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Efficient *in vivo* transfection and safety profile of a CpG-free and codon optimized luciferase plasmid using a cationic lipophosphoramidate in a multiple intravenous administration procedure

Abstract

As any drug, the success of gene therapy is largely dependent on the vehicle that has to selectively and efficiently deliver therapeutic nucleic acids into targeted cells with minimal side-effects. In the case of chronic diseases that require a life-long treatment, non-viral gene delivery vehicles are less likely to induce an immune response, thereby allowing for repeated administration. Beyond the gene delivery efficiency of a given vector, the nature of nucleic acid constructs also has a central importance in gene therapy protocols.

Herein, we investigated the impact of two firefly luciferase encoding plasmids on the transgene expression profile following systemic delivery of lipoplexes in mice, as well as their potential to be safely and efficiently readministered. Whereas pTG11033 plasmid is driven by a strong ubiquitous cytomegalovirus promoter, pGM144 plasmid, which has been designed to avoid inflammation and provide sustained transgene expression in lungs, is CpG-free and is under control of the human elongation factor-1 alpha promoter.

Combined to the efficient cationic lipophosphoramidate BSV4, bioluminescence data showed that both plasmids were mostly expressed in the lungs of mice following a primary injection of lipoplexes. However, mice transfected with pGM144 exhibited a higher and more sustained transgene expression than those treated with pTG11033. Repeated administration studies revealed that several injections of lipoplexes could lead to similar transgene expression profiles if an interval of several weeks between subsequent injections was respected. A transient hepatotoxicity and a partial inflammatory response were caused by lipoplex injection, irrespective of the plasmid used.

Altogether, these results indicate that repeated systemic administration of lipophosphoramidate-based lipoplexes in mice conducts to an effective lung transfection without serious side effects, and highlight the need to use long-lasting expressing and well tolerated plasmids in order to efficiently renew transgene expression by the successive doses.

1. Introduction

The use of nanotechnology in the biomedical field has gained interest in recent years as a versatile strategy for selective drug delivery. Nanosizing a formulation improves the drug dissolution leading to enhanced drug absorption and bioavailability [1]. Depending on their physicochemical characteristics and administration route, nanovectors also provide protection and reduced clearance of easily degraded or short half-life molecules such as nucleic acids, thus prolonging pharmacological effects and organ/cell-specific uptake.

However, the enhancement of drug delivery using nanoparticles could be accompanied by side-effects. Therefore, the pharmacokinetic pattern of the original drug and the encapsulated one may differ. Consequently, it is of importance to monitor pharmaceutics and the biodistribution of these formulations in order to document the efficacy and possible side-effects, especially when they have to be readministered.

Among nanoparticles, cationic liposomes are widely used as non-viral vectors for both *in vitro* and *in vivo* applications. Most of them are composed of three basic domains: a hydrophilic positively-charged headgroup, a hydrophilic domain and a spacer, linking both parts. Inspired from cell membrane phospholipids, our group has focused during the past decade on modulating each structural domain to improve their gene delivery efficacy, and particularly in the lungs [2]. Thus lipophosphoramidates, composed of an arsonium headgroup linked *via* a phosphoramidate linker to two aliphatic chains (particularly phytanyl, oleic and linoleic chains) appeared as a promising compromise between a high transfection efficiency and a moderate toxicity in mice [3–5].

Nevertheless, the liposome-mediated *in vivo* gene delivery has also been limited by a transient transgene expression. Gene therapy of chronic lung diseases such as cystic fibrosis (CF) would require a long-term expression of *CFTR* transgene involving repeated administration of gene therapy products, in order to obtain a sustained gene expression in slowly dividing or terminally differentiated cells of the lungs [6]. Since most of viral vectors become ineffective when repeatedly administered because of the development of an immune response against viral proteins [7], synthetic gene transfer agents (GTAs) appear more suitable for this purpose. However, the suitability of lipophosphoramidate formulations and more broadly, of non-PEGylated formulations for repeated administration, notably through the intravenous (IV) route, has not yet been extensively investigated. Beyond the necessity to use a safe and very efficient GTA that overcomes the pathophysiological barriers, two elements of the plasmid DNA (pDNA) have also recently shown their key roles at the level and in the persistence of the gene expression [8].

First, replacing the commonly used cytomegalovirus (CMV) immediate/early promoter by a non-silenced promoter such as the human elongation 1 alpha (hEF1α) promoter, has shown hopeful results in maintaining the transgene expression in the lungs [9]. Second, the removal of all unmethylated CG dinucleotides (CpGs) in the exogenous pDNA sequence, which are detected by the toll-like receptor 9 (TLR-9) in the endosomes and trigger innate immunity, appeared as a requirement for future non-viral gene therapy protocols [10]. Thus, through an extensive nucleotide optimization, the UK CF Gene Therapy Consortium has generated a *CFTR* expression plasmid, completely depleted of all CpG sequences, which is currently evaluated in a clinical trial in humans using GL67

formulation. The *luc* version of this plasmid (pGM144), utilizing the hCEFI promoter (hybrid of CpG-free versions of the human CMV enhancer (hC) and hEF1α promoter sequences), has shown a direct persistent, high-level transgene expression for at least 6 months in the mouse lung after an aerosol delivery of lipidic and polymeric GTAs, without any side-effects [11,12].

Nevertheless, such a plasmid construct had never been evaluated in a repeated intravenous administration procedure. We thus combined BSV4 lipophosphoramidate with pGM144 plasmid or with the common CMV-driven luciferase encoding pDNA (pTG11033; Transgene), and compared their IV transfection profiles in mice. As future treatments of chronic lung diseases will require efficient repeated dose of GTAs, in order to maintain therapeutic levels of the transgene expression, we have explored the kinetics of *luc* expression using both plasmids after several injections, and monitored the hepatotoxicity and inflammation induced.

2. Materials and methods

2.1. Physicochemical characterization of BSV4-based liposomes and lipoplexes

2.1.1. BSV4 cationic lipid and zetasizer measurements

The cationic lipophosphoramidate 4 (BSV4) was synthesized as previously described [3]. Basically, this compound is characterized by a lipid moiety, consisting of two linoleic chains (C18:2 Δ9 Δ12), and by a trimethylarsonium cationic headgroup linked to the lipophosphoramidate moiety by an ethylene spacer (Figure 1). The diunsaturated lipophosphoramidate BSV4 is slightly more fluid but less fusogenic than its monounsaturated analogue (KLN47) [3], and was mainly structured as a lamellar mesophase in a liposomal solution [4].

To prepare BSV4/DNA complexes, plasmids were first diluted in 0.9 % NaCl and 20 mM HEPES for Zetasizer measurements and *in vivo* transfection assays before being added to the liposomal solution. These mixtures were kept at room temperature for at least 30 min before use, in order to allow the formation of DNA complexes. As previously described, lipoplexes characterized by a single theoretical charge ratio (CR) of 4 were prepared, permitting the total condensation of pDNA [3]. CR is defined as the ratio of the vector positive charge, carried by the arsonium headgroup, to the negative DNA phosphate charges.

The mean particle diameter and zeta potential (ξ) of the liposomes and lipoplexes were measured using a 3000 Zetasizer (Malvern Instruments) at 25°C after an appropriate dilution of the formulations. Briefly, for measurements with lipoplexes, each assay used 50 µg of plasmid DNA mixed in 0.9 % NaCl and 20 mM HEPES with the required quantity of BSV4 liposomes in order to form lipoplexes with CR 4. For the liposome measurements, we used a lipid quantity equivalent to a CR 4 mixture in 20 mM HEPES, 0.9 % NaCl. Similar measurements with BSV4-based lipoplexes were already performed in water [3].

