Enhancement of liver-directed transgene expression at initial and repeat doses of AAV vectors admixed with ImmTOR nanoparticles

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Systemic AAV (adeno-associated virus) gene therapy is a promising approach for the treatment of inborn errors of metabolism, but questions remain regarding its potency and durability. Tolerogenic ImmTOR nanoparticles encapsulating rapamycin have been shown to block the formation of neutralizing anti-capsid antibodies, thereby enabling vector re-administration. Here, we further demonstrate that ImmTOR admixed with AAV vectors also enhances hepatic transgene expression at the initial dose of AAV vector, independent of its effects on adaptive immunity. ImmTOR enhances AAV trafficking to the liver, resulting in increased hepatic vector copy numbers and transgene mRNA expression. Enhanced transgene expression occurs through a mechanism independent of the AAV receptor and cannot be replicated in vivo with free rapamycin or empty nanoparticles. The multipronged mechanism of ImmTOR action makes it an attractive candidate to enable more efficient transgene expression at first dose while simultaneously inhibiting adaptive responses against AAV to enable repeat dosing.

INTRODUCTION

The goal of gene therapy for inherited diseases is to provide lifelong correction of the genetic defect. AAV has become the platform of choice for most in vivo gene therapy applications due to its demonstrated safety and efficacy in human clinical trials, highlighted by the approval of the first two AAV gene therapeutics by the U.S. Food and Drug Administration (1–5). However, questions about the durability of AAV-mediated gene therapy remain because of the nonreplicating, episomal nature of AAV vectors. Colella et al. (6) recently highlighted several challenges in the ability to achieve and maintain therapeutic levels of transgene expression. One issue is the potential waning of transgene expression over time due to cell proliferation in the growing target organ of pediatric patients, resulting in vector genome dilution or to cell turnover as an outcome of dam-

We have previously reported development of ImmTOR nanoparticles encapsulating rapamycin (also called SVP-Rapamycin) that have been shown to induce a tolerogenic immune response to co-administered biologic therapies via induction of tolerogenic dendritic cells and antigen-specific regulatory T cells and reduction in antigen-specific B cell activation (18–20). Moreover, the addition of ImmTOR to AAV gene therapy vectors was recently shown to effectively and specifically inhibit adaptive antibody and T cell immune responses against AAV capsid, thereby enabling successful repeat administration of AAV vectors in mice and nonhuman primates (21). Here, we further demonstrate that co-administration of ImmTOR with AAV-based vectors also enhances transgene expression after the first dose of AAV vector in naïve mice. This beneficial effect of ImmTOR on first dose transgene expression appears to be independent of its immunomodulatory effects on adaptive immunity and cannot be achieved by free rapamycin. Admixing ImmTOR and AAV before injection is important for enhanced transgene expression after the first dose, but it is not required for inhibition of the antibody response to AAV or for repeated administration of AAV vectors. Mechanistically, our data suggest that ImmTOR can enhance trafficking of AAV to the liver and increase transduction of hepatocytes in a manner that appears to be independent of the AAV receptor (AAVR). This multipronged mechanism of ImmTOR action makes it an attractive candidate to enhance systemic gene therapeutic applications, particularly in those clinical indications where repeat vector dosing may be necessary.

RESULTS

ImmTOR elevates AAV-driven transgene expression at initial administration

The ability of ImmTOR to enable productive transgene expression after a second dose was originally demonstrated in a heterologous transduction scheme in which animals were treated with two different AAV8 vectors expressing different transgenes administered 21 days apart. These studies showed that expression of the second...
transgene, human factor IX, could only be detected when the immune response to the first vector dose was inhibited by co-administration of ImmTOR (20). Expression of the first transgene was not evaluated. Here, we evaluated the effects of ImmTOR on repeat administration of the same AAV vector expressing secreted embryonic alkaline phosphatase (AAV8-SEAP). As previously shown, admixing ImmTOR with AAV inhibited the formation of anti-AAV8 capsid immunoglobulin G (IgG) antibodies (Fig. 1A). Co-administration of AAV8-SEAP with ImmTOR enabled a boost in SEAP expression after a second dose administered 56 days after the initial dose, compared to mice treated with AAV8-SEAP alone, which showed no increase in transgene expression after repeat dosing (Fig. 1B). This effect was not dependent on mouse strain or sex (Fig. S1), two factors that are known to affect the levels of AAV-driven transgene expression and immune response polarization (22, 23). Unexpectedly, the addition of ImmTOR to AAV8-SEAP also showed a beneficial effect on transgene expression even after the first dose (Fig. 1B and fig. S1, B and C). This increase in transgene expression was evident as early as 7 days after initial dosing and reached levels approximately 2.5- to 3-fold higher than that observed in mice treated with AAV8-SEAP alone (Fig. 1B and table S1). Transgene expression further increased approximately twofold after a second dose administered at day 56. Thus, the addition of ImmTOR to AAV8-SEAP provided a cumulative benefit of approximately fourfold increased SEAP expression after two doses compared to mice treated with AAV8-SEAP alone. Similar results were observed using different reporter transgenes, such as luciferase and green fluorescent protein (GFP) (fig. S2). The effect of ImmTOR on enhancing first dose transgene expression could not be replicated by using free rapamycin, empty nanoparticles (NP-Empty), or their combination (Fig. 1C and table S2). This effect appeared to be dose dependent (Fig. S3B), with the lower ImmTOR doses being not as effective in AAV antibody suppression (Fig. S3A). Generally, little or no additional benefit of increasing ImmTOR doses beyond 300 μg was seen for either suppression of anti-AAV antibodies or enhancement of transgene expression (fig. S3, C to E). Elevation of transgene expression by ImmTOR was also observed with AAV5 (fig. S3E).

