

# Systemic and local immune responses to intraocular AAV vector administration in non-human primates

Divya Ail,<sup>1</sup> Duohao Ren,<sup>1,2</sup> Elena Brazhnikova,<sup>1</sup> Céline Nouvel-Jaillard,<sup>1</sup> Stephane Bertin,<sup>1,3</sup> Seyed Bagher Mirashrafi,<sup>4</sup> Sylvain Fisson,<sup>1,2</sup> and Deniz Dalkara<sup>1</sup>

<sup>1</sup>Sorbonne Université, Institut de la Vision, INSERM UMRS 968, 17 rue Moreau, 75012 Paris, France; <sup>2</sup>Université Paris-Saclay, Univ Evry, Inserm, Généthon, Intégrare research unit UMR\_S951, 91000 Evry-Courcouronnes, France; <sup>3</sup>CHNO des Quinze-Vingts, INSERM-DGOS CIC 1423, 75012 Paris, France; <sup>4</sup>Department of Statistics, Faculty of Mathematical Science, University of Mazandaran, Babolsar, Iran

**Positive clinical outcomes in adeno-associated virus (AAV)-mediated retinal gene therapy have often been attributed to the low immunogenicity of AAVs and immune privilege of the eye. However, several recent studies have shown potential for inflammatory responses. The current understanding of the factors contributing to inflammation, such as the pre-existence of serum antibodies against AAVs and their contribution to increases in antibody levels post-injection, is incomplete. The parameters that regulate the generation of new antibodies in response to the AAV capsid or transgene after intraocular injections are also insufficiently described. This study is a retrospective analysis of the pre-existing serum antibodies in correlation with changes in antibody levels after intraocular injections of AAV in non-human primates (NHPs) of the species *Macaca fascicularis*. In NHP serums, we analyzed the binding antibody (BAB) levels and a subset of these called neutralizing antibodies (NABs) that impede AAV transduction. We observed significantly higher pre-existing serum BABs against AAV8 compared with other serotypes and a dose-dependent increase in BABs and NABs in the serums collected post-injection, irrespective of the serotype or the mode of injection. Lastly, we were able to demonstrate a correlation between the serum BAB levels with clinical grading of inflammation and levels of transgene expression.**

## INTRODUCTION

Adeno-associated virus (AAV) is a non-enveloped virus with a single-stranded DNA genome.<sup>1</sup> There are several naturally occurring serotypes, each differing in the structure of the capsid that, in turn, affects their tropism. AAV1, AAV4, AAV7, AAV8, and AAV9 have a non-human primate (NHP) origin, whereas AAV2, AAV3, and AAV5 have a human origin.<sup>2</sup> In addition to these, several novel AAV variants are being discovered<sup>3</sup> or generated to fulfill specific needs.<sup>4,5</sup> Both naturally occurring and generated AAVs show distinct transduction profiles that are cell-, tissue-, and even species specific.<sup>6</sup>

The first approved retinal gene therapy consisted of the *RPE65* gene packaged in an AAV2 vector that was delivered by subretinal injections

in patients with Leber's congenital amaurosis (LCA).<sup>7,8</sup> Following this success, there have been several completed or ongoing clinical trials for AAV-based gene therapy, particularly for eye diseases. A major factor that makes the eye an attractive target tissue is its relative immune privilege, which is attributed to the presence of a blood-retina barrier, of local anti-inflammatory agents, and of myeloid cells actively counteracting adaptive immunity.<sup>9</sup> Although the initial reports from the clinical studies showed therapeutic efficacy, vision improvement, and an excellent safety profile with the AAV, follow-up studies revealed inflammatory reactions both in pre-clinical studies and clinical trials.<sup>10</sup> Inflammatory responses can be problematic for several reasons, one of which being the potential clearance of the transduced cells by immune mechanisms bringing into question both the immune privilege and the low immunogenicity of AAV.<sup>7,10</sup>

In humans, AAV exposure is not associated with any pathology, and antibodies against AAVs are prevalent in human populations across the globe. This poses two major issues with respect to their usage for gene therapy. If a patient already has high levels of antibodies against AAVs, then injections with AAVs might trigger a stronger immune response that can potentially contribute to inflammation. Inflammation can result in the clearance of the transduced cells by the immune system that will not just reduce the efficacy of the therapy but could also worsen the condition. The second issue pertains to a subset of these total antibodies called neutralizing antibodies (NABs), which recognize and bind to the virus and neutralize it, preventing transduction and transgene expression and thus reducing efficacy.<sup>11</sup> A study analyzing the prevalence of different AAV serotypes in the human population worldwide revealed that NABs were present against all the tested serotypes, with the prevalence against AAV1 (67%) and AAV2 (72%) being significantly higher than AAV5 (40%), AAV8 (38%), and AAV9 (47%).<sup>12</sup> Another study analyzing over 800 patient samples from 4 continents and 10 countries also

Received 29 September 2021; accepted 20 January 2022;  
<https://doi.org/10.1016/j.omtm.2022.01.011>

**Correspondence:** Deniz Dalkara, PhD, Sorbonne Université, Institut de la Vision, INSERM UMRS 968, 17 rue Moreau, 75012 Paris, France.

**E-mail:** [deniz.dalkara@inserm.fr](mailto:deniz.dalkara@inserm.fr)



concluded that the NABs against AAV1 and AAV2 were higher than anti-AAV7 or anti-AAV8 NABs in humans.<sup>13</sup>

Ocular gene delivery can be done by intravitreal injections as well as by injections in the subretinal space, which is believed to be the less immunogenic mode of injection.<sup>14,15</sup> However, primate studies have shown ocular immune responses following both subretinal and intravitreal modes of injection.<sup>16,17</sup> Other factors that can influence the immune responses are the AAV serotype used,<sup>18</sup> the virus dose injected,<sup>19</sup> whether the injection is re-administrated,<sup>20</sup> and the promoter<sup>21,22</sup> and transgene used.<sup>23</sup>

As part of several completed and ongoing studies conducted on NHPs that involved ocular injections (both subretinal and intravitreal), we collected blood serum samples before and after the ocular AAV injections. In this study, we performed a retrospective analysis of these serum samples from animals we grouped together based on the different AAV serotypes, doses, mode of injection, and promoters used. These NHPs were also monitored for clinical signs of inflammation by slit-lamp examination and fundoscopy. Eye-fundus imaging and optical coherence tomography (OCT) were done to test transgene expression, as well as the structural integrity of the retina, and were co-related to the serum antibody levels. With this retrospective analysis, we confirm in NHPs some trends observed in small animal studies as well as provide new insights that can prove valuable for designing future clinical and large animal studies.

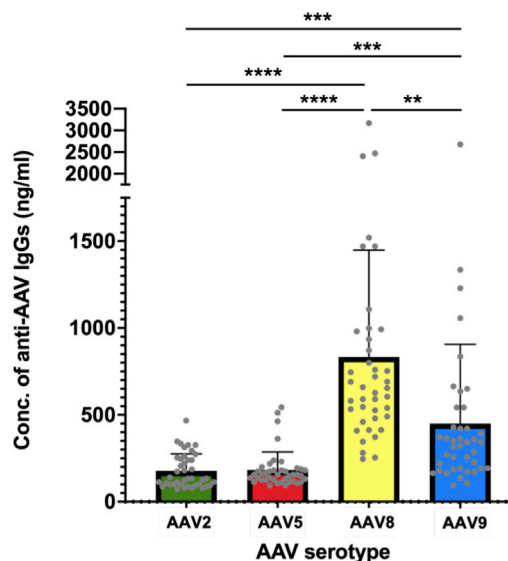
## RESULTS

### Seroprevalence of antibodies against frequently used AAV serotypes in NHP population

Studies analyzing human serums have shown a prevalence of anti-AAV1 and -AAV2 antibodies at a higher level than other serotypes.<sup>12,13</sup> We tested the basal levels of antibodies in 41 NHPs against different serotypes that are commonly used for gene delivery to the retina, AAV2, AAV5, AAV8, and AAV9, by ELISA. We found that in the NHP serums, the level of anti-AAV8 antibodies is significantly higher than in all other serotypes tested. The level of anti-AAV9 antibodies was also significantly higher than AAV2 and AAV5 but lower than AAV8 (Figure 1). There was considerable variability among the individual macaques in the anti-AAV8 and -AAV9 groups, but the intergroup differences were significant (Figure S1). It is worth noting that a majority of the 41 animals received intraocular injections with AAV2-based vectors and, hence, were sometimes pre-selected to have low levels of anti-AAV2 serum antibodies. Hence, differences we observe between anti-AAV2 antibodies and other serotypes are likely skewed. Nonetheless, none of these animals were pre-selected for low antibodies against AAV5 or AAV8, so the difference between these two serotypes reflects the seroprevalence of those serotypes in the Mauritius macaque population.

