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ORIGINAL ARTICLE

Adeno-associated virus serotype 9-mediated pulmonary transgene expression: effect of mouse strain, animal gender and lung inflammation

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Gene therapy holds great potential for the treatment of various acquired and inherited pulmonary diseases. Among various viral vectors, adeno-associated viral (AAV) vectors have been most frequently used in different clinical trials of pulmonary gene therapy. In the present study, we examined the kinetics and duration of transgene expression, vector biodistribution and development of neutralizing antibodies (NAB) in mice after pulmonary application of AAV2/9 vector. The pulmonary route of application did not affect any of the measured parameters. Transgene expression and biodistribution analysis at day 450 post-application confirmed the systemic spread of the vector after pulmonary delivery. Using $SPB^{-/-}$ mice, the study shows that AAV2/9-mediated gene expression is influenced by animal gender but not mouse genotype and is insensitive to the presence of lung inflammation. Lower expression levels were observed in male compared with female mice, and transient immunosuppression with dexamethasone significantly reduced the development of NAB in both genders of mice. The study thus advances this serotype for further development and use as a therapeutic vector.
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INTRODUCTION

Gene therapy holds promise for the treatment of a range of inherited pulmonary diseases like cystic fibrosis, α1-antitrypsin deficiency and surfactant protein deficiencies. Most gene therapy protocols have used viral vectors for their efficiency of gene transfer. Of the many vectors used, adeno-associated virus (AAV)-based vectors hold great promise for efficiently targeting airway epithelium *in vivo*.[1] Major interest in AAV vectors has been evoked due to their lack of pathogenicity, ability to transduce non-dividing cells,[2] prolonged transgene expression and low immunogenicity.[3] Initial studies using recombinant AAV (rAAV) vectors were primarily focused on serotype 2. However, the paucity of AAV2 receptors on the apical surface of airway epithelium and the degradation of AAV2 in the cytoplasm has limited the efficacy of this serotype in clinical trials.[4]

The advent of technology for pseudotyping has provided the means to bypass this limitation of receptors and the prevalence of neutralizing antibodies (NAB) to AAV2.[5,8] Numerous serotypes of rAAV have supported persistent transgene expression after pulmonary gene delivery.[6,9–14] A recent study comparing different AAV serotypes *in vivo* in murine lungs and *in vitro* in human ciliated airway epithelium found AAV1, 6 and 9 to be highly efficient.[14] For AAV9, it has been shown that this serotype selectively targets the alveolar epithelial cells after intrapulmonary delivery,[8,14] thereby making this serotype a promising candidate for diseases like surfactant protein deficiencies, which primarily affect alveolar type II epithelial cells.

The study by Limberis and Wilson[8] was the first report demonstrating the applicability of AAV2/9-based vectors for lung gene transfer. However, in that study, human α1-antitrypsin and nuclear-localized bacterial β-galactosdase (nlacZ) were used as marker genes and lung gene delivery experiments were performed in C57BL/6 mice. These mice are immunotolerant to human α1-antitrypsin[15] and show reduced immune response to bacterial β-galactosidase in comparison with BALB/c mice.[16] Therefore, the results presented by Limberis *et al.*[8,14] are likely to be influenced by the reduced immunogenicity observed in the used mouse strain.

Different mouse genotypes are also most likely to influence the development of NAB, which in turn would determine the efficiency with which viral vectors can be readministered. Production of NAB to AAV capsids hinder successful transduction with subsequent viral doses of the same serotype.[17,18] A number of studies have reported successful repeated administration after pretreatment of the host with immunosuppressing antibodies,[19] and/or immune-modulating agents[20] or broad immunosuppressing agents, such as cyclophosphamide.[21] In contrast to the use of immunosuppressive/modulating regimens, two studies have observed that rAAV may be efficiently administered a second time if the time between administrations is extended beyond approximately 28 weeks and the capsid is derived from AAV5 or AAV9.[8,22] These encouraging results are contradicted by Sumner-Jones *et al.*,[17] where administration of rAAV5 resulted in the production of sustained anti-AAV5 capsid-NAB that substantially lowered lung gene transfer on a second administration and effectively abolished it on a third. Importantly, neither the local expression of CTLA4Ig (immunosuppressing antibody) nor an increase in the period between vector administrations from 8 to 36 weeks improved

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the efficacy of second or third administrations of rAAV5. Different mouse strains used in these studies could be one of the factors influencing the development of immune response post-viral gene delivery. These results further strengthen the putative influence of mouse genotype on the observed immune response induced by rAAV vectors. Additionally, they point to the apparently indispensible need of an effective and clinically acceptable immunosuppressive strategy before rAAV vectors can be further explored in clinical trials for inherited diseases, where repeated administrations would be expected.

Pulmonary diseases like cystic fibrosis and surfactant protein B (SP-B) deficiency are characterized by lung inflammation. Bronchoalveolar lavage from cystic fibrosis patients has been shown to inhibit transduction by rAAV2 vectors.[23] Therefore, it becomes imperative to test the efficiency of rAAV2/9 vectors in an inflamed lung before it can be proposed to be used in a therapeutic setting. To achieve this end, we have performed AAV2/9-mediated gene delivery experiments in conditional SPB$^{-/-}$ mice. These mice are well described[24–26] and are transgenic for human SP-B placed under conditional control of doxycycline via the human surfactant protein C promoter. Adult mice made SP-B deficient by removal of doxycycline and developed lung inflammation within 24 h of doxycycline removal, as evidenced by elevated alveolar interleukin (IL)-6, IL-1β and macrophage inflammatory protein-2 concentrations. These mice died within 4 days due to severe respiratory failure.[26] Selective tropism of AAV2/9 for alveolar epithelium[8,14] makes SPB$^{-/-}$ mice a highly suitable animal model to asses the efficiency of AAV2/9 vector in an inflamed lung. Since animal gender has also been shown to influence AAV-mediated gene delivery, transgene expression and biodistribution,[27,28] experiments in the present study were performed in both male and female mice.

To the best of our knowledge, studies comparing the effect of murine genetic background, lung inflammation and animal gender on rAAV2/9-mediated pulmonary gene transfer have not yet been performed, but warrant immediate attention. The present study investigates the effects of these parameters on transgene expression, biodistribution and immunogenicity of AAV2/9 after pulmonary gene delivery via different pulmonary routes of application.

