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Successful AAV8 readministration: Suppression of capsid-specific neutralizing antibodies by a combination treatment of bortezomib and CD20 mAb in a mouse model of Pompe disease

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Abstract

Background: A major challenge to adeno-associated virus (AAV)-mediated gene therapy is the presence of anti-AAV capsid neutralizing antibodies (NAbs), which can block viral vector transduction even at very low titers. In the present study, we examined the ability of a combination immunosuppression (IS) treatment with bortezomib and a mouse-specific CD20 monoclonal antibody to suppress anti-AAV NAbs and enable readministration of AAV vectors of the same capsid in mice.

Methods: An AAV8 vector (AAV8-CB-hGAA) that ubiquitously expresses human α-glucosidase was used for initial gene therapy and a second AAV8 vector (AAV8-LSP-hSEAP) that contains a liver-specific promoter to express human secreted embryonic alkaline phosphatase (hSEAP) was used for AAV readministration. Plasma samples were used for determination of anti-AAV8 NAb titers. Cells isolated from whole blood, spleen, and bone marrow were analyzed for B-cell depletion by flow cytometry. The efficiency of AAV readministration was determined by the secretion of hSEAP in blood.

Results: In näive mice, an 8-week IS treatment along with AAV8-CB-hGAA injection effectively depleted $CD19^{+}B220^{+}$ B cells from blood, spleen, and bone marrow and prevented the formation of anti-AAV8 NAbs. Following administration of AAV8-LSP-hSEAP, increasing levels of hSEAP were detected in blood for up to 6 weeks, indicating successful AAV readministration. In mice pre-immunized with AAV8-CB-hGAA, comparison of IS treatment for 8, 12, 16, and 20 weeks revealed that the 16-week IS treatment demonstrated the highest plasma hSEAP level following AAV8-LSP-hSEAP readministration.

Conclusions: Our data suggest that this combination treatment is an effective IS approach that will allow retreatment of patients with AAV-mediated gene therapy. A combination IS treatment with bortezomib and a mouse-specific CD20 monoclonal antibody effectively suppressed anti-AAV NAbs in naïve mice and in mice with pre-existing antibodies, allowing successful readministration of the same AAV capsid vector.

Baodong Sun and and Priya S. Kishnani contributed equally to this work.

KEYWORDS
AAV readministration, anti-AAV neutralizing antibodies, B-cell depletion, bortezomib, CD20 mAb, immunosuppression

1 | INTRODUCTION

Adeno-associated virus (AAV)-mediated gene therapy is a promising approach to achieving long-term correction of human genetic diseases as a result of the ability of AAV vectors to transduce multiple tissues and enable sustained expression of the therapeutic genes.[1,2] Clinical trials have demonstrated the efficacy of AAV gene therapy; however, loss of AAV vectors often occurs in patients as a result of cytotoxic T lymphocyte (CTL) responses triggered by the AAV capsids and the therapeutic transgene products, or in pediatric patients, because of the rapid growth of organs. Therefore, readministration of AAV is desirable to achieve the durability of gene therapy. A major challenge for AAV readministration is the formation of anti-AAV capsid neutralizing antibodies (NAbs) following the initial AAV treatment, which blocks the transduction of the same AAV serotype even when NAbs are present at low titers.[3–5] In addition, pre-existing humoral immunity to AAV capsids, which develops early in life as a result of exposure to the wild-type virus, limits many individuals from receiving AAV gene therapy.[6–8] Currently, there is no effective approach in the clinical setting to deplete pre-existing AAV NAbs to allow AAV re-dosing.

It has been reported that frequent sessions of plasmapheresis reduced AAV NAbs to undetectable levels in human serum samples with low (1:20 or less) pretreatment titers, but only partially decreased AAV NAb titers in samples with high-level pretreatment antibodies.[9] In preclinical studies, various approaches have been attempted in mice or non-human primates (NHPs) for AAV readministration, such as alternating AAV serotypes,[10] capsid engineering (pseudotyped AAV serotypes),[11] capsid decoys,[12] capsid-specific plasmapheresis,[13,14] IgG-cleaving endopeptidases,[15,16] and tolerogenic nanoparticles encapsulating rapamycin.[17] However, these approaches are either ineffective, are in early stages of development and not clinically approved, or are likely to cause unwanted side effects. By contrast, immunosuppressive approaches such as that evaluated here have proven safe and effective in a highly vulnerable pediatric population.

Indeed, our team has shown remarkable success in the setting of enzyme replacement therapy for patients with Pompe disease using bortezomib, methotrexate, and rituximab to deplete high and sustained titers of antibodies to the therapeutic recombinant human acid α-glucosidase (hGAA). This immunosuppression (IS) regimen resulted in a reversal of high sustained antibody titers and in patients becoming immune tolerant to the therapeutic enzyme. More importantly, these agents have been very well tolerated in patients with Pompe disease with few safety concerns.[18–20] Bortezomib (Takeda Pharmaceutical Company, Tokyo, Japan) is a Food and Drug Administration-approved 26S proteasome inhibitor for the treatment of multiple myeloma and mantle cell lymphoma,[21,22] which has also been widely used as an immune suppressive agent for patients with other conditions.[23–29] Bortezomib has broad effects on the immune system including the suppression of IgG antibody production, inhibition of T-cell proliferation, induction of T-cell apoptosis, preservation of natural regulatory T cells, and prevention of antigen presentation on MHC class I molecules to $CD8^+$ T cells.[30] Bortezomib is particularly effective in targeting and depleting mature antibody-producing plasma cells with high rates of immunoglobulin synthesis through induction of apoptosis by the rapid accumulation of unfolded proteins.[31–33] A recent study demonstrated that a 20-week treatment with bortezomib alone at 1 mg/kg markedly reduced AAV-specific IgG titers in wild-type mice pre-immunized with AAV8, but this alone was insufficient to allow AAV re-dosing. This was likely caused by the failure of bortezomib to deplete memory B cells, which were activated upon AAV re-challenge.[34] This study suggests that bortezomib, in combination with a B-cell depleting agent, may be effective in suppressing anti-AAV antibodies to enable AAV re-dosing.

B-cell depletion therapies with CD20 monoclonal antibodies (mAbs) have been developed as effective treatments for multiple autoimmune diseases and B-cell malignancies.[35] CD20 mAbs deplete B cells through monocyte-mediated Fc-γ receptor-dependent cellular cytotoxicity and/or phagocytosis.[36] Rituximab, a chimeric CD20 mAb with established efficacy in depleting B cells was the first mAb drug approved for clinical use for cancer therapy.[37–40] Rituximab binds the CD20 cell surface molecule, which is expressed late during pre-B and immature B-cell development through memory B cells, but is lost from the cell surface as B cells differentiate into plasma cells.[41] Rituximab targets the CD20 molecule to alter B-cell progression and induce B-cell apoptosis.[42–44] Numerous clinical studies have demonstrated its beneficial effects on immune tolerance induction through B-cell depletion.[45–50]

In the present study, we tested the ability of a combination IS treatment with bortezomib and a mouse-specific CD20 mAb (MB20-11) to suppress anti-AAV capsid NAbs and to enable re-dosing of an AAV vector of the same serotype in naïve mice and in mice with pre-existing anti-AAV capsid NAbs using a mouse model of Pompe disease (GAA-KO mice). This strategy, if successful in mice, should be considered in the design of human AAV gene therapy clinical trials where it is well known that readministration will be required.

2 | MATERIALS AND METHODS

2.1 | AAV vectors

The AAV-CB-hGAA vector was designed to express hGAA under the transcriptional control of a CMV enhancer/chicken β-actin promoter as previously described.[51] The AAV-LSP-hSEAP was designed to express the human secreted embryonic alkaline phosphatase (hSEAP) under the control of the liver-specific promoter (LSP) that was

previously shown to induce immunotolerance to hGAA in GAA-KO mice.[51,52] To construct AAV-CB-hSEAP, a DNA fragment containing the hSEAP cDNA was PCR-amplified from the original CMV-SEAP plasmid (Plasmid #24595; Addgene, Cambridge, MA) and subcloned into the AAV-CB-hGAA vector at the *Kpn*I and *Eco*RI sites to replace the hGAA cDNA. To construct AAV-LSP-hSEAP, the *Sca*I-*Kpn*I fragment containing the LSP from the AAV-LSP-hGAA vector[51] was cloned into the AAV-CB-hSEAP to replace the CB promoter. The primers used for hSEAP amplification were forward: 5′-CTGAGGTACCGCCACCATGCTGGGGCCCTGCATG-3′ and reverse: 5′-GGCGAATTCCCACGGGTTAACCGGGGTG-3′. Both the AAV-CB-hGAA and AAV-LSP-hSEAP were packaged as AAV8 at the Vector Core of the University of North Carolina.

