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BRIEF REPORT

Blockade of the costimulatory CD28-B7 family signal axis enables repeated application of AAV8 gene vectors

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Abstract

Adeno-associated virus serotype 8 (AAV8) gene therapy has shown efficacy in several clinical trials and is considered a highly promising technology to treat monogenic diseases such as hemophilia A and B. However, a major drawback of AAV8 gene therapy is that it can be applied only once because anti-AAV8 immunity develops after the first treatment. Readministration may be required in patients who are expected to need redosing, eg, due to organ growth, or to boost suboptimal expression levels, but no redosing protocol has been established. We have developed a preventive immune-suppressive protocol for a human factor IX (FIX) vector with an intended dose of ~5×10^{11} vg/kg that inhibits the development of anti-AAV8 neutralizing-antibody (NAb) responses and anti-AAV8 T-cell responses using CTLA4-IgG (abatacept). In a preclinical model, transient treatment with abatacept during initial human FIX gene therapy efficiently inhibited the generation of AAV8-specific cellular and humoral responses, and thus permitted redosing of FIX. Furthermore, our data suggest that by suppression of anti-AAV8 NAb responses after the second higher dose (4×10^{12} vg/kg) this protocol can be used to enable redosing up to such high doses. An additional advantage of CTLA4-IgG blocking CD28-mediated signals is its potential suppression of AAV8-specific cytotoxic CD8 T-cell responses, which are believed to kill transduced hepatocytes and might interfere with a successful readministration. Redosing protocols using approved drugs would be beneficial for patients because they could effortlessly be applied in clinical trials and enable safe and efficient treatment options for patients undergoing AAV8 gene therapy.

KEYWORDS

CTLA-4-Ig, gene therapy, hemophilia B, immunosuppression, neutralizing antibodies

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1 | INTRODUCTION

Several clinical trials have shown that adeno-associated virus serotype 8 (AAV8) gene therapy is a promising treatment for patients with monogenic diseases such as hemophilia.[1-3] However, overcoming anti-AAV8 immunity is considered a major challenge. AAV8 immunity results from anti-AAV8 neutralizing antibodies, which can efficiently block transgene transfer even at low titers,[4,5] and anti-AAV8 T-cell responses, which may kill transduced hepatocytes.[6] Treatment with AAV8 vectors also induces novel anti-AAV8 neutralizing-antibody (NAb) responses, and potentially anti-AAV8 T-cell responses, thereby precluding readministration to patients.[1,7]

Repeated AAV8 vector administrations may be required to achieve sufficient transgene expression levels in patient populations which are negative for AAV8 NAbs and are expected to have low transgene expression after the first treatment. This could include patients treated with low-vector doses for safety reasons[1] or young patients with declining transgene expression because of body or organ growth.[8] To enable readministration of the same vector, we here evaluated transient and preventive immune suppression using abatacept. This approach focused on enabling readministration in AAV8-factor IX (FIX) gene therapy or similar gene therapies with low vector doses of about 5 × 10^{11} vg/kg. Abatacept was chosen because its immunosuppressive efficacy has been described in transplantation and rheumatoid arthritis.[9-11] Abatacept blocks the costimulatory interaction between CD28 and CD80/CD86, thereby inhibiting CD4 and CD8 T-cell differentiation and, consequently, the development of anti-AAV8 NAb-secreting plasma cells.[11]

Essentials

- AAV gene therapy can be only applied once due to anti-AAV neutralizing antibody formation.
- Abatacept blocks anti-AAV8 T cell- and neutralizing antibody-response in a preclinical model.
- CTLA4-IgG enables redosing with AAV8 vectors by blocking neutralizing antibodies.
- CTLA4-IgG could be used in patients to enable redosing and control anti-AAV8 T cell responses.

