A sensitive and reproducible cell-based assay via secNanoLuc to detect neutralizing antibody against adeno-associated virus vector capsid

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Most gene therapy clinical trials that systemically administered adeno-associated virus (AAV) vector enrolled only patients without anti-AAV-neutralizing antibodies. However, laboratory tests to measure neutralizing antibodies varied among clinical trials and have not been standardized. In this study, we attempted to improve the sensitivity and reproducibility of a cell-based assay to detect neutralizing antibodies and to determine the detection threshold to predict treatment efficacy. Application of the secreted type of NanoLuc and AAV receptor-expressing cells reduced the multiplicity of infection (MOI) for AAV transduction and improved the sensitivity to detect neutralizing antibodies with a low coefficient of variation, whereas the detection threshold could not be improved by the reduction of MOI to <100. After human immunoglobulin administration into mice at various doses, treatment with high-dose AAV8 vector enabled evasion of the inhibitory effect of neutralizing antibodies. Conversely, gene transduction was slightly influenced in the mice treated with low-dose AAV8 vector, even when neutralizing antibodies were determined to be negative in the assay. In conclusion, we developed a reliable and sensitive cell-based assay to measure neutralizing antibodies against AAV and found that the appropriate MOI to detect marginal neutralizing antibodies was 100. Other factors, including noninhibitory antibodies, marginally influence in vivo transduction at low vector doses.

INTRODUCTION

Adeno-associated virus (AAV) is a promising platform for delivering a transgene that has low pathogenicity and long-term transgenic expression. Clinical studies on gene therapy based on the AAV vector have been extensively developed in recent years and are being touted as the next-generation treatment to cure several inherited and degenerative diseases.1,2 Although AAVs do not cause any disease in humans, a major limitation of the AAV vector is the presence of pre-existing neutralizing antibodies (Nabs) against the AAV capsid secondary to latent infection. The presence of Nabs is well established to inhibit gene transfer mediated by systemic injection of the AAV vector. Indeed, the seropositivity of Nabs has been the main exclusion criterion for enrolling patients in several clinical trials.4 The detection of Nabs is important in the application of AAV-mediated gene therapy for the treatment of human diseases.

The methods for measuring Nabs were different among various clinical trials and are currently not standardized.1–7 Although the prevalence of Nabs relatively varied from 5% to 60% among the AAV serotypes, previous studies were incomparable because of their different assay sensitivities.8–12 There are two main assays to detect anti-AAV antibodies in vitro; these include direct detection of total antibody by ELISA-based capture assays and by a cell-based transduction assay. Total antibody detection is easy to set up; however, it represents all immunoglobulins that bind to the AAV capsid. Therefore, the assay does not necessarily characterize the neutralizing activity that can inhibit virus vector transduction by immunoglobulins.13 Cell-based assays have been widely employed to detect Nabs through assessment of the reduction of vector transduction to the cells after incubation of the AAV vectors with the patient’s serum. Compared with ELISA-based capture assays, cell-based assays can directly assess the inhibition of vector transduction but are less sensitive.5,16 There are several reasons for the limited sensitivity of the cell-based assay. First, poor transduction of the cell with several AAV serotypes in vitro requires a high vector genome for the assay, resulting in a relatively low detection sensitivity for Nabs.13 Furthermore, the selection of reporter transgene affects the sensitivity of the assay.17 For example, the assay based on green fluorescent protein has a limited sensitivity and can lead to false-negative results.18 Accordingly, highly sensitive and reproducible cell-based assays are warranted for standardization.19

In this study, we report an improved cell-based assay to detect Nabs against AAV. Furthermore, we determined the appropriate vector...
copy number to detect marginal Nabs in vitro and examined whether the results reflected in vivo transduction efficacy in mice.

