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Treatment of acute lung inflammation by pulmonary delivery of anti-TNF-α siRNA with PAMAM dendrimers in a murine model

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ABSTRACT

To improve the efficacy of nucleic acid-based therapeutics, *e.g.*, small interfering RNA (siRNA), transfection agents are needed for efficient delivery into cells. Several classes of dendrimers have been found useful as transfection agents for the delivery of siRNA because their surface can readily be functionalized, and the size of the dendriplexes they form with siRNA is within the range of conventional nanomedicine. In this study, commercially available generation 3 poly(amidoamine) (PAMAM) dendrimer was investigated for pulmonary delivery of siRNA directed against tumor necrosis factor (TNF) α for the treatment of acute lung inflammation. Delivery efficiency was assessed *in vitro* in the RAW264.7 macrophage cell line activated with lipopolysaccharide (LPS), and efficacy was evaluated *in vivo* in a murine model of LPS-induced lung inflammation upon pretreatment with TNF-α siRNA. The PAMAM dendrimer-siRNA complexes (dendriplexes) displayed strong siRNA condensation and high cellular uptake in macrophages compared with non-complexed siRNA. Q-PCR analyses showed that the dendriplexes mediated efficient and specific TNF-α silencing *in vitro*, as compared to non-complexed siRNA and dendriplexes with negative control siRNA. Also *in vivo,* the PAMAM dendriplexes induced efficacious TNF-α siRNA inhibition, as compared to non-complexed siRNA, upon pulmonary administration to mice with LPS-induced lung inflammation. Hence, these data suggest that PAMAM dendrimers are promising for the local delivery of TNF-α siRNA in the treatment of lung inflammation via pulmonary administration.

1. Introduction

Oligonucleotide-based therapeutics, including siRNA, antisense oligonucleotides and miRNA, are applied to intervene with the expression of specific target genes and are thereby thought to mediate more specifically disease treatment than therapeutics based on small molecules or peptides/proteins. Inflammatory lung diseases are complex disorders that are usually treated with medications, which are relatively unspecific in their mode of action and are administered systemically, resulting in increased drug exposure but also undesired side effects [1,2]. Here, local siRNA-based treatment may provide a much more specific and safe therapy in which certain inflammatory pathways can be targeted [3]. Of the many pro-inflammatory cytokines involved in lung inflammation processes, TNF-α is believed to play a central role in most inflammatory lung conditions, *e.g.*, chronic obstructive pulmonary disease (COPD), asthma, acute respiratory distress syndrome (ARDS) and acute lung injury (ALI) [4]. More recently, it was shown to be a major target in the treatment of inflammatory flares in covid-19 infection-related ARDS [5]. Hence, TNF-α is a promising target for siRNA-based therapy against both acute and chronic lung inflammation.

A siRNA-based therapeutic targeting lung inflammation can be administered locally to the airways, either via inhalation or via nasal administration, where it can exert its effect directly in the inflamed tissue. Local pulmonary delivery displays several therapeutic advantages, compared with systemic delivery, including (i) a quick onset of action, (ii) a reduced therapeutic dose required, and (iii) reduced side effects [6]. Besides, inhalation and nasal administration represent non-invasive routes of administration, which increases patient compliance, and the fast renal clearance of siRNA, observed after systemic administration, is reduced after local delivery [7].

Although numerous promising therapeutic targets and oligonucleotide sequences have been identified during the past years, which have resulted in a handful of recently marketed or in advanced clinical trial products, there are still significant challenges associated with their delivery [8,9]. Generally, siRNA displays poor chemical stability against nucleases and exhibits low cellular uptake and transfection [10]. Hence, it is pertinent to identify efficient delivery systems that can

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protect the siRNA from degradation, facilitate its transport across the cell membrane and mediate endosomal escape to achieve successful siRNA delivery to the cytosol [11,12]. Numerous transfection agents have been identified for nucleic acid delivery, and they include, amongst others, cationic polymer- and lipid-based nanocarriers, which are very efficient for cellular delivery, but they are often associated with toxicity, even at relatively low doses [13,14].

Dendrimers are synthetic globular polymers displaying a high degree of surface functionality and numerous possibilities for customizing their physicochemical properties, and they have shown great potential for pharmaceutical applications, including the delivery of nucleic acid-based therapeutics [15–17]. Cationic dendrimers like the commercially available polyamidoamine (PAMAM) dendrimers have been shown to mediate efficient cellular uptake and transfection of siRNA *in vitro* in multiple studies [18]. Although widely used *in vitro*, there are only a few studies that have tested the ability of PAMAM dendrimers for siRNA transfection *in vivo* [19,20], and to date, no studies have been performed evaluating pulmonary delivery of PAMAM dendrimers *in vivo*.