2.2. Luciferase-encoding plasmids

For *in vivo* transfection studies, two different firefly luciferase encoding plasmids were used: pTG11033 (9.6 kb) and pGM144 (3.7 kb; also called pG4-hCEFI-soLux) [11] kindly provided from Transgene (France) and the UK Cystic Fibrosis Gene Therapy Consortium respectively. Plasmid pTG11033 carries the luciferase gene expression cassette starting with the cytomegalovirus immediate early 1 (CMV-IE) enhancer/promoter region followed by the intron 1 of HMGCoAR gene for enhanced transgene protein synthesis *via* an increased mRNA export from the nucleus, the firefly luciferase gene and the simian virus 40 (SV40) poly(A) signal. Plasmid pGM144 [11,12] is entirely CpG-free and bears a codon-optimized form of luciferase cDNA, the hybrid promoter hCEFI [11], (CpG-free versions of the human elongation factor 1 alpha promoter and the human CMV enhancer), and a CpG-free form of R6K origin of replication that supports pDNA replication in *E. coli* hosts containing R6K *pir* gene product. The CpG-free construct was designed to avoid lung inflammation after the intra-pulmonary delivery of lipoplexes, but it also appears to mediate a sustained transgene expression in the airways [11,12]. Plasmids pTG11033 and pGM144 were amplified in *Escherichia coli*

DH5α and GT115 strains respectively, and purified using the Qiagen Giga Prep Plasmid Purification protocol (Qiagen, Germany). GT115 strain contains a mutant *pir* gene encoding the π protein, which allows a positive selection of plasmids containing R6K gamma origin of replication. Plasmid purities were checked by electrophoresis on 1.0 % agarose gel. DNA concentrations were spectroscopically estimated by measuring the absorption at 260 nm and confirmed by gel electrophoresis. Only plasmids with an A_{260}/A_{280} ratio between 1.8 and 2 were used.

2.3. In vivo transfection assays

2.3. 1. Animals and administrations

Six to nine weeks old female Swiss mice (Janvier breeding center, Le Genest Saint Isle, France) were housed and maintained at the University animal facility; they were processed in accordance with the Laboratory Animal Care Guidelines (NIH publication #85–23 revised 1985) and with the agreement of the regional veterinary services (authorization FR; 29–024). BSV4-based lipoplexes were prepared at room temperature, in 0.9% NaCl and 20 mM HEPES. The mice were held under a beaker, and 300 µL of complexes incorporating 50 µg of pDNA were injected into the tail vein to each mouse within 5 to 10 sec., using a ½ inch 26-gauge needle and a 1 mL syringe. A total of 8 mice received BSV4/pGM144 lipoplexes, and 8 mice BSV4/pTG11033 lipoplexes, both characterized by a CR of 4 (required for completely condensing pDNA) [3]. Additionally, 4 mice received 50 µg of naked plasmid DNA (pTG11033), and 4 mice received BSV4-based liposomes (quantity equivalent to a CR of 4). Repeated administration experiments were performed using carefully the same protocol conditions.

2.3. 2. In vivo bioluminescence: non-invasive imaging of luciferase activity

Mice to be imaged first received an intraperitoneal injection of D-luciferin (4 mg in 200 µL HEPES buffer (20 mM); Interchim, France). Three minutes later, the animals were anesthetized with a 4 % air-isofluran blend. Once laid in the acquisition chamber, the mice were maintained anesthetized with a 2 % air-isofluran mixture at room temperature all along the experiment.

Five minutes after the luciferin injection, luminescence images were acquired using an *in vivo* imaging system (NightOWL NC320, Berthold) and associated software (WinLight 32 Berthold) with a binning of 8 x 8 and an exposure time of 4 min. Luminescence images were then superimposed onto still images of each mouse. Signal intensities were quantified within the regions of interest as photons/sec units. Several luminescent acquisitions were taken one after the other without readministration of luciferin, in order to get (1) a ventral view, and (2) lateral views of the luminescent signals. It allows to more easily follow the location and level of the transgene expression over time.

2.3. 3. Luciferase activity in tissue homogenates and plasma samples

Two days after administration, 3 mice from each group were sacrificed by cervical dislocation and their multiple organs and tissues (lungs, liver, spleen, heart, leg muscles, abdominal fat, salivary glands, kidneys, and brain) were removed for analysis. The luciferase expression was evaluated as previously described [4]. Briefly, tissue pieces were washed in 1X PBS and disrupted using gentleMACS™ Dissociator (Miltenyi Biotec, Germany) in 1X Passive Lysis Buffer (PLB; Promega). The supernatant

was quantified by a luminometer (MLX® Microtiter Plate Luminometer-Dynex, Guyancourt, France), measuring the light emission over a 15-s reaction period. The total protein content of each supernatant was quantified using the BC assay kit (Interchim, France). Results were expressed as total relative light units per mg of total proteins (Total RLU/mg of tot. prot.). In parallel, blood samples (≈100 µL) were collected from the saphenous vein of the remaining treated mice on a heparin tube before and at regular time points after administration of the different formulations. Tubes were then centrifuged at 10,000 g for 2 min at +4°C and 25 µL of blood plasma were harvested to measure the luciferase activity. Results were expressed as total relative light units per mL of plasma (RLU/mL).

2.4. In vivo hepatotoxicity

Using the same blood collection protocol, plasma samples of the treated mice were also harvested to measure ALT (alanine aminotransferase) and AST (aspartate aminotransferase) levels. The transaminase activities were quantified using a commercial kit (Elitech, France) according to the manufacturer's instructions. This method uses the absorbance kinetics at 340 nm reflecting the oxidation of NADH into NAD operating when the transaminases are in contact with their respective substrates**.** The results were expressed as International Units per Liter (IU/L), and reflected the potential injuries caused by the various formulations, notably of the liver. Adapted for small blood volumes, this method allowed performing the kinetics of transaminase activities on each animal, limiting the difficulties due to inter-individual variations.

2.5. Quantification of the inflammatory and immune responses

The inflammatory and immunogenic responses to transfection with BSV4/pTG11033 and BSV4/pGM144 were evaluated using the Proteome Profiler kit (R&D Systems, USA) according to the manufacturer's instructions. The kit permits quantification of the relative levels of 40 cytokines/chemokines (BLC, C5a, G-CSF, GM-CSF, I-309, eotaxin, sICAM-1, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-13, IL-12 p70, IL-16, IL-17, IL-3, IL-27, IP-10, I-TAC, KC, M-CSF, JE, MCP-5, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, SDF-1, TARC, TIMP-1, TNF-α and TREM-1) in the plasma of the treated mice. The plasma samples were first diluted and mixed with a cocktail of biotinylated detection antibodies. The sample/antibody mixture was then incubated with the Mouse Cytokine Array Panel A to bind any cytokine/detection antibody complex present by its cognate immobilized capture antibody on the nitrocellulose membrane. Following a wash to remove unbound material, Streptavidin-Horseradish Peroxidase and chemiluminescent detection reagents were sequentially added. Light produced at each spot, and proportional to the amount of cytokine bound, was finally quantified by densitometry (BIO-1D, Vilber Lourmat) and compared with the results obtained from naive mice (results were expressed as the Mean Signal Intensity of the cytokines, representing the average of the duplicate spot signals in relation to the positive control intensities of their respective membrane).

