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Research paper

# CpG-free plasmid DNA prevents deterioration of pulmonary function in mice

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ABSTRACT

Nonviral gene vectors have been shown to be therapeutically effective in various animal models of inherited and acquired lung diseases. Although an acute unmethylated CG dinucleotide (CpG)-mediated inflammatory response has been previously observed for first-generation plasmids, its effect on pulmonary function has not been investigated to date. Here, we present data on lung functional parameters together with histopathology, cellular and inflammatory events in response to pulmonary administration of DNA-containing particles. We show that aerosol delivery of polyethylenimine gene vectors containing a first-generation CpG-rich plasmid induced an inflammatory response which was associated with a decrease in lung compliance. In contrast to these observations, aerosol application of CpG-free plasmid DNA prevented immune response and impairment of pulmonary function. These results demonstrate that aerosol delivery of CpG-free plasmid DNA is critical to avoid alteration of pulmonary function. Therefore, we suggest to use CpG-free pDNA for gene delivery to the lungs in future.



## 1. Introduction

Nonviral gene delivery to the lung has been extensively investigated within the previous years. Therapeutic response has been achieved in several mouse lung tumor models using plasmids which coded for the p53 tumor suppressor gene [1–4], as well as in clinical trials for example in cystic fibrosis, where gene delivery techniques are currently being performed [5–7].

For effective therapy, target-specific drug delivery in vivo is a major requirement. Gene transfer to respiratory tissue is possible either via intravenous injection, aerosol inhalation or intratracheal and intranasal instillation. The pulmonary route allows delivery of the therapeutic agent directly to the lungs, bypassing several biological and physiological barriers encountered by intravenously administered agents [8]. Nonviral vectors offer several advantages compared to replication-deficient viruses since they are nonpathogenic, less toxic and less immunogenic [9,10]. Synthetic gene transfer agent polyethylenimine (PEI) is a cationic polymer which has been shown to have remarkable in vitro and in vivo transfection activity and is widely used for gene transfer to respiratory and alveolar tissue [11–13]. PEI is available commercially in a variety of molecular weights and configurations (linear and branched) and interacts with pDNA electrostatically to form discrete nanoparticles. Branched PEI stabilizes pDNA during nebulization [14], resulting in a higher level of pulmonary transgene expression than many cationic lipid based formulations [15]. PEI–pDNA particles delivered via instillation tend to form bolus in lower regions of the lung, while inhaled PEI–pDNA complexes deposit evenly in the lung, distributing within alveolar and bronchiolar epithelium and presumably bind to proteoglycans on cell surfaces [16]. Subsequently, inhaled complexes are endocytosed predominantly by bronchiolar epithelial cells in conducting airways [12,13]. Although the transfection properties of PEI are well characterized, there is limited data on its in vivo toxicity and it has not yet been used in human inhalation studies. Besides, changes in lung function parameters after gene delivery have not been investigated and compared to concomitant histopathology, cellular and inflammatory events. PEI is known to be less biocompatible than other cationic polymers, for example chitosan [17], and bears toxicity that may have a negative impact on the lung. Applied for transfection on mammalian cell cultures, PEI provides high rates of transgene expression, still causing toxic effects and provoking secretion of pro-inflammatory cytokines [18,19]. Several studies have shown that instillation of PEI into murine lungs causes alterations in

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pulmonary functioning and toxic effects, although this was dependent on the type of PEI administered [19–21].

On the other hand, bacterial plasmids which are used most frequently in gene delivery contain unmethylated CpG motifs which are immune stimulatory and provoke diverse effects on the lungs [22–26]. Both innate and adaptive immune systems are activated by CpG motifs through "pattern-recognition receptors", of which the Toll-like receptor (TLR) family is most thoroughly studied. TLR9 is one of the most well-understood members of the family, which is seemingly expressed in the endosomal compartment of dendritic- and B-cells [27] and plays an essential role for viral and bacterial DNA recognition [28]. After B-cells or dendritic cells are exposed to CpG-containing olygonucleotides, the latter appear to enter the endosomal compartment within minutes, leading to activation of cell-signalling pathways, expression of costimulatory molecules and cause cell trafficking to the T-cell zones in the lymph nodes [29]. Therefore, pulmonary gene therapy with nonviral vectors delivered either by instillation or via intravenous injection has been associated with the induction of cytokine response attributed to bacterial plasmids [30]. It has been previously demonstrated that a single CpG dinucleotide in the pDNA is sufficient to induce an inflammatory response in the lungs [31]. Besides, aerosol delivery of PEI–pDNA complexes induced tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1ß) in lung tissue and bronchoalveolar lavage fluid (BALF), but to a much lower extent than cationic lipid/DNA complexes [30].

Against this background, we investigated lung mechanics of mice after inhalation of PEI–pDNA complexes, to further elucidate a conceivable association of inflammatory response with pulmonary function alterations. These results in a small animal model are important in terms of the potential use of PEI-based formulations in future clinical trials for diseases, where the lung is known to be chronically affected or which are associated with deterioration of pulmonary function.

## 2. Materials and methods

### 2.1. Chemicals

Branched PEI (average MW = 25 kDa) was obtained from Aldrich (Deisenhofen, Germany), dialyzed in water (12–14 kDa MW cutoff) on CelluSep membranes (MFPI, Braine-l'Alleud, Belgium) and adjusted to pH = 7. Double distilled endotoxin-free water for injection was purchased from Delta Pharma (Boehringer Ingelheim, Germany).

