

# High concordance of ELISA and neutralization assays allows for the detection of antibodies to individual AAV serotypes

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**Prescreening of participants in clinical trials that use adeno-associated virus (AAV) vectors is required to identify naive participants, as preexisting neutralizing antibodies can limit the efficacy of AAV gene therapies. The presence of antibodies to individual AAV serotypes is typically detected by neutralization assay. To streamline the screening process, we compared an ELISA-based screening method with a neutralization assay for the detection of antibodies against AAV1, AAV8, and AAV9 in a collection of 50 rhesus macaque sera and 20 human sera. We observed a high level of concordance between the two assays (Pearson  $r > 0.8$ ) for all three serotypes in both sample sets. We thus investigated pre- vs post-vector inoculation sera samples from rhesus macaques that received AAV1 or AAV8 vector inoculations for cross-reactive anti-AAV antibodies. All 12 macaques seroconverted to the vector they received, but many also reacted to the other serotypes. Our results validate an easy-to-use ELISA for reliable detection of antibodies to individual serotypes of AAV. Our results also demonstrate that an antibody response post-AAV inoculation may partially cross-react with other AAV serotypes. Overall, these results suggest that either assay can be used by academic labs for prescreening samples for preexisting anti-AAV antibodies.**

## INTRODUCTION

Adeno-associated virus (AAV) vectors bring promise for treating diseases through gene therapy strategies. With the U.S. Food and Drug Administration (FDA) approval of Luxturna in 2017 and Zolgensma in 2019, the interest in using AAV vectors continues to grow. Clinical trials using AAV vectors have targeted hemophilia, alpha 1 antitrypsin deficiency, Duchenne muscular dystrophy, and Pompe disease.<sup>1</sup> Our labs have been developing AAV vectors to express HIV-1 broadly neutralizing antibodies and antibody-like inhibitors to prevent and treat HIV-1 infection.<sup>2–9</sup> These studies have relied extensively on testing AAV vectors in rhesus macaques to evaluate their efficacy against SHIV and SIV infection. Thus, we have screened

hundreds of macaques for preexisting anti-AAV antibodies before conducting the studies.

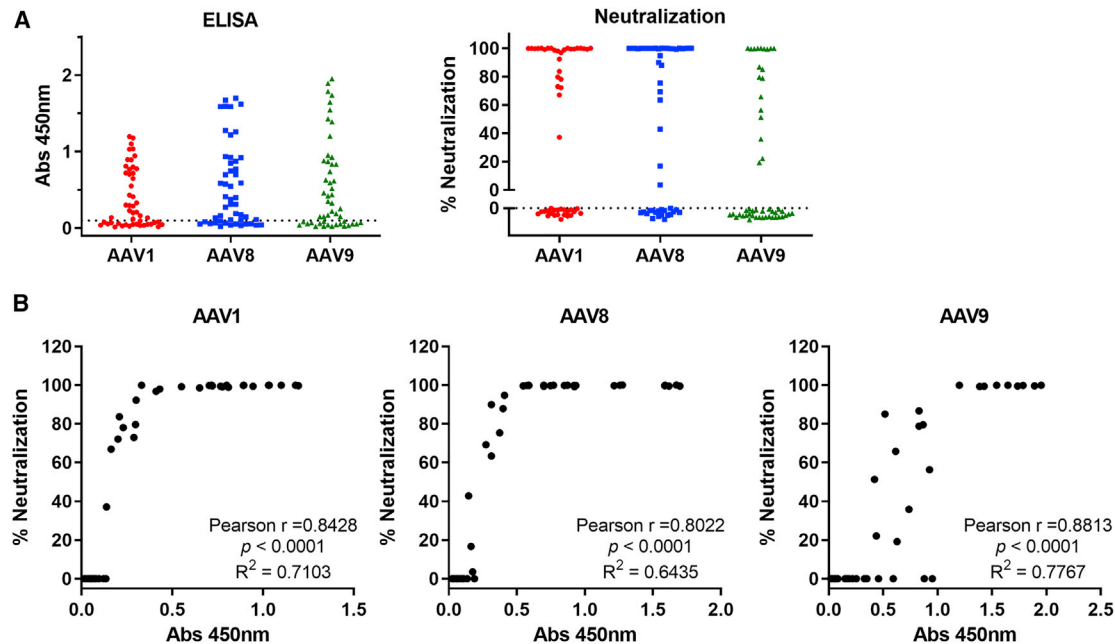
With the popularity of AAV vectors growing, more groups will need to evaluate their therapies in preclinical animal models. This includes the use of nonhuman primates (NHPs) such as rhesus macaques (*Macaca mulatta*). Numerous studies have observed preexisting antibodies against various AAV serotypes in human cohorts,<sup>10–15</sup> and these findings ultimately affect the ability to deploy AAV vectors for therapy. We have observed similar limitations in our selection of rhesus macaques for our studies. However, as the need for NHP models increases, so does the ability to screen NHPs prior to conducting a study.

Here we describe two different screening methods our labs use to determine AAV-negative rhesus macaques for the AAV1, AAV8, and AAV9 serotypes. One method identifies macaques with binding antibodies to intact virions as determined using ELISA. The second method identifies macaques on the basis of an *in vitro* neutralization assay. In the studies described here, we compared these two assays for their performance in identifying preexisting anti-AAV antibodies. For a group of 50 rhesus macaques, we observed a high degree of correlation between the two assays from serum samples tested against AAV1, AAV8, and AAV9 vectors. Correlation of the results from the two assays was also observed for these three vectors when human serum samples were tested. Additionally, both assays were able to identify cross-reactive anti-AAV antibodies after an AAV1 or AAV8 intramuscular (i.m.) vector inoculation. These data indicate that either assay could be used equivalently or together by academic labs to identify seronegative macaques for their preclinical studies.