3. Results

3.1. Physicochemical studies of the liposomes and lipoplexes

The liposomal and lipoplex solutions were characterized by dynamic light scattering (DLS – size analyzer) and zeta potential determination.

First, we observed that BSV4-based liposomes alone (*i.e.* without pDNA) were characterized by a mean diameter of approximately 105 nm and a positive charge (zeta potential, ξ) of +66 mV (Table 1**Error! Reference source not found.**). These data are consistent with the previous measurements [3].

As indicated in Table 1**Error! Reference source not found.**, BSV4-based lipoplexes were characterized by higher mean diameters (>800 nm) than the liposomes, whereas their zeta potentials were of the same order of magnitude (with pGM144) or higher (with pTG11033). By adding nucleic acids into cationic liposomes, zeta potential normally decreases due to the negatively charged phosphate groups in pDNA. In our experiments, BSV4-based lipoplexes exhibited higher (pTG11033; +91.7 mV) or slightly lower (pGM144; +64.6 mV) zeta potentials than liposomes. It is likely that the zwitterionic HEPES buffer and sodium chloride interfere in the measurements, producing rough zeta potential values. Of note, at low ionic strength, the sizes of the BSV4-based lipoplexes are comprised between 175 and 200 nm, with a polydispersity index below 0.4, and a zeta potential of approximately +50 mV [3].

3.2. In vivo transfection experiments

3.2.1. Bioluminescence imaging and luciferase activity in organ/tissue homogenates and in plasma samples

As indicated in Materials and Methods, BSV4-based lipoplexes carrying the luciferase-expressing plasmid pGM144 or pTG11033 were administered to Swiss mice *via* a tail vein injection. A transgene expression was first detected, localized and quantified by bioluminescence imaging (BLI) *in vivo* (values expressed as photons/sec).

As shown in Figure 2A, whatever the plasmid used, a significant luciferase activity was observed 24 h following the injection of BSV4-based lipoplexes. However, whereas the bioluminescent expression issuing from pTG11033 was only detectable into the lung area (Figure 2 A2), mice treated with BSV4/pGM144 also exhibited other bioluminescent signals. Indeed, although the lung was the organ with the highest expression of luciferase, *luc* expression was also observed in several other areas including the abdomen and spleen areas (Figure 2 A1).

To further assess and localize the *in vivo* luciferase activities of both plasmids, some mice were sacrificed 24 h following lipoplex administration, and luciferase activity was measured in isolated organ and tissue homogenates (data expressed as RLU/mg of total proteins).

The measurements first confirmed BLI data showing that, whatever the plasmid used, the lung exhibited the highest luciferase activity among the various organs (Figure 2 B1). Moreover, *luc* activity in the lungs was higher in mice treated with pGM144 ($8.6x10^4 \pm 5.6\ x10^4$ RLU/mg of tot. prot.) than with pTG110033 plasmid ($3.1x10^3 \pm 2.9x10^3$ RLU/mg of tot. prot.), an observation at least partially explained by higher pGM144 copies delivered for a same mass of DNA (50 µg of pDNA contain $1.2x10^{13}$ copies of pGM144 and $4.7\ x10^{12}$ copies of pTG11033).

However, whereas a small luciferase activity was also found in the heart and spleen of both groups of treated mice (with higher values in BSV4/pGM144 group), the luciferase was also curiously detected in the leg muscles, the abdominal fat, and residually in the liver, the salivary glands and the kidneys of BSV4/pGM144 treated mice.

Due to the diffuse BLI signals and to the presence of luciferase in the abdominal fat after BSV4/pGM144 injection, we wondered if the luciferase could be present in the bloodstream. Thus, we quantified the luciferase in the plasma of the treated mice. As shown in Figure 2 B2, a significant luciferase activity was found in the plasma of both groups of treated mice 24 h post-injection, with higher levels in BSV4/pGM144 group. The luciferase substantially decreased in the plasma of BSV4/pGM144 treated mice at day 2, a small activity remaining detectable at day 5. No luciferase was detected in the plasma of BSV4/pTG11033 treated mice by day 2 post-administration. These data confirmed that the luciferase was circulating in the bloodstream, probably due to its release from the transfected cells.

3.2.2. Bioluminescence kinetics and repeatability of BSV4/pDNA systemic delivery

As previously described, transgene expression was detected, localized and quantified by BLI *in vivo* up to the end of the bioluminescence detection. The suitability of BSV4-based lipoplexes for repeated intravenous administration was also explored using both types of luciferase encoding plasmids.

As expected, the detectable expression of pTG11033 in the lungs of mice was transient, lasting approximately 3 days, with a peak of *luc* expression at 24 h ($2.7x10^5 \pm 9.0x10^4$ ph/sec) (Figure 3). PGM144-containing lipoplexes reached a peak of the transgene expression at 48 h ($7.9x10^6 \pm 7.7x10^5$ ph/sec), approximately thirty-fold higher than that observed for pTG11033 expression at 24 h. Thereafter, the levels decreased by one magnitude (from day 2 to day 3) and remained steady for up to 10 days. From day 16 to day 24, pGM144 *luc* expression decreased slowly towards background level, the last signals being always detected in the lung area (Figure 3B).

We then explored the feasibility of *luc* re-expression after a second dose of lipoplex injection. Since *luc* activity often remains in the lungs of the treated mice while BLI signal has just decreased under background level, we empirically decided to wait for 1 month after the end of pGM144 expression detection before the subsequent treatment.

It appeared that both lipoplexes were able to mediate a second luciferase expression in the lungs. The second pTG11033 expression was nevertheless slightly lower ($3.7x10^4 \pm 1.9x10^4$ ph/sec) than the first one ($2.7x10^5 \pm 9.0x10^4$ ph/sec) and lasted 2 days only (Figure 3A). In contrast, pGM144 mediated a second expression similar to the first one, the decrease between the peak and the plateau going however slower (6 days, between days 58 and 64) than after the first injection (1 day, between days 2 and 3) (Figure 3A). The time course of the second transgene expression was also longer as the signal disappeared approximately 30 to 40 days after the second injection. However, it is noteworthy that the chart represents a mean BLI intensity, and most of the treated mice had BLI kinetics similar to those obtained after the first injection. Differences between the bioluminescence signals of mice receiving the same treatment can be explained by some variability in the *in vivo* experimental conditions (*e.g.* a

progressive adiposity leading to photon absorption as well as a variety of other physiological parameters).

We then investigated the feasibility of re-treating the same mice quickly after extinction of the BLI signal, while *luc* activity in the lungs was presumed to remain. Thus, a third injection of BSV4/pGM144 lipoplexes was performed 10 days after extinction of the second BLI signal, leading to the same lung transfection efficiency as well as the same kinetics of transgene expression (Figure 3A; data not entirely shown). The third injection of BSV4/pTG11033 was not performed because two mice died of old age.