### 2.2. Plasmid

pCMVLuc containing the firefly luciferase cDNA driven by the CMV promoter was generously provided by Dr. E. Wagner (Department of Pharmacy, University of Munich, Germany). pCpGLuc was constructed by Manfred Ogris (Department of Pharmacy, Ludwig-Maximilians University, Munich, Germany). Briefly, CpG-free plasmids, pCpG-mcs and pMOD-LucSh, were obtained from Invivogen (Toulouse, France). Luciferase cDNA was excised from pMOD-LucSh by BglII–NheI digestion and cloned into respective sites of pCpG-mcs to generate pCpGLuc. pCpG-mcs was used as backbone plasmid, containing no reporter genes. Both plasmids were propagated in *Escherichia coli* and provided in a highly purified form (LPS content $\leqslant$0.1 EU/μg DNA) by PlasmidFactory (PlasmidFactory GmbH, Bielefeld, Germany). According to the supplier's guarantee, the amount of supercoiled pDNA was $\geqslant$98% ccc (covalently closed circular, ccc grade; assessed by capillary gel electrophoresis).

### 2.3. Animals

Fourteen week old female BALB/c mice were purchased from Janvier (Elevage Janvier, Le Genest St. Isle, France) and maintained under specific pathogen-free conditions. All experiments were approved and controlled by local ethic committee and conducted according to the guidelines of the German law of protection of animal life.

### 2.4. Preparation of PEI–pDNA complexes

Formulation of polyplexes and aerosol application was prepared as reported previously [32]. Two mg of pDNA was complexed with branched PEI. DNA and branched PEI were diluted in distilled water to 4 ml, resulting in concentrations of 0.5 mg/ml DNA and 0.66 mg/ml PEI, respectively (corresponding to an N/P ratio of 10). The DNA solution was pipetted into the PEI solution and mixed gently by pipetting up and down to yield a final DNA concentration of 0.25 mg/ml. DNA solution was pipetted into the PEI solution to yield a final DNA concentration of 1.0 mg/ml. The complexes were incubated for 20 min at ambient temperature before use. Size and zeta-potential of pDNA complexes with PEI was measured via photon correlation spectroscopy (PCS) on a Zetasizer 3000HS (Brookhaven Instrument Corporation, Holtsville, USA). For the determination of the particle size, 600 μl of suspension of complexes prepared for aerosol delivery was pipetted in a plastic cuvette (Greiner, Frickenhausen, Germany) and measured 10 min by dynamic light scattering. The size of complexes was controlled in triplicate prior to every administration. Stability of complexes was measured in a separate series of experiments, which were performed in triplicate for every plasmid. Following the procedure described earlier, PEI–pDNA complexes were prepared in a final volume of 600 μl. Size distribution of the obtained particles was measured every 10 min for 1.5 h. All measurements were analyzed with ZetaPALS Particle Software (Version 3.42). For the measurement of zeta-potentials, 1.6 ml of complexes solution was prepared according to the standard procedure, was placed in a plastic cuvette, and a palladium electrode was immersed into the liquid. The following settings were used: 10 sub-run measurements per sample; viscosity for water 0.89 cP; beam mode $F(Ka) = 1.50$ (Smoluchowsky); temperature 25 °C and Version 3.42 of ZetaPALS Particle Software. Each of complexes was measured in duplicate. The complexes were prepared fresh for every measurement. The sizes of PEI–pCMVLuc, PEI–pCpG-Luc and PEI–pCpG–backbone complexes were 93 ± 1 nm, 123 ± 1 nm and 95 ± 1 nm, respectively, and remained constant for 1.5 h. The zeta-potentials of the complexes were 35–41 mV.

### 2.5. Aerosol application of PEI–pDNA polyplexes

Twenty female BALB/c mice inhaled 8 ml aqueous solution of PEI–pCMVLuc or PEI–pCpGLuc in a whole-body nebulization chamber connected to a jet nebulizer (PARI BOY jet nebulizer, PARI GmBH, Germany) through an aerosol spacer placed in a horizontal position as described previously [32]. The bottom of the spacer was covered with about 150 g silica gel. This leads to a shift of the aerosol size distribution towards smaller diameters due to aerosol drying. The nebulization was performed in the presence of 5% $CO_2$ in air to increase gene vector deposition in the lungs by enhancing the breathing rate of mice during nebulization.

### 2.6. Bronchoalveolar lavage fluid (BALF)

Mice were sacrificed, intubated, and bronchoalveolar lavage (BALF) of mice lungs was performed with ten 0.7 ml aliquots of phosphate-buffered saline. Samples were centrifuged for 10 min

at 160 *g* at 4 °C, and the cell pellet was resuspended in 500 μl PBS. Recovered fluid was stored on ice until further processed.

### 2.7. Quantification of cell type composition of BALF cells

Pellets of BALF cells, resuspended after centrifugation, were used for the preparation of cytospin slides (140 g, 10 min), which were subsequently dried at room temperature, stained with May–Grünwald stain and counterstained with Giemsa stain according to the standard procedure for blood smears. For the quantification of cell types ratio, 500 recognisable cells were taken from different fields of view. Three major cell types were taken into account. Ratios were quantified individually for every experimental animal.

