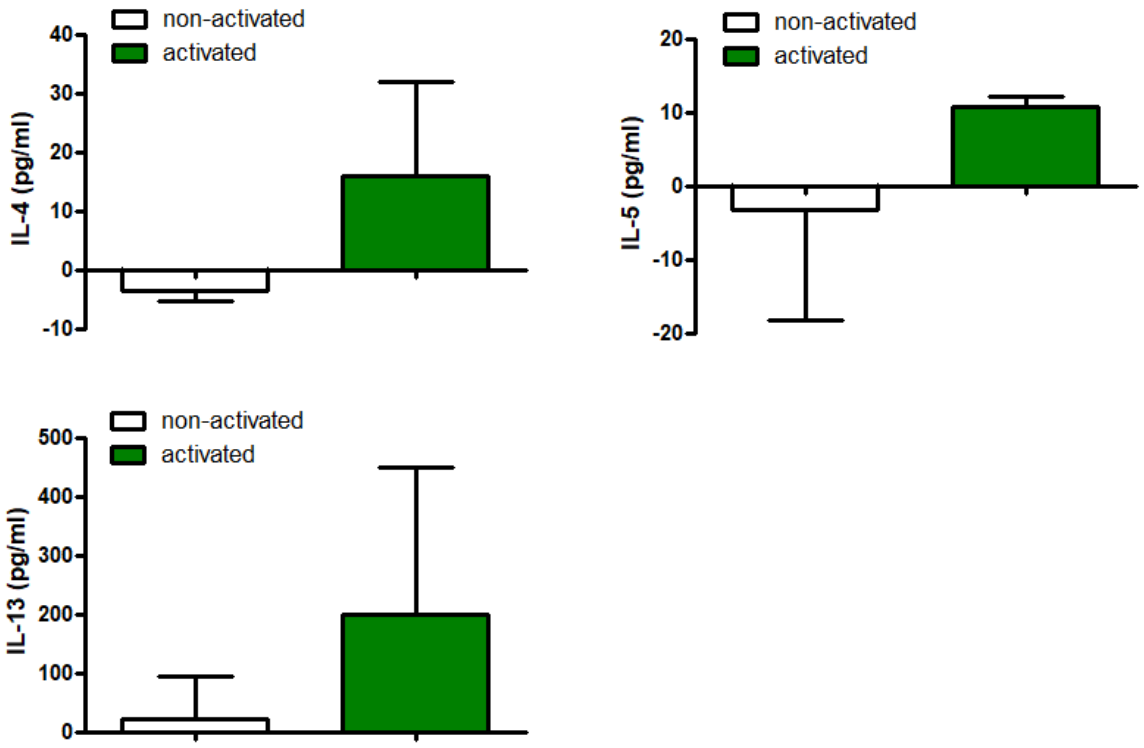


Figure 3. Cytokine concentrations of interleukin (IL)-4, IL-5, and IL-13 in the supernatant of human primary $CD4^+$ T cells after 48 h of activation with A) beads and B) soluble antibodies as measured by sandwich ELISA. White bars represent non-activated cells as controls.

2.2. Optimization of GATA3 siRNA sequences

For optimization of siRNA sequences which downregulate human GATA3, several different sequences were screened in preliminary experiments using lipofectamine as the transfection reagent and MCF-7 cells as an easy-to-transfect model cell line. **Figure 4A** depicts GATA3 expression 24 h after transfection with the four most promising different GATA3 sequences compared to untreated blank cells as well as cells treated with a scrambled negative control sequence. While sequences GATA3_1 and GATA3_7 did not result in any considerable knockdown effect, the two sequences GATA3_8 and GATA3_9 were found to induce significant gene silencing compared to the negative control sequence NC-siRNA. Consequently, these two sequences were combined in a 1:1 mix and applied together at the same final concentration as in the screening

experiments (Figure 4A), resulting in an even more distinct knockdown effect, showing significance compared to both the negative controls and the untreated blank samples ($p<0.05$, **Figure 4B**).

A) B)

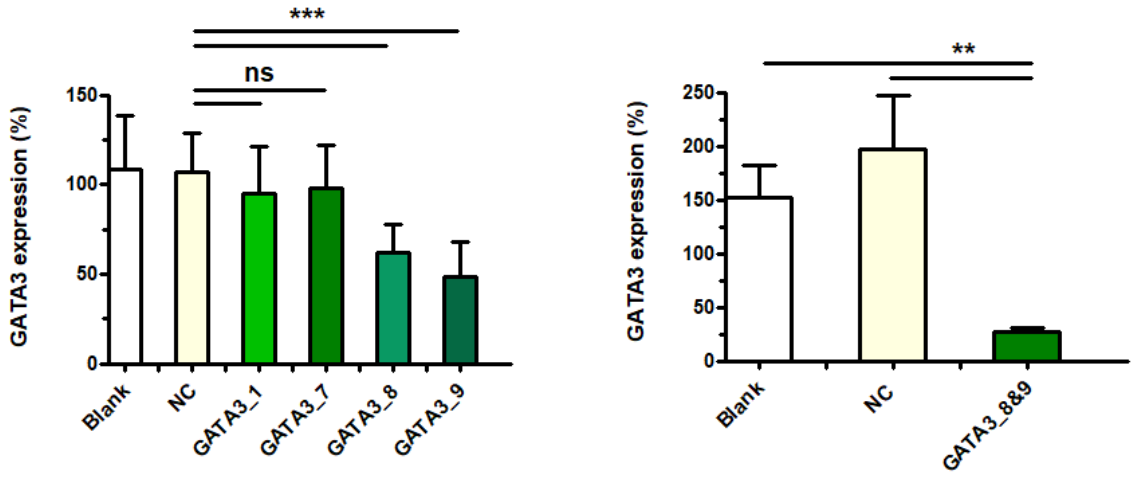


Figure 4. *GATA3* knockdown in MCF-7 cells transfected with lipoplexes consisting of lipofectamine and A) siRNA sequences directed against GATA3: GATA3_1, GATA3_7, GATA3_8, and GATA3_9 and B) a 1:1 mixture of both sequences GATA3_8 and GATA3_9 as measured by qRT-PCR and normalized against *β-Actin* expression. Control cells were left untreated (blank) or transfected with lipoplexes containing scrambled siRNA as negative control (NC). (Data points indicate mean ± SD, n = 3; One-way ANOVA, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$).