### Increase in both the BAB and NAB levels post-injection

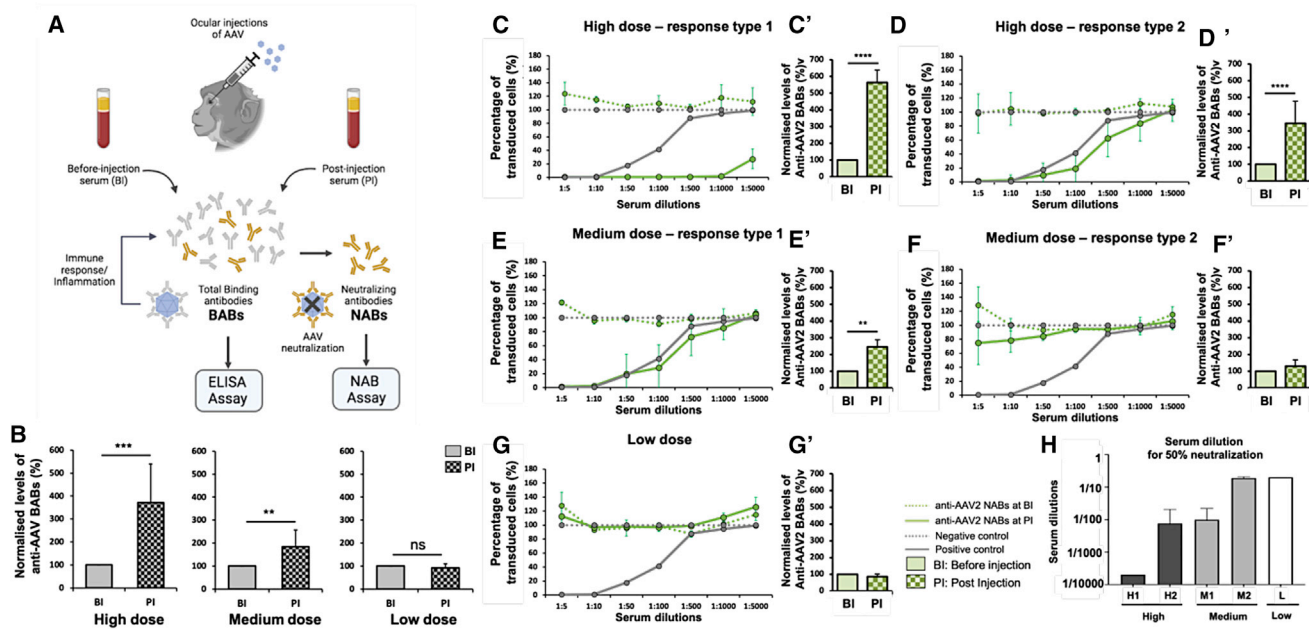
Next, we wanted to test the change in the anti-AAV binding antibodies (BABs) and NABs post-injection. Toward this goal, we collected the blood sample before ocular injections (BI) and post-in-



**Figure 1. Higher basal levels of BABs against AAV8 and AAV9 in NHP serums**

Mean  $\pm$  SD of the concentration of BABs against AAV2 (green), AAV5 (red), AAV8 (yellow), and AAV9 (blue) in serums from 41 NHPs before injection. Significance between individual serotypes was tested using Student's t test (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

jection (PI) and isolated and stored the serum component of the blood for further analysis. At each time point, these serum samples were tested for BABs by ELISA and for NABs by a cell-based NAB assay (Figure 2A). The NHPs received bilateral injections, and the serum samples were grouped according to the total dose received. The animals in the high dose ( $1-6 \times 10^{12}$  vg) group had a 3.7-fold increase in serum BAB levels post-injection, whereas the increase was 1.8-fold in case of the animals that received a medium dose ( $1-6 \times 10^{11}$  vg), and there was no significant difference in the animals that received the low dose ( $1-6 \times 10^{10}$  vg) (Figure 2B). This trend was observed irrespective of the serotype injected as well as when a combination of serotypes was injected (Figure S2). To test the NABs, we need a cell line that is effectively transduced by the different serotypes. We are able to perform NAB assays for AAV2 using HEK293T cells and for AAV9 using Lec2 cells.<sup>24</sup> In our cohort of animals, a majority were injected with AAV2 or AAV2-7m8, a variant of AAV2, so we grouped these by the dose of AAV2 they received and tested the levels of NABs in the serum pre- and post-injection. In the high-dose group, there was an increase in the level of NABs post-injection in all animals tested, with 3 out of 19 animals showing very high levels (type 1 response) that were able to neutralize the AAV even at a 5,000-fold dilution (Figure 2C), and 16 animals had an increase comparable to the positive control (type 2 response) (Figure 2D). The BAB levels in these groups showed a 5.6- and 3.5-fold increase, respectively (Figures 2C' and 2D'). Even in the group receiving the medium dose, two types of responses were observed: 3 animals showed an increase in NABs comparable to the positive control (type 1 response) (Figure 2E), whereas 4 animals did not seem to



**Figure 2. Dose-dependent increase in levels of BABs and NABs post-injection**

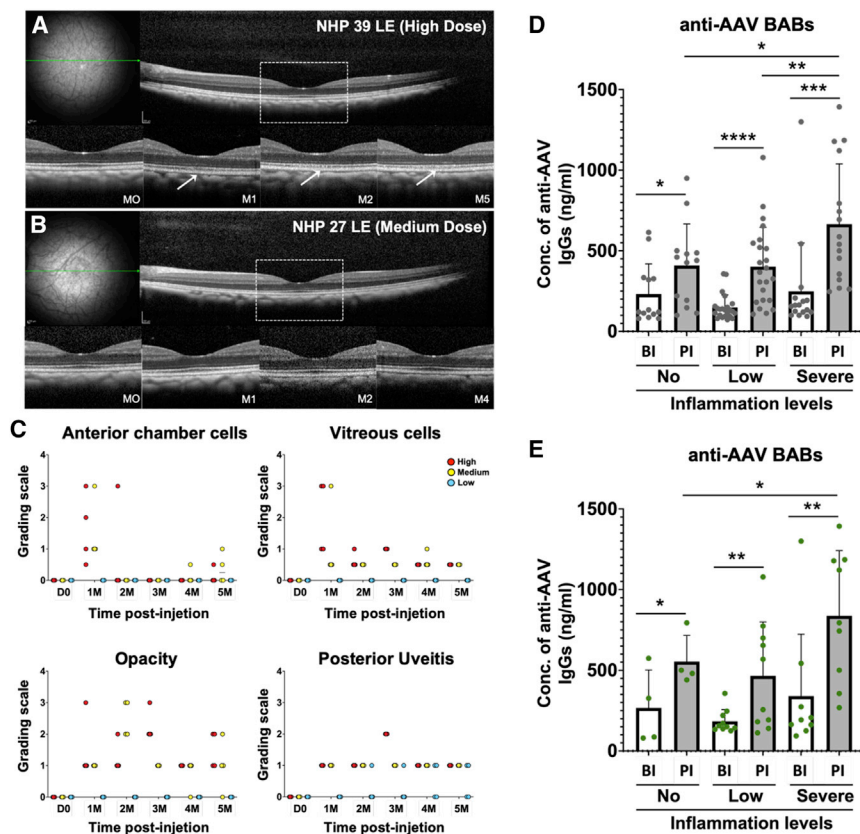
(A) Schematic representation of the experimental protocol showing the serum collection points before injection (BI) and post-injection (PI) from which the total binding antibodies (BABs) are isolated for testing by ELISA, and a subset of these, the neutralizing antibodies (NABs), are tested by NAB assay. (B) Mean fold change in the concentration of BABs against AAV in  $n = 26$  NHPs at a high dose,  $n = 9$  NHPs at a medium dose, and  $n = 4$  NHPs at a low dose. (C–G) Change in the levels of anti-AAV2 NAB levels and (C'–G') BAB levels in (C and C')  $n = 3$  NHPs with high-dose response type 1, (D and D')  $n = 16$  NHPs with high-dose response type 2, (E and E')  $n = 3$  NHPs with medium-dose response type 1, (F and F')  $n = 4$  NHPs with medium-dose response type 2, and (G and G')  $n = 2$  NHPs with low-dose response. (H) Serum dilution at which 50% of the AAVs are neutralized (H1 and H2: NHPs with high-dose response types 1 and 2, respectively; M1 and M2: NHPs with medium-dose response types 1 and 2, respectively; L: NHPs with low dose). The values for BABs are normalized relative to the BI level (set to 100) and are shown as mean  $\pm$  SD. The values for NABs at each dilution are normalized relative to the negative control (set to 100) and are shown as mean  $\pm$  SD. Significance between individual time points was tested using Student's *t* test (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ). High dose:  $10\text{--}60 \times 10^{11}$  vg, medium dose:  $1\text{--}6 \times 10^{11}$  vg, low dose:  $0.1\text{--}0.6 \times 10^{11}$  vg.

