Intramuscular administration of AAV overcomes pre-existing neutralizing antibodies in rhesus macaques

Jenny A. Greig, Roberto Calcedo, Rebecca L. Grant, Hui Peng, C. Angelica Medina-Jaszek,
Omua Ahonkhai, Qiuyue Qin, Soumitra Roy, Anna P. Tretiakova, and James M. Wilson*

ABSTRACT

The seroprevalence of neutralizing antibodies (NAbs) to adeno-associated viral (AAV) vector capsids may preclude a percentage of the population from receiving gene therapy, particularly following systemic vector administration. We hypothesized that the use of intramuscular (IM) administration of AAV vectors might circumvent this issue. IM injections were used to administer AAV8 vectors expressing either secreted or non-secreted transgenes into mice and the influence of NAbs supplied by pre-administration of pooled human IgG on transgene expression was evaluated. We then studied the impact of naturally occurring pre-existing AAV8 NAbs on expression of a secreted transgene following IM vector delivery in rhesus macaques. Finally, we evaluated the ability to readminister AAV vectors via IM injections in rhesus macaques. In mice, the presence of AAV8 NAbs had no effect on gene expression in the injected skeletal muscle. However, liver transgene expression following hepatic distribution of the vector was ablated. In rhesus macaques, naturally occurring pre-existing AAV8 NAb titers of $\leq 1:160$ had no effect on expression levels of a secreted transgene after IM delivery of the vector. Additionally, readministration of AAV vectors was possible by IM injection into the previously injected muscle groups, with no effect on transgene expression by the original vector. Therefore, the presence of pre-existing NAbs in the human population should not preclude subjects from receiving gene therapy by IM administration of the vector so long as sufficient levels of secreted transgene expression can be produced without the involvement of liver.

Key words: vector, antibody, AAV, muscle

38 Abbreviations: adeno-associated viral (AAV), alpha-1-antitrypsin (AAT), cytomegalovirus
39 (CMV), cytotoxic T lymphocyte (CTL), enzyme-linked immunosorbent assay (ELISA), firefly
40 luciferase (ffLuc), genome copies (GC), human immunodeficiency virus (HIV), immunoadhesin
41 (IA), intramuscular (IM), Institutional Animal Care and Use Committee (IACUC), intravenous
42 (IV), intravenous immunoglobulin (IVIG), knockout (KO), monoclonal antibody (mAb),
43 neutralizing antibodies (NAbs), non-human primate (NHP), recombinase activation gene (RAG),
44 simian immunodeficiency virus (SIV), simian-human immunodeficiency virus (SHIV), truncated
45 muscle creatine kinase (tMCK)

46

INTRODUCTION

Gene therapy has been heralded as the future of medicine for many genetic diseases, and with the approval of the adeno-associated viral (AAV) vector Glybera[®] for the treatment of lipoprotein lipase deficiency; it is now becoming a reality. A limitation to the effective use of gene therapy is the prevalence of pre-existing neutralizing antibodies (NAbs) to AAV capsids in humans. Currently, early phase clinical trials of systemically delivered vectors are precluding participation of subjects with high pre-existing NAbs.

The impact of pre-existing NAbs potentially could be minimized by using less commonly recognized AAV serotypes, such as AAV8, to which there are less pre-existing NAbs in humans compared to other serotypes [1, 2]. In addition, the route of vector administration clearly influences the impact of NAbs. Following intravenous (IV) administration of vector, NAbs towards the vector capsid significantly influence transgene expression, where NAb titers of >1:10 preclude an individual from receiving IV gene therapy [3]. In contrast, pre-existing NAbs have been shown to have less impact on gene transfer following intramuscular (IM) delivery [4-6]. For example, in a Phase I clinical trial for alpha-1-antitrypsin (AAT) deficiency two subjects had pre-existing NAbs to the vector that was used for gene delivery, AAV1 (NAb titers of 1:80 and 1:160). These subjects produced similar AAT levels as those who were sero-negative prior to IM vector injection [4, 5]. Therefore, IM administration of an AAV vector is less susceptible to pre-existing NAbs.

