Biomaterials 33 (2012) 5618–5627

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The use of CpG-free plasmids to mediate persistent gene expression following repeated aerosol delivery of pDNA/PEI complexes

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ARTICLE INFO

Article history:
Received 2 March 2012
Accepted 7 April 2012
Available online 8 May 2012

Keywords:
Gene therapy
Lung
Cytokine
Cystic fibrosis
Polyethylenimine
Aerosol

ABSTRACT

Aerosol gene therapy offers great potential for treating acquired and inherited lung diseases. For treatment of chronic lung diseases such as cystic fibrosis, asthma and emphysema, non-viral gene therapy will likely require repeated administration to maintain transgene expression in slowly dividing, or terminally differentiated, lung epithelial cells. When complexed with plasmid DNA (pDNA), the synthetic polymer, 25 kDa branched Polyethylenimine (PEI), can be formulated for aerosol delivery to the lungs. We show that pDNA/PEI aerosol formulations can be repeatedly administered to airways of mice on at least 10 occasions with no detectable toxicity. Interestingly, peak reporter gene activity upon repeated delivery was significantly reduced by up to 75% compared with a single administration, despite similar pDNA lung deposition at each subsequent aerosol exposure. Although the precise mechanism of inhibition is unknown, it is independent of mouse strain, does not involve an immune response, and is mediated by PEI. Importantly, using a dosing interval of 56 days, delivery of a fourth-generation, CpG-free plasmid generated high-level, sustained transgene expression, which was further boosted at subsequent administrations. Together these data indicate that pDNA/PEI aerosol formulations offer a versatile platform for gene delivery to the lung resulting in sustained transgene expression suitable for treatment of chronic lung diseases.

1. Introduction

Lung gene therapy is being evaluated for a variety of acute and chronic lung diseases including cystic fibrosis (CF), cancer and emphysema. The relative accessibility of the pulmonary epithelium makes aerosol delivery of gene therapy formulations attractive for non-invasive application to target cells in the lung, whilst minimising potential risks associated with systemic delivery. Unfortunately, due to the forces required for aerosol generation, few gene transfer agents (GTAs) have so far proven suitable for aerosol administration [1]. One non-viral GTA that continues to demonstrate promise for aerosol gene therapy is the cationic polymer polyethylenimine (PEI), which efficiently condenses plasmid DNA (pDNA) molecules to form polyplexes capable of mediating high-level gene expression both *in vitro* and *in vivo*. In contrast to the majority of GTAs, pDNA/PEI complexes remain stable during nebulisation [2,3], leading to robust gene expression in rodent [2] and sheep lungs [4]. Specifically, gene expression has been observed in ciliated epithelial cells [5] and alveolar type I pneumocytes [6], both of which are important target cell populations for lung diseases.

Chronic lung conditions such as CF are likely to require long-term treatment involving repeated administration of gene therapy formulations in order to achieve sustained gene expression over a period of months or years. Lung epithelial cells tend to be slowly dividing or terminally differentiated [7], and unless gene transfer agents can be integrated into progenitor populations, they will eventually be lost from the epithelium. Unlike the repeated

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doi:10.1016/j.biomaterials.2012.04.019

application of viral vectors, which is often ineffective due to the development of neutralizing antibodies to viral proteins, non-viral GTAs appear less immuno-stimulatory and hence more suitable for repeated administration [8]. Successful re-administration, without significant loss of efficacy, has been reported with non-viral, lipid-based formulations in mice [9,10]. Only one clinical trial has evaluated multiple doses of non-viral vector in the airways; three doses of pDNA/liposomes were successfully delivered to the nasal epithelium of CF patients without any apparent loss of efficacy on the third dose [11]. However, the suitability of PEI formulations for effective repeated administration has not yet been demonstrated.

Transgene expression mediated by pDNA/PEI complexes in the lung is often transient, with initial robust levels falling to background levels typically within 1–2 weeks [12,13]. For therapeutic benefit, the duration of transgene expression achieved following each aerosol delivery must be increased, together with the potential for building expression levels with repeated administrations. A key determinant of the final level and persistence of gene expression is modification of the pDNA vector, including the selection of the promoter/enhancer sequ ence employed to direct expression of the transgene [14]. The commonly used viral promoter elements, such as the Cytomegalovirus (CMV) immediate/early promoter, which have been utilised in the majority of PEI studies to date [2,5], result in robust yet transient expression *in vivo* [15], but replacement of viral promoters with mammalian sequences in lipid-based formulations can prolong transgene expression in the lung. Furthermore, unmethylated CG dinucleotides (CpGs) in the plasmid sequence are immune stimulatory [16] and are recognised by the innate immune system via Toll-like receptor 9 (TLR-9) in the endosomes. A reduction in the number of CpGs in plasmid vectors can reduce acute inflammation from lipid-based formulations [17] and complete removal of all CpGs resulted in inflammation-free, transgene expression which persisted for >6 months in the mouse lung [18].

We have investigated the potential for repeated aerosol administration of pDNA/PEI complexes to generate sustained levels of transgene expression in the mouse lung. In addition, we have explored the potential benefits of using modified, CpG-free pDNA vectors incorporating novel promoter elements, to further enhance the level and duration of gene expression, a requirement for future treatment of chronic lung diseases.

2. Materials and methods

2.1. Animals

Female mice, aged 8–16 weeks at time of procedure, were used in this study and included: inbred BALB/c, inbred C57BL6, outbred MF1 and immunocompromised B6 RAG1 knockout (KO) mice [22], all purchased from the Biomedical Services Unit (University of Oxford, Oxford, UK). Mice were housed in accordance with UK Home Office ethical and welfare guidelines and fed on standard chow and water *ad libitum*. All procedures were carried out under UK Home Office approved project and personal licences for performing experiments on animals under the terms of the Animals (Scientific Procedures) Act 1986.

2.2. Plasmid DNA

The first-generation (G1) plasmids pCIKLux, pCIKCAT and pCIKEGFP contain the firefly luciferase gene, the Escherichia *coli* chloramphenicol acetyl transferase (CAT) gene and the Enhanced Green Fluorescent Protein (EGFP) gene respectively under the control of the human cytomegalovirus virus (CMV) immediate/early promoter/enhancer [18,21]. Second-generation (G2) plasmids, containing reduced numbers of CpGs, and CpG-free fourth-generation (G4) plasmids, all expressing luciferase, are as described [18]. Endotoxin-free pDNA was prepared using EndoFree Plasmid Mega Kit (Qiagen, Crawley, UK) or purchased from Bayou Biolabs (Harahan, LA, USA). Plasmids were re-suspended in endotoxin-free water for injection (B. Braun Medical Ltd, Sheffield, UK).