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**Figure 1. Correlation between ELISA and neutralization assay for rhesus macaque samples**

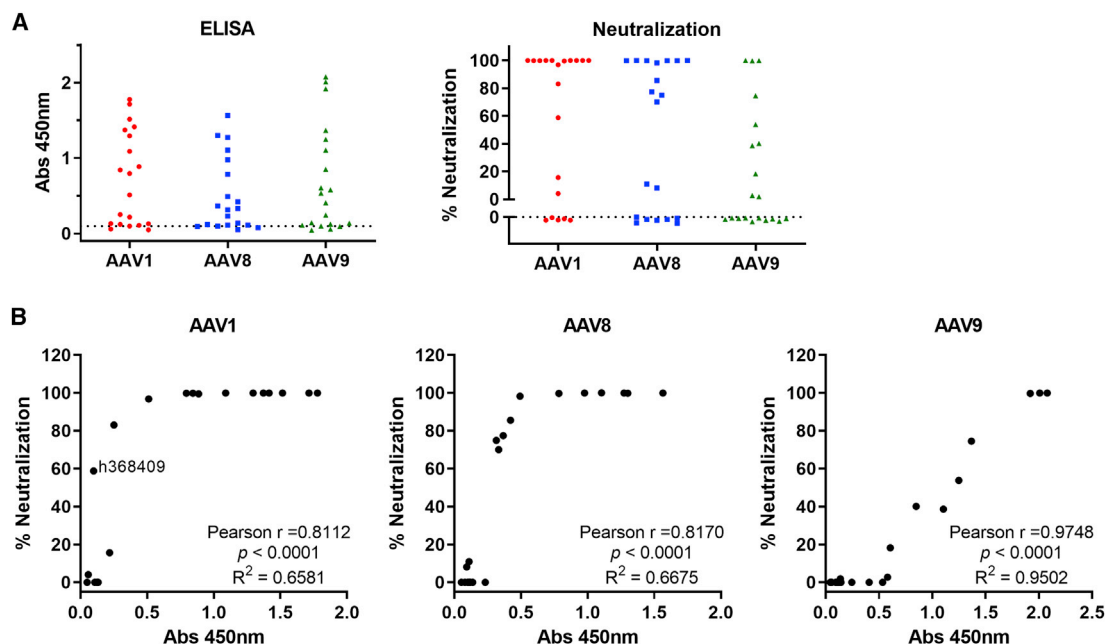
(A) Fifty rhesus macaque samples were screened for preexisting antibodies against the indicated AAV serotype using ELISA (left) and neutralization assay (right). For the ELISA, sera samples were diluted 1:20 and added to plates coated with the indicated serotype. Binding antibodies were determined using an anti-rhesus IgG secondary antibody. For the neutralization assay, sera samples were diluted 1:10 and mixed with an equal volume of the indicated vector. HEK293T cells were then added to the mixture, and luciferase was quantified after 24 h. See also [Table S1](#). (B) Correlation plots comparing the screen results from the ELISA and neutralization for the indicated serotype. Pearson r values, p values (two tailed), and R<sup>2</sup> values are included for each comparison.

## RESULTS

The goal of this study was to compare ELISA- and neutralization-based sample screening methods for preexisting antibodies against different AAV serotypes. ELISA plates were coated with intact virions at a concentration of  $1 \times 10^{10}$  vector genomes (vg)/mL of AAV1 vectors,  $5 \times 10^9$  vg/mL AAV8 vectors, or  $8 \times 10^9$  vg/mL AAV9 vectors, and the *in vitro* neutralization assays used  $7 \times 10^9$  vg/mL of AAV1 vectors,  $2 \times 10^{10}$  vg/mL AAV8 vectors, or  $2 \times 10^{11}$  vg/mL AAV9 vectors. The varying amounts of vectors in the neutralization assay were used to obtain a readout of >10,000 relative light units (RLUs) from AAV transduction of HEK293T cells. Using both these assays, we assessed 50 rhesus macaque serum samples for preexisting AAV1, AAV8, and AAV9 binding and neutralizing antibodies. Binding antibodies were determined using ELISA, and neutralizing antibodies were determined using an *in vitro* cellular assay ([Figure 1A](#)). Because these methods are used to screen a large number of samples, for these studies, binding is defined as the absorbance value at 450 nm at 1:20 dilution, and neutralization is defined as the decrease of transduction signal (firefly luciferase) at 1:10 sample dilution. Results from the ELISA showed varying degrees of antibody binding to the three different AAV serotypes. We observed absorbance values at 450 nm ranging from <0.1 to >1.0 ([Table S1](#)). Similar to the ELISA results, varying degrees of neutralizing antibodies ranging from 0% to >99% neutralizing activity were observed in our sample set.

We next compared the results from the two screening methods ([Figure 1B](#)). For all three serotypes, we observed Pearson r values > 0.8, indicating a high degree of correlation between the results obtained from ELISA and the neutralization assay ( $p < 0.0001$ ). Interestingly, we observed high degrees of neutralization when screening for AAV1 and AAV8 antibodies in samples that had absorbance values > 0.2. For AAV9, there was a higher degree of neutralization variability for samples with absorbance values between 0.1 and 1.0. However, all samples with absorbance values > 1.0 had >99% neutralization against AAV9. Overall, these data demonstrate that either method, ELISA or neutralization assay, can be used to identify rhesus macaques with preexisting AAV antibodies and generate similar screening results.