As traditional vaccine strategies have not succeeded in the prevention of human immunodeficiency virus (HIV) infection, passive immunization using viral vectors expressing

broadly NAb to HIV has been developed as an alternative therapy approach [7, 8]. This strategy removes the need for repeated parenteral delivery of NAb as a traditional biotherapeutic. Studies have shown that IM administration of 1.3×10^{10} genome copies (GC) to humanized mice (a dose of approximately 3×10^{11} GC/kg) resulted in serum expression levels of an immunoadhesin (IA) version of the broadly NAb to HIV, VRC01, at 8.3 µg/ml. This was sufficient to provide protection against subsequent challenge with HIV [8, 9], highlighting the potential of this approach.

As other strategies have been successfully used to administer and readminister AAV vectors locally [10], and as there is less influence by AAV NAb following IM injection, we predict that readministration by IM injection will be possible. The ability to readminister vector will enable a variety of scenarios, including increasing transgene expression by injection of a second dose or expanding the number of HIV NAb expressed to prevent viral escape. In this study, we investigated the influence of AAV NAb on transgene expression following IM administration of AAV8, first in mice and then in non-human primates (NHPs).

MATERIALS AND METHODS

AAV vector production

AAV vectors were produced by the Penn Vector Core at the University of Pennsylvania as previously described [11].

Mice

Male C57BL/6 and recombinaise activation gene (RAG) knockout (KO) mice 6-8 weeks of age were purchased from Charles River Laboratories (Wilmington, MA, USA) and The Jackson Laboratory (Bar Harbor, ME, USA), respectively. Mice were housed under pathogen-free conditions at the University of Pennsylvania's Translational Research Laboratories. Animal procedures and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania. IM vector administration and passive transfer experiments were performed as described previously [12, 13]. Pooled human IgG (intravenous immunoglobulin [IVIG]) was purchased from Carimune NF (CSL Behring, Bern, Switzerland).

Non-human primates

Rhesus macaques (Chinese origin, captive bred, 2.8-7.7 kg) were housed at the NHP Research Program Facility. Studies were performed according to a study protocol approved by the Environmental Health and Radiation Safety Office, Institutional Biosafety Committee, and IACUC of the University of Pennsylvania. Studies in African green (USA born, captive bred, 1.8-2.4 kg) and squirrel monkeys (Bolivian origin, captive bred, 0.7-0.8 kg) were conducted at Covance (Princeton, NJ, USA) in accordance with their IACUC. IM vector administration was performed as described previously [12].

NAb titration assay

NAb assays were performed on serum as described previously [2].

Detection of secreted proteins

AAT and antibody transgene expression were measured in serum from mice and NHPs using an enzyme-linked immunosorbent assay (ELISA) as described previously [1, 12]. For NHP samples, ELISA plates were coated with gp120 proteins from strains of simian-human immunodeficiency virus (SHIV) or simian immunodeficiency virus (SIV) to which the antibodies bind diluted in phosphate-buffered saline (PBS, Immune Technology Corp., New York, NY, USA).

Statistical analysis

All analyses were performed in Prism (GraphPad Software, San Diego, CA, USA) using either an unpaired Student's t-test or analysis of variance for significance (Tukey's or Dunnett's multiple comparisons test).

RESULTS

Pre-existing vector immunity ablates liver gene expression

To create a model of gene expression in the presence of pre-existing vector immunity, passive transfer experiments were conducted. C57BL/6 mice were administered IV with 200 µl of pooled human IgG (intravenous immunoglobulin [IVIG]) or naïve mouse serum two hours prior to AAV vector injection. IVIG contained NAbs to AAV8 of 1:320 [2]. Mice were then administered with 10^{10} GC of an AAV8 vector expressing firefly luciferase (ffLuc) driven by a modified chicken beta-actin promoter (AAV8.CB7.ffLuc) into the right gastrocnemius muscle. On day 7, mice were imaged to quantify ffLuc expression.

In mice pre-administered with naïve mouse serum (denoted as 0 mg IVIG), IM administration of AAV8 resulted in highly transduced skeletal muscle with substantial hepatic targeting (Figure 1A). Quantification of ffLuc revealed high transgene expression in muscle and liver, although the absolute expression of ffLuc could not be compared as the efficiency of imaging in these two organs differs (Figure 1C). The proportion of liver gene expression following IM vector administration was greater than we reported previously [12], likely due to greater hepatic activity of the CB7 promoter compared to the previously used cytomegalovirus (CMV) promoter.