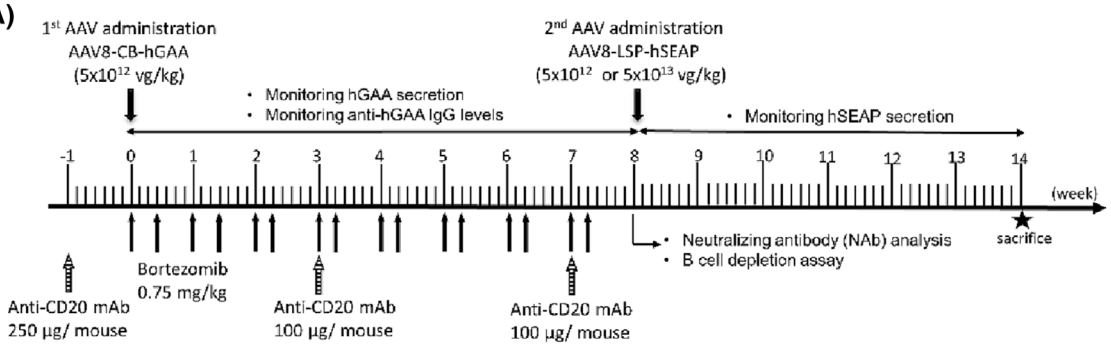
2.2 | Combination IS treatment in näive GAA-KO mice

Animal care and experiments were conducted in accordance with Duke University Institutional Animal Care and Use Committee-approved guidelines. A mouse-specific CD20 mAb (MB20-11) was generated in Dr Tedder's laboratory at Duke University.[53] Bortezomib was purchased from EMD Millipore Corp. (Burlington, MA, USA). To determine the ability of the combination IS treatment to prevent the formation of anti-AAV8 NAbs in a naïve setting, 3-month-old male GAA KO mice[54] were divided into three groups: Group 1 received intravenous (i.v.) injection of the AAV8-CB-hGAA at a dose of 5×10^{12} vg/kg at week 0 along with the combination IS treatment consisting of intraperitoneal injection of anti-CD20 mAb monthly and i.v. injection of bortezomib twice a week for 8 weeks (Figure 1A); Group 2 received the AAV8-CB-hGAA injection at week 0 only with no IS treatment; Group 3 was mock-treated with PBS at week 0 with no IS treatment (Figure 1B). At week 8, one-third of the mice in each group were sacrificed to collect plasma samples for determining anti-AAV8 NAb titers and to isolate cells from whole blood, spleen, and bone marrow for analyzing B-cell depletion by flow cytometry. The remaining mice in each group were re-dosed with AAV8-LSP-hSEAP at a dose of 5×10^{12} or 5×10^{13} vg/kg (i.v.) to determine the transduction efficiency of the second AAV8 vector by monitoring the level of secreted hSEAP in the blood and by determining the AAV genome numbers in the liver.

2.3 | Combination IS treatment in GAA-KO mice with pre-existing anti-AAV8 NAbs

To determine the ability of the IS treatment to deplete pre-existing NAbs, 3-month-old male GAA-KO mice were pre-immunized with

(A)



(B)

Group	AAV-CB-hGAA ($5x10^{12}$ vg/kg)	IS for 8 weeks	NAb and B cell depletion monitoring at week 8	AAV-LSP-hSEAP ($5x10^{12}$ vg/kg)	AAV-LSP-hSEAP ($5x10^{13}$ vg/kg)
1	+ (n=14)	+ (n=14)	+ (n=5)	+ (n=4)	+ (n=5)
2	+ (n=15)	- (n=15)	+ (n=6)	+ (n=4)	+ (n=5)
3	- (n=16)	- (n=16)	+ (n=7)	+ (n=4)	+ (n=5)

FIGURE 1 Experimental design for the naïve setting study in GAA-KO mice. (A) Outline of AAV injections and immunosuppression (IS) treatment regimens for Group 1. Three-month-old GAA-KO mice received i.v. administration of AAV8-CB-hGAA at a dose of 5×10^{12} vg/kg at week 0 and were re-challenged with AAV8-LSP-hSEAP at week 8. For IS treatment, mice were intraperitoneally injected with a mouse-specific anti-CD20 mAb (MB20-11) at 250 µg/mouse at week 1, then at 100 µg/mouse at week 3 and week 7. Bortezomib (0.75 mg/kg) was i.v. injected twice a week (0 h, 48 h) every week for 8 weeks starting from week 0. (B) Summary of the treatment groups. Group 1 mice were injected with AAV8-CB-hGAA vector at week 0 along with combination IS treatment for 8 weeks. Group 2 were injected with AAV8-CB-hGAA at week 0 without IS treatment. Group 3 mice were mock-treated with PBS at week 0. At week 8, one-third of mice in each group were killed to analyze anti-AAV8 neutralizing antibody and B-cell depletion. The remaining mice in each group were i.v. injected with low-dose (5×10^{12} vg/kg) or high-dose (5×10^{13} vg/kg) AAV8-LSP-hSEAP to evaluate the efficacy of AAV re-dosing by monitoring hSEAP secretion from liver. CB, CMV enhancer/chicken β-actin promoter; hGAA, human acid α-glucosidase; hSEAP, human secreted embryonic alkaline phosphatase; LSP, liver-specific promoter.

AAV8-CB-hGAA (i.v., 5×10^{12} vg/kg). After 24 weeks, the mice were divided into two big groups: Group 1 mice received IS treatment (CD20 mAb monthly + bortezomib twice a week) for 8 (Group 1A), 12 (Group 1B), 16 (Group 1C) or 20 weeks (Group 1D), to define an optimal IS treatment duration and evaluate the risk–benefit profile; Group 2 mice did not receive the IS treatment. An additional group of treatment-naïve mice was used as positive controls for AAV re-dosing (Group 3). After the IS treatment period, mice in each group were injected with a second AAV8-LSP-hSEAP vector (i.v., 5×10^{13} vg/kg) at the indicated time points (for details, see Figure 4A).

2.4 | Measurement of AAV8 capsid-specific NAbs in plasma

Anti-AAV8 NAb titers were measured using an in vitro cell-based assay at the Immunology Core of the University of Pennsylvania.[55] The NAb titer (IC_{50}) was determined as the highest sample dilution at which 50% inhibition of transduction is observed compared to a non-inhibition control.

2.5 | Cell preparations

Blood was collected from inferior vena cava with 25-G syringe (BD Biosciences, San Jose, CA, USA) when mice were sacrificed at Week 8. Peripheral blood mononuclear cells were collected by centrifugation over a layer of Ficoll-Paque Premium (MilliporeSigma, St Louis, MO, USA). For isolation of splenocytes, spleens were disrupted into single-cell suspensions and erythrocytes removed by lysis using a commercially available ammonium chloride-based lysing buffer (BD Biosciences). Bone marrow cells were collected by flushing femur with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. After washing, cell preparations from the blood, spleen, and bone marrow were resuspended in a 90% FBS (Gemini, Sacramento, CA, USA) and 10% dimetylsulfoxide (MilliporeSigma) freezing solution and viably cryopreserved and stored in vapor phase liquid nitrogen.

2.6 | Flow cytometry

Surface staining was performed by first incubating cells with a viability dye, Zombie Aqua (Biolegend, San Diego, CA, USA), for 15 min at room temperature. After a wash with FACS Wash containing PBS with 2% FBS, cells were stained 25 min at 4°C with a staining cocktail that included the antibodies: anti-CD20, anti-CD3, anti-BP-1, anti-CD43, anti-B220, anti-IgD, anti-IgM, anti-CD19, and anti-CD24. Cells were washed two times with FACS Wash and fixed with 1% paraformaldehyde and then analyzed with a BD LSRII flow cytometer (BD Biosciences) in the Duke Immune Profiling Core. Data were analyzed using FlowJo software (BD Biosciences). Antibodies against CD20, CD3, IgD, and CD24 antibodies were purchased from BioLegend (San Diego, CA, USA), antibody against BP-1 was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany), antibodies against CD43 and B220 were purchased from BD Biosciences, and antibodies against IgM and CD19 were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.7 | Measurement of plasma hSEAP level

Blood was collected from maxillary of mice, and plasma samples were obtained by centrifugation at 2000 *g* at 4°C for 10 min and maintained at −80°C. Plasma samples were diluted with PBS (1:5) and SEAP levels were measured using the Tropix® Phospha-Light™ System (#T1017; Applied Biosystems, Bedford, MA, USA) in accordance with the manufacturer's instructions.