In this study, we investigated generation 3 PAMAM dendrimers as transfection agents for pulmonary delivery of siRNA targeting TNF-α and examined their efficacy and safety in a murine acute lung inflammation model. Generation 3 PAMAM dendrimers were selected because they display very good efficiency for dendriplex formation They were prepared at different dendrimer-siRNA ratios and were characterized *in vitro* and *in vivo* concerning complexation, cellular uptake, cytotoxicity, *in vitro* transfection efficiency and *in vivo* therapeutic efficacy at the RNA and protein levels, respectively.

2. Materials and methods

2.1. Materials

2′-*O*-Methyl modified dicer substrate asymmetric siRNA duplex directed against TNF-α was provided by Integrated DNA Technologies Inc. (IDT, Coralville, IA, USA) as dried, purified and desalted powder. The duplex was re-annealed in a buffer consisting of 30 mM 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES) and 100 mM potassium acetate, pH 7.5. The TNF-α siRNA consisted of the following sequences:

TNF-α sense 5′-pGUCUCAGCCUCUUCUCAUUCCUGct-3′, and
antisense 5′-AGCAGGAAUGAGAAGAGGCUGAGACAU-3′,

where the underlined capital letters represent 2′-*O*-methylribonucleotides, lower-case letters represent deoxyribonucleotides and p represents a phosphate residue [21]. A negative control siRNA sequence was purchased from Eurogentec (Eurogentec, Angers, France). The sequence of this negative control is not disclosed by the supplier. Fluorescently labeled siRNA with the dye TYE™ 665 was provided by IDT with a sequence targeting luciferase:

Sense 5′-pGGUUCCUGGAACAAUUGCUUUUAca-3′,
Antisense 5′-UGUAAAAGCAAUUGUUCCAGGAACCAG-3′

Primers for PCR were acquired from Eurofins MWG Operon (Ebersberg, Germany). PAMAM dendrimer (ethylenediamine core, generation 3.0) solution was acquired from Sigma-Aldrich (St Quentin Fallavier, France). RNAse-free diethyl pyrocarbonate (DEPC)-treated water was used for all solutions containing siRNA and dendrimers. All experiments with siRNA and dendrimers were performed at 25 °C in 10 mM HEPES buffer (pH 7.4) unless otherwise stated. Lipopolysaccharide (LPS) (γ-irradiated, < 1% protein) was obtained from Sigma-Aldrich (St Quentin Fallavier, France). All additional chemicals used were obtained commercially and were of analytical grade.

2.2. Preparation of dendriplexes

Dendriplexes were prepared in 10 mM HEPES buffer at a nitrogen-to-phosphate (N/P) ratio of 5, 10, 20 and 40, respectively. Dendrimer solutions of 20 μl were added to a siRNA solution of 80 μl and subsequently vortexed, resulting in a 5 μM final siRNA concentration and 37, 75, 150 and 300 μM final dendrimer concentration at N/P ratios 5, 10, 20 and 40, respectively. The mixtures were left for 10 min at room temperature to allow complex the formation. A siRNA concentration of 500 nM was used for dynamic light scattering measurements, and a siRNA concentration of 5 μM was used for gel electrophoresis studies. Amicon Ultra-0.5 ml centrifuge tubes (Thermo-Fisher, Villebon, France) were used for upconcentrating the dendriplex suspensions by centrifugation of the samples at 13,000 g for 20 min.

2.3. Characterization of dendriplexes

The particle size distribution and polydispersity index (PDI) of the dendriplexes were determined by dynamic light scattering using the photon correlation spectroscopy technique, and their zeta potential was estimated by using laser-Doppler micro-electrophoresis using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) equipped with a 633 nm laser and 173° detection optics. The complexation of dendrimers and siRNA into dendriplexes was assessed using gel electrophoresis. Electrophoresis was carried out applying 1% (w/v) agarose gels (Promega, city, USA) containing 5 μl 2.5 mg/mL ethidium bromide solution, and samples consisting of 0.1 nmol siRNA were loaded into each well of the gels. The gels were run for 20 min at 100 V in Tris-borate-EDTA (TBE) buffer (pH 8.2). Visualization of the siRNA bands was performed using an MF-ChemiBIS gel imaging system (DNR Bio-Imaging Systems, Neve Yanim, Israel).

2.4. Cell culture

A murine macrophage cell line RAW264.7 was purchased from the American Type Culture Collection (ATCC, Molsheim, France) and cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Aldrich, St Quentin Fallavier, France) supplemented with 10% (v/v) fetal bovine serum (PAA Laboratories, Pasching, Austria) and 100 U/ml penicillin, 100 μg/ml streptomycin. Cells were grown under a controlled atmosphere with 5% CO_2/95% O_2 at 37 °C and were sub-cultured twice per week by washing and gently scraping the cells from the culture flask. Cells were used between passages 5 and 12.

