Immunological ignorance allows long-term gene expression following perinatal rAAV-mediated gene transfer to murine airways

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Short title: Long-term expression after perinatal gene transfer
Abstract

Gene therapy of the lung has the potential to treat life-threatening diseases such as cystic fibrosis (CF) and α-1-antitrypsin or surfactant deficiencies. A major hurdle for successful gene therapy is the development of an immune response against the transgene and/or viral vector. We hypothesized that by targeting the airways in the perinatal period, induction of an immune response against the vector particle could be prevented due to immaturity of the immune system, in turn allowing repeated gene transfer later in adult life to ensure long-term gene expression. Therefore, we readministered recombinant adeno-associated viral vector serotype 5 (rAAV2/5) to mouse airways at 3 and 6 months after initial perinatal gene transfer. Our findings demonstrate that perinatal rAAV2/5-mediated gene transfer to the airways avoids a strong immune response. This immunological ignorance allows the readministration of an autologous vector later in adult life, resulting in efficient and stable gene transfer up to 7 months, without evidence for a decrease in transgene expression. Together, these data provide a basis to further explore perinatal gene therapy for pulmonary conditions with adequate gene expression up to 7 months.
**Introduction**

Recent breakthroughs in gene therapy for congenital blindness and adrenoleukodystrophy demonstrate the feasibility of successful and safe gene therapy (reviewed in Sheridan, 2011). Although gene therapy for pulmonary disorders (i.e. cystic fibrosis, CF) has been at the forefront of the gene therapy field, efficient gene transfer to the airways remains more challenging than anticipated at first. Initial viral vector-mediated gene therapy trials for inherited disorders of the airways were based on adeno- (AdV) (Zabner et al., 1993; Crystal et al., 1994) and later adeno-associated viral vectors (rAAV) (Wagner et al., 1999; Flotte et al., 2003). In contrast to promising results in preclinical animal models where long-term gene expression was demonstrated (Limberis and Wilson, 2006; Sumner-Jones et al., 2006; Liqun Wang et al., 2009), clinical trials have only shown limited successes so far due to transient and/or inefficient gene expression in the human airways (Harvey et al., 1999; Wagner et al., 2002; Moss et al., 2007). This could be attributed to the non-integrating nature of the vector, the lack of appropriate receptors at the apical side of the respiratory epithelium, induction of an immune response against the transgene or vector particle and the presence of pre-existing immunity to the vector (reviewed in Mingozzi and High, 2011). Further preclinical research is therefore a prerequisite to investigate how to overcome the obstacles encountered in clinical trials.

rAAV are promising viral vectors for gene therapy as many different AAV serotypes with a specific tissue tropism exist (Gao et al., 2002; Gao et al., 2004). Several of these serotypes (i.e. AAV5, AAV6, AAV6.2 and AAV9) have been shown to target the airway epithelium (Zabner et al., 2000; Auricchio et al., 2002; Limberis and Wilson, 2006; Limberis et al., 2009; Carlon et al., 2010).
rAAV vectors are regarded as safe gene delivery vehicles as they are devoid of all viral genes and the wild-type (WT) adeno-associated virus (AAV) they are derived from has, to date, not been associated with any known human pathology (Dismuke et al., 2013).

A major hurdle for gene therapy is the development of an immune response against the transgenic protein and/or the viral vector itself. This was illustrated by a cellular immune response raised against the vector capsid reducing FIX expression in hemophilia B patients following rAAV2/2-based gene therapy (Manno et al., 2006). Also, in clinical gene therapy studies conducted in adults, an adaptive immune response against the rAAV capsid, induced after the first vector dose, hampered repeated administrations (Moss et al., 2004; Mueller and Flotte, 2008). Expression of foreign proteins in the fetus, before the immune system is fully developed, may avoid immune activation. Proof-of-principle fetal gene therapy studies have shown long-term expression both of reporter and therapeutic proteins and induction of immune tolerance against the transgene in small and large animals (Tran et al., 2001; Waddington et al., 2003; Sabatino et al., 2007). Other theoretical advantages of fetal gene transfer include the administration before the onset of pathological changes that hamper gene delivery (e.g. mucus in CF), a longer contact time of the vector, a higher vector-to-target-cell ratio and the presence of expanding stem and progenitor populations. As long as a phenotypic correction is not required before birth, these concepts also stand for gene transfer applications at the early neonatal stage. Although most theoretical advantages relate to a fetal approach, the benefits of the intervention must be weighed against the inherent risk for complications of the procedure (Deprest et al., 2009), rendering a neonatal approach favorable and more easily accepted from an ethical point of view.
In previous work, we demonstrated efficient reporter gene expression in the lungs up to 4 weeks after rAAV delivery to fetal airways (Carlon et al., 2010). In the current study, we validated the efficiency of rAAV2/5-mediated perinatal (fetal and neonatal) gene delivery to the murine airways and monitored long-term reporter gene expression. Transduction of actively dividing tissue, such as the airway epithelium, with a non-integrating rAAV vector requires repeated vector administration(s) to achieve long-term gene correction. We hypothesized that due to the immaturity of the immune system during perinatal gene transfer, an immune response against the vector particle could be prevented, allowing repeated administration(s) in adult life. Our results indeed demonstrate long-term gene expression after perinatal gene transfer and subsequent readministration. Analysis of capsid-specific neutralizing Ab (nAb) indicated low immunoreactivity against the rAAV vector after perinatal gene transfer. This allowed effective vector readministration in adult life, resulting in efficient reporter gene expression in the murine airways up to 7 months. Since multiple readministrations were not feasible, we conclude that immunological ignorance rather than tolerance underlies successful readministration of an autologous vector.
Results

Efficient but transient gene expression in murine airways after perinatal rAAV2/5-based gene transfer

