Pharmaceutical Research, Vol. 25, No. 12, December 2008 (© 2008)
DOI: 10.1007/s11095-008-9693-1

Research Paper

Systemic Delivery of DNA or siRNA Mediated by Linear Polyethylenimine (L-PEI) Does Not Induce an Inflammatory Response

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Received May 26, 2008; accepted July 21, 2008; published online August 16, 2008

Purpose. The success of nucleic acid therapies depends upon delivery vehicle's ability to selectively and efficiently deliver therapeutic nucleic acids to target organ with minimal toxicity. The cationic polymer polyethylenimine (PEI) has been widely used for nucleic acid delivery due to its versatility and efficiency. In particular, the last generation of linear PEI (L-PEI) is being more efficient *in vivo* than the first generation of branched PEI. This led to several clinical trials including phase II bladder cancer therapy and human immunodeficiency virus immunotherapy. When moving towards to the clinic, it is crucial to identify potential side-effects induced by the delivery vehicle.
Materials and Methods. For this purpose we have analyzed the production of pro-inflammatory cytokines [tumor necrosis factor-α, interferon (IFN)-γ, interleukin (IL)-6, IL-12/IL-23, IFN-β and IL-1β] and hepatic enzyme levels (alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase and alkaline phosphatase) in the blood serum of mice after systemic injection of DNA or siRNAs delivered with L-PEI.
Results. Our data show no major production of pro-inflammatory cytokines or hepatic enzymes after injection of DNA or oligonucleotides active for RNA interference (siRNAs or sticky siRNAs) complexed with L-PEI. Only a slight induction of IFN-γ was measured after DNA delivery, which is probably induced by the CpG mediated response.
Conclusion. Taken together our data highlight that linear polyethylenimine is a delivery reagent of choice for nucleic acid therapeutics.

KEY WORDS: hepatic enzyme; polyethylenimine; pro-inflammatory cytokine; siRNA/DNA delivery; sticky siRNAs (ssiRNAs).

INTRODUCTION

Nucleic acid therapy, which consists in introducing exogenous genes, gene segments or oligonucleotides (antisense, siRNA, miRNA, ...) into the cells of a patient remains one of the main therapeutic challenges for the next decade. It has been developed for the treatment of both acquired and inherited diseases. The principle of gene therapy is based on the correction of the disease by delivery of a plasmid encoding a gene, which either restore the function of a deficient gene or kill a specific cell type such as cancer cells (1). Another strategy consists in inhibiting the expression of the gene which is responsible of a disease. This can be achieved by introducing either antisense DNA oligonucleotides, which block the expression of the protein of interest (2,3) or chemically synthesized small interfering RNAs (siRNAs) of 19–21 nucleotides which are able to recognize and induce the degradation of complementary mRNA sequences (4,5). The mechanism of RNA interference can also be achieved by the introduction of plasmids which express *in situ* dsRNA from RNA polymerase III promoters.

The success of nucleic acid therapies relies on the ability to efficiently deliver the appropriate therapeutic material into the target tissue or cells with the least toxicity and without inducing an immune response. Vehicles, which have successfully delivered exogenous nucleic acid *in vivo*, can be divided into two major groups: viral and non-viral vectors (1). Although viral vectors are very efficient, they have also limitations including mutational insertions, carcinogenesis and induction of immune response (6). Non-viral vectors such as polymers, cationic liposomes or naked DNA offer an attractive alternative. Amongst *in vivo* non-viral gene delivery reagents, linear polyethylenimine (L-PEI) is being widely used for its versatility (7–9) and comparatively high transfection efficiency (10,11). L-PEI has been used to efficiently deliver genes *in vivo* into a wide range of organs such as lung (12,13), brain (9,14), pancreas (15), retina (16), bladder (17) as well as tumor (15,17,18). Moreover, clinical trials using linear PEI and a mannosylated derivative as delivery reagent are underway for the treatment of bladder

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ABBREVIATIONS: ALAT, alanine aminotransferase; ALP, alkaline phosphatase; ASAT, aspartate aminotransferase; LDH, Lactate dehydrogenase; L-PEI, linear polyethylenimine; ssiRNA, sticky siRNA.

cancer[1] (18) and human immunodeficiency virus (HIV) therapy[2] (19,20) respectively. There are also a number of studies that have demonstrated effective delivery of siRNAs with L-PEI *in vivo* (8,21,22). Furthermore, we have shown recently the ability of L-PEI to efficiently condense, stabilize and deliver modified siRNAs [sticky siRNAs (ssiRNAs)] *in vitro* and *in vivo* (23). These 3′-modified siRNAs are able to reversibly form oligomers in the presence of L-PEI thus enhancing the stability of the complexes in the presence of blood or serum and increasing delivery efficiency of siRNAs by L-PEI.

However, these studies are missing an important aspect. When moving towards the clinic it is crucial to identify potential side-effects induced by the delivery system. For example it was shown that production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-6 and IL-12 cause liver damage (24,25). These studies also demonstrated that the induction of an immune response can influence hepatic toxicity as well as gene expression.

In this study we have measured the levels of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-6, IL-12/IL-23, IL-1β, and IFN-β,) and of hepatic enzymes [alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP)] in the blood serum of mice after systemic injection of DNA, siRNAs or ssiRNAs delivered with L-PEI. Our data show no major production of pro-inflammatory cytokines or hepatic enzymes after injection of nucleic acids complexed with L-PEI highlighting the potency of this polymer for nucleic acid-based therapies.

MATERIAL AND METHODS

Transfection Reagent and Nucleic Acids

In vivo-jetPEI™ (optimized cationic linear PEI-based transfection reagent for *in vivo* experiments) was from Polyplus-transfection (Illkirch, France). *In vivo*-jetPEI™ is provided as a ready to use solution at 150 mM nitrogen concentration and contained less than 0.1 EU/ml endotoxin as determined using a Limulus amebocyte lysate assay (Cambrex, Charles City, IA, USA). A well controlled polymerization and purification process was used providing polymers with MW around 22±2 kDa with a low polydispersity index between 1.1–1.3. Polyacrylamide gel electrophoresis-purified oligonucleotides (siRNAs or ssiRNAs) were purchased from Eurogentec (Seraing, Belgium). Annealing was performed in annealing buffer (Eurogentec, ×0.4 final concentration) for 2 min at 95°C followed by slow cooling. Sequences were as follows:

GL3Luc siRNA sense: 5′-CUUACGCUGAGUACUUCGA-$(dT)_2$, GL3Luc siRNA antisense: 5′-UCGAAGUACUCAGCGUAAG-$(dT)_2$, GL3Luc ssiRNA sense: 5′-CUUACGCUGAGUACUUCGATT-$(dT)_8$, and GL3Luc ssiRNA antisense: 5′-UCGAAGUACUCAGCGUAAG-$(dA)_8$.

