Readministration of high-dose adeno-associated virus gene therapy vectors enabled by ImmTOR nanoparticles combined with B cell-targeted agents

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Abstract

Tolerogenic ImmTOR nanoparticles encapsulating rapamycin have been demonstrated to mitigate immunogenicity of adeno-associated virus (AAV) gene therapy vectors, enhance levels of transgene expression, and enable redosing of AAV at moderate vector doses of 2 to 5E12 vg/kg. However, recent clinical trials have often pushed AAV vector doses 10-fold to 50-fold higher, with serious adverse events observed at the upper range. Here, we assessed combination therapy of ImmTOR with B cell-targeting drugs for the ability to increase the efficiency of redosing at high vector doses. The combination of ImmTOR with a monoclonal antibody against B cell activation factor (aBAFF) exhibited strong synergy leading to more than a 5-fold to 10-fold reduction of splenic mature B cells and plasmablasts while increasing the fraction of pre-/pro-B cells. In addition, this combination dramatically reduced anti-AAV IgM and IgG antibodies, thus enabling four successive AAV administrations at doses up to 5E12 vg/kg and at least two AAV doses at 5E13 vg/kg, with the transgene expression level in the latter case being equal to that observed in control animals receiving a single vector dose of 1E14 vg/kg. Similar synergistic effects were seen with a combination of ImmTOR and a Bruton's tyrosine kinase inhibitor, ibrutinib. These results suggest that ImmTOR could be combined with B cell-targeting agents to enable repeated vector administrations as a potential strategy to avoid toxicities associated with vector doses above 1E14 vg/kg.

Keywords: gene therapy, adeno-associated virus, AAV redosing, immunogenicity, immune tolerance

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Significance Statement

Gene therapies delivered with adeno-associated virus (AAV) vectors have shown great promise in the treatment of rare genetic diseases, but can be limited by immune responses which prevent vector redosing and which can cause toxicities at high vector doses. Here, we demonstrate that rapamycin-containing ImmTOR nanoparticles combined with B cell-targeted therapies act synergistically to mitigate AAV immunogenicity and enable readministration of high vector doses. Such combination therapies could potentially be used to allow dose titration of AAV therapy to avoid high vector dose toxicity.

Introduction

Gene therapy involving systemic administration of adeno-associated virus (AAV) vectors has scored some remarkable successes in the clinic, resulting in recent approvals of gene therapies for spinal muscular atrophy, Duchenne muscular dystrophy, and hemophilia A and B, as well as promising therapeutic candidates for a number of other rare genetic diseases (1, 2). A potential limitation of AAV gene therapy is that it can only be administered once systemically due to the formation of high levels of long-lasting neutralizing anti-AAV antibodies that preclude redosing (3). The inability to redose AAV remains a considerable hurdle for the field (4). AAV genome copies, which are nonreplicating, may be reduced over time due to vector

elimination or cell turnover, resulting in loss of transgene expression. This issue is of particular concern for pediatric applications, where the target organ may increase in size with age, while vector genome copies do not increase, resulting in loss of efficacy (5–7). Infection, inflammation, or tissue damage may also result in cell turnover that adversely affects transgene expression. Elevation of serum transaminase levels, indicative of liver inflammation, is often a harbinger of reductions in transgene expression in the liver (8). The ability to redose AAV would not only be important for re-storing therapeutic benefit, but could enable dose titration of AAV therapies to avoid or mitigate toxicities associated with high vector doses (4, 9). Preclinical strategies to mitigate anti-capsid antibody responses include the use of

immunosuppressants (10–15), immune tolerizing therapies (16, 17), IgG-specific proteases (18–20), and IgG depletion through plasmapheresis (21) or affinity absorption (22, 23), but vector redosing has been limited to moderate vector doses of less than 1E13 vg/kg. Overcoming the considerable challenge of mitigating antibody responses to high vector doses may require combination therapies.

Recently, we have described the use of ImmTOR nanoparticles, which encapsulate rapamycin, to induce a tolerogenic response to coadministered vectors that enable successful vector redosing (7, 16, 17, 24). ImmTOR primarily targets the antigen-presenting cell—T cell axis by inducing a tolerogenic phenotype in antigen-presenting cells which results in the induction of antigen-specific regulatory T cells (Tregs) when coadministered with a target antigen (25–27). We reasoned that Treg-selective effects of ImmTOR could be complementary to B cell-targeted therapies. Here, we report that combining ImmTOR with a monoclonal antibody (MAb) against B cell activating factor (BAFF), a B cell survival factor, showed a profound synergistic effect on mitigating anti-AAV IgM and IgG antibody responses against a high AAV dose of 5E13 vg/kg and enabled successful vector readministration, resulting in transgene-encoded protein levels similar to those attained by a single administration of 1E14 vg/kg.

Results

Residual anti-AAV IgM antibodies can prevent redosing

We have earlier shown that a single dose of 100 to 300 µg of ImmTOR is effective in suppressing anti-AAV IgG induction and enabling efficient AAV transduction after initial and the second dosing of viral vector within the range of 0.5 to 2.5E12 vg/kg (7, 16, 17, 24). Here, we evaluated a low dose (50 µg) of ImmTOR coupled with a low AAV8-SEAP vector dose of 5E11 vg/kg. ImmTOR treatment resulted in substantially increased SEAP expression even after the first dose compared to control treatment with vector alone, as previously described (24) (Fig. 1A). A single dose of ImmTOR completely suppressed anti-AAV8 IgG antibodies through Day 89 (Fig. 1B), enabling successful redosing of vector on Day 93 which resulted in a further increase in SEAP expression (Fig. 1A). Anti-AAV8 IgG antibodies remained low through Day 125, 32 days after the second dose of AAV8-SEAP + ImmTOR, after which 2 of 5 animals developed anti-AAV8 IgG antibodies. Although 3 of 5 animals remained seronegative for anti-AAV8 IgG at Day 174, administration of a third dose of AAV8-SEAP + ImmTOR on Day 176 did not result in any further increase in transgene expression in any animal (Fig. 1A). As expected, most control animals treated with vector alone showed high levels of IgG to AAV8 and no significant changes in transgene expression with redosing.

The lack of transgene elevation after the third dose of AAV combined with ImmTOR was puzzling since it did not inversely correlate with anti-AAV8 IgG levels. To further investigate, we inoculated a large cohort ($n = 45$) of mice with vector + a suboptimal dose of ImmTOR and assessed anti-AAV8 IgG levels and SEAP expression before and after vector redosing. To eliminate a possible confounding contribution of an immune response against SEAP affecting SEAP levels, we used a heterologous redosing schema wherein AAV8 expressing red fluorescent protein (AAV8-RFP) was dosed at Day 0 and AAV8-SEAP was dosed on Day 86 (Supplementary Fig. S1A). Thirteen animals developed anti-AAV8 IgG responses by Day 72, while the remaining 32 animals remained IgG seronegative (Supplementary Fig. S1B). As expected, animals that had seroconverted prior to administration of AAV8-SEAP showed little or no expression of SEAP (Supplementary Fig. S1C). However, even among the 32 animals that were IgG low or negative at Day 72, only three showed robust expression of SEAP after vector redosing with others remaining SEAP-negative without noticeable increases in anti-AAV8 IgG levels (Supplementary Fig. S1C). These results suggested that some factor other than IgG antibodies was inhibiting vector transduction.