To study stability of gene expression after perinatal rAAV2/5 delivery in the upper (nose) and lower (lungs) murine airways, we compared gene expression over time after fetal and neonatal rAAV2/5 delivery of firefly luciferase (fLUC) (1.5 E+10 genome copies (GC)/animal) and β-galactosidase (β-gal) (1 E+10 GC/animal), n=3-4 per time point per group. Analysis of lung sections showed average transduction efficiencies (% β-gal positive cells) of 6.6 ± 1.1% in the fetal, and 8.9 ± 1.5% in the neonatal group at 1 month post-injection (pi) (Figure 1A). A 6-fold decrease was noted between 1 and 3 months in the neonatal group (p<0.001), in contrast to the fetal group where the transduction efficiency did not change significantly (1.7-fold decrease, p=0.18). Similar data were obtained for fLUC until 3 months pi (Figure S1). In parallel, we quantified the efficiency of nasal transduction (Figure 1B). Starting at 11.6 ± 1.5% and 20.5 ± 3.6% β-gal positive cells 1 month after gene delivery for fetal and neonatal treatment respectively, the number of transduced cells decreased significantly over the next 2 months for both groups (p<0.001 for fetal and p<0.01 for neonatal), resulting in a residual transduction level at 3 months of 0.3 ± 0.1% in the fetal group compared to 4.0 ± 1.1% in the neonatal group. Although the airways were efficiently transduced, gene expression markedly decreased by month 3, providing grounds for vector readministration to establish long-term expression.

Successful readministration of rAAV2/5 after initial perinatal gene transfer

Next, we wanted to evaluate if readministration of an autologous vector was successful after initial perinatal gene transfer. In Figure 2A, a schematic overview is given of the experiment
designed to study the feasibility of rAAV2/5 readministration to the murine upper and lower airways. rAAV2/5 encoding fLUC (1.5 E+10 GC/animal) was delivered by intra-amniotic (I.A.) injection to fetal FVB/N mice at E18 (term E19.5, n=19) and by sniffing to neonatal pups at day 3 (n=22). At 3 months pi, a second dose of the same vector was given by intratracheal (I.T.) instillation to approximately half of the animals (readministration group, n=11 for the fetal group, n=12 for the neonatal group), whereas the other half of the group was kept as non-readministration controls (n=8 for the fetal group, n=10 for the neonatal group). Gene expression levels were monitored using bioluminescence imaging (BLI) at monthly intervals till 4 months post primary administration (Figure 2B,C and E,F for lung and nose, respectively). An adult control group (2-3 months of age, n=6) was included to control for the maximum level of gene expression expected in the upper and lower airways after a single I.T. instillation of rAAV2/5-fLUC (Figure 2D,G). At 1 month pi, a comparable BLI signal was detected in the lungs between the groups (Figure 2B,C). The photon flux however declined significantly in both groups at 3 months pi (5.3-fold, p<0.001 for the fetal and 5.8-fold, p<0.001 for the neonatal group). Also for the nose, a significant decrease was observed between 1 and 3 months, resulting in a 5.4-fold decrease for the fetal group (p<0.001) and a 7.3-fold decrease for neonatal animals (p<0.001).

At this point (3 months post primary administration), rAAV2/5-fLUC (1.5 E+10 GC/animal) was readministered by I.T. instillation (indicated by a red arrow, Figure 2B,C,E,F). fLUC activity showed a significant 5.8-fold increase in lung signal after readministration to the fetal group (3.2 ± 0.5 E+06 p/s, n=11, compared to 5.5 ± 2.8 E+05 p/s for non-readministered controls, n=8, p<0.001, Figure 2B); likewise, the signal in the nose increased 10.3-fold in readministered fetal animals (6.3 ± 1.7 E+06 p/s) compared to controls (6.1 ± 2.8 E+05 p/s, p<0.01, Figure 2E). For
the neonatal group, a 5-fold increase was noted in the lung (1.1 ± 0.2 E+06 p/s, n=12, compared to 2.2 ± 0.3 E+05 p/s, n=10, p<0.001, Figure 2C), whereas no increase was observed in the nose (2.0 ± 0.8 E+06 p/s compared to 2.8 ± 1.7 E+06 p/s, p=0.98, Figure 2F). When comparing the BLI signal after readministration to that obtained in adult control mice receiving a single vector dose (Figure 2D,G), animals in the fetal group reached comparable transduction efficiencies as adult controls for lung and nose, whereas the signal in neonatal animals was significantly lower (34%, p<0.01 and 20%, p<0.001 for lung and nose signal, respectively).

Comparable results were obtained in an independent experiment with a similar set-up (Figure S2). Only here, rAAV2/5-β-gal (1.0 E+10 GC/animal) was co-injected to evaluate vector distribution in tissue sections. At 3 months, a subset of animals was readministered with rAAV2/5-β-gal (2.0 E+10 GC/animal) and rAAV2/5-fLUC (3.0 E+10 GC/animal). fLUC activity in the lung increased 16.2-fold in the fetal and the neonatal group (n=4 and 3, respectively) compared to non-readministered animals (n=4 and 6, p=0.08 and p=0.12, respectively) (Figure S2A,B). Whereas a 33.3-fold increase in nose signal was obtained for the fetal group (p=0.24), only a minimal increase of 4.4-fold was measured in the neonatal group (p=0.65) (Figure S2D,E). On X-gal staining, an increase in β-gal positive cells was noted in the conducting airways 1 month post readministration both in the fetal as well as in the neonatal group (Figure S3). In the nasal respiratory epithelium however, an increase was only noted after readministration in the fetal group (Figure S4).