### 2.8. Determination of macrophage activation level

Freshly prepared cytospin slides of BALF cells were fixed and stained with benzidine dihydrochloride containing myeloperoxidase stain by method of Kaplow [33] and counterstained with Giemsa stain. Five hundred macrophages were taken for quantification from different fields of view.

### 2.9. Cytokine arrays of BALF fluid samples

For the estimation of cytokine concentration in BALF fluid, "Mouse Inflammation Antibody Array 1 Analysis Tool" kit (RayBiotech, Hölzel Diagnostika, Cologne, Germany) was used according to the manufacturer's instructions. Briefly, samples of the BALF fluid were diluted 1:10 with PBS and pipetted into an array plate, afterwards the array membranes were carefully inserted into the liquid. After 30 min of incubation at room temperature, chemiluminescence-based signal imaging was performed using Diana III system (Raytest, Straubenhardt, Germany). The obtained signals were aligned with the help of "RayBio® antibody array analysis tool" (RayBiotech, Hölzel Diagnostika, Cologne, Germany) and normalized according to the signal densities obtained from control (untreated) animals.

### 2.10. Histological examination

For histological examination, lung tissue samples were fixed in 4% buffered formalin and embedded in paraffin. The 4 μm sections were stained with hematoxylin–eosin evaluated according to morphological changes.

### 2.11. Ventilation and lung mechanical measurement

Pulmonary function of mice was measured with a small animal piston-ventilator (SAV-Flexivent, SCIREQ Inc., Montreal, Canada) at indicated time points as previously described [34]. Prior to the measurements, mice were sacrificed by intraperitoneal injection of 50 mg/kg pentobarbital, weighed and subsequently intubated through a tracheostomy. The flexiVent® software allowed continuous monitoring of tidal volumes and airway pressure. During tidal ventilation, the respirator was set to a volume controlled, pressure limited ventilation mode ($V_t$ = 10 μl/g, $P_{max}$ = 30 cm $H_2O$, PEEP = 4 cm $H_2O$) at 2 Hz and 100% oxygen. Measurements were taken in 5-min intervals for 25 min at indicated times after whole-body aerosol application of PEI–pDNA complexes or instillation. We also determined lung function parameters of mice at indicated time points after aerosol application of distilled water or intranasal instillation of 50 μl PEI–pCMVLuc (50 μg) complexes and distilled water, respectively. Additionally, lung function of five untreated mice was measured. In all animals, lung mechanics reached a plateau after 15–20 min after the initiation of mechanical ventilation. Thus, data at 25 min were taken for comparison between groups.

Pressure transducers were calibrated by two-point calibration, and ventilator tubing and cannula were accounted for by open and closed calibration of the system for all perturbations, prior to each experiment. Dynamic mechanics of the respiratory system, compliance ($C_{rs}$) and resistance ($R_{rs}$), and lung input impedance were measured following a recruitment manoeuvre (two inflations to 15 μL/g over 1 s) to provide a standard volume history. For oscillatory measurement, ventilation was halted at PEEP-level. At each time point after treatment, a group of five mice was examined.

To determine impedance of the respiratory system ($Z_{rs}$) by forced oscillation technique (FOT), a forcing signal, consisting of an 8 s pseudorandom oscillatory signal, was applied with amplitude of 3 ml/kg. The forcing signal contained frequencies between 0.5 and 19.6 Hz [35]. Data were collected at 256 Hz and analyzed within 4 s windows with 66% overlap. Lung impedance data were displayed as resistance (real part, $R_{rs}$) and reactance (imaginary part, $X_{rs}$) of the respiratory system within the frequency domain.

Lung impedance data ($Z_{rs}$) were partitioned, applying the constant phase model of the lung, according to suggestion of Hantos et al. [36].

In this model, $Z_{rs}$ consists of airway resistance ($R_{aw}$), airway inertia ($I_{aw}$), tissue elastance ($H_L$), imaginary unit ($j$) and tissue damping ($G_L$) according to the equation:

$$Z_{rs} = R_{aw} + j\omega I_{aw} + (G_L - jH_L)/\omega^{\alpha}$$

with $\omega$ being the angular frequency and $\alpha$ the frequency dependence of $Z_{rs}$ ($\alpha = (2/\pi)\tan^{-1}(1/\eta)$). In this model, lung hysteresivity (eta) = $G_L/H_L$ is a measure for lung tissue composition taking into account both tissue damping and tissue elastance [37,38].

For each measurement, the fitting of the constant phase model is automatically tested. Fitting quality is displayed as coherence of determination (COD). Data were rejected when COD was below 0.85.

### 2.12. Statistical analysis

Results are reported as percent ratio from controls (untreated animals) ± standard error of the mean ($n$ = 5). The statistical analyses between different groups were determined by ANOVA and subsequent post hoc analyses as appropriate (Bonferroni) (SPSS, V.15.0.1, Chicago, Illinois, USA), $p < 0.05$ was considered significant.

