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Toxicity Pathway Focused Gene Expression Profiling of PEI-Based Polymers for Pulmonary Applications

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Abstract: Polyethylene imine (PEI) based polycations, successfully used for gene therapy or RNA interference *in vitro* as well as *in vivo*, have been shown to cause well-known adverse side effects, especially high cytotoxicity. Therefore, various modifications have been developed to improve safety and efficiency of these nonviral vector systems, but profound knowledge about the underlying mechanisms responsible for the high cytotoxicity of PEI is still missing. In this *in vitro* study, we focused on stress and toxicity pathways triggered by PEI-based vector systems to be used for pulmonary application and two well-known lung toxic particles: fine crystalline silica (CS) and nanosized ZnO (NZO). The cytotoxicity profiles of all stressors were investigated in alveolar epithelial-like type II cells (LA4) to define concentrations with matching toxicity levels (cell viability >60% and LDH release <10%) for subsequent qRT-PCR-based gene array analysis. Within the first 6 h pathway analysis revealed for CS an extrinsic apoptotic signaling (TNF pathway) in contrast to the intrinsic apoptotic pathway (mitochondrial signaling) which was induced by PEI 25 kDa after 24 h treatment. The following causative chain of events seems conceivable: reactive oxygen species derived from particle surface toxicity triggers TNF signaling in the case of CS, whereby endosomal swelling and rupture upon endocytotic PEI 25 kDa uptake causes intracellular stress and mitochondrial alterations, finally leading to apoptotic cell death at higher doses. PEG modification most notably reduced the cytotoxicity of PEI 25 kDa but increased proinflammatory signaling on mRNA and even protein level. Hence in view of the lung as a sensitive target organ this inflammatory stimulation might cause unwanted side effects related to respiratory and cardiovascular disorders. Thus further optimization of the PEI-based vector systems is still needed for pulmonary application.

Keywords: Toxicity; gene expression profiling; poly(ethylene imine); inflammation; pulmonary application

Introduction

Viral and nonviral vectors have widely been used in gene therapy as delivery systems for nucleic acids. The transfer of viral vector technology to clinical gene therapy trials has raised safety issues such as unexplained cytotoxicity and immunogenicity in target cells and tissues despite their high

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transfection efficiency.[1,2] Therefore, nonviral vector systems were promoted as promising and safer alternatives for gene and siRNA delivery.[3,4] So far, the main focus of gene therapy and RNA interference was on increasing efficiency and bioavailability while decreasing toxicity on a systemic and cellular level.[5−8]

In this context the identification of mechanisms and pathways underlying cellular perturbation and toxicity caused by exposure of target cells and tissues to polymeric nonviral delivery systems would help to improve their safety profile dramatically. In particular toxicogenomics has proved to be a powerful tool for the direct monitoring of patterns of cellular perturbation by various exogenous agents at the molecular level.[9] To study alteration of gene expression caused by the interaction between the structure and activity of a defined stressor and a selected cellular system can be an effective method to gain a first insight into this complex biological response. One huge issue of PEI-based nonviral vector systems is their strong cytotoxicity.[10,11] In fact PEI-based vector systems have been shown to cause considerable changes of gene expression *in vitro*, effects that might also have the potential to enhance off-target effects also *in vivo* as has been observed for siRNA targeting.[12,13] Indeed in the literature cationic polymers, dendrimers, and lipid-based transfection reagents were shown to alter cellular gene expression, but the consequences of these expression changes on biological systems remain largely unknown at present.

The lung represents a promising target organ for noninvasive local therapy of pulmonary diseases including asthma, chronic obstructive pulmonary disorder, cystic fibrosis, ischemic reperfusion injury and infection with respiratory viruses. Hence this study focuses on the purity of poly(ethylene imine) (PEI) polymers intended to be used for pulmonary delivery of nucleic acids like siRNA. The study only investigated the pure polymers as a "worst case scenario" because it is recently well described that polyplexes normally served lower toxicity than the pure polymers[6,14−17] and normally there is an excess of free PEI necessary for optimal delivery of nucleic acid.[18,19] Respective PEI polymers were selected from previous studies[20] because of their

(1) Howarth, J. L.; Lee, Y. B.; Uney, J. B. Using viral vectors as gene transfer tools (Cell Biology and Toxicology Special Issue: ETCS-UK 1 day meeting on genetic manipulation of cells). *Cell Biol. Toxicol.* **2010**, *26*, 1–20.

(2) Lufino, M. M.; Edser, P. A.; Wade-Martins, R. Advances in high-capacity extrachromosomal vector technology: episomal maintenance, vector delivery, and transgene expression. *Mol. Ther.* **2008**, *16* (9), 1525–38.

(3) Akinc, A.; Thomas, M.; Klibanov, A. M.; Langer, R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *J. Gene Med.* **2005**, *7* (5), 657–63.

(4) Gao, K.; Huang, L. Nonviral methods for siRNA delivery. *Mol. Pharmaceutics* **2009**, *6* (3), 651–8.

(5) Kleemann, E.; Dailey, L. A.; Abdelhady, H. G.; Gessler, T.; Schmehl, T.; Roberts, C. J.; Davies, M. C.; Seeger, W.; Kissel, T. Modified polyethylenimines as non-viral gene delivery systems for aerosol gene therapy: investigations of the complex structure and stability during air-jet and ultrasonic nebulization. *J. Controlled Release* **2004**, *100* (3), 437–50.

(6) Petersen, H.; Fechner, P. M.; Martin, A. L.; Kunath, K.; Stolnik, S.; Roberts, C. J.; Fischer, D.; Davies, M. C.; Kissel, T. Polyethylenimine-*graft*-poly(ethylene glycol) copolymers: influence of copolymer block structure on DNA complexation and biological activities as gene delivery system. *Bioconjugate Chem.* **2002**, *13* (4), 845–54.

(7) Zintchenko, A.; Philipp, A.; Dehshahri, A.; Wagner, E. Simple modifications of branched PEI lead to highly efficient siRNA carriers with low toxicity. *Bioconjugate Chem.* **2008**, *19* (7), 1448–55.

(8) Rudolph, C.; Lausier, J.; Naundorf, S.; Muller, R. H.; Rosenecker, J. In vivo gene delivery to the lung using polyethylenimine and fractured polyamidoamine dendrimers. *J. Gene Med.* **2000**, *2* (4), 269–78.

(9) Poma, A.; Di Giorgio, M. L. Toxicogenomics to improve comprehension of the mechanisms underlying responses of in vitro and in vivo systems to nanomaterials: a review. *Curr. Genomics* **2008**, *9* (8), 571–85.

(10) Xia, T.; Kovochich, M.; Liong, M.; Zink, J. I.; Nel, A. E. Cationic polystyrene nanosphere toxicity depends on cell-specific endocytic and mitochondrial injury pathways. *ACS Nano* **2008**, *2* (1), 85–96.

(11) Fischer, D.; Li, Y.; Ahlemeyer, B.; Krieglstein, J.; Kissel, T. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* **2003**, *24* (7), 1121–31.

(12) Jackson, A. L.; Bartz, S. R.; Schelter, J.; Kobayashi, S. V.; Burchard, J.; Mao, M.; Li, B.; Cavet, G.; Linsley, P. S. Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* **2003**, *21* (6), 635–7.

(13) Fedorov, Y.; Anderson, E. M.; Birmingham, A.; Reynolds, A.; Karpilow, J.; Robinson, K.; Leake, D.; Marshall, W. S.; Khvorova, A. Off-target effects by siRNA can induce toxic phenotype. *RNA* **2006**, *12* (7), 1188–96.

(14) Beyerle, A.; Hobel, S.; Czubayko, F.; Schulz, H.; Kissel, T.; Aigner, A.; Stoeger, T. In vitro cytotoxic and immunomodulatory profiling of low molecular weight polyethylenimines for pulmonary application. *Toxicol. in Vitro* **2009**, *23* (3), 500–8.