2.3. GATA3 Gene Silencing

GATA3 gene silencing as achieved by using the two siRNA sequences GATA3_8 and GATA3_9 but formulated as Tf-Mel-PEI polyplexes rather than lipoplexes was confirmed in Jurkat cells. **Figure 5** illustrates GATA3 expression normalized to β-Actin 24 h after treatment with Tf-Mel-PEI polyplexes containing either only sequence GATA3_8 or GATA3_9 or a 1:1 mixture of both sequences, always formulated at the same final siRNA concentration and with N/P ratio of 10. For comparison, again, an untreated blank sample as well as the scrambled NC-siRNA were used. Here, both GATA3 sequences alone already resulted in a significant gene knockdown, while the 1:1 combination again

achieved an even enhanced silencing effect. This formulation was consequently chosen to be used for further knockdown experiments.

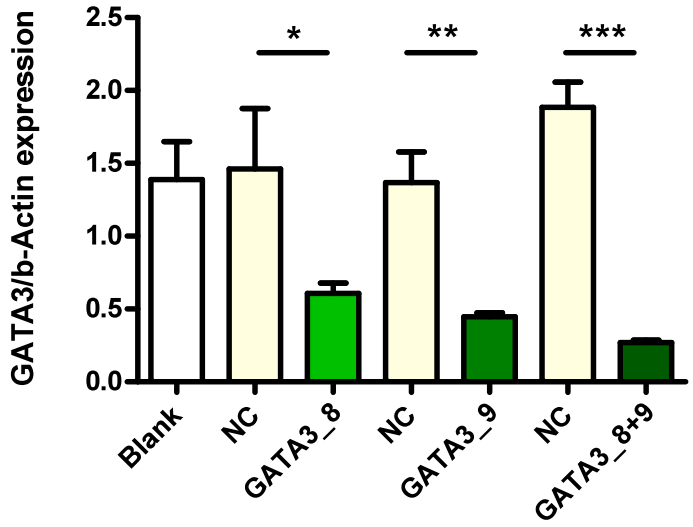


Figure 5. *GATA3* knockdown in Jurkat cells transfected with Tf-Mel-PEI polyplexes containing different GATA3 siRNA sequences at N/P 10 as measured by qRT-PCR and normalized against *β-Actin* expression. Control cells were left untreated (blank) or transfected with respective polyplexes containing scrambled siRNA as negative control (NC). (Data points indicate mean ± SD, n = 3; One-way ANOVA, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$).

The optimized GATA3 sequences were then evaluated for gene knockdown in both Jurkat cells and primary $CD4^+$ T cells within polyplexes prepared with unmodified PEI, the previously described Tf-PEI conjugate, as well as the Tf-Mel-PEI conjugate blend. **Figure 6** shows GATA3 expression normalized to β-Actin after treatment with polyplexes at N/P 10 for 24 h. Neither in Jurkat cells (Figure 6A) nor in primary T cells (Figure 6B) did PEI treatment achieve efficient gene silencing. While Tf-PEI polyplexes did result in a visible downregulation of GATA3 in Jurkat cells, this effect was, however, not significant compared to treatment with scrambled negative control siRNA. In primary T cells, on the contrary, Tf-PEI in fact induced a significant gene knockdown. The Tf-Mel-PEI blend polyplexes were found to be the only treatment able to significantly and specifically silence GATA3 in both cell types, resulting in a knockdown

efficiency of approximately 65 % in Jurkat cells and 70 % in human CD4+ primary T cells, compared to negative controls, respectively.

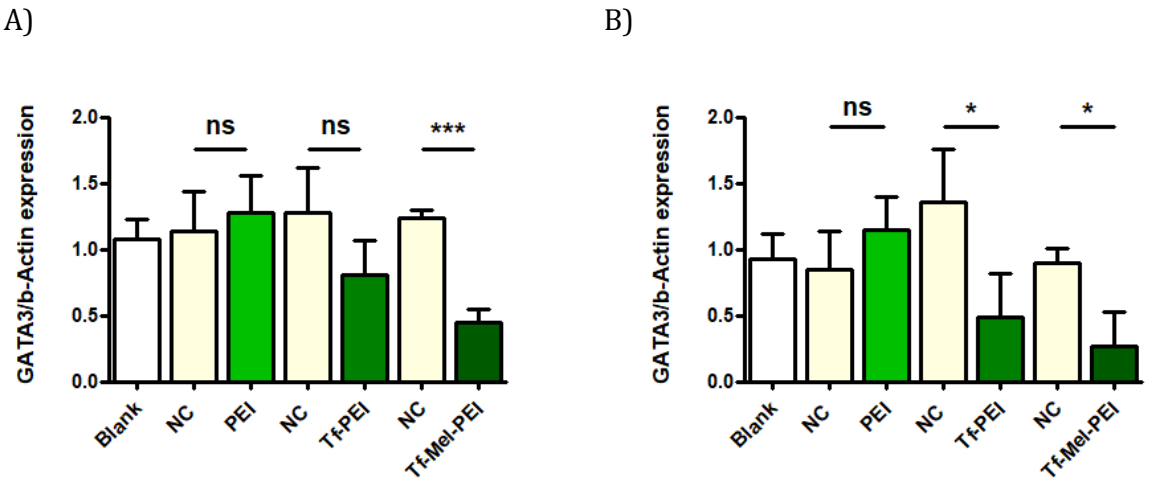


Figure 6. *GATA3* knockdown in A) Jurkat cells and B) human primary CD4+ T cells after transfection with a 1:1 mixture of siRNA sequences GATA3_8 and GATA3_9 encapsulated within PEI, Tf-PEI, or Tf-Mel-PEI, respectively. Control cells were left untreated (blank) or transfected with respective polyplexes containing scrambled siRNA as negative control (NC). (Data points indicate mean ± SD, n = 3; One-way ANOVA, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$).