[1] http://clinicaltrials.gov/ct2/results?spons=%22BioCancell+Therapeutics+Ltd%22&spons

[2] http://clinicaltrials.gov/ct2/show/NCT00270205?term=Phase+I%2FII+%28ACTG+5176%29&rank=1

The pCMV Luciferase-expressing plasmid (pCMVLuc) was originally obtained from Promega (Charbonnieres, France). It was amplified from *Escherichia coli* DH5α competent cells transformed by electroporation. The purification was performed by Tebu-bio laboratories (Le Perray-en-Yvelines, France), by Nucleobond PC 10 000 EF column (Macherey-Nagel, Düren, Germany). The endotoxin level was determined and certified being lower than 30 EU/mg of DNA.

Animal Experiments

All animal studies were conducted in accordance to the French Animal Care guidelines and protocols were approved by the Direction des Services Vétérinaires. OF1 female mice 22–24 g were obtained from Charles River Laboratories (Lyon, France) and subjected to a week quarantine and acclimation period before use. Animals were maintained under conventional housing conditions (12 h light/12 h night, 22°C). Except for LPS (intra-peritoneal) and CCl_4 (subcutaneous) all formulations were intravenously injected through the retro-orbital sinus within 2 s. The weight of the mice was monitored every day after injection during 7 days. Twenty-four hours after injection, liver samples were processed by standard histopathological techniques for cryosections and stained with haematoxylin and eosin.

L-PEI Polyplex Preparation

Forty micrograms of DNA, siRNAs or ssiRNAs were diluted in 100 μl of 5% glucose solution; 6.4 μl of L-PEI (*in vivo*-jetPEI™, N/P ratio of 8) were diluted in 100 μl of 5% glucose solution and mixed by vortexing for 10 s. The L-PEI solution was added to the nucleic acid solution, mixed by vortexing for 10 s and incubated for at least 15 min at RT before injection in mice.

Lipoplex Preparation

DOTAP (1,2-Dioleoyl-3-trimethylammonium-propane) (Sigma/Aldrich, Saint Quentin Fallavier, France) and Cholesterol (Sigma/Aldrich) were dissolved in chloroform at a molar ratio of 1:1. The mixture was vacuum-desiccated, and resuspended in 5% glucose solution at 10 mM concentration. Forty micrograms of pCMVLuc were diluted in 100 μl of 5% glucose solution. Thirty micrograms of DOTAP:Chol were diluted in 100 μl of 5% glucose solution and mixed by vortexing for 10 s. The DOTAP:Chol solution was added to the nucleic acid solution, mixed by vortexing for 10 s and incubated for at least 15 min at RT before injection in mice.

Positive and Negative Controls for Mice Injections

As negative controls we injected either 5% glucose solution, 40 μg of DNA, 40 μg of siRNAs or 40 μg of ssiRNAs without L-PEI in the same conditions as before. As positive controls inducing a strong inflammatory response, we used 50 μg of *E. coli* LPS (Product # L 6143, Sigma/Aldrich) diluted in 1 mL phosphate-buffered saline (PBS; ×1) injected intra-peritoneally or 60 μg of polyinosinic acid:polycytidylic acid (poly(I:C); Sigma/Aldrich) complexed with *in vivo*-jetPEI™ at N/P=8 in 200 μl of 5% glucose solution and injected

retro-orbitally. CCl_4 was used as positive control for hepatic enzyme production in serum. A mixture 1:7 (*v*/*v*) of CCl_4 (Riedel-de Haehn, Seelze, Germany) and olive oil (4 ml/kg) was injected subcutaneously.

Luciferase Assay in Organs upon Nucleic Acid Delivery

Twenty-four hours after injection, mice were anesthetized by intra-peritoneal injection of pentobarbital (40 mg/kg, Ceva, Libourne, France). The organs of interest were dissected, rinsed in PBS (×1) and mixed with an ultra-thurax homogenizer in 1 ml for spleen, kidney and heart and in 2 ml for lung and liver of lysis buffer ×1 (Promega). Each organ mix was frozen at −80°C, thawed and an aliquot of 0.5 ml was taken for luciferase analysis. The aliquot was centrifuged for 5 min at 14,000×*g*. Luciferase enzyme activity was assessed on 5 µl of organ lysate supernatant using 100 µl of luciferin solution (Promega). The luminescence (expressed as RLU) was integrated over 10 s by using a luminometer (Centro LB960, Berthold, Thoiry, France) and normalized per milligram of organ protein with bicinchoninic assay (Pierce, Woburn, MA, USA).

Cytokine Level Determinations

Blood was collected by retro-orbital puncture at 1, 2, 3, 6, 12 or 18 h after injection, incubated for 30 min at 37°C and overnight at 4°C. Samples were then centrifuged at 14,000×*g* for 5 min, and the supernatants were collected. Serum TNF-α, IFN-γ, IL-6, IL-12/IL-23, IL-1β and IFN-β concentrations were determined by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. TNF-α, IFN-γ, IL-6, IL-12/IL-23, IL-1β kits were purchased from ebioscience (San Diego, CA, USA) and IFN-β kit was from Biosource international (Camarillo, CA, USA). The amount of cytokines was determined on 100 µl of ×100 diluted serum loaded in duplicate. For IFN-β and IL-1β level determination, the serum was diluted ×10.

Hepatic Enzyme Level Determinations

Hepatic enzyme levels were determined at the 'Mice Clinical Institute', Illkirch, France. Briefly, 24 h after complex injection, blood was collected by retro-orbital puncture. About 200 µl of blood were collected into heparinized tubes. The blood was centrifuged at 5,000×*g* during 10–15 min within 1 h after collection. Plasma samples were separated and transferred in Eppendorf tubes for analysis. Plasma enzymatic activities were determined using an Olympus analyser (AU 400, Tokyo, Japan) using kits and controls supplied by Olympus. ASAT: Olympus OSR 6109 Kinetic UV test IFCC (without PP) ALAT: Olympus OSR 6107 Kinetic UV test IFCC (without PP) LDH: Olympus OSR6126 Kinetic UV test Pyruvate to Lactate ALP: Olympus OSR6104 Photometric UV test IFCC (pNPP).

