

# High Levels of Persistent Expression of $\alpha$ 1-Antitrypsin Mediated by the Nonhuman Primate Serotype rh.10 Adeno-associated Virus Despite Preexisting Immunity to Common Human Adeno-associated Viruses

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$\alpha$ 1-Antitrypsin ( $\alpha$ 1AT) deficiency is a genetic disorder causing emphysema if serum  $\alpha$ 1AT levels are  $<570 \mu\text{g/ml}$ . We have shown that intrapleural administration of an AAV5 $\alpha$ 1AT vector yielded persistent therapeutic  $\alpha$ 1AT serum levels. Since anti-AAV2 and -AAV5 antibodies prevalent in humans may limit the use of these common serotypes in gene therapy, we screened 25 AAV vectors derived from humans and nonhuman primates for  $\alpha$ 1AT expression following intrapleural administration to mice. The rhesus AAVrh.10 serotype yielded the highest levels and was chosen for further study. Following intrapleural administration, 77% of total body transgene expression was in the chest wall, diaphragm, lung, and heart. Intrapleural administration of AAVrh.10 $\alpha$ 1AT provided long-term, therapeutic  $\alpha$ 1AT expression in mice, although higher doses were required to achieve therapeutic levels in female mice than in male mice. Intrapleural administration of AAVrh.10 $\alpha$ 1AT produced the same levels in AAV2/AAV5-preimmune and naive mice. In mice administered with AAV5 $\alpha$ 1AT and subsequently "boosted" with the AAVrh.10 $\alpha$ 1AT vector, serum levels were increased by 300%. These data indicate that AAVrh.10 is the most effective known AAV vector for intrapleural gene delivery and has the advantage of circumventing human immunity to AAV.

**Key Words:**  $\alpha$ 1-antitrypsin, gene therapy, pleura, adeno-associated virus, neutralizing antibodies, serotypes

## INTRODUCTION

$\alpha$ 1-Antitrypsin ( $\alpha$ 1AT) deficiency is an autosomal recessive disorder associated with early onset emphysema [1–4]. While gene transfer is an appealing strategy to treat  $\alpha$ 1AT deficiency, high circulating levels of  $\alpha$ 1AT are required to bathe the lung in sufficient amounts of  $\alpha$ 1AT to protect the fragile alveolar structures from destruction by neutrophil elastase, the natural substrate of  $\alpha$ 1AT [1–5].

We have recently observed that intrapleural administration of an adeno-associated virus serotype 5 vector expressing the human  $\alpha$ 1AT cDNA mediated persistent high serum and lung levels of  $\alpha$ 1AT, sufficient to treat  $\alpha$ 1AT deficiency [6]. Before we consider this approach for a human trial, we have asked two questions: (1) are there other AAV vectors that are more efficient than the AAV5 serotype in expressing  $\alpha$ 1AT when administered by the

intrapleural route and (2) based on the knowledge that 45 to 80% of humans have antibodies against the common human AAV serotypes 2 and 5 [7–12], is there an AAV vector that will be highly effective in delivering  $\alpha$ 1AT, but also will circumvent preexisting humoral immunity against the common human AAV serotypes? Based on the concept that immunity against gene transfer vectors can be circumvented by switching serotypes [13–20], we hypothesized that the most direct answer to these questions was to screen all available nonhuman primate gene transfer vectors expressing  $\alpha$ 1AT, choose the serotype that yielded the highest levels of  $\alpha$ 1AT when administered via the intrapleural route, and evaluate whether this serotype could circumvent preexisting immunity against the common serotypes 2 and 5 human adeno-associated viruses. The screen of 16

nonhuman primate and 9 human adeno-associated viruses showed that the nonhuman primate rh.10 serotype with AAV2 inverted terminal repeats (ITRs) was the most efficient, yielding persistent  $\alpha$ 1AT serum levels threefold above that mediated by AAV5 with an identical genome. As has been observed by others in liver with AAV vectors with AAV2 ITRs [21–23], a vector with an AAVrh.10 capsid and AAV2 ITRs expressing  $\alpha$ 1AT administered by the intrapleural route was severalfold more effective in expressing  $\alpha$ 1AT in male mice than in female mice. Importantly, the AAVrh.10 vector expressing human  $\alpha$ 1AT could circumvent anti-AAV2 and -AAV5 humoral immunity and could provide a significant “boost” in  $\alpha$ 1AT levels following prior  $\alpha$ 1AT gene transfer mediated by an AAV5 vector.

## RESULTS

### Comparison of Efficacy of Intrapleural Gene Transfer by Human and Nonhuman Primate AAV Vectors

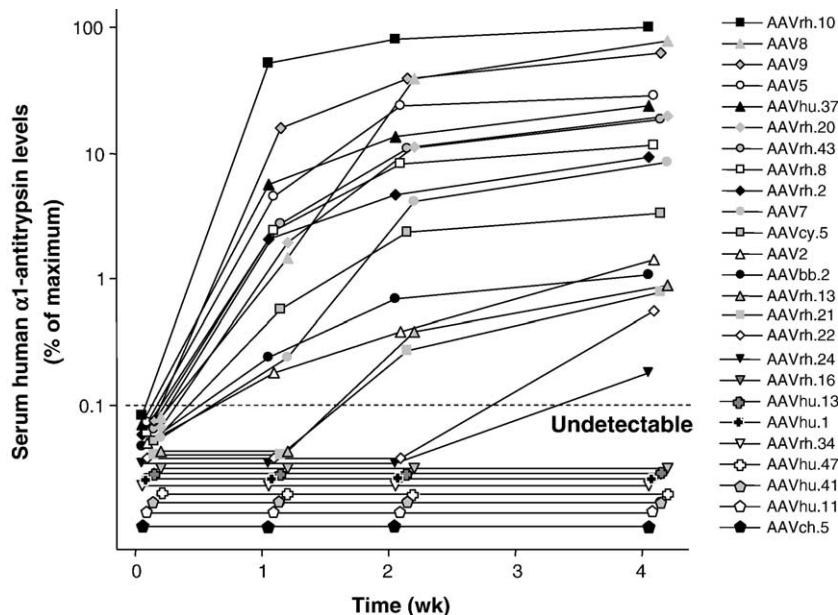
We have previously shown that intrapleural administration to mice of an AAV5-based vector expressing  $\alpha$ 1AT mediated serum  $\alpha$ 1AT levels of  $900 \pm 50 \mu\text{g/ml}$ , 1.6-fold higher than the accepted therapeutic level of  $570 \mu\text{g/ml}$  [3]. To determine if other AAV serotypes are more efficient for gene transfer via this delivery route, we tested the efficiency of intrapleural gene transfer of a battery of alternative human and nonhuman primate AAV serotypes, including several novel serotypes [16,24]. We administered 25 pseudotyped AAV serotypes (16 nonhuman primate, 9 human;  $5 \times 10^{10}$  genome copies for each serotype, all using the AAV2 ITRs) expressing human  $\alpha$ 1AT to male C57BL/6 mice ( $n = 5/\text{group}$ ) by intrapleural administration. Thirteen of these pseudotyped viruses