Next, we assessed the possibility of maintaining the *luc* expression after repeated lipoplex injection at several short time intervals. For that purpose, we reduced the injected lipoplex quantity (30 µg of pDNA, CR4) in order to get a significant *luc* signal without reaching any saturation of the bioluminescence values. We first observed that a second close administration of BSV4/pTG11033 lipoplexes (1 week after the first injection) did not lead to any second bioluminescent signal (data not shown). In the same way, the second administration of pGM144-containing lipoplexes did not increase the lung signal stemming from the first injection (Figure 4A). We injected the third dose at day 10, while *luc* expression in the lungs was steady. Interestingly, the transgene expression in the lungs remained unchanged whereas BLI signal appeared in the spleen, lasting approximately 3 days (days 11 to 13; Figure 4B). The fourth injection occurring 8 days after the second one (Day 20; Figure 4A) led to the same observation, *i.e.* a small and short *luc* expression in the spleen without any variation at the level of the transgene expression in the lungs (Figure 4B). Indeed, the bioluminescent signal in the lungs decreased under background level by day 25, similar to the kinetics observed after a single lipoplex delivery. An additional lipoplex administration at day 26 (shortly after the end of detectable *luc* expression) did not restore any transgene expression.

3-3. *In vivo* hepatotoxicity

As already stated above, with a view to clinical gene therapy, we also investigated the eventual *in vivo* side effects of both types of lipoplexes following repeated administration. As we had previously reported some liver injuries after a single systemic administration procedure, we decided to evaluate the liver reaction induced by either BSV4-based liposomes alone (“BSV4 alone”), BSV4/pGM144 and BSV4/pTG11033 lipoplexes by measuring the levels of liver enzymes AST and ALT in the plasma of the three groups of treated mice over time.

Whereas no variation in the transaminase levels was observed when administrating the liposomes alone (as previously reported) [4], systemic administration of BSV4/pDNA lipoplexes resulted in a fast but transient increase in the transaminase activities, reaching a peak at 24 h post-administration, and recovering baseline values at 72 h (Figure 5). Thus, systemic administration of BSV4 lipoplexes resulted in an early, transient liver toxicity in mice. However, there was no statistical difference in the level of toxicity induced by either pTG11033-containing lipoplexes or pGM144-containing lipoplexes, indicating that plasmid sequence as well as luciferase protein protein did not contribute directly to liver toxicity.

BSV4/pGM144 second and third administrations led each time to an increase in the transaminase activities which lasted approximately 3 days. As illustrated by the standard deviations in Figure 5, there were large inter-individual variations in the enzymatic measurements. Consequently, although an increase in the intensity of the liver enzyme activities seems to occur after each administration, there is no statistical evidence of such a correlation.

3-4. *In vivo* inflammation and cytokine profile

To further understand the physiological responses to transfection with both pDNA contructs after several intravenous administrations of lipoplexes, we used the “Mouse Cytokine Antibody Array Panel A” kit (R&D Systems) to quantify numerous inflammatory and immune cytokines in the plasmas of the treated mice.

We first compared the cytokine profiles at day 1 (Figure 6 A1) and day 3 (Supplementary information; Figure S1) after the first injection of lipoplex containing either the CpG-containing plasmid pTG11033 or the CpG-free plasmid pGM144, and at day 1 after the second administration of lipoplexes (Figure 6 A3). We did not screen the inflammation induced by the liposomes and pDNA separately since our previous results showed that naked pTG11033 was not inflammatory, and that the injection of lipophosphoramidates without pDNA only caused a moderate and very short-term inflammation [4]. Whereas no markers of the immunogenic response were detected in any conditions and at any time in the present experimental conditions, intravenous administration of both types of pDNA-containing lipoplexes led to a sharp and quite similar increase in several pro-inflammatory cytokines levels such as G-CSF, IP-10, I-TAC, JE, MCP-5, MIG, TIMP-1, and also to a slight increase in KC, M-CSF, and IL1-ra levels (D1; Figure 6 A1). Whatever the plasmid used, the inflammatory response was highly transient as most of the cytokines went back to their baseline levels at day 3 (Supplementary information; Figure S1). Similar cytokine profiles were obtained after the second lipoplex injection, confirming that no adaptative response occurred (Figure 6 A2).

Of note, C5a, which is a strong chemoattractant involved in the recruitment of inflammatory cells, was however systematically downregulated by day 1 (Figure 6 A1, A2). Although C5a went back to normal values at day 3 with pTG11033, it remained at low levels with pGM144 and may thus be related to the level of transgene expression.

In contrast, no significant increase in IL-1, IL-2, IL-6, TNF-α or IFN-γ levels was observed at any time and in any conditions. Nevertheless, it is likely that some cytokines had a brief peak of expression in the next hours following the injection, and that our protocol design was not adapted to detect an early inflammatory response. Interestingly, I-TAC expression was only induced by BSV4/pTG11033 administrations. Nevertheless, IP-10 and MIG, which bind the same receptor as I-TAC (CXCR3) were up-regulated with both treatments, indicating that the high level of I-TAC expression might have no major impact on the inflammation process.

In order to investigate their implication in the absence of cumulative transfection, we then followed the cytokine profiles in mice treated with lower doses of BSV4/pGM144 (30µg of pDNA; CR4) at short intervals.

We first observed that the cytokine production decreased by delivering lower quantities of lipoplexes (Figure 6B). Whereas M-CSF displayed the same level of stimulation after each injection, the level of

the inflammatory markers was slightly higher after the first administration than after the subsequent ones, the cytokines recovering their baseline values prior to each next injection (D8(3), Figure 6B). These data indicate that the inflammatory response following lipoplex injection is dose-dependent and is related to an effective gene transfer.

4. Discussion

The development of non-viral vectors for gene therapy is a fast growing field of research. Among their properties, they have been shown to induce less immune response than viral vectors, which is a crucial point when repeated administration is required, as for CF treatment. Since one of our recent work showed that cationic lipophosphoramidates were able to mediate the transgene expression in alveolar epithelial cells of the lungs after IV delivery [4], we decided to evaluate their transfection potential in combination with an optimized reporter plasmid (pGM144). This plasmid contains the same backbone and regulatory elements as the CFTR-encoding plasmid pGM169 used in an ongoing non-viral gene therapy clinical trial of CF [13]. The CMV-driven *luc* plasmid pTG11033 was used as a control. We also intended to test whether or not the lipoplexes could be safely and efficiently readministered using a systemic delivery procedure.

As shown in previous studies, the systemic delivery of the luciferase reporter plasmid pTG11033 complexed to BSV4 lipophosphoramidate mediated a high but only short-term lung transgene expression (up to 3-4 days) [3,4]. Many factors were discussed as being responsible for the lack of sustained transgene expression, but this phenomenon appeared to be directly related to the attenuation of CMV promoter activity [14–16], and more precisely to the silencing of CMV promoter [17–20] due to partial methylation of CpG dinucleotides [21–23]. Using the codon-optimized and CpG-free luciferase encoding plasmid (pGM144) under the same conditions as pTG11033, the luciferase was detected by BLI for more than 20 days (Figure 3). The human elongation factor-1 alpha promoter (EFIα) driving *luc* expression has already been extensively described in several *in vivo* studies as a non-silenced promoter [11,12,19,24,25], but it was the first time that such CpG-free plasmid was evaluated with a cationic lipophosphoramidate in a repeated systemic administration procedure. Of note, under similar experimental conditions, the slightly less efficient monounsaturated lipophosphoramidate derivative KLN-47 gave rise to approximately 13 days of transgene expression in lungs after a single intravenous injection [26].