2.8 | AAV bio-distributions by real-time PCR

Genomic DNA was extracted from frozen tissues using Wizard Genomic DNA Purification Kit (Promega, Medison, WI, USA). A real-time PCR was performed using SYBR Green (#1725124; Bio-Rad, Hercules, CA, USA) and primer pair 5′-ACATGTGCCAGACAGTGGAG-3′ and 5′-GTTGCACTGGTTAAAGCGGG-3′ for hSEAP and 5′-AGAGGGAAATCGTGCGTGAC-3′ and 5′-CAATAGTGATGACCTGGCCGT-3′ for mouse beta-actin. The AAV-CB-hGAA or AAV-LSP-hSEAP plasmids were used to generate standard curves for viral vector genome number calculation.

2.9 | Statistical analysis

Statistical significance was determined by a one-way non-parametric analysis of variance (Kruskal–Wallis) with a post-hoc test (Dunn) using Prism (GraphPad Software Inc., San Diego, CA, USA). Data are presented as the mean ± SD. $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | The bortezomib–CD20 mAb combination IS effectively suppressed the formation of anti-AAV8 NAbs to enable AAV re-dosing in naïve GAA-KO mice

We first evaluated the ability of the combination IS to inhibit the formation of anti-AAV NAbs and allow AAV readministration in naïve GAA-KO mice. Previously, we reported that i.v. administration of an AAV8 vector expressing hGAA driven by the ubiquitous CMV enhancer-chicken β-actin (CB) promoter (AAV8-CB-hGAA) in GAA-KO mice provoked both humoral and

cellular immune responses toward hGAA.[51] The use of AAV8-CB-hGAA as the first AAV vector for initial AAV administration would allow us to evaluate the effect of the combination IS treatment on both anti-AAV capsid and anti-hGAA antibodies in GAA-KO mice. There were three groups of mice in this experiment as shown in Figure 1A,B.

3.1.1 | Suppression of anti-AAV8 NAbs and anti-hGAA IgG antibodies

Eight weeks after the injection of AAV8-CB-hGAA, appreciable titers of anti-AAV8 NAbs were observed in Group 2 mice that did not receive IS treatment (titers 1:187 ± 147); in contrast, the Group 1 IS-

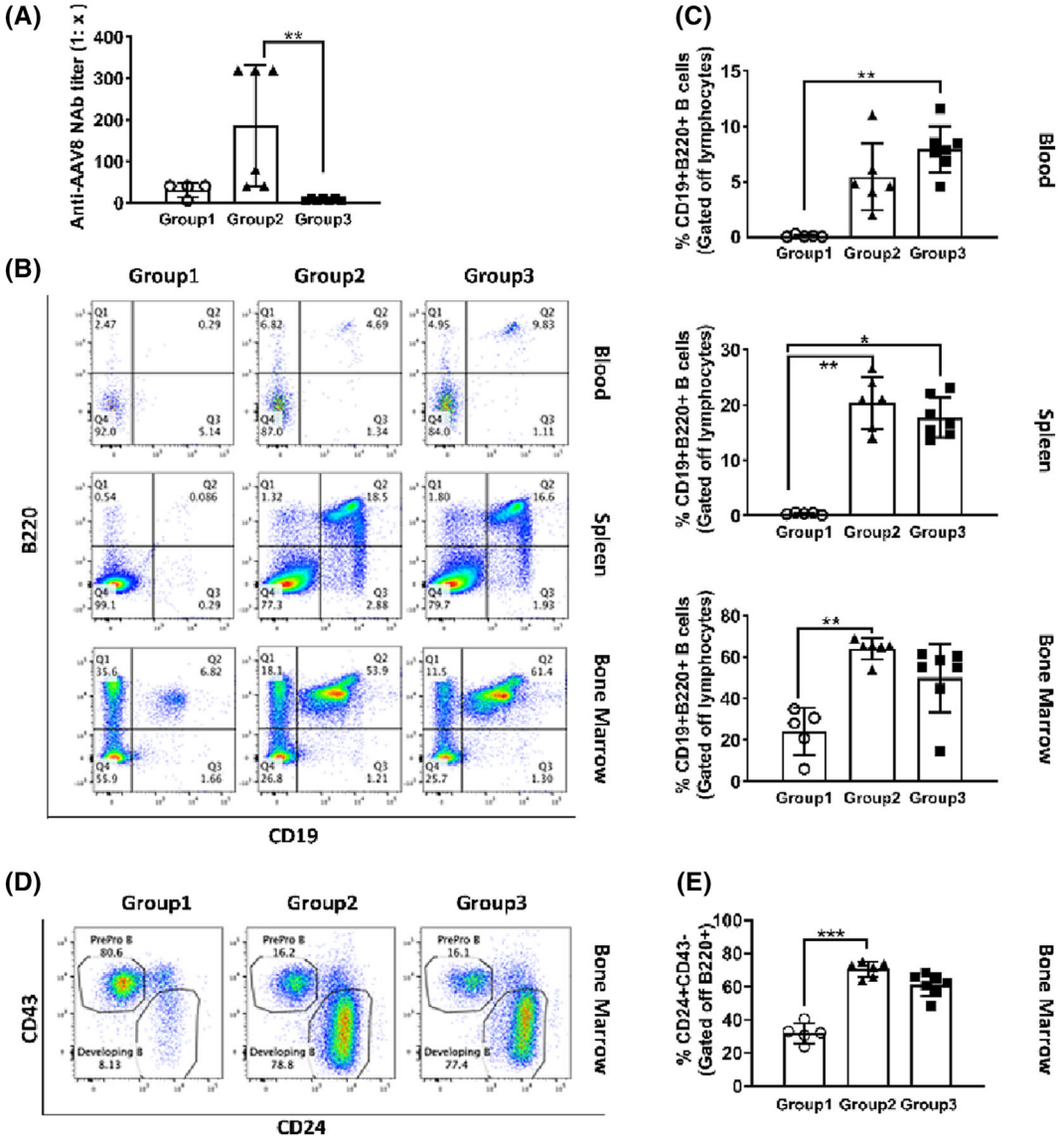


FIGURE 2 Prevention of anti-AAV8 neutralizing antibody formation and depletion of B cells in naïve GAA-KO mice by the 8-week combination IS treatment. (A) Analysis of anti-AAV8 neutralizing antibody titers. Plasma samples were collected from mice in Group 1 (AAV8-CB-hGAA + IS, $n = 4$), Group 2 (AAV8-CB-hGAA, no IS, $n = 6$), and Group 3 (mock-treated with PBS, no IS, $n = 7$) at week 8 following the IS treatment. x, fold dilution of plasma samples. Data shown are the mean ± SD. $^{**}p < 0.01$. (B) Representative flow cytometry plots illustrating the depletion of B cells ($CD19^{+}B220^{+}$) by the IS treatment from the blood, spleen, and bone marrow from mice in Group 1 compared to mice in Groups 2 and 3. Peripheral blood mononuclear cells, splenocytes, and bone marrow cells were collected at week 8 following the IS treatment, and the B cells were quantitated by flow cytometry analysis. B-cell depletion was evaluated by the frequency of B cells as a fraction of viable lymphocytes. (C) Composite data from individual mice in B showing the frequency of B cells ($CD19^{+}B220^{+}$) in the three treatment groups ($n = 5$ for Group 1, $n = 6$ for Group 2, and $n = 7$ for Group 3). Data shown are the mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$. (D) Representative flow cytometry plots of $B220^{+}$ B cells in the bone marrow cells in (B) and (C) showing a loss of B developing B cells ($CD24^{+}CD43^{-}$) in Group 1. (E) Composite data from individual mice in (D) showing diminished frequency of $CD24^{+}CD43^{-}$ developing B cells in the bone marrow ($n = 5$ for Group 1, $n = 6$ for Group 2, and $n = 7$ for Group 3). Data shown are the mean ± SD. $^{***}p < 0.001$.

treated mice showed very low levels of anti-AAV NAbs (titers 1:31 ± 18), and Group 3 mice (naïve) had titers at or below the limit of detection (1:5) (Figure 2A). We also evaluated the effect of the IS treatment on anti-hGAA IgG antibody formation and hGAA secretion. Anti-hGAA IgG antibody levels were increased in Group 2 (AAV8-CB-hGAA without IS) mice, but not in Group 1 (AAV8-CB-hGAA + IS) and Group 3 mice (the mock-treated group) (see Supporting information, Figure S1A). Plasma GAA activities (hGAA secreted from liver) were significantly higher in Group 1 mice than those in Groups 2 and 3 (see Supporting information, Figure S1B). These results suggest that the combination IS treatment was effective in suppressing the formation of both anti-AAV8 and anti-hGAA antibodies.