2.3. Formulation of plasmid DNA complexes

PEI polymers with different molecular weights were obtained from Sigma (Sigma–Aldrich Company Ltd, Poole, UK), with the exception of 22 kDa PEI, which was a kind gift of Dr Carsten Rudolph, University of Munich, Germany. Prior to use, PEI was re-suspended at 4.3 mg/ml in sterile water and pH adjusted to 7.4. Unless otherwise stated, pDNA/PEI formulations were prepared at a final pDNA concentration of 0.2 mg/ml and with a PEI nitrogen to DNA phosphate (N:P) molar ratio of 10:1. Complexes were formed by slowly adding equal volumes of appropriately diluted aqueous pDNA to aqueous PEI whilst vortexing. Complexes were allowed to stand at room temperature for 15–20 min prior to use.

2.4. Administration of pDNA complexes to the mouse airways

Studies in which complexes were delivered by nasal instillation were conducted using female BALB/c mice (8–16 weeks old). Plasmid DNA/PEI complexes were instilled under isoflurane anaesthesia (University of Oxford Vet Services, Oxford, UK) in a total volume of 100 μl of formulation per mouse (20 μg pDNA dose). Aerosol delivery of pDNA/PEI formulations was performed using a continuous, unrestrained whole body exposure protocol. Mice ($n = 6–36$) were placed into an 8 L Perspex chamber and exposed to aerosol generated using an Aerotech II nebuliser (CIS-US Inc, Bedford, MA, USA) operating at 40 psi and with 5% CO_2 in air as the driving gas (BOC, Bristol, UK). Unless otherwise stated, a total of 20 ml of complexes (containing 4 mg of pDNA) was aerosolised into the chamber during each pDNA/PEI exposure. For experiments involving multiple groups exposed to different numbers of aerosols, groups were introduced sequentially into a weekly dosing regimen such that all mice were housed together and exposed to the final pDNA/PEI aerosol simultaneously. Similarly, in all studies examining the effects of pre-treatments on subsequent reporter gene expression, all groups received the final pDNA/PEI aerosol simultaneously. Unless otherwise stated, all animals were sacrificed 24 h following the final aerosol exposure and whole lung luciferase expression determined.

2.5. Analysis of luciferase gene expression

Animals were killed by cervical dislocation and the lungs and tracheas removed *en bloc* and stored at −80 °C in reporter lysis buffer (Promega, Southampton, UK). After thawing, tissues were homogenised using a FastPrep FP120 sample disrupter (Thermo Electron Corporation, Milford, MA, USA) incorporating Lysing Matrix D sample tubes (MP Biomedicals, Illkirch, France) and reporter activity was measured using the Luciferase assay system (Promega) in conjunction with a Glomax 20/20 luminometer (Promega). Total lung protein was determined using the detergent compatible DC-protein assay (Bio-Rad, Hemel Hempstead, UK) and luciferase activity in relative light units (RLU) was normalised for protein content before graphing. To aid comparison with other studies, standards of recombinant luciferase protein (Promega) were also routinely analysed; for comparative purposes 100 RLU/mg total lung protein corresponds to 2467 ng recombinant luciferase per mg total lung protein.

2.6. Collection of bronchoalveolar lavage (BAL) fluid

Mice were euthanized by cervical dislocation at 24 h following treatment and the trachea was exposed by blunt dissection. A small tear was made in the trachea and a cannula was inserted. Lungs were washed three times with 1 ml BAL fluid solution (1 × PBS, 0.1% w/v BSA, 0.05 mM EDTA). Cells from BAL fluid were removed by centrifugation and counted [18]. The supernatant was collected to determine cytokine levels.

2.7. Quantification of plasmid deposition using TaqMan PCR

Immediately following aerosol delivery of pDNA/PEI complexes, mice were killed by cervical dislocation and the lungs and trachea removed *en bloc* and total DNA was prepared from each tissue using the QIAGEN DNeasy kit (QIAGEN). Samples were stored at −80 °C until required, then thawed, minced and processed according to the manufacturer's instructions. Plasmid DNA present in the total DNA extracted from each mouse lung was quantified using an ABI PRISM 7700 Sequence Detector (TaqMan) (Applied Biosystems, Warrington, UK) and primer and fluorogenic probe combinations as described previously [19].

2.8. Bio-Plex

The levels of 23 cytokines (CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL11 (Eotaxin), CXCL1 (KC), G-CSF, GM-CSF, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, IFNγ and TNFα) in BAL fluid were determined [25] using a Bio-Plex multiplex cytokine assay (Bio-Rad Ltd., Hemel Hempstead, UK) according to manufacturer's instructions. Samples were analysed using a Bio-Plex 100 or a Bio-Plex 200 system with Bio-Plex Manager software v5.0 (Bio-Rad).

2.9. Cytotoxicity assay

Cytotoxicity was determined using the CytoTox96 assay system (Promega) according to the manufacturer's instructions. Briefly, HEK 293T cells were seeded at 20,000 cells/well in a 96-well plate and incubated with PEI at concentrations ranging from 0.0001 to 4 mg/ml in Opti-MEM (Invitrogen, Paisley, UK) and 5% foetal calf serum. After 48 h cellular toxicity was assayed by quantification of lactate dehydrogenase (LDH) release into the cellular supernatant. LDH in the supernatant samples and retained in intact cells was assayed separately for each sample and cellular toxicity expressed as the percentage of total LDH detected in the supernatant.

2.10. Statistical analysis

Error bars on graphed data represent mean ± standard error of the mean (SEM) for all data sets. Where appropriate, two groups of data were compared using Student's *t*-test. Multiple groups were analysed using one-way ANOVA followed by Tukey's multiple comparison test or Dunnett's multiple comparison test when data was compared to a single control group. Data were considered to be significantly different when $p < 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$). GraphPad Prism software v5 (GraphPad, San Diego, CA) was used for the analyses.

3. Results

3.1. Repeated aerosol delivery of pDNA/PEI complexes

The suitability of pDNA/PEI complexes for repeated administration to the lung was explored in a BALB/c mouse model. Plasmid pCIKLux, expressing firefly luciferase under control of the CMV immediate/early promoter, was complexed with 25 KDa PEI and delivered to the lungs of BALB/c mice via instillation or aerosol. Lungs were harvested at the time-points indicated and luciferase reporter gene expression measured in homogenised whole lung tissue. As expected, transgene expression under the control of the CMV promoter was transient in the mouse lung [13,20]. Luciferase activity was high at 24 h after dosing, but rapidly decreased to background levels by day 8 (Fig. 1a). To investigate transgene expression after repeated administration, these data were used to define a 7-day interval between doses, to allow for loss of luciferase expression from the first dose before a subsequent dose was delivered. Unexpectedly, repeated delivery of pCIKLux/PEI aerosols at 7-day intervals did not appear to restore luciferase gene expression to the levels observed after a single dose (Fig. 1b). Luciferase activity in mice 24 h after exposure to a single aerosol dose was 68 ± 9 RLU/mg compared with 49 ± 4 RLU/mg after the second dose, with a highly significant reduction in luciferase activity after the third (15 ± 2 RLU/mg) and fourth (13 ± 2 RLU/mg) aerosol doses compared with initial levels ($p < 0.001$). Utilising this dosing strategy, whereby gene expression was measured in mice culled after each of four separate aerosols, it is impossible to exclude variability in individual aerosol exposures as a confounding factor in the observed changes in lung luciferase expression. To overcome this potential source of variability, an alternative dosing