We next assayed 20 blinded human samples obtained commercially from BioIVT to determine if there was also a correlation between ELISA and neutralization assays with these samples ([Figure 2A](#); [Table S2](#)). Again, we observed a high degree of correlation between the two assays for all three serotypes tested, with Pearson r values > 0.8 ( $p < 0.0001$ ) ([Figure 2B](#)). Like the rhesus macaque samples, human samples with absorbance > with by ELISA usually resulted in a high neutralization response against AAV1 and AAV8. Yet for AAV9, we observed a linear correlation between ELISA absorbance value and neutralization response ( $R^2 = 0.9502$ ,  $p < 0.0001$ ). Importantly, no samples that were positive for anti-AAV antibodies by ELISA



**Figure 2. Correlation between ELISA and neutralization assay for human samples**

(A) Twenty human samples were screened for preexisting antibodies against the indicated AAV serotype using ELISA (left) and neutralization assay (right). Both the ELISA and neutralization assays were performed as described in Figure 1A. See also Table S2. (B) Correlation plots comparing the screen results from the ELISA and neutralization for the indicated serotype. Note that sample h368409 is indicated as the only sample for which binding antibodies were not detected, yet the sample had neutralizing activity. Pearson  $r$  values,  $p$  values (two tailed), and  $R^2$  values are included for each comparison.

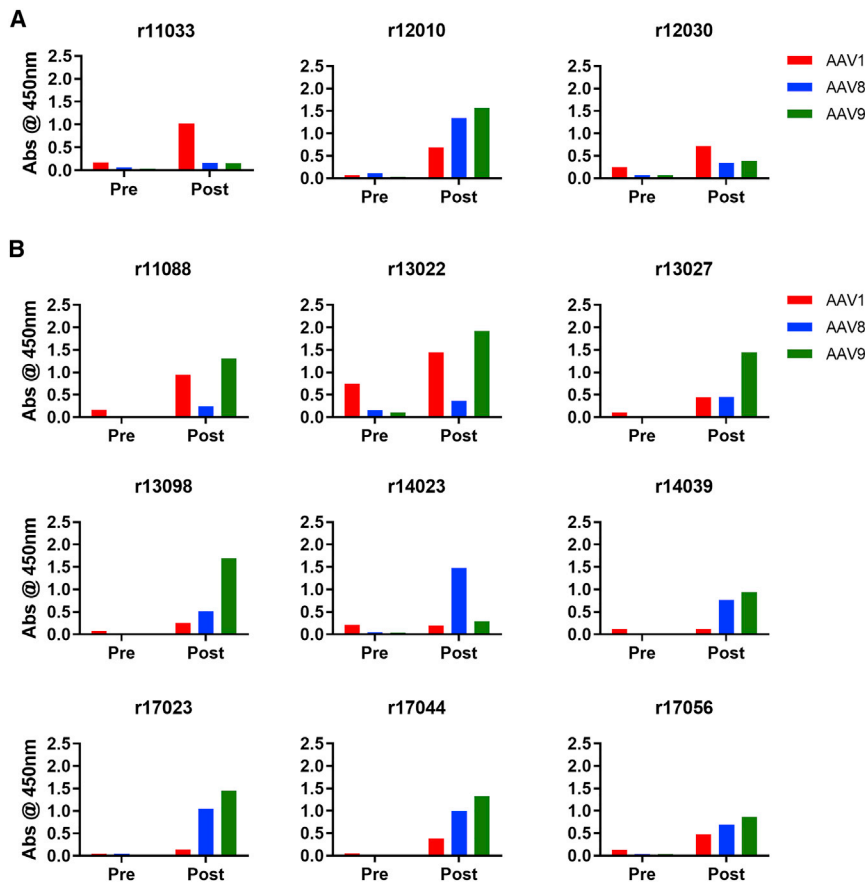
were negative for neutralizing antibodies against that same serotype, and vice versa.

We also examined the ability of both assays to detect anti-AAV responses after inoculation of a particular AAV serotype. An anti-AAV response after inoculation typically limits the transduction of a second inoculation with that same AAV capsid.<sup>16</sup> For this study, we used 12 rhesus macaque samples from a previous study.<sup>3</sup> Three samples came from macaques that received AAV1 i.m. inoculations and nine from macaques that received AAV8 i.m. inoculations. When assessed by ELISA, all three macaques that received AAV1 inoculations had increases in binding antibodies against AAV1 (Figure 3A). Additionally, two animals in this group (r11033 and r12010) had increases in signal against AAV8 and AAV9 serotypes. We observed similar results in the nine macaques that received AAV8 inoculation, with all nine having increases in ELISA signal against AAV8 postinoculation (Figure 3B). We also observed an increase in signal against AAV1 and AAV9 in six of the macaques in this group. The results from the neutralization assay showed similar trends. In the group of three macaques that received AAV1 inoculations, we observed >99% neutralizing activity postinoculation (Figure 4A). Two animals generated neutralizing antibodies against AAV8, and one macaque had neutralizing antibodies against AAV9. As expected, all nine macaques that received AAV8 inoculations developed neutralizing antibodies against AAV8 (Figure 4B). Neutralizing antibodies were observed in seven macaques against

AAV1 and AAV9. Interestingly, r14023 did not generate neutralizing antibodies against either AAV1 or AAV9 after AAV8 inoculation, suggesting that some neutralizing epitopes that are present on AAV8 are not present on AAV1 and AAV9.

The data in Figures 3 and 4 were then compared to determine the correlation between the two assays (Figure 5). We included all 24 data points taken from the 12 macaque samples for the pre- and post-AAV inoculation results. Similar to the results in Figures 1 and 2, we observed a high degree of correlation between the ELISA and neutralization assay results for all three capsids tested (Pearson  $r > 0.8$ ,  $p < 0.0001$ ). Together, these data highlight that rhesus macaques generate binding/neutralizing antibodies against the inoculated serotype in every instance postinoculation. More important, there appears to be a high likelihood that a host will also generate cross-reactive antibodies against other AAV serotypes following inoculation. On the basis of the correlation observed in Figure 5, these cross-reactive antibodies can be observed by both the ELISA and neutralization assay.