## 3. Results

### 3.1. Lung function measurements in mice after aerosol application of PEI–pDNA gene vectors comprising a first-generation plasmid

Dynamic lung mechanics was measured at different time points after aerosol application of PEI–pDNA complexes. Untreated mice as well as animals treated with vehicle (water for injection) were used as controls. Aerosol application of vehicle reduced lung compliance ($C_{rs}$) to 68% of untreated controls one hour after administration, which returned to normal after 24 h (Fig. 1A). Aerosol application of PEI gene vectors with first-generation plasmid (PEI–pCMVLuc) reduced $C_{rs}$ to 72% of untreated controls one hour after administration, which returned to normal after 24 h but then progressively declined to 60% of untreated controls seven days after administration (Fig. 1A). Aerosol application of vehicle and PEI–pDNA complexes caused no alterations in the respiratory system resistance ($R_{rs}$) (Fig. 1B). Results from lung impedance measurements are outlined in Fig. 1C–F. Tissue damping (GTiss), reflecting resistive forces of lung tissue, was not significantly different from control values after PEI–pDNA aerosol application.

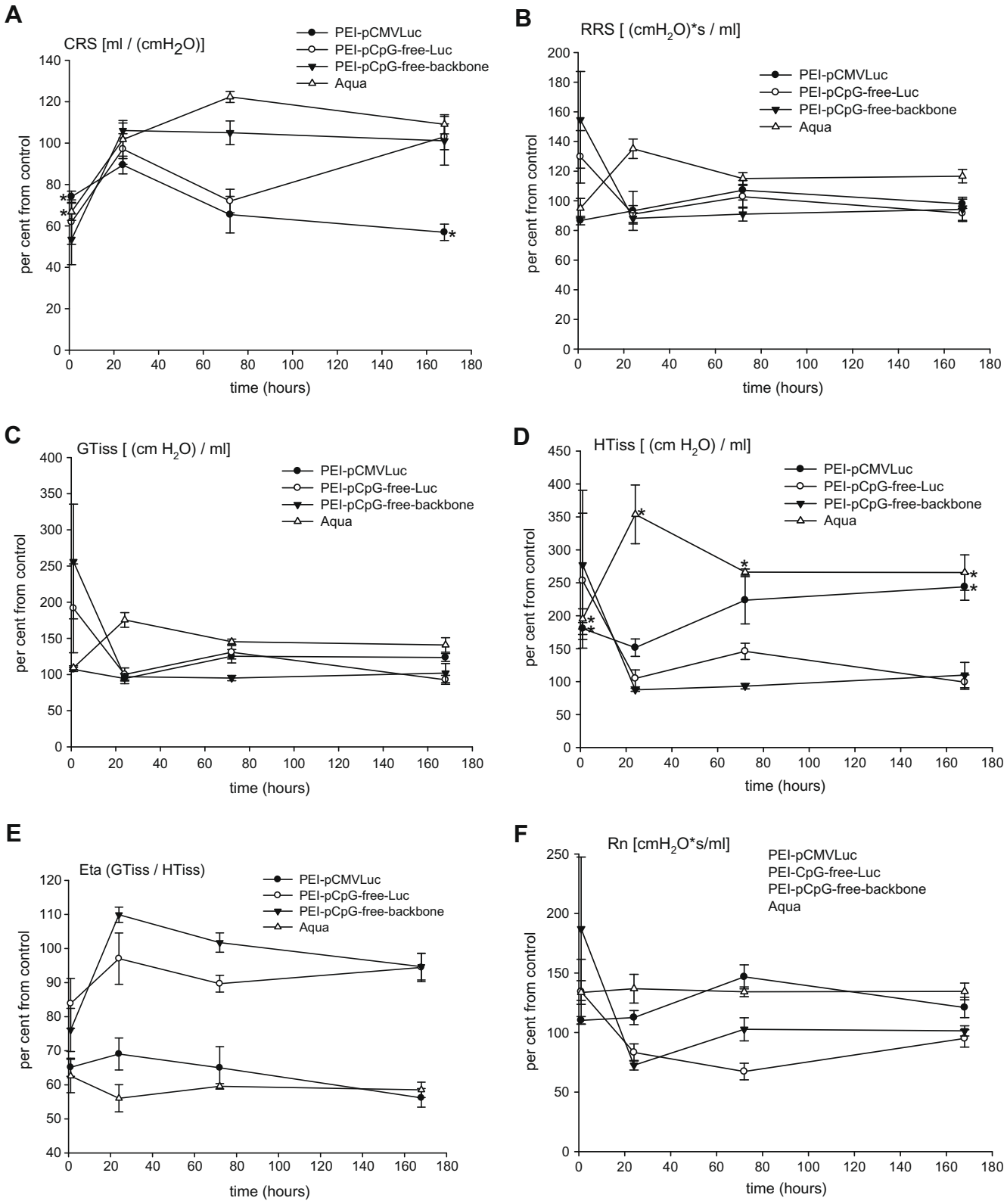


**Fig. 1.** Lung function of mice measured at indicated time points after nebulization of PEI–pDNA particles and distilled water. At indicated time points after nebulization, pulmonary function of each mouse was measured. (A) Compliance of mice lungs ($\Delta V/\Delta p$) is given as [ml/(cm $H_2O$)]. (B) The respiratory system resistance is shown [(cm $H_2O * s$)/ml]. Additionally, we detected tissue damping (GTiss, C) and elasticity of collateral lung (HTiss, D), hysteresivity (Eta, E) and Rn (F). Results are reported as percent from control (untreated mice) ± standard error of the mean. Statistically significant differences from lung function parameters of untreated control mice to those of treated mice are denoted with an asterisk ($p < 0.05$).

