Pharmaceutical Research, Vol. 25, No. 7, July 2008 (© 2008)
DOI: 10.1007/s11095-008-9558-7

Research Paper

Plasmid CpG Depletion Improves Degree and Duration of Tumor Gene Expression After Intravenous Administration of Polyplexes

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Received November 20, 2007; accepted February 11, 2008; published online March 4, 2008

Purpose. Tumor gene expression after the intravenous (i.v.) administration of current polymer-based gene delivery systems is generally low and short-lived. Immune stimulatory CpG dinucleotides, present within the plasmid DNA of the polyplexes are likely to contribute to this. The effect of CpG replacement on the levels of transgene expression was studied, after the i.v. administration of polyethylenimine (PEI) polyplexes.
Methods. Tumor transfection and immune stimulation of PEI polyplexes containing plasmid DNA encoding for luciferase and rich in CpG motifs was monitored and compared to polyplexes containing the same gene but devoid of CpG motifs. Lipoplexes based on 1,2-dioleyl-3-trimethylammonium-propane/dioleoylphosphatidylethanolamine liposomes were included as a control.
Results. The replacement of CpGrich DNA by CpGfree DNA did neither affect the physical properties of the DNA complexes nor did it affect their *in vitro* transfection activity or cytotoxicity. The immune stimulation (interleukin-12) after i.v. administration of the PEI DNA complexes was low and unaffected by the presence of CpG motifs. The absence of CpG motifs within the different DNA complexes improved the degree and the duration of organ and tumor gene expression.
Conclusion. The depletion of CpG dinucleotides within the plasmid DNA of polyplexes enhances the degree and duration of *in vivo* transgene expression.

KEY WORDS: CpG dinucleotides; gene delivery; gene expression; immune stimulation; intravenous; lipoplexes; plasmid DNA; polyplexes; tumor.

INTRODUCTION

The emerging field of gene therapy offers a number of exciting potential strategies for treating cancer (1). Cancer gene therapy aims to achieve the sustained expression of a variety of anti-tumor proteins (like e.g. tumor-suppressor proteins, antigens, cytokines and suicide proteins) (2,3). Systemic application is often to be preferred as it enables to reach tumor sites which cannot be reached by local injection (3). The tumor transfection activity of systemically administered plasmid DNA can be greatly increased upon complexation with non-viral carrier systems. Complexation of plasmid DNA with cationic polymers or cationic liposomes leads to the formation of positively charged nano-sized complexes (further referred to as polyplexes and lipoplexes, respectively) protecting the complexed DNA against nuclease activity. In addition, the positively charged DNA polyplexes are readily taken up by target cells and have demonstrated to enhance transfection activity of the complexes compared to uncomplexed DNA (4). Nevertheless, intravenous (i.v.) administration of non-viral gene delivery systems generally results in low and transient tumor transgene expression levels (5–7).

Bacterial CpG dinucleotides have been identified to be major contributors to the low and short-lived transgene expression in vertebrates after non-viral gene delivery (8). CpG dinucleotides are immunostimulatory motifs which are present within bacterially produced plasmid DNA. Whereas CpG dinucleotides in bacterial DNA are unmethylated, in vertebrate DNA they are methylated and occur less frequent (9). After i.v. administration of the DNA complexes, they are captured and endocytosed by macrophages and other immune cells. CpG motifs within the plasmid DNA are recognized as a pathogen-associated molecular pattern upon acidification of the endosome, by the toll-like receptor 9 (TLR-9) which is present at the endosomal membrane (10). TLR9-mediated intracellular signaling leads to a pleiotropic inflammatory response that includes the production of large amounts of pro-inflammatory cytokines, among which tumor necrosis factor-alpha, type I interferons, and interleukin 12 (IL-12), and the activation of a variety of immune cells of the innate and adaptive immune system (e.g. natural killer-cells, T-cells, macrophages) (9). The increased production of pro-inflammatory cytokines increases the risk for systemic toxicity.

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Bacterial CpG motifs impair transgene expression after non-viral gene delivery via different mechanisms. The induced pro-inflammatory cytokines exert a direct inhibitory effect on transgene transcription by initiating plasmid promoter shutdown (11). Moreover, high local levels of pro-inflammatory cytokines can initiate apoptosis of transfected cells. In addition, activation of immune cells has been reported to eliminate transfected cells and transgene products (12–14). Finally, transgene expression of episomal plasmids can be limited by de novo methylation of the unmethylated bacterial CpG motifs. Cytosine methylation is a naturally-occurring DNA modification in vertebrates which interferes with transgene transcription, resulting in transcriptional silencing (15–17).