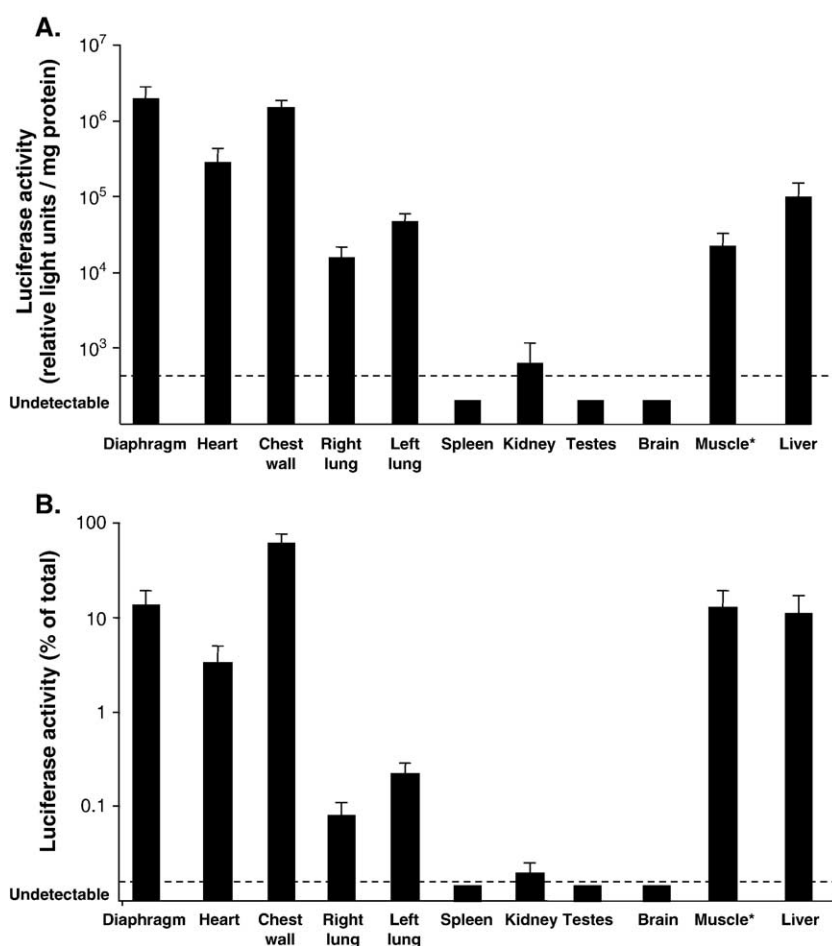
were derived from rhesus macaques (AAV7, AAV8, AAVrh.2, AAVrh.8, AAVrh.10, AAVrh.13, AAVrh.16, AAVrh.20, AAVrh.21, AAVrh.22, AAVrh.24, AAVrh.34, and AAVrh.43) [24], and 1 was derived from cynomolgus macaque (AAVcy.5), 1 from baboon (AAVbb.2), 1 from chimpanzee (AAVch.5); 9 were isolated from humans (AAV2, AAV5, AAV9, AAVhu.1, AAVhu.11, AAVhu.13, AAVhu.37, AAV hu.41, and AAVhu.47). Nine of these AAV serotypes resulted in  $\alpha$ 1AT levels below the level of detection of the assay, while other serotypes resulted in the production of variable levels of human  $\alpha$ 1AT (Fig. 1). The AAV serotype that resulted in the highest levels of  $\alpha$ 1AT following intrapleural administration was the novel rhesus macaque-derived AAVrh.10. This was followed by AAV8, AAV9, AAV5, AAVhu.37, AAVrh.20, AAVrh.43, and AAVrh.8, all mediating levels of  $\geq 10\%$  of that mediated by AAVrh.10 at week 4 following administration.

### Transgene Delivery to Chest Structures Following Intrapleural Administration of AAVrh.10 Vectors

To determine the pattern of organ distribution of transgene expression directed by the AAVrh.10 serotype following intrapleural administration, we injected male C57BL/6 mice ( $n = 4$ ) intrapleurally with an AAVrh.10 vector expressing luciferase, a nonsecreted transgene ( $5 \times 10^{10}$  genome copies). Six weeks after vector administration, the highest levels of luciferase expression, expressed as relative light units per milligram of protein, were in the organs and structure of the chest, i.e., chest wall, diaphragm, lungs, and heart (Fig. 2A). When expressed as the total amount of luciferase expressed in each organ, and relative to 100% of total body luciferase activity recovered, we observed the same distribution (Fig. 2B). Considered as a group, the expression of luciferase in the

**FIG. 1.** Evaluation of intrapleural gene transfer efficiency of human and nonhuman primate AAV-based vectors expressing human  $\alpha$ 1AT. The AAV vectors ( $5 \times 10^{10}$  genome copies) with the AAV2 inverted terminal repeats and pseudotyped as indicated and containing the human  $\alpha$ 1AT expression cassette were administered intrapleurally to male C57BL/6 mice ( $n = 5/\text{group}$ ). Serum  $\alpha$ 1AT levels were measured by ELISA at the time of vector administration (week 0) and at weeks 1, 2, and 4 following vector administration. The data shown are percentages of means of five mice, with the levels at week 4 of AAVrh.10 used as the 100% point. The human-derived vectors are AAV2, AAV5, AAV9, AAVhu.1, AAVhu.11, AAVhu.20, AAVhu.37, AAVhu.41, and AAVhu.47; all others are derived from nonhuman primates. The dashed line represents the level below which human  $\alpha$ 1AT is undetectable.





**FIG. 2.** Organ distribution of transgene expression following intrapleural administration of AAVrh.10 vector expressing the luciferase reporter transgene. The AAVrh.10 luciferase vector ( $5 \times 10^{10}$  genome copies) was administered intrapleurally to male C57BL/6 mice ( $n = 4$ ). After 6 weeks, the mice were sacrificed, the organs were collected, and the weight of each organ was determined. Samples of each organ were homogenized and the organ distribution of luciferase activity was determined and normalized to protein concentration. (A) Luciferase activity in relative light units/mg of protein (mean  $\pm$  standard error). (B) Percentage luciferase activity per organ relative to total body luciferase activity. The total luciferase activity was determined as the sum for all organs derived by multiplying the weight of each organ by the luciferase activity in relative light units/mg protein. \*For muscle, the left quadriceps was analyzed; for (B), the total luciferase activity in total body muscle was estimated as detailed under Methods. The dashed line represents the level below which luciferase is undetectable.

chest structures (chest wall, diaphragm, lungs, and heart) represented 77% of the total luciferase expressed in the mouse. Consistent with the luciferase data, assessment of chest structures following administration of AAVrh.10GFP showed expression in the chest wall, diaphragm, and lung, including the mesothelium and submesothelial structures (endothelium, cells in connective tissue). We also saw expression in hepatocytes.