Together, our experiments, carried out twice, demonstrate that readministration of an autologous vector 3 months after fetal or neonatal rAAV2/5 delivery results in efficient re-transduction of the lungs, but results only in an increase in nasal gene expression after initial fetal rAAV2/5 delivery.
Analysis of nAb against the rAAV2/5 capsid after perinatal gene transfer and after readministration

To study the immunological mechanism underlying this successful readministration, we evaluated whether an adaptive immune response against the rAAV2/5 capsid was induced after perinatal gene transfer and after readministration by analyzing nAb against the capsid in serum and bronchoalveolar lavage fluid (BALF) (Figure 3). As controls, we included non-treated animals (neg, n=5) and adult control mice that received a single vector dose (adult ctrl, n=6). At 4 months after perinatal gene transfer, only a weak capsid-specific humoral immune response was observed in serum of the fetal group (n=8; inhibition of 28.8%, 12.8% and 6.7% at 1/40, 1/160 and 1/640 serum dilutions; Figure 3A, left segment), whereas in the neonatal group (n=10) inhibition reached 99.8%, 89.9% and 56.9%, respectively. For comparison, serum of adult controls inhibited transduction completely at a 1/640 dilution 1 month pi (99.1% inhibition).

1 month after vector readministration however (Figure 3A, right segment) a complete inhibition of transduction (≥97% inhibition at 1/640 serum dilution) was measured for both the fetal (fetal-R; n=11) and neonatal group (neonatal-R; n=12), in line with adult control mice (Figure 3A, right segment). Of note, further serum dilutions allowed rAAV2/5-fLUC transduction to the same extent in all 3 treatment groups, indicating comparable serum nAb concentrations (Figure S5). These results indicate that after fetal administration only a weak capsid-specific humoral immune response is induced, whereas after neonatal gene transfer a more pronounced immune response is observed. However, after readministration, nAb levels increased in both groups to comparable levels as in the adult mice that received a single vector dose.
In addition, we explored the local immune response in the lungs by sampling the BALF. 4 months after fetal ($n=4$) or neonatal ($n=5$) vector delivery, capsid-specific nAb were undetectable in BALF samples (Figure 3B, left segment). However, 1 month after readministration, nAb increased in both treatment groups (fetal-R, $n=6$; neonatal-R, $n=7$), comparable to that of adult controls ($n=6$) (Figure 3B, right segment).

Together, these results indicate that although only low immunoreactivity against the rAAV2/5 capsid was present after perinatal rAAV2/5 vector delivery either in serum or BALF, a strong capsid-specific humoral immune response was induced upon readministration to the same extent as a single administration in adult animals (compare fetal-R and neonatal-R to adult ctrl, Figure 3A,B), arguing against the establishment of tolerance to the viral vector.

**Increased immune cell infiltration in the re-transduced lung after rAAV2/5 readministration**

We then studied the local immune response in the re-transduced lung in more detail. First, we analyzed relative differences in B cell and regulatory T cell (Treg) populations in BALF (Figure S6). As controls, we included BALF of non-treated animals ($n=5$) and adult controls ($n=6$). At 4 months after perinatal treatment, B cell numbers (B220+) in the fetal ($n=4$) and neonatal ($n=5$) group were not different compared to negative controls (Figure S6A, left segment). Likewise, the increase in B cells in readministration animals (fetal-R, $n=6$; neonatal-R, $n=7$) was comparable to adult controls (Figure S6A, right segment). Treg (CD4+ Foxp3+) levels increased in the perinatal treatment groups after readministration (Figure S6B, right segment); they were significantly higher than in negative controls ($p<0.01$ for both groups), but not different compared to adult controls. This response can be attributed to the fact that Tregs accumulate in
regions undergoing tissue inflammation (Kamikozuru et al., 2009). H&E stainings on lung sections (Figure 4A) demonstrated infiltration of mononuclear cells around conducting airways and blood vessels after readministration (Figure 4A, middle and bottom row), a signal which was absent in non-readministered animals (Figure 4A, top row). Scoring of lung sections for inflammation after perinatal rAAV2/5 administration (Table 1) showed minimal infiltration, comparable to negative animals. Readministration resulted in increased lymphocytic infiltration around the conducting airways and blood vessels comparable to adult controls. More detailed analysis detected CD4+ as well as CD8+ T cells in the lymphocytic infiltrates (Figure 4B,C, bottom row).

These data demonstrate that despite low immunoreactivity in the transduced lung after perinatal rAAV2/5 delivery, a strong local adaptive immune response is induced upon re-transduction of the lung, comparable to a single rAAV2/5 administration in adult animals. This indicates that immunological ignorance rather than tolerance is at play. The increase in Tregs after readministration is likely secondary to induced tissue inflammation and does not play a role in the induction and maintenance of immunological tolerance.

A second vector readministration does not increase gene expression after perinatal gene transfer

In a next step, we evaluated a second readministration of rAAV2/5-fLUC (1.5 E+10 GC/animal) at 6 months after perinatal treatment or 3 months after the first readministration (n=5 for the fetal and neonatal group) (Figure 5A). After an increase in BLI signal measured 1 month post primary readministration (described previously in Figure 2, but shown again in Figure 5 in grey dots), only a minor decrease in BLI signal was noted for the fetal and neonatal group between 4
and 6 months (1.4-fold, p=0.47 (fetal) and 1.2-fold, p=0.77 (neonatal) for lung (Figure 5B,C); 1.9-fold, p=0.80 (fetal) and 3.1-fold, p=0.50 (neonatal) for nose (Figure 5E,F)). Following the second readministration (orange arrow in Figure 5B,C and E,F), lung and nose signals remained unchanged when analyzed 1 month later both in the fetal group (p=0.79 for lung; p=0.79 for nose) (Figure 5B,E) and in the neonatal group (p=0.08 for lung; p=0.13 for nose) (Figure 5C,F). Compared to adult controls receiving a single vector dose, the lung signal after the second readministration in the fetal group was only 32% (p<0.01, Figure 5D) and the nose signal only 16% (p<0.001, Figure 5G). For the neonatal group, 31% and 5% of the lung and nose signal of adult control mice was obtained (p<0.01 and p<0.0001, respectively, Figure 5D,G).