2.5. Cell viability

The cellular viability of the RAW264.7 cells was assessed by using the 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. Cells were prepared in suspension, counted and seeded in 96-well plates at a density of 8×10^3 cells per well and left to attach overnight. The cells were subsequently incubated with dendriplexes (N/P ratio 5) at different concentrations for a period of 24 h. To each well with 100 μl culture medium, 10 μl of MTT-solution (5 mg/ml in PBS, pH 7.4) was added and the plate was incubated for 2 h at 37 °C. Subsequently, the medium was replaced with 200 μl dimethylsulfoxide, and the absorbance was measured at 570 nm, using a microplate reader (FLUOstar OPTIMA, BMG labtech, Germany).

2.6. Cellular uptake

Cellular uptake of siRNA was assessed in RAW264.7 cells using fluorescence microscopy and flow cytometry, respectively. For fluorescence microscopy, the cells were seeded in chambered cover slides μ-Slide 8 well (Ibidi, Planegg, Germany) at a density of 5×10^4 cells per well and cultured for 12 h. The cells were then treated with fluorescently labeled siRNA for 4 h (at an N/P ratio of 5 for dendriplexes and a concentration of 100 nM siRNA), washed with PBS and fixed using 4% (v/v) paraformaldehyde. Microscopy was performed using a Zeiss LSM 510 (Carl Zeiss, Jena, DE) fluorescence microscope equipped with a 1 mW helium–neon laser and a plan-apochromat 63X objective lens

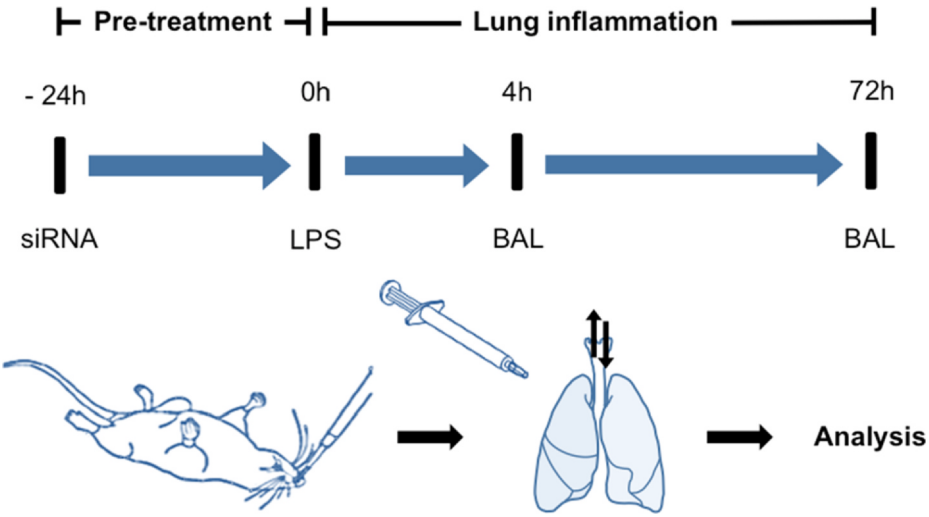

Fig. 1. Schematic illustration of the *in vivo* experimental procedure. Mice were prophylactically administered with treatments 24 h before inflammation induction by LPS. BAL was collected either at 4 or 72 h for TNF-α determination.

(numerical aperture 1.40, oil immersion). Images were captured at a 63X magnification and overlayed with differential interferential contrast (DIC) images. For flow cytometry, the cells were cultured in 12-well plates at a density of 5×10^4 cells per well. The cells were treated with fluorescently labeled siRNA for 4 h (at an N/P ratio of 5 for dendriplexes and a concentration of 100 nM siRNA) and subsequently resuspended and analyzed using an Accuri C6 (BD biosciences, Franklin Lakes, NJ, USA) flow cytometer. The mean fluorescence intensity (MFI) was used to determine the relative cell uptake (the ratio between treated and non-treated samples), expressed in arbitrary units.

2.7. Cell transfection

Cell transfection studies were performed as described previously [1722]. Briefly, RAW264.7 cells were seeded at a density of 1×10^6 cells per well in 6-well culture plates. Dendriplexes prepared at an N/P ratio of 5 were transferred to culture plates to achieve a final siRNA concentration of 100 nM, and they were incubated for 24 h. Three hours before collection, the cells were exposed to lipopolysaccharide (LPS) dispersed in PBS to obtain an LPS concentration of 5 ng/ml. Negative controls were only given PBS, and positive controls only received LPS, but were not treated with siRNA. For collection, the culture medium was removed from the wells, 1 ml ice-cold TRIzol (Thermo Fisher, Villebon-sur-Yvette, France) was added to each well, and the cells were scraped and homogenized by pipetting.