RESULTS

Comparison of transduction efficiency among different AAV serotypes in several cell lines

We first attempted to select the cell lines that had high transduction efficiency for each AAV serotype. Because 10,000 relative light units (RLU) of luciferase has been reported to be important for reproducible Nab assays,15 the multiplicity of infection (MOI, vector genomes [vg]/cell) of the AAV vector genome that can obtain 10,000 RLU was considered the minimal threshold for transduction to detect Nabs. We generated several AAV vectors expressing luciferase, and the transduction efficiency of each serotype was examined in HEK293 cells. We found that luciferase expression was efficient by transduction with AAV1, AAV2, and AAV6 but was less efficient by transduction with AAV5 and AAV8 (Figure S1A). To find an efficient cell line for AAV5 and AAV8 transduction, we employed other cell lines, such as CHO-K1, HT1080, and Huh-7 cells. AAV5 and AAV8 showed better gene transfer efficiency in CHO-K1 cells and Huh-7 cells, respectively (Figures S1B and S1C). We used CHO-K1 and Huh-7 cells for further cell-based assays for Nabs against AAV5 and AAV8, respectively.

Comparison of the ability to detect protein expression among the reporter genes

Next, we attempted to further increase the transduction sensitivity by AAV5 and AAV8, whose transduction efficiencies were inefficient in vitro, to alter the reporter genes. We compared the threshold of the AAV vector genome to detect transduction among enhanced green fluorescent protein (EGFP), luciferase, and the secreting type of NanoLuc (secNanoLuc). NanoLuc has been demonstrated to offer improved performance for Nabs analysis16–18 because of its stability and sensitivity. SecNanoLuc, which is a NanoLuc variant with a secretory signal peptide, was shown to enable the detection of marginal Nabs19,20 due to its superior sensitivity to NanoLuc. In this study, NanoLuc conjugated with an interleukin (IL)-6 signal peptide (secNanoLuc) was shown to enable the detection of protein expression in the supernatant.22–24 We generated the AAV5 and AAV8 vectors harboring EGFP, luciferase, or secNanoLuc reporters under the control of the CAG promoter (Figure 1A) and transduced the CHO-K1 or Huh-7 cells at different MOI values (Figures 1B–1D). We detected only 5%–15% of EGFP-positive cells even in the high vector genome (MOI of 10,000) (Figure 1B). Efficient EGFP expression could not be obtained at an MOI of <1,000 (Figure 1B). The application of luciferase resulted in efficient reporter expression in the cytoplasm at an MOI of 1,000 (Figures 1C and 1D). No luciferase reporter was expressed in the supernatant; however, >10-fold higher secNanoLuc activity was noted in the supernatant (Figures 1C and 1D). The sensitivity of secNanoLuc in the supernatant was 10- to 100-fold higher than that of luciferase, and significant expression was detected even at an MOI of 10 (Figure 1D). The signal of secNanoLuc was stably detected within 30 min, whereas that for luciferase was more unstable (Figure S2).

Influence of the AAV vector genome number on the detection of Nabs

Next, we examined whether differences in MOI affected the detection of AAV Nabs. The AAV5 or AAV8 vector harboring luciferase or secNanoLuc was incubated with a serial dilution of human immunoglobulin or anti-AAV monoclonal antibody (mAb); this mixture was then added to the cell culture. The titer of Nabs was expressed as the dilution ratio that obtained 50% inhibition of transgene expression (ND50) (Figure 2). At the same dilution rate, the detection of Nabs was better with a lower MOI; ND50 increased more at an MOI of 100 than at an MOI of 1,000 (Figure 2). Both luciferase and secNanoLuc yielded similar results (ND50 is higher at an MOI of 100), but the
detection system using secNanoLuc had better interassay and intraassay % coefficient of variation (CV) (Tables 1 and 2). In addition, secNanoLuc had a consistently high RLU of >10,000 (Figure 1).

Application of the AAV receptor-expressed cell line
To obtain maximum transduction efficacy, we generated HEK293 cells expressing the AAV receptor (AAVR). HEK293 cells were transduced with the plasmid vector harboring the AAVR cDNA by controlling the EF1α promoter, followed by selection of the transduced cells by the addition of G418 (Figure 3A). We obtained HEK293 cells that expressed AAVR at a high level after limited dilution (Figure 3A).

