MSD-based assays facilitate a rapid and quantitative serostatus profiling for the presence of anti-AAV antibodies

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Adeno-associated virus (AAV) vector applications are often limited by capsid-directed humoral immune responses, mainly through neutralizing antibodies (NAbs), which are present throughout the human population due to natural AAV infections. Currently, antibody levels are often quantified via ELISA-based protocols or by cellular NAb assays and less frequently by in vivo NAb assays in mice. These methods need optimization for each serotype and are often not applicable to AAV variants with poor in vitro transduction. To tackle these limitations, we have established Meso Scale Discovery (MSD)-based assays for the quantification of binding antibodies (BAbs) and NAbs against the three most commonly used AAV serotypes, AAV2, AAV8, and AAV9. Both assays detect anti-AAV-IgG1 with high sensitivity and consistency as shown in a screen of sera from 40 healthy human donors. Subsequently, BAb and NAb titers were determined for identification of seronegative animals in a non-human primate (NHP) cohort. Moreover, the MSD-based BAb assay protocol was extended to a panel of 14 different AAV serotypes. In summary, our platform allows a rapid and quantitative assessment of the immunological properties of any natural or engineered AAV variant irrespective of transduction efficiency and enables high-throughput screens.

INTRODUCTION
Adeno-associated virus (AAV) represents a valuable platform for the development of recombinant viral vector-based gene therapies by combining efficient gene delivery with a favorable safety profile. Within the last decade, the attractiveness of AAV vectors was highlighted by positive results in various clinical trials and the recent market approval of Luxturna and Zolgensma by the US Food and Drug Administration (FDA) and later by the European Medicines Agency (EMA). The successful use and increasing number of AAV vectors in pre-clinical and clinical settings also raises the need for robust protocols to determine pre-existing anti-AAV antibodies. Many humans encounter a natural infection with AAVs, which triggers the secretion of neutralizing antibodies (NAbs) by plasma B cells for substantial periods of time even after clearance of the initial infection. This is further stimulated upon re-encounter with AAV capsids. NAbs to AAV have a very high prevalence in the adult human population and are frequently cross-reactive to different AAV serotypes. This poses a substantial hurdle for therapeutic applications of AAV-based vectors in the gene-therapy field, since even low NAb titers can adversely affect transgene expression. Therefore, clinical treatment requires extensive pre-screening of patient cohorts to determine and monitor their NAb status.

Different strategies have been developed to overcome these hurdles and enable inclusion of seropositive individuals into clinical trials, such as transient immune suppression, immunoglobulin G (IgG) clearance or cleavage (plasmapheresis, empty capsid decoys, Ides protease treatment), or engineering of recombinant AAVs with immune escaping properties as well as switching to non-human serotypes in novel vector-design strategies. Nonetheless, pre-screening and close monitoring of the patient immune status remains pivotal for effective and long-lasting therapeutic gene expression as well as for potential vector re-administration.

Non-human primates (NHPs) are a relevant species for testing AAV-mediated gene therapies intended for human treatment. Like humans, NHPs are natural hosts for AAVs and often possess NAbs against different AAV serotypes that are commonly used in (pre-)clinical research. Thus, pre-screening of NHP cohorts for their antibody status is required in gene-therapy studies before vector administration.

Different assays are currently being used for the determination of total antibody or NAb titers. These include ELISA-based approaches as well as NAB assays in cell culture and mouse models. Both cell-culture based assays and mouse experiments are cumbersome, costly and time consuming and require a highly optimized protocol for individual AAV capsids of choice, while ELISA-based assays may require validation with an orthogonal method.

We have developed a Meso Scale Discovery (MSD)-based assay for the quantitative assessment of binding antibodies (BAbs) that...
potentially can be used for any AAV subtype of interest. Our method is fast and scalable and requires small amounts of serum samples, consumables, and virus stocks. In addition to its broad dynamic assay range, it combines the sensitivity of an ELISA-based approach with the accuracy of cellular NAb assays and is universally applicable to natural and recombinant AAVs alike.