RNA extraction was performed following the stepwise instructions provided with the TRIzol reagent, and the total RNA content of the extracts was assessed using a Biomate 3 UV spectrophotometer (Thermo Fisher) with a TrayCell ultra-micro cell (Hellma Analytics, Paris, France). Additional quality control of the extracts was performed with an RNA LabChip, using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Reverse transcription was performed for 1 µg RNA extracts using a mix of primers [17] and an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, (Hercules, CA, USA). The cDNA was diluted 1:10 (v:v) in PCR-grade water and stored at −80 °C until further use. TNF-α mRNA silencing was assessed by real-time reverse transcription-polymerase chain reaction (RT-PCR), essentially as previously described [22]. Real-time PCR was performed using a C1000 Thermal Cycler instrument with a CFX96 Real-Time System (Biorad) with the following cycling conditions: Initial denaturation step at 95 °C for 5 min, followed by 35 cycles including (i) denaturation at 95 °C for 10 s, (ii) annealing at 60 °C for 10 s, and (iii) elongation at 72 °C for 10 s. The CFX manager software 3.0 (Biorad) was used for crossing point (CP) analysis, and the values were normalized against the average of the two reference genes ribosomal protein, large P2 (RPLP2) and glucoronidase b (GUSb). The reference genes were selected based on a screening study of eight reference candidates [17].

2.8. In vivo studies

All animal experiments were conducted following the European rules (86/609/EEC and 2010/63/EU) and the Principles of Laboratory Animal Care and the national French legislation (Decree No. 2013–118 of February 1, 2013). All experimental procedures were refined to provide for maximal comfort and minimal stress to the animals. Female Swiss CD-1 outbred mice were purchased from Envigo (Gannat, France), and all experiments were performed using mice at the age of 6–8 weeks. The mice were housed in groups of four with access to water and food *ad libitum* and kept at a constant temperature (19–22 °C) and relative humidity (45–65%).

2.8.1. Acute lung inflammation model

A well-known procedure for LPS-induced lung inflammation [23–25] was modified using Swiss CD-1 mice. The mice were anesthetized by intraperitoneal (i.p.) injection of 200 µl Ketamine (3.5 mg/ml)/Xylazine solution (5 mg/ml). Subsequently, LPS solution (3.2 mg/kg) was administered to the mice *via* intranasal administration (1.2 µl LPS solution / g of mouse weight). For extraction of the bronchoalveolar lavage (BAL), the mice were euthanized *via* an overdose of pentobarbital (i.p. injection of 200 µl 20 mg/ml pentobarbital solution). Subsequently, the trachea was cannulated with a catheter, and the lungs were flushed twice with 0.3 ml PBS. The BAL was centrifuged at 400 g for 10 min, the supernatant was stored at −20 °C for protein quantification and TNF-α determination.

2.8.2. Prophylactic siRNA treatment

Mice were dosed with siRNA as a prophylactic treatment before inducing lung inflammation (Fig. 1). Non-complexed siRNA and dendriplexes (N/P ratio of 5) were administered *via* intranasal administration similar to LPS administration at a siRNA concentration of 1.0 mg/kg using an average volume of 30 µl siRNA solution 24 h before LPS challenge. The BAL was extracted 4 and 72 h, respectively, after the LPS challenge, and a minimum of five mice was included in each sample group. Negative controls were dosed twice with PBS, and positive control mice were first dosed with PBS and subsequently treated with LPS.

2.8.3. TNF-α measurements

The TNF-α protein levels were assessed by using the Cytometric Bead Array - Mouse inflammation kit (BD biosciences). The supernatants of BAL samples were diluted in assay diluent from the kit, and the samples were prepared following the manufactureŕs instructions. The BAL TNF-α levels were quantified using an Accuri C6 flow cytometer, where 2000 ROI counts were measured for each sample. All samples were prepared in duplicates and analyzed using the BD Accuri C6 software.

2.9. Statistics

The data of the *in vitro* studies are reported as mean values ± SD, and the data for the *in vivo* studies are reported as mean values ± SEM. The statistical significance was determined using either a two-tailed, unpaired Student's *t*-test or ANOVA, with the statistical significance set at * $p < 0.05$, ** $p < 0.01$.

3. Results and discussions

3.1. Dendriplex formation

All dendriplexes displayed an average size between 127 and 153 nm and a PDI between 0.19 and 0.27 (Table 1). There was no clear correlation between the N/P ratio of the dendriplexes and their resulting sizes and PDIs although it has previously been shown that the sizes of PAMAM dendriplexes decrease with an increase in N/P ratio [26]. Positive zeta potential values were observed for all N/P ratios, indicating a net positive surface charge and condensation of siRNA. The zeta potential increased significantly ($p < 0.05$, ANOVA) as a function of the N/P ratio, which can be attributed to the increase in charge ratio, and it also indicates improved siRNA condensation at higher N/P ratios.