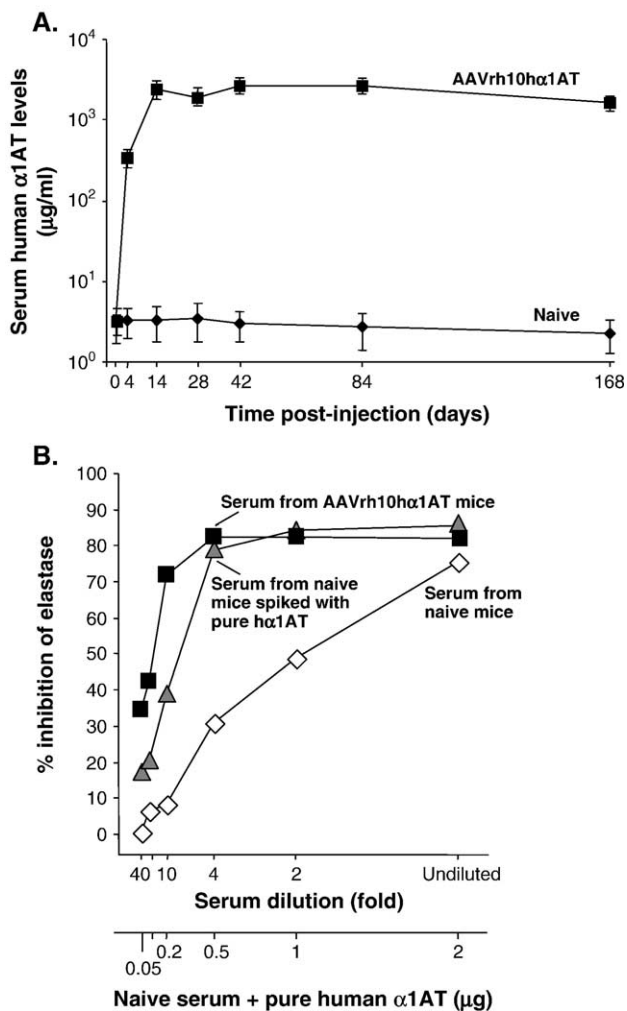
#### Sustained High Serum Levels of $\alpha$ 1AT Following Intrapleural Administration of AAVrh.10 $\alpha$ 1AT

Since the ultimate goal of these studies is to characterize an AAV vector of potential usefulness in the treatment of  $\alpha$ 1AT deficiency, we assessed the levels of serum  $\alpha$ 1AT produced by the AAVrh.10 $\alpha$ 1AT vector after intrapleural delivery of  $10^{11}$  genome copies of vector, as a function of time (male C57BL/6 mice,  $n = 4$  group). The data show that sustained therapeutic levels ( $>2.5$ -fold above the minimum of 570  $\mu$ g/ml) of human  $\alpha$ 1AT were achieved starting on day 4 and up to 168 days (24 weeks) post-vector administration (Fig. 3A).

We tested the ability to inhibit neutrophil elastase activity by the  $\alpha$ 1AT produced by intrapleural administration of the vector using an *in vitro* assay. The ability of the vector-produced  $\alpha$ 1AT present in the serum to inhibit neutrophil elastase was comparable to that of purified human  $\alpha$ 1AT (Fig. 3B).

#### Therapeutic Serum Levels of $\alpha$ 1AT Can Be Achieved in both Male and Female Mice Despite Impact of Sex on AAV-Mediated Transgene Expression

The level of transgene expression following intravenous administration of AAV2 vectors to mice has been observed to be dependent on the sex of the experimental animals used, with male mice expressing 5- to 13-fold higher levels than females with the same dose of vector [21,22,31]. We tested whether there is a difference in serum  $\alpha$ 1AT levels between male and female mice following intrapleural administration with the nonhuman primate-derived AAVrh.10 $\alpha$ 1AT vector. We injected male and female C57BL/6 mice by the intrapleural route with  $5 \times 10^{10}$  genome copies of AAVrh.10 $\alpha$ 1AT and assessed serum  $\alpha$ 1AT levels. The



**FIG. 3.** Time course and function of serum human  $\alpha$ 1AT levels in mice following intrapleural administration of AAVrh.10 $\alpha$ 1AT vector. (A) Time course. The AAVrh.10 $\alpha$ 1AT vector ( $10^{11}$  genome copies) was administered intrapleurally to male C57BL/6 mice ( $n = 4$ /group). Serum human  $\alpha$ 1AT levels were measured at the time of vector administration (day 0) and at days 4 to 168 following vector administration. Serum  $\alpha$ 1AT levels were measured by ELISA; values shown are means  $\pm$  standard error. (B) Function. Assessment was made of inhibition of neutrophil elastase by human  $\alpha$ 1AT in the serum following intrapleural administration of AAVrh.10 $\alpha$ 1AT 6 weeks previously. The % inhibition of elastase activity is shown on the ordinate. The top abscissa shows the serum dilution, and the bottom abscissa shows the amount of human  $\alpha$ 1AT (in  $\mu$ g) present in the positive control, i.e., spiked naive serum, or present in the serum of mice injected with the AAVrh.10 $\alpha$ 1AT vector. Undiluted mouse serum following AAVrh.10 $\alpha$ 1AT administration had human  $\alpha$ 1AT levels of 2  $\mu$ g/ $\mu$ l. The serum of naive mice, which does not contain human  $\alpha$ 1AT, was used as a negative control; as expected, the endogenous murine  $\alpha$ 1AT was capable of inhibiting neutrophil elastase.

data show that there is an impact of sex on serum  $\alpha$ 1AT levels following intrapleural administration of the AAVrh.10 $\alpha$ 1AT vector (Fig. 4A). The serum  $\alpha$ 1AT levels at 28 days were  $1360 \pm 250$   $\mu$ g/ml in male mice and  $480 \pm 58$   $\mu$ g/ml in female mice, representing a 2.8-fold difference ( $P < 0.05$  male vs female). Since the

female mice did not achieve therapeutic levels of human  $\alpha$ 1AT at a dose of  $5 \times 10^{10}$  genome copies, we administered a higher dose of the vector ( $10^{11}$  genome copies) intrapleurally to male and female mice and assessed the levels of  $\alpha$ 1AT. The data indicate that at a dose of  $10^{11}$  genome copies, therapeutic serum  $\alpha$ 1AT levels are achieved in both male ( $2450 \pm 102$   $\mu$ g/ml at 28 days) and female mice ( $1070 \pm 57$   $\mu$ g/ml, at 28 days, almost 2-fold the required therapeutic threshold of 570  $\mu$ g/ml).