To determine if the neutralizing activity was due to a soluble factor, sera from AAV8 + ImmTOR-treated mice that remained anti-AAV8 IgG-negative but did not support vector redosing (group circled in blue in Supplementary Fig. S1C) were pooled and passively transferred to naïve recipients prior to AAV8-SEAP administration. The passively transferred anti-AAV8 IgG-negative sera inhibited in vivo AAV8-SEAP transduction in a dose-dependent manner (Fig. 2A). Interestingly, mice passively immunized with higher amounts of IgG-negative serum prior to AAV8-SEAP administration showed attenuated levels of de novo anti-AAV8 IgG but not IgM antibodies (Fig. 2B and C). The anti-AAV8 IgG-negative serum pool was nearly as effective as the anti-AAV8 IgG-positive pool in blocking AAV8 transduction in vitro (Fig. 2D). However, heat inactivation (63°C, 10 min) of the sera partially eliminated neutralizing activity in the anti-AAV8 IgG-negative pool but not the IgG-positive pool. Heat inactivation is known to affect IgM but not IgG, suggesting that the neutralizing activity was due to anti-AAV8 IgM antibodies.

Ibrutinib combined with ImmTOR leads to dose-dependent inhibition of anti-AAV IgM and IgG antibodies, enabling successful vector readministration, the efficiency of which inversely correlates with anti-AAV8 IgM levels prior to redosing

Marginal zone B cells and B1 B cells in the mouse can produce IgM antibodies in a T cell-independent (TI) manner (28). Such TI-IgM antibodies are unlikely to be affected by ImmTOR which acts primarily at the antigen-presenting cell-T cell axis. We reasoned that B cell-targeted agents that affect B cell maturation could act synergistically with ImmTOR in blocking both IgG and IgM antibody responses against AAV capsid. We tested two agents which have been reported to attenuate IgM responses: ibrutinib, an irreversible Bruton's tyrosine kinase (BTK) inhibitor known to affect the signal transduction pathway downstream of B cell receptor (BCR) (29), and a MAb against B cell activating factor (BAFF), a critical B cell differentiation and survival cytokine (30). We chose these two pathways as there are available marketed drugs, ibrutinib and belimumab, targeting BTK and BAFF, respectively. In the clinic, ibrutinib, a small molecule drug, is administered orally using a daily regimen due to its relatively short half-life (31). Conversely, the anti-BAFF MAb has a relatively long half-life, as expected for an antibody, and belimumab is administered clinically via intravenous infusion at 2-week intervals for the first 3 doses and at 4-week intervals thereafter (32). We designed dosing schemes for both agents accordingly, with ibrutinib administered daily over 17-day periods and an anti-mouse BAFF MAb administered intravenously (i.v.) every 14 days or 28 days, as described below. Pilot studies showed that ibrutinib alone had little or no effect on anti-AAV IgM or IgG responses and did not enable vector redosing (Supplementary Fig. S2). We then investigated the ability of ImmTOR and ibrutinib to act synergistically to inhibit
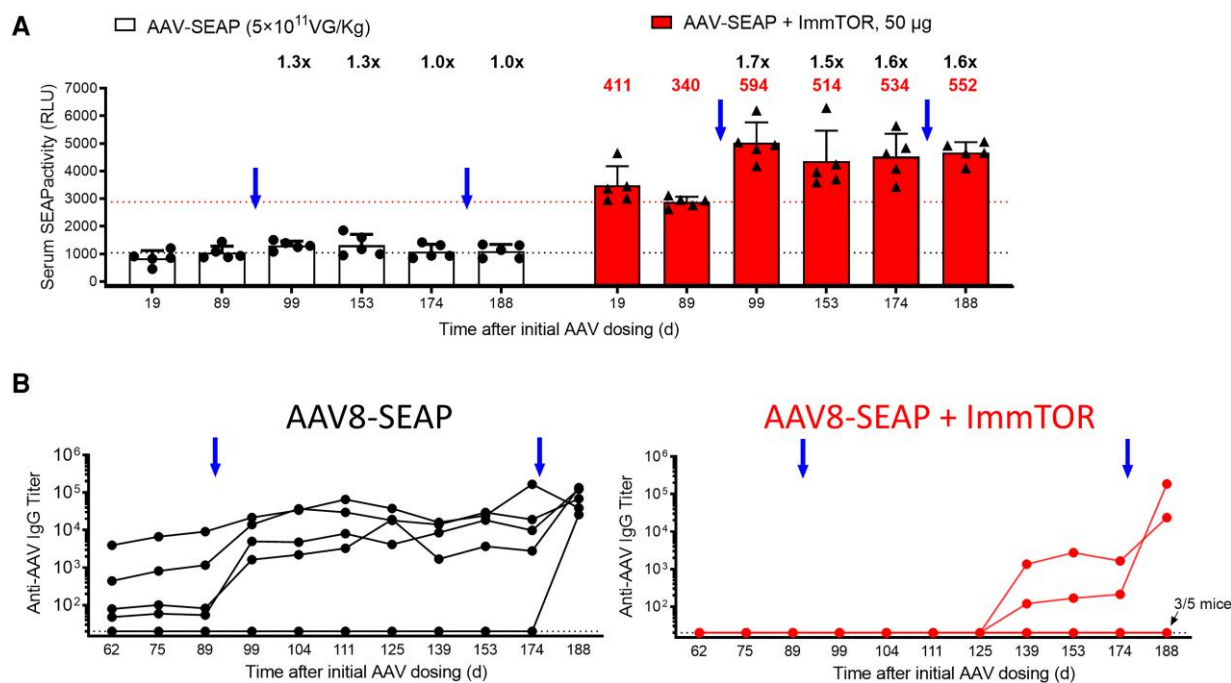
Fig. 1. Low-dose ImmTOR inhibits anti-AAV8 IgG and enables increased SEAP expression after two AAV8-SEAP vector doses but has no effect at the third vector injection. A) Serum SEAP dynamics. Mice (5/group) were injected 3 times with AAV8-SEAP (5E11 vg/kg) on days 0, 93, and 176 either alone or coadministered with low-dose ImmTOR (50 µg). AAV readministration days are indicated by arrows. Fold increases in SEAP expression postsecond dose (d99–d188) vs. preredosing (d89) are shown (upper text above columns) and percent increase in SEAP expression vs. day 19 untreated AAV-SEAP group are shown for all the time-points in the AAV8-SEAP + ImmTOR-treated group (lower text above columns). SEAP levels prior to the second vector dose are indicated for the AAV8-SEAP group (lower-dotted line) and the AAV8-SEAP + ImmTOR group (upper-dotted line). B) AAV8 IgG dynamics. Anti-AAV8 IgG titers are shown for days 62 to 188. AAV readministration days are indicated by arrows. The number of mice that remain seronegative at day 188 is indicated.