Though the immunostimulatory effect of DNA complexes containing unmethylated CpG motifs is being exploited in cancer immunotherapy (18–20), it should be minimized in gene therapy strategies where high and long-lasting transgene levels are desired (12,21). Several strategies have been studied to limit the immunostimulatory response to i.v. administrated DNA complexes (22) (1) directing the DNA complexes to the target cells (23,24), (2) co-administering macrophage depleting agents, immunosuppressant agents, or inhibitors of endosomal acidification (25–27), and (3) modifying the plasmid DNA by the *in vitro* methylation of unmethylated CpG motifs, by the removal of these motifs, or by the addition of so-called neutralizing motifs (25,26,28–30). Whereas all three strategies have shown to result in a reduced inflammatory toxicity of cationic lipoplexes, CpG removal appears to outweigh all other strategies when the effects on transgene expression are considered. Yew *et al*. (26, 29) showed that a reduction of the number of unmethylated CpG motifs within i.v. administered lipoplexes not only improved the degree but also the duration of transgene expression in liver and lung up to 49 days post-injection (p.i.). Similar studies have demonstrated the effects of CpG motif depletion in naked DNA formulations on transgene expression (31,32). Recently, Park *et al*. (33) demonstrated the advantage of CpG motif reduction in polymer-based formulations, using minicircle plasmid DNA. The immunogenicity of minicircle plasmids is strongly reduced by the selective excision of non-functional or immunostimulatory plasmid elements. Nevertheless, in these plasmids there is still a number of CpG motifs present. To our knowledge, no study addressed the potential benefits of complete CpG depletion in polymer-based formulations. The aim of the present study was to evaluate the effect of CpG depletion on the levels of transgene expression, after i.v. administration of PEI22 polyplexes. PEI22 (Mw 22 kDa) is a linear cationic polymer and is considered as the gold standard within the field of non-viral i.v. gene delivery (34). As a control, we included lipoplexes based on cationic liposomes composed of 1,2-dioleyl-3-trimethylammonium-propane/dioleoylphoshatidylethanolamine (DOTAP/DOPE), a lipoplex formulation which is frequently used in i.v. gene delivery (35,36).

MATERIALS AND METHODS

Materials

The plasmid pCpGvitro neomycin–luciferase (neo–luc), further referred to as pCpGfree, is a 7,114-bp plasmid devoid of CpG dinucleotides, and was purchased from Cayla SAS (Toulouse, France). The plasmid contains the coding sequence for firefly luciferase and is under transcriptional control of a composite promoter consisting of a mouse cytomegalovirus (mCMV) enhancer and the eukaryotic elongation factor-1 alpha (EF-1α) core promoter. EF-1α is an enzyme catalyzing the guanosine triphosphate-dependent binding of tRNA to ribosomes and is abundantly expressed in almost all kinds of mammalian cells (6). The plasmid has been synthesized by replacing all CpG dinucleotide containing codons for codons devoid of CpG dinucleotides, corresponding to the same amino acid. By these means, a new codon-optimized gene was created encoding for proteins whose amino acid sequence remains identical to the wild-type counterparts. Porcine cytomegalovirus (pCMV)-Luc, a plasmid containing the coding sequence for firefly luciferase, was purchased from Elim Biopharmaceuticals (St. Hayward, US). The 6,201-bp plasmid is under transcriptional control of the cytomegalovirus (CMV) immediate promoter and contains 638 CpG motifs. The QuickChange Site-Directed Mutagenesis kit and Pfu polymerase were purchased at Stratagene (La Jolla, US), primers for the site-directed mutagenesis were ordered at Eurogentec (Seraing, Belgium). The restriction enzymes PstI, SacI, NcoI, and T4 DNA ligase were purchased from Fermentas (St. leon-Rot, Germany). PacI was ordered at Westburg B.V. (Leusden, The Netherlands). The Nucleobond PC 10,000 EF DNA purification (Macherey-Nagel) was purchased from Bioke (Leiden, The Netherlands). The lipids DOTAP and DOPE were ordered at Avanti Polar lipids (Alabaster, USA) and Lipoid (Ludwigshafen, Germany), respectively. Linear polyethylenimine (PEI22, Mw 22 kDa) was a generous gift from Prof. Dr. E. Wagner (Ludwig-Maximilians-Universität, München, Germany). Luciferase assay reagent, reporter gene lysis buffer, and Quantilum recombinant luciferase were obtained from Promega (Leiden, The Netherlands). The Quantikine mouse IL-12 p70 immunoassay was purchased from R&D systems (Abingdon Park, UK).

Methods

Plasmid Construction and Purification

A CpGrich control plasmid, bearing 542 CpG dinucleotides (further referred to as pCpGrich) was constructed by replacing the promoter/5′-untranslated region (UTR) of the purchased vector pCMV-Luc with the composite enhancer/core-promoter and 5′UTR region of pCpGfree. To enable excision of the original promoter/5′-UTR region, a PstI restriction site was introduced into pCMV-Luc, by replacing the TT dinucleotide to CA, at position −8 relative to the transcription start. A site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit and two 40 bp primers(5′-CATTAGCATTCCGG TACTGCAGGTAAAATGGAAGACGCCA-3′ and 5′-TGGCGTCTTCCATTTTACCTGCAGTACCGGAATGC TAATG-3′, bold letters refer to added PstI site). The original promoter/5′-UTR region of the modified pCMV-Luc was excised by digestion with SacI and PstI (−750 to −6 relative to the transcription start). The linearized vector, devoid of promoter, was isolated by preparative gel electrophoresis,

then blunted and dephosphorylated to prevent self-ligation. The insert, consisting of the enhancer/promoter/5′-UTR region of pCpGfree (position −1,258 to −1, relative to the transcription start) was excised by digestion of pCpGfree with PacI and NcoI. The insert was isolated by preparative gel electrophoresis, blunted with Pfu polymerase and a deoxynucleotide triphosphate mix, and ligated into the blunted vector, resulting from the excised pCMV-Luc to create pCpGrich, at a 1:3 vector to insert ratio, using T4 DNA ligase. *E. coli* XL-1 Blue supercompetent cells (Stratagene) were transformed with the resulting plasmid and screened for the proper insert direction by restriction enzyme digestion.

pCpGfree and pCpGrich were grown in *E. coli* and purified to an endotoxin free grade (<0.1 EU/μg DNA), using a Nucleobond PC 10,000 EF kit.