(15) Fischer, D.; von Harpe, A.; Kunath, K.; Petersen, H.; Li, Y.; Kissel, T. Copolymers of ethylene imine and N-(2-hydroxyethyl)-ethylene imine as tools to study effects of polymer structure on physicochemical and biological properties of DNA complexes. *Bioconjugate Chem.* **2002**, *13* (5), 1124–33.

(16) Glodde, M.; Sirsi, S. R.; Lutz, G. J. Physiochemical properties of low and high molecular weight poly(ethylene glycol)-grafted poly(ethylene imine) copolymers and their complexes with oligonucleotides. *Biomacromolecules* **2006**, *7* (1), 347–56.

(17) Mao, S.; Shuai, X.; Unger, F.; Wittmar, M.; Xie, X.; Kissel, T. Synthesis, characterization and cytotoxicity of poly(ethylene glycol)-*graft*-trimethyl chitosan block copolymers. *Biomaterials* **2005**, *26* (32), 6343–56.

(18) Boeckle, S.; Fahrmeir, J.; Roedl, W.; Ogris, M.; Wagner, E. Melittin analogs with high lytic activity at endosomal pH enhance transfection with purified targeted PEI polyplexes. *J. Controlled Release* **2006**, *112* (2), 240–8.

(19) Fahrmeir, J.; Gunther, M.; Tietze, N.; Wagner, E.; Ogris, M. Electrophoretic purification of tumor-targeted polyethylenimine-based polyplexes reduces toxic side effects in vivo. *J. Controlled Release* **2007**, *122* (3), 236–45.

(20) Beyerle, A.; Merkel, O. M.; Stoeger, T.; Kissel, T. PEGylation affects cytotoxicity and cell-compatibility of poly(ethylene imine) for lung application: structure-function-relationships. *Toxicol. Appl. Pharmacol.* **2010**, *242*, 146–54.

promising cytotoxic profile, and CS and NZO particles were chosen due to their well-known lung toxicity as benchmarks. CS is a long established lung carcinogen which induces severe pulmonary inflammation often resulting in fibrosis.[21,22] For NZO several studies described the high proinflammatory potential, possibly caused by the fraction of soluble Zn ions released in acidic cell compartments like endosomes.[23] Moreover exposure to zinc fumes can cause metal fume fever, an illness usually related to inhalation of fumes from welding, cutting, or brazing, on galvanized metal.[24,25] The detected gene expression patterns were classified on the basis of the *hierarchical oxidative stress model*[26,27] developed for ambient particulate matter and nanoparticles. Nel and colleagues introduced a three-tier hierarchy that subdivided oxidative stress caused by (nano)particles deposited in the lung into three categories. Tier 1 represents the lowest oxidative stress level characterized by expression of genes important for the cellular antioxidant response and which are regulated via the antioxidant response element binding transcription factor Nrf2. Tier 2 describes subsequent ongoing inflammatory processes based on the ability of unbalanced reactive oxygen species (ROS) to induce redox-sensitive signaling pathways such as MAP kinases and NFκB cascades. Finally the highest oxidative stress level is named tier 3, where cytotoxic events occur like alterations of the mitochondrial activity, cell cycle progression and apoptotic or necrotic cell death pathways. First, the cytotoxic profile was analyzed to define the optimal doses for each of the five stressors, which cause moderate and comparable level of cytotoxicity, and therefore allow us for subsequent comparative mechanistic investigations based on mRNA and protein expression levels. Second, we evaluated alterations in gene expression pattern after treatment of alveolar epithelial-like type II cells with the two lung benchmark particles and the three different PEI-based polymers applying a qRT-PCR-based pathway focused gene array covering 84 genes related to oxidative stress, inflammation, cell growth and proliferation, or cell death (necrosis and apoptosis). Since upon treatment with PEG-modified PEI copolymers significant inductions of proinflammatory gene expression were detected, we followed the inflammatory response on the protein level by cytokine quantification.

Experimental Section

Particles. Branched poly(ethylene imine) (PEI) with a molecular weight of 25 kDa (Polymin, water-free, 99%) was a gift of BASF, Ludwigshafen. The polyethylene imine-*graft*-poly(ethylene glycol)s (PEI-PEG) with a PEG content of approximately 50% (w/w) were synthesized as previously described[6,28] by grafting linear PEG of 0.55 kDa and 2 kDa, respectively, onto branched PEI 25 kDa. These graft copolymers were designated using the following nomenclature: PEI(25k)-*g*-PEG(*x*)*n*. The number in parentheses (25k or *x*, where $x = 0.55$k, 2k) represents the molecular weight of PEI or PEG block in daltons, and the index *n* is the average number of PEG blocks per PEI molecule. The number was calculated on the basis of ^{1}H NMR spectra as described previously.[28] Min-U-Sil 5 (crystalline silica) was obtained from U.S. Silica Company, Berkerly Springs, WV, with a median diameter of 1.7 μm as specified by the manufacturer. Zinc oxide (CAS-No: 1314-13-2) was obtained from Alfa Aesar (A Johnson Matthey Company, Karlsruhe, Germany) with a nominal average diameter of 70 nm; it is designated as nanosized ZnO (NZO).

Polymers were diluted in sterile sodium chloride (150 mM) solution and vigorously vortexed directly before use. Crystalline silica and nanosized ZnO suspensions were prepared in sterile, double-distilled water. Stock solutions (10 mg/mL) were sonicated for 15 min prior to serial dilution, and each suspension was sonicated for 10 min directly before use.

Cell Culture. Cell culture experiments were performed using the murine alveolar epithelial-like type II cells (LA4; ATCC No. CCL-196). Cells were grown in HAM's F12 medium with stable Glutamax containing 15% fetal bovine serum (FBS, Gibco, Germany) and 1% nonessential amino acids and 100U/mL penicillin and 100 mg/mL streptomycin at 37 °C and 5% CO_2. All cells were passaged every 2−3 days. All reagents were obtained from Biochrom AG, Seromed, Germany, or otherwise signed. LA4 cells were seeded at a density of 0.5×10^6 cells/well (2 mL) in 6-well plates (FALCON, Germany) and were allowed to adhere overnight in an incubator at 37 °C and 5% CO_2. After 24 h cell culture medium was replaced and the appropriate amount of the particle dilution was added to the well in a final volume of 2 mL/well. Cells were treated with the polymers and particles for 6 h and 24 h.

Cell Viability. Cell viability was determined using the Cell Proliferation Reagent WST-1 (Roche Diagnostics, Germany)

(21) Thakur, S. A.; Beamer, C. A.; Migliaccio, C. T.; Holian, A. Critical role of MARCO in crystalline silica-induced pulmonary inflammation. *Toxicol. Sci.* **2009**, *108* (2), 462–71.

(22) Castranova, V. Signaling pathways controlling the production of inflammatory mediators in response to crystalline silica exposure: role of reactive oxygen/nitrogen species. *Free Radical Biol. Med.* **2004**, *37* (7), 916–25.

(23) Xia, T.; Kovochich, M.; Liong, M.; Madler, L.; Gilbert, B.; Shi, H.; Yeh, J. I.; Zink, J. I.; Nel, A. E. Comparison of the mechanism of toxicity of zinc oxide and cerium oxide nanoparticles based on dissolution and oxidative stress properties. *ACS Nano* **2008**, *2* (10), 2121–34.

(24) Gordon, T.; Fine, J. M. Metal fume fever. *Occup. Med.* **1993**, *8* (3), 504–17.

(25) Lindahl, M.; Leanderson, P.; Tagesson, C. Novel aspect on metal fume fever: zinc stimulates oxygen radical formation in human neutrophils. *Hum. Exp. Toxicol.* **1998**, *17* (2), 105–10.

(26) Li, N.; Xia, T.; Nel, A. E. The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles. *Free Radical Biol. Med.* **2008**, *44* (9), 1689–99.