have developed anti-AAV2 NABs post-injection (type 2 response) (Figure 2F). The level of BABs in this group also co-relates to the NAB level showing a 2.5-fold increase in the medium-dose response type 1 group (Figure 2E'), and no change in the BAB levels in the medium-dose response type 2 group (Figure 2F'). Hence, this range of the dose seems to be a threshold, such that doses above this will definitely elicit a serum humoral response whereas doses below this limit will most likely not, when all other factors are kept constant. Two animals were injected with a low dose of AAV2, and both did not have an increase in NABs or BABs (Figures 2G and 2G'). The NAB level in serum is defined by the serum dilution at which 50% of the cells are neutralized. This value could not be determined for the 2 low-dose samples and the 3 high-dose samples with response type 1, so these were set at the limits of our experiment at 1/5 and 1/5,000, respectively. For the high-dose response type 2, the medium-dose response type 1, and the medium-dose response type 2 groups, this value was between 1/100 and 1/500 and, more precisely, at 0.0085, 0.01, and 0.19, respectively (Figure 2H).

#### Local signs of inflammation correlate to serum antibody levels

The dose also has an impact on the retina, as deposits and structural abnormalities were observed in some of the animal eyes that received

a high dose, whereas these signs did not appear in animals that received a medium or low dose. In the left eye of NHP39 that was injected with a high dose ( $1 \times 10^{12}$  vg), structural changes were observed 1 month post-injection, which persisted until 5 months (arrows pointing at disruptions in the outer retina in Figure 3A). These punctuate disruptions of the outer retinal layers are not observed in NHP27 that received a medium dose ( $5 \times 10^{11}$  vg). NHP27 and NHP39 were injected intravitreally with an AAV2 capsid containing the same promoter and transgene (Figures 3A–3B). Further, clinical signs of local immune responses were graded using the Standardization of Uveitis Nomenclature (SUN) working group and the NIH grading scales.<sup>25</sup> We counted the anterior chamber/vitreous cells and evaluated signs of posterior uveitis. A summary of 4 eyes, the left and the right eye (from 2 NHPs in each case injected with AAV2), for each dose is shown (Figure 3C). In NHP24 and NHP39, which received the high dose ( $1 \times 10^{12}$  vg), there were cells in the anterior chamber that subsided eventually, but the cells in the vitreous persisted until 5 months, albeit at a low level. Opacity of the eye or vitreous haze was only observed in eyes that received the high dose or, in the case of NHP25 and NHP27, that had received a medium dose ( $5 \times 10^{11}$  vg) but not in the eyes of NHP34 or NHP41, which received low dose ( $5.5 \times 10^{10}$  vg) injections. Posterior uveitis



**Figure 3. Increase in the serum levels of BABs correlates with clinical signs of inflammation**  
 (A and B) OCT images of the retina of (A) NHP 39 that received a high-dose injection and (B) NHP 27 that received a medium-dose injection. (C) Fundus image of the injected area, cross-section of the retina close to the fovea indicated by the green line, and the inset demarcated by the dotted lines is shown at day of injection (M0), 1 month (M1), 2 months (M2), and 4 months (M4) or 5 months (M5) post-injection as indicated; arrows point to disruptions in the outer retina. (D) Clinical grading of ocular immune response by evaluation of anterior chamber cells, vitreous cells, opacity, and posterior uveitis from the day of injection (D0) to 5 months post-injection (5M). (E) Serum levels of anti-AAV BABs in NHPs BI and PI grouped by the level of ocular inflammation observed. (F) Serum levels of anti-AAV BABs in NHPs grouped by the level of ocular inflammation and transgene expression (NHPs from Table 1). The values for BABs are shown as mean ± SD. Significance between individual time points was tested using Student's t test (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001).

at a very low level of grade 1 was observed in many of the animals tested (Figure 3C).

Based on this clinical grading for local ocular immune responses, as well as on monitoring of the health of the retina by imaging and retinal structure by OCT, we divided the animals into 3 groups. The first group showed no signs of inflammation, the second group showed some signs of inflammation, and the third group included animals that we described as having severe inflammation. The animals in the first group required no intervention, the ones with low inflammation were given local triamcinolone acetonide injections 2–5 days post-AAV injections, and the animals that had severe inflammation were given additional triamcinolone acetonide treatment and, in some cases, intramuscular injections of short-term systemic corticosteroids. In each group, there was a significant increase in the level of BABs post-injection compared with before injection: 1.8-fold increase for the no inflammation group, 2.4-fold increase for the low inflammation group, and 2.7-fold increase for the severe inflammation group. Interestingly there was a 1.6-fold difference between the post-injection levels of the no inflammation and severe inflammation groups, which was significant (Figure 3D). Some of the animals in these groups were injected with a transgene that contained a GFP reporter. Hence, for these animals, we looked at the transgene expression by imaging the fundus (Table 1). Some animals (NHP6, NHP8, and NHP11) in the severe inflammation group did not express

the transgene, whereas the animals that received the same serotype of AAV at the same dose but had a low level of inflammation showed good reporter gene expression (NHP6, NHP7, and NHP10). The level of BABs in these animals showed a trend like the one observed in all animals combined (Figure 3E). We performed some additional statistics to test the influence of each of different factors on the outcome (inflammation categorized as No, Low, and Severe) by Pearson's chi-squared test. The “Serum BAB level” was highly correlated, the “Dose” had a moderate correlation, and the “Promoter” correlated weakly with the outcome of inflammation. The results of the chi-squared test were not significant in the case of the “Serotype” and “Mode of injection.” The correlation coefficient and the significance for each factor are listed in Table 2.

**The mode of injection and its impact on the increase of anti-AAV antibodies in blood sera**

The vitreous is composed of a gelatinous substance composed of water and a network of collagen and hyaluronan.<sup>11</sup> It has been shown that intravitreal injection results in the increased spreading of AAV particles into systemic circulation leading to adaptive responses.<sup>15</sup> We tested the difference in BAB and NAB levels in animals that received AAV by different modes of intraocular injection. We observed a 3.6-fold increase in the level of BABs post-injection in the 6 animals that received bilateral subretinal injections, a 4.1-fold increase in the 12 animals that received intravitreal injections, and a 4.4-fold increase in the animals that received a combination of subretinal and intravitreal injections (Figure 4C). Some of these animals were injected with AAV2 and hence tested for the NAB levels prior to injection. In each group, we observed that post-AAV injection, there was an increase in NABs, which was comparable to the positive

**Table 1. Effect of local signs of inflammation on transgene expression**

NHP ID	Age	Gender	Eye	Capsid serotype	Viral genomes injected	Mode of injection	Transgene expression
<b>No inflammation</b>							
NHP14	5	M	RE	AAV2-7m8	$5 \times 10^{11}$	IVT	yes, low
			LE	AAV2-7m8	$5 \times 10^{11}$	IVT	yes
NHP15	8	F	RE	AAV2-7m8	$5 \times 10^{11}$	IVT	yes, very good
			LE	AAV2-7m8	$5 \times 10^{11}$	IVT	yes, very good
NHP23	4	M	RE	AAV2	$5 \times 10^{11}$	IVT	yes, very good
			LE	AAV2	$5 \times 10^{11}$	IVT	yes, very good
NHP33	5	M	RE	AAV2-7m8	$1 \times 10^{11}$	SR	yes
			LE	AAV2-7m8	$1 \times 10^{11}$	SR	yes
<b>Yes-low</b>							
NHP1	8	F	LE	AAV9	$1 \times 10^{11}$	SR	yes, very good
			RE	AAV9-7m8	$1 \times 10^{11}$	SR	yes, very good
NHP2	9	F	RE	AAV2-7m8	$1 \times 10^{12}$	SR	yes, low
			LE	AAV2-7m8	$1 \times 10^{12}$	IVT	yes, low
NHP5	16	F	RE	AAV5	$5 \times 10^{11}$	SR	yes, low
NHP6	6	M	LE	AAV9-7m8	$5 \times 10^9$	SR	yes
NHP7	5	M	RE	AAV9-7m8	$5 \times 10^9$	SR	yes
			LE	AAV9-7m8	$5 \times 10^9$	SR	yes
NHP10	7	M	RE	AAV9-7m8	$1 \times 10^9$	SR	yes
			LE	AAV9-7m8	$5 \times 10^9$	SR	yes
NHP32	5	M	RE	AAV9-7m8	$1 \times 10^{11}$	IVT	yes, very good
			LE	AAV2-7m8	$1 \times 10^{11}$	IVT	yes
<b>Yes-severe</b>							
NHP3	13	F	RE	AAV9-7m8	$1 \times 10^{12}$	IVT	yes, low
			LE	AAV9	$1 \times 10^{12}$	IVT	yes, low
NHP6	6	M	RE	AAV9-7m8	$5 \times 10^9$	SR	no
NHP8	9	M	RE	AAV9-7m8	$5 \times 10^9$	SR	no
			LE	AAV9-7m8	$5 \times 10^9$	SR	yes
NHP9	9	M	LE	AAV9-7m8	$5 \times 10^9$	SR	yes
NHP11	6	M	RE	AAV9-7m8	$1 \times 10^9$	SR	no
			LE	AAV9-7m8	$1 \times 10^9$	SR	yes, low
NHP36	9	F	RE	AAV2-7m8	$1 \times 10^{12}$	SR	yes, low
			LE	AAV2-7m8	$1 \times 10^{12}$	SR	yes, low

M, male; F, female; RE, right eye; LE, left eye.

control (Figures 4B, 4D, and 4F), and there was no difference in the serum dilution at which 50% neutralization is obtained (Figure 4E).