2.4. Evaluation of Downstream Effects

Downstream effects of GATA3 gene silencing on respective cytokine secretion by primary T cells was evaluated using ELISA. Concentrations of the three interleukins IL-4, IL-5, and IL-13 were measured in the supernatant of treated cells 48 h after activation and subsequent transfection with Tf-Mel-PEI polyplexes containing either scrambled control NC-siRNA or siRNA directed against GATA3 (GATA3_8 and GATA3_9 in a 1:1 mixture) at N/P ratio 10. **Figure 7** summarizes the results of respective sandwich ELISAs. Secretion of all three interleukins was significantly downregulated via GATA3 targeted treatment compared to negative controls, indicating that GATA3 knockdown can indeed affect appropriate downstream signaling. The best results were obtained for IL-5 and IL-13, wherein GATA3 knockdown resulted in a consequent decrease of interleukin production of 50 % and 35 %, respectively, compared to negative controls. For IL-4, negative values were obtained based on data interpolation resulting in not

fully conclusive results. Nevertheless, a general trend towards decreased cytokine concentration could be observed after GATA3 treatment.

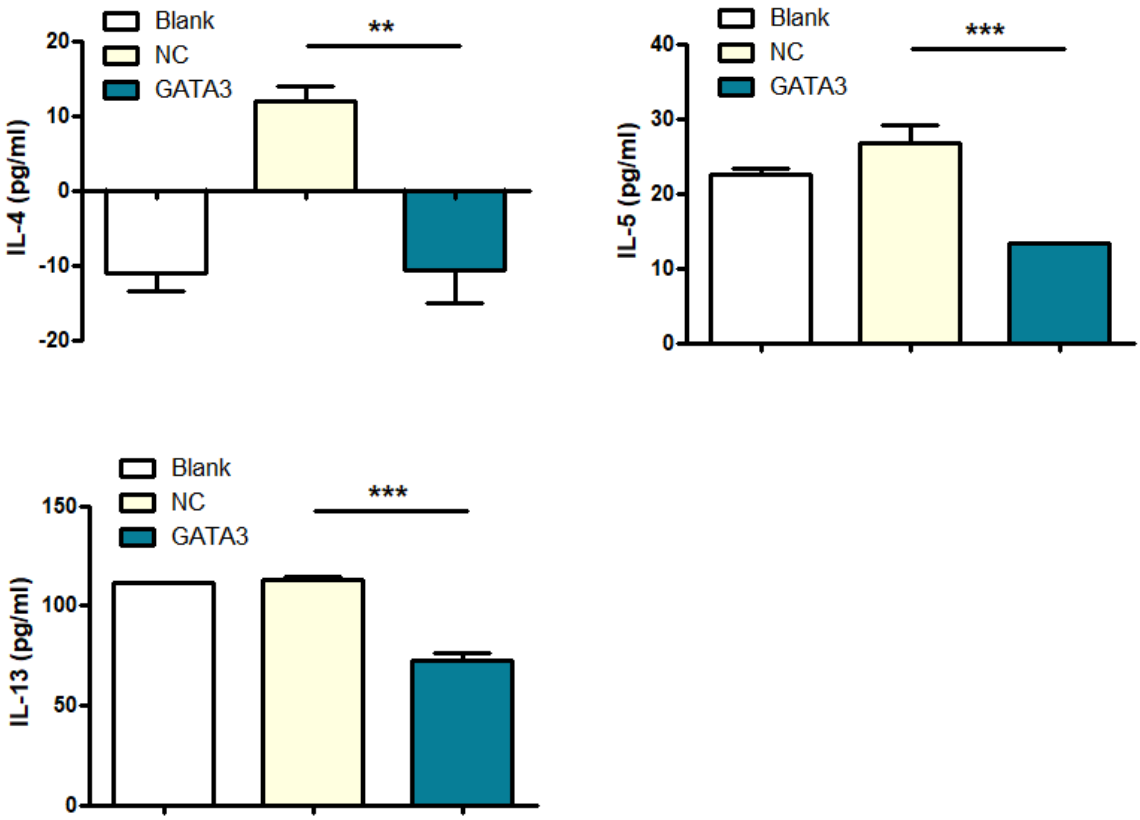


Figure 7. Cytokine concentrations of A) IL-4, B) IL-5, and C) IL-13 in the supernatant of human primary $CD4^+$ T cells after 48 h of activation and subsequent transfection with Tf-Mel-PEI polyplexes containing a 1:1 mixture of siRNA sequences GATA3_8 and GATA3_9. Control cells were left untreated (blank) or transfected with respective polyplexes containing scrambled siRNA as negative control (NC). (Data points indicate mean ± SD, n = 3; One-way ANOVA, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$).