(27) Nel, A.; Xia, T.; Madler, L.; Li, N. Toxic potential of materials at the nanolevel. *Science* **2006**, *311* (5761), 622–7.

(28) Petersen, H.; Fechner, P. M.; Fischer, D.; Kissel, T. Synthesis, Characterization, and Biocompatibility of Polyethylenimine-*graft*-poly(ethylene glycol) Block Copolymers. *Macromolecules* **2002**, *35* (18), 6867–6874.

according to the method of Mosmann.[29] Briefly, LA4 cells were seeded at a density of 0.25×10^6 cells/well/2 cm^2 in 24-well plates in cell culture medium containing 15% FBS and grown overnight in an incubator at 37 °C and 5% CO_2. For the treatment, the cell culture medium was replaced by freshly, prewarmed, serum-reduced (2% FBS) cell culture medium without antibiotics. Particles were exposed to the cells for 24 h, and the relative viability [%] related to control samples (untreated cells) was calculated by following equation: cell viability = $(OD_{sample}/OD_{control}) \times 100$. All data represent at least three independent experiments. In addition IC_{50} values were determined following a 72 h incubation of LA4 cells (8000 cells/well) in 96-well plate with particle and polymer concentrations in the range from 0.001 to 1 mg/mL. After 72 h treatment metabolic activity was detected as described above and IC_{50} values were calculated as concentration which inhibits cell viability by 50% relative to untreated control cells according to Minko.[30] The results of optical density measurements were fitted logistically by the Levenberg−Marquardt method of least-squares minimization for nonlinear equations under default conditions using Origin 7.0 (OriginLab Software, Northampton, MA) by the following equation: $Y = Y_0 + (Y_m - Y_0)/(1 + (C/C_0))$, where C_0 is the IC_{50} dose, Y is the optical density in a well containing a particular polymer/extract of concentration C. Y_0 and Y_m are the optical densities corresponding to 0% viability and 100% viability, respectively.

Cytotoxicity. For detection of the cytosolic enzyme lactate dehydrogenase (LDH) in the culture supernatant, a characteristic of membrane damage, we used the Cytotoxicity Detection Kit (Roche Diagnostics, Germany) according to the manufacturer's protocol. The experiments were carried out according to the conditions of WST-1 assay. After 24 h the LDH concentration in the cell culture supernatant was spectrophotometrically determined in an ELISA reader (Labsystems iEMS Reader MF) at a wavelength of 492 nm. According to the manufacturer's protocol cells treated with 2% (w/v) Triton X-100 serving as control and set as maximum of LDH release (100%). The relative LDH release is defined by the ratio of LDH released over total LDH in the intact cells (high control). Less than 10% LDH release was regarded as a nontoxic effect level in our experiment.[31] All data represent at least three independent experiments.

RNA Isolation. After 6 h and 24 h incubation RNA was isolated from the cells by using RNeasy Mini Kit (Qiagen, Hilden, Germany). Briefly, cell culture medium was removed and cells were washed two times with prewarmed, sterile PBS without Mg^{2+} and Ca^{2+}, pH 7.4. Cells were lysed with 350 μL of RLT buffer containing 1% (v/v) 2-mercaptoethanol. The cell lysate was collected in an Eppendorf tube, and the tube was vigorously vortexed. 350 μL of cold (4 °C) ethanol (70%) was added to each cell lysate to precipitate the RNA. 700 μL of lysate was placed onto a Qiagen column and centrifuged at 15000*g* for 1 min. The flow-through was discarded, and DNase digestion was carried out using DNase I (Qiagen, Hilden, Germany). 350 μL of RW1 buffer was added to the column, and the column was centrifuged at 15000*g* × 1 min. The flow-through was discarded, and the column was placed in a new collection tube. In the same way 500 μL of RPE buffer containing ethanol was added twice and centrifuged first at 15000*g* for 1 min and a second time at 15000*g* for 2 min. The column was placed in a new tube, and the RNA was eluted from the column with 30 μL of RNase-free water by centrifugation at 15000*g* for 1 min. RNA was directly frozen at −80 °C and stored for further investigations. The quality of RNA was checked in an agarose gel (1%) containing SYBRGold (Invitrogen, Germany, Karlsruhe), and the RNA amount was quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) at absorbance ratios of 260 and 280 nm.

Gene Expression Profiling. cDNA synthesis was performed using RT^2 First Strand Kit (Superarray, Catalog no. C-03) following the instructions of the manufacturer. Briefly, genomic DNA was eliminated using Genomic DNA Elimination Mixture provided in the kit. RT cocktail was prepared, and first strand cDNA synthesis reaction was carried out according to the manufacturer's instructions.

For detailed information regarding the genes applied on this array RT^2 Profiler PCR Array Mouse Stress & Toxicity Pathway Finder (Superarray, Cat. no. APMM-003A), see Supporting Information S1.

Array was performed according to the instructions of the manufacturer, and the qRT-PCR was carried out with ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Briefly, cDNA was diluted in an appropriate amount of master mix (RT^2 Real-Time SYBR Green/ROX PCR Master Mix) and RNase-free water and loaded onto the array plate. The qRT-PCR was performed using a two-step cycling program with an initial heating for 2 min at 50 °C followed by two stages with one cycle of 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Immediately after the cycling program a melting curve program was run to generate a first derivative dissociation curve.

Genes were clustered according to the seven clusters of the RT^2 Profiler PCR Array Mouse Stress & Toxicity Pathway Finder provided by the manufacturer, and the gene expression pattern was illustrated in a heat map (Supporting Information S2) using free software called CARMAweb from Graz University of Technology, Institute of Genomics and

(29) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65* (1−2), 55–63.

(30) Minko, T.; Kopeckova, P.; Pozharov, V.; Kopecek, J. HPMA copolymer bound adriamycin overcomes MDR1 gene encoded resistance in a human ovarian carcinoma cell line. *J. Controlled Release* **1998**, *54* (2), 223–33.

(31) Choksakulnimitr, S.; Masuda, S.; Hideaki, T.; T., Y.; Mitsuru, H. In vitro cytotoxicity of macromolecules in different cell culture systems. *J. Controlled Release* **1995**, *34*, 233–214.

Bioinformatics,[32] https://carmaweb.genome.tugraz.at/carma/. Log2 ratios were used as input, with red colors indicating upregulation (treated vs control) and green colors indicating downregulation of the respective genes. Biological network interactions of genes regulated more than 2-fold were analyzed using the Ingenuity Systems pathway software (http://www.ingenuity.com), which is based on an expert curated interaction database. For clarity, some interactions were not shown. A small number of relations were added manually based on literature research, Figure 2A,B.

Cytokine Release. Eight cytokines/chemokines were detected simultaneously in the cell culture supernatant using Luminex technology (Linco Research, St. Charles, MO). In this study, the secretion of the following cytokines/chemokines was investigated: IL-1α, IL-6, TNF-alpha, G-CSF, CXCL1 CXCL10, CCL2, and CCL3. The assay was performed as described previously.[33] The mean fluorescence intensity (MFI) was detected by the Multiplex plate reader (Luminex System, Bio-Rad Laboratories, Germany) for each sample (50 μL) with a minimum of 100 beads per region being analyzed. The raw data (MFI) were captured using the Multiplex plate reader software (Bioplex Manager, Version 2.0). For data analysis, a 5-parameter logistic curve fit was applied to each standard curve and sample.

Statistics. All values are presented as mean ± standard error (SEM) of at least three independent experiments. Significant differences between two groups were evaluated by Student's *t* test or between more than two groups by one-way ANOVA followed by Tukey's multiple comparison test. Statistical analysis was performed using the program STATGRAPHICS PLUS Version 5.0 (Statpoint, Inc., Warrenton, VA).

Results

Cytotoxicity Evaluation. For all particles and polymers a clear dose-dependent cytotoxicity was found after 24 h treatment (Figure 1A−F).