The failure to re-transduce the airways efficiently by a second readministration (Figure 5) can be attributed to the presence of capsid-specific nAb, measured both 1 month (described previously in Figure 3) and 3 months post primary readministration (i.e. prior to the second readministration, Figure S7), supporting the inhibitory role of nAb in preventing re-transduction after the second readministration (Figure 5).

In conclusion, our results demonstrate long-term gene expression up to 7 months after initial perinatal gene transfer followed by a single readministration in adult life. Analysis of capsid-specific nAb indicated low immunoreactivity against the rAAV vector after perinatal gene transfer, but a significant increase in nAb levels after readministration. Additionally, multiple readministrations were not feasible, demonstrating that immunological ignorance rather than tolerance was induced against rAAV2/5 capsid after perinatal gene transfer.
Discussion

Transduction with a non-integrating rAAV vector results in transient gene expression in actively dividing cells and remains a challenge in many gene therapy studies (Flageul et al., 2009; Wang et al., 2012). Currently, many methods are evaluated for rAAV readministration, e.g. by serotype switching (Weinstein et al., 2010; Wang et al., 2012), capsid engineering (Vandenberghe et al., 2009) or immune modulation and tolerance induction to prevent immune responses to the vector and/or transgene (Goudy et al., 2011; Wang et al., 2011). Alternatively, the viral vector can be administered to an immature immune system by perinatal gene therapy (Sabatino et al., 2007; Sinn et al., 2008; Hu et al., 2011). We report here for the first time the successful readministration of rAAV2/5 to the upper and lower murine airways after initial perinatal gene transfer.

In an effort to better understand the underlying immunological mechanism, we detected a significantly lower level of capsid-specific nAb after perinatal treatment compared to adult gene delivery (Figure 3). These findings reflect the difference in immune system maturity at the moment of vector delivery, explaining successful rAAV2/5 readministration in adult life after initial perinatal gene transfer. However, 1 month after the first readministration, an increase in capsid-specific nAb, B cells and Tregs was observed in serum and BALF for both the fetal and neonatal treatment group (Figures 3 and Figure S6), comparable to adult control mice that received a single vector dose. These data demonstrate that a primary immune response against the rAAV2/5 capsid was generated only after the first readministration, indicating that immunological ignorance rather than tolerance is induced against the vector particle. Similar to our observations, adenoviral fLUC-vector (AdV-fLUC) delivery to the pre-immune fetus by
intrahepatic injection in CD-1 mice did not induce immune tolerance to fLUC or AdV proteins (Lipshutz et al., 2000). These results demonstrate that fetal administration of a viral vector does not necessarily lead to induction of tolerance against foreign proteins, such as vector or transgenic proteins, despite the immaturity of the immune system.

We demonstrated that readministration to the mouse airways was feasible after both fetal and neonatal rAAV2/5 gene delivery. However, significantly higher expression levels were obtained after fetal compared to neonatal gene transfer. This might be explained by the degree of immune system maturity at the time of vector administration. While the immune system of the mouse fetus is still immature at E18, 2-3 day old neonatal pups are on the border of developing a relatively mature immune system (Darrasse-Jeze et al., 2005; Fontenot et al., 2005; Liston and Rudensky, 2007). This would suggest that full immune ignorance was induced after fetal gene delivery, in contrast to only partial ignorance after neonatal gene transfer at day 3. Although immune system immaturity of neonatal mice less than 3 days of age resembles that of fetal mice, the human immune system is relatively well developed in utero by gestational week 23 (West, 2002), supporting the importance to further study and optimize fetal gene therapy in a preclinical context.

Although this study did not focus on a potential immune response against the transgenes, CD4+ and CD8+ T cells were detected in peribronchial and perivascular infiltrates in the lung after retransduction following initial fetal and neonatal gene transfer, in line with the adult controls (Figure 4 B,C). This demonstrates that not only a humoral immune response was mounted against the foreign proteins (rAAV capsid and/or transgenes), but also a cellular immune response. However, we cannot discern whether CD4+ or CD8+ T cells infiltrated the lung as part of an immune reaction towards the transgene or the rAAV capsid. Of note, gene expression
remained stable over the span of 7 months (the end point of our study) after initial perinatal gene transfer and subsequent readministration in the airways. Therefore, despite the presence of CD8\(^+\) T cells in the peribronchial and perivascular infiltrates, there was no apparent cytotoxic response giving rise to a loss of transduced cells. In clinical trials using rAAV for hemophilia B patients, the stability of gene expression was hampered by a capsid-specific cytotoxic T cell response (Mingozzi et al., 2007; Nathwani et al., 2011), which was not predicted by preclinical animal studies (Li et al., 2007; Wang et al., 2007). This emphasizes the need to study the cellular immune response against the transgene and the rAAV capsid in more detail in different animal models.

The marked reduction in transduced cells in the upper and lower airways 3 months after fetal and neonatal gene transfer (Figure 1) can be explained by profound proliferation of airway epithelium early in life. Although the turn-over of airway epithelium in adult mice is typically considered to be about 100 days (Rawlins and Hogan, 2006), the proliferation rate in the growing mouse lung is presumably a lot higher, although detailed information is lacking to date. Cell proliferation will lead to dilution of the episomal rAAV genomes. This is supported by the fact that contrary to the strong decrease in lung signal at 3 months post perinatal treatment (Figure 1, 2), the same dose administered to adult mice resulted in stable gene expression (unpublished observations), in line with data published by Wilson and colleagues (Wang et al., 2012).