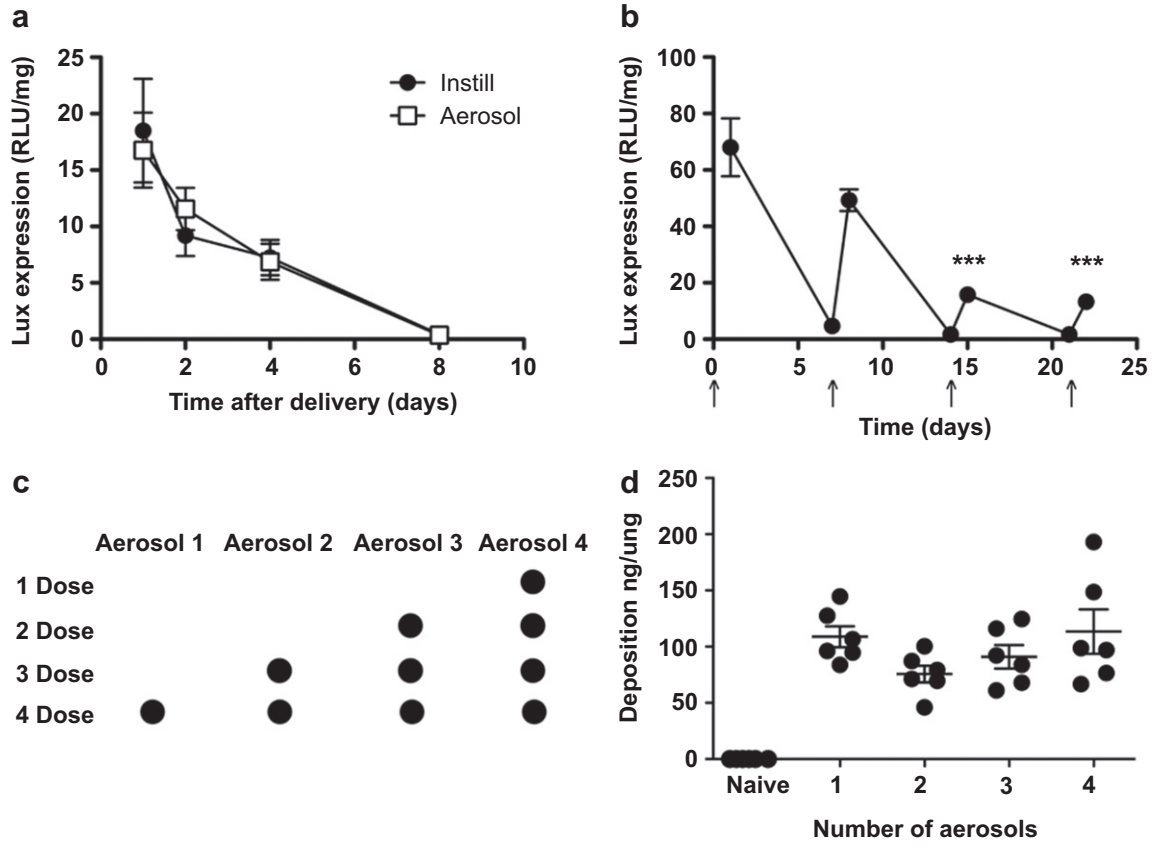

Fig. 1. PEI-mediated gene expression in the mouse lung (a) Duration of luciferase gene expression in whole mouse lung ($n = 6$ per group) following nasal instillation (100 μl) or exposure to aerosol (10 ml) containing 0.2 mg/ml pCIKLux complexed with 25 kDa PEI. (b) Repeated administration of pCIKLux/PEI aerosols to the mouse lung. Female BALB/c mice ($n = 49$) were repeatedly exposed to aerosols (20 ml) containing 0.2 mg/ml pCIKLux/PEI at 7-day intervals. Arrows indicate aerosol exposures. Groups of mice ($n = 7$) were sacrificed 1 day and 7 days after each aerosol and whole lung luciferase expression determined and compared with gene expression measured following a single aerosol exposure ($^{***}p < 0.001$; ANOVA and Dunnett's post hoc analysis). (c) Dosing schedule for repeated aerosol exposure. To minimise variability between groups of mice receiving different numbers of aerosol doses, all mice were exposed to the final test aerosol simultaneously with assessment of luciferase gene expression 24 h later. Dots indicate the presence of test group within the exposure chamber. (d) Plasmid pCIKLux deposition in the mouse lung. Groups of $n = 6$ female BALB/c mice were exposed to between one and four (20 ml) pCIKLux/PEI aerosols delivered at 7-day intervals. Plasmid deposition in each mouse was assessed immediately following final exposure using TaqMan quantitative PCR. Bars represent mean ± SEM for each group.

strategy was employed. Groups of mice were introduced sequentially into a weekly dosing regimen such that all mice (irrespective of treatment history) were housed together and exposed to the fourth and final pCIKLux/PEI aerosol simultaneously (Fig. 1c). When groups of mice were exposed to multiple pCIKLux/PEI aerosols in this manner reporter gene expression was similar to that observed previously (Fig. 1b), with mice exposed to three or four aerosols demonstrating significantly lower lung gene expression than mice exposed to only a single aerosol (data not shown). This dosing strategy was employed for subsequent repeat administration studies. To determine if these observations were due to reduced inhalation of the complexes during repeat administration, possibly due to reduced lung function or behavioural changes in the mice during later doses, lung deposition of pDNA was measured in mice immediately following the final aerosol dose using quantitative TaqMan PCR (Fig. 1d). As observed in other studies [5,13], only relatively small quantities of pDNA could be detected in the lungs of exposed mice, but importantly, there was no reduction in aerosol deposition in mice previously exposed to pCIKLux/PEI aerosols compared with mice exposed to aerosol for the first time ($p = 0.16$ ANOVA).

3.2. Repeated aerosol delivery in different mouse strains

Levels of transgene expression in the mouse lung can vary depending on the strain of mouse [13,21], therefore it was possible that the inhibition of gene expression observed after repeated aerosol delivery was also strain-specific. We compared the reporter gene expression after multiple aerosol administrations in inbred BALB/c mice (Fig. 1b) with that observed in alternative mouse strains, including inbred C57BL6, outbred MF1 and immunocompromised B6 RAG1 KO mice. Groups of mice were exposed to between one and four aerosols of pCIKLux/PEI delivered at 7-day intervals and lung luciferase activity was measured 24 h after each dose (Fig. 2a). As expected, the level of luciferase activity varied between strains, but was still inhibited after repeated aerosol doses, indicating that this was not simply a strain-dependent phenomenon. The reduction in luciferase activity was significant for each subsequent aerosol ($p < 0.01$ ANOVA and Dunnett's post hoc analysis), with expression typically reduced to around 20–30% of initial levels by the third exposure. Furthermore, the immunocompromised B6 RAG1 KO mice are deficient in mature B and T cells [22] suggesting that it is unlikely that the observed inhibition of reporter activity is due to an immune response. To confirm that the inhibition was not due to host responses to luciferase expression, BALB/c mice were exposed to consecutive aerosols containing PEI complexed with plasmids expressing alternative reporter genes, delivered at 7-day intervals, followed by a final pCIKLux/PEI aerosol (Fig. 2b). Luciferase activity was significantly lower in these mice compared with those receiving only the final pCIKLux/PEI aerosol ($p < 0.001$), and was similar to luciferase activity in mice exposed to three consecutive pCIKLux/PEI aerosols. The data in Fig. 2b also show that a similar inhibition of luciferase activity was observed in mice exposed to aerosols containing an equivalent dose of 25 kDa PEI without any pDNA. Together these data suggest that the inhibition of gene expression upon repeated administration of PEI complexes is independent of the transgene and is mediated solely by the presence of PEI.