Considering a mass-based dose metric, NZO was the most cytotoxic agent, resulting in cell viability reduction of over 90% and cell membrane damage (LDH release) of 15% already at a dose of 5 μg/mL. Particles and polymers show decreasing cytotoxicity as estimated by their IC_{50} values in the following order: PEI 25 kDa > NZO > PEI(25)-PEG(2)10 > PEI(25)-PEG(0.55)30 > CS (Table 1). In comparison to PEI 25 kDa, PEGylated PEI copolymers showed significantly reduced cytotoxicity ($p < 0.05$) (Figure 1C,F and Table 1). For PEI 25 kDa the highest concentration of 50 μg/mL caused severe toxicity yielding cell viability levels below detection and significantly elevated levels of LDH (15.03 ± 2.86, $p < 0.05$). To ensure sufficient RNA integrity for the subsequent qRT-PCR-based toxicity pathways focused gene array we used conditions resulting in low to moderate cytotoxicity levels as characterized by the WST-related toxicity below 40%, and the membrane toxicity (LDH) below 10%. The following doses were selected in the gene array study: 400 μg/mL for CS, 2.5 μg/mL for NZO, and 5 μg/mL for each of the three PEI-based polymers.

Toxicological Focused Gene Expression Profiling. We selected a pathway focused design with a representative set of genes indicative for a broad range of toxicological responses covering seven different stress and toxicity pathway clusters: metabolic and oxidative stress (1), heat shock (2), proliferation and carcinogenesis (3), growth arrest and senescence (4), inflammation (5), necrosis or apoptosis: DNA damage and repair (6) and necrosis or apoptosis: apoptosis signaling (7) (Supporting Information S1).

Two time points were investigated to detect acute and prolonged effects, namely 6 h and 24 h after treatment. From the 84 genes analyzed the expression of 35 genes (42%) was altered for all particles and polymers investigated at both time points (Supporting Information S2). For a more comprehensive arrangement of the observed expression changes we categorized them according to the three tier levels of the hierarchical oxidative stress model from Nel and colleagues[27] (Table 2).

Lung Toxic Benchmarks. The highest number of changes in gene expression was observed upon treatment with CS after 6 h and 24 h (Table 2). The following genes were more than 2-fold upregulated after 6 h treatment, and the corresponding tier levels are indicated: cytotoxicity, *NfkBia*, *E2f1*, *Tnfsf10*, *Gadd45*, *Cdkn1a*, *Ddit*, *Bax*, *Tnfrsf1a*, and *Ccng*; inflammation, *Cxcl10*, *Casp1*, *Nos2*, *NfkBia*, *Il-18*, and *Lta*; oxidative stress: *Ugt1a2*, *Cryab*, and *Hspa1b*. Most genes regulated after 6 h of treatment were related to inflammation and/or to cell survival/apoptosis pathways. After 24 h treatment, only five genes showed elevated mRNA levels which were either related to a sustained proinflammatory state (*Cxcl10* and *Serpine1*) or to an oxidative stress response (*Mt2* and *Hmox1*).

In contrast NZO treatment induced expression for oxidative and heat shock stress genes after 6 h (*Hspa1b* and *Hspb1*, *Ugt1a2*, *Hmox1*, *Mt2*) but returned to baseline levels (or even below) after the 24 h treatment. Exceptions were *NfkBia* and *Cxcl10*, which are be indicative of inflammation.

PEI-Based Nonviral Carrier Systems. Within the first 6 h PEI 25 kDa induced only for a few gene expression changes above a factor of 2 with the most notable markers indicating oxidative stress (*Ugt1a2* and *Mt2*), and to a lesser extent also antiapoptotic, survival and proinflammatory (*Cxcl10*, *Csf2*, *Nfkbia*) responses. The response pattern changed considerably for the second phase of analysis with an increasing contribution of apoptosis-related markers. Compared to the PEG-modified PEI copolymers after 24 h PEI 25 kDa treatment was the only polymer causing obvious

(32) Rainer, J.; Sanchez-Cabo, F.; Stocker, G.; Sturn, A.; Trajanoski, Z. CARMAweb: comprehensive R- and bioconductor-based web service for microarray data analysis. *Nucleic Acids Res.* **2006**, *34* (Web Server issue), W498–503.

(33) Prabhakar, U.; Eirikis, E.; Reddy, M.; Silvestro, E.; Spitz, S.; Pendley, C., 2nd; Davis, H. M.; Miller, B. E. Validation and comparative analysis of a multiplexed assay for the simultaneous quantitative measurement of Th1/Th2 cytokines in human serum and human peripheral blood mononuclear cell culture supernatants. *J. Immunol. Methods* **2004**, *291* (1−2), 27–38.