In agreement with the observations reported in early studies relative to the *in vivo* systemic administration of lipoplexes [27,28], our own previous fluorescence biodistribution experiments showed that BSV4-based liposomes and lipoplexes displayed a preferential tropism for the lungs of mice after IV delivery [3]. In addition to the anatomical parameter, the underlying lung transfection mechanism may involve interactions between the cationic lipoplexes and the negatively charged serum components leading to a lipoplex aggregation [29]. Given that the entire cardiac output passes through the pulmonary circulation, which is a rich network of capillary segments as small as a few micrometers, the lipoplex aggregates are likely to accumulate in the lung microcirculation during their first passage [30]. Both types of lipoplexes (BSV4/pTG11033 and BSV4/pGM144) exhibited equivalent physicochemical characteristics and may thus have a similar behavior once in the blood circulation.

Nevertheless, the lipoplex aggregates also displayed heterogeneous sizes, probably including some particles small enough to escape the lung microcirculation and passively target other organs such as the heart and the spleen where the luciferase was also detected (Figure 2B). Their capture by phagocytotic cells of the mononuclear phagocyte system (MPS) may also be related to the luciferase activity in the spleen, although there is often no direct connection between biodistribution and transgene expression [31,32].

If the luciferase activity was systematically higher in organs of mice treated with pGM144 than those treated with pTG11033 plasmid, it is at least in part explained by the differences in plasmid size. Indeed, for an equivalent injected mass of both plasmids, almost three times as many copies of pGM144 were delivered to mice. Although this difference makes very difficult to compare the qualities of the two plasmids in terms of expression levels, it does not impact the duration of transgene expression which is mostly related to the plasmid sequence [9].

Probably due to the high transfection rates and/or high luciferase production rates by the transfected cells, the luciferase was excreted and remained also detectable in the plasma of mice, the activities being higher and more sustained when using pGM144. Thus, the luciferase detection in organs other than the lungs may also come from the protein release and its accumulation in local niches. It would consequently be necessary to establish a clear correlation between the plasmid content and the expression activity in positive tissues. Such a luciferase release raises important issues since an adaptive immune response against the transgene product has already been observed by others [25,33]. More precisely, high levels of transgene expression within the first weeks after transfection can result in clearance of the transgene expressing cells [25,34]. In our case, since the second and third administrations of BSV4/pGM144 lipoplexes gave rise to similar reporter gene expression profiles than after the first injection, it is likely that such a phenomenon does not occur. It would nonetheless be interesting to perform the same experiments with immunodeficient mice lacking functional B- and T-cells, or using an immune suppression agent. However, the second pTG11033 *luc* expression in the lungs appeared to be slightly lower than the first one, and rapidly silenced. Because both pDNA preparations were purified using the same plasmid purification protocol, differences in impurity levels (*e.g.* residual bacterial genomic DNA) between pDNA productions could be ruled out for a silencing phenomenon. It would thus be interesting to check if CpG content and/or CMV promoter are involved in this process.

The long-term expression of pGM144 plasmid in the lungs was in agreement with the previous reports obtained after nebulization of lipidic and polymeric formulations [11,12,35]. Nonetheless, the period of transgene expression was longer after nebulization, lasting more than 4 months [35]. Beyond the less sensitivity of BLI technique compared to quantitative PCR and quantitative reverse transcriptase PCR, many hypotheses can be suggested to explain the *luc* expression difference in term of expression. The route of administration can lead to the transfection of different cell types. Indeed, the nebulization of non-viral gene delivery systems often led to the transfection of epithelial cells such as ciliated epithelial cells [36,37] or alveolar type 1 pneumocytes [37,38] which are both slowly dividing or terminally differentiated [6]. Although we have shown that IV injection of lipoplexes could also conduct to a gene transfer in type 1 and type 2 pneumocytes [4], the systemic delivery procedure often leads to

a transgene expression in various cell populations including endothelial cells [39,40], macrophages and others that may have higher rates of turnover.

Moreover, albeit pGM144 is a CpG-free plasmid, CpG islands in residual bacterial genomic DNA can remain in pDNA preparations as minor contaminants, induce inflammation and reduce reporter gene expression [41,42].

Several groups including ours have already observed a transient hepatic toxicity after a systemic delivery of lipoplexes [4,26,43–46], the liver being the second most irrigated organ after the lungs. We thus measured the liver enzyme levels (ALT, AST) in the plasma of mice from the two treatment groups. We observed that each subsequent lipoplex injection caused transient liver injuries (2-3 days), irrespective of the plasmid used (Figure 5). These data verified that the phenomenon was neither due to the transgene expression nor to CpG content. Of note, the correlation between the transaminase activities and histopathological changes has been previously confirmed [4].

Many genetic diseases such as CF are likely to require a long-term expression of the therapeutic gene, involving repeated administration of gene therapy formulations. As the lungs of mice can be efficiently transfected by a second dose of BSV4-based lipoplexes, we then assessed whether the transgene expression level could be maintained or scaled up by close subsequent reduced doses of pGM144-containing lipoplexes (intervals between 2 and 10 days), while a luciferase activity was still detectable in the lungs.

We observed here that a second lipoplex injection (containing 30 µg of pGM144) 2 days after the first treatment did not scale up the bioluminescence signal. Repeated injections between 8 to 10 days apart did not make any variation in the transgene expression level, while usual clinical signs of toxicity such as short piloerection were observed in the few days following the injection. Similar to a single administration, the luciferase activity finally dropped under background between 3 and 4 weeks. These data demonstrated that a refractory period between two successful transfections exists. To our knowledge, only one study has shown a stable transgene expression in the lungs of mice (without any increase) following repeated IV injection of non-viral GTAs (small amounts of pCMV-luc plasmids complexed to protein/PEI) [47]. Our results are consistent with most of the other reports, pointing out a refractory period of about 1 to 2 weeks, during which the repeated intravenous lipofection was ineffective [39,48]. The duration of unresponsiveness was closely related to the dose of DNA as it was shortened by decreasing the dose of pDNA [48], and was mainly attributed to the immunostimulatory effects (*e.g.* production of TNF-α, IFN-γ or IL-1β cytokines) of unmethylated CpG sequences [42,48]. It is noteworthy that the use of CpG-free plasmid DNA does not completely abolish the transient inflammatory response induced lipoplex delivery in sheep [49] as well as in the airways of patients [13], contrary to mice [11]. In our experimental conditions, no strong difference between the immunostimulatory activity of CpG-rich and CpG-free lipoplexes was noticed. As the levels of inflammatory markers correlated with the transfection level (the markers were systematically upregulated after an injection leading to transfection), the passage of lipoplexes through the endothelium is likely to be the major cause of this phenomenon. Nevertheless, the hepatic and splenic impairments probably also contribute to the cytokine production [50]. Consequently, monitoring the hepatotoxicity induced by closely repeated lipoplex injections and quantifying the inflammatory

markers directly into organs of the MPS (*i.e.* lungs, liver and spleen) that may capture the lipoplexes that was not internalized (and before their elimination *via* the urinary tract) can help us to understand the origin of inflammation. Such a refractory period was also observed using another route of administration since the second intranasal delivery of PEI 22 kDa complexes, 7 days after the initial treatment, only gave around 20% of the initial gene expression level, and this event was not caused by any antibody production against the reporter gene product [51].Using PEI 25 kDa, Dames *et al* pointed out that a repeated aerosol application of polyplexes, in a 3-day time interval, could maintain the gene expression at high levels compared with a single application. Nevertheless, the third dose (which was administered 6 days after the first one) did not totally restore the initial gene expression level [52]. More recently, Davies and colleagues pointed out the time-dependent refractoriness period to a second efficient gene transfer to the lungs using pGM144 plasmid [12].
Although different routes of administration were used, the similarities between the transgene expression profiles after multiple close administrations suggest that common cellular mechanisms are probably involved. It is likely that the transfected cells are occupied by the previous dose and became resistant, at least in part, to a second treatment. The systemic transfection procedure, where the lipoplexes have to cross the endothelium and the basal layer, may also lead to a healing phenomenon, leading to the accumulation of fibroblasts which may act as an additive barrier to gene transfer agents.