Gel electrophoresis studies using EtBr to stain the siRNA were performed to assess qualitatively the binding between siRNA and dendrimer at different N/P ratios. These studies show binding between siRNA and dendrimers at all N/P ratios (Fig. 2). In all cases, the major part of the siRNA was retained inside the dendriplexes in the presence of EtBr, as compared to free, non-complexed siRNA. Based on the size and binding results of the dendriplexes, subsequent experiments were performed at an N/P ratio of 5 as a compromise between the complexation and high siRNA loading as shown by gel retardation assay. Moreover, this ratio was selected to reduce the cytotoxicity as much as possible.

3.2. Cell viability and uptake

Cell viability studies using the MTT assay showed that the dendriplexes were well tolerated up to a siRNA concentration of 800 nM (N/P ratio 5) (Fig. 3A). A similar profile was observed for non-complexed dendrimers (not shown). The cytotoxicity measured for the dendriplexes is relatively low, as compared to other cationic transfection agents, *e.g.*, polyethyleneimine (PEI) and poly-l-lysine [27], which indicates that PAMAM dendriplexes are well tolerated by RAW264.7 cells. Cellular uptake assessed at a subtoxic concentration of 100 nM siRNA using flow cytometry showed that the uptake of non-complexed siRNA was low and did not increase over time (Fig. 3B). In contrast, the cellular uptake of the dendriplexes increased gradually with time, resulting in a six-fold higher uptake after 4 h, as compared to non-complexed siRNA. Confocal fluorescence microscopy images captured after 4 h incubation showed no visible fluorescence inside the cells incubated with non-complexed siRNA, which also suggests a very low uptake of siRNA (Fig. 4). Cells incubated with dendriplexes showed substantial fluorescence inside the cells with numerous red dots distributed within every cell (Fig. 4). The confocal fluorescence microscopy images indicate efficient dendrimer-mediated cellular uptake of siRNA, which support the flow cytometry results.

Table 1
Z-average, polydispersity index (PDI) and zeta potential of dendriplexes prepared in HEPES buffer (10 mM, pH 7.4). Data points represent mean values ± SD ($n \geq 3$). Zeta potential values were significantly different from each other (ANOVA, $p < 0.05$).

N/P ratio	*Z*-average (nm)	PDI	Zeta potential (mV)
5	152 ± 6	0.22 ± 0.04	36 ± 2
10	141 ± 4	0.19 ± 0.02	39 ± 1
20	127 ± 7	0.27 ± 0.03	45 ± 4
40	153 ± 11	0.24 ± 0.03	46 ± 4

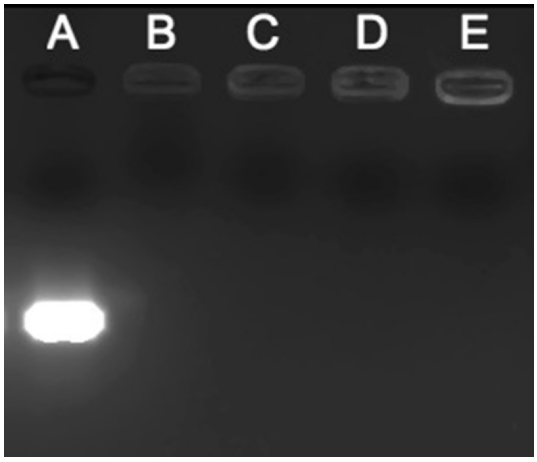

Fig. 2. Agarose gel electrophoresis of dendriplexes (0.9 μg siRNA per lane). A: Non-complexed siRNA; B-E: dendriplexes prepared at N/P ratios of 5, 10, 20 and 40, respectively.

3.3. TNF-α silencing in cells

Transfection using TNF-α siRNA and negative control siRNA in macrophages activated with LPS was evaluated at the mRNA level using qPCR (Fig. 5). An siRNA containing 2′-*O*-methylated nucleotides in the antisense strand was selected to minimize the innate immune response that occurs with the administration of siRNA [28]. Another reason is provided by the greater stability of this chemically modified siRNA [29]. Non-complexed TNF-α siRNA mediated little but statistically significant inhibition (26%) of the TNF-α mRNA expression ($p < 0.05$), as compared with the negative control siRNA, and 28% inhibition, as compared to the LPS-challenged positive control ($p < 0.05$). In contrast, the PAMAM dendriplexes with TNF-α siRNA mediated 86% inhibition of TNF-α expression, as compared to the LPS-challenged positive control ($p < 0.001$) and 58% inhibition compared with the PAMAM dendriplexes with negative control siRNA ($p < 0.01$), which represents a substantially greater inhibition compared with non-complexed siRNA. Hence, PAMAM dendriplexes display high potential for TNF-α silencing, but some unspecific silencing of PAMAM dendriplexes with negative control siRNA was also observed. The transfection data support the observations from the cell uptake studies, indicating the improved performance of dendriplexes, as compared with non-complexed siRNA.