After aerosol application of vehicle, GTiss significantly increased compared to untreated controls 24 h after treatment. Seven days after aerosol application, these values returned to control levels (Fig. 1C). Tissue elastance (HTiss) was significantly increased after aerosol administration of PEI–pDNA and vehicle (Fig. 1D). The hysteresivity (eta), which is the ratio of GTiss to HTiss, was equally re-

duced for each treatment group compared to untreated lungs (Fig. 1E). Newtonian resistance $R_n$, reflecting central airway resistance, was not significantly affected by any treatment (Fig. 1F).

These results demonstrate that aerosolized PEI–pDNA gene vectors containing a first-generation plasmid led to alteration of lung compliance of mice one week after administration.

### 3.2. Lung function measurements in mice after aerosol application of PEI–pDNA gene vectors comprising CpG-free plasmid DNA

The observed decrease in lung compliance after aerosol administration of PEI–pDNA gene vectors containing a first-generation plasmid could have been caused by immunogenic unmethylated CpG motifs in plasmid DNA or by a putative immune response to luciferase transgene. We therefore performed analogue experiments with a CpG-free luciferase reporter plasmid (PEI–pCpG-free-Luc) and its backbone (PEI–pCpG-free backbone) without reporter gene, respectively. For each of the analyzed lung function parameters, values were not significantly different from untreated controls at time points later than 24 h (Fig. 1A–F). Lung function abnormalities were only observed one hour after application, which may have resulted from transient airway epithelial swelling caused by distilled water inhalation.

### 3.3. Analysis of BALF cells after aerosol delivery of PEI–pDNA complexes comprising a first-generation plasmid and CpG-free plasmid DNA

Aerosol application of PEI–pCMVLuc particles led to 2-fold increase in number of lung resident cells as long as to seventh day of observation, while nebulization of distilled water rose their amount approximately 4-fold (data not shown). Similar results were found for the numbers of alveolar macrophages (AMs) in BALF (Fig. 2A). The number of activated AMs peaked 24 h after aerosol administration of PEI–pCMVLuc complexes and was 7-fold higher than control values. Macrophage activation levels observed 7 days after aerosol delivery of PEI–pCMV complexes were significantly higher than those in untreated mice (Fig. 2B). The other three treatment groups (administration of PEI–pCpG-free-Luc, PEI–pCpG-free backbone and aqua) did not differ from control. Number of resident neutrophils did not increase significantly after either aerosol treatments (Supplementary Fig. 1). Also, the number of lymphocytes was not altered by aerosol administration of PEI–pDNA complexes (Supplementary Fig. 2). Mice which received PEI–pDNA complexes with CpG-free plasmid DNA by aerosol administration did not show significant differences to untreated controls (Fig. 2A and B, Supplementary Figs. 1 and 2).

### 3.4. Analysis of cytokine content in BALF fluid samples

BALF of every mouse was tested for concentration of 40 different cytokines and pro-inflammatory factors. For the majority of them, no significant difference from control levels was observed (data not shown). Still secretion of seven cytokines, namely GM-CSF, IL10, IL12p40p70, IL12p70, IL13, MIG and MIP1a, increased significantly 1 h or 24 h after administration of PEI–pDNA particles (Fig. 3). We have observed a 5-fold increase in GM-CSF factor already 1 h after PEI–pCMVLuc aerosolization, whereas the administration of PEI–pCpG-free-Luc increased its secretion almost 10-fold ($p < 0.05$). A 3- to 4-fold increase was shown for other above mentioned factors at 1 h time point compared to untreated control. Whereas after aerosolization of PEI–pCpG-free-Luc gene vectors, all values returned to normal after 24 h, cytokine levels remained elevated after aerosol application of PEI–pCMVLuc gene vectors.