RESULTS

Effect of DNA/L-PEI Delivery on Pro-inflammatory Cytokines Production

The efficiency of *in vivo* gene delivery with non-viral vectors may be drastically decreased by the production of pro-inflammatory cytokines which can lead to toxicity. It has been shown previously that intravenous injection of DNA complexed with cationic liposomes induce production of pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-12 (25–29). The purpose of our study was to determine the levels of pro-inflammatory cytokines produced upon PEI-based polyplex injection in mice. Linear polyethylenimine (L-PEI), due to its "proton sponge" effect (7) is among the most efficient vectors for gene delivery *in vitro* (30) as well as *in vivo* (9,31, 32). We used the firefly luciferase reporter gene (pCMVLuc plasmid) in order to determine the amount of DNA which gives the highest transfection efficiency with L-PEI in various organs in mice. Increasing amounts (20 to 100 µg) of pCMVLuc/L-PEI complexes at N/P ratio of 8 were injected intravenously through the retro-orbital sinus. Complexes were prepared in 5% glucose in order to generate stable complexes (of at least 24 h) whose size is adapted to *in vivo* experiments, *i.e.* around 100 nm in diameter [personal data and (33)]. The N/P ratio of 8 was used since it gives the highest transfection efficiency (data not shown). Organs (spleen, liver, heart, kidney and lung) were harvested 24 h after injection of the complexes and luciferase expression was determined in each organ (Fig. 1). These organs showed the highest luciferase expression, whereas almost no luciferase expression was observed in other organs such as pancreas, uterus or intestine, except salivary glands (data not shown). Our data showed that increasing the amount of DNA above 40 µg did not significantly increase gene transfer efficiency. While all mice survived with 40 µg DNA, delivery of 100 µg of pCMVLuc resulted in appearance of toxicity (40% mice survival). We therefore performed all the subsequent experiments using the optimal conditions, *i.e.* 40 µg of DNA (1.6 mg/kg) complexed with L-PEI at N/P=8 (3 mg/kg). A cationic lipid formulation (DOTAP:Chol) previously used for *in vivo* gene delivery (34,35) was also tested. We found the DNA/DOTAP:Chol ratio of 1:5 (*w*/*w*) to be optimal for gene transfer efficiency with 40 µg of DNA (data not shown). The level of luciferase measured in the lung was 5.10^6 RLU/mg of protein using 40 µg of DNA. A weight loss was observed within the first 2 days after injection (data not shown) in all

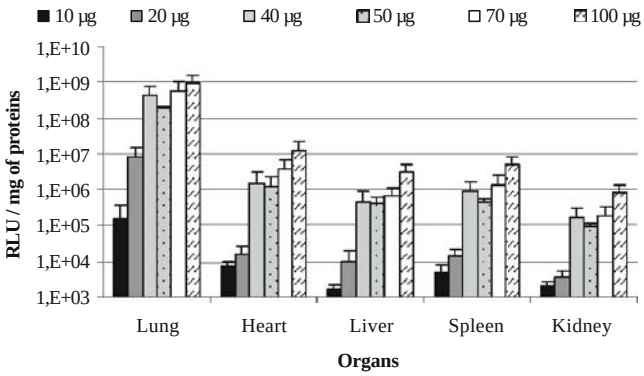


Fig. 1. Effect of the injected DNA amount on the luciferase level in various organs. Different amount of pCMV Luciferase expressing plasmid (pCMVLuc), respectively 10, 20, 30, 40, 50, 70 and 100 µg complexed with *in vivo*-jetPEI™ at N/P=8 were injected by i.v. through the retro-orbital veins. The level of luciferase was measured in each organ 24 h after the injection. Mean ± SD were calculated and expressed relative to the protein level present in each organ (n=6).

mice including control mice. We believe that it is due to blood puncture, stress of injection in addition to the injected compounds. The weight recovery was slightly faster after polyplex than after lipoplex injection (after 3 days, total weight recovery for L-PEI alone, average weight loss of 8% for DNA/L-PEI complexes and 11% for DNA/DOTAP:Chol complexes).

We then analyzed the induction of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-6, IL-12/IL-23) in mice serum, at different time points (1, 3, 6, 12 and 18 h) upon DNA/L-PEI injection. Complex solution (*i.e.* 5% glucose) as well as L-PEI alone, DNA alone, LPS and DNA/DOTAP:Chol were used respectively as controls. As expected, a strong TNF-α induction (approximately 5,000 pg/ml after 1 h) was observed after LPS injection (Fig. 2A). However, no TNF-α (<4 pg/ml) expression was observed after DNA/L-PEI or DNA/DOTAP:Chol complexes delivery, nor after L-PEI alone. Transient IFN-γ expression, between 6 and 12 h, after injection of either polyplex or lipoplex was observed (Fig. 2B). Levels were similar for both types of complexes (Fig. 2B) and significantly decrease 18 h after injection. High expression level of IL-12/IL-23 was observed after LPS delivery (Fig. 2C). Injection of DNA/DOTAP:Chol complexes resulted in transient induction of IL-12/IL-23 pro-inflammatory cytokine (Fig. 2C). IL-12/IL-23 induction (<10 ng/ml) was also observed after DNA/L-PEI injection (Fig. 2C) but it remained significantly lower than after lipoplex or LPS injection, respectively ×2 and ×3.5 lower. IL-6 expression was drastically induced after LPS injection in mice (Fig. 2D). In contrast, compared to LPS, 13.6 and 3.2 fold lower transient induction of IL-6 was measured respectively 3 and 6 h after injection of polyplex (Fig. 2D). For lipoplex delivery, this induction was respectively four and five-fold lower at 3 and 6 h, compared to LPS. Finally L-PEI (Fig. 2) as well as DOTAP:Chol (data not shown) vehicles alone did not induce any pro-inflammatory cytokines.