3.3. Dose dependence of repeat aerosol inhibition

To further understand the inhibition process, mice were exposed to aerosols containing increasing quantities of pCIKLux/PEI formulation, specifically two aerosols containing 1, 4 or 8 mg complexes at 7-day intervals, followed by a single aerosol containing 4 mg pCIKLux/PEI (Fig. 3a). A dose-dependent reduction in luciferase activity compared with control mice exposed to a single 4 mg/ml pCIKLux/PEI aerosol was observed, with the greatest inhibition seen in mice pre-exposed to the greatest quantity of pCIKLux/PEI. These data, and those shown in Fig. 2b, are consistent with the observed inhibition of transgene expression being mediated by prior exposure to PEI.

The 25 kDa PEI used here is a highly promising GTA for aerosol gene delivery, but alternative forms of PEI with different molecular weights and chemical properties are also available commercially. To determine if the inhibition of gene expression is common to all forms of PEI, groups of mice were pre-treated with two aerosols generated with un-complexed PEI polymers of molecular weight

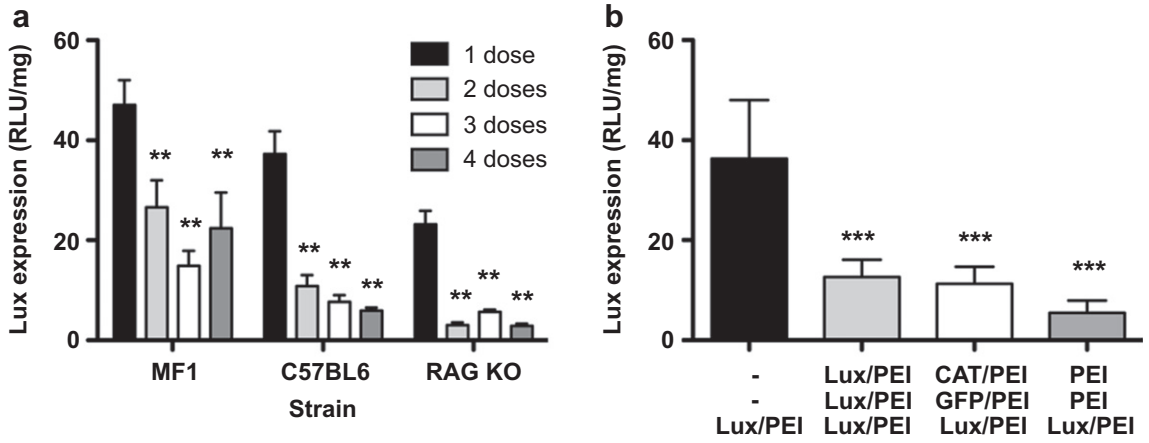

Fig. 2. Comparison of mouse strains and reporter gene expression (a) Effect of mouse strain on repeat PEI administration. Groups of outbred MF1 mice, inbred C57BL6 mice and immunocompromised B6 RAG1 knockout (KO) mice were exposed to between one and four (20 ml) pCIKLux/PEI aerosols delivered at 7-day intervals. Whole lung luciferase expression in each group was determined 24 h following the final exposure. Data represents mean ± SEM for MF1 and C57BL6 ($n = 6$ per group) and B6 RAG1 KO ($n = 4$ per group). Expression upon repeat exposure was compared with expression observed following a single aerosol for each strain (black bars) (**$p < 0.01$; ANOVA and Dunnett's post hoc analysis). (b) Groups of BALB/c mice ($n = 6$ per group) were exposed to two treatment aerosols followed by a test aerosol containing pCIKLux/PEI. Treatment aerosols consisted of PEI complexed with pCIKLux, or PEI complexed with pCIK based vectors incorporating alternative transgenes (CAT: chloramphenicol acetyl transferase; GFP: Green Fluorescent Protein), or an equivalent dose of un-complexed 25 kDa PEI. All aerosols (20 ml) were delivered at 7-day intervals and whole lung luciferase expression was analysed 24 h following the final pCIKLux/PEI test aerosol and compared (***$p < 0.001$; ANOVA and Dunnett's post hoc analysis) with mice exposed to the final aerosol only.