anti-AAV antibody responses and enable vector redosing. Ibrutinib was dosed at 20, 100, or 500 µg once a day orally for 17 days starting at 2 days prior to each AAV8-SEAP ± ImmTOR dose administered on Days 0, 93, and 161 (Fig. 3A). The control group administered AAV vector alone showed high levels of anti-AAV IgM antibodies in the initial period after the first dose (Fig. 3B) which declined as animals seroconverted to produce anti-AAV IgG antibodies (Fig. 3C). Interestingly, IgM levels in 3 of 5 animals in the control group increased after each subsequent administration of AAV despite having already seroconverted to IgG (Fig. 3B). Ibrutinib combined with ImmTOR suppressed IgM responses more effectively than ImmTOR alone in the 2-week interval after initial AAV administration (Fig. 3B, Supplementary Fig. S3A to C). Following vector readministration on Day 93, animals treated with ImmTOR + high-dose ibrutinib continued to show lower levels of anti-AAV IgM antibodies compared to animals treated with ImmTOR alone (Supplementary Fig. S3D to F), although all ImmTOR and ImmTOR + ibrutinib-treated animals remained IgG seronegative through Day 104 (Fig. 3C). However, by Day 153, some animals in the ImmTOR and ImmTOR + ibrutinib-treated groups seroconverted to IgG antibodies (Fig. 3C). Anti-AAV IgM antibodies increased after the third vector dose in some animals treated with ImmTOR or ImmTOR combined with 20 or 100 µg doses of ibrutinib, but remained low in the group treated with ImmTOR + 500 µg ibrutinib (Fig. 3B, Supplementary Fig. S3G to I). Similar synergistic activity of ImmTOR and ibrutinib on anti-AAV IgM and IgG responses was observed when reducing the regimen of ibrutinib from daily oral dosing to 3 times per week (Supplementary Fig. S4A to C).

The benefit of inhibiting anti-AAV IgM and IgG antibodies can be inferred from increased expression of the SEAP transgene product following repeat administration. As expected, control animals treated with AAV8-SEAP alone showed no increase in transgene expression after vector readministration, correlating with the development of anti-AAV antibodies. In contrast, coadministration of ImmTOR provided an approximately 2-fold increase in transgene expression after the second vector dose (Fig. 3D). The addition of ibrutinib provided a further increase in SEAP expression after vector redosing that reached a maximum benefit at 100 µg ibrutinib. The efficiency of AAV readministration was inversely correlated with the early IgM response after the initial vector administration as well as after vector readministration at multiple time-points (representative graphs shown in Supplementary Fig. S5A and B). A further increase in transgene expression was observed following the third vector dose, although the magnitude of increase was less marked perhaps due to the development of anti-AAV IgG antibodies in some animals. A similar inverse correlation was observed between immediate IgM levels after the third AAV8-SEAP dose and subsequent SEAP expression levels (Supplementary Fig. S5C). SEAP expression was approximately 5-fold higher in the groups treated with ImmTOR + 100 or 500 µg ibrutinib compared to vector alone at Day 203 following three doses of AAV-SEAP (Fig. 3D).

Anti-BAFF MAb combined with ImmTOR inhibits B cell maturation and their differentiation into plasmablasts leading to efficient control of anti-AAV IgM and IgG antibodies

An initial evaluation of an anti-BAFF MAb (aBAFF) showed modest blunting of the anti-AAV8 IgG response with little or no effect on IgM antibodies (Supplementarty Fig. S6A to C). Moreover, aBAFF
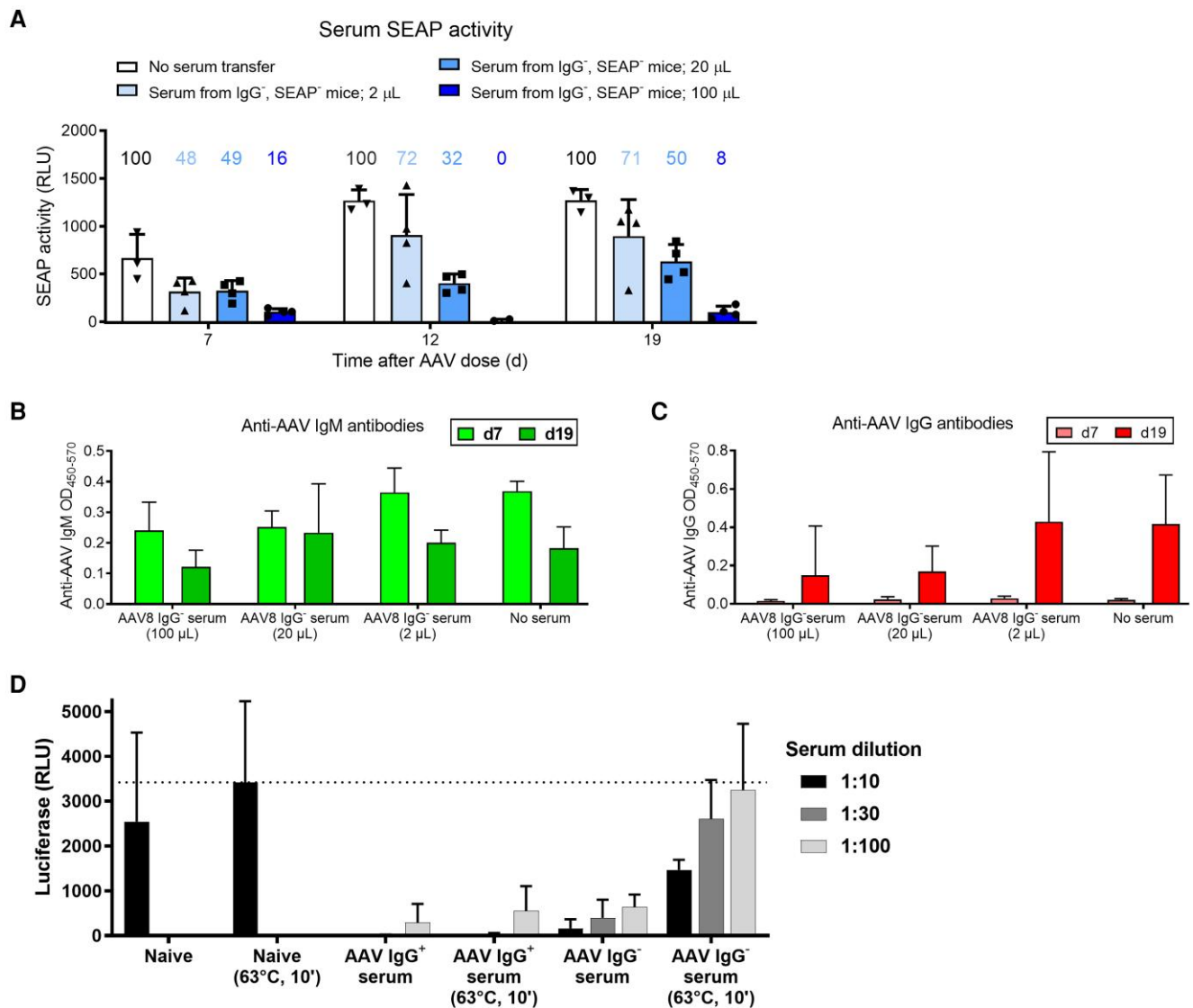
Fig. 2. IgM against AAV is the key factor determining lack of effective redosing in ImmTOR-treated mice with no detectable IgG to viral capsid. A–C) Passive immunization with anti-AAV8 IgG-negative serum prevents AAV transduction. Pooled sera from selected mice treated with ImmTOR + AAV8-SEAP were used to passively immunize naïve recipient mice 24 h prior to AAV8-SEAP administration. All individual serum samples used for the pool had low anti-AAV8 IgG (OD of 0.017 to 0.038 by AAV8 IgG ELISA) and no SEAP activity (described in Fig. S1B to D). Recipient mice were inoculated with 2, 20, or 200 µl of pooled sera, as indicated. A) SEAP dynamics. Relative SEAP levels vs. non-immunized control normalized to 100 are shown above each bar. B and C) De novo induction of anti-AAV8 IgM B) and IgG C) antibodies following AAV8-SEAP treatment in mice passively immunized with IgG-negative serum. D) Inhibition of in vitro AAV8-Luc transduction by untreated or heat-inactivated anti-AAV8 IgG-positive and IgG-negative serum. Pooled sera (from the studies described in Figs. 1 and S1) either possessing high levels of IgG against AAV8 or those determined to be IgG seronegative were maintained on ice or heat-inactivated at 63°C for 10 min and then incubated with AAV8-Luc at various dilutions as indicated. The AAV8-Luc/sera admixture was then used to transduce Huh-7 cells in vitro, and luciferase activity was assessed 24 h later.