Hepatic Enzyme Activity after DNA/L-PEI Complex Injection

In order to evaluate the toxicity in the liver, the level of hepatic enzymes (LDH, ASAT, ALAT and ALP) was determined. It is generally accepted that increased cytosolic

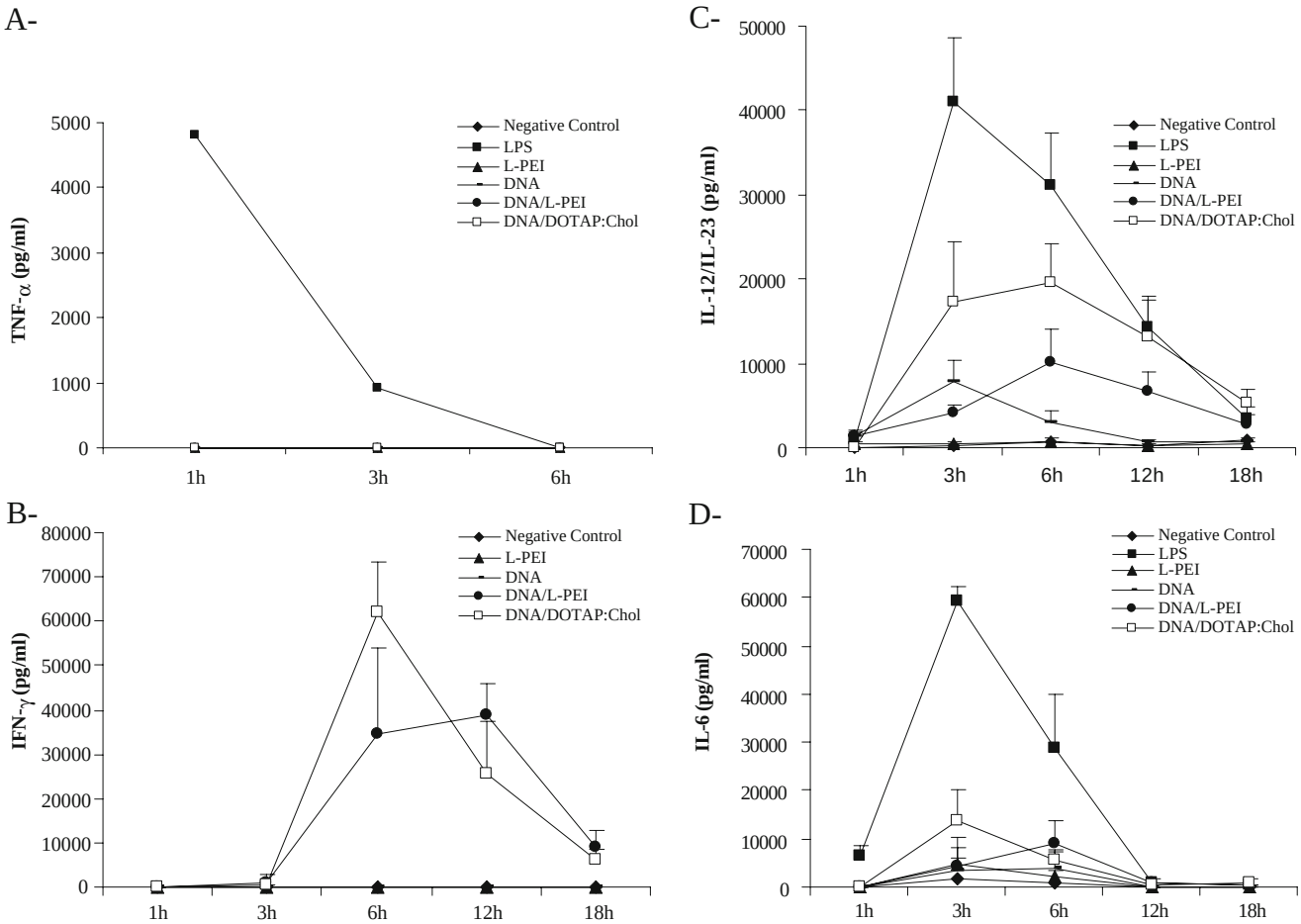


Fig. 2. Serum TNF-α (**A**), IFN-γ (**B**), IL-12/IL-23 (**C**) and IL-6 (**D**) concentration after intravenous injection of DNA/L-PEI polyplex. Forty micrograms of pCMVLuc complexed with L-PEI (N/P=8) in 5% glucose solution were injected intravenously through the retro-orbital sinus. L-PEI alone (6.4 μl, corresponding to N/P=8) and DNA alone (40 μg) were also injected. As a negative control, 5% Glucose solution was used. As positive control, 50 μg of *E. coli* LPS in PBS was i.p. injected. Forty micrograms of DNA complexed with a cationic lipid (DOTAP:Chol) at a lipids/DNA ratio of 5:1 (*w*/*w*) were also injected as control for pro-inflammatory induction after lipoplex delivery. Blood was collected 1, 3, 6, 12 and 18 h after injection, and serum was extracted. Level of pro-inflammatory cytokines was determined by ELISA. Each value represents the mean ± SD (n=4 for controls and n=8 for specific assays).

enzyme activity in the blood occurs secondarily to hepatic damage or necrosis (36). We checked the liver surface after abdominal operation for necrosis and no damage was observed within 24 h. Slight damage of hepatic lobules was observed 24 h after each injection of either polyplex (Fig. 3A) or lipoplex (data not shown). DNA/L-PEI delivery reversibly affects the integrity of the plasma membrane. This slight damage of hepatic lobules observed may be the results of the effect on the integrity of the plasma membrane. However this damage is reversible as the liver looks normal 48 h after injection of the complexes. Histological sections were performed 24 h after injection and showed that the damage is superficial (Fig. 3B). No necrosis zones were observed after polyplex delivery. High amounts of DNA (>70 μg) complexed with L-PEI or DOTAP:Chol increase liver damage, leading to lethality above 100 μg of DNA. Delivery of DNA with L-PEI leads to a strong transgene expression in the lung and to a lesser extent in the liver. However transient tissue damage is only observed in the liver and no damage was visible upon dissection in the lung and other organs.

The level of hepatic enzymes was evaluated 24 h after polyplex or lipoplex injection. CCl_4 was added as positive control for hepatic damage. No major induction of hepatic enzyme was determined after injection of L-PEI alone (Fig. 4) or DOTAP:Chol alone (data not shown). As shown in Fig. 4, no significant ALP induction (<300 U/l) was observed after delivery of DNA/L-PEI or DNA/DOTAP:Chol as well as reagents (L-PEI or DOTAP:Chol) alone. The levels of ALAT and ASAT were found to be respectively lower than 300 and 500 U/l after polyplex or lipoplex injection, while CCl_4 injection leads to much higher level (7,700 and 6,800 U/l respectively). A slight increase in LDH was also observed after polyplex and lipoplex injection (respectively 2,000 and 1,400 U/l), but this induction is 11-fold lower than with CCl_4. Taken together, our data showed no serious hepatic toxicity after DNA/L-PEI complex delivery.