IM administration of 10^{10} GC of vector in the presence of 12 mg IVIG completely ablated liver ffLuc expression (Figure 1B). Dose-titration of IVIG revealed a threshold level of pre-existing NAbs above which hepatic gene expression was ablated as pre-administration of 1.2 mg IVIG produced a 150-fold reduction in liver ffLuc expression and higher doses abrogated expression to background levels (Figure 1C). In contrast, muscle gene expression was not affected by any dose of IVIG studied. The influence of IVIG on gene expression in skeletal muscle was vector dose-dependent as liver gene expression was significantly reduced at 1.2 mg IVIG with a concomitant decrease in muscle expression when the vector dose was decreased ten-fold (Supplementary Figure 1). At the lower vector dose, transgene expression was completely ablated in muscle and liver at 40 mg IVIG. Thus, the ability to express an AAV gene therapy product in the presence of pre-existing vector immunity is influenced by both the level of NAbs and the AAV vector dose injected.

Pre-existing vector immunity reduces secreted protein expression in mice

As the presence of pre-existing AAV NAbs significantly affected liver gene expression of a non-secreted protein (ffLuc) following IM injection, we investigated the effect of NAbs on expression of a secreted transgene. Human AAT was used as C57BL/6 mice have previously been shown to be tolerant to this non-self transgene [1, 14]. Administration of 1.2-4 mg IVIG prior to IM injection of 10^{10} GC of AAV8.CB7.AAT decreased AAT expression eight-fold, whereas higher doses of IVIG attenuated gene expression (Figure 1D). Following IV administration of vector, we observed ablation of gene expression in the presence of IVIG (Figure 1D). These data indicate that the majority of secreted product from AAV8 with the highly active and constitutive CB7 promoter is from liver following IM injection, with muscle expression accounting for 10-20% of that produced from liver.

Promoter comparison for expression of secreted transgenes in mice and NHPs

Since we observed a high level of transgene expression in C57BL/6 mice using the CB7 promoter following IM administration, we next examined the ability of CB7 to express a transgene in a large animal model, rhesus macaques. In addition, we investigated two additional promoters: the ubiquitous CMV and the muscle-specific truncated muscle creatine kinase (tMCK) promoter [15] (Figure 2). To minimize macaque immune responses to the transgene product, we used a rhesus macaque-derived monoclonal antibody (mAb) sequence, 2.10A mAb, isolated following challenge with SHIV [16]. First, the vectors were evaluated in immunodeficient RAG KO mice following IM administration of 10^{11} GC per mouse ($\sim 5 \times 10^{12}$ GC/kg) of AAV8 vectors expressing 2.10A mAb from the CB7, CMV, or tMCK promoter. Peak 2.10A mAb expression levels of 5040, 466, and 250 $\mu\text{g/ml}$ were observed by the CB7, CMV,

and tMCK promoters, respectively (Figure 2A). To our knowledge, this level of antibody production by vector expression has not been previously reported.

To translate expression from mice to NHPs, the same vectors were then administered IM at a dose of 3×10^{12} GC/kg into rhesus macaques (Figure 2B). All macaques were screened and determined to have pre-existing AAV8 NAb titers of $<1:5$ prior to vector administration. The peak transgene expression levels were 1264, 633, and 115 $\mu\text{g/ml}$ from the CB7, CMV, and tMCK promoters, respectively (Figure 2B). Although the CB7 promoter produced the highest expression levels in both mice and rhesus macaques, translation to a larger animal model resulted in a four-fold drop in expression. Expression by the tMCK promoter also decreased (2.2-fold) from mice to macaques, whereas CMV promoter-driven expression increased 1.3-fold in macaques. Overall, the translation of vector and promoter activity from mice to NHPs was impressive.

Expression of secreted proteins in three species of NHPs

To demonstrate that the high expression levels observed in rhesus macaques were not species specific, we studied two additional NHP species (squirrel and African green monkeys). As the CMV promoter produced similar expression levels in mice and rhesus macaques, we produced AAV8 vectors using this promoter expressing the rhesus macaque-derived 201Ig IA transgene [12]. Rhesus macaques with no detectable AAV8 NABs were administered IM with 3×10^{12} GC/kg of AAV8.CMV.201Ig IA; these animals expressed 458 $\mu\text{g/ml}$ 201Ig IA at peak, which was ~two-fold higher than what was seen in immunodeficient mice [12]. Expression plateaued and remained stable at ~85 $\mu\text{g/ml}$ for over two years (Figure 3A). One rhesus macaque (090-

0286) died unexpectedly one year after vector administration due to hypertrophic cardiomyopathy.