3. Discussion and Conclusion

Current asthma treatment focusses on palliating symptoms while the underlying inflammatory pathways involved in the disease remain largely uncontrolled. Consequently, GATA3, the central transcription factor in Th2 mediated asthmatic airway inflammation offers a promising target for respective medications, however, is challenging to reach. Transferrin was found to be a suitable option to achieve the entry into otherwise hard-to-transfect T cells and targeted delivery to activated disease-related T cells in particular. [40] As early as in 1999, the group of Wagner et al. described how coupling of transferrin to polycationic vectors such as PEI results in stable carriers for efficient and target-specific gene delivery to subcutaneously growing tumors. [450] This strategy was thenceforward constantly advanced and refined and applied for various cancer targeting approaches [41], but eventually also found suitable for further applications other than tumor targeting. The transferrin receptor (TfR) as entry port for iron transport is not only overexpressed on different cancer cell types, but also on the membrane of cells involved in inflammatory cascades, to meet their elevated iron requirements for enhanced cell proliferation and differentiation. [451] It can therefore also be used to target disease-causing cell subsets in inflammatory diseases such as asthma. In our previous work, we developed a Tf-PEI based carrier system for specific delivery of siRNA to activated T cells as a potential novel asthma therapy. Despite showing optimal particle characteristics and being able to successfully and preferentially knock-down particular genes *in vitro*, [40] Tf-PEI polyplexes lacked efficient *in vivo* gene silencing capability. [448] In a follow-up study, the original Tf-PEI formulation was therefore modulated by blending with a PEI conjugate with the endosomolytic peptide melittin in order to increase endosomal escape of the polyplexes which was hypothesized to hinder the full potential of *in vivo* effects. Indeed, the addition of melittin distinctly improved polyplex transport from the endosomes to the cytosol, resulting in a significantly enhanced knockdown effect *in vitro* and *ex vivo*. [449] While this latter study concentrated on the characterization and optimization of the Tf-Mel-PEI polyplexes, their endosomal escape abilities, and their binding specificity towards the TfR as measured by several proof-of-concept experiments and knockdown of surrogate genes due to lack of available validated human GATA3 siRNA sequences, the focus of the work presented here was achieving therapeutically relevant gene

knockdown in activated T cells, specifically of GATA3 and evaluation of respective downstream treatment effects.

The first step was to gain more precise insights into the T cell activation process and optimize respective processes to develop a suitable model mirroring the characteristics of activated, disease-related T cells in asthmatic patients. Therefore, two different activation procedures were tested, namely the standard lab protocol using soluble antibodies directed against CD3 and CD28 and the application of so-called Dynabeads®, which are magnetic ferric oxide beads covalently coupled to anti-CD3 and anti-CD28 antibodies. These beads have a diameter of 4.5 μm resembling the size of antigen presenting cells and, moreover, are chemically inert and superparamagnetic, meaning that they can easily be removed from activated cell cultures using a magnet. CD3 and CD28 were chosen as the typically used primary and costimulatory signals for T cell activation partially mimicking *in vivo* stimulation via antigen-presenting cells (APC). [452] The activation process is generally initiated by recognition of peptide-loaded major histocompatibility complexes (MHCs) on APCs by T cell receptors (TCRs). These APCs provide at the minimum two signals required for T cell activation, one via the TCR/CD3 complex and one additional signal through one or more costimulatory cell interactions such as via CD28. [453] Exclusively in the presence of an appropriate costimulatory signal, the primed T cell is capable of starting a productive immune responsive characterized by differentiation, proliferation, IL-2 production and following steps, for example recruitment of eosinophils, stimulation of B cells to enhance their antibody production and activation of natural killer cells. [454]

To evaluate and compare activation processes in both continuous cell lines and primary cells, Jurkat cells, a human T lymphocyte cell line, were chosen as a model cell line, and human $CD4^+$ primary T cells were isolated from freshly obtained buffy coats via magnetic bead separation. Initial tests were performed to optimize cell viability during the activation process, concerning parameters such as cell number and concentration or well plate format (data not shown). Consequently, three parameters were determined to optimize the activation procedure itself and its resulting outcome. As a first activation marker, expression of TfR was chosen and measured at different activation time points. The transferrin receptor plays a crucial role in this work, being both the targeting and entry port of Tf-Mel-PEI polyplexes for efficient transfection of activated T cells. In our

previous experiments, the targeting specificity of Tf-Mel-PEI towards TfR was confirmed by surface plasmon resonance spectroscopy (SPR) measurements and the preferential uptake in activated vs. naïve T cells was highlighted. [449] While in Jurkat cells 100 % of the cells express the receptor constitutively, primary T cells only show a negligible TfR expression at naïve levels. Here, the receptor expression was distinctly upregulated via both activation procedures: with soluble antibodies and with antibody coated beads. Dynabeads, in particular, increased TfR expression resulting in a CD71 positive cell population accounting for 45 % compared to almost no positive cells at naïve status. This upregulation could furthermore be maintained for additional 48 h, indicating that the activation process and subsequent receptor expression proceeds in a sustained and reproducible way. In comparison, T cell activation with soluble CD3 and CD28 antibodies resulted in a lower, but still distinct upregulation of TfR expression, reaching a maximum of 30 % CD71 positive cells after 3 days. Concerning receptor expression, the beads can, therefore, be concluded as the more effective option.

Since the ultimate aim of this study is to generate a novel asthma therapy on the basis of downregulating the transcription factor GATA3 in activated T cells, GATA3 expression levels were determined as the second surrogate marker for the T cell activation status. Comparing soluble antibodies and beads in Jurkat as well as primary T cells, again, both activation procedures led to a distinct activation with a subsequent increase of respective expression. While the maximally reached GATA3 expression in Jurkat cells was comparable for the two activation methods, in primary $CD4^+$ T cells, the beads clearly surpassed the soluble antibodies, resulting in a steady and activation time dependent increase in gene expression. A significant GATA3 expression was also found in mice by Yagi et al. after naïve T cell activation, eventuating in Th2 differentiation. [455] Overall these results give reason to assume that the selected activation procedures are able to achieve increased GATA3 levels and thereby simulate respective inflammation processes orchestrated by activated T cells in the lungs of asthmatic patients.