Adaptive immunity and complement play no role in enhancing first dose AAV transgene expression by ImmTOR

The rapid enhancement of transgene expression after a single dose of AAV + ImmTOR in naïve mice suggested that the effect of ImmTOR on first dose transgene expression in naïve mice was independent of its immunomodulatory effects on the adaptive immune response. This hypothesis was further evaluated using immunodeficient Rag2-knockout (Rag2-KO) mice that are devoid of T and B lymphocytes. The addition of ImmTOR to AAV-SEAP vector enhanced SEAP expression in Rag2-KO mice to a degree similar to that observed in wild-type mice, indicating that T and B cells were not necessary for the first dose benefit of ImmTOR (fig. S4A). As expected, Rag2-KO mice, but not wild-type mice, treated with AAV-SEAP alone showed an increase in transgene expression after the second dose, confirming that the adaptive immune response was responsible for preventing vector redosing in wild-type mice. However, Rag2-KO mice treated with AAV-SEAP + ImmTOR showed an even higher cumulative expression of SEAP due to the enhancement of SEAP expression after the first dose. Depletion of complement before AAV administration, either alone or combined with ImmTOR, did not further increase transgene expression (fig. S4B).

Immediate ImmTOR effect on AAV vector is dependent on their delivery as an admix

Because ImmTOR is known to accumulate in the liver (22), we next determined whether admixing might enhance trafficking of AAV to the liver. Potential interaction between ImmTOR and AAV particles was assessed by dynamic light scattering. The smaller 20-nm AAV particles could be distinguished from larger 150-nm ImmTOR particles; however, after admixing, the AAV peak and the ImmTOR peaks coalesced into a single peak (fig. S5). A similar effect was seen using NP-Empty of the same polymer composition and near-identical surface charge (−8.6 ± 0.1 mV in NP-Empty versus −8.9 ± 0.1 mV in ImmTOR), indicating that the interaction between virions and ImmTOR was not directly dependent on rapamycin cargo. However, the surface charge (as measured by zeta potential) of the resulting ImmTOR and AAV complex changed more markedly and was different from that of NP-Empty and AAV (−1.1 ± 0.2 mV versus −2.9 ± 0.1 mV, respectively), indicating the potential for stronger association of ImmTOR with AAV.

We next evaluated the effects of admixing of AAV + ImmTOR before retro-orbital administration versus sequential administration of ImmTOR in one eye followed by administration of AAV in the other eye. We also assessed whether the effect of ImmTOR on first dose transgene expression was related to more efficient transduction or to posttransduction steps such as mRNA transcription or protein expression of the transgene. Admixing of AAV-SEAP and ImmTOR showed a substantial increase in viral genome copy number as early as 72 hours after injection compared to sequential administration of AAV-SEAP and ImmTOR or dosing with AAV-SEAP alone. The increased transduction with admixed AAV-SEAP + ImmTOR was followed by higher levels of transgene mRNA in the liver and SEAP activity in the blood (Fig. 2, A to D, and table S3). Thus, there was a clear correlation between higher hepatic vector genome copies, hepatic transgene transcription, and transgene-encoded protein expression. The mode of ImmTOR administration (admixed versus non-admixed) made no substantial difference to the suppression of anti-AAV IgG antibodies (Fig. 2E), again indicating that the mechanism of first dose enhancement of transgene expression was distinct from that involved in the inhibition of adaptive immunity that allows vector redosing.

Admixing of ImmTOR to AAV vector leads to increased AAV trafficking to hepatocytes