Results on the feasibility of repeated rAAV delivery vary to a large extent and may depend on the host, delivery route, target organ, time point of administration, AAV serotype or vector dose tested (Halbert et al., 1997; Beck et al., 1999; Halbert et al., 2000). Although a single readministration was successful in our experimental set-up, a second readministration 3 months later did not lead to an increase in gene expression (Figure 5). This failure correlated with an
increase in capsid-specific nAb after the first readministration which persisted until the time point of the second readministration (Figure S7). Also in a CF clinical trial, repeated administration of rAAV2/2 (3 doses, one month apart) was unsuccessful (Moss et al., 2004). However, there is preclinical evidence that in certain settings, rAAV vectors can be readministered (Auricchio et al., 2002; Limberis and Wilson, 2006), for example by delaying the time point of readministration (Limberis and Wilson, 2006).

In conclusion, perinatal gene transfer to the airways using rAAV2/5 does not evoke a strong immune response. In our hands, this immunological ignorance allowed readministration of an autologous vector, resulting in efficient and stable gene transfer up to 7 months, without evidence for a decrease in gene expression (Figure 5). For permanent gene correction, later time points should be monitored to evaluate the stability of gene expression. However, our data already provide a basis to study perinatal gene therapy for pulmonary conditions where temporary gene expression is sufficient, e.g. bronchopulmonary dysplasia. Furthermore, the current preclinical gene therapy protocol can prove useful to delay pathological changes which already occur in utero until other therapeutic approaches can be applied after birth. As we did not observe a decrease in gene expression after 7 months, this gene therapy model also offers perspectives to study gene transfer for pulmonary diseases that require permanent genetic correction. Moreover, this mouse model allows basic immunological studies on immune tolerance and ignorance in the context of gene therapy.
Materials and methods

rAAV2/5 production

rAAV2/5 CBA-eGFP-P2A-fLUC or CBA-β-gal were produced and purified as previously described (Van der Perren et al., 2011). Vector titers were determined by real time PCR using the primer probe set for the polyA sequence (Limberis et al., 2009). rAAV titers are presented as DNase resistant GC/mL (Gao et al., 2002).

Vector delivery to perinatal or adult murine airways

For all experiments, FVB/N mice were used (Janvier, Le Genest St Isle, France). Fetal vector delivery by I.A. injection (60 μL of vector suspension) was performed as previously described (Carlon et al., 2010; Carlon et al., 2012). Neonatal vector administration to the airways was performed by sniffing at day 3 post partum. Pups were immobilized in upright position and vector (60 μL vector suspension) was administered drop per drop into one nostril over a time period of 2 h while pups were kept warm by placing them on a heating pad in between intranasal vector administrations. I.T. vector instillation was performed in adult mice 8-12 weeks of age. After exposure of the trachea through a midline incision, 160 μl of vector diluted in PBS was instilled into the trachea with a 26 G PTFE catheter (BD Vasculon Plus, VWR, Vienna, Austria) using a stereoscopic zoom microscope (x3 magnification). All animal procedures were carried out under “Biosafety level 2” conditions and approved by the KU Leuven Biosafety Committee (laboratory accreditation number LA1210579; ethical committee approval number P065/2009).
Bioluminescence imaging

BLI was performed as previously described (Carlon et al., 2010). Measurements are reported as the total photon flux from a 4.3 cm² rectangular region of interest (ROI) for the lung and a 2.0 cm² circular ROI for the nose.

X-gal staining for β-gal expression in tissue sections

Processing of lung samples and quantification of transduction efficiency in the conducting airways was determined as previously described (Carlon et al., 2010). Briefly, transduction efficiency of the conducting airways was determined using a computerized image analysis system (StereoInvestigator; MicroBrightField, Magdeburg, Germany). Cell type status was assigned based on morphology of β-gal positive cells counterstained with Mayer’s paracarmine. Three sections, spaced approximately 200 µm apart, were analyzed per animal and the data presented as the number of β-gal positive cells relative to the total number of cells in the conducting airways. To determine the percentage of positive cells in the conducting airways, the trachea, bronchi or bronchioles were delineated and all β-gal positive cells counted, followed by quantification of all the epithelial cells.

Noses were processed and stained with X-gal solution (Sigma, Diegem, Belgium) as described (Limberis et al., 2007), followed by Mayer’s paracarmine counterstaining. The number of transduced cells was counted in three standard cross-sections covering the proximal, central and distal nose region as described previously (Parsons et al., 1998). Cross-sections were analyzed by counting the number of β-gal positive cells in the epithelium around the entire perimeter of the nasal airway in the three sections relative to the total amount of epithelial cells. Only respiratory epithelial cells (from all three section levels) were included in the cell counts as the
respiratory epithelium possesses similarities to lung conducting airway respiratory epithelium (Grubb and Boucher, 1999). β-gal positive cells were visualized using a Leica Biopoint 2 light microscope. Brightness and contrast were optimized using Adobe Photoshop CS3.

**Immunohistochemistry and H&E staining on lung sections**

Frozen sections (6 µm) were fixed in cold (−20°C) acetone for 15 min. Sections were stained with a rat mAb specific for CD4 (GK1.5; in-house supernatant) and a rat mAb specific for CD8 (clone 2.43; in-house supernatant). For immunofluorescence, the following detection Ab were used: Alexa Fluor488–donkey anti-rat (A21208; Molecular Probes, Gent, Belgium) and DAPI (D1306; Molecular Probes). Images were acquired with an LSM 510 Meta confocal microscope (Zeiss). Adjacent lung sections of 6 µm were stained with H&E to visualize immune cell infiltration using a Leica Biopoint 2 light microscope.

**rAAV transduction inhibition assay**

Individual blood samples were collected at different time points from animals by retro-orbital bleeding. BALF samples were collected at the time point of harvest (1 month after the first or second readministration) by flushing 2 mL of PBS through the trachea. Serum and BALF samples were heat-inactivated for 45 min at 56°C. HEK293T cells were seeded at 5.0 E+04 cells/well into 96 well-plates on day 1. The next day, 4-fold dilutions of serum or BALF samples in DMEM without FCS were prepared. Wells with the same volume of DMEM served as no-serum or no-BALF controls. Approximately 1.0 E+09 GC/well of rAAV2/5-CBA-eGFP-P2A-fLUC in DMEM were added to serum or BALF samples or control wells, incubated at 37°C for 60 min and added to the cells (100 µL final volume). 1 h later, 100 µL/well DMEM + 20% FCS was added. 24 h later, the transduction efficiency was analyzed by quantifying fLUC activity.
The percentage of inhibition was calculated relative to positive (i.e. no-serum or no-BALF) controls.