Lack of Pro-inflammatory Cytokine Induction after siRNA or ssiRNA/L-PEI Complex Injection

Therapeutic siRNAs require the use of efficient and safe delivery vehicles. To enhance stability in serum, the siRNAs are sometimes chemically modified. Recently, several studies have shown the potency of L-PEI to deliver siRNAs *in vivo* (8,21,22,37). Moreover, we have shown recently that ssiRNAs display increased stability and silencing efficiency over siRNAs when complexed with L-PEI (23). In the present study, we questioned whether injection of siRNAs or ssiRNAs or their complexes with L-PEI could induce the production of pro-inflammatory cytokines. This would adversely influence therapeutic applications since immune stimulation would be associated with off-target effects and toxicity. Similarly to DNA studies, 40 μg of siRNAs or ssiRNAs complexed or not with L-PEI were injected intravenously. Based on the DNA study, we determined the optimal time point for each cytokine dosage (*i.e.* 1 h for TNF-α, 6 h for IFN-γ and 3 h for IL-6 and IL-12/IL-23). For IL-1β the optimal time point was chosen according to the literature (38,39). As shown in Fig. 5, no TNF-α (<4 pg/ml), IFN-γ (<15 pg/ml) nor IL-1β (<8 pg/ml) was induced after siRNA- or ssiRNA-containing polyplex injection. The induction of IL-6 and IL-12/IL-23 observed after siRNA/L-PEI or ssiRNA/L-PEI injection is minimal compared to LPS (18 to 32 fold lower for IL-12/IL-23 and 20 fold less for IL-6).

Since siRNAs and ssiRNAs are double-stranded RNAs, they have the potential to induce IFN-β production through Toll-like receptor 3 or 7 (TLR3, TLR7) (39). This could be especially true for ssiRNAs, as they have the capacity to reversibly form oligomers in the presence of L-PEI. We studied the level of IFN-β in mice after siRNA/L-PEI and ssiRNA/L-PEI complex injection. Polyinosinic acid:polycytidylic acid (poly(I:C)), a double-stranded RNA, which is recognized by TLR3 and induces immune responses in mice (40,41), was used as positive control. As expected from the previous cytokine measurements, L-PEI alone failed to induce an IFN-β response (Fig. 6). As shown in Fig. 6, siRNAs and ssiRNAs complexed with L-PEI elicited only a weak IFN-β response, 10-fold weaker than poly(I:C) complexed with L-PEI.

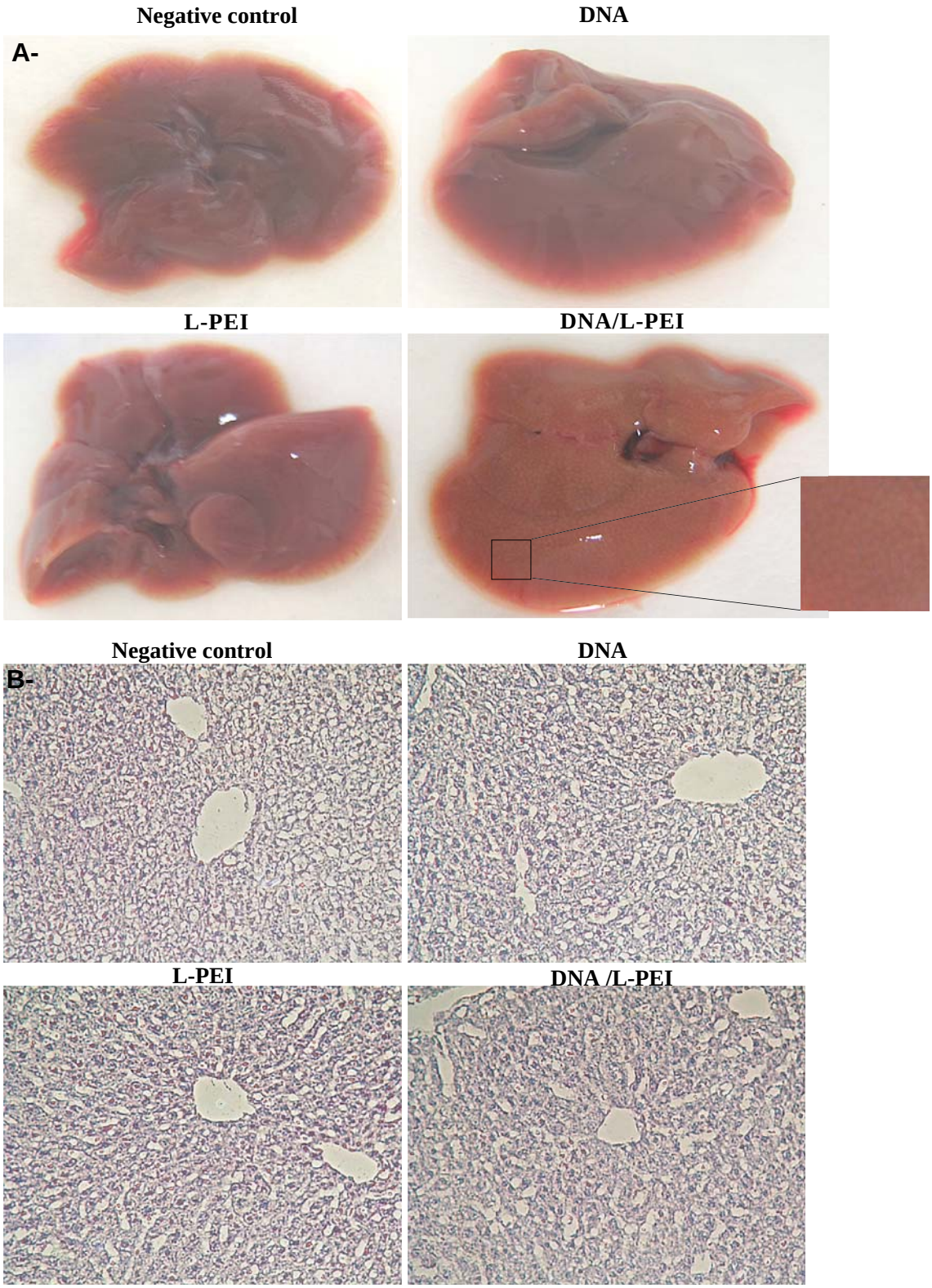
Level of Hepatic Enzymes after siRNA and ssiRNA/L-PEI Complexes Injection