treatment did not enable effective readministration of AAV (Supplementary Fig. S6D). We next evaluated whether aBAFF would have synergistic activity with ImmTOR when coadministered with a total of four doses of AAV (Fig. 4A). The combination of ImmTOR and aBAFF showed synergistic effect in inhibiting the anti-AAV IgM antibody response (Fig. 4B). This effect was dose-dependent with respect to the ImmTOR dose level and the aBAFF dose regimen. Specifically, IgM levels in the group treated with 150 µg of ImmTOR and eight doses of aBAFF (bottom panel) were statistically lower than those in the group treated with 150 µg of ImmTOR alone (third panel from the bottom), from day 108 to the end of the study at day 179, with $P < 0.05$ on days 108 to 112 and $P < 0.01$ from day 118 onwards (Fig. 4B). Stronger inhibition of the anti-AAV8 IgM response translated to the better

control of IgG seroconversion (Fig. 4C). A suboptimal dose of 50 µg ImmTOR provided strong inhibition of IgG responses through Day 91, 2 months after the second vector administration. However, breakthrough IgG responses increased with each subsequent vector dose. The addition of anti-BAFF to low-dose ImmTOR provided robust inhibition of anti-AAV IgG responses through Day 179, while the combination of anti-BAFF with 150 µg ImmTOR completely inhibited anti-AAV IgG responses. The strong inhibition of anti-AAV antibodies by ImmTOR and aBAFF enabled increases in SEAP transgene expression after each successive AAV dose, with SEAP levels that were up to 5-fold to 7-fold higher than in control mice treated with AAV8-SEAP alone (Fig. 4D and E). As observed with the combination of ImmTOR and ibrutinib, there was a strong inverse correlation between IgM and SEAP
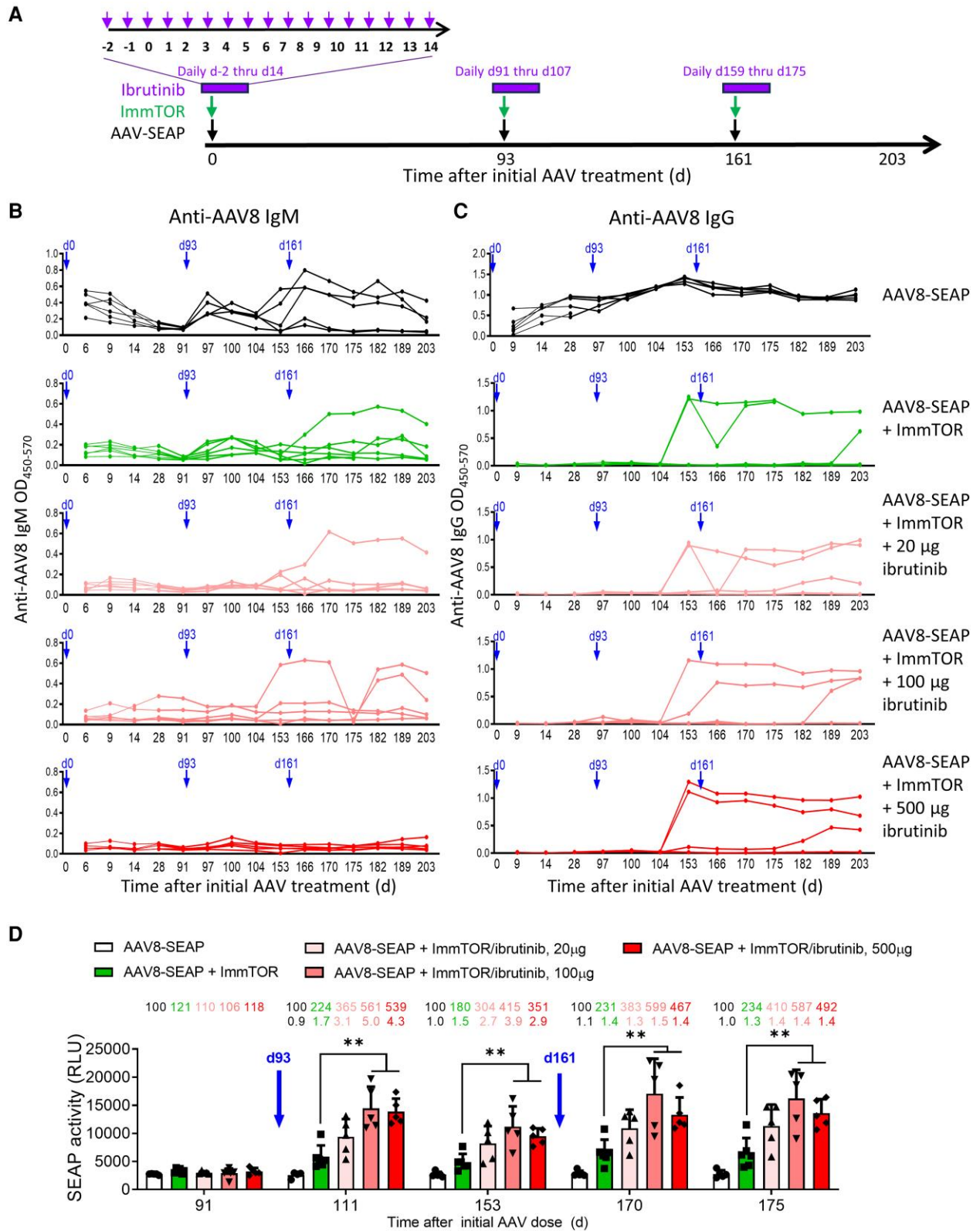
Fig. 3. Ibrutinib combined with ImmTOR inhibits anti-AAV IgM responses in a dose-dependent fashion and enables successful vector readministration. A) Experimental scheme. Five groups of mice (6 each) were injected with 5E11 vg/kg AAV8-SEAP on days 0, 93, and 161. One group was treated with ImmTOR alone (100 μg) and three groups received ImmTOR combined with ibrutinib (17 daily injections at 20, 100, or 500 μg/day, starting 2 days before through 14 days after each AAV injection). B and C) Anti-AAV8 IgM B) and IgG C) dynamics after three AAV administrations. AAV8 dosing days are indicated by arrows. D) SEAP expression dynamics after two AAV8-SEAP redosings. AAV8 redosing days are indicated by arrows. Percent increase in SEAP activity at each time-point vs. untreated group as 100 is indicated (top line). Transgene boosting efficacy with each dose (ratio of post-redosing SEAP expression vs. that immediately before redosing) for each group is also shown (bottom line). Time-points with statistical difference between SEAP expression in groups treated with ImmTOR combined with ibrutinib and ImmTOR alone are indicated (**P < 0.01).