RESULTS

Pulmonary routes of application compared in BALB/c mice with respect to transgene expression, NAB development and vector biodistribution

AAV2/9-luc was administered to BALB/c mice (10^{11} genome copies (GC) per mouse) either via intranasal (IN) sniffing or intratracheal (IT) instillation. At different time points post-application, luciferase expression was measured by *in vivo* bioluminescence imaging. Using either of the application routes, luciferase expression could be detected as early as day 7 post-vector application and was sustained without any significant decline till the end of the experiment, that is day 450 post-application (Figures 1a and b). Quantification of expression revealed no significant difference at any time point between either of the two application routes (Figure 1c).

Blood was collected at day 450 and serum prepared for NAB assay. Serum from untreated BALB/c mice served as control. A 10-fold dilution of the serum from the AAV2/9-luc-treated mice completely neutralized the viral vector, whereas a 100-fold dilution resulted in approximately 98% reduction in luciferase expression compared with serum from untreated mice (Figure 2a). Diluting the serum further did not affect the vector infectivity. Similar to the luciferase expression data, pulmonary route of application did not influence the NAB titer. Thus, a single dose of AAV2/9-luc resulted in stable long-term expression (>1 year) in BALB/c mice after pulmonary gene delivery. However, it also resulted in the development of NAB, which persisted longer than 1 year. Both transgene expression and NAB development were independent of pulmonary route of application.

In addition to blood, visceral organs were isolated from the mice for biodistribution analysis. Total DNA was isolated from the harvested organs and the amount of vector DNA quantified by real-time PCR using luciferase-specific primers as described previously.[29] With both routes of application, maximum delivery to the lungs, when compared with the other organs, could be confirmed (Figure 2b; $P \leqslant 0.001$). However, a trend showing lower delivery to the lungs combined with higher amounts of viral vector delivery to spleen and liver was observed after IN application. Gonads were also tested for vector DNA and were all negative (data not shown). This trend was further supported by luciferase activity measured in the isolated tissues from these mice. Approximately twofold higher luciferase expression was observed in the lungs after IT delivery (303 925 ± 136 824 relative light unit per mg tissue) compared with after IN delivery (170 486 ± 53 437 relative light unit per mg tissue). Expression levels in other organs were 100–1000-fold lower compared with the lungs. Gonads were not screened for expression as they were negative for vector genomes by real-time PCR. For this reason, IT was chosen as the route of viral application in further experiments.

Effect of dose reduction on transgene expression, NAB development and biodistribution in BALB/c mice

Luciferase expression images and subsequent biodistribution analysis at day 450, both by real-time PCR and luciferase activity quantification, provided evidence for the systemic spread of the viral vector post-application. In our subsequent experiments, we attempted to minimize this systemic distribution by reducing the applied dose from 10^{11} to 10^{10} and 10^{9} GC per mouse. These doses were applied IT and luciferase expression monitored over time. Figure 3a shows images of mice from the three groups (10^{11}, 10^{10} and 10^{9}) at 28 days post-vector delivery. Though the systemic distribution of the viral vector could be curtailed, reducing the applied dose also resulted in significantly lower expression. A 10-fold reduction of the dose (10^{10} GC per mouse) resulted in approximately 50-fold lower expression, whereas with a 100-fold reduced dose (10^{9} GC per mouse), luciferase expression was only marginally above background (Figure 3b).

We further investigated if reducing the dose had any influence on NAB titers. Blood was isolated from the 10^{10} and 10^{9} group mice at day 230 post-application and serum was used in NAB detection assay. Similar to the results obtained with the 10^{11} group, a 10-fold dilution of the serum from both 10^{10} and 10^{9} group resulted in complete neutralization of the viral vector. Diluting the serum 100-fold resulted in 65 and 10% reduction in luciferase expression, respectively, compared with serum from untreated mice (Figure 4a). Any further dilution did not affect the vector infectivity.

Since expression with the 100-fold lower dose (10^{9} GC per mouse) was barely detectable, biodistribution analysis was performed only for the mice of the 10^{10} group. These mice were euthanized at day 230 post-vector application, internal organs were harvested and used for DNA isolation. Vector DNA was quantified using real-time PCR. Similar to the results obtained with mice from 10^{11} group, maximum deposition was observed in the lungs compared with the other organs (Figure 4b), while the gonads were negative for vector DNA (data not shown). Nonetheless, reproducible detection and quantification of vector DNA from the visceral organs reflected the failure of dose reduction in containing viral vector distribution. Thus, a lower vector dose though reducing NAB development, also significantly reduced expression and