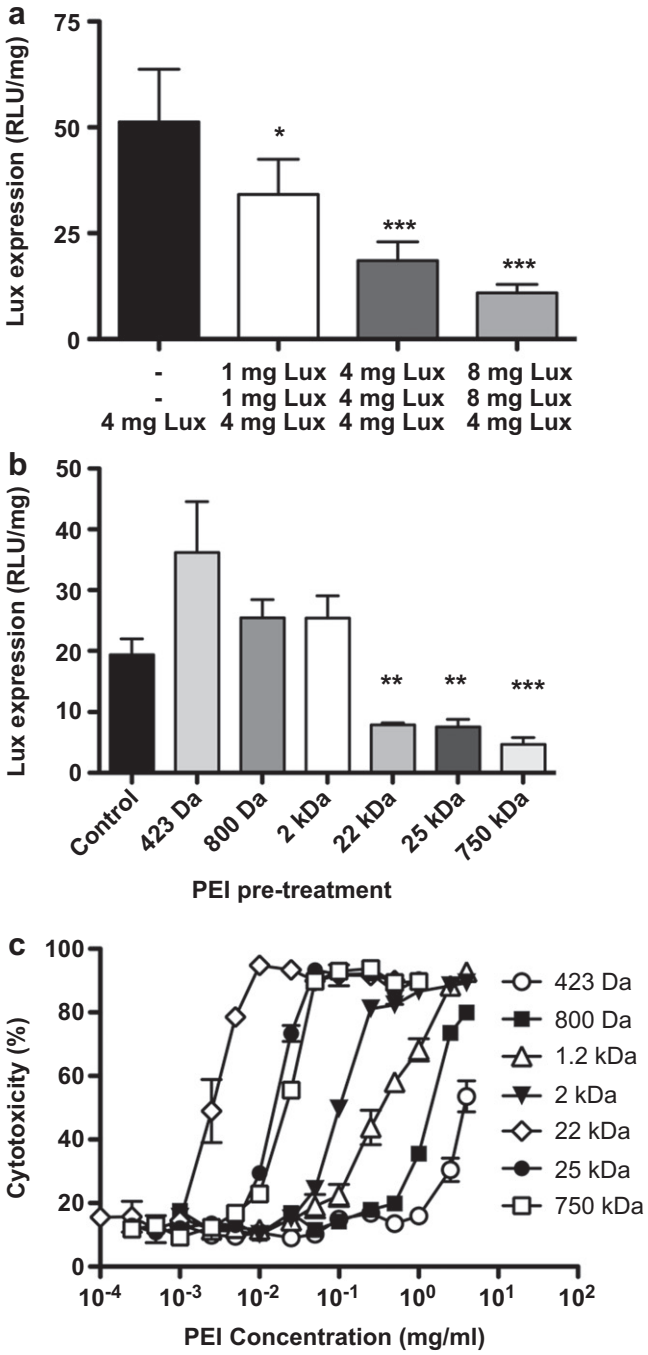

Fig. 3. Role of PEI dose and molecular weight (a) Dose dependence of inhibition. Groups of mice ($n = 6$ per group) were exposed to two 20 ml treatment aerosols (7-day interval) containing either 1 mg, 4 mg or 8 mg of pCIKLux complexed with PEI at an N:P ratio of 10:1. All mice were subsequently exposed to a single test aerosol containing 4 mg pCIKLux/PEI and luciferase expression was determined 24 h later and compared ($^{*}p < 0.05$, $^{***}p < 0.001$; ANOVA and Dunnett's post hoc analysis) with control mice exposed to the final aerosol only (black bar). (b) Effect of PEI molecular weight. Groups of mice ($n = 6$ per group) were exposed to two 20 ml treatment aerosols (7-day interval) containing un-complexed PEI polymers ranging in MW from 423 Da to 750 kDa (equivalent to 0.2 mg/ml pDNA/PEI aerosol). All mice were subsequently exposed to a single test aerosol containing pCIKLux complexed with 25 kDa PEI with luciferase expression determined 24 h later and compared ($^{**}p < 0.01$, $^{***}p < 0.001$; ANOVA and Dunnett's post hoc analysis) with control mice exposed to the final aerosol only (black bar). (c) Cytotoxicity of PEI polymers. HEK 293T cells were cultured in the presence of increasing concentrations of PEI polymers ranging in MW from 423 Da to 750 kDa. Cytotoxicity was determined by the release of cellular lactate dehydrogenase into the tissue culture supernatant over a period of 48 h. Each data point represents mean ± SEM for $n = 3$ replicates.

(MW) ranging from 423 Da to 750 kDa, at 7-day intervals, followed by a final aerosol of pCIKLux complexed with 25 kDa PEI; and luciferase activity was measured in the lungs. Fig. 3b shows that inhibition of luciferase expression was directly correlated with the MW of the PEI polymer used. Low MW PEI polymers (423 Da, 800 Da and 2000 Da) did not result in a significant loss of luciferase activity compared with mice receiving a single pCIKLux/PEI aerosol ($p > 0.05$ ANOVA and Dunnett's post hoc analysis), whereas prior treatment with high MW PEI polymers (22 kDa, 25 kDa and 750 kDa) resulted in a significant reduction in luciferase activity compared with controls. When assessed in cell culture, a clear correlation between MW and cellular toxicity was observed

(Fig. 3c); the high MW PEI polymers were significantly more cytotoxic than the lower MW polymers, the latter demonstrating no inhibition of luciferase activity upon repeat administration of pDNA/PEI aerosols (Fig. 3b). These data suggest that the cytotoxicity associated with high MW PEI polymers could be involved in the inhibition of luciferase gene expression.

3.4. Toxicity and dosing interval

Striking toxicity has been reported following direct instillation of pDNA/PEI complexes into the mouse lung, including weight loss and dose-dependent inflammatory changes [6,23], but delivery of an equivalent formulation by aerosol led to robust transgene expression with no detectable toxicity [6]. To look for potential cumulative lung toxicity associated with extended repeated administration, mice were exposed to between one and 10 aerosols of pCIKLux/PEI delivered at 7-day intervals, and several toxicological markers examined. There were no significant differences observed in animal weight (Fig. 4a), or tissue histology, which was normal (Supplementary Fig. S1). There was no increase in cell infiltrates detected in bronchoalveolar lavage (BAL) fluid from mice receiving any number of aerosols compared with the naïve animals (Fig. 4b). Similarly, when BAL fluid was analysed for cytokines associated with inflammatory changes in the lung, only IL12-p40, which is often elevated in response to the presence of CpGs in the lung [18], showed a slight increase above naïve levels (Fig. 4c), confirming that aerosol delivery of pDNA/PEI complexes is benign. These data are in sharp contrast to animals that were directly instilled with the formulation, leading to increased cell infiltration (Fig. 4b) and a significant elevation of 11 out of 23 cytokines measured (Fig. 4c). However, despite the convincing lack of detectable toxicity following aerosol delivery, Fig. 4d shows that luciferase activity after 10 aerosols (3.9 ± 0.6 RLU/mg) was still considerably lower than in mice exposed to a single aerosol (41.3 ± 10.1 RLU/mg).

3.5. Expression from CpG-free plasmids

To investigate the duration of the PEI-mediated inhibition of gene expression after repeat administration the interval between consecutive aerosol doses was increased. Mice were exposed to a total of three pCIKLux/PEI aerosols delivered at dosing intervals of between 7 and 120 days. Luciferase expression was measured 24 h following the final exposure and expressed as a percentage of that observed in age-matched mice exposed only to the final pCIKLux/PEI aerosol (Fig. 5a). Three aerosols delivered at 7-day intervals resulted in only 31% of the levels seen after a single dose, whilst an interval of 120 days allowed almost 80% recovery of expected luciferase activity. This indicated that the PEI-mediated inhibition was transient, with an estimate of around 157 days between doses being required for 100% recovery of gene expression (linear regression analysis, R squared 0.96; Pearson correlation $p < 0.0005$).

In an attempt to benefit from the improved transgene expression observed with modified plasmid vectors, 25 kDa PEI was complexed with a range of novel plasmids and the resultant formulations delivered as an aerosol to the lungs of mice. Fig. 5b shows luciferase expression from the first-generation pG1-UbC-Lux plasmid containing the mammalian polyubiquitin C (UbC) promoter, compared with pCIKLux (also known as pG1-CMV-Lux [18]). The UbC promoter led to increased persistence of luciferase activity detectable up to 28 days after a single aerosol, but the level was still low (1.7 ± 0.5 RLU/mg) compared with the initial high levels achieved the CMV equivalent (16.8 ± 8.3 RLU/mg). A range of promoter elements was then tested in the context of a second-generation (G2) plasmid with reduced numbers of CpGs [18]; in this context the UbC promoter led to sustained luciferase activity with increased expression at 28 days (22.3 ± 1.6 RLU/mg) (Fig. 5c). Fourth-generation (G4) plasmids that are completely devoid of CpGs [18] were also tested, although the promising UbC promoter could not be evaluated in a CpG-free context because removal of all CpGs from the UbC promoter resulted in loss of activity (data not shown). Instead, we tested three synthetic CpG-free promoters and showed that when complexed with PEI (Fig. 5d), plasmid pG4-hCEFI-soLux containing the hCEFI promoter element generated luciferase activity that was both persistent and high level (92.9 ± 18.3 RLU/mg at day 28) compared with earlier generations (Fig. 5e). It was therefore hypothesised that the use of pG4-hCEFI-soLux, combined with a suitable interval between repeated doses, could compensate for the refractory dosing period observed with PEI. Fig. 5f shows that when pG4-hCEFI-soLux/PEI complexes were delivered as an aerosol to the mouse lung with a dosing interval of 56 days, high-level luciferase activity (168.3 ± 19.7 RLU/mg at day 7) was sustained after the first dose and was boosted on the second (382.3 ± 28.9 RLU/mg) and third (744.2 ± 213.2 RLU/mg) doses. This demonstrates that not only can the use of modified plasmids augment expression levels from a single aerosol dose of PEI complexes, but can further boost expression on repeated administration for long-term gene expression (Fig. 5f).