#### Delivery of $\alpha$ 1AT by AAVrh.10 $\alpha$ 1AT is Efficient Even in the Presence of Preexisting Immunity against Common AAV Serotypes

Immunity against the human serotypes AAV2 and AAV5 has been reported to be prevalent in the human population [7–12]. To assess whether the AAVrh.10 $\alpha$ 1AT vector, derived from a virus isolated from a rhesus macaque to which humans are presumably not exposed, functions in the presence of preexisting immunity against the common human serotypes AAV2 and AAV5, we immunized male C57BL/6 mice by subcutaneous injection in the footpad with  $2 \times 10^{10}$  genome copies each of AAV2 and AAV5 expressing green fluorescent protein (GFP) and boosted them with the same dose 2 weeks later. Two weeks after the second boost (day 28), robust levels of anti-AAV2 and anti-AAV5 neutralizing antibody titers were present in animals immunized with the combination AAV2 and AAV5 GFP vectors, as assessed for the ability of mouse immune serum to block *in vitro* gene transfer by the reporter vectors AAV2LacZ and AAV5LacZ (Fig. 5A). Also on day 28, the naive animals and immunized animals ( $n = 3$ /group) were injected intrapleurally with  $10^{11}$  genome copies (gc) of AAV2<sub>CU</sub> $\alpha$ 1AT or AAV5<sub>CU</sub> $\alpha$ 1AT or  $5 \times 10^{10}$  gc of AAVrh.10 $\alpha$ 1AT. The differences in genome copies used in the study was based on the differences in gene transfer efficacy resulting from these vectors [6]. Thus, to achieve production of comparable levels of human  $\alpha$ 1AT with the AAV5 and AAVrh.10 vectors, we used twice as much AAV5-based vector. At 2 and 4 weeks post-administration of the AAV2<sub>CU</sub> $\alpha$ 1AT, AAV5<sub>CU</sub> $\alpha$ 1AT, and AAVrh.10 $\alpha$ 1AT vectors, we assessed serum human  $\alpha$ 1AT levels by ELISA. The data show that the AAVrh.10 $\alpha$ 1AT vector is capable of directing similarly high levels of human  $\alpha$ 1AT both in naive animals, as expected, and in animals with preexisting immunity to AAV2 and AAV5 ( $P > 0.9$ , comparing levels 4 weeks after AAVrh.10 $\alpha$ 1AT administration in AAV2/AAV5 preimmune mice vs naive mice). In contrast, there was a 980-fold lower level of serum  $\alpha$ 1AT at 4 weeks in AAV2/AAV5 immune mice receiving AAV5<sub>CU</sub> $\alpha$ 1AT compared to naive recipients. Similarly, there were undetectable  $\alpha$ 1AT levels in AAV2/AAV5-immune mice injected with AAV2<sub>CU</sub> $\alpha$ 1AT compared to a level of 12  $\mu$ g/ml in naive mice receiving the same vector (Fig. 5B).

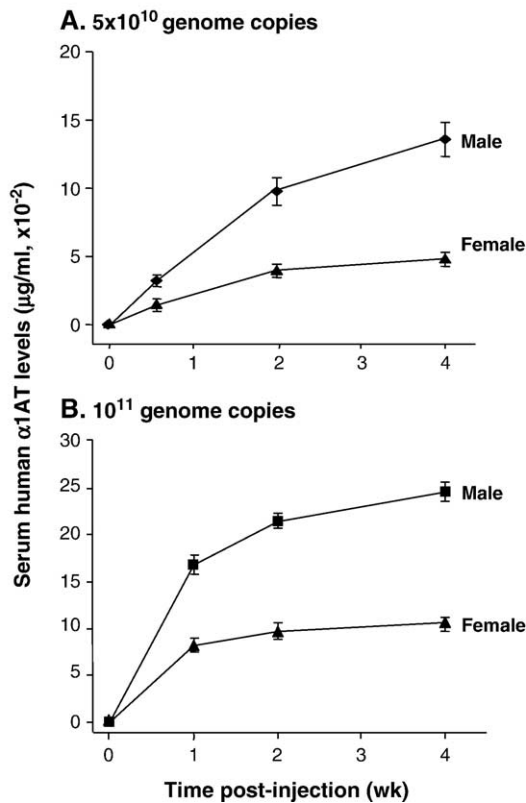


FIG. 4. Impact of sex on efficiency of transgene expression following AAVrh.10-mediated gene transfer. Male and female C57BL/6 mice were administered AAVrh.10 $\alpha$ 1AT by the intrapleural route. At various time points, the serum human  $\alpha$ 1AT levels were assessed by ELISA. Data are plotted as means  $\pm$  standard error for  $n = 4$  mice/group. (A)  $5 \times 10^{10}$  and (B)  $10^{11}$  genome copies.

#### Serum $\alpha$ 1AT Levels in Mice with Prior $\alpha$ 1AT Gene Transfer by an AAV5 Vector Are Significantly Boosted by AAVrh.10 $\alpha$ 1AT Following Intrapleural Administration

Given that the AAVrh.10 $\alpha$ 1AT vector can circumvent anti-AAV2 and anti-AAV5 humoral immunity, we assessed whether this vector could boost human  $\alpha$ 1AT levels even in the case of prior  $\alpha$ 1AT gene transfer mediated by an AAV5-based vector. To test this hypothesis, we injected C57BL/6 mice by the intrapleural route with  $10^{11}$  gc of AAV5<sub>CU</sub>h $\alpha$ 1AT ( $n = 6$ ) or  $5 \times 10^{10}$  gc of AAVrh.10 $\alpha$ 1AT ( $n = 6$ ) and assessed serum human  $\alpha$ 1AT levels and anti-AAV5 and anti-AAVrh.10 neutralizing antibody titers at 6 weeks post-vector administration. The animals receiving the AAVrh.10 $\alpha$ 1AT vector developed neutralizing antibodies against AAVrh.10, and those receiving the AAV5<sub>CU</sub>h $\alpha$ 1AT vector had neutralizing antibodies against the AAV5 serotype (Fig. 6A). The animals that had been injected with either vector were split into two groups of  $n = 3$ , one that received no further treatment and another that received a boost with

$5 \times 10^{10}$  gc of AAVrh.10 $\alpha$ 1AT. We determined anti-AAV5 and -AAVrh.10 neutralizing antibody titers. At 2 and 4 weeks postadministration (boost) with the AAVrh.10 $\alpha$ 1AT vector, we assessed serum human  $\alpha$ 1AT levels by ELISA. The results show that intrapleural administration of AAVrh.10 $\alpha$ 1AT to animals previously injected with the AAV5<sub>CU</sub>h $\alpha$ 1AT vector resulted in a boost of serum h $\alpha$ 1AT levels of 300% ( $P < 0.05$  compared to unboosted animals at