We further investigated the impact of admixed ImmTOR on AAV liver trafficking using ImmTOR containing an encapsulated Alexa488 fluorescence probe combined with either Alexa647 fluorophore-conjugated AAVAnc80 virions or an AAVAnc80-enhanced GFP (eGFP) reporter construct. When Alexa488-labeled ImmTOR and Alexa647-labeled AAVAnc80 were admixed before injection, an elevation of Anc80 virion trafficking to hepatocytes was observed at 48 hours after inoculation compared to mice injected with Alexa647- AAVAnc80 alone (Fig. 3A). Because conjugation of a fluorescent probe to the surface of AAV could potentially affect its interaction with ImmTOR or its ability to transduce cells, we also assessed the effect of ImmTOR on expression of a GFP transgene when admixed with an AAVAnc80-GFP vector. An increase in GFP expression was evident in hepatocytes of mice treated with the admix compared to mice treated with AAVAnc80-GFP alone (Fig. 3B).
Fig. 1. ImmTOR suppresses induction of IgG against AAV and elevates transgene expression after initial and repeat co-administrations with AAV vector. (A and B) C57BL/6 mice \((n = 6)\) were injected on days 0 and 56 with AAV8-SEAP \((5 \times 10^{11} \text{ vg/kg})\) alone or admixed with 100 \(\mu\)g of ImmTOR. OD, optical density; RLU, relative luminescence unit. Anti-AAV IgG (A) and SEAP activity (B) were measured at times indicated. SEAP activity in the AAV8-SEAP + ImmTOR–treated group is shown as fold increase over the group receiving AAV8-SEAP alone (top line), and its ratio after AAV re-administration (red arrow) versus that before redosing (d46) is also shown (bottom line; untreated, nonbold; ImmTOR-treated, bold). (C) ImmTOR, but not free rapamycin or NP-Empty, enhances transgene expression after co-administration with AAV vector. BALB/c males \((n = 6)\) were injected with AAV8-SEAP \((5 \times 10^{11} \text{ vg/kg})\) alone or combined with ImmTOR \((200 \mu\)g), equivalent mass of NP-Empty, or empty NP combined with 200 \(\mu\)g of free rapamycin. SEAP activity in the ImmTOR-treated group is shown as fold increase over that group receiving AAV8-SEAP alone. AAV IgG and SEAP levels between the test groups (A and B) or between group treated with ImmTOR and all test groups (C) were different at every time point \((P < 0.01, \text{ multiple t test})\).
Fig. 2. ImmTOR admixed to AAV provides higher transduction and mRNA and protein expression but similar inhibition of IgG response. (A to D) Groups of C57BL/6 female mice (n = 20) were injected with AAV8-SEAP (5 × 10^{11} vg/kg) alone or combined with 100 μg of ImmTOR, administered either as an admix or sequentially (non-admix) as shown in experimental scheme (A). Livers were harvested from five mice per group at each time point and analyzed for vector DNA (B) and SEAP mRNA (C). Sera from the same animals were analyzed for SEAP activity (D). Vector genome copies (vg per cell) and SEAP mRNA fold increase [normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] over naïve mice. (E) C57BL/6 female mice (n = 10 per group) were injected with AAV8-SEAP (5 × 10^{11} vg/kg) alone or combined with 100 μg of ImmTOR, either as an admix or administered sequentially. Hepatic vector DNA and SEAP mRNA and serum SEAP levels in the group receiving AAV8-SEAP admixed to ImmTOR versus the other two groups were statistically different at every time point (P < 0.01, except for the 3-day and 7-day time points for vector DNA at P < 0.05, multiple t test), as were anti-AAV IgG levels in groups treated with AAV8-SEAP combined with ImmTOR versus the group receiving AAV8-SEAP alone (P < 0.01, multiple t test).
to mice treated with AAVAnc80-GFP alone by day 5 and became well pronounced by day 7 (Fig. 3B). The hepatocytes from mice treated with the admix showed a population of cells that were positive for GFP expression but negative for Cy5-ImmTOR (GFP+/ImmTOR−) and a population of cells that were double positive (GFP+/ImmTOR+) (Fig. 3C). The population of GFP+/ImmTOR− hepatocytes in mice treated with the admix was similar in size to the population of GFP+ cells in mice treated with AAVAnc80-GFP alone, suggesting that the increased percentage of GFP+ cells in the admix-treated mice was due to hepatocytes that took up both AAVAnc80-GFP and ImmTOR. 

Fluorescence intensity per cell was not increased after treatment with AAVAnc80-GFP and ImmTOR (1.67 ± 0.5) compared to AAVAnc80-GFP alone (2.32 ± 0.63). In contrast, admixing AAV to ImmTOR had little or no effect on the percentage of hepatocytes that endocytosed ImmTOR (fig. S6). NP-Empty had modest effects compared to ImmTOR on GFP transgene expression when admixed to AAVAnc80-GFP (fig. S7), and the number of double-positive cells (GFP+Cy5+), representing colocalization of AAV and nanoparticle, was substantially greater with ImmTOR-Cy5 compared to NP-Empty–Cy5 (fig. S7, A, D, and E), further indicating that ImmTOR uniquely affects both AAV trafficking and transgene expression.

**Admixing of ImmTOR to AAV restores transgene expression after AAVR knockdown in vitro and enhances AAV4 transduction in vivo**

We next assessed the effect of ImmTOR in an in vitro transduction assay to determine whether ImmTOR could directly facilitate increased transduction of hepatocytes by AAV. AAVAnc80 transduction is known to be dependent upon the AAVR receptor (24). Expression of AAVR in Huh-7 cells was knocked down by treatment with small interfering RNA (siRNA) specific for AAVR (Fig. 4A). Huh-7 cells expressing approximately 20% of normal AAVR (Fig. 4A, lane 9) showed a 50% reduction in AAVAnc80-luciferase expression (Fig. 4B). However, admixing of AAVAnc80-luciferase with ImmTOR restored transduction of Huh-7 cells treated with AAVR...
siRNA, and yielded luciferase expression 35% above the nontreated cells (Fig. 4B). These results suggested that ImmTOR may facilitate transduction of hepatocytes by AAV in a manner that is independent of AAVR.