Preparation of the DNA Complexes

DOTAP/DOPE liposomes were prepared by dissolving an equimolar amount of DOTAP and DOPE in chloroform and methanol. The solution was evaporated under reduced pressure and the lipid film was subsequently hydrated in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) 10 mM, pH 7.4 (total lipid concentration 20 mM). The resulting liposome dispersion was extruded several times through two stacked polycarbonate filters (Poretics, Livermore, USA, 200 and 100 nm pore size) with a high-pressure extrusion device until the desired diameter was obtained (100–120 nm).

DNA complexes were made at different molar ratios of cationic and anionic charge (N/P ratio), corresponding to the ratios of nitrogen of the polymer or lipid and the anionic DNA phosphates. A mass per charge of 43, 698 and 330 Da was used for PEI22, DOTAP and DNA, respectively. DNA complexes for *in vitro* use were prepared at a final DNA concentration of 10 μg/ml in Hepes (20 mM Hepes, pH 7.4) or in Hepes-buffered saline (20 mM Hepes, 0.9% *g*/*v* NaCl, pH 7.4). Lipoplexes were prepared by adding one volume of a DNA solution to one volume of a liposome dispersion, followed by mixing. Polyplexes were prepared by adding four volumes of the polymer solution to one volume of DNA solution and subsequent mixing. DNA complexes for *in vivo* studies were prepared at a final DNA concentration of 150 μg/ml in 20 mM Hepes, pH 7.1. Lipoplexes were prepared at an N/P ratio of 4, as aforementioned. Polyplexes were prepared at an N/P ratio of 6, by adding one volume of a polymer solution to one volume of a DNA solution and subsequent mixing. After preparation, one tenth a volume of a 50% *g*/*v* D-glucose in 20 mM Hepes, pH 7.1 stock was added to obtain an isotonic dispersion (37). All complexes were incubated for 30 min at room temperature, prior to use.

Complex Characterization

The average hydrodynamic diameter and the zeta potential of the DNA complexes was determined using dynamic light scattering (ALV CGS-3 system, Malvern Instruments, UK) and electrophoretic mobility (Zetasizer Nano-Z, Malvern instruments) measurements, in 20 mM Hepes pH 7.4, at a DNA concentration of 10 μg/ml. Both instruments were calibrated using polystyrene latex beads of defined size and zeta-potential.

Complex cytotoxicity and in vitro transfection activity

Neuro 2A cells (Murine Neuroblastoma, ATCC CCL-131) were cultured in RPMI 1640 medium completed with bovine calf serum (10%) and antibiotics/antimycotics (100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) at 37°C at a 5% CO_2 humidified atmosphere.

Transfection activity and cytotoxicity of the complexes was determined 24 and 72 h after incubation of the complexes with the cells (1 h), in serum containing medium, as described earlier (38). Experiments were carried out twice in triplicate in two separate 96-well plates. One series was tested for luciferase expression (38), the other series was tested for cell viability using an XTT based colorimetric assay (38,39). Luciferase activity of the cells was determined after cell lysis using a Fluostar Optima Fluorimeter equipped with a luminescence light guide (BMG LabTech, Germany). Transfection activity of the complexes was expressed as relative light units (RLU), corrected per microgram total protein (Micro BCA Protein Assay, Pierce, The Netherlands). Cytotoxicity of the complexes was determined by assessing the percentage of viable cells after transfection, normalized to values obtained after incubation with buffer only (38).

In Vivo Studies

The animal experiments were performed according to national regulations and approved by the local animal experiments ethical committee. Six-week old male A/J mice (Harlan, The Netherlands) were inoculated with 1×10^6 Neuro 2A cells in the left flank. Seven to 12 days after inoculation, at an average tumor volume of 0.4–0.9 cm^3, the different formulations were injected into the tail vein of the mice (0.2 ml, corresponding with 30 μg DNA).

At 3, 5 and 7 h post-injection, blood was collected by saphenous venepuncture. Blood samples were allowed to coagulate at 4°C for 4 h and then centrifuged at 4,000×*g* for 10 min. Serum was collected, diluted with phosphate-buffered saline and kept at −80°C until analysis. IL-12 serum levels were determined using a Quantikine mIL-12 immunoassay.

One, 5 or 11 days post-injection, mice were sacrificed by cervical dislocation and luciferase levels of the tumor and the different organs was assessed as previously described (40). Luciferase levels were expressed as relative light units per organ (or per gram tumor). In this setting, 6×10^3 RLU corresponds to 1 pg of recombinant luciferase.

Statistical Analysis

Statistical analysis was performed using GraphPad InStat 3.06. A p-value of < 0.05 was considered to be significant (two-tailed). The Grubb's test for detecting outliers was used for identifying outliers.