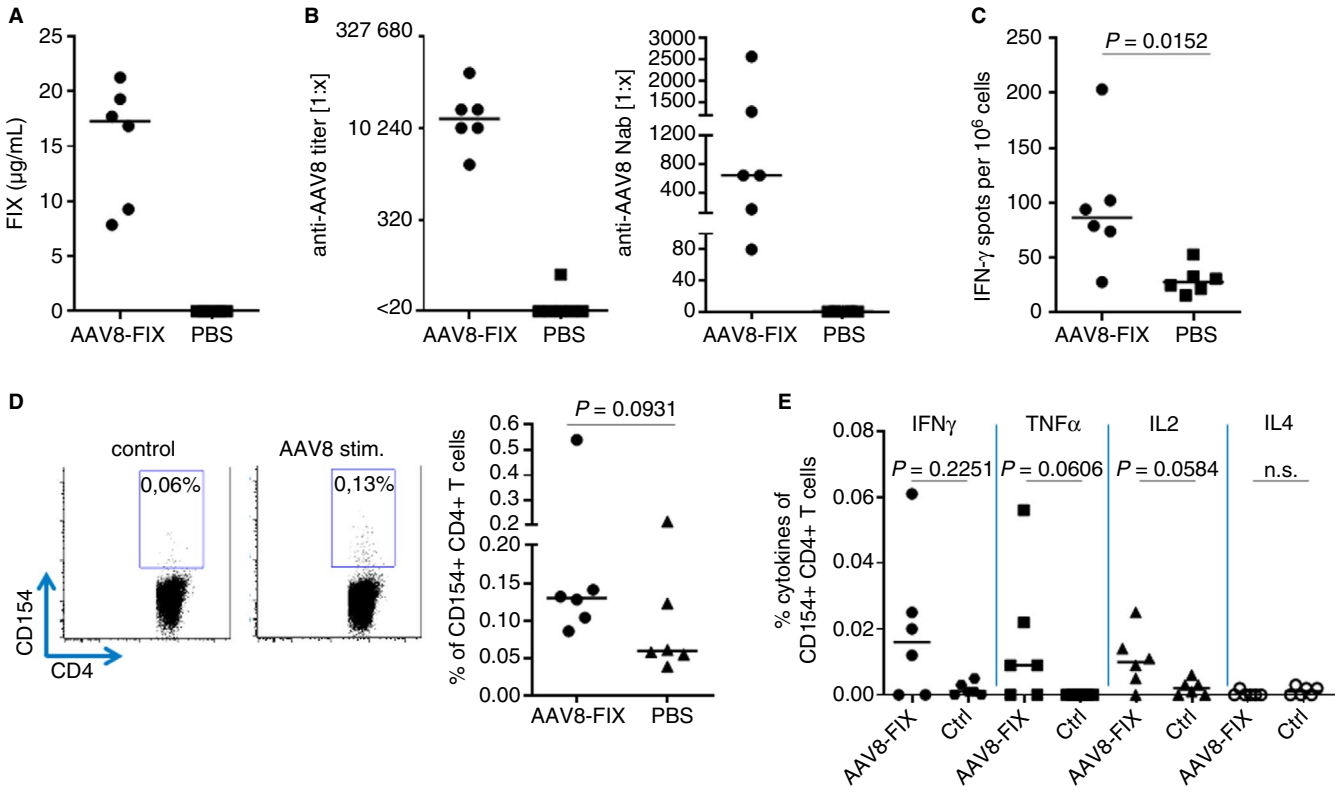

FIGURE 1 Adeno-associated virus serotype 8 (AAV8)-factor IX (FIX) gene therapy elicits robust anti-AAV8 T-cell and antibody responses, preventing redosing. Mice were immunized with AAV8-FIX (FIXR338L) or buffer and killed on day 28 for analysis of FIX expression and anti-AAV8 immunity. A, FIX expression on day 28. B, Anti-AAV8 binding and neutralizing antibody titer at day 28 after AAV8 challenge. C, T-cell response was characterized after in vitro restimulation with AAV8 peptide pools by IFN-γ ELISpot. AAV8-specific $CD4^+$ T-cell responses were analyzed by fluorescence-activated cell sorting (D, E). Ctrl, Control; PBS, phosphate-buffered saline (significant $P > .005$; n.s. = not significant)

2 | RESULTS AND DISCUSSION

To characterize the anti-AAV8 immune response, mice were injected intravenously with an AAV8-FIX vector bearing the FIXR388L transgene. At day 28, the treatment resulted in high FIX plasma levels in all mice receiving the AAV8-FIX vector, demonstrating the effectiveness of AAV8-based vectors to deliver transgenes (Figure 1A). Treatment with AAV8-FIX elicited AAV8-specific binding antibodies (BAbs), including anti-AAV8 NAbs that would block a subsequent gene therapy treatment (Figure 1B). At the same time, a weak but statistically significant AAV8-specific T-cell response was detected by an IFN-γ secretion ELISpot (Figure 1C) and by flow cytometric assessment of the activation marker CD154, defining antigen-specific $CD4^+$ T cells (Figure 1D).[12] The cytometric data showed that the cellular response elicited against AAV8 is mediated mostly by $CD4^+$ T cells as the frequencies of IFN-γ, CD107a, and TNF-α elicited by $CD8^+$ T cells were in the same range as the controls (data not shown). Further analysis of the cytokine expression revealed that AAV8 promotes a typical antiviral Th1-cell polarization because the $CD154^+CD4^+$ T cells coexpressed predominantly IFN-γ and TNF-α, but no IL-4 (Figure 1E).