To test this possibility, we conducted an in vivo study with AAV4, an AAV serotype that does not use AAVR (24). While liver transduction by AAV4-GFP alone was inefficient, with very low genome copy numbers seen in recipient mouse hepatocytes and no fluorescence detected in them in excess of background levels, administration of AAV4-GFP admixed to ImmTOR led to higher virus genome copy number and detectable GFP fluorescence in hepatocytes of recipient animals (Fig. 4C).

**Admixing of ImmTOR to AAV shields AAV from partial neutralization by immune sera**

The question of whether ImmTOR particles could partially shield AAV virions from neutralizing antibodies was explored by admixing AAV, ImmTOR, and neutralizing antibodies pooled from anti-AAV–positive mouse sera before in vivo administration in naive mice. The order of addition of immune serum and ImmTOR to AAV made a clear difference in subsequent in vivo transgene expression. Specifically, if neutralizing sera were added to AAV before the addition of ImmTOR, the neutralizing antibodies inhibited transgene expression, as expected (Fig. 5A, group 1 versus group 2).

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**Fig. 4.** ImmTOR restores AAV8-Luc transgene expression after DsiRNA knockdown of AAVR in vitro and enhances AAV4 transduction in vivo. (A) Huh-7 cells were left untreated or transfected with scrambled DsiRNA (Scr) or AAVR-specific DsiRNA and analyzed for AAVR expression at 72 hours (Western blot, quantified by densitometry). Lane 1, untreated; lane 2, Scr; lanes 3 to 5, 6 to 8, and 9 to 11, treated with 20, 10, and 1 nM AAVR DsiRNAs 1, 2, and 3, respectively. (B) Huh-7 cells were left untreated or transfected with Scr or AAVR-specific DsiRNA 3 (20 nM, 72 hours), then transduced with Anc80-luciferase alone or admixed with ImmTOR, and, 24 hours later, assayed for luciferase activity (12 to 18 replicates per group in each individual study), normalized to the average activity in controls (no DsiRNA) (****P < 0.0001, one-way ordinary ANOVA). Experiment was repeated twice with similar outcomes, and the representative result is shown. (C) Mice were injected (intravenously) with AAV4-GFP (6.25 × 10¹² vg/kg) alone or admixed to ImmTOR (300 µg), and 9 days later, livers were analyzed for GFP fluorescence flow cytometry or vector genome copies (*P < 0.05, **P < 0.01, unpaired t test). Dotted green line indicates the level of background autofluorescence in naïve mice.
However, if ImmTOR was added to AAV before the addition of neutralizing sera (Fig. 5B), transgene expression was rescued to levels on par with naïve mice treated with AAV alone or with controls injected with AAV preincubated with normal sera (Fig. 5B, group 2 versus groups 3 and 5), although expression levels were still lower than that observed in mice treated with nonneutralized AAV admixed to ImmTOR (Fig. 5B, groups 4 and 6). Similar effects were observed when AAV admixed with ImmTOR was injected in mice that had received passive transfer of a low level of anti-AAV mouse sera (Fig. 5C, groups 1 and 3). Admixing of ImmTOR and AAV before injection was important, as non-admixed ImmTOR was unable to elevate transgene expression in this setting (Fig. 5C, groups 1 and 2). Notably, if high levels of anti-AAV sera were used, then the admixture of ImmTOR to AAV was unable to overcome suppression of AAV transduction by the neutralizing antibodies.

These data were confirmed using human donor sera (Fig. 6A and B). In this case, a panel of individual sera was selected representing different levels of neutralizing antibodies and anti-AAV IgG antibodies (Fig. 6A). Serum sample 35, one of the serum samples with an intermediate level of neutralizing activity, showed approximately 50% inhibition of Huh-7 transduction at a serum dilution of 1:100. The human sera were passively transferred into naïve recipient mice, which were then inoculated with AAV either alone or with ImmTOR. Serum SEAP was measured on day 3 (A and B) or 35 (C) and is shown as the percentage of the untreated control; readings from groups receiving ImmTOR are in bold (A and B). Experiments were repeated two to three times with similar outcomes, and representative results are shown. Statistical difference between groups admixed or not admixed to ImmTOR is shown (*P < 0.05; ns, not significant; Mann-Whitney test).

**Fig. 5. Partially neutralizing mouse anti-AAV sera can be overcome by admixing to ImmTOR.** (A) AAV8-SEAP was incubated in vitro with anti-AAV8-positive (1 and 2), normal (3 and 4), or no (5 and 6) serum, then either left untreated (1, 3, and 5) or admixed with 50 μg of ImmTOR (2, 4, and 6), and injected into groups of mice (n = 4). (B) AAV8-SEAP was admixed to 50 μg of ImmTOR (2, 4, and 6) or left untreated (1, 3, and 5), then incubated in vitro with anti-AAV8-positive (1 and 2), normal (3 and 4), or no (5 and 6) serum, and injected into groups of mice (n = 4). (C) Mice were passively immunized with 0.2 μl of anti-AAV8-positive (titer = 1:100,000) serum (1 to 3) or mock-treated (4) and then injected with AAV8-SEAP either alone (1 and 4) or with 50 μg of non-admixed (2) or admixed (3) ImmTOR. Serum SEAP was measured on day 3 (A and B) or 35 (C) and is shown as the percentage of the untreated control; readings from groups receiving ImmTOR are in bold (A and B). Experiments were repeated two to three times with similar outcomes, and representative results are shown. Statistical difference between groups admixed or not admixed to ImmTOR is shown (*P < 0.05; ns, not significant; Mann-Whitney test).