RESULTS AND DISCUSSION

Plasmid DNA Characteristics

pCpGvitro neo-luc (further referred to as pCpGfree) is a commercially available 7,114-bp plasmid devoid of CpG dinucleotides. It contains the coding sequence for firefly

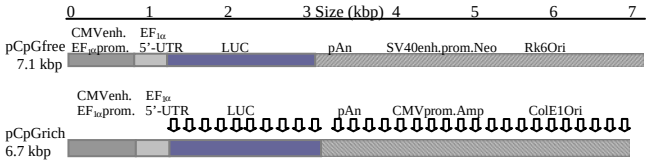

Fig. 1. Linearized diagram of pCpGfree and pCpGrich. *CMVenh. EF1$_\alpha$prom .*: composite promoter based on cytomegalovirus enhancer and mouse elongation factor-1 alpha core promoter. *EF1$_\alpha$ 5′-UTR*, 5′ untranslated region of mouse elongation factor-1 alpha; *LUC*, exon encoding for firefly luciferase; *pAn*, polyadenylation signal; *Neo*, neomycin resistance; *Amp*, ampicillin resistance; *Ori*, origin of replication. One *arrow* represents approximately 20 unmethylated CpG dinucleotides.

luciferase and is under transcriptional control of a composite promoter consisting of a mCMV enhancer and the EF-1α core promoter (Fig. 1).

A control vector, a 6,724 bp plasmid bearing 542 CpG motifs (further referred to as pCpGrich) was constructed by replacing the promoter/5′-UTR region of a purchased vector pCMV-Luc with the composite core-promoter/enhancer and 5′UTR region of pCpGfree.

Characterization of the DNA Complexes

pCpGfree and pCpGrich were complexed with PEI22 (Mw 22 kDa) and the cationic liposome formulation DOTAP/DOPE. PEI22 has proven to be one of the most efficient cationic polymers for *in vitro* and *in vivo* gene transfer (41). DOTAP is a cationic lipid, commonly used for liposome-mediated transfection (36). Co-inclusion of the helper lipid DOPE improves the lipoplex transfection activity by facilitating endosomal membrane destabilization (42).

The physical characteristics of the PEI22- and DOTAP-complexes prepared with pCpGrich or pCpGfree were determined. Under the experimental conditions, size and zeta potential of the complexes was not affected by the type of plasmid used (Tables I and II, $p \geq 0.08$). Positively charged polyplexes were formed after condensation with PEI22 at a DNA concentration of 10 μg/ml (zeta potential approximately +25–+31 mV, Table I). Polyplexes were smallest at an N/P ratio of 6 and 9. Complexation of DNA with DOTAP liposomes at an N/P ratio of 2 resulted in lipoplexes bearing a strong negative zeta potential. Likely, at this lipid to DNA ratio, the amount of cationic lipid is inefficient to complex all DNA present. Lipoplexes prepared at an N/P of 4 and 8 demonstrated a strongly positive zeta potential (+48–+57 mV). The mean size of the lipoplexes was relatively large (320–610 nm) when compared to that of the polyplexes (120–170 nm). Complexes were stable over a period of several days at room temperature.

Complex Cytotoxicity and *In Vitro* Transfection Activity

The effect of plasmid replacement (pCpGrich to pCpGfree) on the cytotoxicity (Fig. 2) and *in vitro* transfection activity (Fig. 3) of PEI22 and DOTAP complexes was studied, using Neuro 2A cells. Relative cell viability after incubation with the different DNA complexes was not affected by the type of plasmid used. PEI22 complexes showed considerable cytotoxicity (Fig. 2A). A 50% reduction in cell viability was observed at an N/P ratio of 6, 24 h after transfection. Incubation of the cells with the DOTAP complexes did not affect cell viability, at any of the N/P ratios investigated (Fig. 2B). Similar results were observed at 72 h after incubation (data not shown).

Replacement of pCpGrich by pCpGfree within both DNA complexes did not influence their respective *in vitro* transfection activities (Fig. 3). Highest levels of luciferase transgene expression were mediated by PEI22 polyplexes, prepared at an N/P ratio >6 (Fig. 3A). Transfection activity of DOTAP lipoplexes was highest at an N/P ratio of 2–4 (Fig. 3B), being tenfold lower than observed for the PEI22 polyplexes. The high ionic strength of the transfection medium favors the formation of aggregates at these specific N/P ratios (data not shown). Aggregate formation of the DNA complexes has likely contributed to the enhanced transfection activity as it promotes cellular contact and internalization (43). The similar short-term gene expression profiles observed for the two different plasmid types was expected as they bear the same transcription initiation sites. Apparently, the extensive sequence differences within the plasmid regions upstream of the EF1α 5′-UTR did not substantially affect the initial transcription rate of the plasmids.

In Vivo Inflammatory Response

The immunostimulatory activity of the different CpGfree and CpGrich formulations were tested by determining serum IL-12 levels after i.v. injection (30 μg DNA). As a result of the high inter individual differences in onset and level of inflammatory response, the IL-12 levels measured were

Table I. Mean Particle Size (nm) and Zeta Potential (mV) of pCpGrich and pCpGfree Complexes at a DNA Concentration of 10 μg/ml

Formulation		pCpGrich		pCpGfree	
	N/P ratio	Size	Zeta potential	Size	Zeta potential
PEI22	3	590 (±210)	31 (±7.4)	990 (±1,000)	25 (±15)
	6	170 (±20)	27 (±0.4)	170 (±10)	27 (±0.5)
	9	120 (±0)	25 (±1.2)	130 (±10)	25 (±0.2)
DOTAP	2	320 (±20)	−58 (±1.6)	320 (±10)	−59 (±0.9)
	4	540 (±10)	51 (±0.8)	610 (±90)	48 (±3.3)
	8	430 (±10)	55 (±1.4)	460 (±20)	57 (±0.5)

Mean (±SD), $n=3$. Polydispersity values of the complexes were within acceptable limits (<0.30), indicating rather monodisperse formulations.