We characterized our in-house MSD BAb assay with 40 human serum samples against the highly abundant AAV serotypes, AAV2, AAV8, and AAV9, and subsequently used it to screen the sera of 42 NHPs for BAbs against any of the given serotypes. Furthermore, we extended the analysis to include IgA and IgM assessment for the human cohort. Comparison of the determined antibody titers with a cellular NAb assay resulted in a highly coherent data set with 38/40 sera being classified as either positive or negative in both assays and the remaining 2 outliers being at the limit of detection in either assay, indicating that MSD-based BAb quantification reflects serum NAb levels. Our study further demonstrates the suitability of the MSD-based assays for the quantification of IgGs against any given AAV capsid, as shown with a panel of 14 different AAV serotypes. Thus, the presented MSD-based assays allow for rapid and reliable determination of anti-AAV antibody levels and therefore provide a valuable tool for a wide range of pre-clinical research that can easily be extended to clinical applications.

RESULTS

Establishment of MSD-based in vitro BAb and NAb assays

First, signal detection was quantified for BAb assays of AAV2, AAV8, and AAV9 with different coating conditions between $5 \times 10^3$ to $5 \times 10^9$ viral genomes (vg) per well using monoclonal antibodies specific to respective serotypes (Figure S1). While coating of higher virus concentrations resulted in an overall increase in the electrochemiluminescence (ECL) signal, no major differences have been observed in the resolution of different antibody concentrations. Coating of $5 \times 10^8$ vg/well was therefore chosen for all subsequent assays to enable a sensitive readout, particularly in the lower range, without compromising on absolute sensitivity and minimizing virus need. The assay range was subsequently quantified by titrating different amounts of monoclonal antibodies ranging from 1 to 2,500 ng/mL (Figure 1A). The same assays were performed with intravenous Ig (IVIG) ranging from 10 to 5,000 mg/mL (Figure 1B). The monoclonal antibodies all have a sensitivity resolution below 1 ng/mL (which is in accordance with thresholds previously determined by Falese et al. for AAV5-based assays31) and show a low coefficient of variation (%CV = 2.66–5.53) but have major differences in their signal intensity ($10^2$–$10^4$ for AAV2 and $10^3$–$10^5$ for AAV8 and AAV9) and concentration range. In contrast, IVIG had a more uniform signal intensity ranging from $10^3$–$10^4$ irrespective of the serotype (see also Figures S2 and S3). We continued with IVIG for assay establishment since it reflects the polyclonality of an IgG response targeting multiple epitopes and can be read out using the same anti-human/NHP detection antibody together with unknown samples. Moreover, the raw data (Figure S2) showed no background noise for monoclonal antibodies and a dose-dependent increase in unspecific signals for IVIG assays in wells coated with AAV buffer only. We observed the same effect for some, but not all, serum samples tested. It is therefore essential to include background subtraction for all samples at the respective

![Figure 1](https://www.moleculartherapy.org/)

Figure 1. Establishment of binding and neutralizing antibody assays with monoclonal antibodies and IVIG

(A) Binding antibody (BAb) assay quantification with monoclonal antibodies for AAV2 (A20), AAV8 (ADK8), and AAV9 (ADK9). (B) Binding antibody assay quantification with intravenous immunoglobulin (IVIG). Please see Figure S2 for data before normalization. (C) AAV2 neutralizing antibody (NAb) assay with A20. (D) AAV2 neutralizing antibody assay with IVIG. Data are represented as mean ± SD of triplicate experiments, and the coefficient of variation (%CV) is indicated within the corresponding graphs. For comparison of different coating conditions, please see Figure S1. For replicate BAb assays with AAV2, AAV8, and AAV9, please see Figure S3.
concentrations individually to obtain the amount of anti-AAV-specific IgG.