#### Pre-existing antibodies and cross-reactivity across serotypes affect antibody levels post-injection

An important consideration for injections is the pre-existing levels of BABs and NABs.<sup>26,27</sup> We grouped the animals based on the presence of pre-existing antibodies and then compared the post-injection increase in BABs to the levels before injection. As expected, there was an increase post-injection that was slightly higher in the group with pre-existing BABs, but there was no significant difference between

the post-injection levels of the groups with and without pre-existing BABs (Figures 5A–5C). The NAB and BAB levels against AAV2 increased in both NHP18 (without pre-existing NABs) and NHP33 (with pre-existing NABs). Similarly, the NAB and BAB levels against AAV9 increased in both NHP9 and NHP8 (Figures 5D–5G).

Some studies have shown that there is cross-reactivity across serotypes wherein an injection with one serotype resulted in an increase in NABs against another serotype.<sup>26,28</sup> To test this, we grouped the animals that had received AAV2 injections based on their pre-injection levels of BABs against AAV5, AAV8, and AAV9. We observed a significant

**Table 2. Influence of factors on the outcome of inflammation**

Factor	Co-efficient	p value	Correlation
Dose	0.45	0.005	moderate
Serotype	0.32	0.34	no
Mode of injection	0.07	0.9	no
Promoter	0.32	0.05	weak
Serum BAB levels	0.52	0.01	high

increase in anti-AAV9 BABs in the group of 20 animals that had pre-existing anti-AAV9 BABs but were injected with AAV2 (Figures 6A–6C). NHP8 had low pre-existing anti-AAV2 NAB and BAB levels, and this level remained low post-injection of a combination of AAV5 and AAV9 (Figures 6D and 6E). On the other hand, NHP9 had some pre-existing anti-AAV2 NABs and BABs that further increased post-injection with AAV5 and AAV9 (Figures 6F and 6G).

#### Influence of promoter and long-term monitoring of antibody levels

After we established the impact of dose on serum BAB and NAB levels, we investigated whether there are other factors that can potentially have an effect. A group of animals that were injected intravitreally with the same AAV serotype (AAV2) in each eye at the same dose ( $5 \times 10^{11}$  vg) were evaluated for their BAB levels post-injection. The only difference between these vectors was the promoter driving the transgene expression. Four animals had a ubiquitous CAG promoter, whereas 2 animals had a ganglion-cell-specific promoter, SNCG.<sup>29</sup> Three out of the 4 animals with the ubiquitous promoter had a higher level of BABs post-injection compared with the 2 animals with the ganglion-cell-specific promoter (Figure S3).

We monitored the same animals (Figure S3) long term by analyzing the BAB levels in serums collected at time points of 1 month until 6 months post-injection. In 5 out of the 6 animals, the BAB levels increased 1 month post-injection, and this level stayed stable until 6 months. Two NHP samples that were tested at time points later than 6 months (up to 1 year) also showed that the BABs could be detected until much later in the serums post-injection (data not shown).

There is an interest in the re-administration of AAV<sup>20,30</sup> and so, lastly, we examined the effect of double and sequential injections on the post-injection antibody levels. In our cohort, two animals received double injections: NHP1 received two injections in both eyes combining AAV2 along with AAV9, and NHP2 received two injections in both eyes of AAV2. In both cases, there was an increase in BABs post-injection, which stayed at the same level or increased only slightly after the second injection. This increase was comparable to two animals (NHP3 and NHP21) that had received similar serotypes and doses but as a one-time injection (Figure S4).

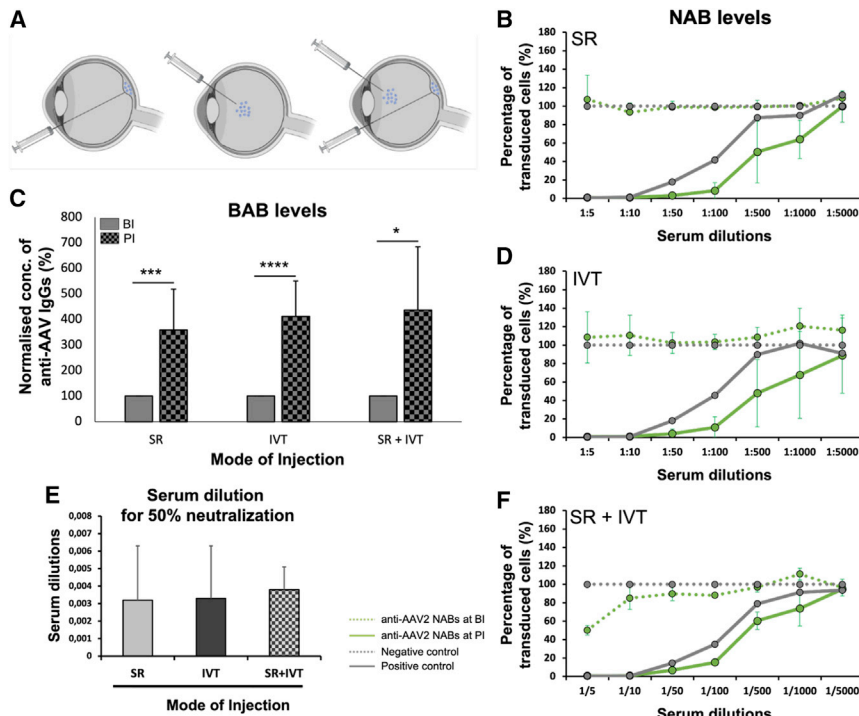
## DISCUSSION

The pre-existing levels of anti-AAV antibodies in the serum have potentially important implications in study outcomes involving intra-

ocular AAV administration. In this work, we first examined the basal levels of anti-AAV antibodies against commonly used AAVs in the sera of macaques that have been used for pre-clinical testing of various gene therapy strategies. In humans, anti-AAV2 NABs were found to be much higher than anti-AAV7 or -AAV8.<sup>12,13</sup> AAV4, AAV7, AAV8, and AAV9 originate from monkeys, whereas AAV2, AAV3, and AAV5 are believed to have a human origin.<sup>31,32</sup> Thus, it is not surprising that the levels of anti-AAV8 and -AAV9 antibodies were higher than the levels of anti-AAV5 in the 41 NHPs we tested. As mentioned earlier, the levels of AAV2 in our samples may have an exclusion bias, since very often animals in our studies are pre-selected to be AAV2-NAB-negative prior to their enrollment in AAV-based studies. Nonetheless, the anti-AAV5 levels in all samples tested were low, like what is observed in human samples. But, unlike humans who have low seroprevalence of AAV8 and AAV9, the anti-AAV8 and -AAV9 levels were higher in NHPs.<sup>12</sup> Also, when the capsid sequence homology is compared, AAV5 is the most distantly related to all other serotypes (Figure 6H).