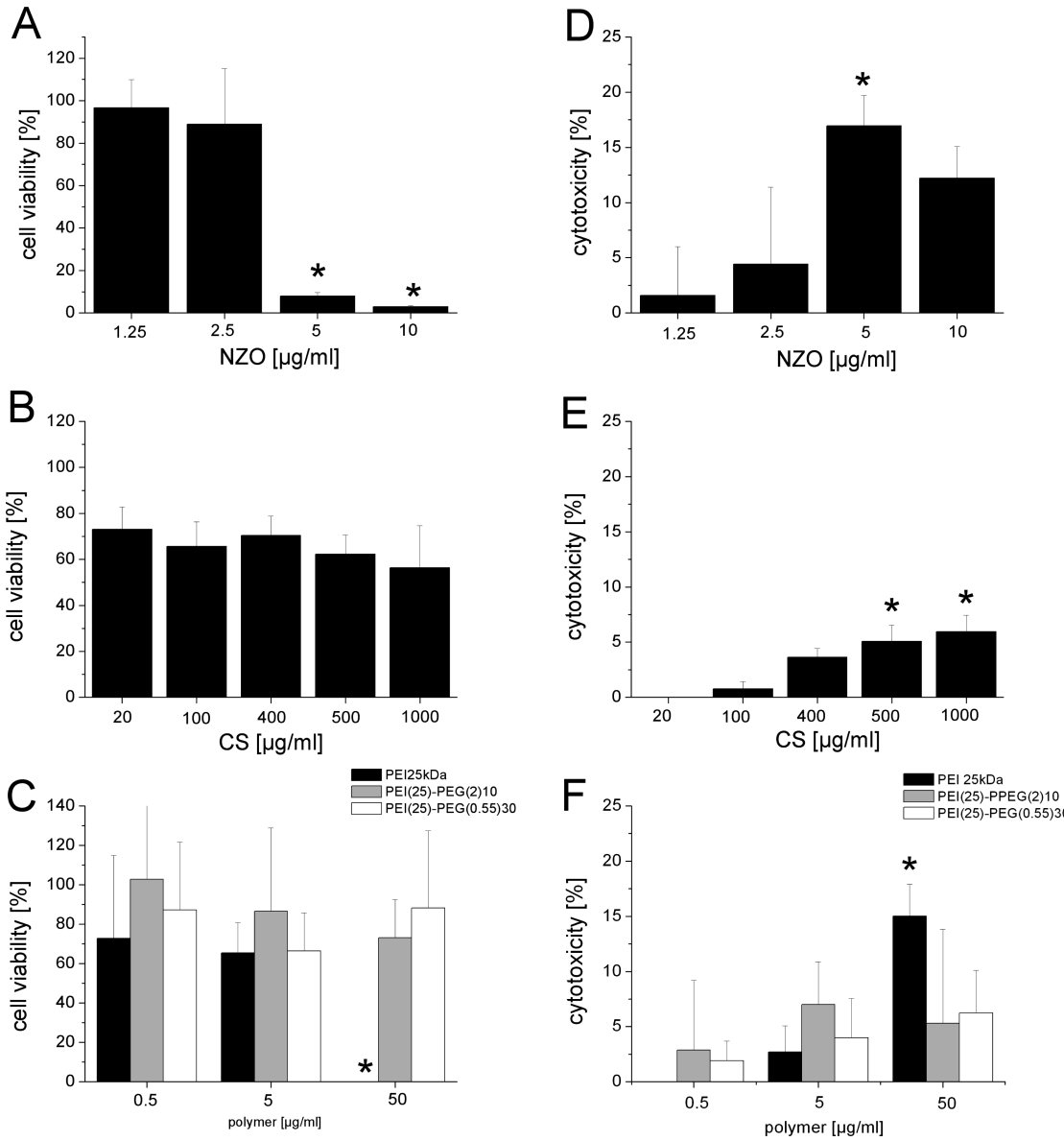

Figure 1. Cytotoxicity evaluation. The left panel (A−C) shows cell viability and the right panel (D−F) cytotoxicity (LDH release) in LA4 cells after 24 h treatment with nanosized ZnO (NZO), crystalline silica (CS), PEI 25 kDa (black), PEI(25)-PEG(2)10 (gray), and PEI(25)-PEG(0.55)30 (white). Values are given as mean ± SEM, and experiments were repeated three times in the case of the PEI-based polymers and five times in the case of NZO and CS. Statistically significant changes were represented by an asterisk with p value < 0.05 vs control (untreated cells).

Table 1. IC_{50} Values[a]

particle/polymer	IC_{50} [mg/mL]
CS	>1
NZO	0.014 ± 0.003
PEI 25 kDa	0.003 ± 0.000
PEI(25)-PEG(2)10	0.022 ± 0.009
PEI(25)-PEG(0.55)30	0.033 ± 0.004

[a] IC_{50} values were presented as mean ± SD of three independent experiments. Cell proliferation in LA4 cells was analyzed using WST-1 assay after 72 h particle exposure, and IC_{50} values were calculated using sigmoidal fitting using the Boltzmann equation.

apoptotic gene expression (*Bcl2l1*, *Bax*, *Anxa5*, *Ercc4* and *Atm*), a pattern not at all observed for the two lung toxic benchmark particles NZO and CS after 24 h treatment, but after 6 h for CS.

During the early response after 6 h, highly elevated expression levels of *E2f1*, a cell cycle regulating transcription factor, and *NfkBia*, an important component of the NFκB machinery, involved in many proinflammatory and apoptosis/survival signaling pathways, were detected for all three PEI polymers, as well as oxidative stress indicating phase II metabolizing enzyme *Ugt1a2*.

Overall, during the first 6 h treatment PEGylated copolymers but not PEI 25 kDa caused mainly an upregulation of proinflammatory genes including *Cxcl10*, *Caps1*, *Nos2*, and *Nfkbia*. The cellular stress response markers *Cryab* and *Hsp1b* showed a similar expression pattern with high levels for Hspa1b only

Table 2. Gene Expression Profiling[a]

pathway	gene name	GenBank	fold change early response (6 h)	fold change late response (24 h)	pathway	gene name	GenBank	fold change early response (6 h)	fold change late response (24 h)
				CS					
cytotoxicity	E2f1	NM_007891	3.7	0.6	inflammation	Nfkbia	NM_010907	4.4	1.1
cytotoxicity	Tnfsf10	NM_009425	3.4	nd	inflammation	IL18	NM_008360	3.9	0.0
cytotoxicity	Gadd45a	NM_007836	3.0	0.7	inflammation	Lta	NM_010735	3.0	0.6
cytotoxicity	Cdkn1a	NM_007669	2.4	0.8	inflammation	Serpine1	NM_008871	−3.3	3.9
cytotoxicity	Ddit3	NM_007837	2.3	0.7	inflammation	Csf2	NM_009969	1.0	2.9
cytotoxicity	Bax	NM_007527	2.2	0.4	ox. stress	Ugt1a2	NM_013701	14.9	0.3
cytotoxicity	Tnfrsf1a	NM_011609	2.0	0.3	ox. stress	Cryab	NM_009964	6.1	0.1
cytotoxicity	Ccng1	NM_009831	2.0	0.2	ox. stress	Hspa1b	NM_010478	2.2	0.5
inflammation	Cxcl10	NM_021274	11.6	6.6	ox. stress	Mt2	NM_008630	1.8	2.3
inflammation	Casp1	NM_009807	8.0	0.1	ox. stress	Hmox1	NM_010442	1.7	2.1
inflammation	Nos2	NM_010927	7.8	0.8					
				NZO					
inflammation	Nfkbia	NM_010907	3.9	0.4	ox. stress	Ugt1a2	NM_013701	6.8	0.6
inflammation	Cxcl10	NM_021274	2.2	0.5	ox. stress	Hmox1	NM_010442	3.0	0.4
ox. stress	Hspb1	NM_013560	9.8	0.4	ox. stress	Mt2	NM_008630	2.2	0.3
				PEI 25 kDa					
cytotoxicity	E2f1	NM_007891	2.1	1.5	inflammation	Csf2	NM_009969	2.4	0.8
cytotoxicity	Bcl2l1	NM_009743	−1.5	3.2	ox. stress	Ugt1a2	NM_013701	10.0	0.7
cytotoxicity	Bax	NM_007527	1.0	2.4	ox. stress	Mt2	NM_008630	2.1	0.8
cytotoxicity	Atm	NM_007499	−1.3	2.2	ox.s tress	Hspa1b	NM_010478	−1.4	4.7
cytotoxicity	Ercc4	NM_015769	−1.3	2.0	ox. stress	Cyp1b1	NM_009994	−2.8	2.6
cytotoxicity	Anxa5	NM_009673	1.3	2.0	ox. stress	Hmox2	NM_010443	−2.5	2.4
inflammation	Nfkbia	NM_010907	3.3	1.5					
				PEI(25)-PEG(2)10					
cytotoxicity	E2f1	NM_007891	2	0.7	inflammation	Nos2	NM_010927	2.0	0.9
inflammation	Cxcl10	NM_021274	4.1	0.1	ox. stress	Ugt1a2	NM_013701	7.1	0.4
inflammation	Nfkbia	NM_010907	3.5	1.7	ox. stress	Cryab	NM_009964	3.0	0.9
inflammation	Casp1	NM_009807	2.1	1.0	ox. stress	Hspa1b	NM_010478	1.6	3.1
				PEI(25)-PEG(0.55)30					
cytotoxicity	E2f1	NM_007891	3.0	0.9	inflammation	Serpine1	NM_008871	2.0	1.4
inflammation	Nfkbia	NM_010907	7.4	2.7	inflammation	IL1b	NM_008361	1.3	5.9
inflammation	Nos2	NM_010927	4.5	2.8	ox. stress	Ugt1a2	NM_013701	10.0	0.8
inflammation	Casp1	NM_009807	4.1	1.1	ox. stress	Cryab	NM_009964	2.5	1.2
inflammation	Cxcl10	NM_021274	2.5	0.3	ox. stress	Hspa1b	NM_010478	1.7	4.8
inflammation	Mif	NM_010798	2.1	0.8					

[a] Genes were clustered in three tier levels (tier 3, cytotoxicity; tier 2, inflammation; tier 1, oxidative stress) according to the hierarchical oxidative stress model from Nel and colleagues.[27] Shown are genes with change of expression of more than 2-fold after 6 h and 24 h treatment with CS (Min-U-Sil 5 400 μg/mL), NZO (2.5 μg/mL), PEI 25 kDa (5 μg/mL), PEI(25)-PEG(2)10 (5 μg/mL), and PEI(25)-PEG(0.55)30 (5 μg/mL). Fold changes (linear) were calculated using comparative cycle threshold (CT) method and normalized to the housekeeping gene *Hprt 1*.

after 24 h for both PEGylated PEIs. Only PEI(25)-PEG(0.55)30 induced particular high levels of *Mif* and *Serpine1* after 6 h and *Il1b*, *Nos2* and *Nfkbia* after 24 h, all well-described proinflammatory marker genes (Figure 2A and B).