In summary, several major conclusions can be drawn from the current study. First, the regulatory elements of pGM144 plasmid (*e.g.* EFIα promoter and hCMV enhancer) appear very promising for multiple intravenous transfections, as they give rise to a long-term transgene expression in the lungs, permitting re-attainment of an equivalent gene expression level once it has decreased to background. This is all the more important since multiple close lipoplex injections do not maintain or scale-up the transgene expression level. Inflammatory and hepatoxicity events were systematically highly transient and appeared to be more related to an effective transfection than to CG dinucleotides or transgene expression kinetics. All these results strongly support the further development of non-viral formulations, as well as tools and methods to follow the physiological response to transfection, particularly after closely repeated injections. Such elements would considerably help to find suitable conditions for a sustained transgene expression in the target cells, in order to avoid having to resort to integrating DNA vectors.

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Table 1

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Table 1

Liposomes	Size (nm)	Polydispersity index	Zeta potential (mV)
BSV4	105.5	0.233	+67
Lipoplexes CR = 4	**Size (nm)**	**Polydispersity index**	**Zeta potential (mV)**
BSV4/pTG11033	> 800	-	+91.7
BSV4/pGM144	> 800	-	+64.6

Figure 1

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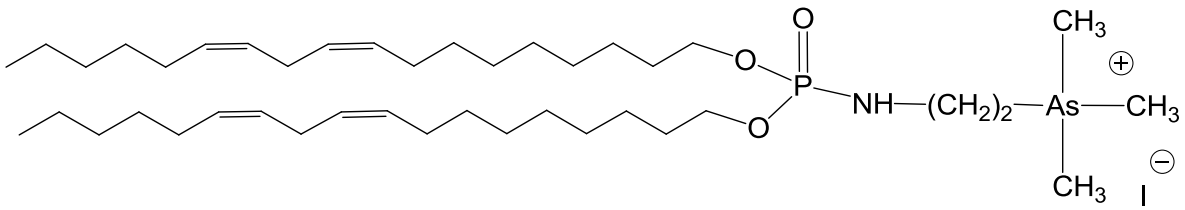

Figure 1

Figure 2

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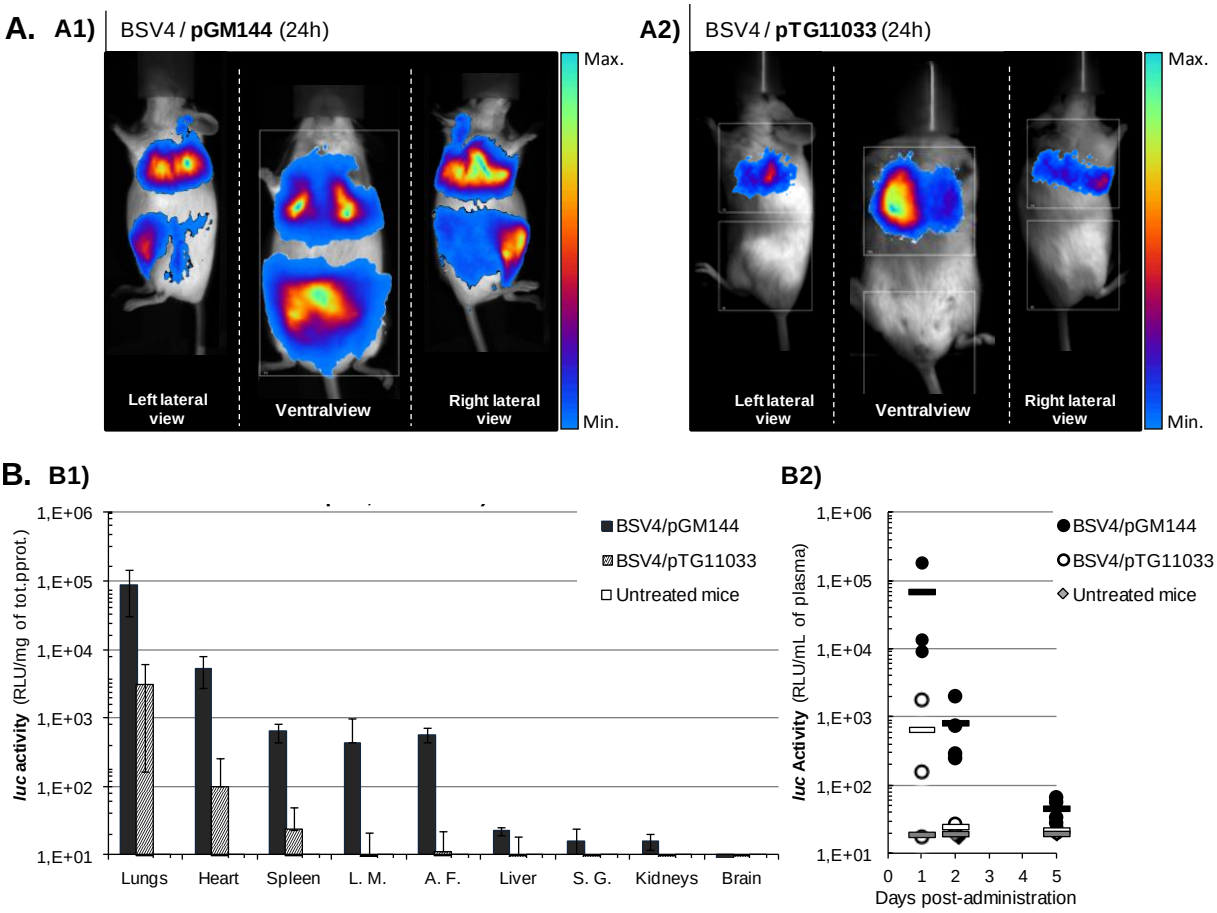