In our study, we found that the most important factor that influences the rise in serum antibody levels is the dose injected in the retina (Figure 2) irrespective of the serotype (Figure S1) or the mode of injection (Figure 4). In the vitreous space, there are higher chances of exposure to systemic circulation,<sup>15</sup> whereas the subretinal space does not expose AAV to systemic circulation, owing to the blood-retina barrier.<sup>9,33</sup> Hence, intravitreal AAV injections are expected to be more immunogenic than subretinal injections.<sup>18,34,35</sup> A study showed that an intravitreal (IVT) injection in one eye results in an immune response in the contralateral eye. This prevented a second IVT injection in the fellow eye, but a subretinal (SR) injection was possible.<sup>14</sup> On the other hand, a primate study concluded that SR injections of AAV8 in NHPs induced both innate and adaptive immune responses.<sup>16</sup> In our study, we did not find a difference in the serum NAB and BAB levels in the animals that were injected by different modes of injection. There was an increase post-injection in both cases (Figure 3), but we only tested the antibody levels in the serum. It is possible that the differences (if any) may be more prominent locally than at a systemic level. Although this study has shown a positive co-relation ( $R = 0.3$ ) between the NABs detected in the vitreous fluid and the NABs in the serum, it did not make a comparison of BABs.<sup>26</sup> It may be worthwhile to test BABs in the vitreous fluid of NHPs to ascertain if there are indeed any differences between antibody levels after SR and IVT injections. This could pose a technical challenge though, as extracting the vitreous fluid is an invasive procedure that can affect the intraocular pressure, cause cataracts, or even result in retinal detachment.<sup>36</sup> Performing vitreous sampling before and after AAV injections could result in more problems than the AAV injection itself.

An increase in dose refers to an increase in capsids, transgenes, and promoters. Each of these individually or collectively could be responsible for the increase in serum antibody titers.<sup>11,19,37</sup> We observed that the response to capsid depends on the pre-existing levels of a particular serotype, which differs by species. The response to the transgene could



**Figure 4. No impact of the mode of ocular injection on the serum levels of BABs and NABs**

(A and B) Schematic representation of the modes of injection. SR, subretinal, IVT, intravitreal. (B–F) Anti-AAV2 NAB levels in (B)  $n = 2$  NHPs that received SR injections, (C and D)  $n = 12$  NHPs that received IVT injections, and (F)  $n = 2$  NHPs that received a combination of SR and IVT injections. (C) BAB levels in  $n = 6$  NHPs that received SR injections,  $n = 12$  animals that received IVT injections, and  $n = 4$  NHPs that received SR + IVT injections. (E) Serum dilution at which 50% of the AAVs are neutralized. The values for NABs are normalized relative to the negative control (set to 100) and are shown as mean  $\pm$  SD. The values for BABs are normalized relative to the BI level (set to 100) and are shown as mean  $\pm$  SD. Significance between individual time points was tested using Student's *t* test (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

1 year post-injection, which has to be taken into consideration if repeat injections are required.

An immune response to viruses and the generation of antibodies is a sign of a healthy functioning immune system, so the serum antibodies are not a cause for concern unless they cause

inflammation.<sup>11,21,40,41</sup> One of the important findings of our study is that in the case of animals that developed severe local inflammation, the serum antibodies were significantly higher than for the animals that did not have any inflammation. This also correlated with clinical signs of inflammation (Figure 3) and with the transgene expression (Table 1). Hence, the serum antibody levels can be used as a relatively less invasive indicator for ocular inflammation.

not be evaluated, as the samples were collected from different studies (most projects have 2–5 assigned NHPs) and were injected with different combinations of serotype-promoter-transgene-dose-mode of injection; hence, we rarely have more than 2 animals that have the exact same combinations. Furthermore, most of these studies were funded by industrial partners, and the transgene information is proprietary; hence, we did not attempt to analyze the impact of the transgene as a single variable on inflammation, as we are not able to reveal the transgene-related information. But we were able to make a comparison of promoters in a group of macaques. Studies have shown that the promoter influences the AAV injection outcome, where ubiquitous promoters have been linked to a higher toxicity affecting both the transgene expression and the function of the retina.<sup>19,21,22</sup> We observed a trend of higher serum antibodies in NHPs injected with ubiquitous promoters compared with cell-specific promoters (Figure S4).

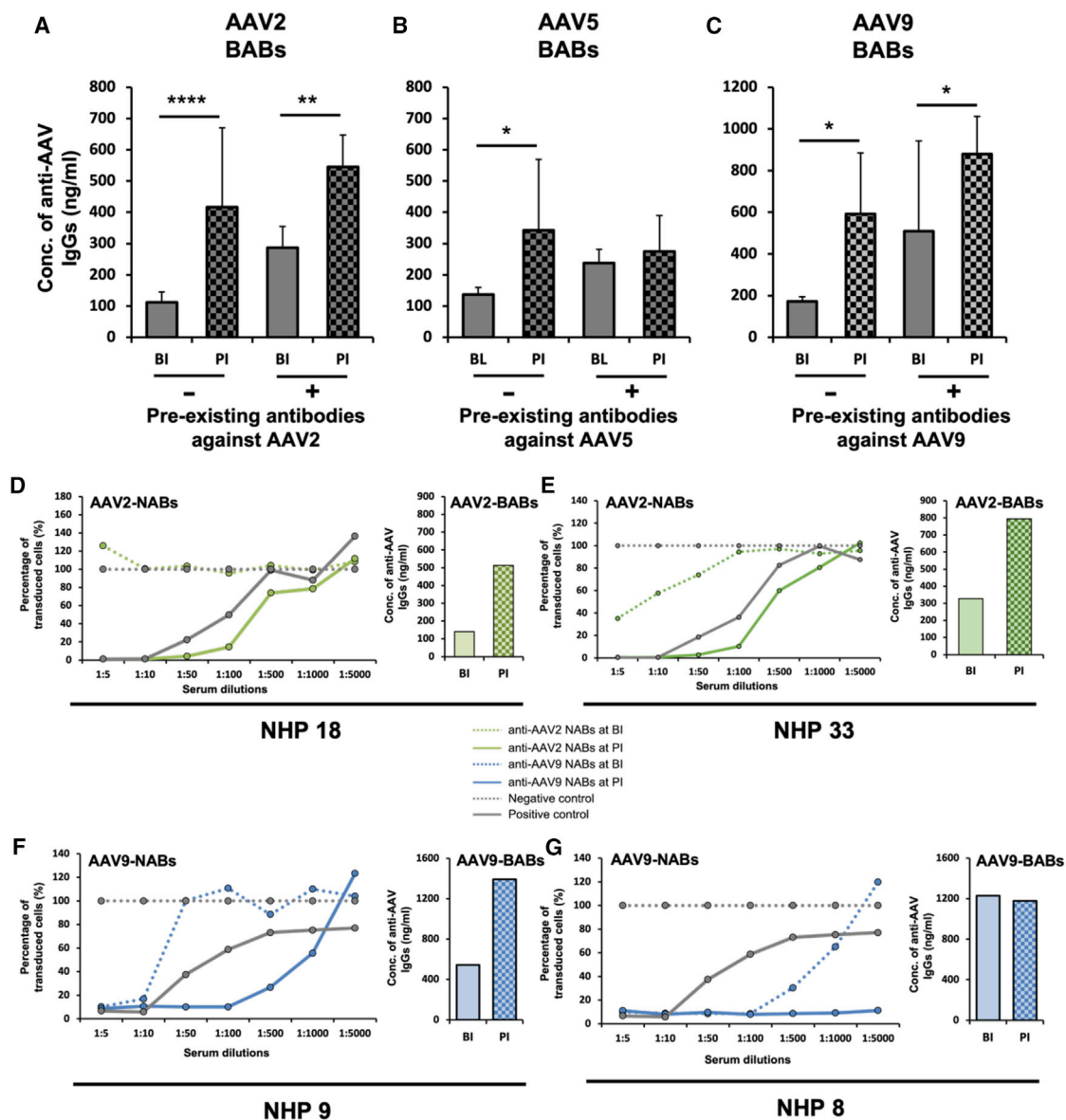
Owing to ethical issues, high costs, and inter-individual variability, it is challenging to achieve statistical significance in studies involving NHPs. Nonetheless, with our comprehensive retrospective analysis of 41 NHPs used in pre-clinical gene therapy studies over a period of 10 years, we were able to make some statistically significant observations and show some trends that, when considered in the context of other studies, provide meaningful insights for future pre-clinical studies involving AAV-mediated gene delivery.