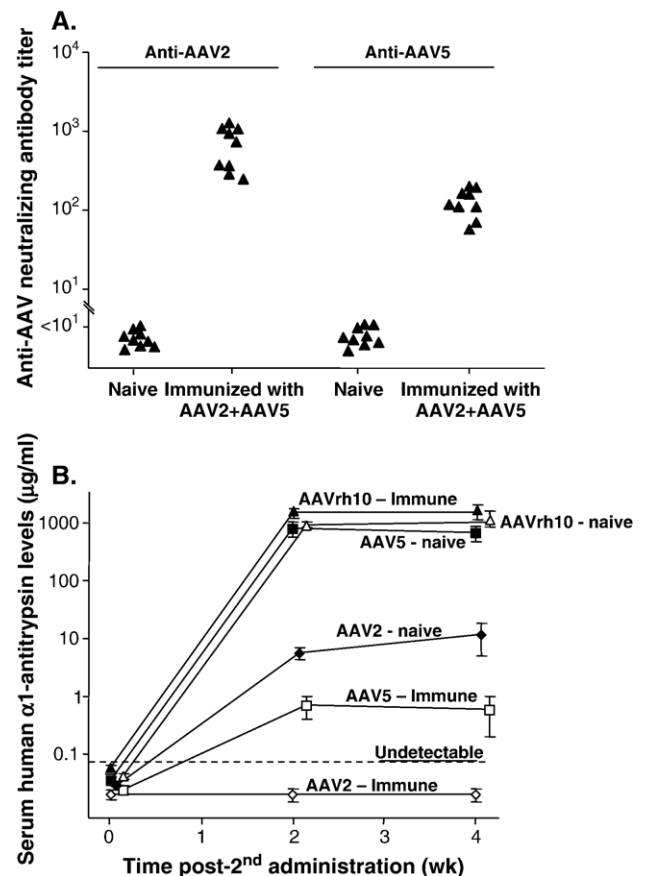
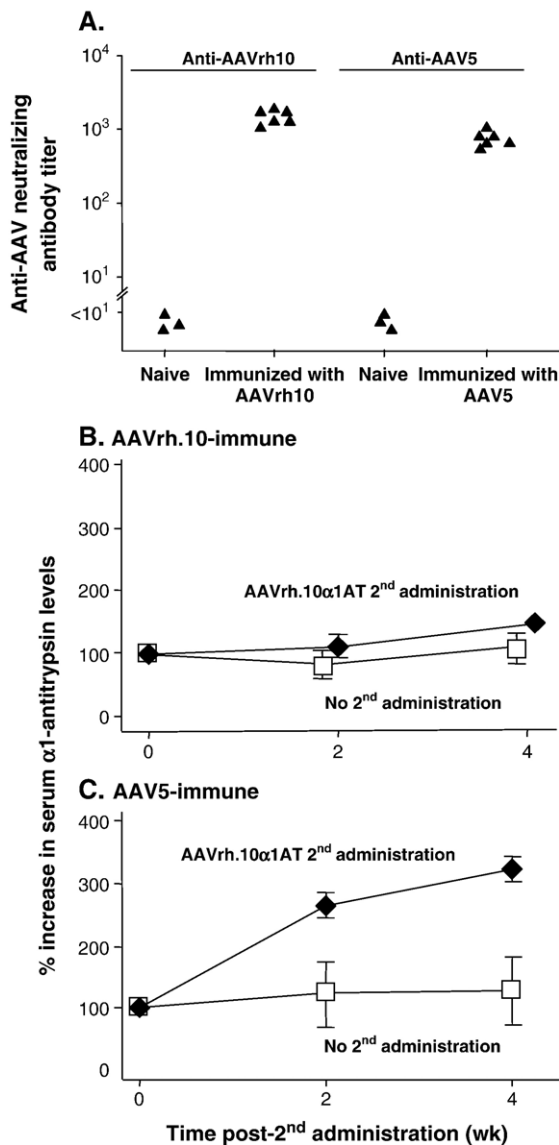


FIG. 5. Impact of preexisting anti-AAV2 and -AAV5 immunity on gene transfer using AAV2, AAV5, and AAVrh.10 vectors. Male C57BL/6 mice were immunized by two subcutaneous injections of  $2 \times 10^{10}$  genome copies (gc) each of AAV2 and AAV5 expressing GFP. After 4 weeks, the immune animals and naive controls ( $n = 3$ /group) were administered  $10^{11}$  gc of AAV2<sub>CU</sub>h $\alpha$ 1AT or AAV5<sub>CU</sub>h $\alpha$ 1AT or  $5 \times 10^{10}$  gc of AAVrh.10 $\alpha$ 1AT following assessment of anti-AAV2 and anti-AAV5 neutralizing antibody titers. The differences in genome copies were purposefully chosen to achieve production of comparable levels of human  $\alpha$ 1AT with the AAV5 and AAVrh.10 vectors; as previously reported [6], the AAV2-based vector is much less efficient at expressing human  $\alpha$ 1AT than AAV5. (A) Anti-AAV2 and -AAV5 neutralizing antibody titers. Serial dilutions of serum were assessed for the ability to block *in vitro* gene transfer by the reporter vectors AAV2LacZ and AAV5LacZ and used to determine neutralizing antibody titers. (B) Serum human  $\alpha$ 1AT levels following intrapleural administration of AAVs expressing human  $\alpha$ 1AT cDNA in naive and anti-AAV immunized mice. At 2 and 4 weeks postadministration in naive or AAV2 and AAV5 immunized mice, serum human  $\alpha$ 1AT levels were assessed by ELISA and expressed in  $\mu\text{g/ml} \pm$  standard error.



**FIG. 6.** Repeat administration of AAV vectors of same and different serotypes expressing  $\alpha$ 1AT. C57BL/6 mice were injected by the intrapleural route with  $10^{11}$  gc of AAV5<sub>CU</sub>h $\alpha$ 1AT or  $5 \times 10^{10}$  gc of AAVrh.10 $\alpha$ 1AT. After 6 weeks, serum human  $\alpha$ 1AT levels and anti-AAV5 and anti-AAVrh.10 neutralizing antibody titers were assessed. The animals were then boosted with  $5 \times 10^{10}$  gc of AAVrh.10 $\alpha$ 1AT or left unboosted, as indicated. (A) Anti-AAV5 and -AAVrh.10 neutralizing antibody titers. Serial dilutions of serum were assessed for the ability to block *in vitro* gene transfer by the reporter vectors AAV5LacZ and AAVrh.10Luc and used to determine neutralizing antibody titers. (B) AAVrh.10 $\alpha$ 1AT followed by AAVrh.10 $\alpha$ 1AT. At 0, 2, and 4 weeks postadministration (boost), serum human  $\alpha$ 1AT levels were assessed by ELISA. (C) AAV5 $\alpha$ 1AT followed by AAVrh.10 $\alpha$ 1AT. At 0, 2, and 4 weeks postadministration (boost), serum human  $\alpha$ 1AT levels were assessed by ELISA. For (B) and (C), the impact of the boost administration on serum h $\alpha$ 1AT levels is expressed as percentage change using serum h $\alpha$ 1AT level of unboosted AAV5-administered mice at time 0 as 100% ( $\pm$  standard error).