3.4. In vivo silencing and modulation of inflammation

To induce gene silencing in the lung, siRNAs were delivered using PAMAM dendrimers. Many studies have examined the effect of PAMAM dendrimers on the lung. At first, it was shown that 83% of the PAMAM dendrimers dosed were still remained in the lung 6.5 h post pulmonary administration to mice [30]. Several studies have stressed the lung biocompatibility of PAMAM dendrimers and the absence of any

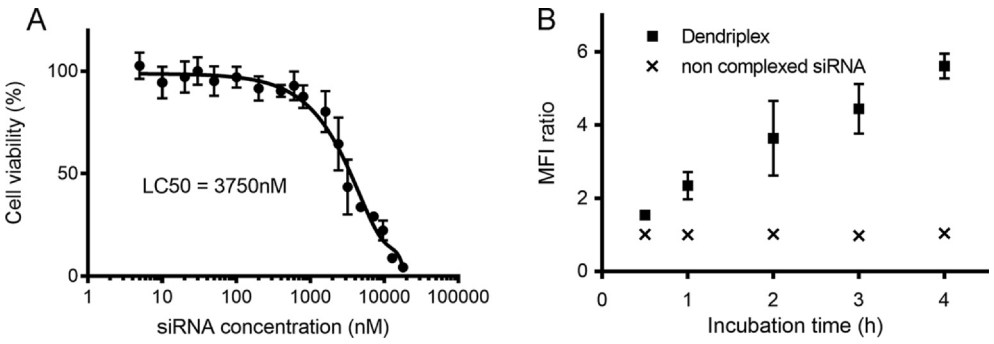

Fig. 3. Cell viability of RAW 264.7 cells as a function of siRNA concentration, measured after 24 h incubation with dendriplexes (A). Cellular uptake of siRNA (100 nM) in RAW 264.7 cells as a function of incubation time, measured as siRNA mean fluorescence intensity (MFI) using flow cytometry (B). Data points represent mean values ± SD ($n \geq 3$). For non complexed siRNA, the error bars are smaller than the points.

inflammatory response in the lung [31,32].

In several studies, it has been shown that intranasal challenge with LPS results in lung inflammation characterized by an increase in the levels of TNF-α as well as other pro-inflammatory cytokines in the lungs [33]. Besides, the massive recruitment of macrophages and neutrophils takes place rapidly, and the inflammatory process lasts for several days [34,35]. Chronic inflammation is usually characterized by occasional flare-ups, including COPD, asthma, and cystic fibrosis, which represent the most common reason for contact between the patient and the health care system [36]. These flare-ups can be triggered by both external and internal stimuli, *e.g.*, exposure to pollutants and increased stress levels. Hence, the optimal treatment strategy may be a prophylactic measure taken to reduce the response to such stimuli. Therefore, in this study, we investigated the therapeutic effect of a prophylactic treatment with siRNA given 24 h before inducing an inflammatory response with LPS.

TNF-α levels were compared for mice treated with LPS (Control +) as well as untreated mice (Control -) and were evaluated to assess the potential inflammatory response of the non-complexed siRNA and the dendriplexes. Dendriplexes were administered at a siRNA dose of 1 mg/kg, based on previous siRNA studies on pulmonary delivery, where doses ranging between 0.6 and 3 mg/kg were administered [37–39].

The TNF-α concentrations in the BAL were measured 4 h and 72 h after exposing the mice to LPS. The TNF-α levels measured after 4 h (Fig. 6A) indicated a significant ($p < 0.05$) reduction in TNF-α for subjects treated with dendriplex TNF-α siRNA as compared to negative control or to free TNF-α siRNA, whereas mice treated with non-complexed TNF-α siRNA did not show any significant difference from Control + . Mice treated with dendriplex TNF-α siRNA demonstrated 24% TNF-α silencing compared with those treated with LPS-treated positive control and a 24% TNF-α silencing compared with the dendriplex negative control siRNA. Mice treated with non-complexed TNF-α siRNA showed a mere and non-significant 2% TNF-α silencing compared with the LPS-treated control and a 7% TNF-α silencing compared with the non-complexed control siRNA. This indicates the need for a transfection agent for the delivery of siRNA to the lungs and that