The cross-reactivity between certain serotypes such as AAV2 and AAV9 could be because they are more closely related both in terms of genome sequence homology as well as capsid sequence homology (Figure 6H). Eighty-four percent of genome sequence homology exists between AAV2 and AAV9, whereas the sequences of AAV2 and AAV5 have a 54% homology and AAV9 and AAV5 have a 46% homology.<sup>38,39</sup> This may have consequences in selecting the serotype that can be used in cases where there is a need for a second injection. For example, after a first injection with AAV2, a follow-up injection with AAV5 might entail less risks than a subsequent injection with AAV9. Our data, consistent with previous studies,<sup>26</sup> reveal that anti-AAV antibodies persist in the serum even after 6 months to

MATERIAL AND METHODS

**AAV production**

AAV vectors containing transgenes were packaged by co-transfection in HEK293 cells (ATCC, CRL-1573), harvested 3 days post-transfection, and purified by iodixanol gradient ultracentrifugation. The 40% iodixanol fraction was collected after a 90 min spin at  $354,000 \times g$ . Concentration and buffer exchange were performed against PBS containing 0.001% Pluronic.<sup>32</sup> AAV vector stocks titers were then determined based on quantitative real-time PCR titration method using ITR primers and SYBR Green (Thermo Fisher Scientific) as described earlier.<sup>42</sup>



**Figure 5. Effect of pre-existing antibodies on BAB and NAB production**

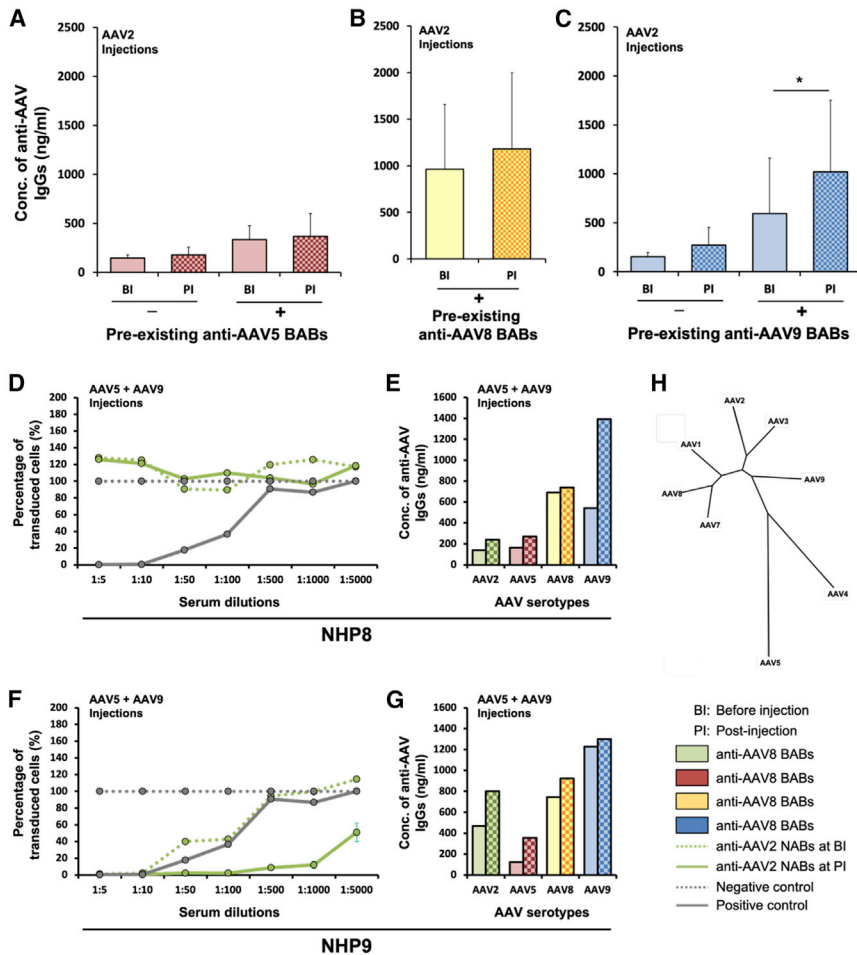
(A–C) Serum concentration of BABs against (A) AAV2 in n = 22 NHPs without (–) and n = 5 NHPs with (+) pre-existing BABs, (B) against AAV5 in n = 7 NHPs without (–) and n = 2 NHPs with (+) pre-existing BABs, and (C) against AAV9 in n = 4 NHPs without (–) and n = 7 NHPs with (+) pre-existing BABs. (D and E) Serum concentration of NABs and BABs against AAV2 in (D) NHP18 and (E) NHP33. (F and G) Serum concentration of NABs and BABs against AAV9 in (F) NHP9 and (G) NHP8. In each case, the BABs and NABs are tested against the same serotype that the animals were injected with. The values for BABs are shown as mean ± SD. The values for NABs at each dilution are normalized relative to the negative control (set to 100) and are shown as mean ± SD. Significance between individual time points was tested using Student’s t test (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001).

**Animals and intraocular injections**

All NHPs in this study were *Cynomolgus Macaca fascicularis* and originated from Mauritius. Prior to injections, they were anesthetized with an intramuscular injection of 10 mg/kg ketamine (Imalgene 1000, Merial), and 0.5 mg/kg xylazine (Rompun 2%, Bayer). Anesthesia was maintained with an intravenous injection of propofol at 1 mL/kg/h (PropoVet Multidose 10 mg/mL, Zoetis). Pupils were

dilated using 1% tropicamide (Mydraticum, Théa Pharmaceuticals, Clermont-Ferrand, France), and the eyelids were kept open using eyelid speculums. A 1 mL syringe equipped with a 32 mm, 27G needle was used for IVT injections by insertion into the sclera approximately 2 mm posterior to the limbus to deliver between 50 and 100 µL of the viral vector solution. For SR AAV injections, two 25G vitrectomy ports were set approximately 2 mm posterior to the limbus, one for



**Figure 6. Cross-reactivity across serotypes**

(A–C) NHPs injected with AAV2 and tested BI and PI for serum concentration of binding antibodies (A) against AAV5 in  $n = 18$  NHPs without (–) and  $n = 9$  NHPs with (+) pre-existing BABs, (B) anti-AAV8 in  $n = 27$  NHPs with (+) pre-existing BABs and (C) anti-AAV9 in  $n = 7$  NHPs without (–) and  $n = 20$  NHPs with (+) pre-existing BABs. (D–G) NABs against AAV2 and (E and G) BABs against AAV2, AAV5, AAV8, and AAV9 in (D and E) NHP8 and (F and G) NHP9 BI and PI of AAV5 + AAV9. Shown are mean values  $\pm$  SD. Significance at individual time points was tested using Student's *t* test ( $p < 0.05$ ). (H) Phylogeny tree generated from AAV capsid sequences of different serotypes, where the branch lengths are proportional to the evolutionary change (calculated using ClustalW).

diluted in blocking buffer (6% milk in  $1 \times$  PBS) for ELISA and in cell culture medium (DMEM + 10% fetal bovine serum) for NAB assays. The before-injection samples were collected 1–6 months prior to injection, and the post-injection samples were collected 2 months post-injection in the case of the majority of the NHPs, but in some cases, this time point was later, up to 6 months post-injection.

### NAB assay

NAB assay for AAV2 was performed using HEK293T cells (ATCC, CRL-1573) and for AAV9 using Lec2 cells (ATCC, CRL-1736) as described before.<sup>24</sup> Briefly, HEK293T cells were plated at a density  $7 \times 10^5$ /well and Lec2 cells were plated at a density of  $6.5 \times 10^5$ /well

in a 96-well plate and placed in an incubator ( $37^\circ\text{C}/5\% \text{CO}_2$ ) for 4 h. In another plate, serum dilutions in DMEM + 10% fetal bovine serum were prepared. To these serum dilutions, AAV2 at a multiplicity of infection (MOI) of 6,400 and AAV9 at an MOI of 20,000 were added and incubated for 2 h at  $4^\circ\text{C}$ . The serum-virus mix was added to the cells in duplicate and incubated overnight at  $37^\circ\text{C}$ . Twenty-four h later, the cells were lysed using cell culture lysis buffer (Promega, E1531) and mixed with luciferase assay reagent (Promega, E1501) as per the manufacturer's protocol. The luminescence was measured using the Spark Multimode microplate reader (TECAN, Männedorf, Switzerland). The positive controls in our assays consisted of a mix of NHP serums that tested positive with high NAB titers in our previous assays. These serum samples were pooled together, aliquoted, and frozen (for single use). The negative control consisted of media without serum.

### ELISA

A 96-well plate (Nunc Maxisorp, Thermo Fisher Scientific, 442404-21) was coated with  $1 \times 10^9$  vg AAV/well. To generate a standard curve, a dilution series using the Monkey immunoglobulin G (IgG)-UNLB antibody (Southern Biotech, 0135-01) was coated on each

the endo-illumination probe and the other for the SR cannula. A 1 mL Hamilton syringe equipped with a 25G SR cannula with a 41G tip was used for the injection (DeJuan/Awh 25ga, Synergetics, O'Fallon, MO, USA). The endo-illumination probe and cannula were introduced into the eye. Fifty to one hundred  $\mu\text{L}$  of the viral vector solution was injected subretinally to create a bleb either below or above the fovea. After SR or IVT vector administration, ophthalmic steroid and antibiotic ointments (Fradexam, TVM) were applied. All animal experiments and procedures were ethically approved by the French "Ministère de l'Éducation, de l'Enseignement Supérieur et de la Recherche" and were carried out according to institutional guidelines in adherence with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as well as the Directive 2010/63/EU of the European Parliament.