We next evaluated expression in squirrel and African green monkeys. Once again, we only included animals with NAb titers of <1:5 in the study. Interestingly, none of the 58 squirrel and 40 African green monkeys screened had AAV8 NAb titers greater than background. These data contrast with the high seroprevalence seen in rhesus macaques, where up to 80% of rhesus macaques had AAV8 NAb titers [1]. We administered squirrel and African green monkeys with the same vector dose as used above for rhesus macaques. The peak expression in squirrel monkeys was comparable to in rhesus macaques (297 µg/ml 201Ig IA; Figure 3B), whereas the peak expression was ~four-fold lower in African green monkeys (Figure 3C). One African green monkey (017-1868) appeared to develop an immune response to the transgene as expression dropped at four weeks and remained at low but detectable levels (Figure 3C). Therefore, AAV8 transduced skeletal muscle in two NHP species at efficiencies equal to or greater than that observed in mice.

Threshold effect for impact of NAb titers following IM vector administration

To evaluate the influence of pre-existing NAb titers on gene expression, we injected rhesus macaques with naturally occurring AAV8 NAb titers of 1:20 (3), 1:160 (1), and 1:320 (1) with 3×10^{12} GC/kg of AAV8.CMV.201Ig IA (Figure 4). The peak expression in animals with a NAb titer of 1:20 was slightly higher than expression in naïve macaques (580 µg/ml compared to 458 µg/ml). Higher pre-existing NAb titers decreased peak expression, as animals with titers of 1:160 and 1:320 had 201Ig IA levels of 71 and 117 µg/ml, respectively. At two years after vector administration,

there was no significant difference in steady-state serum 201Ig IA expression from macaques with $\leq 1:160$ NAb titers prior to AAV8 administration (Figure 4). As AAV8 NAb titers of 1:160 would completely block hepatocyte transduction, this result suggests that muscle contributes more to overall systemic production of the IA than liver [3].

AAV8 NAb titers were monitored throughout the course of the study for macaques that had pre-existing immunity titers of $<1:5$ (Figure 4B), 1:20 (Figure 4C), and $>1:160$ (Figure 4D). All groups of animals showed a marked increase in AAV8-specific NAb titers following vector administration. In animals that were naïve for AAV8 prior to vector administration NAb titers decreased over time to a median titer value of 1:160 at ~2 years post-AAV8 vector administration (Figure 4B). In comparison, macaques with naturally occurring pre-existing NAb titers of 1:20 and $>1:160$ rapidly plateaued at a titer 16- to 32-fold greater than that preceding injection of vector and remained at those high levels for at least a year (Figures 4C and 4D).

Vector readministration results in expression equivalent to that seen in naïve NHPs

As IM vector administration with naturally occurring NAb titers of $\leq 1:160$ did not influence expression of a secreted transgene, we investigated the ability to readminister AAV vectors IM. We evaluated three rhesus macaques previously injected with AAV8.CMV.201Ig IA for cross-reacting NAb titers to AAV9. Two animals (090-0264 and 090-0273) had AAV9 NAb titers of 1:10 and one macaque had an AAV9 NAb titer of 1:40 (090-0290). On day 770 after AAV8 administration, we performed IM administration of AAV9 at the same dose (3×10^{12} GC/kg). To determine expression from both the AAV8 and AAV9 vectors within the same macaque, we used an AAV9 vector expressing a heterologous transgene, 2.10A mAb, differentiated from

201Ig IA by its binding specificity. A rhesus macaque (RQ9151) naïve for AAV8 and AAV9 was injected with the same dose of the AAV9 vector for comparison of 2.10A mAb expression in the absence of AAV8 pre-injection (Figure 5A).

Two of the three readministered rhesus macaques expressed significantly higher levels of 2.10A mAb than the naïve animal on day 28 (Figure 5A). Later time points suggest that this may be a function of natural variation in the macaques, as 2.10A mAb expression by the naïve animal plateaued between that of the readministered macaques. Following an initial peak of 2.10A mAb expression in macaque 090-0290, expression remained low but detectable (Figure 5A). This expression pattern could indicate an immune response against 2.10A mAb. Expression of 201Ig IA in all three rhesus macaques was not influenced by vector readministration (Figure 5B). These data clearly show that readministration by IM injection is possible for heterologous serotypes when NAb titers to vector capsids are $\leq 1:160$. In addition, readministration had no effect on a previously expressed transgene or the level of expression that can be produced for a second transgene.