**Luciferase assay**

Cells were lysed in 70 µL of lysis buffer (50 mM Tris pH 7.5, 200 mM NaCl, 0.2% Nonidet P-40 (NP-40), 10% glycerol) and the lysate was assayed according to the manufacturer’s protocol (ONE-Glo luciferase assay system; Promega, Madison, WI). Luciferase activity was normalized to total protein determined by BCA assay (Pierce Biotechnology, Rockford, IL).

**FACS analysis of BALF samples**

After BALF collection, samples were centrifuged at 400 g for 5 min to collect the cell pellet. Lymphocytes were surface stained for 30 min at 4°C with phycoerythrin-conjugated anti-CD45R (B220; RA3-6B2; eBioscience, Vienna, Austria) and allophycocyanin-H7-conjugated anti-CD4 (GK1.5; BD Biosciences, Erembodegem, Belgium), before fixation and permeabilization with the eBioscience Foxp3 Fixation/Permeabilization kit (cat.nr. 00-5523-00, eBioscience). Cells were then stained for 30 min at 4°C with anti-Foxp3-APC (FJK-16s; eBioscience). Analysis was performed using a BD FACS Canto II flow cytometer (BD Biosciences) with FlowJo version 10.0.6 analysis software (Tree Star, Inc.)

**Statistical analysis**

General changes in BLI signal over time were analyzed using repeated measures ANOVA. Comparisons between groups at specific time points were performed using a one-way ANOVA followed by a Tukey HSD post-hoc test. Changes in β-gal signal in each group separately over time were analyzed using a mixed model ANOVA, including animal ID as a random effect,
group as a fixed effect and time as a covariate. For FACS analysis and rAAV transduction inhibition data, statistical significance was evaluated using ANOVA to compare different treatment groups. Student's t-test was subsequently used for pair-wise comparisons. A p-value < 0.05 was considered statistically significant. Data are presented as mean ± SEM, unless stated otherwise. STATISTICA (version 8.0) software (StatSoft, Tulsa, OK, United States) was used for statistical analysis.
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Author Disclosure Statement

No competing financial interests exist.
References


Li H., Murphy S. L., Giles-Davis W., Edmonson S., et al. (2007). Pre-existing AAV capsid-specific CD8+ T cells are unable to eliminate AAV-transduced hepatocytes. Mol Ther 15, 792-800.


Figure legends
Figure 1: Perinatal rAAV2/5-mediated gene transfer to murine airways

(A,B) β-gal expression in the airways after fetal and neonatal rAAV2/5 administration (1.0 E+10 GC/animal) at 1 month pi. (A) Lower airways (lung), representative images of the conducting airways (trachea, bronchi and bronchioles) at 200x magnification. (B) Upper airways (nose), showing representative images of the proximal nose region (left images, 50x) and a portion of the nasal epithelium (right images, 200x). The graphs visualize the average transduction efficiency in the conducting airways or nasal epithelium. For both the lower and upper airways,
the relative contribution of the different regions to the total transduction efficiency is visualized by the different shadings described in the legends. Mixed model ANOVA, mean ± SEM, **p<0.01, ***p<0.001. Scale bar = 100 µm for lung sections, scale bar = 200 and 100 µm respectively for low and high magnification nose sections.
Immunological ignorance allows long-term gene expression following perinatal rAAV-mediated gene transfer to murine airways (doi: 10.1089/hum.2013.196).
Figure 2: Successful readministration of rAAV2/5 after initial perinatal gene transfer

(A) Overview of the experiment designed to study the feasibility of rAAV2/5 (1.5 E+10 GC/animal) readministration to murine airways after perinatal gene delivery. fLUC expression was visualized (photos below) and quantified (B-G) over time using BLI. The pseudocolor scale of BL images depicts the photon flux per second, per square centimeter per steradian (p/s/cm²/sr). Total photon flux (p/s) was quantified over time for nose and lung. Measurements of individual animals were plotted as single values and the average BLI signal per group per time point is depicted. The black arrow depicts the first vector dose received as fetus/neonate; the red arrow readministration in adult life. Red circles: BLI signal after readministration, blue triangles: non-readministration controls. (D,G) The lung and nose signal of animals that received a second vector dose after perinatal gene delivery were compared to the signal measured in adult controls who received a single vector dose, 1 month pi. One-way ANOVA and Tukey HSD post-hoc test, **p<0.01, ***p<0.001. Abbreviations: fetal-R or neonatal-R, fetal or neonatal vector delivery and readministration at 3 months.
Immunological ignorance allows long-term gene expression following perinatal rAAV-mediated gene transfer to murine airways (doi: 10.1089/hum.2013.196).