Cytokine Profiling. For lung particle interactions the proinflammatory effect is a sensitive and critical response to foreign material, which is induced already at doses below significant cytotoxicity. To confirm the gene array results the induction of a proinflammatory state was also investigated at the protein level. A panel of eight representative cytokines (IL-1α, IL-6, TNF-alpha, G-CSF, CXCL1 CXCL10, CCL2, and CCL3) was assessed for their release from LA4 cells upon 24 h treatment with the above selected doses. Corresponding to the gene expression data, CS and PEI(25)-PEG(2)10 showed the most prominent proinflammatory response (Figure 3).

Increased levels were mainly detected for the acute-phase cytokines like CXCL1, CXCL10, TNF, IL-6, and G-CSF (CSF2) upon CS and PEGylated PEI treatment, with a more than 10-fold elevated protein release for IL-6, CXCL10 and G-CSF caused by PEI(25)-PEG(2)10. In addition, monocyte chemoattractant protein 1 (MCP-1, also known as CCL2) showed up to 70-fold elevated levels after 24 h treatment with CS. At the concentration used in the qRT-PCR array as well as at higher doses of up to 1000 μg/mL for CS and NZO and 50 μg/mL for PEI 25 kDa (data not shown), both

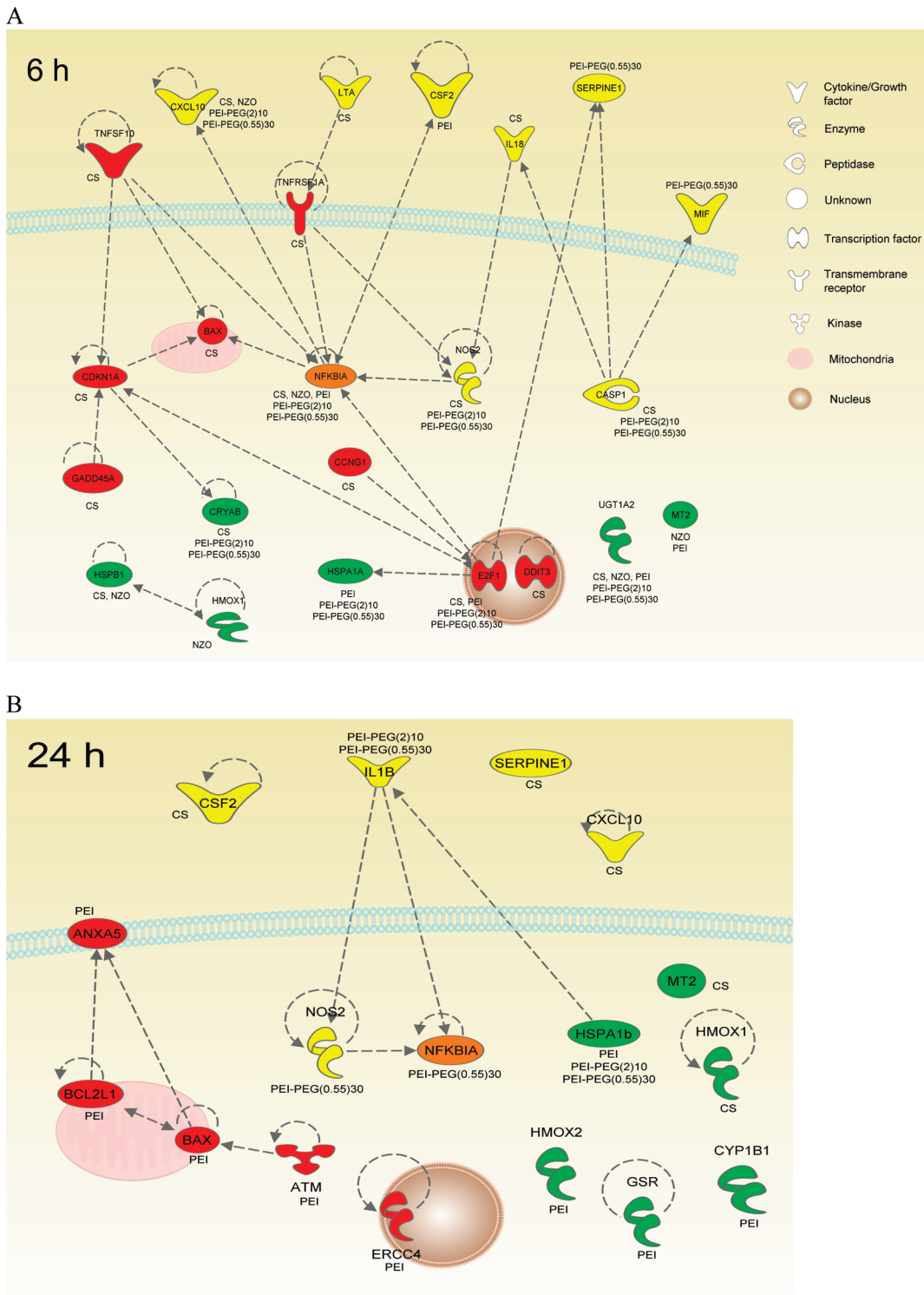

Figure 2. Pathway modeling. Network of all more than 2-fold induced genes at 6 h (A) or 24 h (B). Interactions are based on the Ingenuity pathway software, and the different treatments are indicated. Genes are highlighted according to Nel and colleagues[27] (green, oxidative stress; yellow, inflammation; red, cytotoxicity; orange, inflammation/cytotoxicity overlap).

treatments caused only negligible release levels of the investigated proinflammatory cytokines.

Discussion

Cytotoxicity. To overcome the high cytotoxicity of PEI-based nonviral vector systems is a major challenge toward an improved safety and efficiency of gene therapy and RNAi. Some modifications of standard PEI 25 kDa already yielded promising carriers. For example, as previously described[20] PEGylation of PEI 25 kDa with a PEG chain length of up to 2 kDa and a grafting degree higher than 10 strongly reduced cytotoxicity of PEI 25 kDa. The lung toxic bench-

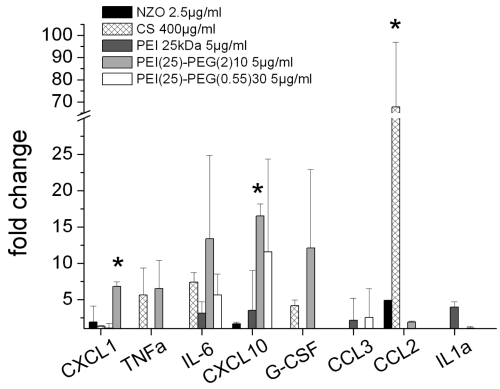

Figure 3. Cytokine release. Cytokine release was determined in LA4 cells after 24 h treatment. Values are represented as fold changes compared to the control (untreated cells; mean ± SEM). Statistically significant changes with p value < 0.05 compared to controls are indicated by an asterisk. Experiments were repeated three times in the case of the PEI-based polymers and five times in the case of NZO and CS.

mark particles showed similar cytotoxicity levels for PEI 25 kDa and NZO, but much less for CS.

For the qRT-PCR pathway-focused gene array particle concentrations with moderate cytotoxic effects were used as clearly shown by the data on membrane damage and metabolic activity. By avoiding a state of full-blown cell death we were able to study underlying toxicity mechanisms under well-defined cell culture setting.

Stress and Toxicity Pathway Finder. *Lung Toxic Benchmarks.* Inhalation of CS has long been associated with lung disease such as silicosis and lung cancer.[34,35] The typical involved processes were generation of oxidative species, release of inflammatory cytokines and chemokines and proliferative factors. In our *in vitro* study we were able to reproduce and distinguish these processes by gene expression profiling and by that validate our system. In accordance with previous results we observed a strong upregulation of genes related to oxidative stress response and detoxification (*Ugt1a2*, *Cryab*, *Mt2*, *Hmox1*), inflammation (*Nfkbia*, *Cxcl10*, *Casp1*, *Nos2*, *Il18*, *Lta*, *Hspa1b*, *Serpine1*, *Csf2*), and cytotoxicity pathways (*Tnfsf10*, *E2f*, *Gadd45*, *Cdkn1a*, *Ddit3*, *Bax*, *Tnfrsf1a*, *Ccng1*).