To assess the reproducibility and sensitivity of the BAb assay, replicate measurements were performed with AAV2, AAV8, and AAV9 on three different plates and repeated on 3 individual days with 2.1–8,000 ng/mL IVIG (Figure S3). While variation was observed for AAV2 assays at IVIG concentrations >1,000 ng/mL, mainly resulting from saturation of binding, an overall low level of variation was found between different plates or replicates. The calculated % CV was between 10.44 and 15.70 for AAV2, mainly due to saturation effects at high IVIG concentrations, and between 5.54 and 12.03 for AAV8 and 8.58 to 12.91 for AAV9 and is indicated on the respective graphs. The sensitivity threshold was defined as the mean value plus three standard deviations of the first concentration with relative luminescence units (RLU) 1 < x < 10 after background signal subtraction (corresponding to 2.1 μg/mL for AAV2, 5.2 μg/mL for AAV8, and 13.1 μg/mL for AAV9). Threshold values are indicated in the respective plots and range between 6.00 and 18.46 RLU. As shown in Figure 1B for AAV8 and AAV9 assays, signals below 10 RLU can still be quantified, however, at the cost of large fluctuations, and it is therefore recommended to apply a more stringent cutoff as described above.

A cellular NAb assay protocol was established, in parallel, to compare BAb levels with their neutralizing capacity. A detailed experimental description is included in the Materials and methods. Briefly, a serial dilution of serum, monoclonal antibody, or IVIG was pre-incubated with AAV2 capsids harboring an anti-fluorescein isothiocyanate (FITC) transgene at 37°C for 30 min and then was used to transduce HEK293-H cells for 72 h. Transduced cells produce and secrete a V5-coupled anti-FITC antibody that can be quantified by an MSD-based readout from cellular supernatant with a Biotin-anti-V5 antibody. The NAb titer is determined at 50% transduction efficiency after normalization to transduced cells without IgGs. The obtained half maximal inhibitory concentration (IC50) value for AAV2 with A20 is 56.96 ng/mL (Figure 1C), in accordance with 65.9 ng/mL determined by Falese et al. for AAV513 and 25.27 μg/mL with IVIG (Figure 1D). Extrapolation of the IVIG NAb IC50 value corresponds to 513.8 RLU in the BAb assay (Figure 1B, left graph) and to values between 151.13 to 248.89 RLU in replicate experiments in Figure S3, which shows that the BAb assay detection limit is about 10-fold lower than the minimal concentration needed for 50% neutralization in a cellular readout.

Examination of a healthy human cohort by MSD-based BAb and cellular NAb assays

The seroprevalence of NAbs against the three most widely used therapeutic AAV serotypes, AAV2, AAV8, and AAV9, is well characterized in literature.6,7,11 After establishment of the BAb and NAb assays with IVIG, a set of 40 human serum samples from healthy donors was tested in BAb and NAb assays for assay comparison (Figure 2 and S4–S6). BAbRs were measured with a starting serum dilution of 1:5. In total, 15 out of 40 donors had a BAb IgG signal for AAV2 >10 RLU, corresponding to 37.5% of all tested samples. A high degree of cross-reactivity was observed for all sera, with most donors being either negative or positive against all tested serotypes, although with lower signal intensities for AAV8 and AAV9 compared with AAV2 (Figure 2A).

In addition, AAV2 NAb assays were performed with serum of the 40 donors. To determine the detection limit of the NAb assay, 15 sera with transduction rates >50% relative to no serum control were selected to obtain a pooled negative serum (PNS) as a matrix control (Figures S4 and S5, top graph). Different amounts of A20 were spiked in ranging from 2.5 to 500 ng/mL, and NAb assays were measured, revealing a dose-dependent neutralization below 50% transduction starting from IC50 2.99 at 50 ng/mL A20 (Figure S4). Therefore, sera with IC50 values below 3 were defined as negative in the human cohort and are labeled as not applicable (n.a.) in Figure 2B. Only minor matrix effects were observed, shifting from IC50 values of 56.96 obtained in the A20 setup (Figure 1C) to 75.04 in the PNS-spiked-in quantification (Figure S4, bottom right).

Exemplary NAb assays of serum S1 (IC50 < 1:4), S34 (IC50 = 4.01), and S37 (IC50 = 7.66) are shown in Figure 2C to highlight the assay’s ability to truly determine titer differences in the lower range as well as examples of highly positive sera with IC50 values above 1:320 (S31 IC50 = 1,444 and S39 IC50 = 13,090). NAb transduction of 1:2, 1:4, and 1:8 diluted human serum and all quantified IC50 values are summarized in Figure S5.