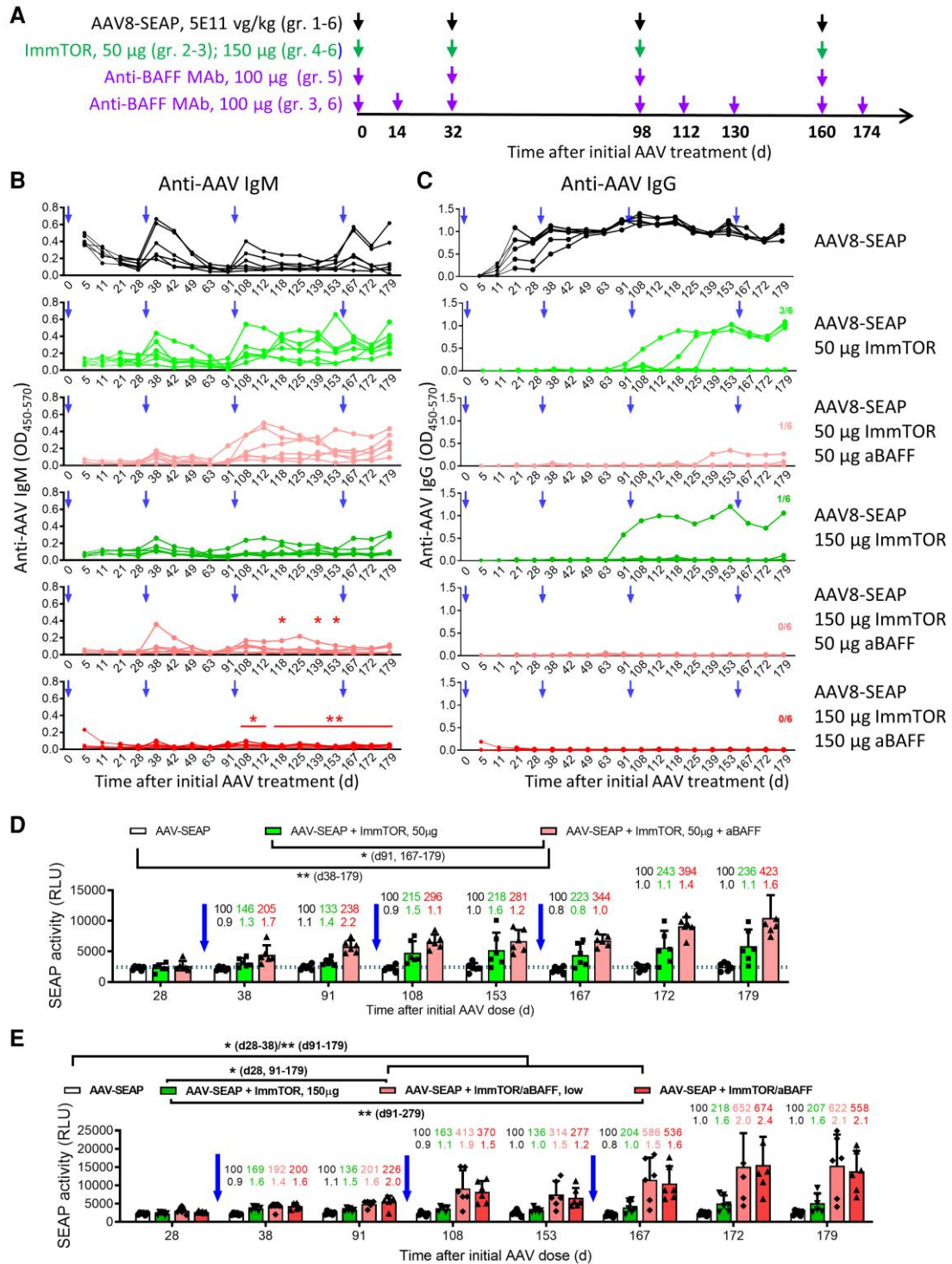
Fig. 4. Quadruple AAV dosing in combination with ImmTOR and aBAFF. A) Experimental scheme. Six groups of mice (6 each) were injected with 5E11 vg/kg AAV8-SEAP 4 times on days 0, 32, 98, and 160. Group 1 received only the four AA8-SEAP injections. Groups 2 and 3 received low-dose ImmTOR (50 µg) concurrently with AAV, either without or with an 8-dose course of aBAFF (100 µg, days 0, 14, 32, 98, 112, 130, 160, and 174), respectively. Groups 4, 5, and 6 received a standard dose of ImmTOR (150 µg) concurrently with AAV without or with a 4-dose course (days 0, 32, 98, 160) or 8-dose course (days 0, 14, 32, 98, 112, 130, 160, and 174) of aBAFF (100 µg), respectively. B and C) Anti-AAV8 IgM B) and IgG C) dynamics after four AAV administrations. Days of AAV8 administration (days 0, 32, 98, and 160) are indicated by arrows; see panel A for details on dose regimen of other agents. Time-points with statistical difference between IgM levels in groups receiving 150 µg ImmTOR alone (third panel from the bottom) vs. those receiving the same ImmTOR dose combined with aBAFF (two bottom panels) are indicated in panel B (*$P < 0.05$, **$P < 0.01$). Numbers of mice (out of total) in each ImmTOR-treated group becoming IgG-positive by day 179 is shown in panel C. D and E) Serum SEAP expression dynamics after three AAV8-SEAP redosings in arms receiving low D) or standard E) dose of ImmTOR with or without aBAFF. AAV8 redosing days (days 32, 98, and 160) are indicated by arrows; see panel A for details on dose regimen of other agents. Relative levels of transgene expression for each experimental group vs. the untreated control (normalized to 100) is shown for each time-point as indicated in the top line above each bar. Transgene boosting efficacy of treatment following each round of AAV readministration is calculated as the ratio of postdosing SEAP expression vs. that immediately before redosing for each treatment group, as indicated in the bottom line above each bar. Time-points with statistical difference between SEAP expression in different groups are indicated (*$P < 0.05$, **$P < 0.01$).
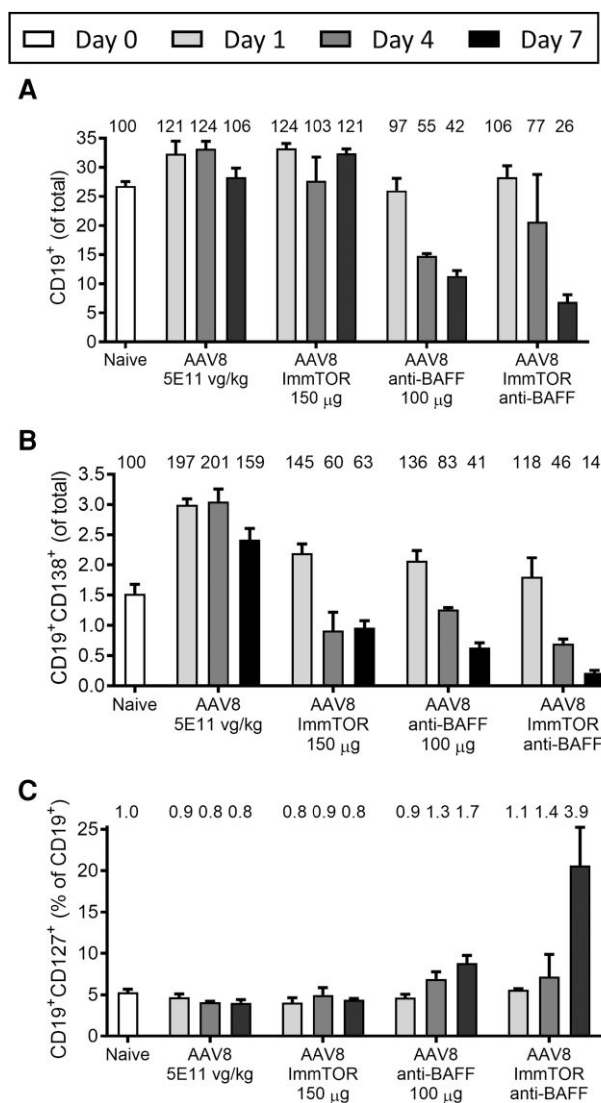
Fig. 5. ImmTOR and aBAFF synergize to decrease splenic B cells, plasmablasts, and elevate pro-/pre-B cells counteracting the activation by AAV antigen. A to C) Size of splenic fractions of CD19$^+$ A), CD19$^+$CD138$^+$ B), and CD19$^+$CD127$^+$ C) cells at 1, 4 or 7 days after inoculation with AAV8 (5E11 vg/kg) alone or combined with ImmTOR (150 μg) and/or aBAFF (100 μg) as indicated. The size of each cell fraction vs. that of naïve mice (day 0, no treatment) as 100 (A, B) or 1.0 C) is shown. Three mice per each time-point in each group have been used.