To further evaluate this assumption, downstream effects of T cell activation and subsequent upregulated GATA3 expression on respective cytokine secretion was chosen as a third parameter to be investigated. Th2 related IL-4, IL-5, and IL-13 are all directly affected by GATA3 expression due to the transcription factor being translocated to the

nucleus upon T cell receptor stimulation [456] and being involved in interleukin promoter activation and Th2 cell differentiation. GATA3 protein is hypothesized to dock on and interfere with promoter regions of the interleukin genes, in turn upregulating their expression. However, this mechanism is not yet fully clarified. [457] IL-4 mainly drives polarization of the $CD4^+$ T cell response in the Th2 phenotype in combination with suppression of interferon (IFN)-γ-producing Th1 cells [458], substantially impacting the Th1/Th2 imbalance in inflammatory cascades in asthma. Moreover, it directs differentiation of T cells into IL-4 producing effector T cells, as even naïve T cells are able to produce IL-4. In this way, a significant population of naïve $CD4^+$ is stimulated to secrete IL-4 after primary activation. [455] IL-4 also controls the growth and differentiation of B cells [459] and the specificity of the immunoglobulin G (IgG) class switching, navigating further disease related processes. IL-5 is known to be key maturation and differentiation factor for eosinophils, leading to increased eosinophil numbers and antibody levels upon over-expression. It regulates expression of genes involved in cell survival, proliferation, and maturation of eosinophils as well as B cells and therefore plays a major role in both innate and acquired immune response as well as eosinophilia. [460] IL-13, on the other hand, intersects with several biological activities of IL-4, both sharing the IL-4 receptor alpha-chain essential for signal transduction. Since T cells, however, do not express functional IL-13 receptors, IL-13, unlike IL-4, is not able to induce Th2 cell differentiation in a feedback loop. [461] The effects of the former comprise goblet cell differentiation resulting in enhanced mucus production, increase of bronchial hyperresponsiveness, activation of fibroblasts as well as B cell antibody switch from IgM to IgE. [462] In this work, analogous to the first two tested factors, TfR and GATA3 expression, a distinct increase was also observed for interleukin secretion upon soluble as well as bead based activation. As experiments in the Jurkat cell line unfortunately yielded inconsistent results, only data obtained from human primary $CD4^+$ T cells are discussed here. The consistent enhancement of production of all three tested cytokines confirms that both activation procedures do not only influence the T cells themselves, but furthermore also their surrounding microenvironments and thereby presumably even further cell types and other downstream processes and cascades. As all three tested interleukins play key roles in the inflammation process underlying allergic asthma pathogenesis, it can hence be expected that this stage of the disease process can properly be illustrated in the

activated T cell model. IL-13 was by far the strongest affected cytokine and will be discussed in more detail below.

In summary it can be stated that both tested activation procedures, beads and soluble antibodies, achieved a distinct activation of continuous as well as primary T cells resulting in an upregulation of TfR, GATA3 as well as secretion of selected interleukins. In comparison, CD3 and CD28 antibody coated beads were shown to be overall more effective. As the ferric oxide beads resemble antigen presenting cells in size, they can mimic these essential helpers in the activation process, thereby leading to more precise signaling. Increasing the activation time from two to three days did not considerably increase activation parameters further and rather resulted in reduced cell viability. Consequently, Dynabead activation for 48 h was chosen as the preferred T cell activation method to achieve a proper model for activated T cells mirroring the status in inflamed airways of asthmatic lungs.

For the knockdown of GATA3, a potent murine siRNA sequence had already been optimized by the group of Garn et al. and was shown to decrease the number of eosinophils and level of airway hyperresponsiveness in models of acute allergic airway inflammation after intranasal administration. [27] For humans, however, to the best of our knowledge, no comparably suitable sequence had been detected to this point. Therefore, the first step towards successful GATA3 knockdown in human cells was to discover an appropriate functional siRNA sequence resulting in significant gene silencing. After testing multiple sequences from different vendors in preliminary screening experiments with lipofectamine as transfection agent in easy-to-transfect MCF-7 cells, two sequences were found to be particularly eligible for this purpose, namely GATA3_8 and GATA3_9. It was then hypothesized that these sequences taken together, at the same final siRNA concentration, could be even more effective as combining more than one siRNA sequence had been shown to have significant advantages over use of the corresponding individual siRNAs [463] and can lower off-target effects of single sequences due to smaller applied concentrations [464]. In fact, a 1:1 mixture of both functional siRNA sequences resulted in approximately 90 % knockdown of GATA3 as compared to the negative control. This combination was subsequently chosen and applied to all further gene silencing experiments.

SiRNA sequences GATA3_8 and GATA3_9 were then further validated in Jurkat cells and within the Tf-Mel-PEI blend polyplexes, confirming the capability of both sequences seperately, and all the more in combination, resulting in a significant silencing of the target gene. The knockdown potential of Tf-Mel-PEI was furthermore highlighted in comparative experiments in Jurkat cells as well as primary human $CD4^+$ T cells involving unmodified PEI and the previously described Tf-PEI conjugate as controls. In both cell types, the superior gene silencing efficiency of the blend polyplexes could clearly be pointed out, again emphasizing the essential role of endosomal escape particularly in T cells for effective siRNA therapy. Although Tf-PEI also resulted in a distinct knockdown effect compared to respective negative controls, only after the addition of melittin, the transport of the siRNA cargo from the endosomes to the cytosol was enhanced and this really maximized the treatment outcome. These results are in line with the previous findings of Olden et al. showing that endosomal acidification in human T cells in general both takes place slower and is less robust than in other cell types, [50] underlining the need for further endosomal escape triggers.