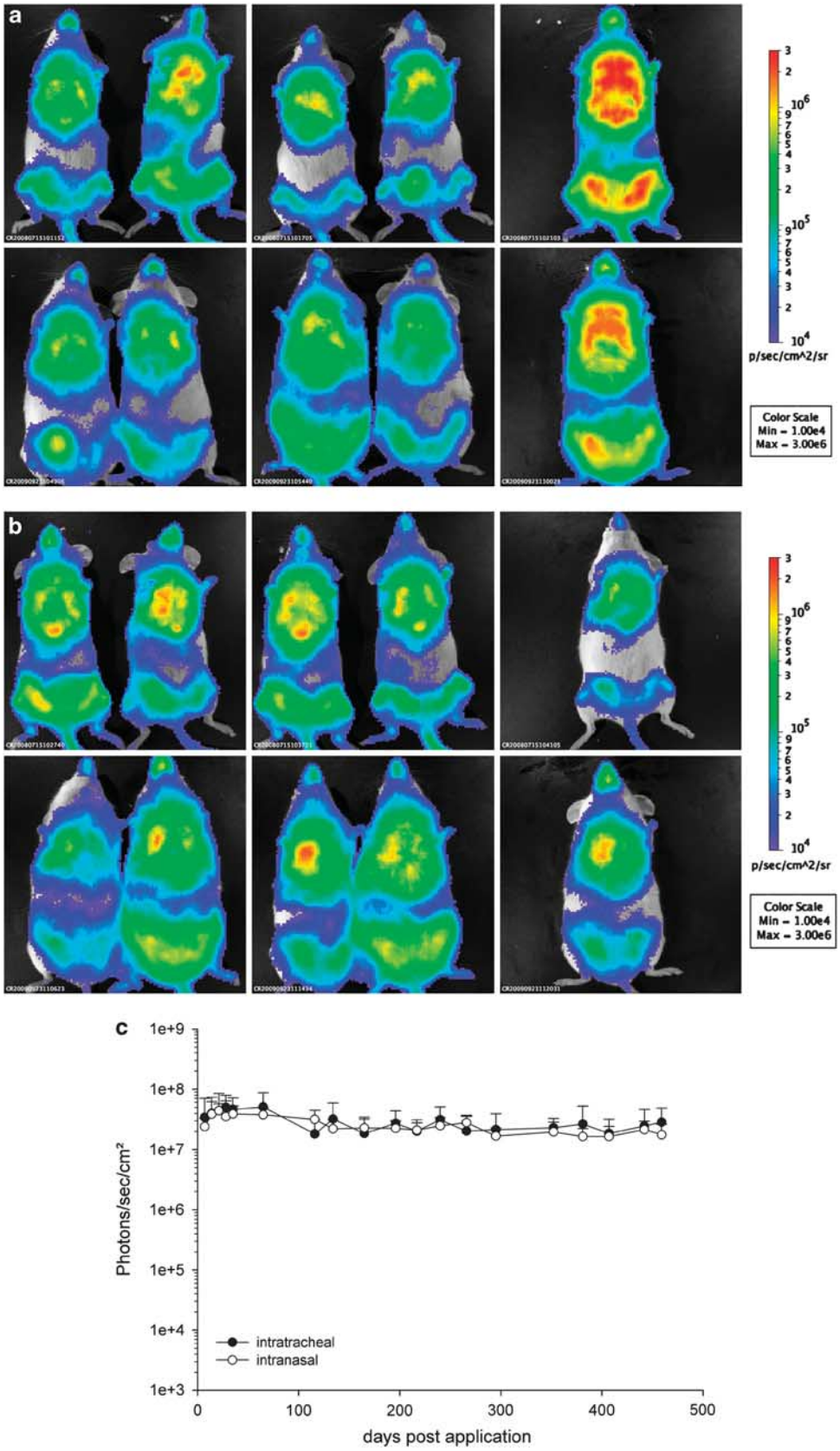

Figure 1 *In vivo* luciferase expression in female BALB/c mice after intratracheal (**a**) and intranasal (**b**) application of 10^{11} GC per mouse (n=5). Upper panel in each figure shows image from day 7, while the lower panel shows day 450 post-vector delivery. *In vivo* bioluminescence images were taken and quantified as described in Materials and methods. Expression was stable and unaffected by the different application routes (**c**). Values represent mean ± s.d.

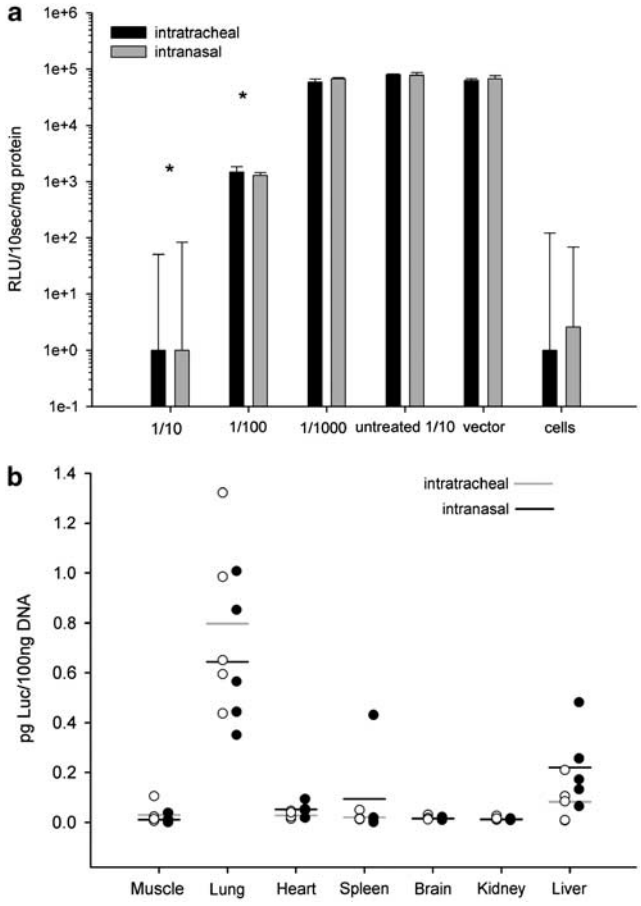

Figure 2 Development of NAB (**a**) and biodistribution (**b**) in female BALB/c mice after intratracheal and intranasal application of 10^{11} GC per mouse (n=5). Prior to euthanizing the mice, blood was taken to prepare the serum and assay for NAB was performed as described in Materials and methods. Serum of untreated mice, viral vector without serum and untreated cells were used as controls. Pulmonary delivery route did not affect NAB development. Significant differences compared with the serum of untreated mice are marked with an asterisk. After euthanizing, internal organs were taken and real-time PCR was performed with isolated DNA. IT delivery presented a trend of higher vector concentration in lungs combined with lower concentrations in liver and spleen. Differences were not significant. Values represent mean ± s.d.

had no beneficial effect on biodistribution. For this reason, all further experiments were performed with the dose of 10^{11} GC per mouse.

Effect of mouse strain, lung inflammation, animal gender and dexamethasone treatment on transgene expression and NAB development

To investigate if transgene expression and NAB development post-AAV2/9-luc-mediated pulmonary gene delivery are affected by the genetic background of the mouse, we performed experiments in $SPB^{-/-}$ mice. On comparing luciferase expression after AAV2/9-luc delivery in female $SPB^{-/-}$ with female BALB/c mice, no significant differences between the two strains, with respect to the kinetics and stability of expression, could be observed (Figure 5a).