ZnO particles and Zn^{2+} ions have been described to stimulate oxygen radical formation and cause metal fume fever.[24,25,36] In particular Zn is a potent and well-known inducer of heat shock protein transcription,[37] a phenomenon that might be seen as a protective mechanism of these chaperons for recovery from stress situations.

In our study we observed a pronounced stress response characterized by heat shock and oxidative stress marker expression like *Hspb1*, *Hmox1*, *Mt2*, *Ugt1a2* as well as minor indications for proinflammatory reactions (*NfkBia* and *Cxcl10*) after 6 h. After 24 h no upregulated genes but rather repressed expression levels were detected (Supporting Information, S2). Even though the transcriptional pathways leading to gene repression are less understood compared to those of induction, the repression of the major oxidative stress marker heme oxygenase 1 (Hmox1) has been discussed as potential defense strategy upon hypoxic conditions. Besides that, Zn exposed cells have been found to react with an overall inhibition of protein synthesis.[38]

PEI-Based Nonviral Vector Systems. PEI-based nonviral vector systems caused all together less alteration of gene expression levels as compared to our lung toxic benchmark particles, but we could still detect a prominent cytotoxic (PEI 25 kDa) as well as proinflammatory response to PEG-modified PEI copolymers, which could be related to the higher molecular weight of PEG-modified PEI-PEG copolymers.

In detail after 6 h treatment several genes related to inflammatory signaling were especially altered upon treatment with PEG-modified PEI-based nonviral vectors. After 24 h notably more genes, mainly related to apoptosis, were induced by PEI 25 kDa (Figures 2 and 4).

NfkBia and *E2f* seem to be key players induced by the inflammatory response after 6 h. *Nfkbia* triggers inflammation mediated by chemokines and cytokines as well as apoptotic signaling pathways a process that is well described in the literature.[39] The transcription factor *E2f* is a potent activator for genes involved in cell cycle regulation, DNA synthesis and replication,[40] but is also known to promote apoptotic signaling.[41,42]

High levels of *Cxcl10* and *Nos2* were observed after treatment with PEG-modified PEI polymers. These genes are well-known for their immunomodulatory potential and are

(34) Donaldson, K.; Borm, P. J. The quartz hazard: a variable entity. *Ann. Occup. Hyg.* **1998**, *42* (5), 287–94.
(35) Sayes, C. M.; Reed, K. L.; Warheit, D. B. Assessing toxicity of fine and nanoparticles: comparing in vitro measurements to in vivo pulmonary toxicity profiles. *Toxicol. Sci.* **2007**, *97* (1), 163–80.
(36) Nemery, B.; Demedts, M. Respiratory involvement in metal fume fever. *Eur. Respir. J.* **1991**, *4* (6), 764–5.
(37) Plank, C.; Mechtler, K.; Szoka, F. C., Jr.; Wagner, E. Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery. *Hum. Gene Ther.* **1996**, *7* (12), 1437–46.
(38) Walthera, U. I.; Fortha, W. Influence of zinc on protein metabolism in various lung cell lines. *Toxicol. in Vitro* **1999**, *13* (6), 905–914.
(39) Hanada, T.; Yoshimura, A. Regulation of cytokine signaling and inflammation. *Cytokine Growth Factor Rev.* **2002**, *13* (4−5), 413–21.
(40) Rogoff, H. A.; Kowalik, T. F. Life, death and E2F: linking proliferation control and DNA damage signaling via E2F1. *Cell Cycle* **2004**, *3* (7), 845–6.
(41) Rogoff, H. A.; Pickering, M. T.; Frame, F. M.; Debatis, M. E.; Sanchez, Y.; Jones, S.; Kowalik, T. F. Apoptosis associated with deregulated E2F activity is dependent on E2F1 and Atm/Nbs1/Chk2. *Mol. Cell. Biol.* **2004**, *24* (7), 2968–77.
(42) Yu, F.; Megyesi, J.; Safirstein, R. L.; Price, P. M. Involvement of the CDK2-E2F1 pathway in cisplatin cytotoxicity in vitro and in vivo. *Am. J. Physiol.* **2007**, *293* (1), F52–9.

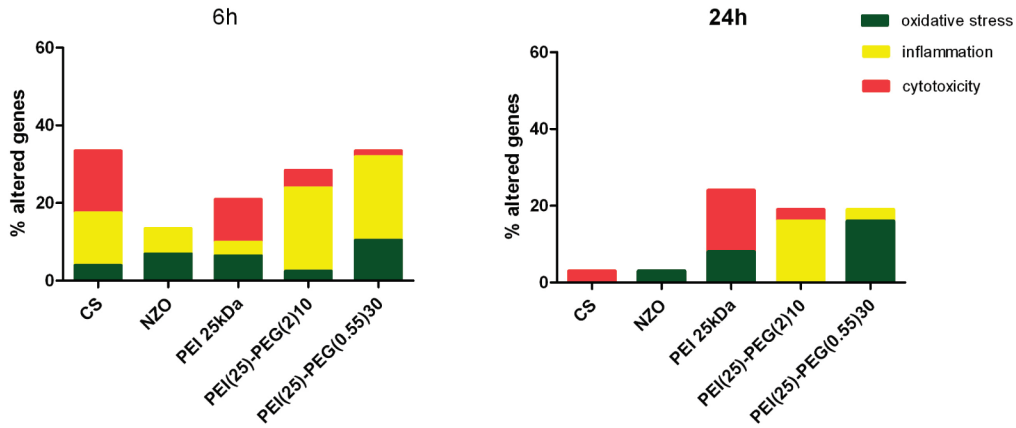

Figure 4. Expression synopsis. Genes were clustered in three tier levels (tier 1 (green), oxidative stress; tier 2, inflammation (yellow); tier 3, cytotoxicity (red)) according to the hierarchical oxidative stress model from Nel and colleagues.[27] The percentage of altered genes (>2-fold) after 6 h (left) and 24 h (right) treatment with CS (400 μg/mL), NZO (2.5 μg/mL), PEI 25 kDa (5 μg/mL), PEI(25)-PEG(2)10 (5 μg/mL), and PEI(25)-PEG(0.55)30 (5 μg/mL) was calculated based on the number of genes more than 2-fold induced, related to the number of genes representing the tier level on the array.

known to be highly induced during inflammation.[43] Both, PEI(25)-PEG(2)10 and PEI(25)-PEG(0.55)30 treatment induced stress response proteins, in particular antioxidant defense pathways represented by *Ugt1a2*, *Mt2*, and *Hmox1*, which are possibly upstream of the inflammatory cascade (*Il18*, *Cxcl10*, *Nfkbia*, *Casp1*, *Nos2*). PEI 25 kDa caused high upregulation of genes like *Bax*, *Bcl2l1*, *Atm*, *Anxa5*, and *Ercc4* related to apoptotic cell death, whereas PEG-PEI copolymers induced high levels of genes related to inflammation like *Casp1*, *Il1b*, *Hspa1b*, *Nos2*, and *NfkBia*. Since the observed long lasting inflammatory potential of CS[44−47] was found to be associated with its hazard to cause chronic lung disease,[35,48] our observation might raise serious concerns about the safety of nondegradable, PEGylated polymers like PEI(25)-PEG(0.55)30.