DISCUSSION

AAV-mediated gene therapy is progressing rapidly into the clinic. NAb to vector capsid affect efficiency of *in vivo* transduction and are influenced by route of administration, dose, and capsid serotype [3]. Pre-existing NAb previously were shown to have a lesser effect on gene expression following IM vector injection than systemic administration [3-5]. In this study, we simulated pre-existing human NAb in mice by passive transfer of human IVIG prior to vector administration. This approach enabled us to evaluate the effect of pre-existing human NAb

compared to NAb arising following immunization, despite AAV not being a pathogen in mice. Our previous work indicated that the serologic response to natural AAV infections has greater breadth than that elicited to vector in immunologically naïve animals [2, 4]. In the current study, pre-existing human NAb had little effect on expression of non-secreted transgenes in muscle. For secreted transgenes, the localization of gene expression is less important than the total level detected in circulation. The lack of liver transgene expression with pre-existing NAb would only be of concern if sufficient gene expression cannot be produced from skeletal muscle alone. Following IM administration of AAV8 expressing AAT in mice, low dose IVIG (1.2-4 mg) produced a significant reduction in AAT expression with attenuation seen with ≥ 12 mg IVIG. In contrast, there was complete absence of AAT expression at all doses of IVIG following IV vector delivery. This suggests that in mice the contribution of liver to systemic production of a secreted protein is ~five-fold higher than the contribution from transduced muscle. This is not the case in NHPs, where muscle dominates the systemic production of a secreted transgene product following IM injection. We note that these conclusions were derived from a vector with a promoter known to have very high activity in both liver and muscle. Thus, the results are likely to be different with promoters that have more specificity to liver or muscle.

Although passive transfer experiments in mice can generate a measurable model of the impact of AAV8 NAb, they cannot predict the impact of naturally occurring AAV NAb in larger animals. Thus, we studied rhesus macaques with naturally occurring AAV8 NAb to better model human subjects. At a high dose of AAV8 (3×10^{12} GC/kg), pre-existing NAb titers of $\leq 1:160$ did not affect gene expression in rhesus macaques. In comparison, studies using IV administration of AAV showed that NAb titers of $> 1:10$ completely abrogated gene expression;

thus this approach is 16-fold more susceptible to NAb [3]. This is highly relevant to the selection of subjects for gene therapy clinical trials via IM administration as, in the US, only 5% of the population have pre-existing NAb titers of $>1:80$ [2]. Therefore, using skeletal muscle as the route of administration increases the amount of subjects who could receive gene therapy up to ~95%.

As lower levels of naturally occurring NABs to the vector capsid did not impact transduction efficiency, we attempted to readminister NHPs with a heterologous serotype vector via the same route of administration. While AAV8 NAb titers were $\leq 1:320$ at the time of readministration (two years after the first vector administration), suggesting that readministration with the same AAV serotype into muscle would be possible, we decided to use AAV9 for the second vector administration. Currently, there are no studies that have explored whether the antibodies generated in response to a naturally occurring AAV infection differ to those generated by administration of a gene therapy product. Cross-reacting AAV9 NABs (serotype used for readministration) of $\leq 1:40$, generated following AAV8 IM delivery, did not impact gene expression of either the first or second transgene. It is a theoretical issue that immune memory could increase the risk of developing a cytotoxic T lymphocyte (CTL) response to the gene therapy product [17]. To limit any potential immune reaction to the transgene product, we used rhesus macaque-derived antibody sequences, minimizing the risk of transgene recognition as non-self that could result in a CTL response. There was no evidence of myositis or loss of transgene expression throughout the three-year study, which would be associated with a destructive cytotoxic response.

After an initial peak in NABs, the titers slowly decreased until prior to injection of AAV9 (Figure 4B, C, and D). Following AAV9 vector administration, a similar NAb response to AAV8 was seen in these animals as seen for AAV9 (data not shown) suggesting a cross-reactive humoral immune response despite a different AAV serotype being used for readministration. A similar phenomenon was observed by Wang *et al.* [18] in a heterologous AAV2 and AAV8 regimen in a canine model of hemophilia B. These data clearly show that readministration by IM injection was possible for heterologous serotypes when NAb titers to vector capsids are $\leq 1:160$. Also, readministration had no effect on a previously expressed transgene or the level of expression that can be produced for a second transgene.