Next, we examined whether the expression of AAVR could enhance AAV vector transduction. AAV1, AAV2, and AAV5 had enhanced transduction efficacy at higher vector doses but not at an MOI of 100 (Figure S3). Conversely, transduction with AAV8 was significantly enhanced by the expression of AAVR in all vector doses (Figure S3). To further enhance the transduction efficacy, we employed secNanoLuc instead of luciferase. When the HEK293 cells expressing AAVR were transduced with the AAV8 vector expressing secNanoLuc, significant expression of the transgene could be detected even at an MOI range of 0.1–1.0 (Figure 3B). Nevertheless, the ND50 values at MOIs of 1, 10, and 100 did not differ in the HEK293 cells expressing AAVR (Figures 3C and 3D). Because the assay CV tended to become worse as the amount of the vector genome decreased (data not shown), an MOI of 100 was the appropriate vector genome to detect the marginal Nab in vitro. Nab titers assessed by the empty capsid affected the detection of Nabs. We purified AAV8 vectors using two methods, i.e., the commercially available purification kit (AAVpro purification kit, Takara Bio, Shiga, Japan) and the cesium chloride-based centrifugation method. We examined the quality of each AAV8 vector by sedimentation velocity analytical ultracentrifugation. The additional peak for empty capsid was observed for AAV8 vectors purified using the purification kit (11.6% and 16.9% at optical density [OD]260, 20.3% and 25.6% at OD280 in two independent vectors), but not for those purified using the chloride-based centrifugation method (Figure 4A). We compared Nab titers assessed by both vectors and found that the purification method did not affect Nab detection at an MOI of 100 in Huh-7 and HEK293 cells expressing AAVR (Figure 4B).

Prediction of AAV vector transduction in vivo
We further investigated whether the detection of Nabs by the cell-based assay would predict in vivo AAV8 vector transduction. We employed AAV8 to assess the effect of Nabs on systemic administration of the vector in vivo because of high transduction efficiency in the mouse liver. The transduction efficiency of mouse liver with AAV5 is 100-fold lower than that with AAV8 (data not shown). We intravenously administered human immunoglobulin or anti-AAV8 mAbs into mice to obtain AAV8 Nabs in the blood (Figure S4A). We extracted blood at 1 h after the injection and measured Nabs using a cell-based assay (Figure S4A). As expected in previous experiments, the sensitivity to detect Nabs was poor at an MOI of 1,000 (Figure S4B). Nabs in mice could be detected after injection of >0.5 mg HEK293 cells expressing AAVR seemed similar to those assessed by Huh-7 cells (see Figure 6), but showed a better CV (Table S1).
of human immunoglobulin or 30 ng of mAbs but not after injection of 0.15 mg of human immunoglobulin or 10 ng of mAbs at an MOI of 100 (Figures S4C and S4D).

To examine whether the presence of Nabs in blood inhibited transduction after systemic injection of the AAV vector, we administered the AAV8 vector harboring secNanoLuc after the injection of human immunoglobulin or mAbs (Figure 5A). After administration of the AAV8 vector at a high dose (1 × 1013 vg/kg), AAV vector transduction was marginally inhibited in mice treated with 1.5 mg of human immunoglobulin (ND50 1.79 at an MOI of 100) (Figure S4C; Figure 5B). However, after administration of the AAV8 vector at a low dose (0.5 × 1012 vg/kg), transduction was completely abolished in mice treated with 0.5 mg of immunoglobulin (ND50 1.28 at an MOI of 100) or 100 ng of mAbs (ND50 1.274 at an MOI of 100) (Figures S4C and S4D; Figures 5C and 5D). The detection of Nabs at 1:1 serum dilution in mice treated with immunoglobulin (0.5 mg) or mAbs (30 ng) showed a significant decrease in vector transduction in vivo (Figures 5C and 5D). In mice treated with 0.15 mg of immunoglobulin, we observed a statistically significant reduction of vector transduction, but we could not detect Nabs in their blood (Figures 5C). We compared the results between the cell-based assay for Nabs and the ELISA-based capture assays for total antibody. The ELISA-based capture assays could detect immunoglobulin levels that could not be detected using the cell-based Nabs assay (Figure S5).