3.1.2 | Depletion of B cells in blood, spleen, and bone marrow

For a cellular assessment of the immunological changes elicited by the combination IS treatment, we performed flow cytometry analysis of B cells from blood, spleen, and bone marrow at 8 weeks following the first AAV vector injection. A representative upstream gating hierarch for analysis of B cells is shown in the Supporting information (Figure S2). Group 1 mice (AAV8-CB-hGAA + IS treatment) showed almost no $CD19^{+}B220^{+}$ B cells in blood and spleen, in marked contrast to Group 2 (AAV8-CB-hGAA without IS) and Group 3 (the mock-treated group) (Figure 2B,C). Interestingly, a small population of $CD19^{+}B220^{+}$ B cells persisted in bone marrow of Group 1 mice, which, on further examination, demonstrated a loss of mature B cells, with a resulting higher frequency of pro-B and pre-B cells as defined by CD24 and CD43 cell surface markers (Figure 2D,E). Thus, the 8-week IS treatment effectively depleted B cells from circulation and spleen and inhibited B-cell maturation in the bone marrow.

3.1.3 | The combination IS treatment enabled successful AAV8 re-dosing, but required a high dose of vector administration

To exclude the effect of transgene-related cellular immune response on AAV8 re-dosing, we chose to use a second AAV8 vector (AAV8-LSP-hSEAP) for AAV readministration that expresses hSEAP, a commonly used reporter for in vivo gene expression under the control of an LSP that was previously shown to induce immunotolerance to hGAA in GAA-KO mice.[51] The efficacy of AAV readministration in

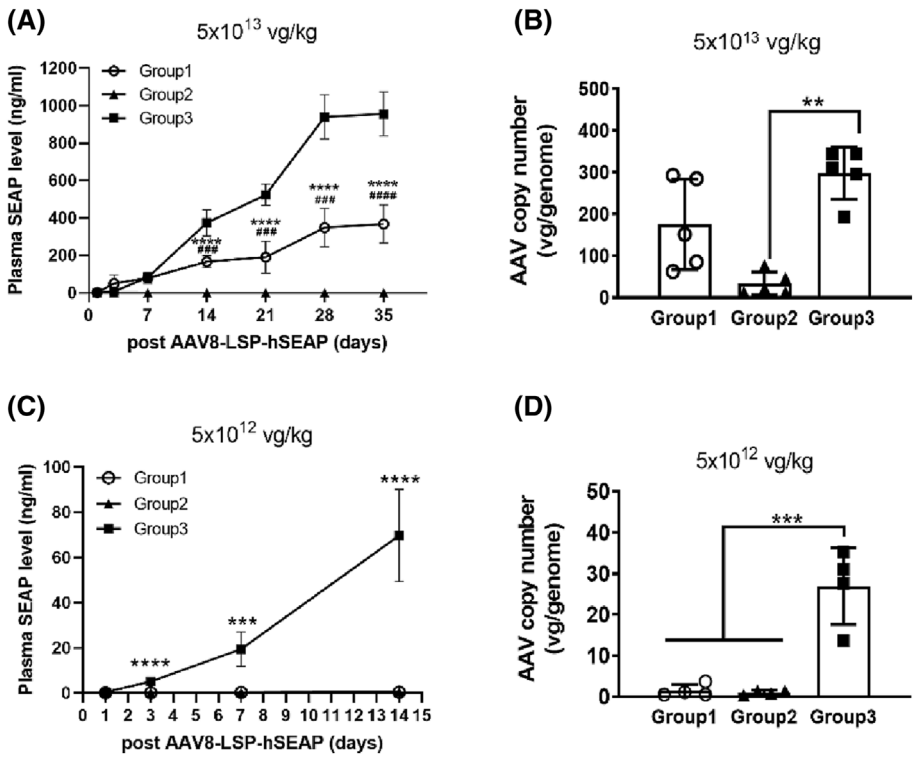


FIGURE 3 Determination of the transduction efficacy of AAV8-LSP-hSEAP after AAV re-dosing in the naïve setting experiment. Eight weeks after the first AAV injection, mice from Group 1 (AAV8-CB-hGAA + IS), Group 2 (AAV8-CB-hGAA, no IS), and Group 3 (PBS, no IS) were re-challenged with the second AAV (AAV8-LSP-hSEAP). Plasma hSEAP levels were monitored at the indicated time points, and AAV-LSP-hSEAP genome numbers in liver were analyzed after 6 weeks. Plasma hSEAP levels (A) and AAV copy numbers in liver (B) following the high-dose (5×10^{13} vg/kg, $n = 5$ each group) AAV-LSP-hSEAP administration demonstrated successful AAV re-dosing in Group 1 mice. By contrast, plasma hSEAP levels (C) and AAV copy numbers in liver (D) following the low-dose (5×10^{12} vg/kg, $n = 4$ each group) AAV-LSP-hSEAP administration showed no AAV transduction in Group 1 mice. Data shown are the mean ± SD. $^{****}p < 0.0001$ versus Group 3; $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ versus Group 2 in (A). $^{***}p < 0.001$, $^{****}p < 0.0001$ versus Group 1 in (C).

mice was determined by monitoring the levels of secreted hSEAP in the blood.

Following the injection of the AAV8-LSP-hSEAP vector, plasma hSEAP levels were monitored for reporter transgene expression in all mice. Following the high-dose (5×10^{13} vg/kg) injection, increasing plasma hSEAP levels were observed in both Group 1 (AAV8-CB-hGAA + IS treatment) and Group 3 (the mock-treated group) mice, with the hSEAP levels in Group 3 being significantly higher than those of Group 1 (Figure 3A); no hSEAP expression was detected in any of the mice in Group 2 (AAV8-CB-hGAA without IS) throughout the duration of the experiment (Figure 3A), indicating that the 8-week IS treatment effectively, but not completely prevented the formation of anti-AAV8 NAbs in Group1 mice. Correlating with the plasma hSEAP level data, copy numbers of AAV8-LSP-hSEAP vector genome were high in the livers of Group 1 (175 ± 108 vg/genome) and Group 3 (298 ± 62 vg/genome) mice, but were extremely low in the livers of Group 2 mice (34 ± 27 vg/genome) 6 weeks after AAV8-LSP-hSEAP injection (Figure 3B). However, after injection of the low-dose (5×10^{12} vg/kg) AAV8-LSP-hSEAP vector, increasing plasma hSEAP levels were observed in only Group 3 mice; no secreted hSEAP was detected in any of the mice in Groups 1 and 2 at the indicated time points (Figure 3C), suggesting the presence of low levels of anti-AAV8 NAbs in Group 2 mice after the 8-week IS treatment, which required a higher dose of AAV8-LSP-hSEAP to overcome. As expected, for the low-dose treatment, high AAV8-LSP-hSEAP genome numbers were detected in the livers of Group 3 mice (27 ± 9), but almost undetectable in the livers of Groups 1 and 2 mice (Figure 3D).

3.2 | The bortezomib–CD20 mAb combination IS effectively depleted anti-AAV NAbs to enable AAV re-dosing in GAA-KO mice with pre-existing NAbs

3.2.1 | Sixteen-week IS treatment is optimal for AAV re-dosing

We next evaluated the ability of the combination IS treatment to enable AAV readministration in GAA-KO mice with pre-existing NAbs. To determine the optimal IS duration, 3-month-old mice were pre-immunized with the AAV8-CB-hGAA at a dose of 5×10^{12} vg/kg. After 24 weeks, Group 1 mice were divided into four subgroups to receive the combination IS treatment for 8, 12, 16, and 20 weeks, respectively (Figure 4A, Groups 1A–D). Immediately following the completion of IS treatment, mice in each subgroup of Group 1 were injected with a second AAV8-LSP-hSEAP vector at a dose of 5×10^{13} vg/kg and monitored for 6 weeks for hSEAP expression. Mice in Group 2 (pre-immunized mice without IS treatment) and Group 3 (mock-treated with PBS) received the same dose of AAV8-LSP-hSEAP at the indicated time point (Figure 4A). As shown in Figure 4B, plasma hSEAP level was undetectable in Group 1A mice receiving IS for 8 weeks, but increased moderately in Group 1B mice receiving IS for 12 weeks. Mice in Group 1D, treated with IS for 20 weeks, showed very low hSEAP level. Mice in Group 1C that received the 16-week IS treatment demonstrated the highest hSEAP level. Correlating with the plasma hSEAP level data, AAV8-LSP-hSEAP copy number (vg/genome) was high in Group 1C (IS for 16 weeks) livers (74 ± 44), low in Group 1B (IS for 12 weeks) livers (17 ± 26) and Group 1D (IS for 20 weeks) livers (17 ± 39), and extremely low in Group 1A (IS for 8 weeks) livers (0.83 ± 1.68). These data suggest that IS for 16 weeks is an optimal treatment duration to enable successful readministration of AAV8 vector.