Last, we analyzed the impact of preexisting AAV antibodies on improving transgene expression after an AAV booster inoculation. Nine of the 12 macaques in Figures 3 and 4 received i.m. AAV1 booster inoculations. Fuchs et al.<sup>3</sup> reported antibody concentrations for these animals before and after the AAV1 booster inoculation. We assessed the correlation of the change in 4L6 concentration after the AAV1 boost with the anti-AAV1 antibodies determined by ELISA



**Figure 3. Screening of anti-AAV antibodies by ELISA after AAV1 or AAV8 inoculation in rhesus macaques**

(A) Sera samples from three rhesus macaques were screened using ELISA for preexisting binding antibodies to the indicated serotypes pre- and post-AAV1 intramuscular vector inoculation. ELISAs were performed as described in Figure 1. (B) Similar to (A), except the nine rhesus macaques were inoculated with an AAV8 vector.

or neutralization assay (Figure 6). We observed a moderate negative correlation (Pearson  $r = -0.3$  to  $-0.7$ ) when comparing the change in 4L6 concentrations and anti-AAV1 binding antibodies determined by ELISA. Additionally, we observed a strong negative correlation when using the neutralization assay. For example, r13022, r13027, and r13098 had an average increase of 4L6 expression after an AAV1 vector boost inoculation of 4.3-fold, even with all three macaques raising AAV1 neutralizing antibodies after an AAV8 i.m. inoculation. This was even more pronounced for r14023 and r14039, two macaques that did not raise AAV1 neutralizing antibodies after an intravenous AAV8 inoculation and were observed to have a >100-fold increase in 4L6 expression. In contrast, r11088 (part of the same group as r14023 and r14039) had only a 6-fold increase in expression despite not raising neutralizing antibodies against AAV1 after an AAV8 intravenous infusion. Additionally, r11033, r12010, and r12030 did not have increases in 4L6 concentrations after an i.m. AAV1 inoculation followed by a second i.m. AAV1 booster inoculation. All three of these macaques raised AAV1 neutralizing antibodies after the first AAV1 inoculation (Figures 3 and 4).

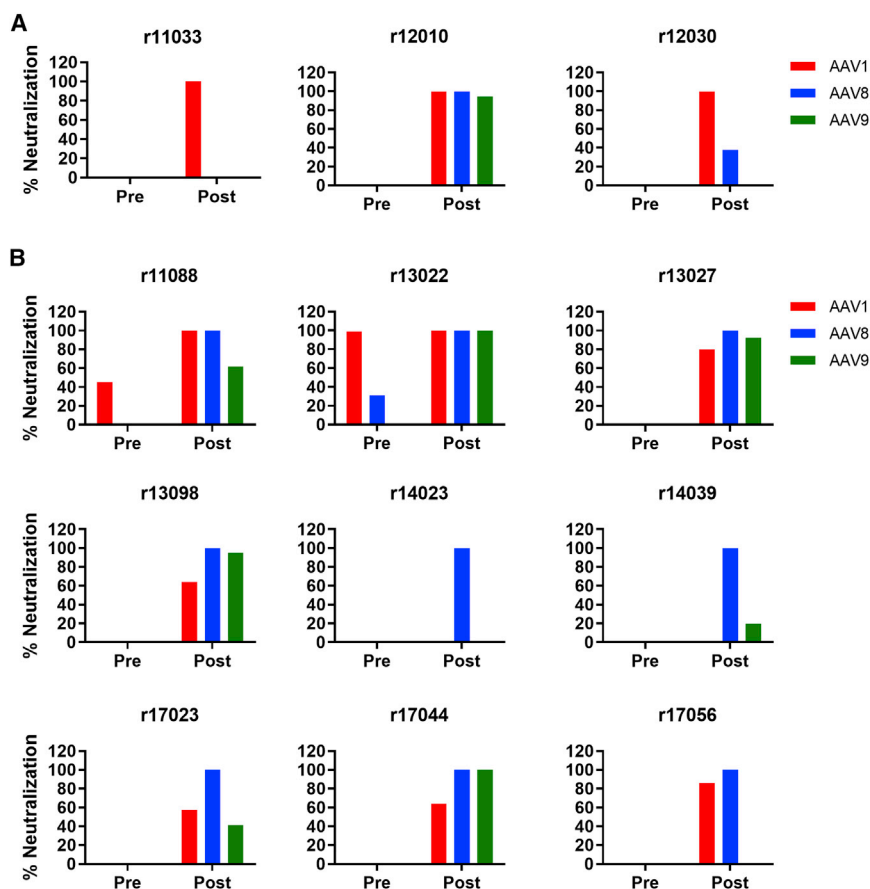
## DISCUSSION

Here we assessed two different strategies for screening samples for preexisting anti-AAV antibodies. Our results show that both methods

can identify NHPs or humans without antibody responses against specific AAV serotypes. Additionally, both the ELISA-based and neutralization-based method can easily be implemented in most academic labs with a high degree of confidence for prescreening samples. Our data show high concordance between the two assays for multiple serotypes using both NHP and human samples. We assayed 70 samples against three different AAV serotypes using both assays, for a total of 210 comparisons. In only one instance (sample h368409 against AAV1 in Figure 2; Table S2) did we observe a false negative by the ELISA, a rate of 0.47%. In this example, we observed neutralizing activity (58.81% neutralization) when binding antibodies were relatively low as determined by the ELISA (<0.1 absorbance value at 450 nm). One explanation could have been that we used a 1:20 dilution of serum samples for the ELISA while using a 1:10 dilution for the neutralization. Normalizing the dilution factor could have identified binding antibodies from this sample.

Despite this one example, the high degree of correlation we observed between the ELISA and neutralization assay is not that surprising. Both assays use intact virions and thus provide the same epitopes for analysis. Additionally, for a neutralizing antibody to function, it must bind the virion. As we demonstrated, neutralization correlated with the presence of binding antibodies in all but one sample tested against AAV1, AAV8, and AAV9. Conversely, there were five instances in which we detected binding antibodies but not neutralizing antibodies. These samples were all in the AAV9 group, possibly indicating that the host was directly exposed to a different AAV serotype and raised cross-reactive binding antibodies that do not bind a neutralizing epitope on AAV9. Future NHP studies would be worthwhile to evaluate the *in vivo* transduction efficiency of AAV vectors in the presence of non-neutralizing anti-capsid binding antibodies.