4 weeks; we used unboosted AAV5-administered mice as 100%, Fig. 6C). As expected, a second administration of AAVrh.10 $\alpha$ 1AT to animals previously injected with the same vector did not result in an increase in serum h $\alpha$ 1AT levels ( $P > 0.3$  comparing AAVrh.10 $\alpha$ 1AT boosted and unboosted animals 4 weeks postboost; Fig. 6B). The data demonstrate that the AAVrh.10 $\alpha$ 1AT vector can potentially be used to boost human  $\alpha$ 1AT levels in subjects previously treated with an AAV5-based gene transfer vector. Of note, C57BL/6 mice are tolerant to human  $\alpha$ 1AT, i.e., they do not develop immunity to the “foreign” protein when boosted with a different serotype vector [32,33].

## DISCUSSION

We have previously described a novel and efficient strategy to treat  $\alpha$ 1AT deficiency in humans by intrapleural delivery of an AAV5-based vector expressing the human  $\alpha$ 1AT cDNA [6]. To identify alternative vectors based on nonhuman primate AAV serotypes that could circumvent preexisting anti-AAV immunity in humans, the present study reports the screening of 16 different nonhuman primate and 9 human AAV vectors expressing  $\alpha$ 1AT administered by the intrapleural route to mice. The data show that: (1) a novel rhesus macaque-derived serotype, AAV rh.10, resulted in the highest serum levels of  $\alpha$ 1AT,  $>2.5$ -fold above the therapeutic level of 570  $\mu$ g/ml, sustained through the 24 weeks of the study, with most of the transgene expression localized to the chest structures, and (2) the AAVrh.10h $\alpha$ 1AT vector can circumvent preexisting immunity to AAV2 and AAV5, two common human serotypes, and has the potential to boost  $\alpha$ 1AT levels even in subjects previously treated with an AAV5 vector expressing  $\alpha$ 1AT.

### Use of Nonhuman Primate AAV Serotypes as Gene Transfer Vectors

AAV-based vectors are useful for gene transfer because of their low toxicity and persistent transgene expression levels [34–41]. With respect to AAV vectors and  $\alpha$ 1AT deficiency, an AAV2 vector expressing the human  $\alpha$ 1AT is currently in a Phase I clinical trial to treat  $\alpha$ 1AT deficiency by intramuscular delivery, based on data showing stable expression of  $\alpha$ 1AT via intramuscular delivery in mice, as well as rodent and nonhuman primate safety studies [33,42–44]. We have shown that direct delivery into the pleural space of an AAV5  $\alpha$ 1AT vector enhances the efficiency of gene therapy for the lung manifestations of  $\alpha$ 1AT deficiency [6]. However, while these AAV-based strategies represent valid options for  $\alpha$ 1AT-deficiency gene therapy, there is an important concern regarding the effectiveness of using AAV2 or AAV5 for human gene therapy: preexisting immunity against these vectors in the human population may dampen their efficacy.

One solution to this dilemma is to use nonhuman primate AAV serotypes to circumvent recognition by neutralizing anti-AAV antibodies prevalent in the human population [7–12,16,24]. For example, vectors pseudotyped with capsids from AAV7 and AAV8, two serotypes isolated from rhesus monkeys, demonstrated high efficiency at transducing muscle and liver, respectively, and since they are immunologically distinct from human AAV serotypes, they cannot be neutralized by antibodies generated against the capsids of the human serotype AAV1, AAV2, or AAV5 [16]. Mice previously immunized with an AAV2 vector expressing  $\alpha$ 1AT expressed high levels of transgene when injected intraperitoneally with an AAV8 vector expressing canine factor IX [16]. To identify an AAV vector with potential use in gene transfer of  $\alpha$ 1AT via the pleura, yet that could circumvent immunity against the common human AAV serotypes, a total of 25 pseudotyped AAV-based vectors carrying the AAV2 inverted terminal repeats and an expression cassette for human  $\alpha$ 1AT cDNA were screened by measuring serum  $\alpha$ 1AT levels in a time-dependent fashion by intrapleural administration in mice [24]. Of these 25 vectors, 16 were pseudotyped with capsids from novel nonhuman primate AAVs and 9 were packaged with capsid proteins from human AAV serotypes [24]. The highest levels of serum  $\alpha$ 1AT were obtained with AAVrh.10, a member of clade E, isolated from a rhesus monkey. The AAVrh.10 vector also resulted in very rapid high levels of expression, producing the highest levels of  $\alpha$ 1AT at 1 week post-vector administration. Because of these advantages over other serotypes tested, the AAVrh.10  $\alpha$ 1AT vector was further investigated by intrapleural administration in mice at time points ranging up to 168 days postinfection. Interestingly, this vector resulted in therapeutic levels of  $\alpha$ 1AT after only 4 days following intrapleural administration, and this was maintained at this level for the duration of the study.

#### Local Expression of $\alpha$ 1AT in the Chest Structures Directed by the AAVrh.10-Based Vector

We have previously shown that administration of an AAV5-based vector directly into the visceral surface of the lung via the pleural space results in local transgene expression in the diaphragm, lungs, and heart [6]. Local production of  $\alpha$ 1AT has the important advantage of efficiency, since the lung is only 2% of body weight, and attempts at systemic delivery of  $\alpha$ 1AT via gene transfer have the challenge of much higher daily production levels to achieve efficacious levels of  $\alpha$ 1AT in the lung [33,42,43,45]. Further, the pleura as a site for gene transfer has little risk of detrimental effects of possible vector-induced inflammation, since even marked pleural inflammation is not associated with lung dysfunction [46]. Transgene delivery via the pleura using the AAVrh.10-based vector demonstrated that at least 75% of the gene expression was in cells of the structures and

organs of the chest, including the chest wall, diaphragm, lung, and heart. Interestingly, in contrast to AAV5 [6], the AAVrh.10 vector delivered by intrapleural administration resulted in approximately 10% of the total expression in the liver, suggesting that this vector can also be used to express transgenes in hepatocytes, without systemic administration. This is relevant for  $\alpha$ 1AT deficiency, since the natural site of production of  $\alpha$ 1AT is the liver [1–4,47]. Consistent with the quantitative data, studies with AAVrh.10 expressing GFP demonstrated GFP expression in the chest wall, diaphragm, lung, and liver. Because of the differences in the sensitivity of the immunohistochemistry in each site, the data cannot be used quantitatively, but expression was observed in the mesothelium and underlying endothelium and cells in interstitial connective tissue. Outside of the chest, it was observed in hepatocytes (not shown).

#### Sex Differences in Transgene Expression Levels Directed by AAV Vectors

Another potential concern with the use of AAV-based vectors for gene therapy is that, at least for some organs in rodents, the sex of the experimental subject determines the level of transgene expression. In studies in male mice, AAV2-, AAV5-, and AAV8-based vectors targeted to the liver effect greater than fivefold higher expression levels than in female mice [21–23]. This effect is thought to be mediated by higher binding of an androgen-dependent host liver nuclear protein to the Rep binding site of the AAV2-derived inverted terminal repeats in males [22]. This sex-dependent effect has been shown to vary from organ to organ [22]. Our data showed that the male serum  $\alpha$ 1AT expression level was greater than that of females when the site of injection was the pleura, which results in higher expression in the chest structures than in the liver. However, despite the greater than threefold lower expression in female mice, therapeutic levels of  $\alpha$ 1AT could be achieved in these animals at a higher vector dose. These data suggest that the use of this efficient serotype may circumvent the lower levels of expression effected by AAV vectors in females.