$SPB^{-/-}$ mice were also used to investigate the efficiency of AAV2/9 in an inflamed lung. Doxycycline was removed from drinking water of these mice either 1 or 2 days prior to vector administration. AAV2/9-luc was applied via the IT route (10^{11} GC per mouse) and mice were put back on doxycycline containing water. Mice, which had been on normal water for two days, did not survive the IT instillation procedure involving anesthesia and died within 24 h post-gene delivery. In mice, which were on normal water for 1 day prior to vector application, luciferase expression was measured 7, 14 and 28 days post-gene delivery. No significant difference could be observed between this group of mice compared with mice receiving doxycycline containing water throughout (Figure 5b). Development of progressive lung inflammation, as evidenced by elevated alveolar IL-6, IL-1β and macrophage inflammatory protein-2 concentration, as early as 24 h post-doxycycline removal, has been reported for these mice by Ikegami *et al*.[26] When followed over time, these mice succumb to severe respiratory failure within 4 days post-doxycycline removal. Similar transgene expression level observed in the compared group of mice indicates that AAV2/9-mediated gene transfer and expression is independent of the presence of SP-B-deficiency-induced inflammation.

In $SPB^{-/-}$ mice, we also investigated the influence of animal gender on transgene expression and NAB development. IT application of AAV2/9-luc (10^{11} GC per mouse) and subsequent luciferase measurement by *in vivo* imaging revealed 10–50-fold higher expression in female mice compared with their male counterparts (Figure 5c). The difference between the two genders persisted till the end of the experiment (day 120). In an attempt to prevent and/or reduce NAB development, separate groups of $SPB^{-/-}$ mice were given dexamethasone intra-peritoneally for 28 days starting at 3 days prior to viral vector application followed by every third day post-vector application. Dexamethasone dosing scheme is diagrammatically presented as Figure 6a. Application of dexamethasone differentially influenced transgene expression in male and female mice. Though not significant, a trend reflecting higher expression in males and lower expression in females with dexamethasone was observed.

In both gender groups, NABs were detected as early as 1 week post-application and their titers increased over time till week 13 post-application (Figures 6b and c). There was no further increase in NAB titer from 13 to 17 weeks. No gender-specific differences with respect to the kinetics and final NAB titers could be observed. Administration of dexamethasone resulted in a significant reduction in the development of NAB in both gender groups.

Effect of animal gender and dexamethasone treatment on interferon-γ and tumor necrosis factor-α

Increase in the NAB titer over time from 1 to 13 weeks prompted us to investigate if there was any parallel increase in T-cell activity. As markers of T-cell-mediated immune response, we quantified proinflammatory interferon-γ and tumor necrosis factor-α in murine serum using specific enzyme linked immunosorbent assays. There were no significant differences in the amounts of these cytokines measured for any of the compared groups (data not shown). Thus, even though NAB titer increased from 1 to 13 weeks post-viral vector application, there was no concomitant increase in T-cell cytokine levels.

Real-time PCR for vector DNA in $SPB^{-/-}$ mice

To investigate if the differences in luciferase expression between male and female mice were due to differential gene delivery by AAV2/9, total DNA was isolated from the murine lungs at day 120 post-application and vector DNA was quantified using real-time PCR. Neither dexamethasone treatment nor animal gender had any effect on AAV2/9-mediated pulmonary gene delivery (data not shown).

DISCUSSION

Gene therapy holds immense potential for the treatment of numerous inherited and acquired pulmonary diseases. Dominance of viral