Although we observed a strong correlation between the ELISA and neutralization assays for screening anti-AAV antibodies, there are some aspects to consider for reproducibility of these assays. One consideration would be the number of freeze-thaw cycles samples undergo when being analyzed, as more cycles may lead to more antibody degradation. At most, the samples used in this study underwent three



**Figure 4. Screening of anti-AAV antibodies by neutralization assay after AAV1 or AAV8 inoculation in rhesus macaques**

(A) The same sera samples from three rhesus macaques in Figure 3 were screened using neutralization assay for preexisting neutralizing antibodies to the indicated serotypes pre- and post-AAV1 intramuscular vector inoculation. Neutralization assays were performed as described in Figure 1. (B) Similar to (A), except the same nine rhesus macaque samples from Figure 3 were inoculated with an AAV8 vector.

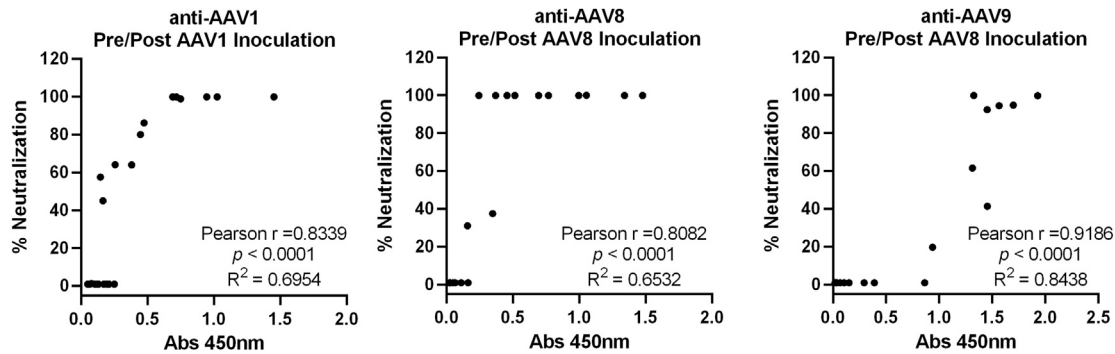
freeze-thaw cycles. A second point to be aware of is the length of time to develop the ELISA plates. Shortening or extending the develop time may change some of the values observed to be middling on the basis of the ELISA. Consistently using the same amount of developing time would be useful to identifying a cutoff for which samples have low binding antibodies. Third is the serum dilution used for screening. Our studies using AAV in rhesus macaques have focused primarily on i.m. inoculations, an administration route that has been shown to tolerate neutralizing titers up to 1:160. However, groups may need to adjust and use lower dilutions (such as 1:2 or 1:5) than the 1:10 and 1:20 dilutions if systemic AAV inoculations will be performed. Last, groups need to consider the amount of vector used for screening. The amounts of vector used in these studies was variable for the various capsids screened, and this could affect the signal observed for either the ELISA or neutralization assay.

The data shown in Figures 3 and 4 have implications for attempts to boost expression of a transgene with a second AAV vector. We have shown that this strategy can be successful in increasing antibody expression from an AAV vector when first using AAV8 vectors to target the liver and AAV1 vectors to target skeletal muscle.<sup>3</sup> Although the data presented in Figure 6 show a negative correlation for higher binding or neutralizing antibodies with greater increases

observed in 4L6 concentrations, caveats must be considered. First is that not all macaques received the same AAV capsid for the first inoculation. Not surprisingly, the three macaques that initially received AAV1 inoculations had the smallest change in 4L6 concentrations upon receiving a second AAV1 inoculation. A second caveat is the route of administration of the AAV inoculation. We observed the greatest change in 4L6 concentrations in animals that received systemic AAV8 inoculations followed by i.m. AAV1 inoculations. A factor that Fuchs et al.<sup>3</sup> suggested for the large increase in 4L6 concentrations is that liver expression of 4L6 before the AAV1 booster inoculation could have generated T regulatory cells that limited anti-drug antibody responses against 4L6. This may be why

r11088 may have had a large increase in 4L6 concentrations despite the presence of AAV1 binding/neutralizing antibodies before the AAV1 inoculation. In general, these data were similar to observations made by Greig et al.<sup>17</sup> In their study, they showed that expression of an anti-SIV antibody when delivered i.m. by an AAV9 vector was not inhibited after an initial AAV8 i.m. vector inoculation. Together, these studies may suggest that a second serotype could be used to boost expression if the neutralization titer is below a certain threshold that remains to be defined. Even with some examples of success with boosting transgene expression from a second serotype, we must remain cautious about this method. Even though AAV serotypes have varying degrees of capsid homology among them,<sup>18</sup> one inoculation could result in binding and neutralizing antibodies against other capsids. Inoculation with one serotype of an AAV vector may limit the ability to use a second AAV vector or require secondary screening after the first inoculation to identify serotypes that are not recognized by the anti-AAV antibodies. These results do highlight the need to keep developing new synthetic vectors<sup>19,20</sup> or discovering new AAV serotypes from other species that are more distant from humans and NHPs.<sup>21-24</sup>

Several publications have reported findings similar to what we describe here. Ito et al.<sup>25</sup> reported 2 of 20 human sera positive



**Figure 5. Correlation between EISA and neutralization assays for rhesus macaque samples pre- and post-AAV inoculation**

Correlation plots for ELISA and neutralization assay results presented in Figures 3 and 4 for AAV1, AAV8, and AAV9. All 24 data points are included, 12 from pre-AAV inoculation and 12 from post-AAV inoculation. Pearson  $r$  values,  $p$  values (two tailed), and  $R^2$  values are included for each comparison.