After setting up the model and establishing that abatacept can suppress anti-AAV8 immune responses sufficiently, we performed two independent experiments to show that prevention of anti-AAV8 immunity enables redosing: A redosing scenario was mimicked to show the impact of AAV8-FIX–induced immunity on subsequent treatment with AAV8-FIX gene therapy. Mice immunized with low-dose AAV8-FIX without immunosuppression (Figure 2A) showed that FIX expression on day 28 could not be further boosted to reach the expression levels of the controls (about 20 μg/mL on day 77;

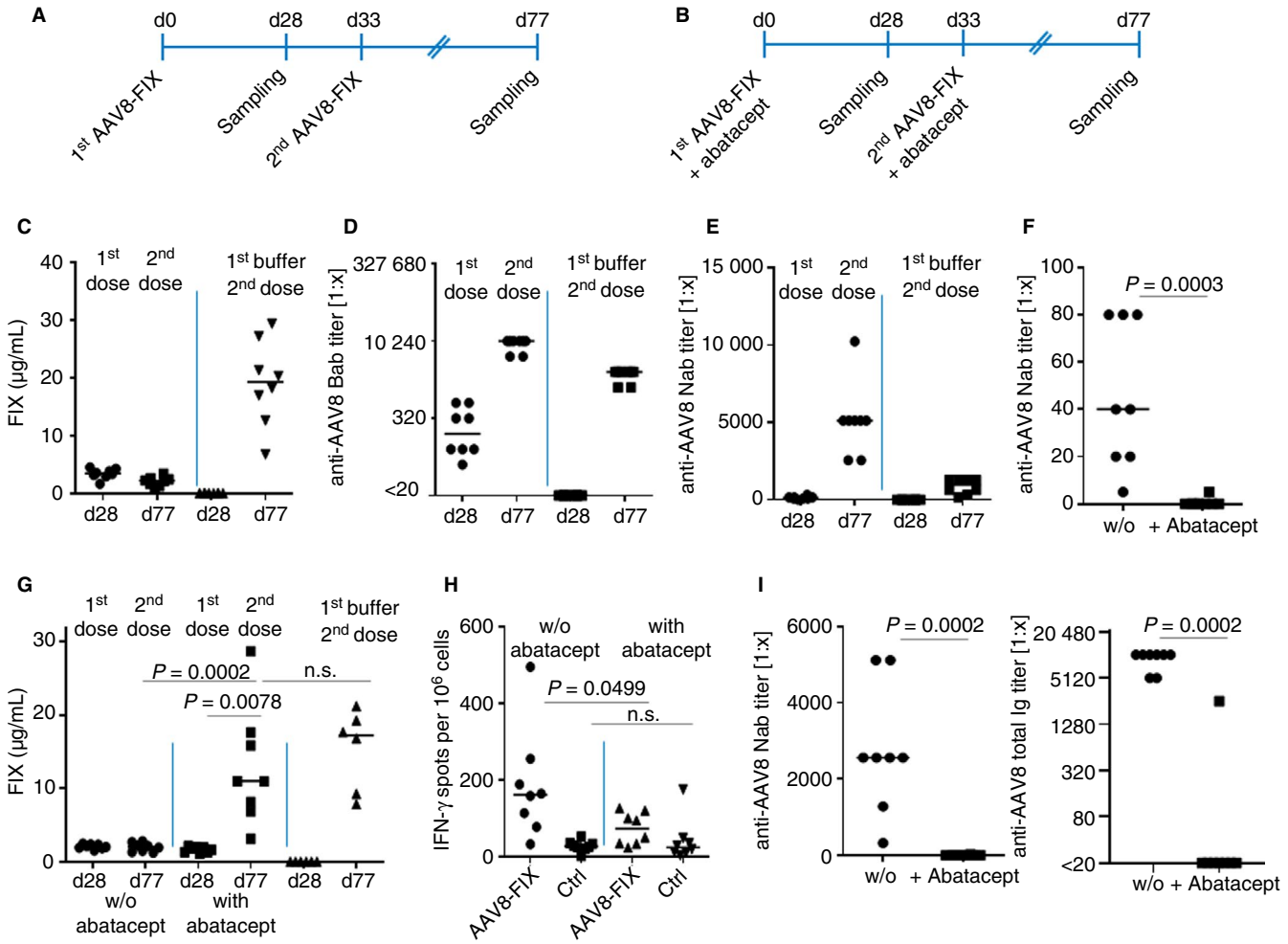

FIGURE 2 Abatacept efficiently suppresses anti-adeno-associated virus serotype 8 (AAV8) T-cell and antibody responses, enabling redosing with AAV8-factor IX (FIX). Mice were treated according to the indicated scheme once (A) or twice (B) with AAV8-FIX or buffer. For repeated treatment, 5×10^{11} vg/kg AAV8-FIX and a subsequent dose of 4×10^{12} vg/kg were used with and without immunosuppression. Treatment in the absence of abatacept showed that AAV8-induced immune responses inhibit the application of the second treatment, thus blocking the boosting of FIX expression on day 77 after the second dose (C). Lower titers of AAV8 antibodies were induced after the first dose and boosted after the second (D, E). Abatacept suppresses anti-AAV8 NAb responses (F), enabling redosing and boosting of FIX expression (G). Concomitant abatacept treatment during the second vector application suppresses the adaptive anti-AAV8 immune response of day 77 (H, I) (significant $P > .005$; n.s. = not significant)

Figure 2C). The boosting of FIX expression was blocked by the adaptive immune responses, the major barrier to redosing (Figure 2D-E). Confirming previous studies, low anti-AAV8 NAbs measured after a first low-dose application appeared to inhibit the boost in FIX transgene expression (Figure 2E).[4] The boosting of anti-AAV8 antibody responses in double-treated mice (day 77; Figure 2D-E) compared with the mice receiving AAV8 only once (day 28; Figure 2D-E) demonstrated that the mice receiving both applications developed a classic prime-boost immune reaction against AAV8.

Generation of anti-AAV8 immunity was prevented in two independent experiments by applying abatacept before and after the AAV8-FIX injection (Figure 2B). Concomitant application of abatacept efficiently inhibited the development of AAV8-specific NAb responses on day 28 after treatment (Figure 2F). Repeated application of AAV8 vectors in the presence of abatacept finally boosted FIX plasma levels on day 77 to within the range shown by the controls, which were treated only once with AAV8-FIX (Figure 2G shows a representative experiment). A second application of abatacept was applied to show that anti-AAV8 antibodies and T cells could be suppressed efficiently, even after the second higher dose, potentially facilitating a third AAV8 vector treatment (Figure 2H-I).