Figure 3

% transduction (rel. to no-BALF ctrl)

Neg
Fetal
Neonatal
Fetal-R
Neonatal-R
Adult ctrl

% transduction (rel. to no-serum ctrl)

Neg
Fetal
Neonatal
Fetal-R
Neonatal-R
Adult ctrl

1/32
1/8
1/2

1/160
1/640
1/40
Figure 3: Analysis of nAb against the rAAV2/5 capsid after perinatal gene transfer and after readministration

A rAAV transduction inhibition assay was performed to analyze nAb against the rAAV2/5 capsid in serum and BALF. Samples were analyzed 4 months after fetal or neonatal rAAV2/5 delivery, or 1 month after readministration. Serial dilutions of serum (A) or BALF samples (B) were incubated with rAAV2/5-fLUC and tested for inhibition of transduction in HEK293T cells. Transduction was quantified by measuring RLU per µg protein and is expressed as mean percent transduction relative to no-serum or no-BALF control ± SEM. The dashed line represents 100% transduction as measured by no-serum or no-BALF control. Abbreviations: fetal or neonatal, non-readministration controls 4 months after fetal or neonatal rAAV2/5 delivery; fetal-R or neonatal-R, fetal or neonatal vector delivery and readministration at 3 months, serum/BALF collection 1 month later; adult ctrl, adult controls that received a single vector dose, serum/BALF collection 1 month pi.
Figure 4: Evaluation of lung inflammation after perinatal rAAV2/5 administration or readministration in adult life

(A) Infiltration of immune cells in lung parenchyma was evaluated by H&E staining 4 months after fetal and neonatal rAAV2/5 administration (1.0 E+10 GC/animal of rAAV2/5-β-gal and 1.5 E+10 GC/animal of rAAV2/5-fLUC) and 1 month after readministration at 3 months. Samples of
negative and adult control mice that received a single vector dose were analyzed in parallel. Representative images at different time points depict peribronchial, peribronchiolar and perivascular regions in which infiltration of immune cells was assessed. (B) Anti-CD4-Alexa-488 staining for identification of CD4\(^+\) T cells in lymphocytic infiltrates. (C) Anti-CD8-Alexa-555 staining for identification of CD8\(^+\) T cells in lymphocytic infiltrates. DAPI was used for nuclear staining. Scale bar = 50 µm, except scale bar HE bottom row = 200 µm. Abbreviations: readmin, readministration at 3 months and tissue collection at 4 months; non-readmin, non-readministered controls 4 months after perinatal gene transfer.
Figure 5: Evaluation of a second rAAV2/5 readministration after initial perinatal gene transfer

(A) Overview of the experiment designed to study the feasibility of repeated rAAV2/5-fLUC readministration (1.5 E+10 GC/animal) to murine airways after perinatal gene delivery. (B-G)
fLUC expression was quantified over time using BLI. Total photon flux (p/s) was quantified at different time points for nose and lung. Measurements of individual animals were plotted as single values and the average BLI signal per group per time point is depicted. Measurements in grey were taken over from figure 2. Red and orange circles: BLI signal after the first and second readministration respectively; blue triangles: non-readministration controls. The red and orange arrows indicate the first and second readministration respectively. (D,G) The lung and nose signal of animals that received a second readministration after perinatal gene delivery was compared to the signal measured in adult controls who received a single vector dose, 1 month pi. One-way ANOVA and Tukey HSD post-hoc test, **p<0.01, ***p<0.001. Abbreviations: fetal-RR or neonatal-RR, fetal or neonatal vector delivery and readministration at 3 and 6 months.
Table 1: Scoring of lung inflammation after perinatal rAAV2/5 administration or readministration in adult life

<table>
<thead>
<tr>
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<th>Diffuse (sub-) mucosal infiltrate</th>
<th>Diffuse (sub-) mucosal infiltrate</th>
<th>Alveolar/ venous infiltrate</th>
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<tr>
<td></td>
<td>central airways</td>
<td>peripheral airways</td>
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<td>0 ± 0</td>
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<tr>
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<td>1.7 ± 0.6</td>
<td>0.8 ± 0.3</td>
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</table>

Lung sections were stained with H&E and scored to evaluate the degree of lung inflammation after fetal and neonatal rAAV2/5 administration (1.0 E+10 GC/animal rAAV2/5-β-gal; 1.5 E+10 GC/animal rAAV2/5-fLUC) and readministration at 3 months. Lung sections were scored according to the region as well as the degree of inflammation. Score 0 represents no immune cell infiltration; score 0.5 few infiltrating cells with minimal significance; score 1 mild lymphocytic infiltrate; score 2 moderate lymphocytic infiltrate; score 3 severe lymphocytic infiltrate. Mean ± SD.

a) Non-readministration controls for the fetal and neonatal group 4 months after perinatal rAAV2/5 delivery

b) Readministration animals for the fetal and neonatal group 1 month after readministration
Scoring of lung inflammation after perinatal rAAV2/5 administration or readministration in adult life
Supporting information

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Figure S1: Bioluminescence imaging of perinatal rAAV2/5-mediated gene transfer to murine airways

rAAV2/5-fLUC (1.5 E+10 GC/animal) and rAAV2/5-β-gal (1.0 E+10 GC/animal) were delivered to fetal mice at E18 and to neonatal pups at day 3. fLUC expression was visualized (photos below) and quantified (A-D) using BLI. The pseudocolor scale of BL images depicts the photon flux per second, per square centimeter per steradian (p/s/cm²/sr). Total photon flux (p/s) was quantified at different time points (1-3 months) for nose and lung. Measurements of individual animals were plotted as single values and the average BLI signal per group per time point is depicted. Animals were sacrificed at each time point, explaining the reduction in number of experimental animals over time.
Figure S2: Readministration of rAAV2/5 in adult life is more efficient after initial fetal than neonatal vector delivery

rAAV2/5-fLUC (1.5 E+10 GC/animal) and rAAV2/5-β-gal (1.0 E+10 GC/animal) were delivered to fetal mice at E18 and to neonatal pups at day 3. At three months of age, a second vector dose was given by I.T. instillation to approximately half of the animals, whereas the other half of the group was kept as non-readministration controls. An adult control group was included to control for the maximum amount of gene expression to be expected in the upper and lower airways after I.T. instillation of rAAV2/5-fLUC and -β-gal. fLUC expression was visualized (photos below) and quantified (A-F) over time using BLI. The pseudocolor scale of BL images depicts the photon flux per second, per square centimeter per steradian (p/s/cm²/sr). Total photon flux (p/s) was quantified at different time points (1-4 months) for nose and lung. Measurements of individual animals were plotted as single values and the average BLI signal per group per time point is depicted. The grey circles represent all the different animals from the experiment.
including the ones harvested at different time points (1-3 months). The black arrow depicts the first dose received as fetus/neonate; the red arrow readministration in adult life. Red circles: BLI signal after readministration, blue triangles: non-readministration controls. (C,F) The lung and nose signal of animals that received a second vector dose (i.e. readministration) after perinatal gene delivery was compared to the signal measured in adult control animals who received a single vector dose, 1 month pi. Comparisons between groups at specific time points were performed using a one-way ANOVA followed by a Tukey HSD post-hoc test, *p<0.05, **p<0.01. Abbreviations: fetal-R or neonatal-R, initial fetal or neonatal vector delivery followed by readministration at 3 months.