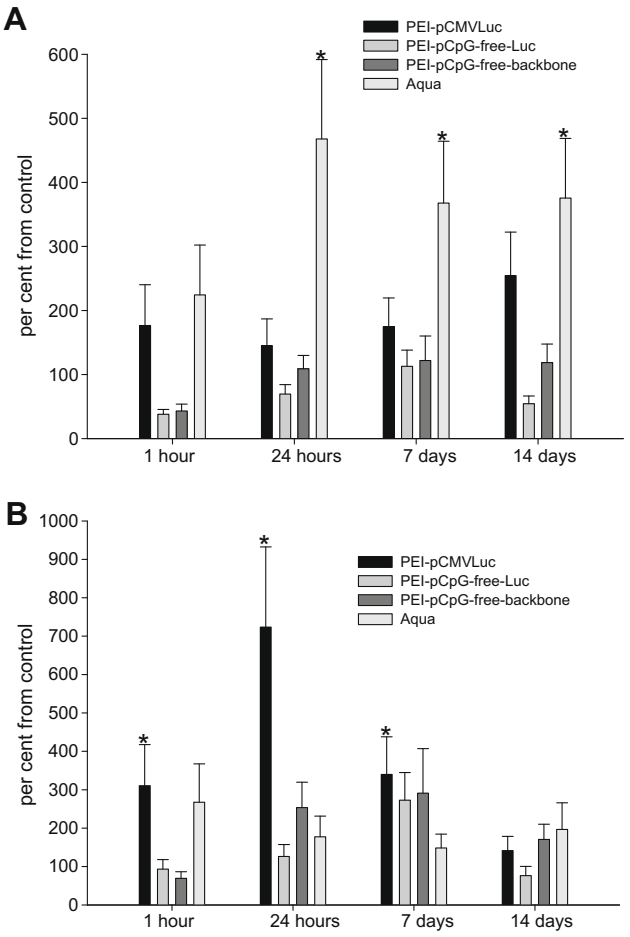


**Fig. 2.** Analysis of lung resident macrophages. At indicated time points, the number of resident alveolar macrophages was estimated using May–Grünwald staining of cytospin preparations of BAL (A). The number of activated alveolar macrophages was estimated using benzidine dihydrochloride staining by Kaplow (B). Results are reported as percent from control (untreated mice) ± standard error of the mean. Statistically significant differences from lung function parameters of untreated control mice to those of treated mice are denoted with an asterisk ($p < 0.05$).

### 3.5. Histological examination of lung tissue after intranasal instillation and aerosol delivery of PEI–pDNA complexes

Mice which received PEI–pCMVLuc complexes via aerosol delivery showed a moderate inflammatory response that was associated with local immune cell infiltration after 24 h (Fig. 4C and D). Seven days after treatment, restricted central parenchymal foci with strong capillary thrombus formation were observed (Fig. 4E and F). Mice which received PEI–pDNA complexes with CpG-free plasmid DNA by aerosol application (Fig. 4G and H) did not show differences to vehicle controls (Fig. 4A and B).

## 4. Discussion

In this study, we demonstrate that aerosol application of first-generation plasmid DNA complexed with PEI affects lung compliance in mice. Aerosol application of pDNA containing unmethylated CG dinucleotides (CpG) leads to adverse effects which were observed as late as one week after treatment. Only the use of CpG-free plasmid DNA was capable of preventing these adverse effects. These results therefore suggest that CpG-free plasmid DNA is critical to minimize potential inflammatory response upon nonviral aerosol gene delivery to the lungs.

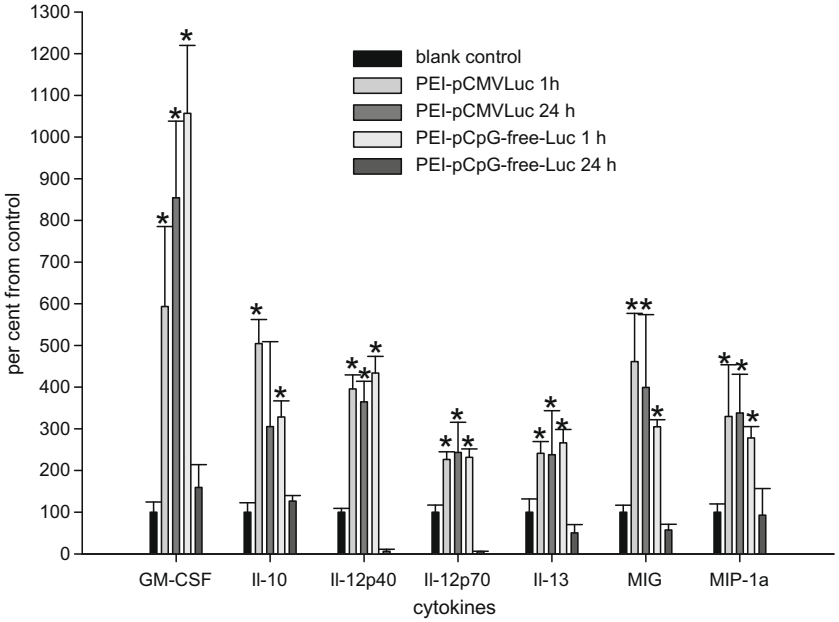


**Fig. 3.** Secretion of cytokines measured in BALF fluid. At indicated time points after aerosol administration, BALF fluid was collected from mice and analyzed for cytokines concentrations using RayBiotech ELISA-based array. Untreated mice were used as control. Results are reported as percent from control ± standard error of the mean. Statistically significant differences from control are denoted with an asterisk ($p < 0.05$).