**ImmTOR permits AAV dose sparing and multiple repeat administrations**

One of the potential advantages of ImmTOR would be to enable AAV dose titration and dose sparing. We observed that a fivefold
increase in AAVAnc80-SEAP vector dose (2.5 × 10^{12} \text{ vg/kg} \text{ versus } 5 \times 10^{11} \text{ vg/kg}) resulted in a ∼2.5- to 3-fold increase in SEAP expression in BALB/c mice (Fig. 7 and table S4). However, the addition of ImmTOR to the lower dose of AAVAnc80-SEAP reduced the gap to ∼1.5-fold. Moreover, after a second injection on day 70, SEAP expression in animals receiving low-dose AAV + ImmTOR increased to a level higher than that observed in animals receiving the high dose without ImmTOR. Thus, ImmTOR enabled dose titration with two low doses to achieve superior transgene expression at 40% of the total vector dose in animals treated with a single high dose without ImmTOR. A further increase of SEAP expression could be observed following a third dose of AAVAnc80-SEAP + ImmTOR at day 167, whereas transgene expression remained the same or decreased with time in mice treated with AAV alone. ImmTOR mitigated the formation of anti-AAV antibodies in groups treated with ImmTOR, even after three vector injections, while a robust IgG response was seen in mice injected with AAV without ImmTOR, especially at high AAV doses (Fig. 7C).

**DISCUSSION**

Colella et al. (6) recently highlighted immunogenicity and vector potency as two key challenges in the ability to achieve and maintain therapeutic levels of transgene expression. Here, we show that the addition of ImmTOR nanoparticles to AAV vectors has the potential to partially mitigate both issues by mediating more efficient transgene expression at the first dose of gene therapy and by enabling vector redosing by preventing the formation of capsid-specific antibodies. The cumulative benefit of enhancing first dose transgene expression and enabling repeat dosing can provide up to a fourfold increase in transgene expression compared to gene therapy with AAV vector alone.

The mechanisms by which ImmTOR affects transgene expression at the first dose and second dose appear to be distinct, as the first dose benefit is tied to admixing of ImmTOR with the AAV vector before injection (Figs. 2 and 5), whereas successful vector redosing is dependent on mitigation of neutralizing antibody formation, which can be attained by sequential (non-admixed) administration of ImmTOR followed by AAV vector (Fig. 2). In particular, redosing efficiency, but not the first dose enhancement of transgene expression, was the same with or without ImmTOR in immunodeficient mice and was equal to redosing efficiency with ImmTOR in wild-type mice (fig. S3). A high ImmTOR to AAV particle ratio [approximately 80:1 to 160:1 for 100 to 200 μg of rapamycin and AAV (5 × 1011 vg/kg) as used in most of the studies described in this report] may also account for the first dose benefit, but not for efficient redosing. Fluorescently labeled ImmTOR showed rapid accumulation in the liver and spleen from intravenous administration (25). Our data suggest that ImmTOR may enhance trafficking of AAV to the liver. The percentage of GFP-expressing hepatocytes is increased approximately twofold when AAVAnc80-GFP is admixed with ImmTOR (Fig. 3). The difference appears to be due to a population of hepatocytes that are double positive for GFP transgene expression and uptake of fluorescent-labeled ImmTOR. The transduction of many AAV serotypes, including AAVAnc80 (24, 26), has been shown to be dependent on the AAVR receptor (27). AAV transduction following knockdown of AAVR by siRNA can be restored by admixing ImmTOR to AAVAnc80 (Fig. 4, A and B), suggesting that ImmTOR may facilitate AAVAnc80 entry into cells through a non-AAVR-dependent mechanism. This was further confirmed by the ability of ImmTOR to elevate in vivo liver transduction efficiency of AAV4 (Fig. 4C), an unusual AAV serotype that does not use AAVR for cell entry (25).