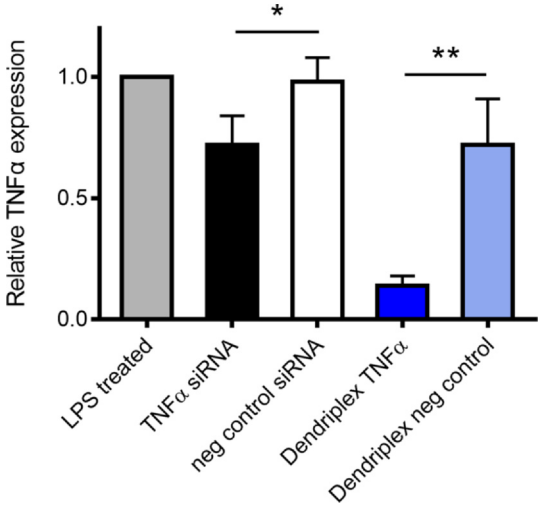

Fig. 5. TNF-α mRNA silencing in RAW 264.7 cells by dendriplexes. Samples were normalized to the LPS-treated cells (Control +) without siRNA, and the results denote the TNF-α mRNA expression level of cells treated with TNF-α siRNA, relative to transfection with negative control siRNA. The samples correspond to a siRNA concentration of 50 nM and an N/P ratio of 5. Results denote mean values ± SD ($n \geq 3$). Statistical significance: * $p < 0.05$, ** $p < 0.01$.

dendriplexes mediate a substantial inhibition of TNF-α expression in the early stage of LPS-induced lung inflammation. The TNF-α levels measured 72 h after challenging the mice with LPS (Fig. 6B) were significantly ($p < 0.05$) reduced for the two groups treated with TNF-α siRNA, as compared to the corresponding groups treated with negative control siRNA. Mice treated with non-complexed TNF-α siRNA demonstrated significant ($p < 0.05$) TNF-α silencing of 34% compared with the LPS-treated positive control, and a significant TNF-α silencing of 23% ($p < 0.05$) compared with the non-complexed negative control

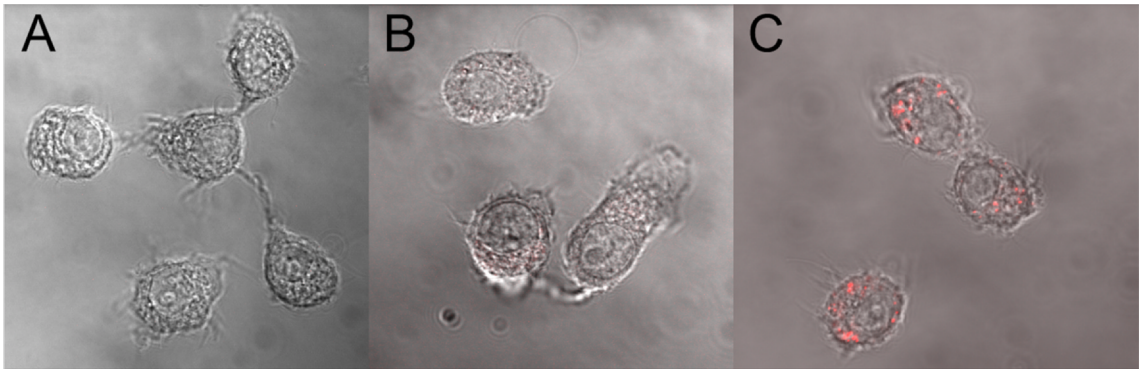

Fig. 4. Representative images of the cellular uptake of dendriplexes (100 nM siRNA) in RAW 264.7 cells after 4 h incubation. Overlay of a bright field and fluorescence image for untreated cells (A) and cells incubated with non-complexed siRNA (B) and dendriplexes (C).