levels in animals treated with ImmTOR + anti-BAFF at both early (day 11) and late (day 160) IgM time-points (Supplementary Fig. S7). To our knowledge, this is the first demonstration of the ability to administer four successive AAV vector doses.

The effects of ImmTOR and aBAFF on various B cell and pre-B cell populations were then investigated. Overall levels of mature B cells and, especially, of CD19$^+$CD138$^+$ plasmablasts were significantly decreased by both ImmTOR and aBAFF and this effect was synergistic (Fig. 5A and B). The plasmablast population decreased 10-fold at 7 days after AAV coadministered with ImmTOR and aBAFF compared to AAV alone (Fig. 5B). ImmTOR alone did not affect mature splenic B cells, while aBAFF affected both mature B cells and plasmablasts and synergized with ImmTOR (Fig. 5A and B). Conversely, the fraction of immature splenic pre-/pro-B cells was elevated in mice treated with ImmTOR and aBAFF (Fig. 5C). Similar effects were observed if

ImmTOR and aBAFF combination treatment was applied without AAV (Supplementary Fig. S8).

ImmTOR + anti-BAFF MAb mitigate immunogenicity and enable vector redosing at a high vector dose of 5E13 vg/kg

Severe adverse effects of AAV gene therapy have been associated with vector doses of 1E14 vg/kg or higher. We next evaluated the ability of ImmTOR + aBAFF to mitigate antibody responses and enable repeat administration of vector doses of 5E13 vg/kg (Fig. 6). A control group receiving a single vector dose of 1E14 vg/kg was compared to test groups receiving two doses of 5E13 vg/kg ± ImmTOR and/or aBAFF (Fig. 6A). Recent studies have shown that three monthly doses of ImmTOR provides more durable inhibition of anti-AAV antibodies in mice and nonhuman primates (33), and monthly injections of ImmTOR have been recently shown to be well tolerated in human clinical trials (34). Therefore, we employed three monthly ImmTOR injections in this study to provide a more effective and clinically applicable regimen to mitigate antibody responses to high vector doses. As expected, animals treated with vector alone showed high IgM levels after dosing which rapidly declined by Day 30 as animals seroconverted to IgG (Fig. 6B and C). Anti-BAFF alone showed no appreciable effect on either IgM or IgG responses to AAV, while ImmTOR alone initially blunted the IgM response and delayed IgG production in most animals but was not able to maintain control after redosing (Fig. 6B and C). In contrast, the ImmTOR and aBAFF combination exhibited good control of the IgM response while preventing seroconversion to IgG after the initial vector dose of 5E13 vg/kg. While IgM levels increased after the second dose, most animals remained IgG-negative at the end of the study (Fig. 6B and C).

Importantly, the ability to inhibit the anti-AAV IgG and IgM responses enabled effective redosing at vector doses of 5E13 vg/kg (Fig. 6D). As expected, the control group administered a single dose of 1E14 vg/kg initially showed higher levels of SEAP expression compared to the test groups after the first dose of 5E13 vg/kg. It is notable that groups treated with ImmTOR showed increased SEAP expression compared to other groups that were administered 5E13 vg/kg AAV8-SEAP, consistent with previous findings that ImmTOR enhances transgene expression at the first dose (17). After the second vector dose of 5E13 vg/kg at Day 56, the group treated with ImmTOR and aBAFF showed a significant increase in transgene expression that reached or exceeded levels observed in the control group dosed with a single vector dose of 1E14 vg/kg. In contrast, all other groups show little or no increase in transgene expression after the second dose. These results demonstrate for the first time the ability to successfully redose AAV at a high vector dose of 5E13 vg/kg.