We next compared the transduction efficiency of AAV8-LSP-hSEAP between Group 1C (pre-immunized with AAV-CB-hGAA + IS treatment for 16 weeks), Group 2 (pre-immunized with AAV-CB-hGAA, no IS), and Group 3 mice (mock-treated with PBS, no IS). Increasing plasma hSEAP levels were observed in both Group 1C and Group 3 mice, but hSEAP level was undetectable in Group 2 mice (Figure 5A). AAV8-LSP-hSEAP copy number (vg/genome) was very high in Group 3 livers (162 ± 35) and low in Group 2 livers (16 ± 30) (Figure 5B). Although the copy number in Group 1C livers was lower than that in Group 3 livers, similar plasma hSEAP level was observed between the two groups at 6 weeks following the AAV8-LSP-hSEAP vector injection (Figure 5).

In an attempt to understand why the 20-week IS treatment was less effective than the 16-week IS treatment, we performed hematoxylin and eosin (H&E) staining of livers from each group. As shown in the Supporting information (Figure S3), livers from groups 1A (IS for 8 weeks) and 1B (IS for 12 weeks) showed variably increased steatosis with minimal inflammation. Group 1C (IS for 16 weeks) livers had less steatosis, with some cases showing more prominent lobular inflammation. Group 1D (IS for 20 weeks) livers had no notable steatosis and showed similar inflammation as seen in Group 1C, with focal necrosis (acidophil bodies and hepatocyte dropout) occasionally seen in some of the mice. Group 2 (AAV without IS treatment) livers showed variably increased inflammation in some cases. Group 3 (the mock-treated group) showed normal murine liver histopathology.

We next compared the transduction efficiency of the AAV8-LSP-hSEAP vector in different tissues between Group 1C and Group 1D mice following AAV re-dosing. Although AAV genome copy number (vg/genome) in Group 1C livers was significantly higher than that in Group 1D livers, there was no significant difference in AAV genome copy number in the skeletal muscle (0.15 ± 0.05 vs. 0.27 ± 0.16) and heart (0.24 ± 0.16 vs. 0.16 ± 0.14) between the two groups (see Supporting information, Figure S4A). We further compared the levels of AAV receptor (AAVR or KIAA0319L), a type I transmembrane protein that has been shown to be the key receptor mediating the entrance of AAV8 into cells,[56] in the liver, quadriceps, and heart between Group 1C and 1D mice. As shown in the Supporting information (Figure S4B), there was no decrease in AAVR levels in any of the tissues of Group 1D mice compared to Group 1C mice.

3.2.2 | Sixteen-week IS treatment effectively reduced pre-existing NAb titers and depleted B cells in AAV pre-immunized mice

Twenty-four weeks after AAV8-CB-hGAA administration, anti-AAV8 NAb titers were extremely low in Group 1C mice (16 weeks IS; titers

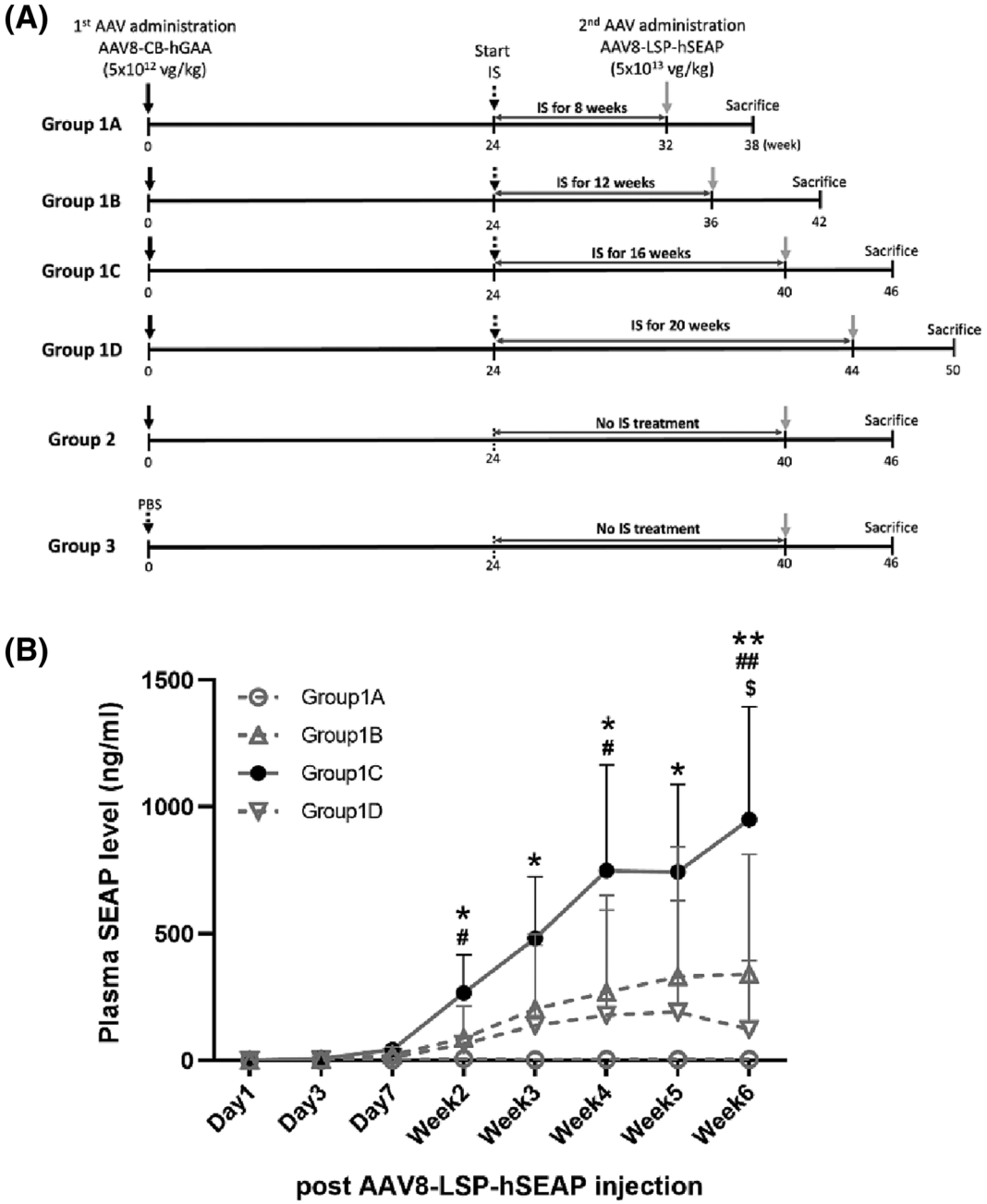


FIGURE 4 Determination of the optimal IS treatment duration in AAV pre-immunized GAA-KO mice for AAV re-dosing. (A) Experimental timeline of AAV injection and combination IS treatment. Three-month-old GAA-KO mice were pre-immunized by i.v. injection of AAV8-CB-hGAA at 5×10^{12} vg/kg at week 0. After 24 weeks, the AAV-treated mice were divided into two groups: Group 1 received IS treatment (intraperitoneal administration of MB20-11 monthly and intravenous injection of bortezomib twice a week) for 8 (Group 1A), 12 (Group 1B), 16 (Group 1C), or 20 weeks (Group 1D) to define an optimal IS treatment duration; Group 2 did not receive IS treatment. An additional group of treatment-naïve mice (PBS, no IS treatment) was used as positive controls for AAV re-dosing (Group 3). After the indicated IS treatment period, mice in each group were i.v. injected with AAV8-LSP-hSEAP at 5×10^{13} vg/kg (gray arrow). (B) Plasma hSEAP level was monitored following AAV8-LSP-hSEAP administration in mice from Groups 1A to 1D to determine the optimal IS treatment duration. Plasma samples were collected at the indicated times ($n = 5$ for Groups 1A–1C, $n = 6$ for Group 1D). Data shown are the mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ versus Group 1A; $^{\#}p < 0.05$. $^{\#\#}p < 0.01$ versus versus Group 1D; $^{\$}p < 0.5$ versus Group 1B.