In this study, we also conducted a quantitative comparison of AAV-mediated, muscle-directed gene transfer across multiple species using different promoters. When AAV8 dosing was scaled according to body mass, there was no difference in expression of a secreted protein from the largely muscle-specific CMV promoter. The primary focus of translation from mice to large animals has been the impact of capsid; an equally important consideration is the relative activity of promoters. Very similar activities of two constitutive promoters and one muscle-specific promoter were seen in mice and macaques. The only discrepancy was enhanced activity of the CB7 promoter in mice, likely due to greater delivery to and expression from AAV8 in mouse liver following IM administration.

The expression levels of rhesus anti-SHIV/SIV antibodies seen in three species of NHPs are unprecedented. Modeling of passive immunization strategies in NHPs using AAV for expression of broadly NABs suggests that this approach could fail due to generation of anti-

341 idotype antibodies, which are seen when expressed primatized antibodies contain human CDR
342 regions [19, 20]. However, we did not observe this when expressing a rhesus antibody in rhesus
343 macaques or other NHP species. Only one African green monkey out of 29 NHP presented here
344 and elsewhere by us [12] developed an immune response to the antibody transgene that
345 prevented detection of gene expression. Therefore, an anti-idiotypic response was likely not
346 simulated in NHPs, and expression of fully rhesus antibodies in NHPs could model the
347 expression of human anti-HIV antibodies in humans.

348
349 In conclusion, these studies provide insights into the clinical translation of AAV8 gene therapy
350 to muscle. First, transgene expression is equivalent when scaled to body mass between mice and
351 NHPs. Second, gene transfer to muscle is more refractory to inhibition by NAbs than gene
352 transfer to liver. Finally, vectors of different serotypes can be effectively readministered to
353 muscle.

354 355 **FUNDING**

356 This research was supported by a grant from the Bill and Melinda Gates Foundation, Seattle,
357 WA [grant number 51061 to J.M.W.].

358 359 **CONFLICTS OF INTEREST**

360 The authors declare having potential competing financial interests J.M. Wilson is an advisor to
361 REGENXBIO, Dimension Therapeutics, and Solid Gene Therapy, and is a founder of, holds
362 equity in, and has a sponsored research agreement with REGENXBIO and Dimension
363 Therapeutics; in addition, he is a consultant to several biopharmaceutical companies and is an

inventor on patents licensed to various biopharmaceutical companies. J.A.G., R.C., R.L.G.,
H.P., C.A.M.J., O.A., Q.Q., S.R., and A.P.T. declare no conflicts.

ACKNOWLEDGEMENTS

We would like to acknowledge the support of the Vector, Immunology, Cell Morphology, and
Animal Models Cores of the University of Pennsylvania Gene Therapy Program. In addition, we
would like to thank Erin Bote, Deirdre McMenamin, and Christine Draper for their invaluable
technical assistance.

REFERENCES

- [1] Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci U S A*. 2002;99:11854-9.
- [2] Calcedo R, Vandenberghe LH, Gao G, Lin J, Wilson JM. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *J Infect Dis*. 2009;199:381-90.
- [3] Wang L, Calcedo R, Bell P, Lin J, Grant RL, Siegel DL, et al. Impact of pre-existing immunity on gene transfer to nonhuman primate liver with adeno-associated virus 8 vectors. *Hum Gene Ther*. 2011;22:1389-401.
- [4] Flotte TR, Trapnell BC, Humphries M, Carey B, Calcedo R, Rouhani F, et al. Phase 2 clinical trial of a recombinant adeno-associated viral vector expressing alpha1-antitrypsin: interim results. *Hum Gene Ther*. 2011;22:1239-47.
- [5] Brantly ML, Chulay JD, Wang L, Mueller C, Humphries M, Spencer LT, et al. Sustained transgene expression despite T lymphocyte responses in a clinical trial of rAAV1-AAT gene therapy. *Proc Natl Acad Sci U S A*. 2009;106:16363-8.
- [6] Manno CS, Chew AJ, Hutchison S, Larson PJ, Herzog RW, Arruda VR, et al. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. *Blood*. 2003;101:2963-72.
- [7] Johnson PR, Schnepf BC, Zhang J, Connell MJ, Greene SM, Yuste E, et al. Vector-mediated gene transfer engenders long-lived neutralizing activity and protection against SIV infection in monkeys. *Nat Med*. 2009;15:901-6.
- [8] Balazs AB, Chen J, Hong CM, Rao DS, Yang L, Baltimore D. Antibody-based protection against HIV infection by vectored immunoprophylaxis. *Nature*. 2012;481:81-4.