To determine whether the achieved knockdown of GATA3 would also result in sufficient interference with respective parameters in downstream cascades orchestrated by the transcription factor, concentrations of the three most relevant cytokines IL-4, IL-5, and IL-13 were measured in cell supernatants by sandwich ELISA. Even if for IL-4, an average mathematically negative value was obtained by extrapolation, a reduction of all three cytokines was observed after treatment with GATA3 siRNA formulated in Tf-Mel-PEI polyplexes. The strongest decrease in secretion was achieved for IL-5, the predominant cytokine involved in eosinophilic activation, causing airway inflammation as a classic feature of severe allergic asthma. [465] IL-5 displays the main growth, differentiation, and activation factor for human eosinophils, highly expressing the IL-5 receptor on their surface membranes. Therefore, the downregulation of IL-5 amongst other cytokines can have particularly positive influences on patients with severe asthma, especially when suffering from eosinophilic inflammation. [466] In fact, several monoclonal antibodies targeting IL-5, namely mepolizumab, reslizumab, and benralizumab have been developed to specifically bind and interfere with the IL-5 receptor on eosinophils and were recently approved for the treatment of adult patients with severe eosinophilic asthma. [466, 467] Beyond IL-5, a significant reduction was

also observed for IL-13 concentration. IL-13 is another central effector of asthmatic processes, most notably inducing goblet cell hyperplasia and mucus hypersecretion [8] and IL-13 signaling was shown to directly result in mucin secretion, airway hyperresponsiveness and pulmonary fibrosis in animal models of asthma [468]. Although glucocorticoids are effective and widely used maintenance therapies in acute and chronic asthma, some patients are non-responsive to steroids maintaining elevated IL-13 levels. [469] For these severe cases, antibodies targeting IL-13 are under investigation as novel targeted add-on treatments. Despite of the benefit of these specific therapies concentrating on single interleukins, the aim of this work is to interfere with GATA3 as the central transcription factor regulating the expression of several different cytokines. Thus, downregulating all of them can be achieved simultaneously, being able to early-on undermine pathologic pathways. The discussed results give reason to assume that GATA3 knockdown via Tf-Mel-PEI polyplexes could indeed be a promising approach to downregulating specific pathways playing essential roles in the inflammation process in asthmatic patients and thereby ameliorating respective symptoms.

For further evaluation of behavior and treatment effects of Tf-Mel-PEI polyplexes under more *in vivo* like conditions, experiments are currently conducted in air-liquid interface cultures of normal as well as asthmatic primary human bronchial epithelial cells resembling the actual status in asthmatic patients. Furthermore, studies in precision cut lung slices are planned to investigate the influence of transfection not only on single cell types, but also on surrounding lung tissue. As a last step, *in vivo* experiments in a murine asthma model are inevitable to gain further insights into distribution, biocompatibility, immunogenicity, and therapeutic effects of polyplexes and will be conducted correspondingly.

In conclusion, within this work, an optimized activation method for both continuous as well as primary T cells has been generated that is suitable to illustrate the inflammation status of activated T cells as a model for asthmatic processes. A functional sequence combination to silence GATA3 gene expression via siRNA-mediated RNAi was found and applied for the first time for specific knockdown in activated T cells. Tf-Mel-PEI polyplexes were confirmed to be an eligible carrier system for targeted GATA3 silencing in activated T cells, resulting in respective alleviation of downstream cytokine

production. Although it was already shown that asthmatic patients can benefit from GATA3 downregulation via specific DNAzymes [28], in this approach, the pharmaceutically active cargo was delivered without any delivery system. For a specific therapeutic application, however, the use of an appropriate carrier such as the one described in this work, offers numerous advantages. Next to the facilitated overcoming of extra- and intracellular barriers, and the ability to precisely influence the release of the loaded drug, a suitable delivery system can also distinctly enhance its stability in the bloodstream. Moreover, a higher drug loading can be achieved, ultimately resulting in lower doses and reduced side effects. Finally, the carrier system can be specifically tailored for individual needs and used to co-deliver other small molecule drugs as well as be equipped with ligands enabling specific targeting of disease-related cell and tissue types. The Tf-Mel-PEI delivery system is currently undergoing testing under more *in vivo* like conditions to ultimately support conclusions on *in vivo* fate and effect of the polyplexes. Overall, the described blend particles display a promising way to deliver siRNA to activated T cells in the lung as a potential new therapy peculiarly for severe cases of allergic asthma.

4. Experimental Section

4.1. Synthesis of Conjugates and Preparation of Polyplexes

Tf-PEI and Mel-PEI conjugates were prepared as previously described. [40] Briefly, Tf was coupled to 5 k PEI (Lupasol® G100, BASF, Ludwigshafen, Germany) via succinimidyl 3-(2-pyridyldithio) propionate (SPDP, Thermo Fisher Scientific, Waltham, USA) to yield the Tf-PEI conjugate with a reducible disulfide bond. Similarly, cysteine modified melittin (Mel, Pepmic, Suzhou, China) was coupled to PEI via 4-polyethylene glycol-N-succinimidyl 3-(2-pyridyldithio) propionate (PEG4-SPDP, Thermo Fisher Scientific, Waltham, USA) after masking of its lytic amine groups with 2,3-Dimethyl-maleic anhydride solution (DMMAn, Sigma-Aldrich, St. Louis, USA) to yield the pH-responsive Mel-PEI conjugate.

For Tf-Mel-PEI blend polyplexes, Tf-PEI and Mel-PEI were mixed in a 1:1 ratio and diluted in 5 % glucose. Defined amounts of siRNA were added to obtain specific amine to phosphate (N/P) ratios according to the following equation for calculating the mass

of polymer required for 50 pmol siRNA: m (PEI in pg) = 50 pmol x 43.1 g/mol x N/P x 52 (protonable unit of PEI = 43.1 g/mol, number of nucleotides of 25/27mer siRNA = 52). Solutions were mixed by pipetting and incubated for 20 min at room temperature before proceeding with respective experiments.

4.2. Cell Culture

Jurkat cells, a human T lymphocyte cell line, were a kind gift from Prof. Dr. Heissmeyer (Institute for Immunology, Biomedical Center Munich). They were cultured in RPMI-1640 cell culture medium (Sigma-Aldrich) supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/l glucose, 10 % (v/v) heat inactivated fetal bovine serum (FBS, Sigma-Aldrich) and 1 x penicillin/streptomycin (Pen/Strep, Sigma-Aldrich). Human and murine primary T cells were cultured in the same medium. MCF-7 cells were cultured in EMEM cell culture medium (Sigma-Aldrich) supplemented with 2 mM L-Glutamine, 1 % non-essential amino acids (NEAA), 10 % (v/v) heat inactivated fetal bovine serum (FBS) and 1 x penicillin/streptomycin (Pen/Strep, all supplements were obtained from Sigma-Aldrich). All cells were grown at a humidified atmosphere with 5 % CO_2 at 37 °C.