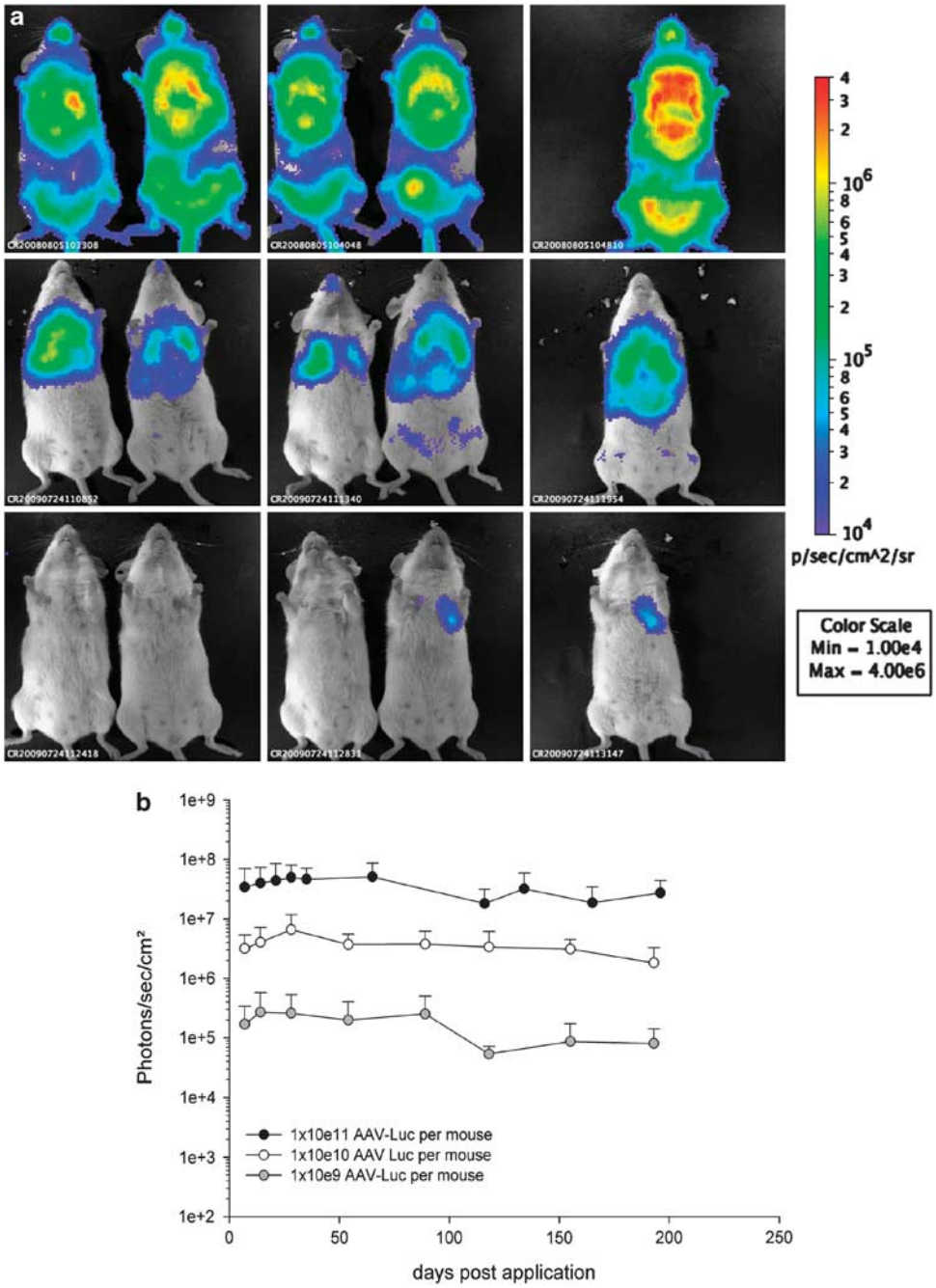

Figure 3 *In vivo* luciferase expression in female BALB/c mice after intratracheal application of 10^{11} (first row), 10^{10} (second row) and 10^{9} (third row) GC per mouse at 28 days post-delivery (n=5) (**a**). *In vivo* bioluminescence images were taken and quantified as described in Materials and methods. Expression was stable in each group. A 10-fold reduction of the dose (10^{10} GC per mouse) resulted in approximately 50-fold lower expression, whereas with a 100-fold reduced dose (10^{9} GC per mouse), luciferase expression was only marginally above background (**b**). Values represent mean ± s.d.

vectors in numerous gene therapy clinical trials can be attributed to their high efficiency of gene transfer. Desirable attributes like lack of pathogenicity, ability to transduce non-dividing cells,[2] prolonged transgene expression and low immunogenicity[3] present AAVs as potential vectors for clinical applications.

The present study successfully demonstrates stable long-term expression using an AAV2/9-based vector in two different mouse strains. No effect of either mouse genotype or pulmonary route of delivery on transgene expression could be observed. $SPB^{-/-}$ mice have an FVB/n background, which is the most commonly used strain for generation of transgenic mice. The use of BALB/c strain in our study was based on the fact that transgenes are more immunogenic in BALB/c mice. In fact, it has been reported that three of the most widely used transgenes lacZ,[16] enhanced green fluorescent protein[30] and firefly luciferase[31] are more immunogenic in BALB/c mice than in C57BL/6 mice. Previous studies reporting long-term expression in the lungs after AAV2/9-mediated delivery were performed in C57BL6 per mice[8,14] using human α1-antitrypsin and nuclear-localized lacZ as marker genes. It is well documented that these mice are immunotolerant to human α1-antitrypsin.[15] Thus, the long-term expression

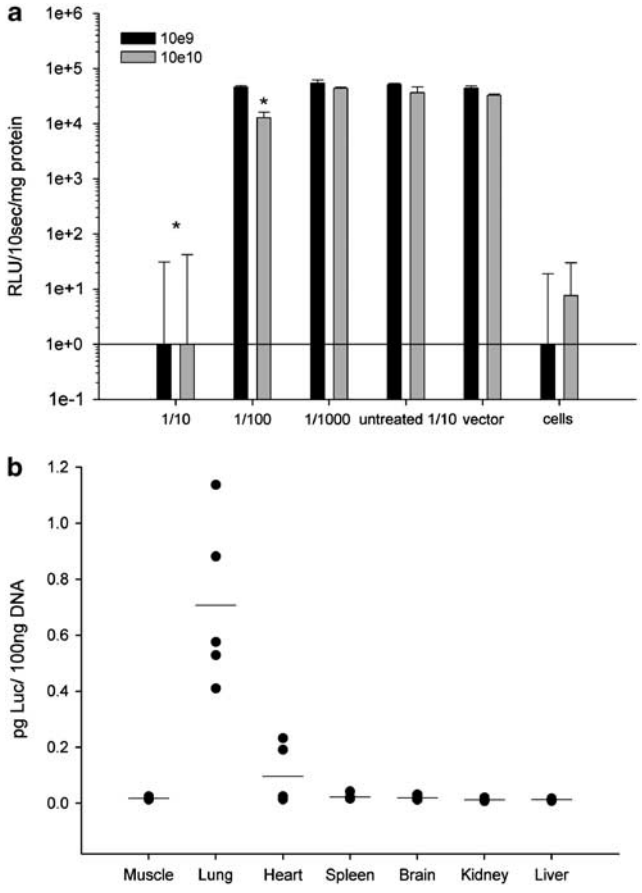

Figure 4 Development of NAB (**a**) and biodistribution (**b**) in female BALB/c mice after intratracheal and intranasal application of 10^{10} and 10^{9} AAV-luc (n=5). Prior to euthanizing, blood was taken to prepare the serum and assay for NAB was performed as described in Materials and methods. Serum of untreated mice, vector without serum and untreated cells were used as controls; 10-fold dilution of the serum from both groups resulted in complete neutralization of the viral vector. Diluting the serum 100-fold resulted in 65 and 10% reduction in luciferase expression, respectively. Significant differences toward the serum of untreated mice are marked with an asterisk ($P \leqslant 0.05$) (**a**). After euthanizing the mice, organs were harvested and real-time PCR was performed with isolated DNA only from mice treated with 10^{10} GC per mouse. Highest vector concentration was observed in lungs (**b**). Values represent mean ± s.d.

observed in these previous studies is most likely to be influenced by the reduced immunogenicity of the transgene (*lacZ*) or the immunotolerant status of the mouse strain. Besides mouse strain, animal gender has also been shown to influence rAAV-mediated gene delivery, transgene expression and biodistribution.[27,28] In a study addressing liver-directed delivery of vector particles, Davidoff *et al*.[27] reported 5- to 13-fold higher transgene expression in male compared with female mice, irrespective of the promoter, cDNA and mouse strain. Moreover, molecular analysis revealed that the rAAV genome was stably retained in male liver at levels that were sevenfold higher than those observed in females. These differences were observed with both rAAV2- and rAAV5-based vectors. In a relatively recent study, Paneda *et al*.[28] reported gender-related differences in rAAV-mediated liver transduction and biodistribution for the serotypes 1, 5, 6 and 8. To the best of our knowledge, there are no prior studies comparing the effect of animal gender on AAV2/9-mediated pulmonary gene transfer. The present study demonstrates gender-specific differences in transgene expression post-AAV2/9-mediated gene delivery. In contrast to the above studies, though there was no difference in gene delivery as measured by real-time PCR, 10–50-fold higher expression was observed in female mice compared with their male counterparts. Such gender-specific differences raise concern over the reported efficiencies of different gene delivery vectors tested in only one animal gender and strengthen the call for future studies investigating gene vector performance in animal models, to be performed in both genders in order to gain a more 'holistic' view of vector potential.