Repeated administration of AAV vectors is required in patients who are expected to have low expression levels due to low vector doses or a decline in transgene expression over time.[8] Hence, various redosing strategies have been suggested such as using different serotypes of vectors with[13] and without[14] preventive immunomodulation. These approaches have the disadvantage that new AAV vectors need to be developed and redosing efficacy might be impacted by cross-reactivity of anti-AAV immune responses. Accordingly, an approach using rapamycin-filled nanoparticles, avoiding the development of new gene therapy vectors, was suggested.[15] Our data indicate that a preventive approach based on CTLA-4IgG would enable redosing without having to develop a new drug. The present protocol focused on AAV8-FIX gene therapy using vector doses up to 5×10^{11} vg/mL. Based on our data, vector doses up to 5×10^{11} vg/mL could be used to enable redosing. However, a second treatment to boost FIX expression using a dose of 4×10^{12} vg/kg in the presence of abatacept showed that abatacept was capable of suppressing anti-AAV8 NAb responses efficiently even at higher vector doses (Figure 2I). As the presence of neutralizing antibodies is the limiting factor for readministration, we conclude that with our immune suppressive regimen, vector doses up to 4×10^{12} vg/kg could be suppressed to enable redosing. Further studies are needed to enable readministration of doses higher than 4×10^{12} vg/kg because higher vector doses are likely to induce stronger anti-AAV8 immunity.

Abatacept and the second-generation product belatacept are approved drugs with known safety profiles that suppress T-cell activation and accordingly primary antibody responses.[9-11] CTLA-4-IgG targets T cells and, hence, antibody-producing plasma cells that do not require T-cell help would not be affected by CTLA4-IgG.[16] Accordingly, preexisting AAV antibody levels would not be reduced by using CTLA4-IgG. Other technologies such as immune adsorption columns could be used to remove preexisting AAV8 antibodies.[17] However, CTLA-4 IgG could play an important role in suppressing preexisting memory T-cell responses that are considered to kill transduced hepatocytes.[18]

In general, in both cases, anti-AAV T-cell immunity could be inhibited more specifically by CTLA-4IgG, reducing the potential side effects of prednisone.[19,20] Accordingly, mice treated with abatacept did not show statistically significant different transgene expressions compared with the control group (Figure 2G), and no treatment-related effects were observed (data not shown). Additionally, a preventive transient immunosuppression with a drug that has a known safety profile is intended, thus decreasing any potential side effects of the immune suppressant. Moreover, if required, abatacept could be safely combined with other immunosuppressants.[21,22] Our data suggest that AAV8 immune responses are T-cell dependent and transient and preventive immune suppression targeting T cells can enable redosing in AAV8 gene therapy.