1:14 ± 13), high in Group 2 mice (no IS; titers 1:248 ± 244), and below the limit of detection in Group 3 mice (naïve; titers 1:5) (Figure 6A). To determine the efficacy of 16-week IS treatment in depleting B cells, we immune profiled B-cell subsets in the blood, spleen, and bone marrow. In Group 1C mice, $CD19^{+}B220^{+}$ B cells were almost completely depleted in the blood and spleen and partially, but significantly decreased in the bone marrow by the 16-week IS treatment, compared to Group 2 and Group 3 mice (Figure 6B,C). To further

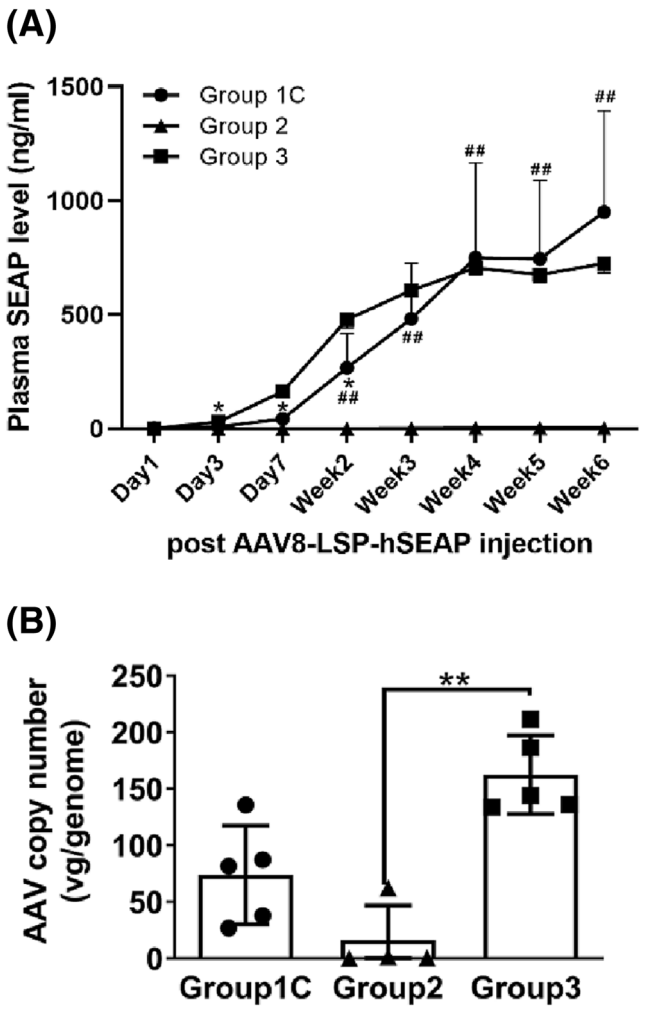


FIGURE 5 Effect of the 16-week IS on the transduction efficiency of the second AAV8 vector. (A) Plasma samples collected at the indicated times following AAV re-dosing with AAV8-LSP-hSEAP were assayed for hSEAP level; $n = 5$ for Group 1C (AAV8-CB-hGAA + IS for 16 weeks), $n = 4$ for Group 2 (AAV8-CB-hGAA, no IS), and $n = 5$ for Group 3 (PBS, no IS). Data shown are the mean ± SD. $^{*}p < 0.05$ versus Group 3; $^{\#\#}p < 0.01$ versus Group 2. (B) Quantitation of AAV genome copy numbers in livers from mice in 6 weeks after the AAV8-LSP-hSEAP administration. Data shown are the mean ± SD. $^{**}p < 0.01$.

understand the effect of the combination IS, we next assessed $CD80^+$ memory B cells and IgD^+ IgM^+ naïve B cells. The numbers of the $CD80^+$ memory B cells (Figure 7A,B) and IgD^+ IgM^+ B cells (Figure 7C,D) were significantly lower in both blood and bone marrow of Group 1C mice than that in Group 2 and Group 3 mice. This suggests that the combination IS treatment decreased anti-AAV neutralizing antibodies by eliminating plasma cells and depleted the population of B cells potentially capable of generating AAV8 NAbs through interfering with the development of B cells from progenitor populations.

Taken together, the results reported in the present study demonstrate that bortezomib and the CD20 mAb combination treatment effectively diminished humoral immunity to AAV capsid and reduced neutralizing activity to AAV following vector readministration.

4 | DISCUSSION

In the present study, we tested the ability of a combination IS therapy with two clinically approved and safe immunosuppressive drugs, bortezomib to target antibody-producing plasma cells (and diminish processing and presentation of antigen) and rituximab (CD20 mAb) to deplete mature B cells, thereby preventing the generation of de novo antibody producing plasma and B memory cells, aiming to prevent anti-AAV NAb formation in naïve mice or deplete pre-existing anti-AAV NAb in mice pre-immunized with AAV.

It was reported that bortezomib alone[34] or in combination with another immunosuppressive agent prednisolone[57] effectively decreased anti-AAV antibodies, but the reduction was insufficient to allow AAV re-dosing in mice. Bortezomib has also been found to impair T-cell-mediated immune responses. Treatment with bortezomib decreased the number of $CD4^+$ and $CD8^+$ T cells in oxazolone-sensitized skin tissue and the draining lymph nodes and induced T-cell apoptosis through inhibition of nuclear factor-kappa B activation during T-cell-dependent inflammatory responses.[58] In NHPs previously injected with an AAV8 vector expressing human factor IX, immunosuppression with rituximab in combination with cyclosporine A did not lower high-titer (> 1:1000) anti-AAV8 or anti-AAV6 NAbs, but, in one animal with a low-titer NAb (1:31.6), the combination decreased anti-AAV6 NAb titers to baseline (1:1) and allowed successful AAV6 readministration.[59] In human rheumatoid arthritis patients with pre-existing anti-AAV NAbs, a single course of rituximab treatment partially decreased NAb titers in a subset of patients with NAb titers < 1:1000 and lowered titers to below 1:5 in a few patients with a titer of 1:31.6 or less.[60]

In the naïve setting study, we demonstrated that an 8-week combination IS treatment along with AAV8-CB-hGAA injection effectively, but not completely, prevented the formation of anti-AAV8 NAbs in GAA-KO mice, which subsequently required a higher dose (5×10^{13} vg/kg) of the second AAV8 vector (AAV8-LSP-hSEAP) administration to achieve efficacy. This suggested that a longer duration of IS treatment might be needed to allow AAV re-dosing at lower doses. In the pre-existing NAb setting study, comparison of IS treatment for 8, 12, 16, and 20 weeks demonstrated that combination IS for 16 weeks was an optimal treatment duration that enabled successful AAV8 readministration (Figure 4B). The 16-week immunosuppression treatment resulted in successful anti-AAV8 NAb depletion and allowed efficacious readministration of AAV vectors without any serious adverse events. However, in this experiment, we tested only the high-dose (5×10^{13} vg/kg) AAV8-LSP-hSEAP for AAV re-dosing based on the result from the naïve setting study. Considering that the 16-week IS treatment was more effective than the 8-week IS treatment in allowing AAV readministration (Figure 4B), it is possible that AAV re-dosing with lower doses of AAV8-LSP-hSEAP will be successful following the 16 weeks of IS treatment. Clinical studies have demonstrated that systemic administration of high doses of AAV vectors equal or above 5×10^{13} vg/kg can cause hepatotoxicity and kidney damage.[61,62] Our future research will focus the optimization of the current IS regimen by adding a third immune suppressive agent such