Besides encouraging, these results in the lungs are also surprising because the primary target cells for AAV2/9 are alveolar epithelial cells,[8,14] which are terminally differentiated and based on normal cell turnover rates,[32,33] the expression should steadily decrease with time. Taking different features of AAV2/9 like its selective tropism for alveolar epithelial cells, stability of resulting transgene expression and the episomal persistence of AAV genomes in transduced cells[3,34] together allows us to speculate that cells transduced by AAV2/9 constitute a stable cell population with very low turnover rates. Experiments to identify the cells targeted using AAV2/9 are currently underway. Studies are also underway to investigate the role of lung inflammation on the cellular tropism of AAV2/9.

Relevant for therapeutic application in the clinic is the efficiency of the gene transfer vector in appropriate disease model. Selective tropism of AAV2/9 for alveolar epithelium[8,14] makes SPB$^{-/-}$ mice a suitable animal model to assess the efficiency of AAV2/9 vector in an inflamed lung. AAV2/9-luc was tested in the diseased state of SP-B deficiency in SPB$^{-/-}$ mice. The deficient phenotype was induced by removing doxycycline from their drinking water. It has been shown that adult mice made SP-B deficient by removal of doxycycline, develop progressive lung inflammation (24 h onward) as evidenced by elevated alveolar IL-6, IL-1β and macrophage inflammatory protein-2 concentration and succumb to severe respiratory failure within 4 days post-doxycycline removal. Restoration of SP-B expression following administration of doxycycline rapidly reversed SP-B-dependent inflammation in the lungs.[26] In the present study, mice, which were fed normal water for 2 days prior to viral vector application, died within 24 h post-gene delivery. In mice on normal water for 1 day, no significant difference in expression was observed compared with the control mice on doxycycline containing water. These results point to the resistance of AAV2/9 to pulmonary inflammation and make this serotype highly favorable for further development as a vector for therapeutic applications. To the best of our knowledge, this is the first report investigating the efficiency of AAV2/9 vectors in any model of lung inflammation.

It is now well established that the immunogenicity of a transgene product can be influenced by various parameters that include the genetic background of the experimental mice, vector dose and target tissue. Previous studies have demonstrated the role of transgene and/or vector-specific T cells in the loss of transgene expression in various tissues.[35] Persistence of expression in BALB/c and SPB$^{-/-}$ mice suggests that there is little or no cellular immune response to the vector capsid or luciferase in these mouse strains after AAV2/9-mediated delivery via the airways. In addition to T-cell-mediated immune responses, NAB development is another major limitation of viral vectors and inhibits vector readministration.[1,3] Previous studies addressing repeated dosing with rAAV vectors have provided contradicting data. Successful repeated administration after pretreatment of the host with immunosuppressing antibodies,[19] and/or immune-modulating agents[20] or broad immunosuppressing agents,[21] has been reported. Independent of immunosuppressive/modulating regimens, two studies have observed that rAAV may be efficiently

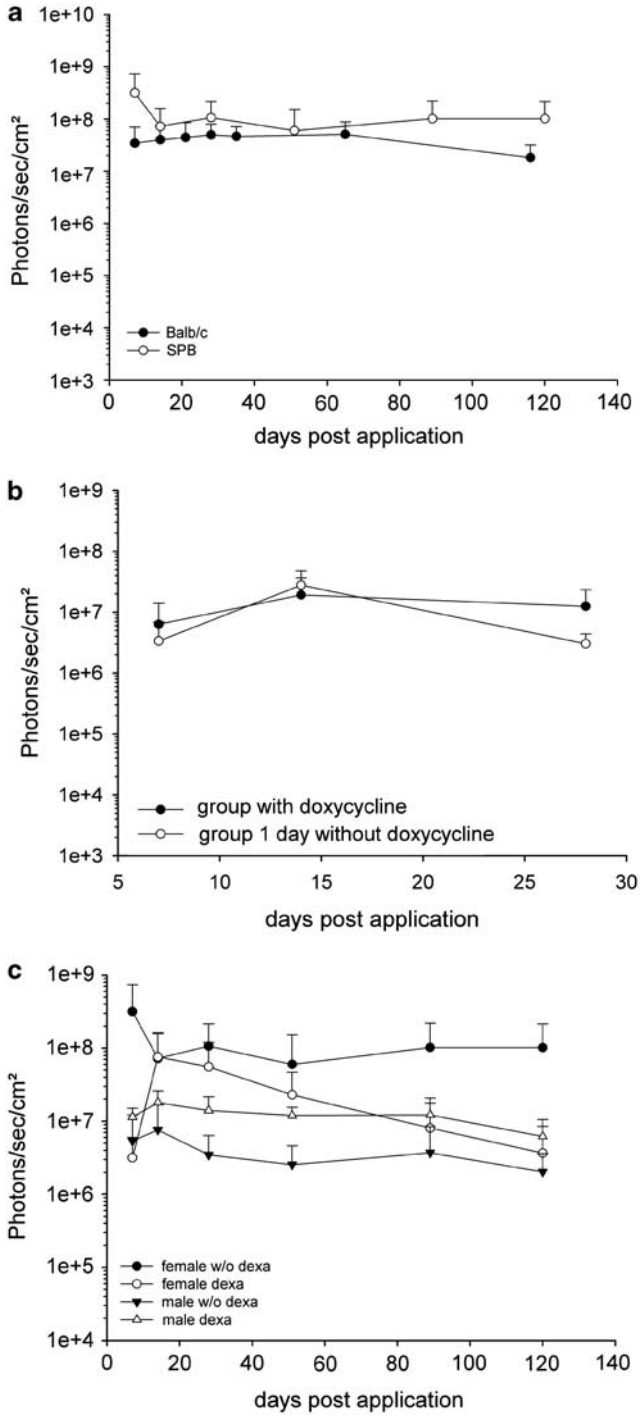

Figure 5 Effect of mouse strain, lung inflammation and animal gender on AAV2/9-mediated transduction. *In vivo* bioluminescence images of mice treated with 10^{11} GC per mouse (n=5) via IT instillation were quantified as described in Materials and methods. Comparison of luciferase expression in female BALB/c and SPB$^{-/-}$ mice (**a**). Influence of lunge inflammation induced by doxycycline removal in male SPB$^{-/-}$ mice (**b**) and influence of gender and immunosuppression with dexamethasone in female and male SPB$^{-/-}$ mice (**c**). Values represent mean ± s.d. Dexamethasone dosing scheme is presented in Figure 6a.