4. Discussion

Gene therapy for chronic lung diseases will probably require repeated application in order to treat target cell populations that are slowly dividing or terminally differentiated, such as the epithelial cells lining the lung. In the mouse, epithelial lung cells appear to be replaced relatively slowly with an average half-life of 17 months [7]. However, unless a vector is integrated into the genome of progenitor cells, gene expression will eventually be lost from the epithelium as transfected cells are gradually replaced. In this study, we investigated the potential for 25 kDa PEI aerosol formulations to be repeatedly administered in order to treat chronic lung diseases.

In a BALB/c mouse lung model, we showed that the activity of a reporter gene after a single dose of pDNA/PEI was transient (Fig. 1a), and that after multiple aerosol doses, reporter levels were significantly reduced compared with a single administration, despite similar pDNA lung deposition at each exposure (Fig. 1). A previous report [13], involving three aerosol doses of a pDNA/PEI formulation to the mouse lung, did not show inhibition on repeat administration, but the short dosing interval (3 days) did not allow reporter activity to fall to baseline before re-administration, and therefore was unlikely to reveal this kind of inhibitory effect. The inhibition observed in our study was minimised when the interval between doses was increased, thereby defining a refractory period for effective repeated administration (Fig. 5a). We showed that the reduction in reporter activity was not due to the strain of mouse, nor to an immune response to the transgene product and was mediated solely by the presence of PEI (Fig. 2). When other commercially available PEI polymers were tested in mice, the larger MW polymers (22 kDa, 25 kDa and 750 kDa) led to an inhibition of reporter activity on repeat administration and, interestingly, also displayed the highest levels of cytotoxicity in cell culture (Fig. 3).

The lung is well-known for its sensitivity to inhaled particles and so careful monitoring of the effects of aerosol delivery is required. Reports in the literature of lung toxicity caused by 25 kDa PEI are principally associated with the delivery method, such that when pDNA/PEI formulations at a concentration of up to 1 mg/ml were directly instilled into the mouse lung, the animals displayed overt