### Serum collection and dilutions

NHP blood was collected from a peripheral vein using a 22G into a red top vacutainer at various time points before and after ocular injections of AAVs. The blood sample was centrifuged at  $800 \times g$  for 10 min, and the serum (supernatant) was transferred into a separate Eppendorf tube and stored at  $-80^\circ\text{C}$  until further use. The serum was

plate and incubated overnight at 4°C. The AAV and antibody were diluted in coating buffer (0.1 M carbonate buffer pH 9.5). The plate was washed with blocking buffer (6% milk in 1× PBS), followed by incubation with serum in triplicates for 2 h, washing with wash buffer (1× PBS + 0.05% Tween), and incubation with the secondary antibody mouse anti-monkey IgG-HRP (Southern Biotech, 4700-05) for 1 h. For visualization, tetramethylbenzidine (TMB) substrate (Sigma Aldrich, T0440) was applied for 10 min, the reaction was stopped using the TMB stop solution (Thermo Fisher Scientific, SS04), and the luminescence measured at 450 nm using the Spark Multimode microplate reader (TECAN, Männedorf, Switzerland).

### Grading for clinical signs of inflammation

After pupil dilation, a Spectralis HRA + OCT system (Heidelberg Engineering) was used to acquire OCT images and fluorescent images of GFP using the Fundus Autofluorescence mode (excitation wavelength of 488 nm and barrier filter of 500 nm).

For grading the “Anterior chamber cells,” the grading system, defined by the SUN working group<sup>25</sup> in which grades are assigned by counting the cells in the field (field size is 1 × 1 slit beam), was used: grade 0: <1, grade 0.5: 1–5, grade 1: 6–15, grade 2: 16–25, grade 3: 26–50, and grade 4: >50 cells in the field. For grading the “Vitreous cells,” we used the NIH grading system: 0: no cells, 0.5: 1–10 cells, 1: 11–20 cells, 2: 20–30 cells, 3: 30–100 cells, and 4: >100 cells.<sup>43,44</sup> For grading the “Posterior Uveitis,” we used the British Medical Journal (BMJ) grading system: grade 0: no vitreous cells, grade 1: vitreous cells present with a clear view of posterior fundus, grade 2: vitreous cells present with a poor view of details and a view of optic nerve and retinal vessels, grade 3: vitreous cells present with a view of optic nerve, and grade 4: vitreous cells present with no view of the posterior segment.<sup>45</sup> For grading the opacity, we developed our own grading system with the aim of having a systematic parlance to better define and understand common features that we observe during ophthalmic exams. Here, 0: nil (no clinical findings), 1: minimal (single filamentary and small cotton-like floaters), 2: mild (multiple filamentary and small cotton-like floaters), 3: moderate (cloud-like floaters), 4: marked (massive partial vitreous opacities like vitreous fibroplasia), and 5: severe (vitreous is completely opaque).

### Statistical analysis

Statistical analysis was performed using Prism 9.0 software (GraphPad, San Diego, CA, USA). All data are presented as mean ± standard deviation (SD). The number of samples (N) used for individual experiments is given in the figure legends. Student’s t tests were used to test the significance between the means of groups. A p value below 0.05 was considered significant and is indicated on graphs as \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, and \*\*\*\*p < 0.0001. In the case of NAB assays, to estimate the serum dilution at which 50% neutralization occurs, we fit a curve between observations using linear interpolation. This computation for interpolation has been performed using the *stats* package built in R.<sup>46</sup>

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtm.2022.01.011>.

## ACKNOWLEDGMENTS

This work was supported by ERC Starting Grant (REGENETHER 639888), the Institut National de la Santé et de la Recherche Médicale (INSERM), Sorbonne Université, The Foundation Fighting Blindness, Agence National de Recherche (ANR) RHU Light4Deaf, \*LabEx LIFESENSES (ANR-10-LABX-65), and \*IHU FORESIGHT (ANR-18-IAHU-01). The authors would like to thank Melissa Desrosiers and Camille Robert for their technical assistance with the production of plasmids and viral vectors. The authors also extend their thanks to Shivani Shah (SAMP, France) and Oudom Kem for writing and implementing the code in R and Anees Mehdi for assistance with statistical methods and discussions. The graphical abstract and schematic illustrations were created on [BioRender.com](https://www.biorender.com).

## AUTHOR CONTRIBUTIONS

Conceptualization, D.D. and D.A.; experiments, D.A. performed ELISA assays, D.R. did the NAB and ELISA assays, E.B. and C.N.-J. did the ocular exams, OCT, and serum collections, and S.B. performed all the ocular injections; analysis, D.A., D.R., and E.B. did the experimental analysis and S.B.M. did the statistical analysis; writing – original draft, D.A.; writing – revision & editing, D.A., D.D., and S.F.; project administration, D.D. and D.A.; funding acquisition, D.D.

## DECLARATION OF INTEREST

D.D. is an inventor on a patent of AAV virions with variant capsid and methods of use thereof with royalties paid to Adverum Biotechnologies (WO2012145601 A2) and on patent applications on non-invasive methods to target cone photoreceptors (EP17306429.6 and EP17306430.4) licensed to Gamut Tx (now SparingVision). D.D. is a founder of Gamut Tx (now SparingVision). All other authors declare no competing interests.

## REFERENCES

- Hastie, E., and Samulski, R.J. (2015). Adeno-associated virus at 50: a golden anniversary of discovery, research, and gene therapy success - a personal perspective. *Hum. Gene Ther.* 26, 257–265.
- Wu, Z., Asokan, A., and Samulski, R.J. (2006). Adeno-associated virus serotypes: vector toolkit for human gene therapy. *Mol. Ther.* 14, 316–327.
- Gao, G.-P., Alvira, M.R., Wang, L., Calcedo, R., Johnston, J., and Wilson, J.M. (2002). Novel adeno-associated viruses from rhesus monkeys. *Proc. Natl. Acad. Sci. U S A* 99, 11854–11859.
- Barnes, C., Scheideler, O., and Schaffer, D. (2019). Engineering the AAV capsid to evade immune responses. *Curr. Opin. Biotechnol.* 60, 99–103.
- Kotterman, M.A., and Schaffer, D.V. (2016). Engineering adeno-associated viruses for clinical gene therapy. *Physiol. Behav.* 176, 139–148.
- Hickey, D.G., Edwards, T.L., Barnard, A.R., Singh, M.S., De Silva, S.R., McClements, M.E., Flannery, J.G., Hankins, M.W., and MacLaren, R.E. (2017). Tropism of engineered and evolved recombinant AAV serotypes in the rd1 mouse and ex vivo primate retina. *Gene Ther.* 24, 787–800.
- Bainbridge, J.W.B., Smith, A.J., Barker, S.S., Robbie, S., Henderson, R., Balaggan, K., Viswanathan, A., Holder, G.E., Stockman, A., Tyler, N., et al. (2008). Effect of gene