4.3. T Cell Isolation and Activation

48-well cell culture plates (Thermo Fisher Scientific) were coated with anti-CD3 antibody (BD Biosciences, Franklin Lake, USA) at a concentration of 5 µg/ml in PBS and incubated for 3 h at 37 °C or overnight at 4 °C. After washing the wells 3 times with rising amounts of PBS, 100.000 (for flow cytometry) or 500.000 (for PCR and ELISA) Jurkat cells or primary T cells, respectively, were added per well in fresh medium. Anti-CD28 antibody (BD Biosciences) was added at a final concentration of 1 µg/ml. For activation via beads, Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation (Thermo Fisher Scientific) were washed and added to the cells in a bead:cell ratio of 1:1 according to the manufacturer's protocol. Cells were activated for 24, 48, or 72 h at a humidified atmosphere with 5 % CO2 at 37 °C before further analysis.

4.4. Transferrin Receptor (TfR1) Expression

Jurkat cells and primary $CD4^+$ T cells from the activation procedure were collected at different days post activation, washed with PBS and resuspended in 100 µl of PBS. Per sample, 10 µl of 1:10 diluted FcR binding inhibitor (eBioscience, Frankfurt, Germany) in PBS was added, mixed and incubated for 5 min at 4 °C. Subsequently, 1 µl of undiluted anti-CD71 antibody (eBioscience) was added to appropriate samples, while isotype controls were stained with IgG1 antibody (eBioscience) and blank samples were left unstained. All samples were vortexed and incubated for another 30 min at 4 °C while protected from light. After washing with PBS 3 times, cells were resuspended in 400 µl PBS with 2 mM EDTA and samples were analyzed using an Attune ® NxT flow cytometer (Thermo Fisher Scientific) with 488 nm excitation and 574/26 nm emission filter. The cells of all samples were gated according to morphology based on forward/sideward scattering, and 10.000 events were evaluated per sample.

GATA3 Expression: Jurkat cells and primary CD4+ T cells from the activation procedure were collected at different days post activation and total RNA was isolated with the PureLink RNA mini kit (Thermo Fisher Scientific) or the RNeasy micro kit (QIAGEN), respectively, according to the manufacturer's protocol with additional DNAse I digestion. From the obtained RNA, cDNA was synthesized and amplified with the Brilliant III ultra-fast SYBR® green QRT-PCR master mix kit (Agilent Technologies, Santa Clara, USA), custom-synthesized GATA3 primers (Thermo Fisher Scientific) and QuantiTect® primer assay Hs_ACTB_2_SG (Qiagen, Venlo, Netherlands) on a qTOWER real-time PCR thermal cycler (Analytik Jena, Jena, Germany). Cycle threshold (Ct) values were calculated using the qPCRsoft software (Analytik Jena), and a 1:5 serial dilution of an untreated blank sample was prepared as a standard curve. Ct values were plotted against the assigned concentration of each point in the curve (1, 0.2, 0.004, 0.0008, and 0.00016). For each sample, gene expression of GATA3 was normalized by corresponding β-Actin expression.

4.5. ELISA

Primary $CD4^+$ T cells from the activation procedure were harvested at different days post activation. Cells were centrifuged for 5 min at 350 x g, and supernatants of the samples were collected and frozen at -80 °C. Interleukin concentrations were quantified with human ELISA kits for IL-4, IL-5, and IL-13 (Invitrogen, Carlsbad, USA), respectively, according to the manufacturer's protocol. Absorbance was recorded at a wavelength of 450 nm using a FLUOstar Omega (BMG Labtech).

4.6. GATA3 Sequence Optimization and GATA3 Gene Knockdown

To find a suitable human siRNA sequence for efficient GATA3 knockdown, different siRNA sequences, namely Hs_GATA3_1, Hs_GATA3_7, Hs_GATA3_8, and Hs_GATA3_9 (QIAGEN) were screened. Therefore, 100.000 MCF-7 cells per sample were seeded in a 24-well plate (Thermo Fisher Scientific), transfected with Lipofectamine™ lipoplexes containing 25 pmol siRNA either directed against GATA3 (siGATA3_1, siGATA3_7, siGATA3_8, siGATA3_9) or scrambled negative control siRNA (siNC), respectively, and incubated for 24 h. Lipoplexes were prepared according to the manufacturer's protocol. Cells were harvested and GATA3 gene expression was quantified as described above. In addition, an analogous experiment was performed with lipoplexes containing a total of 25 pmol of a 1:1 mixture of sequences Hs_GATA3_8 and Hs_GATA3_9.

For GATA3 Gene Silencing experiments, 500.000 Jurkat cells per sample were seeded in a 96-well plate or $1x10^6$ primary T cells were seeded in a 48-well plate (Thermo Fisher Scientific) and activated for 48 h as described above. Cells were transfected with polyplexes containing 25 pmol siRNA either directed against GATA3 (siGATA3_8+9, 1:1 mixture of sequences siGATA3_8 and siGATA3_9) or scrambled negative control siRNA (siNC), respectively, at an N/P ratio of 10. After 24 h of incubation, cells were harvested and GATA3 gene expression was quantified as described above.

4.7. Statistical Analysis

All experiments were performed in triplicates. All results are presented as mean value ± standard deviation (SD). One-way ANOVA and two-way ANOVA with Bonferroni post-hoc post-test were conducted in GraphPad Prism (GraphPad Software, La Jolla, USA) to calculate p-values with 95 % confidence.