Interestingly, serum S6 tested strongly positive in the NAb assay with an IC50 titer of 137 despite being devoid of detectable binding IgG antibodies (blue bar in Figure 2B and NAb assay in Figure 2C). We therefore extended the BAb assay protocol to two additional Ig isotypes (IgA and IgM) and measured the serum samples at 1:4 and 1:10 dilutions (Figure S6). We observed a strong IgM signal for serum S6 (47,546 RLU at 1:10 dilution), which explains the determined NAb response. Additionally, a weaker IgM signal was measured for serum S9 (3,093 RLU at 1:10 dilution), which also had no IgG but a NAb titer of 4.05. A weaker IgA response was obtained for serum S6 (3,093 RLU at 1:10 dilution), which also had no IgG but a NAb titer of 4.05. A weaker IgA response was obtained for serum S5 (2,198 RLU at 1:10 dilution) with a NAb titer of 6.02. No signal was obtained for IVIG controls in these assays, confirming selectivity of the anti-Ig isotype antibodies. We also combined the BAb readout for individual Ig subtypes by detecting binding through secondary antibodies specific for the κ or λ light chain either alone or in combination (Figure S6, right panel), which delivers comparable results but is less specific than the separate readout for individual Ig isotypes.

Taken together, 22/40 sera tested negative and 18/40 sera tested positive in the NAb assay with an IC50 titer of 1:4 and above (Figure S7, top graph), corresponding to a seroprevalence of 45% in the cohort, which is in accordance with previous studies.5,21 No differences were observed between female and male donors. However, a substantial likelihood for seroconversion was seen with increasing age, with
14 out of 18 seropositive individuals being 40 years or older (Figures 2D and S7).

All but one serum without NAbs were also negative in the BAb assay (21/22 samples). S38 was a borderline outlier, with 11 RLU in the IgG analysis. Fourteen sera had IgG titers proportional to their Nab potency and were confirmed positive. Additionally, three serum samples (S5, S6, and S9) had no IgG but did have detectable IgA or IgM titers. S34 remains the only serum with an IC50 of 4.01 that was not found to be related to any antibody-mediated neutralization (Figures S7, bottom graph, and S8). In total, 35/40 donors tested positive or negative by Nab and IgG BAb assays. When taking the signal for other Ig isotypes into account, this value increases to 38/40 sera (95%), with the only two outliers being close to the
detection limit. This confirms the suitability of MSD-based readouts for quantification of binding Ig isotypes as a surrogate assay for NAbs.

**BAb and NAb screening in NHPs**

NAb against AAVs are not only found in humans but are also frequently present in NHP colonies and can negatively impact AAV-mediated transgene expression in pre-clinical primate studies. The established BAb assays were used to screen a cohort of 42 NHPs for their antibody status against AAV2, AAV8, and AAV9 to identify seronegative animals and to test for serum cross-reactivity. A titer of roughly 1:4 was determined in the human cohort as the lowest cutoff for weakly positive sera. This starting dilution was also used in the NHP screen in order to improve detection of low antibody titers. As expected, the majority of screened NHPs had high BAb antibody levels against one or multiple serotypes (Figure 3A). In contrast to the human cohort, no clear cross-reactivity was seen between seropositive samples against AAV2 versus AAV8 or AAV9. Multiple animals had strong BABs against all three serotypes (e.g., NHP23), but individual NHPs were either positive for one serotype (e.g., NHP1 AAV2+/AAV8-/AAV9- or NHP34 AAV2-/AAV8-/AAV9+) or were positive for a subset only (e.g., NHP4 AAV2-/AAV8+/AAV9+). NAb assays against AAV2 were performed for all animals, and the AAV2 BAb profile was confirmed by corresponding NAb titers (Figure 3B). In total, four AAV2-seronegative NHPs were identified (NHP4, NHP20, NHP32, and NHP34) and one additional animal (NHP42) with a NAb titer below 1:4 (Figure 3C). Of these, NHP20, NHP32, and NHP42 had the best profile with undetermined or low (<1:8) antibody titers against all three serotypes. Strong serum effects were observed in all cellular NHP Nab
assays, boosting transgene expression up to 500% of cells without NHP serum addition (no serum control). In addition to the data presented here, it would be valuable to include analysis of additional iso- types, in particular in NHP sera with a low NAb titer.