Discussion

This study extends earlier findings which showed the ability of ImmTOR to enable effective AAV redosing at moderate vector doses of 2.5E12 vg/kg (7, 16, 17, 24). Here, we report that low doses of 50 to 100 μg ImmTOR provided suboptimal activity in vector redosing despite effective control of anti-AAV IgG immune responses. Our data indicate that the residual neutralizing activity is due to incomplete suppression of the anti-AAV IgM response. Mechanistically, ImmTOR has been shown to induce tolerogenic antigen-presenting cells which lead to the induction of antigen-specific regulatory T cells that can inhibit T cell-dependent B cell
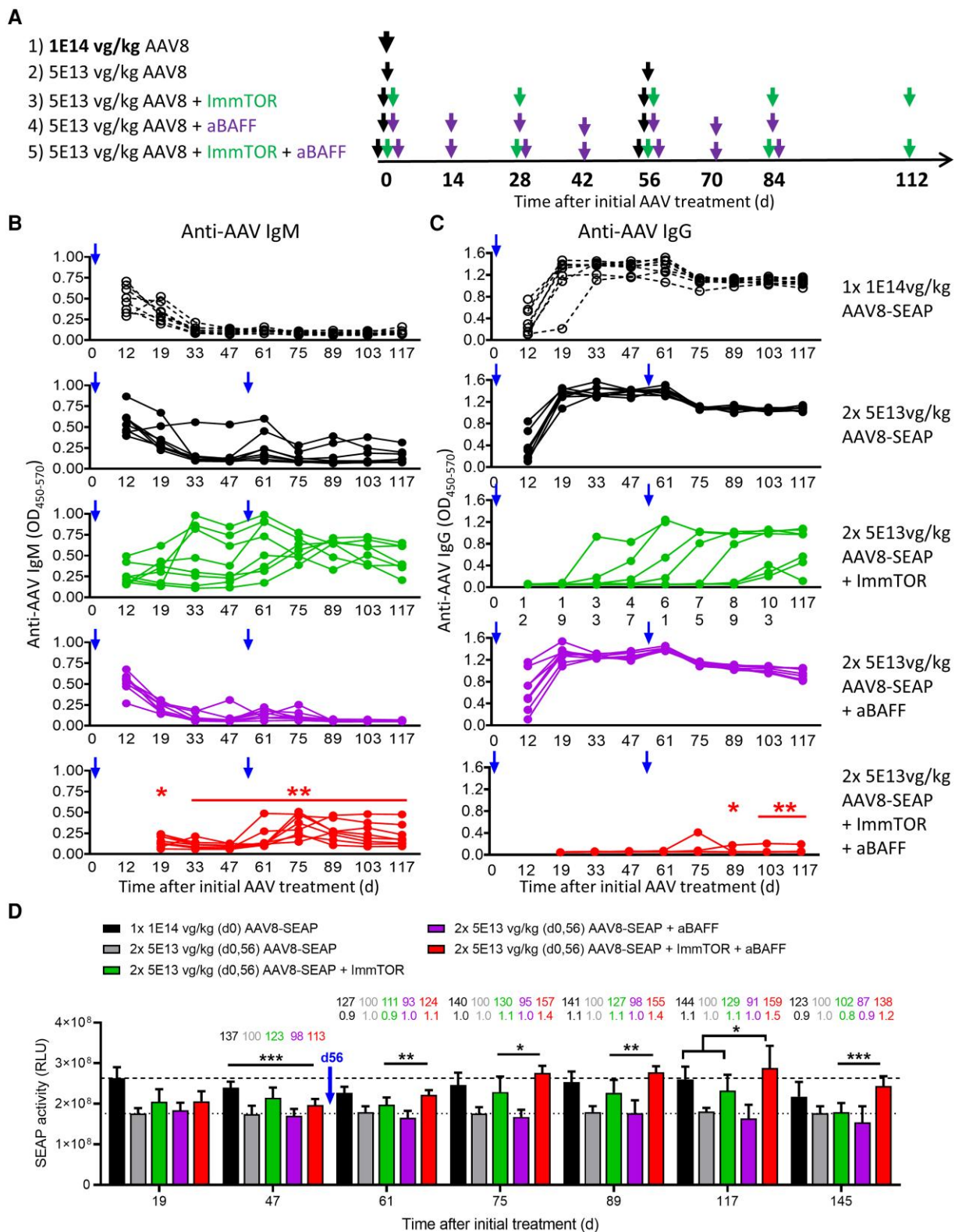
Fig. 6. ImmTOR and aBAFF enables readministration of high doses of AAV. A) Experimental scheme. Five groups of mice (8 each) were injected with either a single dose of 1E14 vg/kg AAV8-SEAP at day 0 (Group 1) or two doses of 5E13 vg/kg at days 0 and 56 (Groups 2–5). Animals receiving two injections of 5E13 vg/kg either received no further treatments (Group 2), monthly doses of ImmTOR (200 μg; Group 3), biweekly doses of aBAFF (100 μg; Group 4) through day 28 followed by monthly doses through day 112, or the combination of ImmTOR and aBAFF (group 5), as indicated. B and C) Anti-IgM B) and IgG C) dynamics (times of AAV8-SEAP dosing indicated by arrows). D) SEAP dynamics from day 19 to the end of the study (day 145). AAV redosing at day 56 is indicated by arrow. Day 19 SEAP level in Group 1 injected with 1E14 vg/kg AAV8 is shown by the dashed black line and that in Group 2 injected with 5E13 vg/kg AAV8 is shown by the dotted gray line. Transgene expression levels for each experimental group at each time-point vs. that in untreated group injected with 5E13 vg/kg AAV8 normalized to 100 are indicated (top line). Transgene boosting efficacy (ratio of postredosing SEAP expression vs. that immediately before redosing) for each group is also shown (bottom line). Statistical significance of differences between groups treated with ImmTOR vs. treated with ImmTOR and aBAFF combination in panels B to D is indicated as is the difference in SEAP levels between group dosed with 1E14 vg/kg AAV vs. group dosed twice with 5E13 vg/kg AAV and treated with ImmTOR and aBAFF ($*P < 0.05$, $**P < 0.01$, $***P < 0.01$).

responses (26, 35). However, the initial production of IgM can occur in a T cell-independent fashion, and the prevention of helper T cell-mediated class-switching to IgG by ImmTOR may lead to prolonged IgM production. The IgM antibodies were shown to be capable of neutralizing AAV transduction both in vitro and in vivo. The magnitude of IgM levels in ImmTOR-treated mice prior to redosing showed an inverse correlation with postredosing transgene expression levels and redosing efficacy. We therefore tested the combination of ImmTOR with two B cell-targeting agents, ibrutinib, a small molecule inhibitor of the B cell receptor-activated Bruton's tyrosine kinase, and an MAb against BAFF, a key component of B cell survival factor, for the ability to mitigate anti-AAV IgM responses and enable more efficient vector redosing.

BTK is a critical component in the signal transduction pathway downstream of surface-expressed BCR. Activated BTK induces cytoplasmic calcium influx and leads to upregulation of many transcription factors including NF-κB (36, 37), which collectively are instrumental for B cell activation, proliferation, differentiation, and survival (38–40). Pharmacological inhibition of BTK in mice showed significant reductions in marginal zone B cell subsets that are involved in T cell-independent antibody responses as well as T2 and T3 but not T1 transitional B cells, leading to inhibition of both IgM and IgG antibody responses. Ibrutinib (PCI-32765) is an oral irreversible BTK inhibitor (41) that is approved for the treatment of several B cell-related malignancies (29, 31, 39).

Xiang et al. (12) reported that ibrutinib with rapamycin provided more effective suppression of anti-AAV antibody responses than either agent alone. Rapamycin alone was only modestly effective at inhibiting anti-capsid IgG responses, even when administered at immunosuppressive daily doses of 25 µg/mouse/day starting 3 to 7 days before AAV administration and continuing for 3 to 4 weeks afterwards. In contrast, single doses of 50 to 100 µg ImmTOR administered at the time of AAV dosing provided effective inhibition of anti-AAV IgG responses. The combination of ibrutinib with rapamycin also required a more aggressive regimen of ibrutinib (25 mg/kg administered 6 times per week for four consecutive weeks starting one week prior to AAV administration) than when combined with ImmTOR (~4 to 5 mg/kg administered 3 times per week for 2 weeks starting at the time of vector administration).

ImmTOR combined with aBAFF was even more effective than the combination with ibrutinib. BAFF is a survival and maturation factor for B cells that belongs to the TNF superfamily (42–44). Belimumab, a humanized anti-human BAFF MAb is approved for the treatment of systemic lupus erythematosus (32). While ImmTOR alone had little or no effect on total splenic B cells or immature pre-B cells, ImmTOR enhanced the activity of aBAFF to inhibit B cell maturation, resulting in fewer mature splenic B cells and an increased fraction of pre-/pro-B cells. The combination also synergized to inhibit B cell proliferation and subsequent transition to Ig-producing plasmablasts. Importantly, the combination of ImmTOR and aBAFF enabled administration of four successive doses of AAV, leading to transgene expression levels that were 5.5 to 7 times higher than in mice treated with AAV alone (Fig. 4E). To our knowledge, this is the first time that AAV has been successfully administered 4 times.