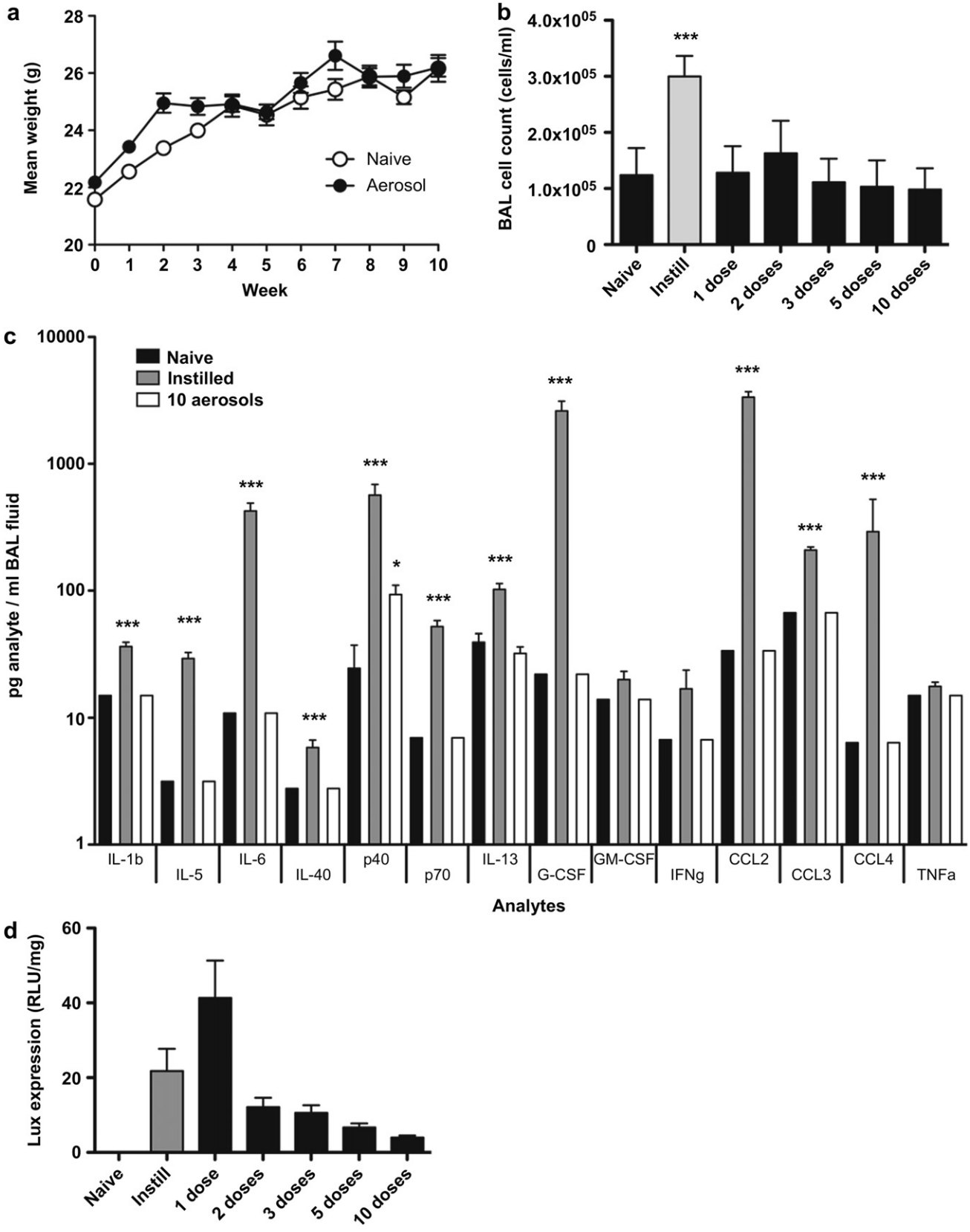

Fig. 4. Pulmonary toxicity of PEI complexes (a) Effect of chronic exposure to PEI aerosols on mouse weight. Groups of BALB/c mice ($n = 6$ per group) exposed to between one and 10 pCIKLux/PEI aerosols delivered at 7-day intervals were weighed 24 h following each aerosol exposure and mean weights (±SEM) were compared with age-matched control mice housed under identical conditions. (b) Inflammatory cells in bronchoalveolar lavage (BAL) fluid. BAL fluid was collected from mice exposed to pCIKLux/PEI aerosols and total nucleated cell counts determined. Results were compared with naïve mice and mice instilled with 100 μl of pCIKLux/PEI formulation. Data represents mean ± SEM for $n = 6$ mice per group with statistical analysis performed relative to naïve animals (***$p < 0.001$; ANOVA and Dunnett's post hoc analysis). (c) Cytokines in BAL fluid. BAL fluid was analysed for the presence of 23 different cytokines. Data represent mean ± SEM for $n = 6$ mice per group with statistical analysis performed relative to naïve animals (ANOVA and Dunnett's post hoc analysis of log transformed data) (black bars). For clarity, not all cytokine data are shown. (d) Luciferase expression. Whole lung luciferase activity was measured in mice exposed to between one and 10 aerosols of pCIKLux/PEI and compared with naïve mice and mice instilled with 100 μl of pCIKLux/PEI formulation. All samples were harvested 24 h after final delivery.

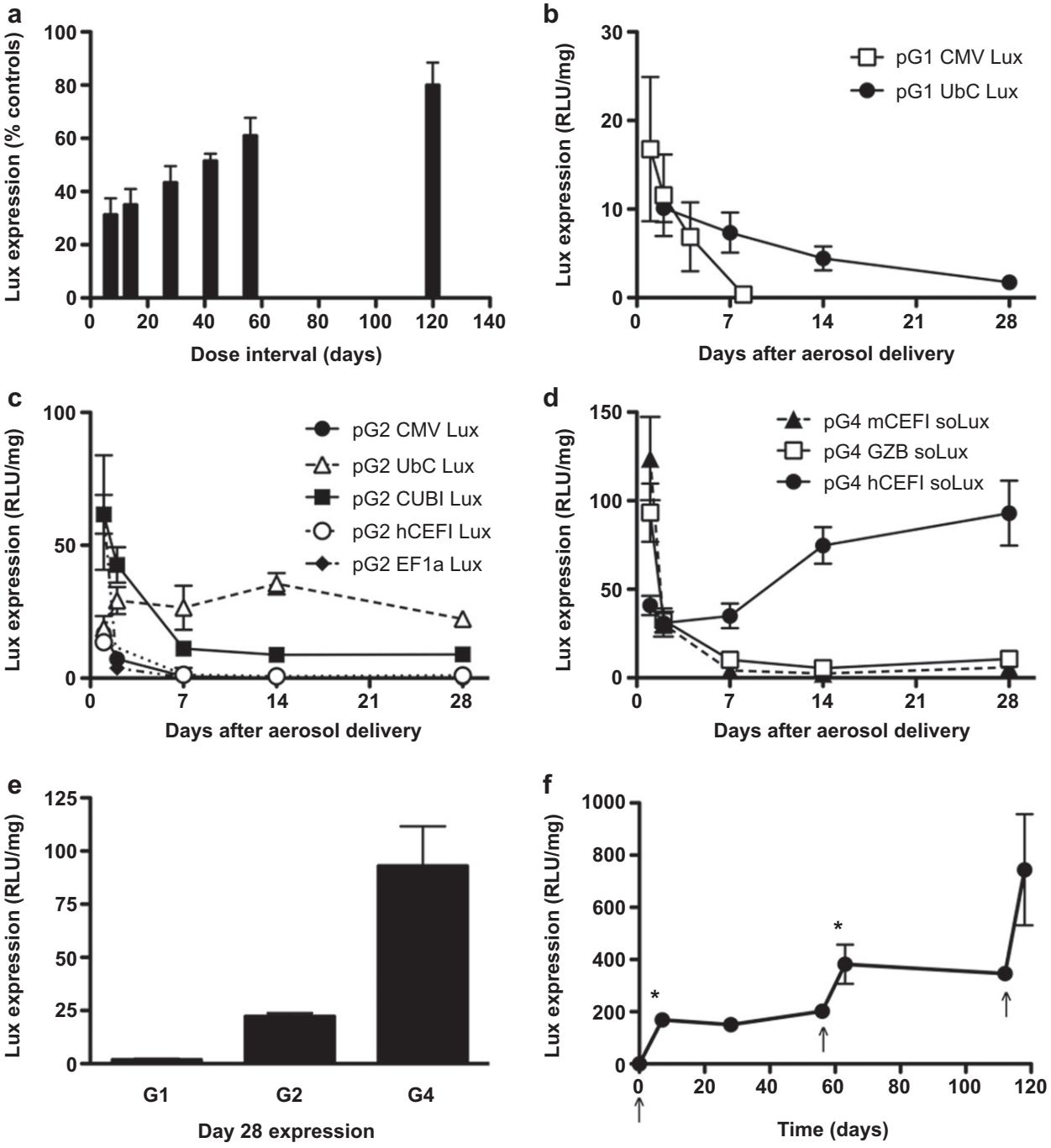

Fig. 5. Optimisation of plasmid DNA expression vectors (a) Refractory period for aerosol delivery. Groups of mice were exposed to three pCIKLux/PEI aerosols (20 ml) delivered at intervals ranging from 7 to 120 days between doses. Whole lung luciferase expression was determined in all mice 24 h following the final aerosol exposure and expressed as a percentage of luciferase expression observed in age-matched control mice exposed simultaneously to a single pCIKLux/PEI aerosol. Data represent mean ± SEM for $n = 6$ mice per group except 120 day interval group where $n = 5$. (b) to (d) Enhanced gene expression using modified plasmid vectors. Plasmids containing a variety of sequences and transcription elements were complexed with PEI (0.2 mg/ml pDNA and N:P ratio of 10:1) and aerosolised (10 ml) to groups of female BALB/c mice ($n = 30$) housed in a single aerosol exposure chamber. Groups of mice ($n = 6$) were harvested at intervals following aerosol exposure and whole lung luciferase expression determined. Data represent mean ± SEM. Luciferase expression profiles are shown for (b) first-generation (G1: CpG-rich), (c) second-generation (G2: CpG-reduced) and (d) fourth-generation (G4: CpG-free) plasmids. (e) Comparison of maximal lung gene expression levels observed with G1, G2 and G4 plasmids 28 days after aerosol exposure. All data represent mean ± SEM for $n = 6$ animals. (f) Enhanced gene expression upon repeated administration of PEI aerosols. Female BALB/c mice were exposed to multiple aerosols (20 ml) containing the fourth-generation CpG-free expression plasmid pG4-hCEFI-soLux complexed with 25 kDa PEI. Aerosols were delivered at 56-day intervals with groups of mice ($n = 6$) harvested at intervals following each aerosol. Arrows indicate aerosol exposure. *indicates statistical significant increase in luciferase expression (t-test) following aerosol exposure.