397 [9] Horwitz JA, Halper-Stromberg A, Mouquet H, Gitlin AD, Tretiakova A, Eisenreich TR, et al.
398 HIV-1 suppression and durable control by combining single broadly neutralizing antibodies and
399 antiretroviral drugs in humanized mice. *Proc Natl Acad Sci U S A*. 2013;110:16538-43.

400 [10] Limberis MP, Wilson JM. Adeno-associated virus serotype 9 vectors transduce murine
401 alveolar and nasal epithelia and can be readministered. *Proc Natl Acad Sci U S A*.
402 2006;103:12993-8.

403 [11] Gao G, Lu Y, Calcedo R, Grant RL, Bell P, Wang L, et al. Biology of AAV serotype
404 vectors in liver-directed gene transfer to nonhuman primates. *Mol Ther*. 2006;13:77-87.

405 [12] Greig JA, Peng H, Ohlstein J, Medina-Jaszek CA, Ahonkhai O, Mentzinger A, et al.
406 Intramuscular injection of AAV8 in mice and macaques is associated with substantial hepatic
407 targeting and transgene expression. *PLoS One*. 2014;9:e112268.

408 [13] Wang L, Wang H, Bell P, McCarter RJ, He J, Calcedo R, et al. Systematic evaluation of
409 AAV vectors for liver directed gene transfer in murine models. *Mol Ther*. 2010;18:118-25.

410 [14] Breous E, Somanathan S, Wilson JM. BALB/c mice show impaired hepatic tolerogenic
411 response following AAV gene transfer to the liver. *Mol Ther*. 2010;18:766-74.

412 [15] Wang B, Li J, Fu FH, Chen C, Zhu X, Zhou L, et al. Construction and analysis of compact
413 muscle-specific promoters for AAV vectors. *Gene Ther*. 2008;15:1489-99.

414 [16] Robinson JE, Franco K, Elliott DH, Maher MJ, Reyna A, Montefiori DC, et al. Quaternary
415 epitope specificities of anti-HIV-1 neutralizing antibodies generated in rhesus macaques infected
416 by the simian/human immunodeficiency virus SHIVSF162P4. *J Virol*. 2010;84:3443-53.

417 [17] Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJ, et al. Successful
418 transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host
419 immune response. *Nat Med*. 2006;12:342-7.

420 [18] Wang L, Calcedo R, Nichols TC, Bellinger DA, Dillow A, Verma IM, et al. Sustained
421 correction of disease in naive and AAV2-pretreated hemophilia B dogs: AAV2/8-mediated,
422 liver-directed gene therapy. *Blood*. 2005;105:3079-86.

423 [19] Gardner MR, Kattenhorn LM, Kondur HR, von Schaewen M, Dorfman T, Chiang JJ, et al.
424 AAV-expressed eCD4-Ig provides durable protection from multiple SHIV challenges. *Nature*.
425 2015;519:87-91.

426 [20] Saunders KO, Wang L, Joyce MG, Yang ZY, Balazs AB, Cheng C, et al. Broadly
427 neutralizing human immunodeficiency virus type 1 antibody gene transfer protects non-human
428 primates from mucosal simian-human immunodeficiency virus infection. *J Virol*. 2015.

429

FIGURE LEGENDS

Figure 1. Impact of pre-existing immunity on expression following IM administration in C57BL/6 mice.