3 | METHODS

3.1 | Mice and gene vectors

C57BL/6J mice, purchased from Charles River Laboratories Inc, were challenged at age 8 to 10 weeks and killed at indicated time points. Purified full AAV8-FIX capsids (90% full capsids) were used at the indicated doses.

3.2 | Experimental gene therapy

Mice were treated with two doses (5×10^{11} vector genomes [vg] per kg of body weight and 4×10^{12} vg/kg) of AAV8-FIX or buffer. The groups received 500 μg abatacept (Bristol-Myers Squibb) intraperitoneally 3 days and 1 day before injection of AAV8 vectors, and at days 1, 3, and 6 after injection. AAV8-FIX was further injected with and without abatacept in the same way. Blood was collected from the tail at the indicated time points. Spleens were harvested on day 77.

3.3 | Cell preparation

Single-cell suspensions were obtained from spleens by passing through a 40-μm filter (BD Biosciences). After lysing erythrocytes with ACK lysis buffer (Thermo Fisher), cells were cultured in RPMI medium supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.3 mg/mL glutamine, 10% inactivated fetal bovine serum (FBS; all PAA Laboratories Inc), and 50 μM β-mercaptoethanol (Sigma-Aldrich).

3.4 | ELISpot

The IFN-γ ELISpot was performed according to the manufacturer's protocol (Cellular Technology Limited) and as described using AAV8

peptide pools (15mer, offset 5, pool 1 = peptide 1-50, pool 2 = peptide 51-100, pool 3 = the remaining peptides; Anita Kruzik, Damir Fetahagic, Bettina Hartlieb, Sebastian Dorn, Frank M. Horling, FS, BMR, and M.d.l.R., manuscript submitted January 2019).

3.5 | In vitro NAb assay

The in vitro NAb assay was performed using AAV8-luciferase reporter constructs and Huh7 cells and a 1:5 cutoff.[4]

3.6 | Detection of FIX protein concentration

FIX protein concentration was measured with an enzyme-linked immunosorbent assay (ELISA) using commercially available polyclonal-paired anti–human FIX antibodies as described.[4]

3.7 | ELISA

Anti-AAV8 IgG1 binding antibodies were detected by ELISA as described.[4] In brief, AAV8 capsids were coated on 96-well plates overnight at 4°C. For antibody detection, horseradish peroxidase-conjugated secondary antibodies specific to murine IgG1 were incubated for 1 hour, and then tetramethylbenzidine used as a substrate. Anti-AAV8 Ig binding antibody ELISA was performed as described above by using a peroxidase-conjugated pan-Ig specific secondary antibody.

3.8 | Fluorescence-activated cell sorting

Single-cell suspensions were stimulated with AAV8 peptide pools for 6 hours and subsequently stained using CytoFix/CytoPerm (BD) and CD4-BV605 (RM4-5), CD8-PerCpCy5.5 (53-6.7), IFN-γ- PeCy7 (XMG1.2), IL-2-APC (JES6-5H4), IL-4-Pe (11B11), TNF-α–A700 (MP6-XT22 all from Biolegend), CD154-FITC (MR1) from Invitrogen, and CD107a-eFluor 450 (eBio1D4B) from eBioscience. Dead cells were discriminated by the Aqua Dead Cell Stain Kit (Thermo Fisher).

3.9 | Statistical analysis

Between-treatment group differences were assessed for endpoints using the non-parametric unpaired Mann-Whitney test and between time points in the same animals a non-parametric, paired Wilcoxon test. The analyses were performed with Graphpad Prism 8.2.

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CONFLICTS OF INTEREST

FS, BMR, and M.d.l.R. are employees of Baxalta Innovations GmbH, a member of the Takeda group of companies and are Takeda stock owners.

AUTHOR CONTRIBUTIONS

MF designed experiments, analyzed and interpreted the data, and wrote the manuscript; ASJ designed and performed experiments; MD performed experiments and established the assays; NM obtained approvals for animal research; AT provided intellectual input and editorial assistance; FS and BMR interpreted the data, facilitated the study, and reviewed the manuscript; M.d.l.R. designed the study, analyzed and interpreted the data, and wrote the manuscript; and all authors reviewed the manuscript critically for intellectual content, participated in drafting and/or revising the manuscript, and approved the final version for submission.

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