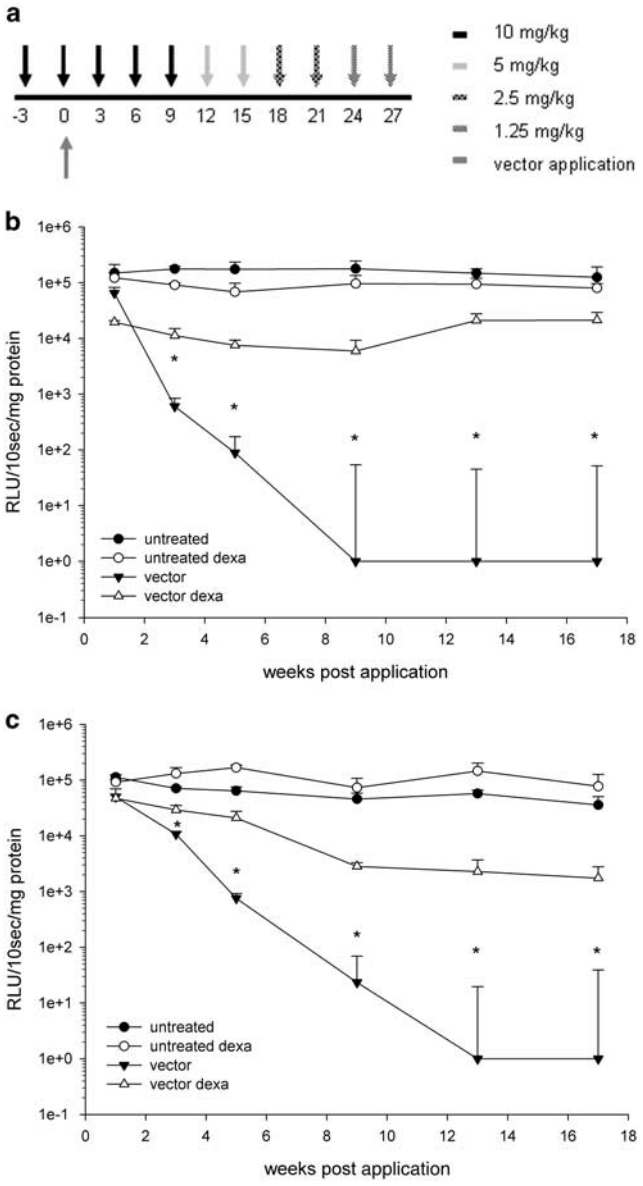

Figure 6 Schematic representation of dexamethasone dosing (**a**) to prevent the development of NAB in female (**b**) and male (**c**) SPB$^{-/-}$ mice after IT instillation of 10^{11} GC per mouse (n=5). At several time-points post-delivery, serum was prepared and NAB assay was performed as described in Materials and methods section. Serum samples of control animals were diluted 10-fold, whereas serum of treated mice was diluted 100-fold. NAB titers increased until 13 weeks post-delivery and animal gender did not affect their development. Immunosuppression with dexamethasone led to significantly reduced NAB titers. Significant differences toward the serum of untreated mice are marked with an asterisk ($P \leqslant 0.05$). Values represent mean ± s.d.

administered a second time if the time between administrations is extended beyond approximately 28 weeks and the capsid is derived from AAV5 or AAV9.[8,22] These results are, however, contradicted by Sumner-Jones *et al.*,[17] where capsid NAB substantially lowered lung gene transfer on a second administration and effectively abolished it on a third dose. Importantly, neither the local expression of CTLA4Ig nor an increase in the period between vector administrations from 8 to 36 weeks improved the efficacy of second or third administrations of rAAV5. In light of such inconsistent data, we are of the opinion that preventing NAB development altogether would be most desirable. In BALB/c mice, we demonstrate that pulmonary route of administration had no influence on NAB development. By using 10- and 100-fold

lower doses, we could significantly reduce NAB titers but lower doses also resulted in drastically reduced transgene expression. In SPB$^{-/-}$ mice, by using transient immunosuppression with dexamthasone, we could significantly reduce the development of NABs in both gender types, thereby overcoming a major hurdle in the way of using AAV-based vectors for gene therapy.

In contrast to transgene expression and NAB development, which were unaffected by the pulmonary route of application, biodistribution studies showed a trend for higher delivery to the lungs combined with lower deposition in spleen and liver when using the IT route. Similar observations have been made previously,[8,12] where >1000-fold higher vector genomes were reported in the lungs compared with other organs. However, no data was presented with respect to the expression in other organs. *In vivo* bioluminescence imaging, vector quantification by real-time PCR and luciferase assays with isolated organs confirm the systemic distribution and persistent expression of the vector in these 'off-target' organs. Though not investigated in the current study, the mechanism of 'transcytosis', as described for different AAV serotypes including AAV5,[36] may be responsible for the systemic spread of AAV2/9 in the present study. Such 'off-target' transgene expression may lead to undesirable effects and, therefore, strategies to prevent this systemic distribution are urgently needed.

These findings bode well for the application of AAV2/9-based vectors, where long-term transgene expression is desired. The performance of AAV2/9 in the presence of lung inflammation puts this serotype close to 'an ideal vector' for use in a therapeutic setting. Prior to clinical application, strategies need to be developed that limit vector biodistribution. Subsequently, AAV2/9 would be an excellent candidate for gene therapy of α1-antitrypsin and surfactant deficiency states, which include neonatal respiratory distress syndrome, congenital pulmonary alveolar proteinosis and surfactant protein(s) deficiencies.