Detection of Nabs in human samples

Finally, we assessed whether our assay accurately selects eligible patients for a clinical trial. We used serum samples obtained from a cynomolgus monkey successfully treated with AAV8 vector expressing coagulation factor IX,25 as a control. We detected extremely high Nab titers in the serum after vector administration, but not before the administration (Figure S6). Next, we assessed Nabs against AAV5 and AAV8 in commercially available human samples (n = 10, 150) or 100 ng of mAbs (ND50 1:2.74 at an MOI of 100) (Figures S4C and S4D; Figures 5C and 5D). The detection of Nabs at 1:1 serum dilution in mice treated with immunoglobulin (0.5 mg) or mAbs (30 ng) showed a significant decrease in vector transduction in vivo (Figures 5C and 5D). In mice treated with 0.15 mg of immunoglobulin, we observed a statistically significant reduction of vector transduction, but we could not detect Nabs in their blood (Figures 5C). We compared the results between the cell-based assay for Nabs and the ELISA-based capture assays for total antibody. The ELISA-based capture assays could detect immunoglobulin levels that could not be detected using the cell-based Nabs assay (Figure S5).

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Discussion

The presence of Nabs is an important factor in determining the success of gene therapy with AAV vectors.26 Cell-based assays have been mainly performed to detect Nabs, but they are not standardized and are less sensitive, compared with the direct detection of total antibody using ELISA-based capture assays.5,6,13 In addition, the required assay sensitivity to predict the success of an AAV-mediated gene therapy remains to be elucidated. In this study, we maximized the sensitivity of cell-based Nab assays and found the appropriate vector genome for the detection of marginal Nabs. Furthermore, we clarified how antibody titers affected the actual transduction of AAV vectors in vivo.

We have developed a sensitive and reproducible cell-based Nab assay. Using the highly sensitive reporter secNanoLuc and cells that were easy to transduce, we could reduce the number of AAV vector genomes required for transduction. Although decreasing the vector dose increased the sensitivity of the assay, we identified an MOI of 100 as the appropriate vector copy number to detect Nabs with a high CV. The reason why the assay sensitivity does not improve with decreasing the MOI to <100 may be explained by the dissociation constant. If the dissociation constant between AAV and Nabs is kept consistent, the binding rate of Nabs to AAVs will deteriorate with the decrease in the concentration of AAV and Nabs in the solution. A lower MOI and Nab resulted in weaker Nab inhibition, and the Nab detection might reach the limitation. The secNanoLuc simplified the test and was expected to improve CVs by direct measurement of the cell supernatant. The employment of secNanoLuc increased the sensitivity of the Nab assays with AAV5 and AAV8, whose transduction could not be efficiently detected in vitro.27,28 The combination with AAVR-expressed cells further enhanced the transduction and allowed evaluation even at a very low concentration of AAV8 vectors. The assay for the detection of Nabs against AAV5 and AAV8 reportedly utilized a relatively high number of vector genomes to detect reporter expression in vitro. A previous report that employed an MOI of 30,000 for the Nab assay against AAV8 suggested the requirement for a more sensitive assay because the presence of a very low Nab below the detection limit impedes the transduction efficacy in vivo.14 Clinical trials on AAV5 employed a MOI of 25,000 for Nab assays of HEK293 cells.5 Another nonhuman primate study on AAV5 used an MOI of 347, but only 1,000 RLU was found to be important in this study. Compared with low vector doses, relatively high doses of the AAV vectors could evade the inhibitory effect of Nabs. This was consistent with the results of our previous experiments on pigs, in which high doses of the AAV8 vectors

Table 1. Interassay CV in ND50 at an MOI of 100

|          | Nab      | ND50 (Mean ± SD) | Interassay CV (%) |
|----------|----------|------------------|-------------------|
| AAV5     | Reporter | luciferase        | secNanoLuc        |
| IVIG     | 87.82 ± 15.62 | 120.23 ± 16.91 | 184.23 ± 64.23 | 17.79 | 14.07 | 34.86 |
| mAb      | 278.45 ± 170.98 | 379.87 ± 32.48 | 8,791.33 ± 1,763.29 | 55.07 | 8.55 | 18.94 |
| AAV8     | Reporter | ND50 (Mean ± SD) | Interassay CV (%) |
| IVIG     | 458.70 ± 252.61 | 379.87 ± 32.48 | 8,041.00 ± 1,523.33 | 15.62 | 17.79 |
| mAb      | 278.45 ± 170.98 | 379.87 ± 32.48 | 8,791.33 ± 1,763.29 | 55.07 | 8.55 | 18.94 |

IVIG, human immunoglobulin; mAb, monoclonal antibody; SD, standard deviation; CV, coefficient of variation.