It is possible that ImmTOR may also enhance transgene expression through other mechanisms. For example, mammalian target of rapamycin (mTOR) inhibitors, such as rapamycin, have been shown to increase AAV transduction efficiency in mice and nonhuman primates through the induction of autophagy (28). In addition, rapamycin can also mitigate acute inflammation through inhibition of the inflammasome pathway that leads to production of interleukin-1β (IL-1β) (29, 30). Preliminary data suggest that ImmTOR can also induce autophagy and inhibit nuclear factor κB (NF-κB)–mediated inflammation in vivo (31, 32).
Increasing transgene expression in humans has been approached through higher vector doses or through design of more efficient vectors. Increasing the vector dose is the easier but less desirable approach due to the high cost of AAV vector manufacturing and desire to minimize potential toxicities, such as liver enzyme elevations that have been observed in multiple clinical trials (10, 14, 33, 34). Engineering more efficient vectors is a promising approach, but it is an inexact science due to the complexity of AAV-mediated gene therapy. Capsids can be engineered and selected for better specificity for the target organ. Likewise, the gene cassette can be designed with stronger promoters and through codon optimization of the transgene sequence. However, vector engineering can have unintended consequences. For example, capsid mutations that increase organ targeting may inadvertently affect capsid stability or manufacturing productivity. Similarly, codon optimization can result in an increased prevalence of CpG islands that can activate Toll-like receptors, thereby increasing the risk of inflammation, or result in cryptic splice sites that create nonproductive mRNA transcripts. Strong promoters, along with high vector doses, have been associated with an increased risk of hepatocarcinoma in mice due to random integration events in the host genome (17). One of the potential advantages of ImmTOR is that it can be added to existing AAV gene therapy candidates without additional modifications. ImmTOR could work together with novel engineered vectors, such as AAVAnc80 (26), to further improve vector transduction efficiency.

Fig. 7. ImmTOR admixed with AAV leads to elevated transgene expression and permits dose sparing and multiple repeat dosing. (A) Anc80-SEAP was administered three times, either twice at 5 × 10¹¹ vg/kg (days 0 and 70) followed by a third injection of 25 × 10¹¹ vg/kg (day 167) or three times at 25 × 10¹¹ vg/kg (days 0, 70, and 167; indicated by arrows) as shown in experimental scheme. The lower dose vector was administered either alone or admixed with ImmTOR (100 μg). (B) SEAP expression levels in groups treated with low-dose AAV + ImmTOR (bold) or with high-dose AAV alone (italics) are shown as the fold increase over the group treated with low-dose virus alone. Baseline expression levels before redosing (day 61) are shown as lines (black dotted, low-dose vector; green dashed: low-dose vector admixed with ImmTOR; blue dash-dotted, high-dose vector). Statistical difference in SEAP levels between the low-dose AAV group treated with ImmTOR and the high-dose AAV group not receiving ImmTOR is shown (*P < 0.05 and **P < 0.01; ns, not significant; multiple t test). SEAP levels in low-dose virus group not receiving ImmTOR were different from both other groups at all time points with P < 0.01. (C) Anti-Anc80 IgG levels were measured by enzyme-linked immunosorbent assay (ELISA).
AAV are prevalent in the human population and can cross-react with most serotypes. Even titers as low as 1:5 can adversely affect AAV transduction, which excludes many patients from being eligible to receive gene therapy. The use of ImmTOR does not directly affect preexisting antibody titers or enable AAV transduction in the presence of high titers of neutralizing antibodies. However, we observed that admixing of AAV with ImmTOR before exposure to low levels of neutralizing antibodies allowed efficient transduction. Admixing AAV with ImmTOR may shield it to some extent from low levels of neutralizing antibodies (Fig. 5A). In addition, the first dose benefit observed with admixing AAV and ImmTOR may partially compensate for the presence of moderately neutralizing levels of antibodies by enhancing transgene expression to levels comparable to that observed in naïve mice treated with AAV alone in the absence of neutralizing antibodies (Figs. 5C and 6B).

AAV redosing is challenging because the doses of AAV typically used in systemic gene therapy invariably result in high titers of neutralizing antibodies that can be maintained for many years (13). There are now multiple strategies exploring various approaches to allow vector re-administration, such as preinjection with decoy empty capsids to bind preexisting antibodies (4, 35), plasmapheresis to adsorb immunoglobulin, and use of conventional immunosuppressants. The use of decoy empty capsids would be expensive, requiring clinical development of empty capsid produced under Good Manufacturing Practices (GMPs), and would subject the patient to additional capsid load that could exacerbate CD8 T cell responses. A case report study showed that plasmapheresis has been shown to be effective in temporarily eliminating modest titers of preexisting antibodies (generally <1:20); however, even five rounds of plasmapheresis were not effective when subjects had preexisting titers of 1:400 or greater (36). Various combinations of potent immunosuppressants have been evaluated in preclinical studies with limited success. There is a case study report of a single patient with Pompe disease that had been chronically immunosuppressed for over 40 months with rituximab-mediated B cell depletion therapy and daily doses of rapamycin before and after receiving AAV gene therapy (37). This patient did not develop anti-capsid antibodies compared to other Pompe patients that were not on immunosuppressive therapy. Now, there is an ongoing prospective clinical trial to evaluate the ability of chronic B cell depletion and daily rapamycin treatment to enable AAV redosing by intramuscular injection (35, 37, 38). Patients will be maintained on immunosuppression for up to 8 months, from 3 weeks before the first AAV dose until 4 months after the second dose administered at 4 months. Such a prolonged immune suppression regimen may not be tolerable for many patient populations. The advantage of ImmTOR is that it is given as a single injection at the time of each administration of AAV and is not associated with chronic immune suppression (18, 21).