signs of toxicity including hunching, piloerection and a 5–10% loss in body weight in the 24 h following dosing [6]. However, in the same study highly concentrated formulations (up to 8 mg/ml) when delivered by aerosol, were well-tolerated, showing no detectable toxicity [6]. Here, we confirmed that instillation of a bolus of pDNA/PEI formulation is highly inflammatory as measured by a significant increase in cell infiltrates (Fig. 4b) and increased inflammatory cytokines in BAL fluid (Fig. 4c), and can result in focal areas of tissue

destruction (Supplementary Fig. S1). By comparison, extensive repeated aerosol delivery (up to 10 doses) was completely benign according to all parameters tested, with histopathology being indistinguishable from naïve controls (Supplementary Fig. S1). In our experience with many different non-viral formulations, aerosol delivery rarely leads to detectable histopathology. To our knowledge there has been only one report showing a histological change, where small areas of lung congestion were observed in one group of mice 7 days after aerosol delivery of pDNA/PEI [24]. However no histopathology was observed at 1 h or 24 h after dosing, and it is not clear how many mice displayed these changes. One possibility is that residual levels of bacterial genomic DNA present in the plasmid preparation could be responsible. Variable levels of lung inflammation [25] and muscle tissue damage [26] have been reported in mice following delivery of non-viral formulations which has been attributed to contaminating levels of bacterial genomic DNA in plasmid preparations. In one study, muscle damage following limb vein injection of naked DNA correlated with the use of plasmid DNA containing as little as 5% bacterial genomic DNA [26]. Importantly, since sheared fragments of bacterial genomic DNA contaminating a plasmid preparation are difficult to visualize, quantitative PCR is required to assess the quality of plasmid DNA used; and Lesina et al [24] did not report levels of genomic DNA present in plasmid preparations used in the study.

As a cationic polymer, PEI is thought to protect pDNA from degradation by buffering endosomal pH [27]. This facilitates the escape of the pDNA from the endosome through osmotic swelling and rupture [28], such that when pDNA is released into the cytoplasm it may be trafficked to the nucleus for transgene expression. In addition to perturbation of the normal endocytotic pathway, the highly cationic PEI molecule can undergo electrostatic and hydrophobic interactions with anionic macromolecules within the cell [29], possibly compromising normal cell function. Indeed, some PEI polymers have demonstrated significant cytotoxicity both in cell culture and *in vivo*; gene expression profiling has shown that relatively high doses of 25 kDa PEI can induce signal early in the apoptotic pathway 24 h after treatment [30]. Although high MW PEIs typically used for gene delivery are associated with cytotoxicity, smaller PEI polymers that are inherently less toxic [31] are also being investigated. Low MW PEIs are intrinsically poor gene transfer agents [31] but generation of higher MW PEI *via* polymerization of small PEIs with biodegradable linkages has resulted in novel reagents that demonstrate efficient gene transfer in the absence of toxicity [31,32]. Such biodegradable PEI polymers, if viable for aerosol delivery, could be used to test whether poor biodegradability is a factor in causing the repeat dosing refractory period observed here.

There have been many reports demonstrating that modification of plasmid vectors can improve the performance of non-viral formulations, both in terms of reducing inflammation and increasing the persistence of transgene expression [14]. When we evaluated a range of novel plasmids including CpG-reduced G2, and CpG-free G4 vectors complexed with PEI, we observed that reporter gene expression levels were significantly increased (Fig. 5). In particular, when the CpG-free plasmid pG4-hCEFI-soLux, which resulted in high level and persistent expression (92.9 ± 18.3 RLU/mg on day 28 after a single dose) (Fig. 5d), was delivered at 56 day intervals, luciferase activity (168.3 ± 19.7 RLU/mg at day 7) was sustained after the first dose and was boosted on the second (382.3. ± 28.9 RLU/mg) and third (744.2 ± 213.2 RLU/mg) doses (Fig. 5f). The use of a similar CpG-free, G4 plasmid, complexed with a cationic liposome, has recently been evaluated in the clinic as a gene therapy for patients with cystic fibrosis (CF) lung disease, generating sustained expression in the nasal epithelium for up to 13 weeks in some patients [33]. A clinical trial involving repeated (monthly) aerosol delivery of this formulation over 12 months is ongoing to determine if this strategy can result in clinical benefit for lung diseases.

Synthetic PEI molecules have been modified in various ways to alter their physiochemical properties and enhance performance, offering a versatile platform for lung delivery of plasmid DNA. The application of PEI formulations has also been extended to encompass delivery of shRNA and siRNA molecules [34] designed to effect knockdown of target genes. For example, complexes of siRNA/PEI for the prophylaxis and therapy of influenza virus infection were directly instilled, or delivered intravenously, to the lungs of mice, resulting in reduced virus production [35]. One obstacle to the clinical progression of non-viral formulations is the potential for physical instability in aqueous suspension, making it difficult to withstand the rigours of storage and shipment [36]. To maintain colloidal stability, PEI formulations are routinely prepared at relatively low concentrations, but for the practical delivery of PEI complexes to the human lung, such formulations will need to be concentrated. Preparation of pDNA/PEI complexes at concentrations >0.5 mg/ml has been associated with precipitation and loss of biological activity, but using a simple ultra filtration process this problem can now be avoided and PEI complexes can be concentrated to >8 mg/ml [6]. Furthermore, such formulations can generate reproducible, high-level transgene expression after nebulisation to the sheep lung, when used as a large animal model of aerosol delivery to patients [6]. A further crucial requirement for clinical application is the reliable and scaleable mixing of components sufficient to guarantee consistent performance; typical small scale mixing performed in laboratories is difficult to standardise resulting in variable physical characteristics of complexes and unreliable performance. However, a pneumatic mixing device is now available for production of large-scale, clinical-grade PEI formulations [37]. Finally, progress has been made in developing a dry powder aerosol formulation for pDNA/PEI delivery using simple sugars as lyoprotectants [38]. Together these studies demonstrate that this non-viral gene transfer agent has significant potential for development as a viable clinical product.

5. Conclusion

Complexes of plasmid DNA and 25 kDa branched PEI polymer can be repeatedly delivered to the mouse lung via aerosol. No histological changes to the lung were observed, even after a total of 10 doses were administered, confirming that this formulation is well-tolerated. One unexpected observation was that transgene expression after repeat doses of pDNA/PEI formulations was lower than that seen after a single dose; an effect mediated by PEI via an unknown mechanism. However, by varying the interval between doses and exploiting recent advances in plasmid design it was possible to circumvent the effects of the refractory period, leading to high-level and sustained transgene expression in the lung. Several advances in controlling the formation and stability of pDNA/PEI complexes have elevated this non-viral gene transfer agent to a stage where it can be considered as a viable clinical formulation for lung gene transfer. The next step is to understand more about the bio-distribution, degradation and excretion pathways for PEI complexes in animal models.

Acknowledgement

This work was funded by a grant from the Cystic Fibrosis Trust to the UK Cystic Fibrosis Gene Therapy Consortium (http://www.cfgenetherapy.org.uk).

Appendix A. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2012.04.019.

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