The data were derived from a mean of three independent experiments.

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Cumulatively, the therapeutic effect of systemic gene therapy may not be necessarily diminished, even if the Nab is determined to be negative on cell-based assays. The therapeutic effects of AAV vectors are known to vary among individuals, and the presence of these factor(s) may be linked to individual differences after vector administration. Binding of antibodies that do not have neutralizing activity may promote antibody-dependent complement activation, which can affect viral clearance from the blood and result in deceased transduction. Meanwhile, serum nonantibody-based neutralizing factors that cannot be detected in a total antibody assay may influence transduction efficiency. From these perspectives, inhibition of transduction efficiency in vivo may vary. Some reports suggested that a Nab assay was more sensitive in vivo than in vitro. Indeed, we could identify the inhibitory effect of marginal antibodies even in the in vivo Nab assay in mice (Figure S7). In future clinical trials, we should carefully discuss the requirement for an in vivo assay from the point of view of animal welfare as well as the complexity of the procedure and standardization.

The detection threshold of our method met the criteria of the US Food and Drug Administration recommendation of at least 100 ng/mL assay sensitivity to detect antidrug antibodies in biological samples, albeit whether the guidance should be adapted for gene therapies remains to be decided. Approximately 100 ng/mL antibodies has been reported to completely prevent disease, and 10 ng/mL provided considerable protection after vaccination against tetanus and diphtheria. Although the clinical desired sensitivity to detect Nabs against AAV has not been revealed, the detection threshold in our present cell-based assay was 50 ng/mL mAbs against AAV. A total antibody assay was reported to detect <100 ng/mL mAbs against AAVs, whereas the previous cell-based Nab assay was less sensitive at 180 ng/mL (low quality control) and 3,000 ng/mL (high quality control). Our data suggested that an in vitro cell-based assay at a MOI of 100 detects the desired level of a mAb and mostly predicted the success of gene therapy.

This study had several limitations that should be addressed in future work. First, we did not assess the influence of clinical sample-related validation (e.g., hemolytic, lipemic, and jaundiced serum). The impact of these validations on the test value should be examined. Second, we could not evaluate the effect of inhibitory factors other than immunoglobulin because we only assessed the effect of purified human immunoglobulin and mAb. We should clarify and carefully assess the importance of serum substances that can inhibit AAV transduction. Furthermore, we assessed only 10 human serum samples in the cell-based assay and did not evaluate whether increasing the sensitivity altered the prevalence of Nabs in a specific population. We previously examined the prevalence of Nabs in Japanese patients and other serious adverse events. In addition, cases of death secondary to hepatotoxicity from high-dose AAV8 (3 × 10^{14}vg/kg) were reported. Considering the safety of AAV-mediated gene therapy, the amount of AAV vectors required to achieve a therapeutic effect should be minimal. Conversely, transduction with relatively low vector doses can be easily affected by the presence of Nabs. Moreover, the existence of marginal antibodies or other unknown factors may slightly inhibit vector transduction in vivo, even if the Nab is determined to be negative on cell-based assays. The therapeutic effects of AAV vectors are known to vary among individuals, and the presence of these factor(s) may be linked to individual differences after vector administration. Binding of antibodies that do not have neutralizing activity may promote antibody-dependent complement activation, which can affect viral clearance from the blood and result in deceased transduction. Meanwhile, serum nonantibody-based neutralizing factors that cannot be detected in a total antibody assay may influence transduction efficiency. From these perspectives, inhibition of transduction efficiency in vivo may vary. Some reports suggested that a Nab assay was more sensitive in vivo than in vitro. Indeed, we could identify the inhibitory effect of marginal antibodies even in the in vivo Nab assay in mice (Figure S7). In future clinical trials, we should carefully discuss the requirement for an in vivo assay from the point of view of animal welfare as well as the complexity of the procedure and standardization.