Table II. Mean Particle Size (nm) of pCpGrich and pCpGfree Complexes at a DNA Concentration of 150 μg/ml

Formulation	N/P ratio	pCpGrich	pCpGfree
PEI22	6	170 (±40)	160 (±60)
DOTAP	4	280 (±20)	310 (±40)

Mean (±SD), $n=3$, Polydispersity values of the complexes were within acceptable limits (<0.30), indicating rather monodisperse formulations.

highly variable. Complexation of pCpGrich plasmid with PEI22 induced low serum IL-12 levels (Fig. 4A). Replacement of pCpGrich by pCpGfree did not affect the IL-12 serum levels observed after administration. IL-12 levels after administration of the CpGrich cationic liposome formulation were higher than the levels observed for the PEI22 polyplexes (Fig. 4B; $p<0.05$). These findings are in line with earlier reports (44,45). CpG depletion significantly decreased the serum IL-12 levels at 3 and 7 h after administration of the liposome formulation, which is again in line with earlier reports (8,26,29,44).

After i.v. administration, lipoplexes and polyplexes accumulate to a high extent in the liver as a result of their efficient uptake by Kupffer cells (46). Previous reports suggest that the systemic cytokines produced after i.v. administered lipoplexes can be attributed to uptake and intracellular processing of the lipoplexes by Kupffer cells (24,47). It is unlikely that the observed differences in immune stimulation by PEI22 and DOTAP complexes originate from major differences in tissue accumulation, with PEI22 polyplexes showing less uptake by Kupffer cells (45). We speculate that differences in the pathway of cellular uptake of the DNA complexes by the immune cells may have contributed to the observed differences in the induced inflammatory response. Whereas lipoplexes are endocytosed exclusively via clathrin-dependent pathways, polyplexes are taken up by clathrin- as well as caveolae-dependent pathways (48,49). It may be that the caveolae-dependent pathway does

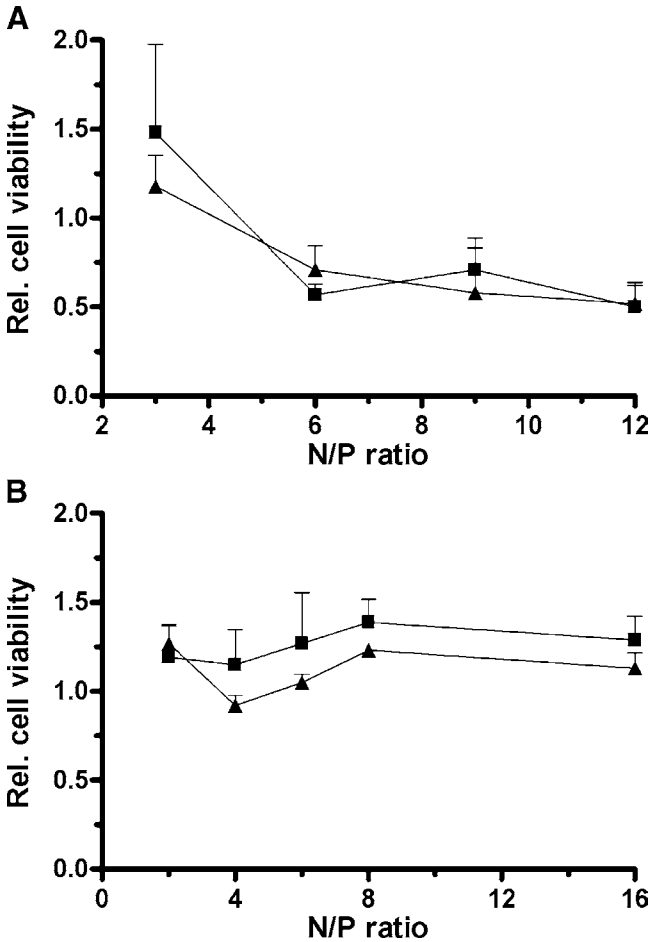

Fig. 2. Relative cell viability of Neuro 2A cells, 24 h after incubation with PEI22 (**A**) and DOTAP (**B**) based DNA complexes. DNA complexes contained CpGrich (*filled square*) or CpGfree (*filled triangle*) DNA. Experiments were carried out twice in triplicate. Values are normalized against buffer treated cells (mean+SD, $n=3$).