#### Strategies for Circumventing Preexisting Immunity against Human AAV Serotypes

The therapeutic value of a gene transfer viral vector depends not only on efficacy and safety parameters, but also on its ability to circumvent the immune system [12–19,26]. This is of particular interest in the case of a viral vector based on a virus to which there is extensive preexisting immunity in the human population, such as AAV2 and AAV5 [12–19,26]. The use of alternative AAV serotypes is a strategy to circumvent preexisting immunity. In that regard, AAV5-pseudotyped vectors have been shown to be effective at gene transfer in the

presence of neutralizing preexisting immunity to AAV2, in muscle and in the brain [11,18]. Similarly, lung transduction by AAV6-pseudotyped vector is unaffected by prior administration of AAV2 or AAV3 vectors [15], and salivary gland administration of recombinant AAV4 can circumvent immunity against AAV2 [17]. The use of AAV2-based ITRs pseudotyped with the capsid proteins from nonhuman primates represents one important avenue to overcome the barrier of preexisting immunity against human AAV serotypes. Consistent with this concept, AAV7- and AAV8-based vectors, isolated from rhesus monkeys, are not recognized by antibodies against human AAV serotypes, and an AAV8-based vector expressing the human factor IX can effect high levels of factor IX expression in mice immunized with AAV2-, AAV1-, or AAV7-based vectors [16]. Anti-AAV5 neutralizing antibodies have been reported [7–12], and thus memory responses would likely be evoked by the administration of AAV2 or 5 vectors, as has been observed in an AAV2-based human trial for factor IX deficiency [48]. Immune responses to AAV vectors following first administration have been shown to preclude readministration of the same vector in terms of efficient transgene expression [31,49–51]. In addition to circumventing the presence of anti-AAV2 and -AAV5 antibodies in the human population at large, it would be useful to identify a second-generation vector that would allow the boosting of  $\alpha$ 1AT levels in individuals who had been already treated with a human AAV5-based vector expressing  $\alpha$ 1AT. In contrast with AAV2 and AAV5, anti-AAVrh.10 neutralizing antibodies are not found in the human population [24]. The AAVrh.10 $\alpha$ 1AT vector described in the present study is capable of effecting gene transfer via the intrapleural route in the presence of neutralizing antibodies against AAV2 and AAV5 and also can boost human  $\alpha$ 1AT levels in mice previously treated with the AAV5 $\alpha$ 1AT vector. Together, these data indicate that AAVrh.10 is the most effective of the known AAV vectors for delivery of genes via the intrapleural route and has the marked advantage over any other serotype in providing therapeutic levels of  $\alpha$ 1AT while circumventing high-level immunity to the common human AAV serotypes. Should re-administration of an AAV vector be necessary, screening of the 25 different serotypes indicated that AAV8 is second to AAVrh.10 in the production of  $\alpha$ 1AT following intrapleural administration, and thus AAV8 might be considered for re-administration should loss of AAVrh.10-mediated expression occur.

## METHODS

**Recombinant AAV vectors.** For screening studies, 16 nonhuman primate and 9 human AAV vectors, all with AAV2 inverted terminal repeats and coding for human  $\alpha$ 1AT driven by a cytomegalovirus enhancer/chicken  $\beta$  actin promoter, were produced in human embryonic kidney 293 cells

(American Type Culture Collection, Manassas, VA, USA) and purified as described by Gao *et al.* [16,24].

AAVrh.10 was chosen for further study based on the screening study demonstrating that intrapleural administration of a vector with AAVrh.10 capsid and AAV2 ITRs yielded the highest serum levels of  $\alpha$ 1AT. All recombinant AAVrh.10 vectors were produced using a three-plasmid system: (1) an expression plasmid (pAAV2<sub>CU</sub> $\alpha$ 1AT for the vectors expressing human  $\alpha$ 1AT or pAAV2-Luc for vectors expressing the luciferase reporter gene); (2) pAAV-44.2, an AAVrh.10 packaging plasmid that provides the AAV Rep proteins (derived from AAV2) needed for vector replication and the viral structural (*cap*) proteins VP1, 2, and 3 derived from AAVrh.10, which determines the serotype of the AAV vector [24,25]; and (3) pAdDeltaF6, an adenovirus helper plasmid that provides the adenovirus helper functions of E2, E4, and VA RNA [26,27].

For AAVrh.10 vector production, the expression plasmid pAAV2-<sub>CU</sub> $\alpha$ 1AT (or the reporter expression plasmid pAAV2 Luc), the AAVrh.10 packaging plasmid pAAV-44.2, and Ad helper plasmid pAdDeltaF6 were cotransfected in 293 cells, which provide the E1 Ad function from an integrated, chromosomal copy of that gene [293 (E1+) cells] [6,16]. The recombinant vector AAVrh.10 $\alpha$ 1AT was produced by Polyfect (Qiagen, Valencia, CA, USA)-mediated cotransfection of 500  $\mu$ g  $\alpha$ 1AT vector plasmid, 500  $\mu$ g AAVrh.10 packaging plasmid, and 1 mg Ad helper plasmid into a 10-Stack Cell Factory (Nunc Brand Products, Rochester, NY, USA) at 80% confluence of low-passage 293 (E1+) cells. Recombinant AAVrh.10Luc and AAVrh.10GFP were produced following the same protocol using 500  $\mu$ g luciferase or 500  $\mu$ g GFP vector plasmid, 500  $\mu$ g AAVrh.10 packaging plasmid, and 1 mg Ad helper plasmid for transfection. Seventy-two hours posttransfection, cells were harvested, and a crude viral lysate was prepared by three cycles of freeze/thaw and clarified by centrifugation. The AAVrh.10-based vectors were purified by iodixanol gradient and Q HP anion-exchange chromatography. All purified rAAVs were concentrated using a BioMax 100 membrane concentrator (Millipore, Billerica, MA, USA) and stored in phosphate-buffered saline, pH 7.4 (PBS), at  $-80^{\circ}\text{C}$ .

The AAV2 and AAV5 vectors (AAV2 or 5 capsid with AAV2 ITRs) expressing GFP or  $\beta$ -galactosidase (LacZ; the vectors are referred to as AAV2GFP, AAV5GFP, AAV2LacZ, and AAV5LacZ) and an AAV5 vector (AAV5 capsid with AAV2 ITRs) expressing human  $\alpha$ 1AT (AAV5 $\alpha$ 1AT) were produced and purified as described by De *et al.* [6].