| Table 2. Intraassay CV in cell-based Nab assay at an MOI of 100 |
|-----------------|-----------------|-----------------|-----------------|
| Nab | Reporter | Dilution rate | % Transduction efficiency (mean ± SD) | Intraassay CV (%) |
|-----|----------|---------------|-------------------------------------|-----------------|
| AAV5 |          |               |                                     |                 |
| IVIG | luciferase | 1:300 | 100.8± 4.61 | 22.99 |
|      |          | 1:100 | 66.48± 21.80 | 35.6 |
|      |          | 1:30 | 3.63± 2.40 | 74.72 |
| secNanoLuc | 1:300 | 95.65± 15.64 | 11.81 |
|      |          | 1:100 | 38.57± 2.50 | 21.44 |
| mAb | luciferase | 1:1,000 | 89.84± 24.05 | 19.91 |
|      |          | 1:300 | 65.23± 25.99 | 19.64 |
| secNanoLuc | 1:1,000 | 97.06± 5.35 | 16.98 |
|      |          | 1:300 | 73.51± 13.56 | 9.80 |
|      |          | 1:100 | 24.18± 14.00 | 18.84 |
| AAV8 |          | 1:3,000 | 87.31± 10.24 | 20.56 |
| IVIG | luciferase | 1:1,000 | 79.28± 17.57 | 18.57 |
|      |          | 1:300 | 46.44± 18.42 | 22.53 |
| secNanoLuc | 1:3,000 | 99.02± 1.37 | 16.73 |
|      |          | 1:1,000 | 84.08± 3.04 | 14.12 |
| mAb | luciferase | 1:30,000 | 91.44± 6.87 | 25.12 |
|      |          | 1:10,000 | 62.08± 1.86 | 29.88 |
| secNanoLuc | 1:3,000 | 83.57± 5.76 | 18.19 |
|      |          | 1:10,000 | 57.89± 9.10 | 12.58 |
|      |          | 1:3,000 | 27.17± 2.55 | 18.81 |

*The data were derived from triplicate samples in one experiment. The data were derived from three independent experiments composed of triplicate samples.*

Notably, although a high-dose vector could evade the inhibitory effect of Nabs, it had a tendency to be associated with hepatotoxicity and prevented the inhibition by Nabs. Clinical trials and preclinical studies on monkeys using AAV5 vectors showed a therapeutic effect even in the presence of Nabs, suggesting that AAV5 is effective even in Nabs-positive patients. Although precise mechanisms including a serotype-specific property have not yet been fully understood, high vector doses (1 × 10^{14}vg/kg or higher) in an AAV5 clinical trial may evade Nabs. For clinical trials that required high vector administration, a sensitive assay to detect Nab may not be necessarily required. Cumulatively, the therapeutic effect of systemic gene therapy in clinical practice is determined according to the AAV vector dose, serotype’s propensity, and Nab titer in the serum. We should carefully determine the specific threshold value for the inclusion criteria of eligible patients based on the vector type and dosage in each clinical trial.
with hemophilia and found that 27.4%–39.7% were positive for Nabs against several AAV serotypes.\textsuperscript{10} Our previous examination employed an MOI of 1,000 for AAV5 and AAV8.\textsuperscript{25} We are now concentrating on examining the prevalence of Nabs in patients eligible for AAV-mediated gene therapy in Japan. Finally, we have shown data only for AAV8 in in vivo mouse experiments in this paper. It is necessary to verify whether the same phenomenon is observed in other serotypes.

In conclusion, we developed a reliable and sensitive cell-based assay for the determination of Nabs against AAV and found an MOI of 100 as the appropriate vector copy number to detect the existence of marginal Nabs. Accurate measurement of Nabs against AAVs can predict the success of systemic AAV vector-mediated gene therapy. Further validation of the assays is required for future clinical application as a laboratory test. In addition, factors other than Nabs may affect the individual differences in treatment response. These factors that influence the therapeutic efficacy of AAV vectors should be clarified to avoid the benefits of gene therapy to many patients with refractory diseases.