for antibodies to AAV2 by both intact virion ELISA and by neutralization testing. Kavita et al.<sup>26</sup> compared a chemiluminescent ELISA method with a neutralization assay for the presence of antibodies to AAV9 in 100 human sera and found a high concordance of the results. Kotterman et al.<sup>27</sup> used a neutralization assay to compare monkey sera pre- and post-AAV inoculation for antibodies against AAV2, AAV5, AAV8, and AAV9. They concluded that although postinjection antibody titers were highest against the serotype administered, antibodies were also cross-reactive against other AAV serotypes (AAV2, AAV5, AAV8, and AAV9). Additionally, natural exposure to an AAV serotype has been documented to lead to cross-reactive AAV antibodies in chimpanzees.<sup>28</sup>

Overall, these results validate the use of ELISA-based screening techniques to identify negative NHPs to be used in preclinical evaluation of AAV vector therapies. Both the ELISA and neutralization assay described here are assays that most academic labs could easily perform, making them less dependent on screening by vector cores. For instance, labs could quickly screen tens or hundreds of samples by ELISA and use the neutralization assay as a secondary means to further examine samples with borderline, marginal, or low ELISA values. Applying these techniques in up-and-coming AAV labs will help more AAV vector therapies reach preclinical evaluation in NHP models.

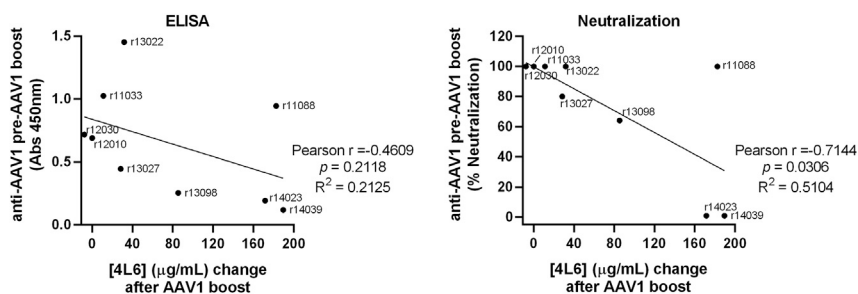
## MATERIALS AND METHODS

### Cell lines and plasmids

HEK293T (ATCC) cells were cultured in growth medium consisting of DMEM supplemented with 10% and 50 U/mL penicillin/streptomycin. Expression plasmids for pAAV Rep2/Cap1, pAAV Rep2/Cap8, pAAV Rep2/Cap9, and pHelper were provided by Guangping Gao. The transfer plasmid for pAAV.CAG.fLuc was obtained from Addgene (#83281).

### Anti-AAV ELISA

Ninety-six-well plates were coated with AAV1, AAV8, or AAV9 vectors produced by the University of Massachusetts Vector Core at the following concentrations:  $1 \times 10^{10}$  vg/mL of AAV1 vectors,  $5 \times 10^9$  vg/mL AAV8 vectors, or  $8 \times 10^9$  vg/mL AAV9 vectors. Vectors were diluted in carbonate-bicarbonate buffer, and plates were incubated overnight at 4°C. Plates were washed 12 times with PBS supplemented with 0.05% Tween 20 (PBS-T) and blotted dry. Plates were blocked with 300  $\mu$ L 5% milk in PBS for 1 h at 37°C. Plates were washed with PBS-T. Serum samples diluted 1:20 in 5% milk in PBS (blocked for 30 min prior to addition) were added to the plates. Five percent milk in PBS was added to the plates as a blank control. Plates were covered and incubated for 1 h at 37°C. Plates were washed 12 times with PBS-T, and horseradish peroxidase (HRP)-conjugated goat anti-rhesus IgG(H + L) secondary antibody diluted 1:5,000 in 5%



**Figure 6. Correlation between anti-AAV1 binding and neutralizing antibodies on changes in 4L6 concentrations after an AAV1 booster inoculation**

Correlation plots comparing the change in 4L6 concentrations from nine rhesus macaques that received AAV1 booster inoculations with AAV1 binding antibodies (ELISA; left) or neutralizing antibodies (neutralization; right). Pearson  $r$  values,  $p$  values (two tailed), and  $R^2$  values are included for each comparison. Note the negative Pearson  $r$  values indicates that higher change in 4L6 concentrations were observed with lower binding or neutralizing antibodies.

milk in PBS was added. After a final 1 h at 37°C incubation, plates were washed 12 times with PBS-T, then developed with TMB solution, uncovered, at room temperature until the positive controls developed (typically 2–3 min). A TMB stop solution was then added to each plate, and absorbance was read at 450 nm.

#### AAV reporter vector production

AAV1-, AAV8-, and AAV9-luciferase reporter vectors were produced by triple transfection in HEK293T cells. HEK293T cells were split the day before transfection to reach a 60%–80% confluency when transfected. Expression plasmids encoding Rep2/Cap (variable depending on which serotype was being made), pHelper, and pAAV.CAG.fLuc were mixed in serum-free OptiMEM in a 1:1:1 ratio at a total of 60 µg DNA per flask being transfected. Transfections were conducted using PEIpro following the manufacturer's instructions (Polyplus Transfection). At 3 days after transfection, cells were harvested by adding 0.5 M EDTA (pH 8.0) to each flask. Cells and medium were centrifuged at 2000 × g for 10 min. Cells were washed twice with PBS and then lysed using an AAV lysis buffer (150 mM NaCl, 2 mM MgCl<sub>2</sub>, 50 mM Tris-HCl [pH 8.0]). Cells in lysis buffer were subjected to three freeze-thaw cycles. Benzonase (50 U/mL) and Triton X-100 (0.01%) were added to the lysate mixture, and the mixture was incubated for 1 h at 37°C. The lysate mixture was centrifuged at 7,000 × g for 1 h, and the supernatant was collected and filter-sterilized with a 0.45 µm filter. POROS GoPure AAVX columns were used to purify AAV1 and AAV8 vectors, and POROS GoPure AAV9 columns were used to purify AAV9 vectors. Eluted vectors were buffer exchanged into sterile PBS and concentrated using Amicon Ultra Centricon Tubes. Vectors were quantified using qPCR with a Roche LightCycler 480ii.