The challenge for human translation is that even neutralizing antibody titers as low as 1:5 can inhibit AAV transduction. Thus, enabling vector redosing requires near-complete inhibition of neutralizing antibody titers. While ImmTOR inhibits anti-AAV antibodies and enables redosing in mice and nonhuman primates, it is possible that ImmTOR may not sufficiently inhibit the formation of antibodies in all patients to enable vector redosing due to heterogeneity of the human population. However, even a substantial reduction of neutralizing antibodies could be beneficial to patients, as antibody titers do naturally decline over time (39). Thus, low to moderate titers of neutralizing antibody titers could decline in a clinically meaningful time period to allow vector redosing. Alternatively, other strategies could be explored, if necessary, to mitigate any residual levels of neutralizing antibodies.

The multipronged mechanism of ImmTOR action makes it an attractive candidate to enhance systemic gene therapeutic applications, particularly in those clinical indications where repeat vector dosing may be necessary. The first dose benefit of adding ImmTOR to AAV gene therapy is immediate, dose dependent, and not mouse strain or capsid specific. It is characterized by increased vector copy number in liver cells and elevated synthesis of transgene-encoded mRNA. The rapid and enhanced transgene expression can enable therapeutic benefit at lower doses of AAV and faster onset of transgene-directed therapeutic effects. Mitigating the formation of neutralizing antibodies by ImmTOR could enable the possibility of vector redosing to achieve, maintain, or restore therapeutic benefit.

**MATERIALS AND METHODS**

**Study design**

The objective was to investigate the effects of ImmTOR (nano-encapsulated rapamycin) on AAV vector transduction in vivo and then the mechanism by which it elevates transgene expression at initial co-administration with vector. Research subjects were animals, their tissues, and blood from healthy human donors. Sample size in animal experiments was determined before the study as per Institutional Animal Care and Use Committee (IACUC) guidelines. No outliers were excluded except for rare individual mice, which were documented not to receive a full dose of experimental item. All experiments were controlled animal studies or controlled laboratory experiments repeated independently at least twice and as many as six times, with representative datasets from individual experiments shown in Results.

**Mice**

Immunologically naïve, male or female BALB/c, C57BL/6, or Rag2-KO female mice in C57BL/6 background aged 28 to 42 days (or 16 to 18 g for females and 18 to 20 g for males) were purchased from Charles River Laboratories (Wilmington, MA, USA). To minimize the potential effects of stress, mice were acclimated to the Animal Care Facility at Selecta for at least 3 days before injection. All the experiments were conducted in strict compliance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and other federal, state, and local regulations and were approved by Selecta’s IACUC.

**Viruses**

AAV8-SEAP, AAV8-luciferase (AAV8-Luc), and Anc80-SEAP were manufactured by SAB Tech (Philadelphia, PA, USA) using proprietary helper plasmid, AAV8AAP or Anc80AAP plasmids, and the plasmid containing the gene of interest. The plasmids were transfected into adherent human embryonic kidney (HEK) 293 cells and harvested 72 hours after transfection by cell lysis. The clarified supernatant from the harvest was purified by CsCl2 gradient, and the AAV8-containing fraction was collected. The viral vector band was assayed by SDS–polyacrylamide gel electrophoresis (PAGE) gel and silver stain to determine a viral titer. Anc80-eGFP was provided by R. Xiao (Massachusetts Eye and Ear Infirmary, Boston, MA, USA). AAV4-GFP was purchased from SignaGen (Frederick, MD, USA). Anc80 empty capsids were generated as by-products of Anc80-SEAP.
samples were analyzed for SEAP expression using the Phospa-
Light SEAP Reporter Gene Assay System (Invitrogen, Carlsbad, CA). Samples were diluted 1:10 in dilution buffer, heat-inactivated at 65°C for 30 min, and cooled over ice. Once the samples reached room temperature, they were added to opaque white assay plates, followed by the assay buffer (5 min) and substrate (20 min) per the manufacturer’s recommendations. Luminescence was read at 477 nm on SpectraMax M5 or SpectraMax L (Molecular Devices, San Jose, CA, USA) and reported in relative luminescence units (RLUs), which are proportional to the concentration of SEAP in the serum. Enzymatic luciferase activity was measured using the Promega Luciferase Assay System (Promega, Madison, WI, USA). Livers were snap-frozen and pulverized using a mortar and pestle. Using the reagents provided, the cells were lysed and spun down, and the supernatant was transferred to a white 96-well plate. A luciferase assay reagent was added to the plate, and the plate was immediately read for luminescence. GFP expression was assessed by fluorescence-activated cell sorting (FACS).

Imaging and quantification of bioluminescence data
Before imaging, mice were injected intraperitoneally with luciferin (150 mg/kg) and ketamine (100 mg/kg) with xylazine (4.5 mg/kg) as an anesthetic. Fifteen minutes after injection, in vivo images were acquired with the In Vivo Imaging System (IVIS) charge-coupled device camera system (Xenogen Corp., Alameda, CA, USA) and analyzed with the LivingImage 2.11 software package (Xenogen Corp.). A region of interest (ROI) was manually selected over the signal intensity. The area of the ROI was kept constant, and the intensity was recorded as maximum (photons s⁻¹ cm⁻² sr⁻¹) within an ROI. Control mice were used to check for background signal.