MATERIALS AND METHODS

Cell culture

HEK293 cells (JRCB Cell Bank, Osaka, Japan), AAVpro 293T cells (Takara Bio), Huh-7 cells (JRCB Cell Bank), and CHO-K1 cells (JRCB Cell Bank) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA) and 2 mM l-glutamine (Thermo Fisher Scientific). HT1080 cells (JRCB Cell Bank) were cultured in DMEM supplemented with 10% FBS, 2 mM l-glutamine, and 1% nonessential amino acids (Wako Pure Chemical, Osaka, Japan).

Generation of AAVR-expressing stable cell line

A codon-optimized human AAVR gene (KIAA0391L: NM_024874) was spliced with FLAG tag at the C terminus was synthesized at GenScript (Piscataway, NJ, USA). The cDNA sequence was cloned into the pBApo-EF1 plasmid (Takara Bio). The linearized pBApo-EF1 plasmid containing AAVR (pBApo-EF1-AAVR) was transfected into HEK293 cells using Lipofectamine 2000 (Thermo Fisher Scientific, CA, USA). To select the transfected cell clones, G418 (Nacalai Tesque, Kyoto, Japan) was added in the culture medium; culture was continued for selection with limited dilution in 96-well plates. We selected the clone that had a high expression of AAVR on flow cytometry.

Plasmid construct and AAV vector production

The cDNA of NanoLuc containing the IL-6 signal peptide (secNanoLuc) was synthesized at GenScript. Firefly luciferase cDNA was derived from pGL3 basic plasmid (Promega, Madison, WI, USA). A CD8 fragment that comprised a chimeric CAG promoter (CMV enhancer, chicken β-actin promoter and splice donor, and rabbit β-globin intron with splice acceptor), a transgene (EGFP, secNanoLuc, or luciferase), and the SV40 polyadenylation signal was introduced between the inverted terminal repeats in the pAAV2 plasmid.

The genes were packaged by triple plasmid transfection of AAVpro 293T cells (Takara Bio) to generate the AAV vector (helper-free system), as described previously.\textsuperscript{36} A plasmid-expressing Rep/Cap (serotypes 1, 2, 5, and 6) and pHelper were purchased from Takara Bio.
The capsid sequence of serotype 8 was synthesized at GenScript. AAV vectors were purified from the transfected cells after 72 h using the AAVpro purification kit (Takara Bio) or CsCl density gradient combined with ultracentrifugation. As previously described, the quality of each AAV8 vector was examined by sedimentation velocity analytical ultracentrifugation at the Gene Therapy Research Institution (Kanagawa, Japan).

AAV vector transduction in vitro

The cell lines were seeded at a density of 10⁴ cells in each 96-well dish coated with poly-L-lysine (Peptide, Osaka, Japan). A vial of vector stock was thawed and diluted in DMEM containing 5% FBS immediately before the transduction experiment and was directly added into each well. AAV vector transduction was measured in triplicate, and the mean value of three measurements was considered as one experiment.

Flow cytometry

To examine EGFP expression, the cells were directly resuspended in stain buffer containing 7-aminoactinomycin D (7-AAD). The expression of EGFP was analyzed on an LSRFortessa (BD Biosciences, Franklin Lakes, NJ, USA). The cells were gated by side scatter (SSC) and forward scatter (FSC), and dead cells were removed on the basis of 7-AAD staining. The mean value of three measurements was considered as one experiment.

Measurement of firefly luciferase and secNanoLuc expressions

Cells were lysed with 100 μL of lysis reagent (Promega) for luciferase assays and were then immediately stored in a deep freezer. To measure secNanoLuc activity, 50 μL of supernatant was directly collected and immediately stored in a deep freezer. After thawing frozen samples at room temperature, 10 μL of lysates or supernatants was added into a 96-well plate (Berthold Technologies, Germany). The 96-well plate was placed in a luminometer (Centro LB 960, Berthold Technologies), and 50 μL of luciferase assay reagent (Promega) or Nano-Glo luciferase assay reagent (Promega) was injected in each well using an automatic injector. The delay and measurement times were set for 2 and 10 s, respectively. All measurements were performed at room temperature and completed within 15 min. The luminescence of the negative sample (cell lysate or supernatant not transduced with AAVs) was approximately 50 RLU and saturated at 1 × 10⁸ RLU. To simplify the method, we did not usually measure protein concentrations in each well. We measured protein concentrations of cell lysate in 10 independent wells and found that the concentration of each well seemed to be identical in each well (Figure S8).