As for DNA delivery with L-PEI, we have evaluated the level of hepatic enzymes (LDH, ASAT, ALAT and ALP) present in serum after siRNA/L-PEI and ssiRNA/L-PEI delivery. Forty micrograms of siRNAs or ssiRNAs complexed (N/P=8) with L-PEI were injected intravenously through the retro-orbital sinus. Buffer solution (5% glucose), LPS, CCl_4, L-PEI and nucleic acid alone were used as controls. No liver damage was observed at any time after complex injection. As shown Fig. 7, no induction of ALP (<300 U/l) was observed irrespective of the formulation or vehicle injected. As expected, CCl_4 induced a strong increase of ALAT, ASAT and LDH (respectively 7,500, 6,800 and 4,000 U/l; Fig. 7). The induction of ALAT, ASAT and LDH observed after polyplex or lipoplex delivery is negligible compared to CCl_4 (below 100 U/l for ALAT and ASAT and 500 U/l for LDH; Fig. 7). No induction of hepatic enzymes was observed after injection of L-PEI (Fig. 7) or DOTAP:Chol alone (data not shown). Thus we conclude that siRNAs- and ssiRNAs-/L-PEI complexes do not induce hepatic toxicity.

DISCUSSION

Among the non-viral vectors used for nucleic acid therapeutics, many pre-clinical and clinical studies, were performed with cationic lipid-based formulations. Unfortunately, most of them induce production of pro-inflammatory cytokines mainly IFN-γ, IL-12 and TNF-α (25,27–29,42). Moreover, leukocyte and thrombocyte counts become elevated as do liver enzymes ALAT and ASAT upon lipoplex delivery (25). More recently, the other class of non-viral vectors, the cationic polymers, came into play, with polyethylenimine being one of the most efficient (10,11).

In this study we explore the production of pro-inflammatory cytokines after nucleic acid (DNA, siRNAs or ssiRNAs) delivery with linear polyethylenimine in immune-competent mice. Currently, L-PEI and a mannosylated derivative of L-PEI are being used in phase II for the treatment of bladder cancer (18) and HIV (19,20) respec-


Negative control
DNA
A-
L-PEI
DNA/L-PEI
Negative control
DNA
B-
L-PEI
DNA /L-PEI

◀ **Fig. 3. A**, **B** Liver structure after intravenous delivery of DNA/L-PEI complex. Forty micrograms of pCMVLuc complexed with L-PEI (N/P = 8) in 5% glucose solution were injected intravenously through the retro-orbital sinus. L-PEI alone (6.4 µl, corresponding to N/P = 8) and DNA alone (40 µg) were also injected. As a control, 5% Glucose solution was used. Twenty-four hours after injection, liver were harvested (**A**) and histological sections were performed. The section was stained with haematoxylin and eosin (**B**).

tively. These clinical trials are performed under local administration (bladder cancer) or topical application on skin (HIV). Earlier phase I studies had shown a good safety profile for these new drugs. However, data regarding toxicity of L-PEI-based polyplexes after systemic injection are scarce (43). We first determined the level of pro-inflammatory cytokines (*i.e.* TNF-α, IFN-γ, IL-6, IL-12/IL-23, IL-1β and IFN-β) after intravenous delivery in mice of nucleic acids complexed with L-PEI.

Among these cytokines, TNF-α is the primary source of toxicity as it induces a septic shock in animals when reaching high serum concentrations (44,45). As shown in our study, no TNF-α production was observed after DNA, siRNA or ssiRNA delivery with linear polyethylenimine.

IL-6 is another major inducer of acute phase reactions in response to inflammation or tissue injury. With TNF-α, IL-6 induces synthesis of acute phase proteins by hepatocytes (46). Compared with the IL-6 production induced after LPS injection, the production of IL-6 induced by DNA delivery with L-PEI complex is low and comparable to the induction observed after DNA/DOTAP:Chol complex. Therefore,

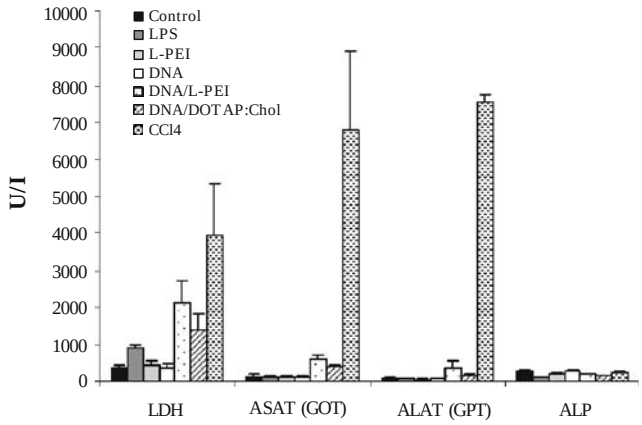


Fig. 4. Serum hepatic enzyme activity after intravenous injection of DNA/L-PEI complex. Forty micrograms of pCMVLuc complexed with L-PEI (N/P=8) in 5% glucose solution were injected intravenously through the retro-orbital sinus. L-PEI alone (6.4 µl, corresponding to N/P=8) and DNA alone (40 µg) were also injected. As a negative control, 5% Glucose solution was used. As positve control, 50 µg of *E. coli* LPS in PBS was injected i.p. and CCl_4, (12.5 µl in 100 µl olive oil) was s.c. administrated. Forty micrograms of DNA complexed with a cationic lipid (DOTAP:Chol) at a lipids/DNA (*w*/*w*) ratio of 5:1 were also injected as control for hepatic enzyme induction after lipoplex delivery. Blood was collected 24 h after injection and level of hepatic enzymes (Alanine aminotransferase, SGPT or ALAT, Aspartate aminotransferase, SGOT or ASAT, Alkaline phosphatase, ALP, and Lactate deshydrogenase, LDH) was determined on serum. Each value represents the mean ± SD (n=4 for controls and n=8 for specific assays).

	siRNA	ssiRNA	siRNA/L-PEI	ssiRNA/L-PEI	LPS
TNF-α	< 4	< 4	< 4	< 4	4425 ± 2391
IFN-γ	< 15	< 15	< 15	< 15	4952 ± 9905
IL-12/IL-23	437 ± 606	253 ± 242	1301 ± 817	732 ± 547	23932 ± 9411
IL-6	58 ± 122	153 ± 166	1204 ± 985	1306 ± 754	24762 ± 27614
IL1-β	< 8	< 8	< 8	< 8	ND

Fig. 5. Levels of TNF-α, IFN-γ, IL-12/IL-23, IL-6 and IL-1β in mice serum after siRNA or ssiRNA/L-PEI complex injection. Forty micrograms of siRNAs or ssiRNAs complexed (N/P=8) with L-PEI in 5% glucose solution were injected intravenously through the retro-orbital sinus. siRNAs and ssiRNAs alone (40 µg) were also injected. Blood was collected at different time points and level of pro-inflammatory cytokines was evaluated by ELISA (1 h for TNF-α dosage, 3 h for IL-12/IL-23 and IL-6 and 6 h for IFN-γ and IL-1β). As positive control, 50 µg of *E. coli* LPS in 1 mL PBS was i.p. injected. Each value represents the mean ± SD (n=4 for controls and n=8 for specific assays). Concentrations are expressed in pg/ml.

siRNA or ssiRNA delivered by L-PEI induced lower levels of IL-6 than those measured after DNA delivery.