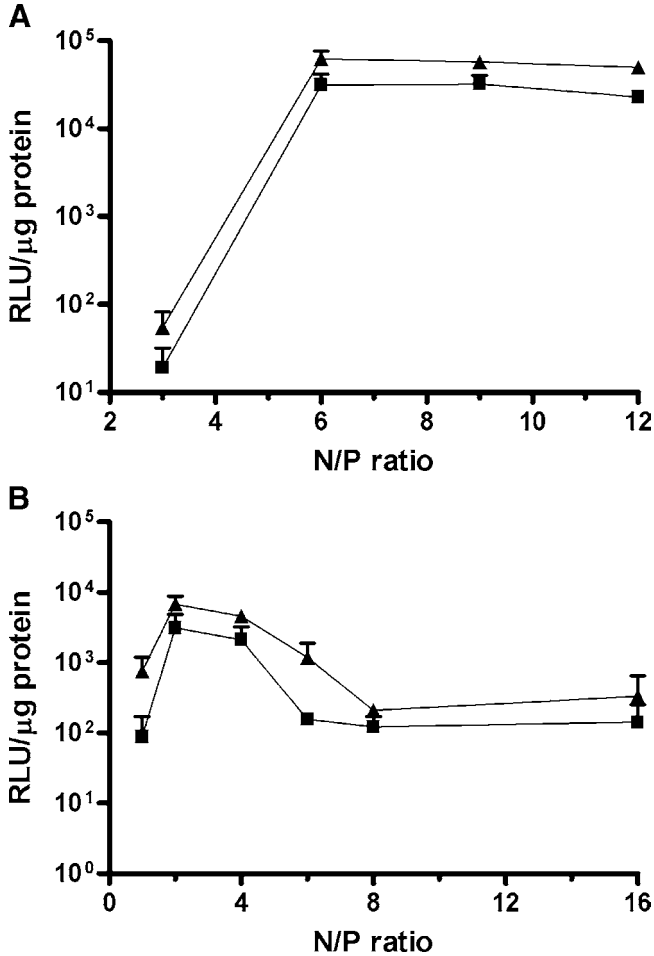

Fig. 3. *In vitro* gene expression in Neuro 2A cells, 24 h after transfection with PEI22 (**A**) and DOTAP (**B**) based DNA complexes. DNA complexes contained CpGrich (*filled square*) or CpGfree (*filled triangle*) DNA. Experiments were carried out twice in triplicate. Values are corrected per microgram protein (mean+SD, $n=3$).

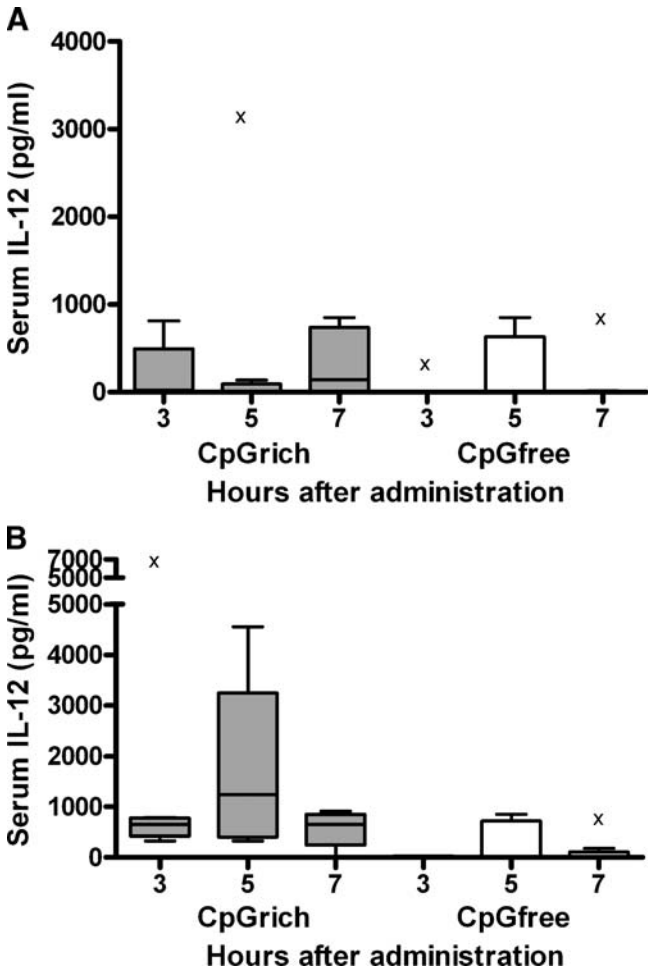

Fig. 4. Box and whisker plot of the serum IL-12 levels of mice after i.v. administration of PEI22 (**A**) and DOTAP (**B**) based DNA complexes in A/J mice (200 µl, containing 30 µg DNA; $n=6$). *X* symbols represent values which were identified as outliers.

not result in TLR9 contact and subsequent CpG-mediated cellular activation.

In Vivo Gene Expression

The effect of plasmid replacement on tumor luciferase expression was determined, at different time-points after i.v. injection of PEI22 or DOTAP complexes in mice, bearing a subcutaneous (s.c.) Neuro 2A tumor (30 µg DNA, Fig. 5). Significant gene expression was detected in the tumor, 1 day after administration of pCpGrich complexed with PEI22 (Fig. 5A). The replacement of pCpGrich by pCpGfree enhanced the degree of gene expression approximately four-fold ($p<0.05$). Moreover, CpG depletion improved the duration of tumor transgene expression. Five days after administration, luciferase levels had dropped 50-fold in the case of pCpGrich-based transfection, whereas luciferase levels were similar to day 1 p.i. in case of pCpGfree-based transfection ($p<0.05$). Eleven days after injection, luciferase levels of both plasmid types had decreased to background levels. Similar effects were observed in case of tumor luciferase expression mediated by DOTAP lipoplexes (Fig. 5B). CpG depletion of DOTAP lipoplexes enhanced the degree of tumor luciferase expression 20-fold, at day 1 p.i. Again, transgene expression endured to at least 5 days, using the CpGfree complexes ($p<0.05$).