Nab assay

We used the following two types of positive control to inhibit AAV transduction: human immunoglobulin (Gammagard, Baxalta, CA, USA) and mAb against AAV (ADK8 against AAV8; ADK5a against AAV5) (Progen, Biotechnik, Heidelberg, Germany). Human immunoglobulin was diluted at a concentration of 50 mg/mL and stored.
at −80°C for future use. mAb was adjusted at a concentration of 50 μg/mL and stored at −80°C for future use.

A Nab assay was essentially performed, as described previously.14,15 Briefly, the cells were seeded at 1 × 10^4 into 96-well culture plates. On the day of transduction, 20 μL of human immunoglobulin or anti-AAV-mAb (ADK8 or ADK5a) was serially diluted with FBS and incubated with 20 μL of the AAV vector at 37°C for 1 h. A portion (7.5 μL) of the mixture was then added into three culture wells. After 48 h, vector transduction was examined based on the expression of luciferase or secNanoLuc. The mean value of the triplicate samples was considered as one experiment. Inhibition of vector transduction by Nabs was expressed as the percentage of transduction; 100% meant no inhibition, whereas 0% meant no transduction.

**AAV vector transduction in vivo**

All animal experiments were approved by the Institutional Animal Care and Concern Committee of Jichi Medical University and were conducted in accordance with the committee’s guidelines. For in vivo studies, 6- to 8-week-old C57BL6/J mice (SLC, Shizuoka, Japan) were used. Anesthetized mice were passively immunized by intravenous administration of 50 μL of human immunoglobulin or anti-AAV mAb (ADK8 or ADK5a) was serially diluted with PBS and incubated with 20 μL of the AAV vector at 37°C for 1 h. A portion (7.5 μL) of the mixture was then added into three culture wells. After 48 h, vector transduction was examined based on the expression of luciferase or secNanoLuc. The mean value of the triplicate samples was considered as one experiment. Inhibition of vector transduction by Nabs was expressed as the percentage of transduction; 100% meant no inhibition, whereas 0% meant no transduction.

**Detection of total antibody by ELISA-based capture assay**

The 96-well microplates were coated with 1 × 10^9 vg of AAV vector in phosphate buffer (pH 9.0) and incubated for 1 h. After washing with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBS-T), the wells were blocked with 5% casein in PBS-T. After three washes with PBS-T, 100 μL of the AAV vector expressing secNanoLuc (B, 1.0 × 10^12 vg/kg; C and D, 5.0 × 10^12 vg/kg) was injected into mice at 1 h after the IVIG or mAb injection. Blood was drawn at 14 days after the administration, and serum secNanoLuc expression was determined by luminescence and expressed as RLU. Values are presented as mean ± SD (n = 5 for B, n = 5–9 for C, n = 4 for D). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (one-way ANOVA, post hoc Turkey test).

**Statistical analysis**

All statistical analyses were performed using GraphPad Prism 8.4.3 (GraphPad, CA, USA). Antibody titers that reached ND50 by human immunoglobulin or anti-AAV mAb were estimated by nonlinear regression (inhibitor versus normalized response, variable slope). A Student’s t test was used to compare firefly luciferase and secNanoLuc expressions in the AAV serotypes on several cell lines. One-way analysis of variance (ANOVA) with a post hoc Turkey test was used for calculations that involved two-group comparisons in the in vivo experiments. CV was calculated as (standard deviation/ mean) × 100 (%). Intraassay CV and interassay CV indicated variations of simultaneous values within one assay and variation of values obtained in three independent experiments, respectively. A p value of <0.05 was considered statistically significant.
SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2021.06.004.

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AUTHOR CONTRIBUTIONS
N.B. designed the study, performed the experiments, analyzed the data, and wrote the manuscript. Y.K. designed the study, performed the experiments, and revised the manuscript. M.H., N.K., T.H., and H.M. designed the study, analyzed the data, and revised the manuscript. T.O. designed the study, analyzed the data, and wrote the manuscript. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS
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