**Figure S3: Evaluation of transgene expression in the lungs after initial fetal and neonatal vector delivery and subsequent readministration in adult life**

β-gal expression in the lower airways (lungs) as detected by X-gal staining after fetal and neonatal rAAV2/5 administration (1.0 E+10 GC/animal) and readministration at 3 months. Representative images of different regions of the conducting airways are given showing the trachea, bronchi and bronchioles at 250x magnification. β-gal expression was visualized at 1 (A,B) and 4 months (C,D) for animals receiving a single dose as fetus or neonate and at 4 months (or 1 month after readministration) for animals that received a second vector dose at 3
months (E,F). (G) β-gal expression in the conducting airways is demonstrated 1 month after I.T. instillation of a single vector dose to adult control mice. (H) Absence of X-gal staining in negative control lungs. Scale bar = 100 µm. Abbreviations: 1 and 4 m, 1 and 4 months after perinatal gene transfer; 4 m-R, 1 month after readministration / 4 months after initial perinatal gene transfer.

Figure S4: Evaluation of transgene expression in the nose after initial fetal and neonatal vector delivery and subsequent readministration in adult life

β-gal expression in the upper airways (nose) as detected by X-gal staining after fetal and neonatal rAAV2/5 administration (1.0 E+10 GC/animal) and readministration at 3 months. (A-
Representative images depict the proximal nose region at 63x magnification for each time point. β-gal expression was visualized at 1 (A) and 4 months (B) for animals receiving a single dose as fetus or neonate and at 4 months (or 1 month after readministration) for animals that received a second vector dose at 3 months (C). (D) β-gal expression in nasal epithelium is demonstrated 1 month after I.T. instillation of a single vector dose to adult control mice. (E) High magnification images (400x) 1 month after fetal and neonatal gene transfer demonstrate β-gal positive cells mainly situated in the respiratory epithelium. A diffuse background staining is present in the olfactory epithelium (arrow). (F) Absence of X-gal staining in negative control nose. Scale bar (A-D) = 200 µm; scale bar (E,F) = 50 µm. Abbreviations: 1 and 4 m, 1 and 4 months after perinatal gene transfer; 4 m-R, 1 month after readministration / 4 months after initial perinatal gene transfer.

**Figure S5: Analysis of nAb against the rAAV2/5 capsid after readministration in adult life**

A rAAV transduction inhibition assay was performed to analyze nAb against the rAAV2/5 capsid in serum. Samples were analyzed 1 month post readministration after initial fetal and neonatal rAAV2/5 delivery (1.5 E+10 GC/animal rAAV2/5-fLUC). Additionally, adult control mice that received a single vector dose were analyzed in parallel. Serial dilutions of mouse sera were incubated with rAAV2/5-fLUC and tested for inhibition of transduction in HEK293T cells. Transduction was quantified by measuring RLU to the total amount of protein and is expressed as mean percent transduction of no-serum control ± SEM. Abbreviations: fetal-R or neonatal-R, initial fetal or neonatal vector delivery followed by readministration at 3 months and serum collection at 4 months; adult ctrl, adult control mice that received a single vector dose, serum collection 1 month pi.
Figure S6: Local adaptive immune response after primary perinatal rAAV2/5 administration or readministration in adult life

BALF samples were analyzed 1 month post readministration after initial fetal and neonatal rAAV2/5 delivery (1.5 E+10 GC/animal rAAV2/5-fLUC). Additionally, samples of animals that only received a single dose as fetus or neonate (i.e. non-readministration controls), as well as samples of negative age-matched mice and adult control mice that received a single vector dose were analyzed in parallel. (A) Analysis of the percentage of B cells (B220+) and (B) Tregs (CD4+Foxp3+) in the total lymphocyte population in BALF. The cells were incubated with fluorochrome-labeled monoclonal antibodies to B220, CD4 and Foxp3 and submitted to FACS analysis. Mean ± SEM, comparisons for each pair using Student’s t-test. Abbreviations: fetal or neonatal, non-readministration controls for the fetal and neonatal group 4 months after perinatal rAAV2/5 delivery; fetal-R or neonatal-R, initial fetal or neonatal vector delivery followed by readministration at 3 months and BALF collection at 4 months; adult ctrl, adult control mice that received a single vector dose, BALF collection 1 month pi.
Figure S7: Analysis of nAb against the rAAV2/5 capsid prior to the second readministration

A rAAV transduction inhibition assay was performed to analyze nAb against the rAAV2/5 capsid in serum before the second readministration. Samples were analyzed 6 months after fetal or neonatal rAAV2/5 delivery (or 3 months after the first readministration). Serial dilutions of serum were incubated with rAAV2/5-FLUC and tested for inhibition of transduction in HEK293T cells. Transduction was quantified by measuring RLU per µg protein and is expressed as mean percent transduction relative to no-serum control ± SEM. The dashed line represents 100% transduction as measured by no-serum control. Abbreviations: fetal or neonatal, non-readministration controls 6 months after fetal or neonatal rAAV2/5 delivery; fetal-R or neonatal-R, a first readministration after fetal or neonatal vector delivery, serum collection 3 months later; adult ctrl, adult controls that received a single vector dose, serum collection 1 month pi.