C57BL/6 mice were administered with 10^{10} GC per mouse of AAV8.CB7.ffLuc in one 10 μ l injection into the right gastrocnemius muscle. Two hours prior to vector administration, mice were injected IV with 200 μ l of naïve mouse serum or pooled human IgG. Mice were imaged using Xenogen whole-body bioluminescent imaging on day 7 post-vector administration. (A) Visualization of ffLuc expression in a mouse pre-injected with naïve mouse serum or (B) 12 mg IVIG. (C) Muscle (open bars) and liver (closed bars) ffLuc expression in photons/second (p/s) were quantified separately for mice pre-injected with a range of doses of IVIG. (D) C57BL/6 mice were administered with 10^{10} GC AAV8.CB7.AAT by IM injection or a 100 μ l IV injection into the tail vein. IVIG or naïve mouse serum was pre-administered. Expression of AAT was measured in serum by ELISA. Values are expressed as mean $\pm$ SEM (n = 4/group). Comparisons between multiple groups were performed using one-way analysis of variance (Dunnett's multiple comparisons test), * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 2. Promoter comparison for expression of a secreted protein following IM vector administration.

(A) RAG KO mice were administered IM with 10^{11} GC per mouse ($\sim 5 \times 10^{12}$ GC/kg) AAV8 vectors using either the CMV, CB7 or tMCK promoter for expression of 2.10A mAb (n = 5/group). (B) Rhesus macaques were administered with the same vectors at a dose of 3×10^{12} GC/kg into the right and left vastus lateralis muscles (n = 2/group). Expression of 2.10A mAb was measured in serum by ELISA and values are expressed as mean $\pm$ SEM. Comparisons

between multiple groups were performed using one-way analysis of variance (Tukey's Multiple Comparison post-test), *** $p < 0.001$.

Figure 3. Long-term expression of secreted transgenes in three species of NHPs.

(A) Rhesus macaques, (B) squirrel monkeys, and (C) African green monkeys were administered IM with 3×10^{12} GC/kg AAV8.CMV.201Ig IA into the right and left vastus lateralis muscles ($n = 4/\text{species}$). Expression of 201Ig IA was measured in serum by ELISA and values are expressed as mean $\pm$ SEM.

Figure 4. Impact of pre-existing immunity to AAV8 on transgene expression following IM administration of vector in NHPs.

(A) 201Ig IA expression in rhesus macaques with naturally occurring NAb to AAV8 of $<1:5$ ($n = 4$, same macaques as shown in Figure 3A), $1:20$ ($n = 3$), $1:160$ ($n = 1$), and $1:320$ ($n = 1$) prior to vector administration. Animals received 3×10^{12} GC/kg AAV8.CMV.201Ig IA by IM administration into the right and left vastus lateralis muscles. Expression of 201Ig IA was measured in serum by ELISA and values are expressed as mean $\pm$ SEM. AAV8 NAb titers were measured at selected intervals following vector administration for macaques which had pre-existing NAb to AAV8 of $<1:5$ (B), $1:20$ (C) and $>1:160$ (D).

Figure 5. Readministration of AAV vectors in NHPs.

Rhesus macaques, which were naïve for pre-existing NAb to AAV8, were administered IM with 3×10^{12} GC/kg AAV8.CMV.201Ig IA. On day 770 post-AAV8 vector administration, animals were readministered IM with AAV9 expressing 2.10A mAb into the right and left vastus lateralis

muscles. (A) Expression of 2.10A mAb from AAV9 vector in readministered macaques (090-0264, 090-0273, and 090-0290) was compared to expression from the same vector injected IM into a naïve rhesus macaque (RQ9151). (B) The time course of 201Ig IA expression in animals that received vector readministration pre- and post-AAV9 injection is shown. Dotted line indicates time of AAV9 administration. Expression of 201Ig IA and 2.10A mAb were measured in serum by ELISA. Values are expressed as mean $\pm$ SEM.

Supplementary Figure 1. Impact of pre-existing immunity of AAT expression following IM administration of a lower vector dose in C57BL/6 mice.

C57BL/6 mice were administered with 10^9 GC per mouse of AAV8.CB7.ffLuc in one 10 μ l injection into the right gastrocnemius muscle. Two hours prior to vector administration, mice were injected IV with 200 μ l of naïve mouse serum or pooled human IgG (intravenous immunoglobulin [IVIG]). Mice were imaged using Xenogen whole-body bioluminescent imaging on day 7 post-vector administration; muscle (open bars) and liver (closed bars) ffLuc expression in photons/second (p/s) were quantified separately for mice pre-injected with a range of doses of IVIG. Values are expressed as mean $\pm$ SEM. Comparisons between multiple groups were performed using one-way analysis of variance (Dunnett's multiple comparisons test), ** $p < 0.01$

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.