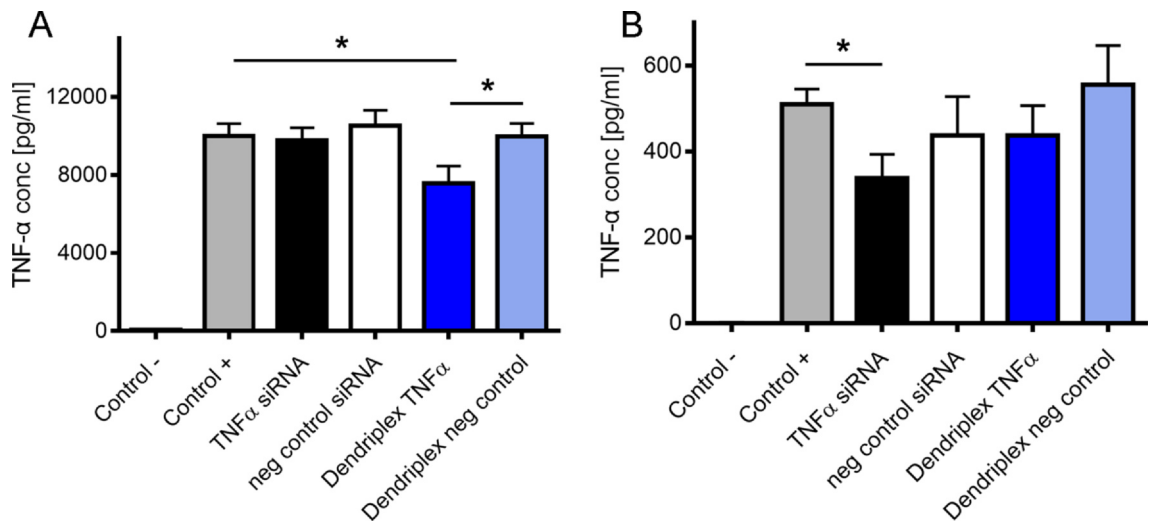

Fig. 6. TNF-α concentration in BAL of mice challenged with LPS 24 h after administration of siRNA treatment and euthanized 4 h (A) and 72 h (B) after the LPS challenge, followed by extraction of BAL. Control - and Control + denote untreated and LPS-treated mice, respectively. Data points represent mean values ± SEM. n ≥ 5. Statistical significance: * $p < 0.05$.

siRNA. In comparison, the dendriplex TNF-α siRNA resulted in a significant TNF-α silencing of 21% ($p < 0.05$) compared with the dendriplex negative control siRNA and a TNF-α silencing of 14% compared with the LPS-treated positive control. This indicates that non-complexed siRNA resulted in slightly more sustained inhibition of TNF-α expression than the dendriplex TNF-α siRNA comparing the expression at 4 h and 72 h after the LPS challenge.

Several polymeric carriers have been investigated for siRNA delivery and many systems have demonstrated successful gene silencing and therapeutic efficacy, including dendrimers. In this study, PAMAM dendrimer was studied as a transfection agent for siRNA delivery to the lungs and assessed in an acute lung inflammation model in mice. The study showed that the dendriplexes provided an efficient delivery *in vitro* with high transfection performance and cellular uptake in RAW264.7 macrophages. Yet, based on *in vivo* studies it was demonstrated that dendriplexes with TNF-α siRNA resulted in improved performance compared with non-complexed TNF-α siRNA at early time e.g 4 h after LPS challenge and lower performance compared with non-complexed TNF-α siRNA 72 h after LPS challenge, meaning that more frequent administrations are needed in patients displaying strong lung inflammation.

In this study, TNF-α was selected as a target because this proinflammatory cytokine plays a central role in lung diseases and its actions are numerous and quite diverse being linked to many lung diseases including asthma, COPD, ALI/ARDS, sarcoidosis, and interstitial pulmonary fibrosis [40]. The data show that a quick response occurs not lasting long which might be related to a very quick dissociation in the lungs. Thanki et al. [7] demonstrated that although part of complexed siRNA was staying in the lungs, around 50% was permeating across the air-blood barrier within 6 h and subsequently excreted via the kidneys [7]. The low stability as well might explain why the efficacy does not stand for longer times. In a previous work [17,41] involving phosphated dendrimers, we compared two dendrimers in a lung injury model using the same anti-TNF-α siRNA. It was clear from these studies that dendriplexes with the lowest Kd were the more active with a long-lasting inhibition effect not only on TNF-α but other cytokines as well. This could be achieved with more frequent lung administration but would also raise the question of chronic toxicity which needs to be further evaluated. Applying higher-generation PAMAM denderimers would have increased the stability and efficacy but again could have induced much higher toxicity. Finally, two factors might explain the lower efficacy of dendriplexes at 72-hour. The first is the high stability of methylated siRNA and the second is the fast elimination of dendriplexes due to the mucociliary clearance, as compared to the clearance of non-complexed molecules that can penetrate in the deep lung without undergoing this elimination process.

4. Conclusion

In the current study, the performance of PAMAM dendrimers was investigated as a delivery system for siRNA transfection, specifically for the local treatment of lung inflammation. We demonstrate a good ability to condensate siRNA, high cellular internalization rate and a specific and efficient gene silencing of TNF-α *in vitro* in macrophages for PAMAM-based dendriplexes with TNF-α targeting siRNA. *In vivo* studies in a murine acute lung inflammation model showed silencing of TNF-α for the dendriplexes although less pronounced compared to *in vitro* performance. The findings suggest that TNF-α targeting siRNA can be used as a local treatment for overall suppression of lung inflammation and can be used as a prophylactic treatment administered one day before an inflammatory trigger.

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