Our work was devoted to the assessment of pulmonary response to aerosol delivery of PEI–pDNA particles. Most of the previous studies addressed inflammatory response after nonviral gene transfer to the lungs by means of standard histology, BALF analysis, measurement of pro-inflammatory cytokines [4,27] and more recently by gene expression profiling [12]. Although together these methods are appropriate to characterize the pattern of the inflammatory response in the lungs as evidenced by infiltrating cells, changes of the lung architecture and genes involved in reaction to stress, they are not capable of assessing any effects on a relevant lung functional level. Therefore, we analyzed if aerosolized PEI gene vectors affected lung function of mice. We demonstrate that forced oscillation technique can be successfully applied to display changes of pulmonary function after nonviral gene delivery to the lungs. Our results demonstrate that even nanogram-amounts of aerosolized PEI gene vectors containing a first-generation plasmid led to a decrease in lung compliance. Interestingly, this effect was not observed at the first day after administration but set in at later time points. The observed toxicity of PEI–pDNA gene vectors could have been caused by either the PEI–pDNA nanoparticles themselves or one of their components, i.e. PEI or pDNA, and the transgene product. To further elucidate the contribution of each of these gene vector components on the observed decrease in lung function, we performed identical experiments with different PEI–pDNA gene vectors by changing their composition. Unmethylated CpG motifs present in the pDNA of the lipid-based gene vectors have been previously shown to largely contribute to the inflammatory response in the lungs. To further assess whether unmethylated CpG motifs present in the pDNA were also the major reason for the observed decrease in lung compliance after aerosol delivery of PEI–pDNA gene vectors, we nebulized PEI–pDNA gene vectors to the mice comprising CpG-free plasmid DNA. However, after aerosol delivery of PEI–pDNA gene vectors comprising CpG-free backbone pDNA (not coding for any reporter gene), we did not observe any decrease in lung function. These observations suggest the critical role of unmethylated CpG motifs present in pDNA of PEI–pDNA gene vectors. To further analyze whether any immune response directed against the expressed luciferase reporter gene may have contributed to the decrease in lung compliance, we further nebulized PEI–pDNA gene vectors to the lungs of mice comprising CpG-free pDNA coding for the luciferase reporter gene but did not observe any decrease in lung function. These observations indicate that unmethylated CpG motifs present in pDNA of PEI–pDNA gene vectors seem to be the major factor which causes the decrease in lung compliance after nebulization. Although we may not entirely exclude any effect of PEI and the nanoparticles themselves, we think that their contribution to the observed decrease in lung compliance is very unlikely because in this case we should have observed a decrease in lung compliance in each treatment group, including PEI–pDNA gene vectors comprising CpG-free pDNA. These observations might be surprising in context with recently reported toxicity observed in vitro and after delivery of PEI and PEI–pDNA gene vectors to the lungs of mice [19–21]. However, the study of Rudolph et al. showed that free branched PEI 25 kDa resulted in a lesser immune response than PEI–pDNA gene vectors. Moreover, if one carefully takes into account that the PEI and PEI–pDNA dose which was administered to the lungs in these studies was in the microgram range, in contrast to our aerosol delivery which is in the nanogram range, it is obvious that in these previous studies the applied dose of PEI–pDNA gene vectors was well above the toxicity threshold. Our results therefore suggest that the amount of PEI present in the PEI–pDNA gene vectors does not lead to toxic effects in the lungs when only nanogram quantities are nebulized to the lungs which are below the toxicity threshold. Interestingly, the absence of PEI-caused toxicity allowed us to focus on negative effects of CpG motifs, which might have been otherwise undiscovered when less harmless administration methods were used. Our results suggest that the toxicity threshold of CpG-rich pDNA is below that of PEI. One may further suggest that differences of properties of the PEI–pDNA gene vectors may have contributed to the alterations of lung compliance observed in our study. However, neither the size nor the surface charge of each of the nebulized PEI–pDNA gene vectors markedly varied.

Neither the number nor the activation levels of lung resident macrophages were increased after administration of PEI complexes with CpG-free plasmids. These observations demonstrate the safety of PEI–pCpG-free-DNA particles aerosol delivery into murine lungs. In our study, we have observed an increase in macrophage