BAb assays as a suitable platform for IgG screening of various AAV serotypes

The BAb assay protocol was finally extended to a panel of 14 different AAV serotypes carrying an EGFP transgene to enable a quantitative comparison of immunological properties, irrespective of AAV cargo or transduction efficiency. AAVs of human and rhesus origin were included in the analysis as well as a porcine AAV (po.1), a known serotype with previously described immune-escape properties. Capsids were incubated with a serial 2-fold dilution of IVIG (Kiovig, 100 mg/mL) starting at 1:10 (corresponding to a range of 10 to 10,000 µg/mL). Duplicate experiments were performed on 2 different plates to ensure reproducibility for all serotypes. All virus stocks were produced with an established purification protocol and capsid protein intensity routinely checked by SDS-PAGE based on ddPCR virus titers to ensure comparable capsid amounts (data not shown). Similar coating efficiency of individual serotypes within a 2-fold range of 5 × 10^8 vg/well was confirmed by ddPCR (Figure S9A). Large differences in IVIG sensitivity were observed between the tested serotypes. While strong antibody binding was detected for AAV1, AAV2, AAV3b, and AAV6–9, less-pronounced IgG binding was measured for AAV5 (Figure 4A). IVIG antibodies reacted with AAV rhesus variant 10 (rh.10) and rh.39 to a similar extent, while hardly any binding occurred for AAV po.1, rh.32.33, and rh.74 until 1,250 µg/mL IVIG (see also Figure S9B). Remarkably, AAV12 proved almost fully inert to IVIG binding compared with the remaining serotypes and showed the most promising profile of all AAVs within the panel. However, a strong dose-dependent increase in IgG was measured even for serotypes with a promising immune-evasive profile (Figure 4B). Thorough characterization of the antibody status in healthy cohorts as well as patients is therefore required for AAV-based gene-therapy vectors of any origin.

DISCUSSION

As gene therapy takes up pace, an increasing number of clinical trials are conducted using recombinant AAV vectors based on naturally occurring serotypes, such as AAV2, AAV5, AAV8, and AAV9, and many additional therapeutic vectors are currently in pre-clinical development, with tailored proprietary AAV capsids for cargo delivery, such as AAV7m8 for the retina or AAVLK03 for the liver. Still, a major hurdle for successful patient treatment is the pre-existence of NAbs in patient cohorts, which can inhibit the therapeutic benefit of gene therapy. A large effort is therefore being made in the field to improve vector design, aiming to avoid NAb recognition. Also, antibody cross-reactivity poses a challenge even with AAVs of non-human origin. It therefore remains pivotal to assess the antibody
status of individual patients prior to treatment. Besides patient screening, pre-screening of seronegative NHPs for animal studies requires a rapid and reliable assay. The MSD-based BAb assays described here are universally applicable to any AAV serotype or recombinant AAV capsid and work irrespective of packaged transgenes or downstream manufacturing processes. They are fast and easy to perform and deliver a reproducible readout with little serum volume. This is of particular advantage for mouse studies and seroconversion experiments, where blood withdrawal might be limited to µL volumes. It further allows batch testing of final products with serum of individual patients. The concept of IgG quantification by MSD has been recently described for AAV5 by Falese et al.\textsuperscript{31} Our BAb assay protocol was extended to three additional serotypes, AAV2, AAV8, and AAV9, for which it reaches a comparable sensitivity below 1 ng/µL with monoclonal antibodies. It enables an unbiased and quantitative comparison across a large panel of different AAV capsids, irrespective of packaged transgenes or differences in transduction efficiency in a label-free manner. In our hands, the BAb assay delivered human and NHP IgG titers that were reproducible in cellular NAb assays in a quantitative way. The results from the human cohort reflect literature data with 45% seroprevalence for anti-AAV2 IgG and a high degree of cross-reactivity to AAV8 and AAV9.\textsuperscript{6,7} The BAb assay setup is therefore suitable for serum pre-screening or monitoring IgG levels after AAV administration with reduced cost and time effort and allows for the fast identification of patients admissible for treatment. Notably, we would advise against titer determination at a single serum dilution, in particular for low dilution cutoffs, as this might severely underestimate the IgG amount through signal quenching as seen for S17 and S39 in the human cohort. Although AAV neutralization mainly occurs through IgG antibodies, co-prevalence of different Ig isotypes has been described previously, e.g., by Kruzik et al.,\textsuperscript{8} and the example of a highly IgM-positive serum S6 even in a small human cohort highlights the need for assessment of different Igs. Conversely, BAb assays detecting Ig light chains tend to show an increase in unspecific signals and might not be the readout of choice for the characterization of low-titer serum samples. Our data emphasize the need for a versatile and quickly adaptable protocol that is suitable to address different aspects of the AAV immune response in a fast and reproducible manner.