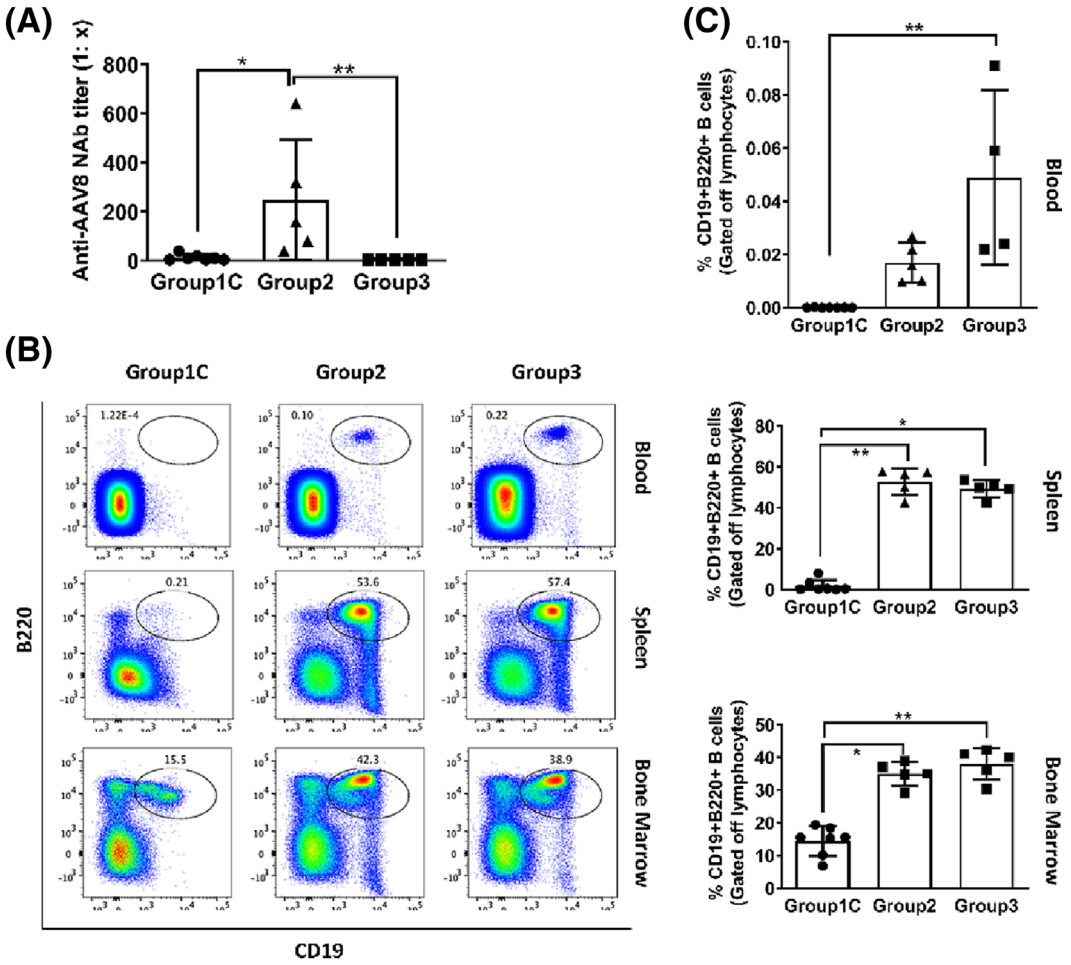


FIGURE 6 Depletion of anti-AAV neutralizing antibodies and B cells by the 16-week IS treatment in AAV pre-immunized GAA-KO mice prior to AAV readministration. (A) Analysis of anti-AAV8 neutralizing antibody titers in plasma. x, fold dilution of plasma samples; $n = 7$ for Group 1C (AAV8-CB-hGAA + IS for 16 weeks), $n = 5$ for Group 2 (AAV8-CB-hGAA, no IS), and $n = 5$ for Group 3 (PBS, no IS). Data shown are the mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$. (B) Representative flow cytometry plots illustrating the depletion of B cells ($CD19^{+}B220^{+}$) by the 16-week IS treatment from the blood, spleen, and bone marrow from mice in Group 1C compared to mice in Groups 2 and 3. (C) Composite data from individual mice in B showing the frequency of B cells ($CD19^{+}B220^{+}$) in the three treatment groups ($n = 7$ for Group 1C, $n = 5$ for Group 2 and Group 3). Data shown are the mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$.

as methotrexate to decrease the required dose for AAV re-dosing and test it in mice and large animal models. To our knowledge, this is the first IS approach using approved drugs that effectively suppressed anti-AAV NAb formation in naïve mice and also depleted pre-existing NAbs in AAV-immunized mice.

Unexpectedly, the 20-week IS treatment was much less effective than the 16-week IS regimen in allowing AAV readministration. We did not observe any obvious side effects (e.g., loss of body weight, changes in liver size and overall hepatic appearance) in the 20-week IS treatment group compared to other treatment groups. Histological analysis of liver sections did not reveal any significant hepatotoxicity related to the IS treatment (Figure S3). Livers from Group 1A (IS for 8 weeks) and Group 1B (IS for 12 weeks) showed minimal inflammation. Group 1C (IS for 16 weeks) livers showed similar results, except more prominent lobular inflammation in some cases. Group 1D (IS for 20 weeks) livers had both inflammation and some focal necrosis (acidophil bodies and hepatocyte dropout), but the foci of liver injury were quite small, amounting to rare individual cells or very rarely areas of cells, and were only present in some of the mice. This minor liver injury was unlikely to be the major cause of the low efficacy of AAV readministration in the 20-week IS treatment group. Comparison of AAV transduction efficiency in different tissues between Group 1C and Group 1D mice following AAV re-dosing demonstrated that AAV genome copy number in Group 1C livers was significantly higher than that in Group 1D livers, but there was no significant difference in AAV genome copy number in the skeletal muscle and heart between the two groups (see Supporting information, Figure S4A). This suggests that the lower AAV transduction in Group 1D livers was unlikely a result of inhibitory effect from anti-AAV8 NAbs because both treatment groups showed similar AAV transduction efficiencies in other tissues (quadriceps and heart). Further comparison of AAVR expression in the liver, quadriceps, and heart between Group 1C and 1D

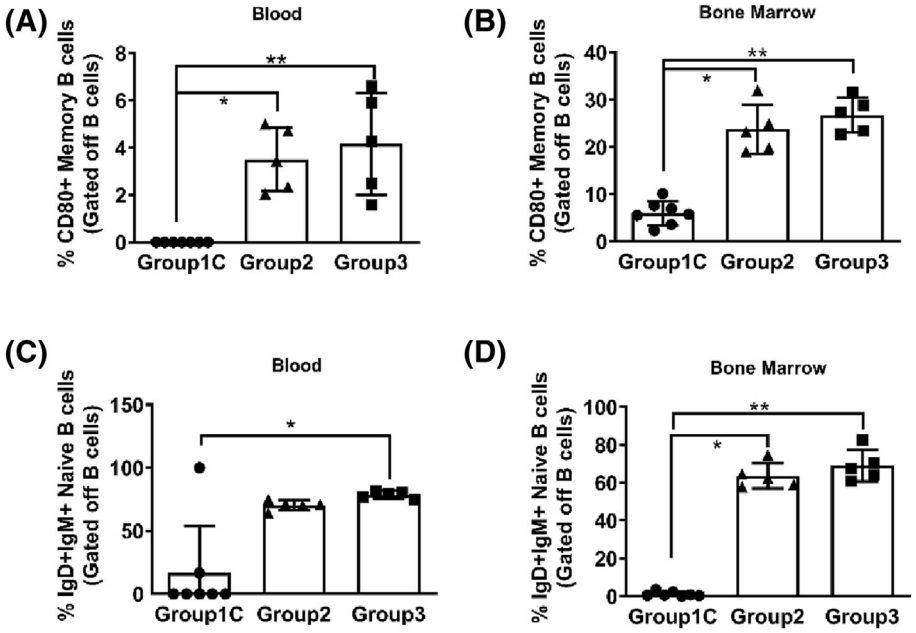


FIGURE 7 The 16-week IS treatment effectively depleted memory B cells and IgD^+IgM^+ naïve B cells from blood and bone marrow in AAV pre-immunized GAA-KO mice. Peripheral blood mononuclear cells and bone marrow cells from mice in Figure 6 were used to quantify the $CD80^+$ memory B cells (A,B) and IgD^+IgM^+ naïve B cells (C,D); $n = 7$ for Group 1C (AAV8-CB-hGAA + IS for 16 weeks), $n = 5$ for Group 2 (AAV8-CB-hGAA, no IS), and $n = 5$ for Group 3 (PBS, no IS). Data shown are the mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$.

mice showed no decrease in AAVR levels in any of the tissues of Group 1D mice, compared to Group 1C mice (see Supporting information, Figure S4B). It is possible that the prolonged IS treatment in the 20-week IS group caused some unknown cellular changes in the hepatocytes that specifically limited the transduction of the second AAV8 vector in liver.

Bortezomib is approved for the treatment of patients with multiple myeloma. Sensory peripheral neuropathy related to bortezomib treatment was reported in some patients with multiple myeloma, but the neuropathy was reversible and normally resolved after the completion of bortezomib treatment.[21] Severe hepatotoxicity because of bortezomib is rare in clinical trials, and bortezomib has been found to increase the risk of liver damage only in patients suffering from severe liver problems.[63] Patients with Pompe disease do not have underlying liver disease, and no hepatotoxicity or neurotoxicity related to bortezomib treatment has been noted in our clinical studies.[18,19,64]

One limitation of this proof-of-concept study is the use of only male mice, which cannot necessarily predict whether IS treatment is also effective in female mice. To understand the role of sex as a biological variable in response to the therapy, we aim to conduct similar experiments in female mice in our future research. Moreover, the findings of the GAA-KO mouse study should be confirmed and extended in a second study with a larger number of mice.