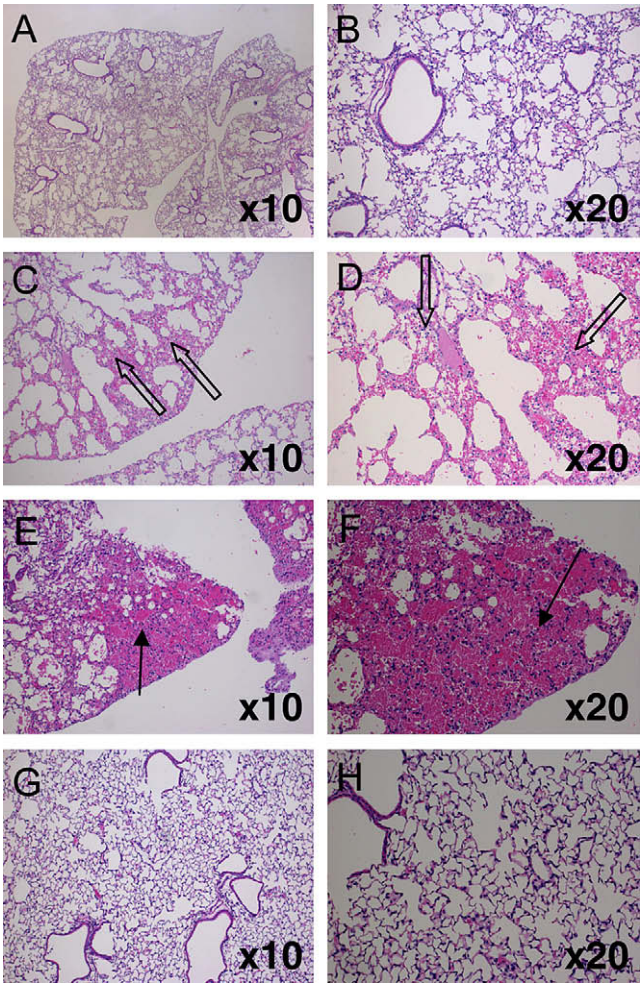


**Fig. 4.** Histological examination of lung tissue at indicated time points after aerosol application and instillation of PEI–pDNA particles and distilled water. Histological examinations were performed using hematoxylin–eosin stain. (A) and (B) Lung tissue of control mice 24 h after distilled water aerosol treatment (10- and 20-fold magnification). (C) and (D) Lung tissue from mice 24 h after PEI–pCMVLuc aerosol treatment (10- and 20-fold magnification). Central parenchymal foci are marked with blank arrows. (E) and (F) Lung tissue from mice 7 days after PEI–pCMVLuc aerosol treatment (10- and 20-fold magnification). Small congestions are marked with filled arrows. (G) and (H) Lung tissue from mice 24 h after PEI–pCpGLuc aerosol treatment (10- and 20-fold magnification). (For interpretation to colours in this figure, the reader is referred to the web version of this paper.)

number after the delivery of distilled water, which was not accompanied with macrophage activation. The absence of sequences which would trigger the pattern-recognition receptors on the surface of macrophages (bacterial DNA-containing CpG motifs [29], or lipopolysaccharides [39]) may explain these observations. Indeed, we have observed significantly higher numbers of activated macrophages after aerosol delivery of PEI–pCMVLuc particles. At the same time, no increase in number of resident phagocytes was detected in this series of experiments. At the moment, we do not have a clear explanation for this data. As far as the nebulized complexes comprised approximately 100 nm in diameter, they might be referred to the class of nanoparticles, which are widely reported to alter the behavior of lung resident cells [40]. Additional experiments, involving PEI–DNA particles of different size and structure, may reveal the influence of aerosol transfection on number of resident macrophages.

Administration of PEI–pCpG-free-Luc and PEI–pCMVLuc complexes influenced the secretion of pro-inflammatory cytokines in the lung, which was measured in BALF fluid 1 h and 24 h after the procedure. We have observed 5-fold and 10-fold increase in GM-CSF concentration 1 h after the delivery of PEI–pCMVLuc and PEI–pCpG-free-Luc, respectively. The secretion of granulocyte macrophage colony-stimulating factor (GM-CSF) occurs in the lung challenged with various particles or bacteria [41,42], and stimulates the production of monocytes, as well as their migration into tissue and following maturation [43]. Interestingly, GM-CSF concentration increased immediately after introduction of complexes with CpG-free DNA but dropped dramatically on the next day, while that after PEI–pCMVLuc delivery remained on the same level. The same tendency was shown for six other cytokines in our assays. Secretion of IL10 and IL12 is reported to play an important role in clearance of bacteria from the infected lung [44,45], while monokine induced by IFN-γ (MIG) as well as macrophage inflammatory proteins (MIPs) seems to influence eosinophils recruitment and induction of their chemotaxis [46]. In our study, their concentration increased significantly after administration of both types of complexes, yet rapid drop to almost normal levels was observed only in case of PEI complexes with CpG-free DNA, whereas PEI–pCMVLuc caused persistently increased levels. The TLR9 is expressed in the endosomal compartment of dendritic- and B-cells [27,29] and is widely reported to play an essential role in recognition of viral and bacterial DNA via activating pro-inflammatory cascades [39]. Also, PEI and its modifications are known to influence cytokine production [47]. Although in our case both PEI and pDNA might have contributed to the initial burst in cytokine secretion, the lung reaction on CpG-free-DNA-containing complexes was rather modest compared to that after PEI–pCMVLuc delivery. Our data clearly show that the introduction of PEI complexes with pCpG-free-Luc causes short-time transient increase in secretion of certain cytokines in the lung, while the effects of PEI–pCMVLuc particles appear to be more persistent. Interestingly, we have not observed any significant increase in TNFalpha production. This inconsistency might be due to the not optimal time points chosen for measurement of cytokines in BALF. Indeed, it was shown that the peak of certain cytokines production in murine lungs takes place approximately between 2 and 12 h after exposure to plasmid DNA [48]. Notably, among 40 cytokines which were tested, none was found responding only to one kind of particles (data not shown), meaning that at least within the first 24 h after administration both types of complexes triggered the same immune response pathway.

In summary, we suggest that neither the PEI–pDNA nanoparticles themselves nor PEI and the luciferase transgene caused lung function abnormalities in our study but that unmethylated CG sequences were the major reason for the observed impairment of lung function. These observations highlight the importance of using CpG-free pDNA for gene delivery to the lungs and demonstrate that the cationic polymer PEI may be used for aerosol gene delivery without inducing alteration of lung function as long as the dose is kept at a low level. An important finding of our study is that forced oscillation technique (FOT) measurements supported the results obtained from standard histology and BALF cell counts. We have shown that an increase in lung compliance after PEI–pDNA aerosol application was accompanied with significant alterations in number of alveolar macrophages and their activation ratio, while aerosol delivery of CpG-motif-free DNA complexed with PEI did not alter lung function and any of these parameters. Applying the constant phase model as suggested by Hantos et al. [36], our data indicate that the inflammatory effect of unmethylated CpG motifs is located to the peripheral lung tissue (GTiss and HTiss) rather than to the central airways.

In summary, we suggest that PEI could be safe for delivery to the lungs and could become a potential candidate for clinical trials in the future. This seems to be important in particular with respect to successful gene expression levels previously observed in a large sheep animal model [49].

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2009.11.013.

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