For NHPs, the antibody profile was very different from the human sera for the three serotypes tested with cross-reactivity between AAV2, AAV8, and AAV9 observed in some, but not all, animals. A recent publication by Gardner et al.\textsuperscript{27} shows that seroconversion of NHPs treated with AAV1 or AAV8 results in a similar profile, with some animals developing serotype-specific antibodies while others have a cross-reactive response to multiple serotypes.

Recent studies reported divergent influence of binding and NABs on AAV transduction.\textsuperscript{35} We observed a strong overlap between MSD-based BAb and NAb assays throughout all in-house measurements in NHP and human sera. However, transduction of cells is inefficient in vitro with many AAVs including AAV8 and AAV9\textsuperscript{26,37} and might require different cell lines for AAV transduction.\textsuperscript{35} The NAB escape effect can be masked by transduction impairment in cellular assays, and even minor changes of the capsid surface residues can negatively affect transduction efficiency. Moreover, serum effects on cellular proliferation can adversely impact NAb assay readout, as observed in our NHP screen. In contrast, the MSD-based BAb assay combines the speed and sensitivity of an ELISA with high comparability with a cell-based NAb assay without adverse serum effects. It is universally applicable to all naturally occurring AAV serotypes and capsid-engineered AAV vectors alike and can easily be adapted to high-throughput screening while requiring little optimization for the testing of different AAV capsids, as demonstrated by the comparison of 14 different serotypes in Figure 4. The favorable immunological profile of rhesus variants rh.32.33 and rh.74 was successfully reproduced in accordance with previously published serological studies of these AAV variants.\textsuperscript{2,39} Moreover, the low sensitivity of AAV12 to IVIG, as described in the original publication by Schmidt et al.,\textsuperscript{40} was confirmed, and AAV12 was found to be superior to all remaining serotypes, which makes it an interesting potential candidate for capsid-engineering approaches. While the gene-therapy field mainly focused on establishment and validation of cellular NAb assays with a functional readout on transduction for first-generation vectors based on natural AAV isolates, pre-clinical research is markedly shifting toward recombinant and even synthetic AAV capsids with tailored properties. This development, in turn, demands new methods of anti-AAV antibody assessment for pre-clinical vector development and patient pre-screening and monitoring, which are more universally applicable and can be easily transferred and compared across different laboratories and gene-therapy programs. Since the impact of the NAb titer on gene therapy largely depends on the route of administration and target tissue, we did not provide definitive cutoff recommendations for a positive BAb assay, as it can be titrated to virtually zero residual background and thresholds need to be individually evaluated. However, the protocols presented here can easily be validated for clinical assessment of anti-AAV BAbs following the guidelines described in a recent review by Gorovits et al.\textsuperscript{41} that is based on the experience of leading gene-therapy organizations and includes regulatory considerations. For perspective, multiplexing either different serotypes or engineered capsids would further extend the Bab MSD platform presented in this study. With high sensitivity, versatility, and reproducibility, MSD-based assays provide an equivalent alternative to ELISA-based methods for studying various aspects of the AAV humoral immune response.