#### Neutralization assay

Serum samples were diluted 1:5 in growth medium, and 50 µL was added to a 96-well plate in duplicate. AAV.CAG.fLuc vector was diluted in growth medium to a concentration that would yield >10,000 RLU (8 × 10<sup>9</sup> vg/mL AAV1, 2 × 10<sup>10</sup> vg/mL AAV8, 2 × 10<sup>11</sup> vg/mL AAV9), and 50 µL was mixed with 50 µL medium only or each diluted serum sample for a final serum dilution of 1:10. Plates were incubated for 1 h at 37°C. HEK293T cells (100 µL) were added to each well at a concentration of 30,000 cells/well. Plates were incubated for 24 h at 37°C. Luciferase was quantified using the britelite Plus Reporter Gene Assay system following the manufacturer's instructions, and plates were read on a BioTek Neo2 plate reader.

#### Statistical analysis

All statistical analyses performed to determine Pearson r values, p values, and R<sup>2</sup> values were done using GraphPad Prism version 9.2.0.

#### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtm.2022.01.003>.

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#### AUTHOR CONTRIBUTIONS

M.R.G. designed the study and performed experiments. D.E.M. and C.P.M. performed experiments. J.M.M.-N. provided critical samples for analysis. S.P.F. performed data analysis. G.G. provided critical reagents. R.C.D. designed and supervised the study. M.R.G. and R.C.D. wrote the manuscript with input and approval from all the coauthors.

#### DECLARATION OF INTERESTS

M.R.G. is a co-founder and consultant for Emmune, Inc. M.R.G. is an inventor on patents with potential royalties licensed to Emmune, Inc. G.G. is a co-founder of Voyager Therapeutics and Aspa Therapeutics and holds equity in both companies. G.G. is an inventor on patents with potential royalties licensed to Voyager Therapeutics, Aspa Therapeutics, and other biopharmaceutical companies. The remaining authors declare no competing interests.

#### REFERENCES

- Mendell, J.R., Al-Zaidy, S.A., Rodino-Klapac, L.R., Goodspeed, K., Gray, S.J., Kay, C.N., Boye, S.L., Boye, S.E., George, L.A., Salabarria, S., et al. (2021). Current clinical applications of in vivo gene therapy with AAVs. *Mol. Ther.* 29, 464–488.
- Fuchs, S.P., Martinez-Navio, J.M., Piatak, M., Jr., Lifson, J.D., Gao, G., and Desrosiers, R.C. (2015). AAV-delivered antibody mediates significant protective effects against SIVmac239 challenge in the absence of neutralizing activity. *PLoS Pathog.* 11, e1005090.
- Fuchs, S.P., Martinez-Navio, J.M., Rakasz, E.G., Gao, G., and Desrosiers, R.C. (2020). Liver-directed but not muscle-directed AAV-antibody gene transfer limits humoral immune responses in rhesus monkeys. *Mol. Ther. Methods Clin. Dev.* 16, 94–102.
- Gardner, M.R., Fellingner, C.H., Kattenhorn, L.M., Davis-Gardner, M.E., Weber, J.A., Alfant, B., Zhou, A.S., Prasad, N.R., Kondur, H.R., Newton, W.A., et al. (2019). AAV-delivered eCD4-Ig protects rhesus macaques from high-dose SIVmac239 challenges. *Sci. Transl. Med.* 11, eaau5409.
- Gardner, M.R., Fetzer, I., Kattenhorn, L.M., Davis-Gardner, M.E., Zhou, A.S., Alfant, B., Weber, J.A., Kondur, H.R., Martinez-Navio, J.M., Fuchs, S.P., et al. (2019). Anti-drug antibody responses impair prophylaxis mediated by AAV-delivered HIV-1 broadly neutralizing antibodies. *Mol. Ther.* 27, 650–660.
- Gardner, M.R., Kattenhorn, L.M., Kondur, H.R., von Schawen, M., Dorfman, T., Chiang, J.J., Haworth, K.G., Decker, J.M., Alpert, M.D., Bailey, C.C., et al. (2015). AAV-expressed eCD4-Ig provides durable protection from multiple SHIV challenges. *Nature* 519, 87–91.
- Martinez-Navio, J.M., Fuchs, S.P., Mendes, D.E., Rakasz, E.G., Gao, G., Lifson, J.D., and Desrosiers, R.C. (2020). Long-term delivery of an anti-SIV monoclonal antibody with AAV. *Front. Immunol.* 11, 449.
- Martinez-Navio, J.M., Fuchs, S.P., Pantry, S.N., Lauer, W.A., Duggan, N.N., Keele, B.F., Rakasz, E.G., Gao, G., Lifson, J.D., and Desrosiers, R.C. (2019). Adeno-associated virus delivery of anti-HIV monoclonal antibodies can drive long-term virologic suppression. *Immunity* 50, 567–575.e565.