Inflammatory Response. Sensitive organs such as the lung are well-known for their high sensitivity to particle treatment, especially for so-called nanosized particles in a size range below 100 nm.[48−50] Exposure of the respiratory system to particles triggers proinflammatory effects and can directly cause tissue damage and alteration of lung function and adverse cardiovascular effects.[37,51] Therefore any inflammatory stimulation caused by inhalation of a therapeutic agent has to be monitored carefully. In our study prominent upregulation of genes related to inflammation (*Cxcl10*, *Nos2*, *IL-1b*, *Casp1*, *NfkBia*, *Csf2*, *Mif*, *Serpine1*, *Lta*, *Il-18*) was observed after treatment with PEI(25)-PEG(2)10, PEI(25)-PEG(0.55)30 and CS. This result is in good accordance with other studies,[35,52,53] where CS was used as positive control for lung inflammation. At protein level PEGylated copolymers and CS caused abundant cytokine release with patterns similar to our qRT-PCR data. The proinflammatory potential

(43) Ichinose, M. Differences of inflammatory mechanisms in asthma and COPD. *Allergol. Int.* **2009**, *58* (3), 307–13.
(44) Hollins, A. J.; Benboubetra, M.; Omidi, Y.; Zinselmeyer, B. H.; Schatzlein, A. G.; Uchegbu, I. F.; Akhtar, S. Evaluation of generation 2 and 3 poly(propylenimine) dendrimers for the potential cellular delivery of antisense oligonucleotides targeting the epidermal growth factor receptor. *Pharm. Res.* **2004**, *21* (3), 458–66.
(45) Regnstrom, K.; Ragnarsson, E. G.; Fryknas, M.; Koping-Hoggard, M.; Artursson, P. Gene expression profiles in mouse lung tissue after administration of two cationic polymers used for nonviral gene delivery. *Pharm. Res.* **2006**, *23* (3), 475–82.
(46) Tagami, T.; Barichello, J. M.; Kikuchi, H.; Ishida, T.; Kiwada, H. The gene-silencing effect of siRNA in cationic lipoplexes is enhanced by incorporating pDNA in the complex. *Int. J. Pharm.* **2007**, *333* (1−2), 62–9.
(47) Omidi, Y.; Hollins, A. J.; Benboubetra, M.; Drayton, R.; Benter, I. F.; Akhtar, S. Toxicogenomics of non-viral vectors for gene therapy: a microarray study of lipofectin- and oligofectamine-induced gene expression changes in human epithelial cells. *J. Drug Targeting* **2003**, *11* (6), 311–23.
(48) Donaldson, K.; Stone, V. Current hypotheses on the mechanisms of toxicity of ultrafine particles. *Ann. Ist. Super. Sanita* **2003**, *39* (3), 405–10.
(49) Stoeger, T.; Reinhard, C.; Takenaka, S.; Schroeppel, A.; Karg, E.; Ritter, B.; Heyder, J.; Schulz, H. Instillation of six different ultrafine carbon particles indicates a surface area threshold dose for acute lung inflammation in mice. *Environ. Health Perspect.* **2006**, *114* (3), 328–33.
(50) Oberdorster, G.; Maynard, A.; Donaldson, K.; Castranova, V.; Fitzpatrick, J.; Ausman, K.; Carter, J.; Karn, B.; Kreyling, W.; Lai, D.; Olin, S.; Monteiro-Riviere, N.; Warheit, D.; Yang, H. Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. *Part. Fibre Toxicol.* **2005**, *2*, 8.
(51) Nemmar, A. H. M. F.; Hoet Peter, H. M. Vermylen Josef, Nemery Benoit. Size effect of intratracheally instilled particles on pulmonary inflammation and vascular thrombosis. *Toxicol. Appl. Pharmacol.* **2003**, *186*, 38–45.
(52) Ovrevik, J.; Lag, M.; Holme, J. A.; Schwarze, P. E.; Refsnes, M. Cytokine and chemokine expression patterns in lung epithelial cells exposed to components characteristic of particulate air pollution. *Toxicology* **2009**, *259* (1−2), 46–53.
(53) Warheit, D. B.; Webb, T. R.; Reed, K. L. Pulmonary toxicity screening studies in male rats with M5 respirable fibers and particulates. *Inhalation Toxicol.* **2007**, *19* (11), 951–63.

of the PEI-based nonviral vector systems seems to be promoted by the PEG-modification of PEI 25 kDa with a PEG chain length of 2 kDa and grafting densities higher than 10.

Two different underlying mechanisms could be highlighted when comparing the cytotoxicity (apoptosis) pathways triggered by CS after 6 h with those by PEI 25 kDa after 24 h as illustrated in Figure 2A,B: For CS it seems to be likely that, during the onset of the cascade, the TNF-receptor has been activated by reactive oxygen species generated by particle surface reactivity. This rapid activation will subsequently be transduced, depending on the cellular stress levels, via the cellular NFκB switchpoint into signaling causing either inflammatory (*Cxcl10*, *Lta*, *Casp1*, *Il18* and *Nos2*) or apoptotic responses (*Tnfsr10*, *Cdkn1a*, *Ccng1*, *Gadd45a*). In contrast to this extrinsic apoptotic pathway, PEI 25 kDa caused high induction of genes related to the intrinsic apoptotic pathway like *Bcl2l1*, *Bax* and DNA damage response like *Atm* and *Ercc4*. This intrinsic response could be induced depending on the endosomal uptake of PEI, which causes a dramatic swelling and rupture of the endosome. While this release process, called "proton sponge effect", seems crucial for the delivery of nucleic acid into the cytoplasm, the thereby generated intracellular stress can induce apoptosis via the intrinsic/mitochondrial pathway.This redox-sensitive caused response process will have to be balanced by the cellular antioxidant and detoxification machinery (*Cyp1b1*, *Cryab*, *Hmox2*, *Gsr*, *Hspb1*, and *Ugt1a2*).

In summary, both benchmark particles revealed significant expression changes only within the first 6 h of treatment. CS induced pathways related to oxidative stress, inflammation and cytotoxicity, and NZO particles yielded a characteristic early stress protein response. The three PEI-based polymers showed certainly related expression pattern, but proinflammatory pathways were primarily induced by PEG-modified PEI-based copolymers. In addition, the response upon PEI 25 kDa could be distinguished through apoptotic pathways triggered in the later phase, after 24 h of treatment (Figure 4). The observed alteration of expression levels of genes involved in apoptosis and cytokine signaling should be avoided on the one hand, but to some extent might also be beneficial for tumor targeting with improved antitumor effects on the other hand.[54−57] The higher inflammatory potential of the PEG-modified PEI copolymers should however be considered as one limitation for their pulmonary application for various lung diseases where repeated and permanent dosing is required. To examine the *in vivo* relevance of the here described alarming proinflammatory PEI-PEG properties, detailed animal studies are currently executed in our lab which shall provide important information about degree and persistence of any relevant inflammatory responses.

Abbreviations Used

CS, crystalline silica; NZO, nanosized ZnO; PEI, poly(ethylene imine); PEG, poly(ethylene glycol); qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; siRNA, small interfering RNA.

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Supporting Information Available: Gene table of the RT^2 Profiler PCR Array Mouse Stress & Toxicity PathwayFinder provided by the manufacturer and heat map. This material is available free of charge via the Internet at http://pubs.acs.org.

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(54) Dufes, C.; Keith, W. N.; Bilsland, A.; Proutski, I.; Uchegbu, I. F.; Schatzlein, A. G. Synthetic anticancer gene medicine exploits intrinsic antitumor activity of cationic vector to cure established tumors. *Cancer Res.* **2005**, *65* (18), 8079–84.

(55) Moroson, H. Polycation- treated tumor cells in vivo and in vitro. *Cancer Res.* **1971**, *31* (3), 373–80.

(56) Akhtar, S. Non-viral cancer gene therapy: beyond delivery. *Gene Ther.* **2006**, *13* (9), 739–40.

(57) Seymour, L. Synthetic Polymers with Intrinsic Anticancer Activity. *J. Bioact. Compat. Polym.* **1991**, *6*, 178.