**MATERIALS AND METHODS**

**Cell culture**

HEK293-11 cells (Thermo Fisher Scientific, Waltham, MA, USA, cat. no. 11631–017) were cultivated at 37°C and 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen Life Technology, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and used for NAb assays until passage 25.

**Serum samples**

Human sera were obtained from 40 healthy blood donors (22 female, 18 male, age 21–63) at the blood donation center on the Boehringer
Ingelheim site in Biberach, Germany. The study was approved by the ethics commission of the state chamber of Baden-Württemberg under AZ: F-2016-121. NHP sera were provided by LabCorp (Münster, Germany) within the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV) approval AZ: 84–02.04.2015.A575. Pooled human IVIG was purchased as a 100 mg/mL stock solution (Kiovig, Takeda, Vienna, Austria), stored at 4°C, and pre-diluted 1:10 in PBS for BAb assays or DMEM for NAb assays.

**AAV vectors**

AAV vector stocks were produced using CELLdiscs by equimolar transfection of Rep-Cap plasmids, pHelper, and pAAV-CMV-EGF or pAAV-CMV-antiFITC plasmids into HEK293-H cells. Purification was performed by polyethylene glycol precipitation, followed by iodixanol density gradient ultracentrifugation and ultrafiltration, as described in detail previously.

**MSD assay for BAb quantification**

The presence of pre-existing total anti-capsid antibodies in NHP or human samples was analyzed using an antigen-capture immunogenicity assay format on the MSD (Meso Scale Discovery, Rockville, MD, USA) platform. Standard Multi-Array MSD plates (L15XA-1) were coated with 5 × 10^8 AAV vg/well in AAV-formulation buffer and were under shaking for 5 min at 750 RPM, unless indicated otherwise in the figure legend. After incubation at 4°C overnight, the plate was washed three times with 300 μL PBS-T (0.05% Tween 20). Blocking was performed using blocking solution (5% MSD Blocker B [R93BB-2] in PBS) for 1 h at room temperature (RT) followed by washing. Serial 2-fold dilutions of human serum (starting dilution 1:5) or NHP serum (starting dilution 1:4) were prepared in 5% Blocker B and incubated for 1 h at RT on coated AAV capsids. IVIG (Kiovig, 100 mg/mL, 1:10 starting dilution) served as an internal control. After washing, bound IgG1 was detected by incubation with anti-human/NHP IgG (MSD D20F-6; 1:1000 dilution in 5% Blocker B) for 1 h at RT followed by washing and readout after addition of 2× MSD Read Buffer (R92TC-2). Within 5 min, ECL was detected by the MSD Meso QuickPlex SQ 120 Imager using Discovery Workbench software v.3.0.18.

Serotype-specific monoclonal antibodies were used for assay establishment at stock concentrations of 50 μg/mL and were diluted between 1 and 2500 ng/mL in 5% Blocker B. These included A20 (anti-AAV2 mouse monoclonal IgG3, Progen, 61055), ADK8 (anti-AAV8 mouse monoclonal IgG2a, Progen, 610160), and ADK9-1R (anti-AAV9 mouse recombinant IgG3, Progen, 610178) and were detected with a goat anti-mouse antibody (MSD #R32AC-1; 1:1000 dilution in 5% Blocker B). For detection of additional Ig isotypes, plates were incubated with anti-human/NHP IgA (MSD D20f-6), IgM (MSD D20P-6), Ig κ (MSD D20TF-6), or Ig λ (MSD D20QG-6) light-chain antibodies (all used at 1:1000 dilution in 5% Blocker B) for 1 h at RT, with the remaining protocol steps unchanged.

Background signal of PBS-coated wells for each serum (or IVIG) dilution was subtracted from each individual AAV-coated sample. BAb assay sensitivity thresholds were calculated as mean plus three standard deviations of the lowest concentration with a signal of 1 to 10 above background values for IVIG and monoclonal antibody assays.