9. Martinez-Navio, J.M., Fuchs, S.P., Pedreño-López, S., Rakasz, E.G., Gao, G., and Desrosiers, R.C. (2016). Host anti-antibody responses following adeno-associated virus-mediated delivery of antibodies against HIV and SIV in rhesus monkeys. *Mol. Ther.* *24*, 76–86.
10. Boutin, S., Monteilhet, V., Veron, P., Leborgne, C., Benveniste, O., Montus, M.F., and Masurier, C. (2010). Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) Types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. *Hum. Gene Ther.* *21*, 704–712.
11. Calcedo, R., Morizono, H., Wang, L., McCarter, R., He, J., Jones, D., Batshaw Mark, L., and Wilson James, M. (2011). Adeno-associated virus antibody profiles in newborns, children, and adolescents. *Clin. Vaccin. Immunol.* *18*, 1586–1588.
12. Calcedo, R., Vandenberghe, L.H., Gao, G., Lin, J., and Wilson, J.M. (2009). Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *J. Infect. Dis.* *199*, 381–390.
13. Fu, H., Meadows, A.S., Pineda, R.J., Kunkler, K.L., Truxal, K.V., McBride, K.L., Flanigan, K.M., and McCarty, D.M. (2017). Differential prevalence of antibodies against adeno-associated virus in healthy children and patients with mucopolysaccharidosis III: perspective for AAV-mediated gene therapy. *Hum. Gene Ther. Clin. Dev.* *28*, 187–196.
14. Kruzik, A., Fetahagic, D., Hartlieb, B., Dorn, S., Koppensteiner, H., Horling, F.M., Scheifflinger, F., Reipert, B.M., and de la Rosa, M. (2019). Prevalence of anti-adeno-associated virus immune responses in international cohorts of healthy donors. *Mol. Ther. - Methods Clin. Dev.* *14*, 126–133.
15. Veron, P., Leborgne, C., Monteilhet, V., Boutin, S., Martin, S., Moullier, P., and Masurier, C. (2012). Humoral and cellular capsid-specific immune responses to adeno-associated virus type 1 in randomized healthy donors. *J. Immunol.* *188*, 6418.
16. Welles, H.C., Jennewein, M.F., Mason, R.D., Narpala, S., Wang, L., Cheng, C., Zhang, Y., Todd, J.-P., Lifson, J.D., Balazs, A.B., et al. (2018). Vectored delivery of anti-SIV envelope targeting mAb via AAV8 protects rhesus macaques from repeated limiting dose intrarectal swarm SIVsmE660 challenge. *PLOS Pathog.* *14*, e1007395.
17. Greig, J.A., Calcedo, R., Grant, R.L., Peng, H., Medina-Jaszek, C.A., Ahonkhai, O., Qin, Q., Roy, S., Tretiakova, A.P., and Wilson, J.M. (2016). Intramuscular administration of AAV overcomes pre-existing neutralizing antibodies in rhesus macaques. *Vaccine* *34*, 6323–6329.
18. Gao, G., Vandenberghe Luk, H., Alvira Mauricio, R., Lu, Y., Calcedo, R., Zhou, X., and Wilson James, M. (2004). Clades of adeno-associated viruses are widely disseminated in human tissues. *J. Virol.* *78*, 6381–6388.
19. Zhang, L., Rossi, A., Lange, L., Meumann, N., Koitzsch, U., Christie, K., Nesbit, M.A., Moore, C.B.T., Hacker, U.T., Morgan, M., et al. (2019). Capsid Engineering overcomes barriers toward adeno-associated virus vector-mediated transduction of endothelial cells. *Hum. Gene Ther.* *30*, 1284–1296.
20. Pekrun, K., De Alencastro, G., Luo, Q.-J., Liu, J., Kim, Y., Nygaard, S., Galivo, F., Zhang, F., Song, R., Tiffany, M.R., et al. (2019). Using a barcoded AAV capsid library to select for clinically relevant gene therapy vectors. *JCI Insight* *4*, 683672.
21. Olson, E.J., Haskell, S.R.R., Frank, R.K., Lehmkuhl, H.D., Hobbs, L.A., Warg, J.V., Landgraf, J.G., and Wünschmann, A. (2004). Isolation of an adenovirus and an adeno-associated virus from goat kids with enteritis. *J. Vet. Diagn. Invest.* *16*, 461–464.
22. Lochrie, M.A., Tatsuno, G.P., Arbetman, A.E., Jones, K., Pater, C., Smith, P.H., McDonnell, J.W., Zhou, S.-Z., Kachi, S., Kachi, M., et al. (2006). Adeno-associated virus (AAV) capsid genes isolated from rat and mouse liver genomic DNA define two new AAV species distantly related to AAV-5. *Virology* *353*, 68–82.
23. Farkas, S.L., Zádori, Z., Benkő, M., Essbauer, S., Harrach, B., and Tijssen, P. (2004). A parvovirus isolated from royal python (*Python regius*) is a member of the genus Dependovirus. *J. Gen. Virol.* *85*, 555–561.
24. Bello, A., Tran, K., Chand, A., Doria, M., Allocca, M., Hildinger, M., Beniac, D., Kranendonk, C., Auricchio, A., and Kobinger, G.P. (2009). Isolation and evaluation of novel adeno-associated virus sequences from porcine tissues. *Gene Ther.* *16*, 1320–1328.
25. Ito, T., Yamamoto, S., Hayashi, T., Kodera, M., Mizukami, H., Ozawa, K., and Muramatsu, S.-i. (2009). A convenient enzyme-linked immunosorbent assay for rapid screening of anti-adeno-associated virus neutralizing antibodies. *Ann. Clin. Biochem.* *46*, 508–510.
26. Kavita, U., Dai, Y., Salvador, L., Miller, W., Adam, L.P., Levesque, P.C., Zhang, Y.J., Ji, Q.C., and Pillutla, R.C. (2018). Development of a chemiluminescent ELISA method for the detection of total anti-adeno associated virus serotype 9 (AAV9) antibodies. *Hum. Gene Ther. Methods* *29*, 237–250.
27. Kotterman, M.A., Yin, L., Strazzeri, J.M., Flannery, J.G., Merigan, W.H., and Schaffer, D.V. (2015). Antibody neutralization poses a barrier to intravitreal adeno-associated viral vector gene delivery to non-human primates. *Gene Ther.* *22*, 116–126.
28. Calcedo, R., and Wilson, J.M. (2016). AAV natural infection induces broad cross-neutralizing antibody responses to multiple AAV serotypes in chimpanzees. *Hum. Gene Ther. Clin. Dev.* *27*, 79–82.