Serious adverse events, including several patient deaths, have been observed at high vector doses of 1E14 vg/kg or higher (9, 45–50). Many of the adverse events associated with systemic AAV gene therapy are thought to be caused by immune responses to the vector, such as liver inflammation associated with capsid-specific CD8 T cells and thrombotic microangiopathy, associated with complement activation. One potential strategy to mitigate safety issues associated with vector doses of ≥1E14 vg/kg is to enable multiple smaller doses of vector (9). Here, we demonstrate for the first time, successful readministration of vector doses of 5E13 vg/kg. Two doses of 5E13 vg/kg administered in combination with ImmTOR and aBAFF resulted in transgene expression similar to or higher than that achieved with a single vector dose of 1E14 vg/kg (Fig. 6D). These results suggest that the combination of ImmTOR + aBAFF may enable dose titration of lower doses of AAV to mitigate toxicities associated with high vector doses.

Materials and methods
ImmTOR, ibrutinib, and anti-BAFF molecules
ImmTOR nanoparticles were prepared as previously described (25, 26). Dosing of ImmTOR was calculated based on the active ingredient, rapamycin (Concord Biotech, Ahmedabad, India), within the range of 200 to 400 µg/mouse. Particle size was determined by dynamic light scattering, and polydispersity index (PDI) was analyzed using a Malvern Zetasizer Nano-ZS ZEN 3600. Antigen-loaded nanoparticles had a particle size distribution within 140 to 155 nm range coupled with a low PDI of <0.15. Ibrutinib (PCI-32765) was purchased from Advanced Chemblocks Inc. (Hayward, CA). Mouse anti-BAFF monoclinal antibody (Sandy-2) was procured from AdipoGen Life Sciences (San Diego, CA).

Viruses
AAV8-SEAP and AAV8-Luc were manufactured as described earlier (SAB Tech, Philadelphia, PA, USA) (24). Briefly, AAV vectors were produced by triple transfection in human embryonic kidney 293 cells and harvested 72 h later. The vectors were purified by $CsCl_2$ gradient centrifugation. Viral titer was determined by SDS-polyacrylamide gel electrophoresis gel followed by silver staining, and, in case of AAV8-SEAP, was confirmed by qPCR using SEAP-specific primers (forward primer, 5′-AGTTTGTCCTTCT TCTGCCCTTT-3′; reverse primer, 5′-TGGGTGCCGCCAAGAA-3′, Thermo Fisher Scientific, Waltham, MA).

Mice
Female C57BL/6 mice (36 to 52 days, 17 to 18 g) were acquired from Charles River Laboratories (Wilmington, MA). Mice were acclimated to the Animal Care Facility at Selecta Biosciences for at least 3 days (and generally, for 7 to 8 days) prior to initial treatment in order to minimize the potential effects of transportation-related stress. All the animal experiments were approved by Selecta's Institutional Animal Care and Use Committee (IACUC) and were conducted in strict compliance with the NIH Guide for the Care and Use of Laboratory Animals and other federal, state, and local regulations.

Animal injections
Mice were injected i.v. (via tail vein or retro-orbital plexus) either with AAV8-SEAP alone or combined as an admixture with ImmTOR nanoparticles in the effective range of 50 to 200 µg. Anti-BAFF MAb was administered intraperitoneally (i.p., 100 µg). Ibrutinib was diluted in propylene glycol to 10 mg/mL for storage, filtered and on the day of injection diluted to desired concentration (2-fold to 50-fold) in 1% Tween 80 in phosphate-buffered saline (PBS) and administered daily (i.p.) at effective range of 20 to 500 µg. For passive immunization studies, pooled anti-AAV8 IgG-negative sera were collected from selected animals treated

with AAV8 + ImmTOR. Naive recipient animals were injected with sera 24 h before AAV-SEAP administration. Bleeds were done via submandibular route using serum collection tubes, samples spun for 5 min at 11,640 rpm, transferred to Eppendorf tubes and placed in −20°C storage for in-house SEAP, IgM and IgG analysis.

SEAP assay

SEAP transgene product levels were analyzed with the Phospha-Light SEAP Reporter Gene Assay System (Invitrogen, Carlsbad, CA) as previously described (24). Samples were read on a SpectraMax M5 or SpectraMax L plate reader (Molecular Devices, San Jose, CA, USA) and the output was reported as relative luminescence units.

Enzyme-linked immunosorbent assay for IgM and IgG against AAV8

Anti-AAV8 IgM and IgG antibodies were analyzed by enzyme-linked immunosorbent assay (ELISA) as previously described (24). IgM antibodies were detected with a donkey anti-mouse IgM-specific horseradish peroxidase (HRP; 1:5,000; Jackson ImmunoResearch, West Grove, PA, USA), and IgG antibodies were detected with a rabbit anti-mouse IgG-specific HRP (1:1,500; Jackson ImmunoResearch). The presence of bound IgM or IgG antibodies was visualized using trimethylboron substrate. The optical density (OD) was measured at 450 nm with a reference wavelength of 570 nm. Serum EC50 (median effective concentration) was determined as described earlier (24) using serial 1:3 dilutions of serum. The EC50 was calculated by four-parameter logistic curve fit (Softmax Pro, Molecular Devices, San Jose, CA). A positive control anti-AAV8 IgG antibody (Fitzgerald Industries, Acton, MA) was used to create a standard curve.

In vitro AAV neutralization

In vitro neutralization of AAV was analyzed as previously described (24). Huh-7 cells were seeded overnight at 50,000 cells per well in 96-well tissue culture plates. The next day, normal or immune mouse serum (1:10 to 1:100 dilution) was admixed with AAV8-Luc (10,000 multiplicities of infection) and then added to the Huh-7 cells after 24 h. Luciferase activity in cell lysates was determined using the Luciferase Assay System (Promega Life Sciences, Madison, WI, USA).

Flow cytometry (murine splenic cell populations)

Splenocytes were harvested by mechanical passage through a 70 μm nylon mesh (Thermo Fisher). Followed by lysis of red blood cells in 150 mM NH_4Cl, 10 mM $KHCO_3$, 10 μM Na_2-EDTA. Cells were washed in PBS containing 2% bovine serum and then filtered again through nylon mesh. Cells were blocked with an anti-CD16/32 antibody (Fc-block, clone 93, BioLegend, San Diego, CA) and then stained with antibodies directed against cell surface receptors: CD19 (clone 6D5), CD138 (clone 281-2), CD127 (clone A7R34), IgM (clone RMM-1), IgD (clone 11-26c.2a), CD21 (clone 7E9), CD23 (B3B4), all from BioLegend. Cells were interogated by flow cytometry (FACSymphony A3 Cell Analyzer; BD Biosciences) and data were analyzed by FlowJo software (TreeStar, Ashland, OR).

Statistical analysis

Pairwise comparison of the mouse experimental groups were analyzed by either multiple t-test (for several time-points) or Mann-Whitney two-tailed test (for a single time-point; individual comparison of two groups presented within the same graph) using GraphPad Prism 9.4.1 software. Significance is indicated in each figure and specified in figure legend (* – $P < 0.05$, ** – $P < 0.01$; *** – $P < 0.001$; **** – $P < 0.0001$; not significant – $P > 0.05$). All summary data for individual experimental groups is presented as mean ± SD (shown as error bars).

Supplementary Material

Supplementary material is available at *PNAS Nexus* online.

Author Contributions

T.K.K. and P.O.I. designed the research, analyzed data, and wrote the paper; C.R., A.M., G.R., and T.C. performed the research and analyzed data; S.S.L. designed the assays and analyzed data.

Data Availability

All data are included in the manuscript and/or Supplementary Material. Materials will be shared upon reasonable request and subject to a materials transfer agreement.

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