Vector genome titers were determined by TaqMan real-time PCR using a CMV-chicken  $\beta$ -actin promoter-specific primer-probe set, designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). Purified rAAV was digested with proteinase K in the presence of 0.5% sodium dodecyl sulfate and 25 mM ethylenediaminetetraacetate at  $55^{\circ}\text{C}$  for 30 min followed by inactivation of the protease at  $95^{\circ}\text{C}$  for 5 min. This material was used as a template for TaqMan analysis using an AAV- $\alpha$ 1AT plasmid DNA standard of known copy number to generate a standard curve. The genome copy number of each recombinant AAV preparation was determined using this standard curve.

**Intrapleural vector delivery.** The recombinant AAV vectors were delivered into the pleural space of the left lung of 6-week-old C57BL/6 mice (the *n* used in each study is given in the figure legends) as previously described [6]. The mice were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg) by intraperitoneal injection. The trachea was cannulated with a 20-gauge angiocatheter (Becton-Dickinson, Franklin Lakes, NJ, USA), and mechanical ventilation was achieved via a small-animal ventilator (Harvard Apparatus, Holliston, MA, USA). Tidal volume and respiratory rate were set at 0.7 to 1.0 ml  $\times$  70/min. An anterolateral chest skin incision approximately 1 cm in length was made using scissors. The thoracic cage was exposed and a thoracotomy was done in the third intercostal space. The different doses of AAV vectors were administered in 100  $\mu$ l of PBS through the exposed pleural space using a 1-ml insulin syringe. After placement of the angiocatheter through the pleural cavity, the thoracic cage was closed with 4-0 absorbable suture (Polysorb, Auburn, NY, USA). The angiocatheter was removed after air was evacuated manually using a syringe. The skin was closed using a second layer of the same sutures, the ventilator was stopped, and the tracheal tube was removed.

**Evaluation of human  $\alpha$ 1AT levels.** To evaluate the levels of human  $\alpha$ 1AT in the serum of mice injected intrapleurally with the  $\alpha$ 1AT-expressing AAV vectors, serum was collected by drawing 250  $\mu$ l of blood from the tail vein at time 0 and at various time points, as indicated under Results. The blood samples were allowed to clot for 1 h at 23°C followed by 30 min at 4°C and then spun in a microcentrifuge at 10,000g for 5 min to collect serum. The levels of human  $\alpha$ 1AT in the serum samples were then determined by using an  $\alpha$ 1-antitrypsin ELISA kit (ALPCO Diagnostic, Windham, NH, USA) and a highly purified  $\alpha$ 1AT standard (courtesy of M. Brantly, University of Florida, Gainesville, FL, USA) [28]. The assay utilizes a peroxidase-conjugated polyclonal anti- $\alpha$ 1AT antibody.

**Inhibition of neutrophil elastase activity by  $\alpha$ 1AT.** To compare the biological activity of serum human  $\alpha$ 1AT following intrapleural administration of AAVrh.10h $\alpha$ 1AT to mice with that of pure  $\alpha$ 1AT, purified from human serum, inhibition of neutrophil elastase activity was assessed *in vitro*. The assay was carried out using N-Suc-Ala-Ala-Ala-*p*-nitroanilide substrate following the manufacturer's protocol (Elastin Products Co., Owensville, MO, USA). Neutrophil elastase (Elastin Products), 0.5  $\mu$ g, from purulent human sputum was used for the assay. Neutrophil elastase was preincubated for 30 min at 25°C with: (1) naive mouse serum, (2) sera from mice intrapleurally administered with AAVrh.10h $\alpha$ 1AT 6 week previously, or (3) naive serum spiked with pure h $\alpha$ 1AT (kind gift from M. Brantly, University of Florida). Preincubation was carried out with different serum dilutions. Substrate (2 mM N-Suc-Ala-Ala-Ala-*p*-nitroanilide) was added and initial reaction rates were measured by monitoring change in absorbance at 410 nm. Human  $\alpha$ 1AT levels in mice serum were determined by ELISA, as described above.

**Organ distribution of AAVrh.10 vector-mediated transgene expression following intrapleural administration.** To evaluate organ distribution of the AAVrh.10 vector following intrapleural administration, AAVrh.10Luc ( $5 \times 10^{10}$  genome copies) in 100  $\mu$ l PBS was administered to the left pleural cavity of C57BL/6 mice ( $n = 4$ ). Six weeks after administration, the animals were sacrificed and tissue samples were collected from each animal: liver, spleen, kidneys, testes, quadriceps muscle, brain, heart, chest walls, right and left lung separately, and diaphragm. The total weight of the organs was determined, and then the organs were analyzed for distribution of luciferase transgene expression. The luciferase enzyme activity in homogenates of the individual organs was assessed, and activities were represented as relative light units quantified by a luminometer (Promega, Madison, WI, USA) and standardized by total protein concentration, using a bicinchoninic acid assay (Bio-Rad, Hercules, CA, USA). The relative light units were also multiplied by total organ weight. To estimate the total amount in muscle, the value for the quadriceps was multiplied by 32, based on the knowledge that the murine quadriceps represents approximately 3.5% of the total murine muscle mass, considering the average total muscle mass of rodents as 40% of total body weight [29]. Organs were also assessed for GFP expression following intrapleural administration of AAVrh.10GFP by immunohistochemistry.

**Immunization with AAV vectors and evaluation of anti-AAV neutralizing antibody titers.** To evaluate the activity of the AAVrh.10-based vector in the presence of preexisting immunity against the AAV2 and AAV5 serotypes, C57BL/6 mice were immunized by two subcutaneous (left foot pad) injections, 2 weeks apart, of AAV2GFP or AAV5GFP ( $2 \times 10^{10}$  genome copies), in 30  $\mu$ l of PBS as vehicle. Neutralizing antibody titer was determined *in vitro* using 293 cells expressing the Ad E4 gene ORF6 in 96-well plates [10,30]. To determine anti-AAV2 neutralizing antibody titer, AAV2LucZ was incubated with serial dilutions of AAV2-administered mouse serum or naive control serum at 23°C for 30 min. 293 cells were infected with serum-treated AAV2LucZ at a multiplicity of infection of  $10^4$  genome copies per cell. At 48 h postinfection,  $\beta$ -galactosidase activity was measured in the cell lysate using a  $\beta$ -galactosidase assay kit (Stratagene, La Jolla, CA, USA). The neutralizing antibody titer was expressed as the reciprocal of serum dilution at which 50% inhibition of AAV2LucZ was observed. Anti-AAV5 neutralizing antibody titer was determined following the same protocol except AAV5LucZ at a multiplicity of infection of  $4 \times 10^4$  genome copies/cell was used. To determine anti-AAVrh.10 neutralizing antibody titer, AAVrh.10Luc at a multiplicity of infection

of  $5 \times 10^4$  genome copies/cell was used as a reporter instead of LacZ; 293 cells were infected as described above and at 48 h postinfection, luciferase activity was measured in the cell lysate using a luciferase assay kit (Promega). Neutralizing antibody titer was defined as described above for the  $\beta$ -galactosidase assay.

**Statistical analysis.** All data are expressed as means  $\pm$  standard error. Comparisons between two groups were performed with the two-tailed Student *t* test.

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