- therapy on visual function in leber's congenital amaurosis. *N. Engl. J. Med.* 358, 2231–2239.
8. Albert, M., Maguire, M.D., Francesca Simonelli, M.D., Pierce, E.A., Pugh, E.N., Jr., Mingozzi, F., Bencicelli, J., Sandro Banfi, M.D., Marshall, K.A., Testa, F., et al. (2009). Safety and efficacy of gene transfer for leber's congenital amaurosis. *J. Med.* 358, 2240–2248.
  9. Streilein, J.W. (2003). Ocular immune privilege: therapeutic opportunities from an experiment of nature. *Nat. Rev. Immunol.* 3, 879–889.
  10. Jacobson, S.G., Cideciyan, A.V., Roman, A.J., Sumaroka, A., Schwartz, S.B., Heon, E., and Hauswirth, W.W.P. (2017). Improvement and decline in vision with gene therapy in childhood blindness. *Physiol. Behav.* 176, 139–148.
  11. Bucher, K., Rodríguez-Bocanegra, E., Daultebekov, D., and Fischer, M.D. (2020). Immune responses to retinal gene therapy using adeno-associated viral vectors – implications for treatment success and safety. *Prog. Retin. Eye Res.* 83, 100915.
  12. Boutin, S., Monteilh, 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.
  13. 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.
  14. Li, Q., Miller, R., Han, P.Y., Pang, J., Dinculescu, A., Chiodo, V., and Hauswirth, W.W. (2008). Intraocular route of AAV2 vector administration defines humoral immune response and therapeutic potential. *Mol. Vis.* 14, 1760–1769.
  15. Seitz, I.P., Michalakos, S., Wilhelm, B., Reichel, F.F., Ochakovski, G.A., Zrenner, E., Ueffing, M., Biel, M., Wissinger, B., Bartz-Schmidt, K.U., et al. (2017). Superior retinal gene transfer and biodistribution profile of subretinal versus intravitreal delivery of AAV8 in nonhuman primates. *Investig. Ophthalmol. Vis. Sci.* 58, 5792–5801.
  16. Reichel, F.F., Daultebekov, D.L., Klein, R., Peters, T., Ochakovski, G.A., Seitz, I.P., Wilhelm, B., Ueffing, M., Biel, M., Wissinger, B., et al. (2017). AAV8 can induce innate and adaptive immune response in the primate eye. *Mol. Ther.* 25, 2648–2660.
  17. Ramachandran, P.S., Lee, V., Wei, Z., Song, J.Y., Casal, G., Cronin, T., Willett, K., Huckfeldt, R., Morgan, J.I.W., Aleman, T.S., et al. (2017). Evaluation of dose and safety of AAV7m8 and AAV8BP2 in the non-human primate retina. *Hum. Gene Ther.* 28, 154–167.
  18. Timmers, A.M., Newmark, J.A., Turunen, H.T., Farivar, T., Liu, J., Song, C., Ye, G.J., Pennock, S., Gaskin, C., Knop, D.R., et al. (2020). Ocular inflammatory response to intravitreal injection of adeno-associated virus vector: relative contribution of genome and capsid. *Hum. Gene Ther.* 31, 80–89.
  19. Beltran, W.A., Boye, S.L., Boye, S.E., Vince, A., Lewin, A.S., Hauswirth, W.W., and Aguirre, G.D. (2011). Complications associated with different promoters. *Gene Ther.* 17, 1162–1174.
  20. Amado, D., Mingozzi, F., Hui, D., Bencicelli, J.L., Wei, Z., Chen, Y., Bote, E., Grant, R.L., Golden, J.A., Narfstrom, K., et al. (2010). Safety and efficacy of subretinal re-administration of a viral vector in large animals to treat congenital blindness. *Sci. Transl. Med.* 2, 1–19.
  21. Khabou, H., Cordeau, C., Pacot, L., Fisson, S., and Dalkara, D. (2018). Dosage thresholds and influence of transgene cassette in adeno-associated virus-related toxicity. *Hum. Gene Ther.* 29, 1235–1241.
  22. Xiong, W., Wu, D.M., Xue, Y., Wang, S.K., Chung, M.J., Ji, X., Rana, P., Zhao, S.R., Mai, S., and Cepko, C.L. (2019). AAV cis-regulatory sequences are correlated with ocular toxicity. *Proc. Natl. Acad. Sci. U S A* 116, 5785–5794.
  23. Ansari, A.M., Ahmed, A.K., Matsangos, A.E., Lay, F., Born, L.J., Marti, G., Harmon, J.W., and Sun, Z. (2016). Cellular GFP toxicity and immunogenicity: potential confounders in *in vivo* cell tracking experiments. *Stem Cell Rev. Rep.* 12, 553–559.
  24. Desrosiers, M., and Dalkara, D. (2018). Neutralizing antibodies against adeno-associated virus (AAV): measurement and influence on retinal gene delivery. *Methods Mol. Biol.* 1715, 225–238.
  25. Jabs, D.A., Nussenblatt, R.B., Rosenbaum, J.T., Atmaca, L.S., Becker, M.D., Brezin, A.P., Chee, S.P., Davis, J.L., Deschenes, J., de Smet, M., et al. (2005). Standardization of uveitis nomenclature for reporting clinical data. Results of the first international workshop. *Am. J. Ophthalmol.* 140, 509–516.
  26. 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.
  27. Calcedo, R., and Wilson, J.M. (2013). Humoral immune response to AAV. *Front. Immunol.* 4, 1–7.
  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.
  29. Chaffiol, A., Caplette, R., Jaillard, C., Brazhnikova, E., Desrosiers, M., Dubus, E., Duhamel, L., Macé, E., Marre, O., Benoit, P., et al. (2017). A new promoter allows optogenetic vision restoration with enhanced sensitivity in macaque retina. *Mol. Ther.* 25, 2546–2560.
  30. Bennett, J., Ashtari, M., Wellman, J., Marshall, K.A., Laura, L., Chung, D.C., Mccague, S., Pierce, E.A., Chen, Y., Bencicelli, J.L., et al. (2012). AAV2 gene therapy readministration in three adults with congenital blindness. *Sci. Transl. Med.* 4, 1–24.
  31. Gao, G., Vandenberghe, L.H., Alvira, M.R., Lu, Y., Calcedo, R., Zhou, X., and Wilson, J.M. (2004). Gao et al-2004-J Virol. *J. Virol.* 78, 6381–6388.
  32. Choi, V.W., Asokan, A., Haberman, R.A., and Samulski, R.J. (2007). Production of recombinant adeno-associated viral vectors for *in vitro* and *in vivo* use. *Curr. Protoc. Mol. Biol.* 78, 1–24.
  33. Campbell, M.H.P. (2012). The blood-retina barrier: tight junctions and barrier modulation. *Adv. Exp. Med. Biol.* 763, 70–84.
  34. Liu, Y.F., Huang, S., Ng, T.K., Liang, J.J., Xu, Y., Chen, S.L., Xu, C., Zhang, M., Pang, C.P., and Cen, L.P. (2020). Longitudinal evaluation of immediate inflammatory responses after intravitreal AAV2 injection in rats by optical coherence tomography. *Exp. Eye Res.* 193, 107955.
  35. Reichel, F.F., Peters, T., Wilhelm, B., Biel, M., Ueffing, M., Wissinger, B., Bartz-Schmidt, K.U., Klein, R., Michalakos, S., and Fischer, M.D. (2018). Humoral immune response after intravitreal but not after subretinal aav8 in primates and patients. *Investig. Ophthalmol. Vis. Sci.* 59, 1910–1915.
  36. Belin, P.J., and Parke, D.W., 3rd (2020). Complications of vitreoretinal surgery. *Curr. Opin. Ophthalmol.* 31, 167–173.
  37. Boyd, R.F., Boye, S.L., Conlon, T.J., Erger, K.E., Sledge, D.G., Langohr, I.M., Hauswirth, W.W., Komáromy, A.M., Boye, S.E., Petersen-Jones, S.M., et al. (2016). Reduced retinal transduction and enhanced transgene-directed immunogenicity with intravitreal delivery of rAAV following posterior vitrectomy in dogs. *Physiol. Behav.* 176, 139–148.
  38. Daya, S., and Berns, K.I. (2008). Gene therapy using adeno-associated virus vectors. *Clin. Microbiol. Rev.* 21, 583–593.
  39. Gao, G., Vandenberghe, L., and Wilson, J. (2005). New recombinant serotypes of AAV vectors. *Curr. Gene Ther.* 5, 285–297.
  40. Vandenberghe, L.H., Bell, P., Maguire, A.M., Cearley, C.N., Xiao, R., Calcedo, R., Wang, L., Castle, M.J., Maguire, A.C., Grant, R., et al. (2011). Dosage thresholds for AAV2 and AAV8 photoreceptor gene therapy in monkey. *Sci. Transl. Med.* 3, 1–11.
  41. Narfstrom, K., Katz, M.L., Bragadottir, R., Seeliger, M., Boulanger, A., Redmond, T.M., Caro, L., Lai, C.M., and Rakoczy, P.E. (2003). Functional and structural recovery of the retina after gene therapy in the RPE65 null mutation dog. *Investig. Ophthalmol. Vis. Sci.* 44, 1663–1672.
  42. Aurnhammer, C., Haase, M., Muether, N., Hausl, M., Rauschhuber, C., Huber, I., Nitschko, H., Busch, U., Sing, A., Ehrhardt, A., et al. (2012). Universal real-time PCR for the detection and quantification of adeno-associated virus serotype 2-derived inverted terminal repeat sequences. *Hum. Gene Ther. Methods* 23, 18–28.
  43. Mahendradas, P., Khanna, A., Kawali, A., and Shetty, R. (2014). Quantification of inflammation in inflammatory eye diseases. *Internet J. Rheumatol. Clin. Immunol.* 2, <https://www.chanrejournal.com/index.php/rheumatology/article/view/102>.
  44. Nussenblatt, R.B., Palestine, A.G., Chan, C.C., and Roberge, F. (1985). Standardization of vitreal inflammatory activity in intermediate and posterior uveitis. *Ophthalmology* 92, 467–471.
  45. Huang, J.J., Elia, M., and Uveitis. (2021). *BMJ Best Practice*, <https://bestpractice.bmj.com/topics/en-us/407>.
  46. R Core Team (2020). R: a language and environment for statistical computing (R Foundation for Statistical Computing), <https://www.R-project.org/>.