The distribution of luciferase expression in the major organs was assessed after i.v. administration of both CpGrich and CpGfree complexes. Transgene expression was highest in the lungs, the liver and the spleen (Fig. 6A–F). Transgene expression in kidney and heart is not shown.

Injection of CpGrich PEI22 complexes mediated highest transgene expression in the lungs (Fig. 6A). CpG depletion did not significantly affect the degree of luciferase expression at day 1, 5 and 11 p.i., although mean levels were higher. In the case of DOTAP-mediated transfection, lung expression was low and only detectable at day 1 and 11 after administration of CpGfree complexes (Fig. 6B, $p<0.05$). Various factors may explain the significant difference in gene expression of polyplexes and lipoplexes, at the different time-points. Aggregation has been reported to be a dominant factor in lung uptake (50–52). Both systems may have differed in the degree of aggregation upon introduction into the bloodstream. A second explanation relates to earlier observations demonstrating that alveolar cells were reached after the intravenous administration of several DNA complexes, as a result of the transient opening of the endothelial

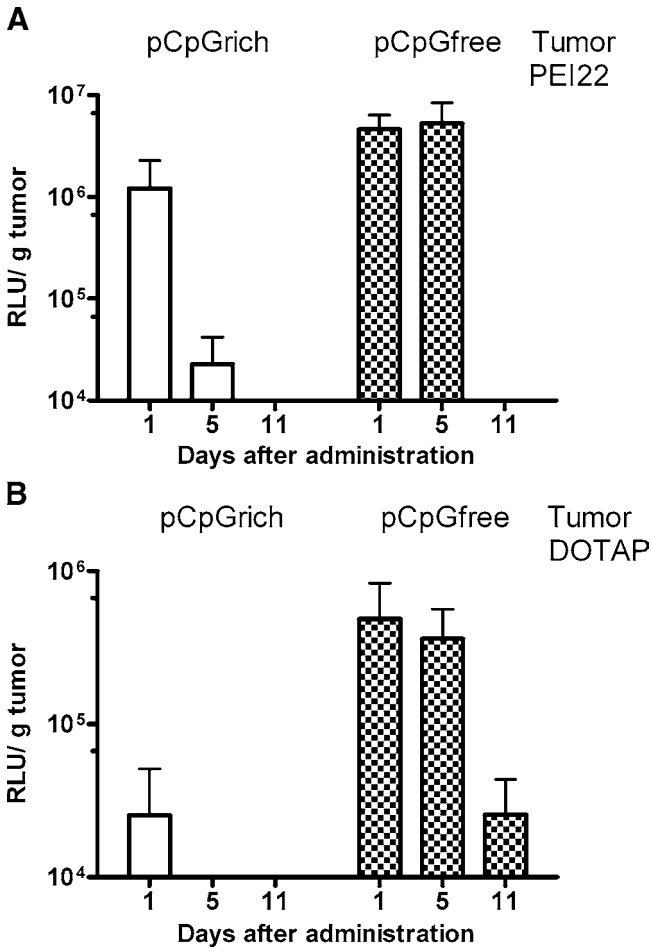

Fig. 5. Tumor gene expression after i.v. administration of PEI22 (**A**) and DOTAP (**B**) based DNA complexes in A/J mice bearing a s.c. Neuro 2A tumor (200 µl, containing 30 µg DNA; mean+SD, $n=3$). Average tumor weight at day 1, 5 and 11 was 0.6, 1.1 and 1.9 g, respectively.