Chapter X

Summary and Perspectives

CHAPTER X - Summary and Perspectives

Within this work, targeted siRNA delivery to activated T cells was evaluated as a way to develop a potential novel treatment for asthma patients suffering from uncontrolled symptoms despite standard mediation compliance. Via initiation of RNAi and subsequent silencing of specific genes, siRNA therapy offers an auspicious basis to target disease-related pathways that otherwise would be difficult or even impossible to tackle. Despite numerous positive attributes such as specificity, potency and the ability to achieve controlled transient gene knockdown, siRNA certainly also encounters several challenges. The unfavorable physiological characteristics such as negative charge and high susceptibility to enzymatic degradation make for difficulties in delivery of intact siRNA to its intracellular targets.

The strategic use of the siRNA carrier system presented in this work, Tf-PEI, combines two approaches to circumvent these hurdles, namely local delivery via pulmonary application and specific targeting of activated T cells. Via targeting of the transferrin receptor, not only the challenging transfection of T cells in general is achieved, but also the directed transfection of disease-related cells orchestrating inflammatory cascades in asthma pathogenesis. This enables a very precise therapeutic intervention leaving cells not impacted by the disease mostly unaffected. Tf-PEI polyplexes showed very favorable characteristics such as particles sizes below 200 nm, slightly negative zeta potentials, and excellent siRNA encapsulation efficiencies and were able to be successfully taken up by activated but not naïve T cells *in vitro* and *in vivo* resulting in sequence specific gene silencing.

As most current asthma treatments mainly focus on palliating symptoms and intervene with the underlying inflammatory processes only towards the end of the inflammatory cascades, the aim of this work was to develop a therapy that would early-on interfere with disease-causing cascades. Rather than downregulating single inflammatory cytokines, such as IL-4, IL-5, or IL-13, their mutual transcription factor GATA3 was chosen as a target. By downregulation of GATA3, all three interleukins can be caught at once, resulting in a distinct decrease of inflammatory messengers and activated immune cells. For this purpose, a suitable model to mimic the activated status of T cells playing a

crucial role in asthma pathogenesis was developed and optimized in order to evaluate downstream treatment effects in a more physiologic setup. A potent GATA3 siRNA sequence combination was found to specifically downregulate gene expression of the zinc finger transcription factor in human cells and previously described sequences were successfully applied for gene knockdown in murine cells, respectively.

In the further course, next to successful cellular delivery, endosomal escape was identified as a second essential hurdle on the way to transfer siRNA mediated gene knockdown into significant *in vivo* effects. Even if siRNA polyplexes were successfully taken up by target cell populations, another hurdle to overcome was efficient release of the therapeutic nucleic acids into the cytosol for an efficient therapeutic impact. Incorporation of siRNA into the RNAi machinery located in the cytoplasm of the cells required endosomal release and was achieved by adding melittin as an endosomolytic compound into the polyplex formulation. To prevent cytotoxic effects of the membrane lytic properties, a pH-responsive shielding was applied to generate a tolerable delivery system. The Tf-Mel-PEI blend polyplexes were designed to combine both the specific targeting of disease-related activated T cells and efficient extra- and intracellular delivery to the site of action in one versatile siRNA carrier system.

One important aspect, however, that still needs further characterization is the exact composition of the blend polyplexes. Despite first studies aiming to obtain more insights into how the particles are built up, it is still not completely clear in which ratio the two single conjugates actually assemble into particles and in which proportions they bind to the encapsulated siRNA. Therefore, fluorescence correlation spectroscopy experiments are planned in order to find out what percentage of each conjugate is bound in the particles or freely diffusing, respectively.

To more specifically investigate the therapeutic effect of the polyplex treatment not only on cultures of immortalized or primary cells, an air-liquid-interface (ALI) culture of bronchial tracheal epithelial cells is currently developed. In this model, parameters such as uptake, gene silencing effect and influence on cytokine levels can be tested under more *in vivo* like conditions involving the presence of mucus, for example. To mimic the physiological environment of asthmatic lungs even more realistically, in addition,

precision cut lung slices will be used in order to demonstrate polyplex effects not only on single cell types, but moreover on the surrounding lung tissue.

Subsequently, more *in vivo* experiments have to be performed to evaluate the tolerability of Tf-Mel-PEI polyplexes beyond the *in vitro* state. Further studies will focus on the therapeutic outcome and investigate influence of the hopefully achieved *in vivo* knockdown of GATA3 on downstream cascades such as levels of inflammatory cytokines and immune cell composition within the lung of asthmatic mice.

In parallel, an inhalable dry powder formulation is currently developed to pack the optimized siRNA polyplexes into so-called nano-in-microparticles with suitable characteristics as a convenient treatment form for asthma patients. This step is crucial for transferring this promising gene silencing approach into a routinely applicable therapeutic dosage form.

Although the Tf-Mel-PEI blend particles were very well tolerated in performed *in vitro* toxicity assays and the transferrin shielding was confirmed to distinctly reduce PEI related inflammatory effects of Tf-PEI *in vivo*, the general toxicity of PEI certainly raises some concerns. Particularly with regards to the fact that siRNA therapy usually has to be applied repeatedly, a biodegradable polymer would be preferable in the long term. Therefore, suitable options are evaluated to replace the cationic polymer in the conjugates with more biocompatible materials such as oligospermines.

Taken together, within this work, efforts were made to realize siRNA mediated gene silencing in diseased lungs of asthma patients as a potential new treatment form by tackling some major hurdles on the way. Tf-Mel-PEI blend polyplexes present a promising base to develop a suitable delivery system achieving specific gene knockdown primarily in activated T cells. Despite the need for further optimization and evaluation, the gathered findings are expected to bring us one step closer to translating siRNA therapy for asthmatic patients from bench to bedside.

Chapter XI

Appendix

CHAPTER XI – Appendix

11.1. References

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