Figure 2

Figure 3

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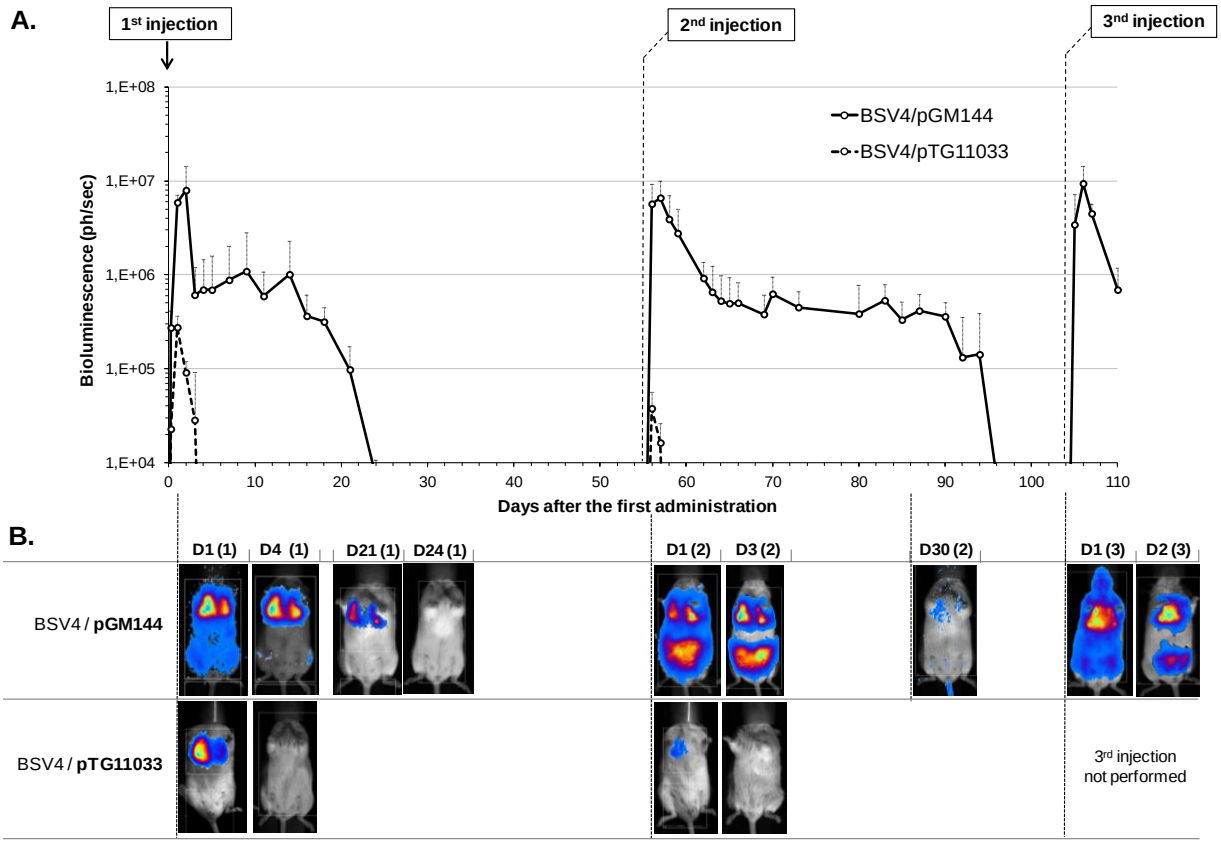

Figure 3

Figure 4

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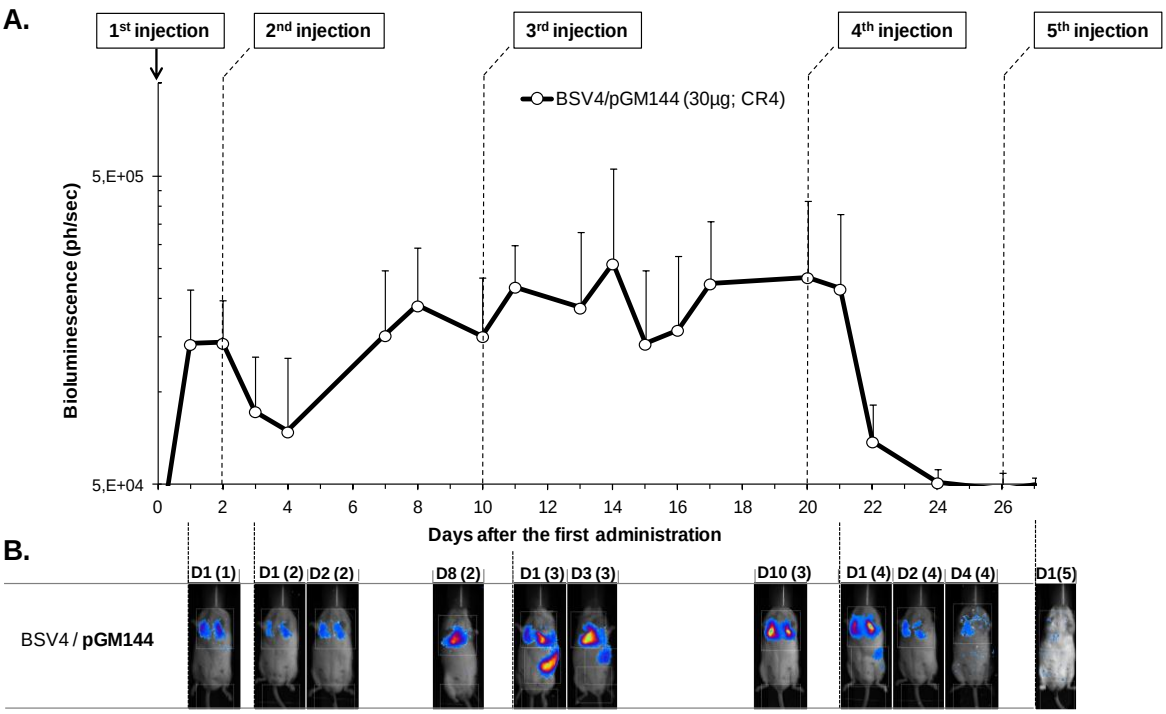

Figure 4

Figure 5

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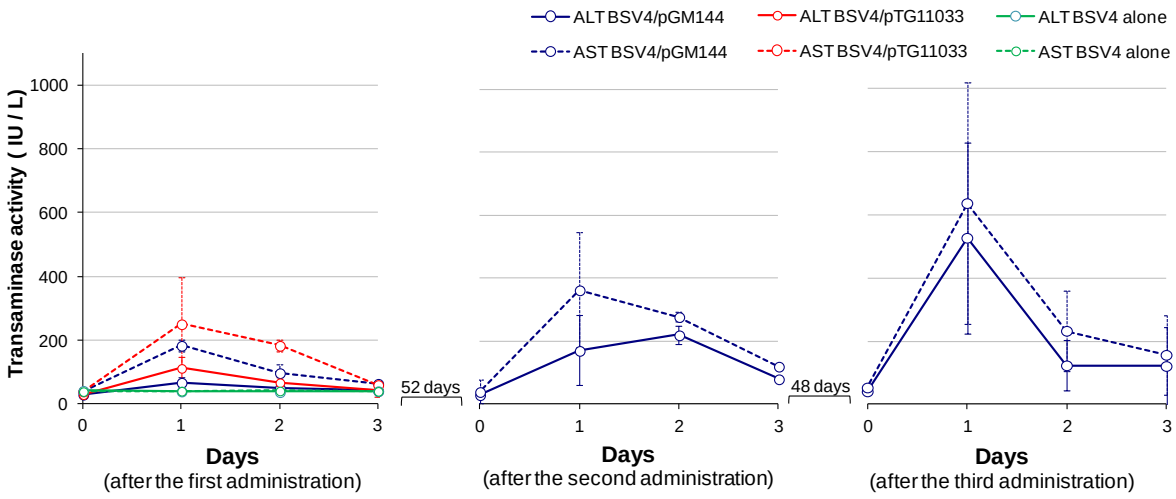