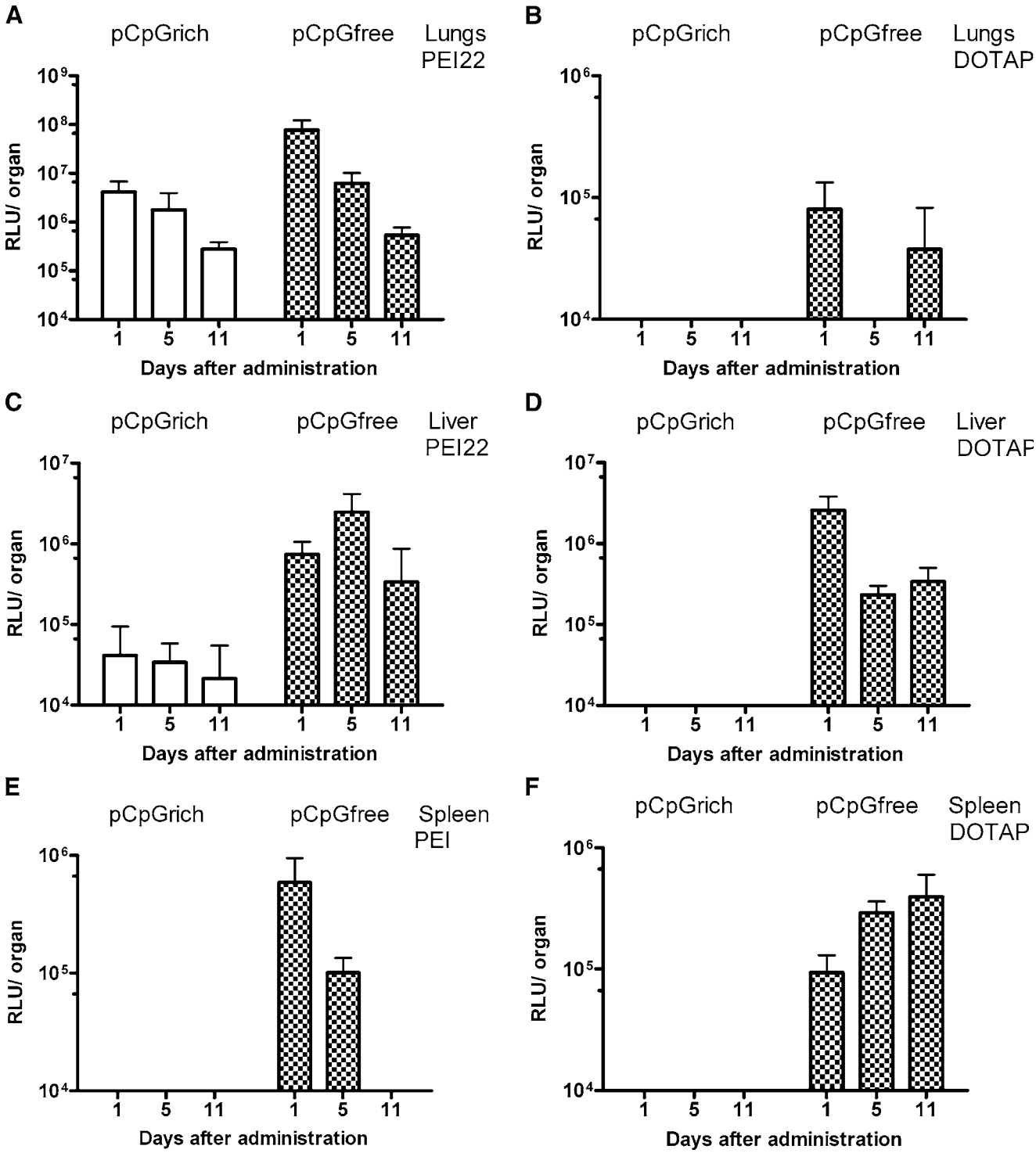

Fig. 6. Organ gene expression after i.v. administration of PEI22 (**A**, **C**, **E**) and DOTAP (**B**, **D**, **F**) based DNA complexes in A/J mice bearing a s.c. Neuro 2A tumor (200 µl, containing 30 µg DNA). Lung (**A**, **B**), liver (**C**, **D**), spleen (**E**, **F**; mean+SD, $n=3$).

cell lining (31,53–55). The DNA complexes investigated in this study might have differed in their ability to penetrate the endothelial cell lining.

Liver (Fig. 6C,D) and spleen (Fig. 6E,F) gene expression were significantly enhanced at all time-points evaluated, upon replacement of pCpGrich by pCpGfree in the different DNA complexes ($p<0.05$). Interestingly, significant luciferase levels in the latter organs persisted over at least 11 days. The prolongation of transgene expression is in good agreement with earlier publications. Yew *et al.* demonstrated transgene liver expression persisting over a period of 28–42 days, after i.v. transfection with lipoplexes with a reduced number of CpG motifs (26). Also, Park *et al.* demonstrated increased transgene adiponectin serum levels over a period of 2–5 days,

after administration of PEI polyplexes consisting of minicircle DNA, compared to PEI polyplexes bearing the complete bacterial expression cassette (33).

The beneficial effects of CpG depletion on the duration of luciferase expression was more pronounced in the liver and in the spleen, when compared to the lung. Luciferase expression observed in these organs is probably originating from a small fraction of the accumulated dose being able to escape from uptake by the non-parenchymal macrophages and to transfect parenchymal cells (e.g. hepatocytes) (56). We hypothesize that the non-parenchymal macrophages have been responsible for extensive suppression of transgene expression in the transfected parenchymal cells, in the case of transfection with CpGrich DNA complexes. Hepatosplenic macrophages have found to be responsible for the capture of a major part of the circulating DNA complexes (24). The TLR9-mediated signaling of CpG motifs by macrophages has shown to result in immune activation and high local levels of pro-inflammatory cytokines (47). The beneficial effect of CpG depletion is likely most obvious in those organs where immune cells are easily accessible (liver and spleen) and therefore are highly involved in the uptake and processing of the complexes, compared to e.g. the lung where immune cells are muss less accessible for interaction with circulating DNA complexes.

The exact mechanism by which CpG depletion improves transgene expression still remains unclear. The superior *in vivo* transgene expression profile of CpGfree DNA complexes might have benefited from the lack of the de novo plasmid methylation (15–17). The results from this study demonstrates that removal of CpG motifs limits the immunostimulatory effects of i.v. administered DNA liposome formulations. A limited immune response may have contributed to the improved transgene expression by alleviating plasmid promoter repression as well as toxicity towards transfected cells and transgene products (26,29,30). The extent to which both mechanisms contribute to the degree and duration of transgene expression is likely dependent on additional factors such as, amongst others, the acute toxicity of the gene delivery system, as well as the type of organ(s) and cell(s) being transfected.

CONCLUSION

This study is the first in demonstrating that the replacement of CpGrich DNA by CpGfree DNA improves the degree and duration of transgene expression after polymer-mediated i.v. gene delivery. The effect is most pronounced in the liver and spleen, and to a lesser extent in the tumor. Therapeutic studies have to clarify whether CpG depletion will enable further progression in tumor gene therapy.

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