MATERIALS AND METHODS

AAV production

The AAV vector (pENN-AAV-CB7-CI) was kindly provided by Julie Johnston (Penn Vector, Gene Therapy Programme, University of Pennsylvania, PA, USA). Briefly, the vector contains AAV2 inverted terminal repeats flanking an expression cassette containing cytomegalovirus-enhanced chicken β-actin promoter, complete chicken β-actin intron and rabbit globin polyA sequence. Firefly luciferase was excised from pGL3-Basic (Promega, Madison, WI, USA) and cloned into the provided vector. Recombinant AAV2/9 expressing luciferase (AAV2/9-luc) was produced and quantified by Arbans Sandhu (Penn Vector).

Animals

Six to 8-week-old female BALB/c mice were obtained from Charles River Laboratories (Sulzfeld, Germany) and maintained under specific pathogen-free conditions. Mice were acclimatized to the environment of the animal facility for at least 7 days prior to the experiments. Conditional SPB$^{-/-}$ mice were kindly provided by Jeffrey A Whitsett (Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA). They were bread in our animal facility and used at the age of 6–8 weeks. These mice were always kept on doxycycline containing water (200 mg l^{-1}) unless and until indicated. All animal procedures were approved and controlled by the local ethics committee and carried out according to the guidelines of the German law of protection of animal life.

In vivo gene delivery

Experiments with BALB/c mice. Before application, AAV2/9-luc was diluted to 10^9, 10^{10} and 10^{11} GC in 100 µl phosphate-buffered solution. The corresponding doses were applied either IN or IT. For IN application, the technique of sniffing[37] was used. For IT delivery, mice were anesthetized (Medetonidin 11.5 µg kg^{-1}, Midazolam 115 µg kg^{-1} Fentanyl 1.15 µg kg^{-1}) and suspended on a plate system (Hallowell EMC) at a 45° angle by the upper teeth. A modified cold-light otoscope Beta 200 (Heine Optotechnik) was used to provide optimal illumination of the trachea. A small spatula was used to open the lower jaw of the mouse and blunted forceps were used to help displace the tongue for maximal oropharyngeal exposure. The tip of the blunt needle was positioned near the carnia (first bifurcation) so that the viral vector was applied directly to the lung lobes. Post-delivery the anesthesia was antagonized using a mixture of Atipamizol 50 µg kg^{-1}, Flumazanil 10 µg kg^{-1} and Naloxon 24 µg kg^{-1}.

Experiments with SPB$^{-/-}$ mice. Both male and female, 6 to 8-week-old conditional SPB$^{-/-}$ mice were used in different experiments. Each experimental group consisted of five animals and AAV2/9-luc (10^{11}GC per mouse) was applied IT. Dosing scheme of dexamethason, starting at 3 days prior and then at different time points post-vector application is presented in Figure 6a.

Male SPB$^{-/-}$ mice were used to investigate the effect of lung inflammation on AAV2/9-mediated gene delivery. To induce lung inflammation, doxycycline was removed from the drinking water at 1 or 2 days prior to vector instillation following which AAV2/9-luc (10^{11}GC per mouse) was applied IT.

In vivo imaging

At subsequent time points post-vector application, mice were anesthetized and D-luciferin substrate (3 mg per 100 µl phosphate-buffered solution per mouse) was delivered intra-peritoneally. After 10 min, bioluminescence was measured (IVIS 100 imaging system; Xenogen, Alameda, CA, USA) using camera settings field of view 10, f1 f-stop, high-resolution binning and exposure time of 1 min. After the measurement, anesthesia was antagonized. Signal in the lung region was quantified and analyzed by background subtraction using the Living Image Software ver 2.50 (Xenogen).

Organ isolation

After the last luciferase measurement mice were euthanized and internal organs (lung, heart, liver, spleen, kidney, gonads, brain and muscle) were isolated for biodistribution analysis. For conditional SPB$^{-/-}$ mice, only lungs were taken to quantify the delivery of viral vector DNA. Organs were snap frozen in liquid nitrogen and homogenized in the frozen state.

In vitro luciferase quantification

Luciferase activity was quantified in the organs isolated from BALB/c mice from the biodistribution experiment. A portion of the homogenized tissue was used for isolating DNA (see below), whereas the rest was used for quantifying lucifearse expression. After addition of 400 µl of lysis buffer (250 mM Tris pH 7.8, 0.1% Triton X-100, Roche Complete Protease Inhibitor Cocktail Tablets) and incubation for 20 min on ice, luciferase activity in the supernatant was measured using a Lumat LB9507 tube luminometer (EG&G Berthold, Munich, Germany). Values represent mean ± s.d. and are expressed as relative light units per mg tissue.

Real-time PCR

Total DNA was isolated using the DNEasy Blood and Tissue Kit (Qiagen; Hilden, Germany). Homogenized tissues (15–25 mg) were digested and purified according to the manufacturer's instructions. Quantitative analysis of AAV2/9-luc DNA was performed with 100 ng of isolated total DNA using an iCycler IQ Real-Time PCR Detection System (Bio-Rad GmbH, Munich, Germany). Luciferase-specific primers and the corresponding real-time PCR have been described and used previously.[29] A serial dilution of pAAV2/9-luc plasmid ranging from 5 fg to 5 ng was used as a standard.

Assay for NAB

Serum from individual mice treated with AAV2/9-luc was prepared at different time points post-viral vector application and aliquots within the same treatment group and time point were pooled. Pooled serum was diluted 10-, 100- and 1000-fold with phosphate-buffered solution in a total volume of 90 µl per dilution. The diluted samples were mixed with equal volume of AAV2/9-luc containing 8×10^9 GC and incubated for 1 h at room temperature. The vector/sera mixture was used to transduce mouse transformed lung epithelial 12 cells (2×10^9 GC per well). These cells were seeded in 24-well plates 1

day before transduction. At 72 h post-transduction, cells were lysed and luciferase activity was determined using a Wallac Victor^2 1420 Multilabel Counter according to Huth *et al.*[38] Results were normalized to total cell protein using Bio-Rad Protein Assay (Bio-Rad, Munich, Germany) with bovine serum albumin as protein standard. Percentage inhibition of transduction was calculated using 10-fold diluted sera from untreated mice as control.

Statistical analysis

Results are reported as mean values ± s.d. Statistically significant differences were evaluated by a non-paired Student's *t*-test. Probabilities of $P \leqslant 0.05$ were considered as significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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