IL-12/IL-23 activate and induce cell proliferation, cytotoxicity and cytokine production by NK cells (46). Strong induction of IL-12/IL-23 was observed after DNA lipoplex injection in mice. This induction is comparable with the induction observed after LPS stimulation. Transient induction of IL-12/IL-23 was also observed after DNA delivery with L-PEI but this induction is between 2 to 4 fold lower than after lipoplex injection. The IL-12/IL-23 induction observed after siRNA/L-PEI or ssiRNA/L-PEI is negligible and injection of L-PEI alone does not induce any IL-12/IL-23 response at all.

In contrast to the immune response inducer, IFN-γ has pronounced antiviral, immunoregulatory and anti-tumoral properties (46). It is induced by nonmethylated CpG sequences present in plasmid DNA or by bacterial genomic contaminations during plasmid preparation (28,29,47–51). The intracellular delivery of DNA complexed with non-viral vectors, as compared to DNA alone, is much increased in some organ cells, mainly liver and lung. Moreover, immune cells, and especially Kupffer cells, internalize large amounts of plasmid without expressing the transgene. This is why the IFN-γ induction is more effective when using delivery vehicles. In our study, we observed transient induction of IFN-γ after polyplex injection. Six hours after injection, this induction is however, lower than the induction observed after lipoplex delivery. Twelve hours after delivery, the induction of IFN-γ due to DNA delivery with polymers or lipids is comparable. Interestingly, since no induction of IFN-γ was observed after siRNAs or ssiRNAs delivered with L-PEI, nor with L-PEI alone, this suggest that the observed induction is due to the plasmid itself rather than to the L-PEI. This was confirmed for other cytokines since injection of L-PEI alone did not induce any pro-inflammatory response. In this study, we have used a plasmid containing CpG sequences, which

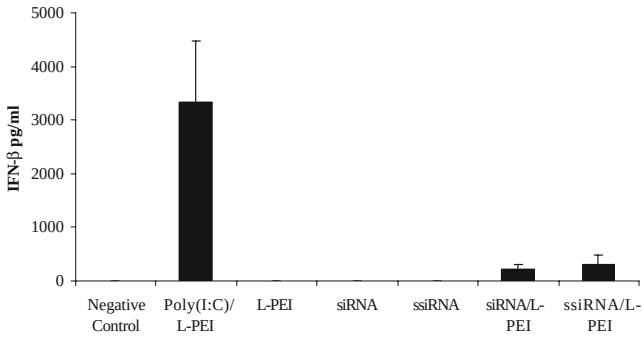


Fig. 6. Level of IFN-β in mice serum after injection of siRNA/L-PEI or ssiRNA/L-PEI complex. Forty micrograms of siRNAs or ssiRNAs complexed with L-PEI (N/P=8) in 5% glucose solution were injected intravenously trough the retro-orbital sinus. As controls, 5% glucose solution, L-PEI alone (6.4 μl, corresponding to N/P=8), siRNAs alone (40 μg), ssiRNAs alone (40 μg) and poly(I:C) (60 μg) complexed with L-PEI at N/P=8 were used. Blood was collected 2 h after complex injection and level of IFN-β was determined on serum by ELISA. Each value represents the mean ± SD (n=4 for controls and n=8 for specific assays).

could explain the observed IFN-γ induction. IFN-γ and other cytokine induction could therefore be reduced by eliminating the DNA CpG motifs or inhibiting these motifs by methylation (52,53).

IFN-β and other pro-inflammatory cytokine production can be induced by siRNAs or long dsRNA. Non-viral reagents, used for the delivery of siRNAs, increase the cellular uptake of nucleic acids and thus have the potential to increase interferon response. Such induction of the interferon response by dsRNA is mediated by signalling through the Toll-like Receptors (39,54–56) and is described as being dependent on the cell type (57), the sequence (39,55,56) and the length of the dsRNA duplex (57). siRNAs, which are short double-stranded RNAs, are in general able to induce specific gene silencing without induction of interferon response. However, sometimes an interferon response is induced after siRNA delivery. This induction can be abolished by appropriate chemical modifications or a new sequence design (58,59). In our previous work (23), we used the sequence of the GL3-Luc siRNA described by Elbashir and collaborators (4) and we showed that GL3-Luc siRNAs and sticky siRNAs (ssiRNAs) failed to induce an IFN-β response *in vitro* when delivered into cells by L-PEI. Here, we confirmed that neither siRNAs nor ssiRNAs induce IFN-β response *in vivo* after systemic delivery mediated by L-PEI. This in turn suggests that transient oligomerization of sticky siRNAs, generating long double-stranded RNAs, upon complexation with L-PEI does not seem to be detected by Toll-like Receptor (TLR3, 7 or 8) and hence fails to induce an IFN-β response.

Taken together our data confirm a previous report showing that no major inflammation is induced after linear PEI-mediated nucleic acid delivery *in vivo* (43). The induction of pro-inflammatory cytokines is lower with L-PEI/DNA complexes compared to cationic lipid-based systems, such as DOTMA:Chol (43) or DOTAP/Chol (as shown here). This decisive advantage over lipids and viruses added to a reasonable gene delivery efficiency may explain the recent developments of several PEI-based vaccines (19,20,60,61). Indeed it was reported in these studies that PEI/DNA immunization did not generate neutralizing antibodies against the delivery vector, even when injected repeatedly (61).