Figure 5

Figure 6

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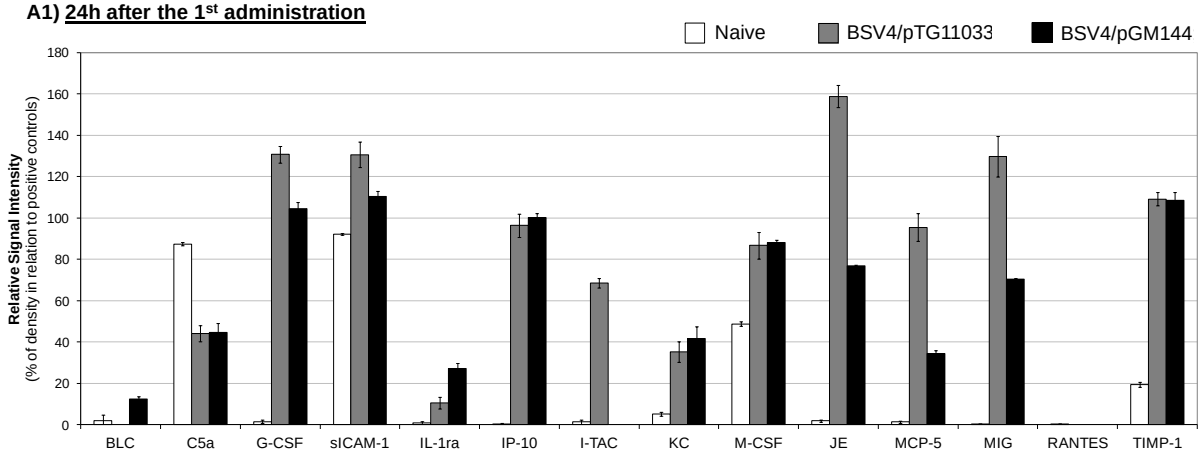
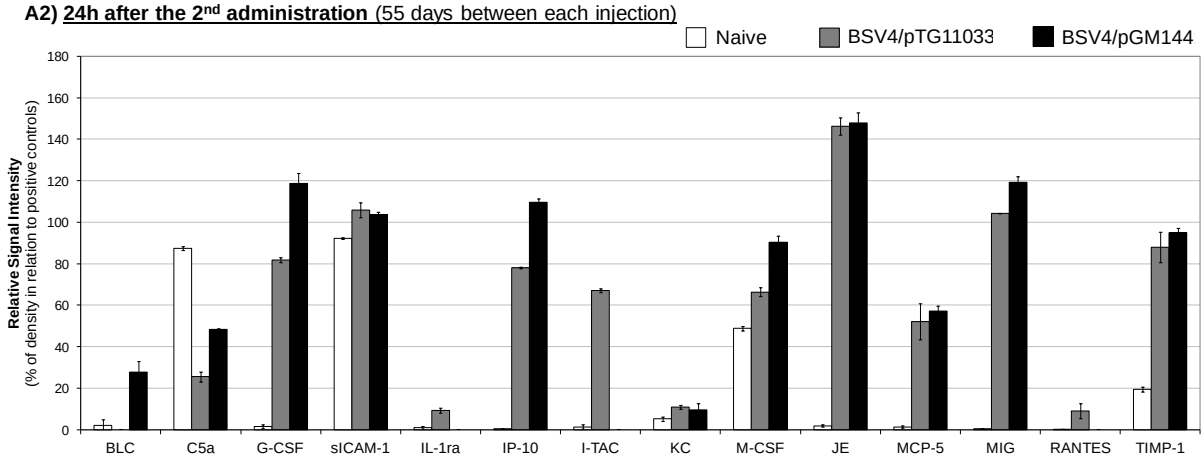
A. Repeated administrations of BSV4/pTG11033 and BSV4/pGM144 (CR4, 50µg of pDNA)

A1) 24h after the 1st administration



A2) 24h after the 2nd administration (55 days between each injection)



B. Multiple close BSV4/pGM144 administrations (CR4, 30µg of pGM144)

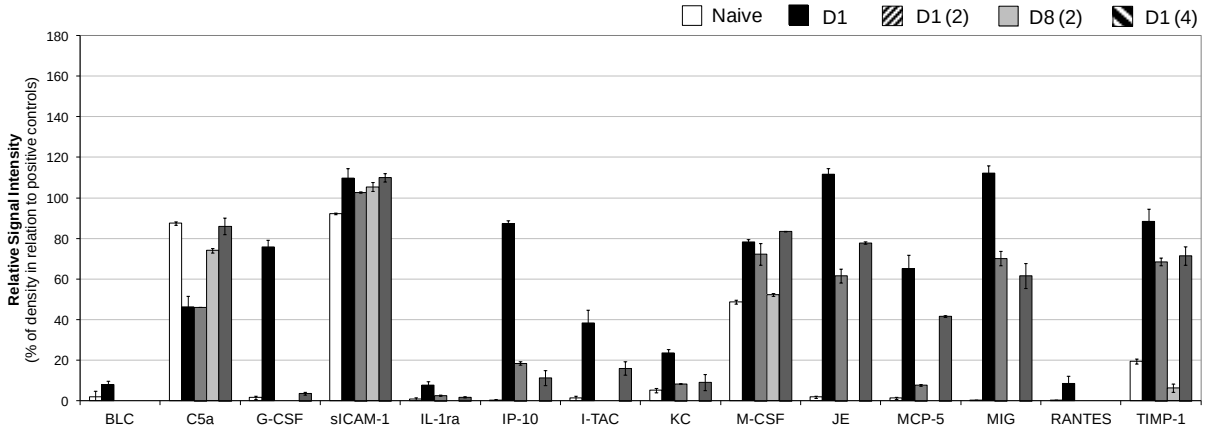


Figure 6

Captions

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Table 1 : Zeta potential and size of BSV4-based liposomes and lipoplexes (Charge Ratio (CR) = 4) formulated in 0.9 % NaCl and 20 mM HEPES.

Figure 1: Chemical structure of lipid 4 (BSV4), a cationic lipophosphoramidate with diunsaturated linoleic chains.

Figure 2: *In vivo* localization of *luc* (A) expression 24 h after intravenous delivery of BSV4/pGM144 (A1) and BSV4/pTG11033 (A2) evaluated by bioluminescence imaging. Luminescence images are superimposed onto still images of each mouse. Luciferase activity measurements (B) on multiple organ and tissue extracts from BSV4/pGM144 (n=3) and BSV4/pTG11033 (n=3) treated mice two days after administration (B1) ("L. M.": Leg Muscle; "A. F.": Abdominal Fat; "S. G.": Salivary Glands), and on plasma of treated mice at three time points (B2).

Figure 3: *In vivo* bioluminescence kinetics of luc expression after three intravenous deliveries of BSV4/pGM144 (50 µg of pGM144, CR4; n=5) and two deliveries of BSV4/pTG11033 (50 µg of pGM11033, CR 4; n=5) quantified by BLI (A). In vivo localization of *luc* expression in a representative mouse of each group at several time points after lipoplex injection by BLI (B; the number of injections is indicated in brackets; "D": Day).

Figure 4: *In vivo* bioluminescence kinetics of *luc* expression after closely repeated intravenous deliveries of BSV4/pGM144 (30µg of pGM144, CR4; n=3) evaluated by BLI (A). Bioluminescence kinetics of *luc* expression in a representative mouse (B; the number of injections is indicated in brackets; "D": Day).

Figure 5: Kinetics of transaminase activities following repeated administration of BSV4/pGM144 (blue lines; n=3), and one administration of BSV4/pTG11033 (red lines; n=3) and BSV4-based liposomes (green lines; n=3). Time intervals between measurements are indicated in the figure.

Figure 6: Densitometric analysis of the proteome profiler blots depicting the expression of cytokines and chimiokines following BSV4/pTG11033 and BSV4/pGM144 (CR4, 50µg of pDNA) systemic delivery (A) (24h after the first (A1) and the second injection (A2)), or multiple close BSV4/pGM144 (CR4, 30µg of pGM144) systemic deliveries (B; the number of injections is indicated in brackets; "D": Day). Only proteins with significant variations at mean expression levels are depicted in the bar graph.

Figure S1

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