**Cellular NAb assay**

The serum titer of NAb was assessed by transduction inhibition assays. Briefly, 96-well plates were seeded with 2.5 × 10^4 HEK293-H cells/well 24 h before transduction. Recombinant AAV vectors (anti-FITC) were diluted in serum-free DMEM and incubated with 2-fold serial dilutions (1:2 to 1:1,024 in serum-free DMEM) of serum samples for 30 min at 37°C. Subsequently, serum-vector mixtures corresponding to 2,500 vg/cell were added to plated cells and incubated for 72 h at 37°C and 5% CO2. Transductions were performed in triplicates. The supernatant was transferred to 96-well plates, cell debris were removed by centrifugation at 200 × g for 5 min, and then the amount of anti-FITC was determined by anti-FITC MSD assay, as described previously. Transduction efficiency was measured as relative counts per well and normalized to a transduced control without serum.

**IC50 determination**

IC50 values were determined by non-linear fitting using GraphPad Prism software v.9.3.1 (GraphPad, San Diego, CA, USA). Dilution values were transformed to a log10 scale, and the “log(inhibitor)” vs. response variable slope function was used to fit IC50 values of triplicate measurements with standard error variation. To minimize effects of serum-mediated increases in transduction, top and bottom constraints were set at 100 and 0 for IC50 calculations, which yielded accurate resolution of neutralizing titers in the spike-in experiment in Figure S4. Extrapolation of NAb IC50 values to AAV2 BAb assays in Figures 1 and S3 was calculated with sigmoidal, 4PL, non-linear fitting (95% CI) in GraphPad Prism.

**ddPCR coating test**

To assess coating efficiency of different AAV capsids, standard Multi-Array MSD plates (L15XA-1) were coated with 5 × 10^8 AAV capsids/well in AAV-formulation buffer under shaking for 5 min at 750 RPM. After incubation at 4°C overnight, the plate was washed three times with 300 μL PBS-T (0.05% Tween-20), and capsid lysis was induced through incubation with 40 μL 1M NaOH and shaking for 30 min at 56°C and 750 RPM. Lysed particles were neutralized with 37 μL 1 M HCl, and 123 μL PCR-grade H2O was added to a final volume of 200 μL. The lysate was diluted 1:1,000- and 1:10,000-fold, and genome quantification was performed by ddPCR using the QX200 system (Bio-Rad, Hercules, CA, USA). Specifically, 9 μL of each sample was added to 10 μL of 2× ddPCR Supermix for Probes (Bio-Rad) and 1 μL of 20× primer-probe sets specific for the cytomegalovirus (CMV) promoter target sequence. The mix was then transferred to a DG8 cartridge, and droplets were generated using the Bio-Rad Droplet Generator and 70 μL of Droplet Generator Oil/well. After carefully transferring 44 μL of droplets to a 96-well plate, the plate was sealed using the Bio-Rad PX1 Plate Sealer and transferred to an Eppendorf ×50s PCR Mastercycler. The cycling conditions were as follows: an initial denaturation step for 10 min at 95°C followed by
40 cycles of 30 s at 95°C and 1 min annealing at 60°C (ramping rate: 2°C/s). Optimal annealing temperature had previously been identified by running a temperature gradient. Following a final heating step of 10 min at 98°C, the plate was cooled down to 10°C and placed into the Droplet Reader. The data were analyzed using the QuantaSoft software (Bio-Rad). Those sample dilutions that showed proper separation of positive and negative droplets were used for the calculation of AAV genomic titers.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2022.04.008.

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AUTHOR CONTRIBUTIONS
J.H., D.B., B.S., S.K., and S.M. contributed to assay development. J.H. and D.B. performed the experiments. J.H. and S.M. designed the experiments and wrote the paper. All authors have read and approved the final manuscript.

DECLARATION OF INTERESTS
All authors listed are employees of Boehringer Ingelheim Pharma GmbH & Co. KG or Boehringer Ingelheim Venture Fund GmbH.

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