Organ toxicity and especially hepatic toxicity is another concern when moving to the clinic. The analysis of cell-specific enzymes in the blood provides information regarding function or integrity of a tissue or organ. Among the hepatic enzymes tested in our study, ALAT and ALP are mostly found in the liver, but are also associated with other organs such as kidney, bone or striated muscle. ASAT is more specific to striated muscle or red blood cells, but is also found in liver. LDH is found in a large quantity of organs such as kidney, heart, muscle, pancreas, spleen, brain, lung, skin, red blood cells or placenta. All of them are increased in case of cell destruction. The overall integrity of all organs was checked 12, 24 and 48 h after injection of lipoplex and polyplex (whether composed of DNA, siRNAs or ssiRNAs). Except for the liver where a slight damage of hepatic lobules was visually observed 24 h after DNA lipoplex or polyplex injection, no injury of any organs was observed. The hepatic damage is reversible, as after 48 h the liver appearance returns to normal. When we analyzed the level of hepatic enzyme in the serum, negligible induction was observed after polyplex injection compared with CCl_4, an hepatotoxic compound that causes functional and morphological changes in cell membrane (62) leading to an elevation of hepatic enzymes (63). Our data clearly showed that neither L-PEI nor nucleic acid/L-PEI complexes are hepatotoxic nor organotoxic as the induction of hepatic enzymes is not significant and restricted to LDH. Cell destruction is leading to cytosolic enzyme leakage. There are two basic hypotheses regarding what degree of cell integrity must be lost before cytosolic enzymes escape from cells (64,65). The first one supports the fact that the release occurs only upon irreversible cell damage. Hence the presence of cytosolic enzyme

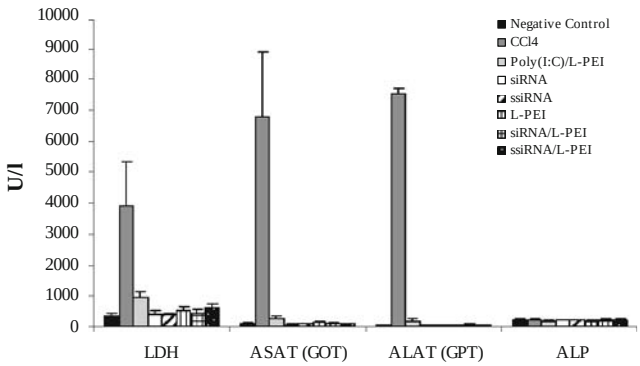


Fig. 7. Level of hepatic enzymes after siRNA/L-PEI and ssiRNA/L-PEI complex injection in mice serum. Forty micrograms of siRNAs or ssiRNAs complexed with L-PEI (N/P=8) were injected i.v. L-PEI alone (6.4 μl, corresponding to N/P=8), siRNAs alone (40 μg) and ssiRNAs alone (40 μg) were also injected. As a negative control, 5% Glucose solution was used. As positive controls 50 μg of *E. coli* LPS in PBS was injected i.p. and CCl_4, (12.5 in 100 μl olive oil) was s.c. administrated. Twenty-four hours after injection, blood was collected. The level of hepatic enzyme (Alanine aminotransferase, SGPT or ALAT, Aspartate aminotransferase, SGOT or ASAT, Alkaline phosphatase, ALP, and Lactate dehydrogenase, LDH) was determined on serum. Each value represents the mean ± SD (n=4 for controls and n=8 for specific assays).

detection in the blood is always an indicator of cell death. The second hypothesis, which is currently more broadly accepted, states that cell release cytosolic enzymes during both reversible and irreversible phases of cell injury. Therefore their serum dosage does not necessarily correlate with cell death (36). The second hypothesis is reinforced by recent experimental studies as well as by a better understanding of reversible cell injury (66). Our data also support the later hypothesis since the slight liver damage observed after DNA/L-PEI complex delivery is reversible and no necrosis zone was observed. The amount of hepatic enzymes observed in the blood serum may therefore be a consequence of transient cell shock consistent with efficient transfection of the carried nucleic acids in the cells. Other studies have reported a reversible and transient liver damage after DNA/L-PEI injection upon administration of high amount of DNA (100 to 150 μg per mouse) or L-PEI (N/P ratio>10; 32,67). Under these non-optimal conditions, inflammation and areas of necrosis are observed in the liver. However patients treated in the on-going clinical trials with nucleic acid delivered with L-PEI receive less than 1 mg/kg of DNA, which proportionally represents a DNA dose of 20 μg for a mouse of 20 g. As for any active treatment, a balance between efficiency, inflammation and toxicity has to be found (68) and we show here that this is attainable. Conflicting conclusions about immune responses attributed to PEI were derived using 'chemical' batches of branched PEI (43,69–71) or ill-defined high molecular weight linear PEI (72). These batches have large and variable polydispersities. Moreover, branched PEI/DNA complexes have larger cationic surface potentials (73), hence are more prone to bind complement proteins (74) or induce formation of microemboli (75). Onset of toxicity was observed using a ten fold larger amount of PEI than required for an optimal transfection (72). Finally, several studies have shown that branched 25 kDa PEI is less efficient in transfection (12,43,69,76,77) than linear PEI. In our study we used a ready to use available formulation of cationic linear PEI optimized for *in vivo* experiments and manufactured under specific quality requirements and specifications. The use of high quality non-viral reagents manufactured with good manufacturing practice guidelines for clinical studies is important as it ensures reproducibility and reduces the production of pro-inflammatory cytokines and toxicity.

CONCLUSION

In summary no major induction of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-6, IL-12/IL-23, IFN-β and IL-1β) was detected after delivery of nucleic acids (DNA, siRNAs or ssiRNAs) with an optimized formulation of linear polyethylenimine. There was a transient increase of IFN-γ after DNA polyplex or lipoplex delivery. Since no induction of cytokines was observed after L-PEI alone or siRNA/L-PEI complex delivery, we believe that this induction could be due the presence of unmethylated CpG in the plasmid used in our experiments. Moreover there was no induction of hepatic enzymes (ALAT, ASAT, LDH and ALP) after DNA or siRNA delivery with linear polyethylenimine showing that L-PEI polyplexes are not hepatotoxic in these conditions. Taken together, our data showed that the use of appropriate formulations of nucleic acid with L-PEI in well-defined conditions gives high transfection efficiency with absence of inflammation and toxicity. As results this linear polymer is well suited for therapeutic approaches using nucleic acids.

ACKNOWLEDGMENTS

These studies were supported by the sixth framework programme, EU-supported research, GIANT Integrated Project N°LSHB-CT-2004-512087 and RIGHT Integrated Project N°LSHB-CT-2004-005276. The authors want to thank Jeanne-Françoise Williamson for critical reading of the manuscript, Jean-Paul Behr for fruitful discussions and Fabrice Stock for the formulation of non-viral reagents.

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