Although AAV vectors are the preferred gene delivery vehicles for long-term gene replacement therapy, clinical studies have demonstrated the limitations of AAV-mediated gene therapy as a result of host immunity to AAV capsid antigens. Dose-dependent anti-AAV capsid cytotoxic T lymphocyte responses were observed in early clinical trials, which were successfully managed by reducing the dose of the AAV vector and/or by treating patients with rising liver enzymes with a short course of immunosuppressive corticosteroids.[65,66] Pre-existing anti-AAV NAbs, even at very low titers, can block viral vector transduction,[3–5] resulting in the exclusion of a significant number of patients from enrollment in clinical trials.[6–8,67] In addition, antibodies raised after initial AAV treatment render readministration of the same vector ineffective. Currently, there is a critical need for safe and effective approaches to modulate humoral immunity to AAV capsids to enable patients with pre-existing antibodies to receive AAV treatment or to allow repeated AAV administration as will be clearly needed to extend the durability of gene therapy.

Methods using alternative serotypes or engineered AAV capsids may evade the pre-existing NAbs,[10–12] but the available serotypes suitable for a given patient will eventually be exhausted, and, indeed, anti-AAV capsid antibodies are known to be highly cross-reactive among different serotypes.[8,68] Frequent sessions of plasmapheresis have been proposed as a strategy to deplete circulating anti-AAV NAbs but appear to be effective only for patients with low titers of antibodies (1:20 or less).[9] Bertin et al.[13] recently reported a capsid-specific plasmapheresis column approach, which selectively and almost completely depleted anti-AAV antibodies from high-titer purified human IgG pools and plasma samples without depleting the total immunoglobulin pool from plasma, decreasing antibody titers to levels that allowed efficient AAV vector administration in mice.

To date, different pharmacological immunomodulation agents, alone or in a combination, have been attempted in mice and NHPs to overcome the barrier of AAV re-dosing,[15–17,59,69–72] as summarized in Table 1. In particular, coadministration of tolerogenic rapamycin nanoparticles (SVP-rapamycin) with AAV vectors effectively inhibited both anti-AA8 capsid cellular and humoral responses in mice and enabled successful AAV readministration in mice and NHPs.[17] However, this approach cannot deplete pre-existing anti-AAV antibodies. Another approach using bacterial IgG-degrading enzymes imlifidase (IdeS) and IdeZ has been shown to be effective in degrading pre-existing anti-AAV NAbs and rescuing AAV transduction in mice and NHPs.[15,16] However, depletion of all IgG antibodies in human patients remains a safety concern. The recent approval of IdeS in Europe for kidney

TABLE 1 Overview of immune modulation approaches used in preclinical studies for AAV readministration

IS regimen	Function	Source	Dose	Serotype	Limitation	Re-dosing	Reference
Mycophenolate mofetil (MMF) Tacrolimus	Inhibits DNA synthesis in lymphocytes Inhibits T-cell activation	NHP	2×10^{13} vg/kg	AAV8	Withdrawal of regimens leads to anti-capsid antibody formation	Did not perform	[69]
Mycophenolate mofetil (MMF) Sirolimus (rapamycin) Daclizumab	Inhibits DNA synthesis in lymphocytes Inhibits mTOR Anti-IL2 receptor antibody	NHP	8×10^{12} vg/kg	AAV2	Treatment with daclizumab increases anti-AAV2 antibody titers	Did not perform	[70]
Rituximab Cyclosporine A	Anti-CD20 antibody Inhibits calcineurin	NHP	2×10^{13} vg/kg 1×10^{13} vg/kg	AAV8 AAV6	Re-dosing requires different serotype Small sample size	Performed, partially effective	[59]
SVP-rapa	Synthetic vaccine particles encapsulating rapamycin	Mouse NHP	4×10^{12} vg/kg 2×10^{12} vg/kg	AAV8	Needs manufacturing development Requires coadministration with AAV	Performed, effective	[17]
IdeS (imlifidase)	IgG-degrading enzyme from *Streptococcus pyogenes*	Mouse NHP	1×10^{12} vg/kg 2×10^{13} vg/kg	AAV8	Potentially induces NAbs to the enzyme itself	Performed, effective	[16]
IdeZ	IgG-degrading enzyme	Mouse NHP	1×10^{13} vg/kg 5×10^{12} vg/kg	AAV8 AAV9	Potentially induces NAbs to the enzyme itself Optimal dose unknown	Did not perform	[15]
Tolerogenic nanoparticles encapsulating rapamycin (ImmTOR)	Induce antigen-specific immune tolerance by targeting rapamycin to immune cells	Mouse	5×10^{11} vg/kg	AAV8	Dose not directly affect pre-existing antibody titers Insufficient to enable AAV transduction in the presence of high titers of NAbs	Performed, effective	[71]
CTLA4-IgG (abatacept)	Blockade of the costimulatory CD28-B7 family signal	Mouse	4×10^{12} vg/kg	AAV8	May not be effective in a pre-existing NAb setting	Performed, effective	[72]

Abbreviations: AAV, adeno-associated virus; NAb, neutralizing antibody; NHP, non-human primate.

transplant patients[73] should facilitate the evaluation of this treatment approach in patients requiring AAV-mediated gene therapy. Recently, Frentsch et al.[72] reported that blockade of the costimulatory CD28-B7 family signal axis by transient treatment with CTLA4-IgG (abatacept) during initial human FIX (AAV8-FIX) gene therapy in mice efficiently inhibited the development of anti-AAV8 capsid NAbs and CTL responses and thus permitted re-dosing of the AAV8-FIX vector. However, this preventive immune-suppressive approach may not be effective in a pre-existing anti-AAV setting.

In the present study, we used two AAV vectors carrying different transgenes to evaluate the efficacy of AAV re-dosing in mice, which is a commonly used method in similar preclinical studies. However, the clinical setting for gene therapy will be more complex with an extra layer of immune responses against the transgene products, especially the CTL response that would result in the elimination of AAV-transduced cells and the loss of gene therapy efficacy. Compared to the challenge of pre-existing anti-AAV capsid NAbs, transgene-related immunity is more manageable using different strategies, such as immunotolerance induction by liver-restricted transgene expression,[51,74–78] prevention of transgene expression in antigen-presenting cells using tissue and cell type specific promoters[79,80] or by incorporating microRNAs targeting sequences in transgene expression cassettes,[81,82] and reduction of innate and CTL immune responses by depleting CpG motifs from transgene cassettes.[83,84]

In conclusion, our data indicate that combination treatment with bortezomib and rituximab is a feasible immune modulation approach to allow patients with pre-existing anti-AAV NAbs to receive AAV gene therapy and thus enable patients to receive repeated AAV treatment when needed. The results from this mouse study warrant investigation of this IS strategy together with additional therapeutics such as methotrexate in NHPs before translation to human patients. Although this combination IS treatment approach is relatively safe and may has a broad spectrum of gene therapy applications, potential side effects caused by bortezomib treatment exist. The risk–benefit to patients must be considered especially for the diseases that have other treatment options or for which gene therapy is not considered a lifesaving intervention.

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CONFLICTS OF INTEREST STATEMENT

JSY was a Duke employee and is currently employed by G1 Therapeutics (Research Triangle Park, NC). AR is employed by EpiVax, Inc. (Providence, RI). PSK and DDK and Duke University might benefit financially if the experimental treatments discussed here prove effective and are successful commercialized in the context of gene therapy. BS has received grant support from Asklepios BioPharmaceutical, Inc. (AskBio) and Actus Therapeutics. PSK has received research/grant support from Sanofi Genzyme and Amicus Therapeutics. PSK has received consulting fees and honoraria from Sanofi Genzyme, Amicus Therapeutics, Maze Therapeutics, JCR Pharmaceutical, and Asklepios Biopharmaceutical, Inc. (AskBio). PSK is a member of the Pompe and Gaucher Disease Registry Advisory Board for Sanofi Genzyme, Amicus Therapeutics, and Baebies. PSK and DDK have equity in AskBio, which is developing gene therapy for Pompe disease. All authors declare that they have no other competing interests.

AUTHOR CONTRIBUTIONS

SJC performed major experiments. BS and PSK designed the study. SJC, BS, and PSK wrote the manuscript. JSY performed flow cytometry experiments and reviewed the manuscript. JAL helped with some of the animal experiments and manuscript writing. TFT provided the mouse-specific CD20 mAb (MB20-11) and reviewed the manuscript. DDK and AR helped with the study design and reviewed the manuscript. AKD reviewed and edited the manuscript. WJ examined histological slides and interpreted the data.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICAL STATEMENT

The present studies in animals were reviewed and approved by Duke University Institutional Animal Care and Use Committee (IACUC) review board (Durham, NC), and animal care and experiments were conducted in accordance with approved guidelines.

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