Flow cytometry (liver cell populations and GFP)
Immediately after euthanizing mice, livers were harvested and rendered into single-cell suspensions via collagenase 4 (Worthington Biochemical Corp., Lakewood, NJ, USA) enzymatic digest according to the manufacturer’s protocol followed by a red blood cell lysis step (5 min at room temperature in 150 mM NH₄Cl, 10 mM KHCO₃, 10 μM Na₂-EDTA). Cells were washed in PBS and 2% bovine serum, filtered on a 70-μm nylon mesh, incubated for 20 min on ice with anti-CD16/32 (Fc-block, clone 93, BioLegend, San Diego, CA, USA), and then stained with antibodies directed toward cell surface receptors: CD11b-PE/Cy7 (clone M1/70), CD68-APC/Cy7 (clone FA-11), and F4/80 (clone BM8), all from BioLegend, and LRP-1 (A2MR-α2) from Invitrogen (Carlsbad, CA, USA). Analysis was performed with a FACSScanto flow cytometer (BD Biosciences, San Jose, CA, USA) with subsequent data analysis using FlowJo software (TreeStar, Ashland, OR, USA).

Vector DNA and transgene mRNA biodistribution
Livers from mice dosed with AAV8-SEAP ± ImmTOR were harvested, and part of tissue was stored in RNA later at −20 ± 5°C until the time of either biodistribution or mRNA expression analysis. Liver tissue homogenization was performed via BeadBug (Benchmark Scientific, Sayreville, NJ, USA). DNA and RNA were isolated from liver homogenate using the QIAamp DNA Mini Kit or RNeasy Mini Kit (Qiagen, Germantown MD, USA), respectively, following the manufacturer’s instructions. The quantity of isolated nucleic acid was measured fluorometrically using either the Qubit dsDNA HS or RNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s protocol. Extracted DNA (100 ng per sample) was used in each quantitative polymerase chain reaction
(qPCR) using TaqMan Genotyping master mix (Thermo Fisher Scientific).

A standard curve was generated using linearized plasmid DNA (SELT01-pFB-CMV-SEAP-SV40pA-Kan) to interpolate AAV8-SEAP viral genome biodistribution (vg per cell) in liver. Complementary DNA (cDNA) was synthesized using 1 μg of RNA with iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA) following the manufacturer’s instructions. Quantitative real-time PCR was performed using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific). The relative expression level (fold change) of each gene was calculated using the 2^(-ΔΔCT) method (40).

**Primers and probes**

Custom-designed transgene-specific primers and probes were used for SEAP DNA biodistribution and mRNA expression assay [forward primer, 5′-AGTTTGACTCCCTTCTGCCCATT-3′; reverse primer, 5′-TGGGTCGCCAGCAAGAA-3′; and MGB probe, 6-FAMCAGAGACGGCACA-MGBNFQ (Thermo Fisher Scientific)]. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the internal control gene for normalization of mRNA expression level.

**Enzyme-linked immunosorbent assay for IgG against AAV8 and Anc80**

The 96-well plates were coated overnight with AAV8 or Anc80, washed, and blocked on the following day, followed by sample incubation (1:40 diluted serum). Plates were then washed, and the presence of IgG was detected using anti-mouse IgG-specific horseradish peroxidase (HRP; 1:1500; Jackson ImmunoResearch, West Grove, PA, USA). The presence of rabbit anti-mouse IgG-specific HRP was visualized using trimethylboron substrate and measured using absorbance at 450 nm with a reference wavelength of 570 nm. The optical density (OD) observed is proportional to the amount of anti-AAV8 IgG antibody in a sample and was reported. Normal human sera were also tested in the same manner using an anti-human IgG-specific HRP detector (1:2000; Southern Biotech, Birmingham, AL, USA). Serum EC50 (median effective concentration) was determined as follows. The positive control anti-AAV8–IgG antibody (Fitzgerald Industries International, Acton, MA) and samples were diluted 1:40 followed by a 1:3 serial dilution. Plates were then processed as described above, and the EC50 was calculated using the four-parameter logistic curve fit function in the Softmax Pro software program (Molecular Devices, San Jose, CA). The positive control anti-AAV8–IgG antibody was used as the standard curve to determine the EC50 for each sample.

**In vitro AAV neutralization by normal human donor serum and AAVR knockdown**

Huh-7 cells were plated in a 96-well tissue culture–treated plate (50,000 cells per well) overnight. The following day, normal human donor sera (1:100 dilution) were preincubated for 1 hour with AAV8-Luc [10,000 multiplicities of infection (MOIs)] and then added to the Huh-7 cells for a 24-hour incubation. The following day, cells were lysed and the luciferase activity was measured using the Luciferase Assay System (catalog no. E1501, Promega Life Sciences, Madison, WI, USA). Virus neutralization by normal donor sera was characterized by the reduction in luciferase activity compared to the controls (cells treated only with AAV8-Luc).

For AAVR knockdown studies, Huh-7 cells were transfected with 20 μg of scrambled DsiRNA as a control, no DsiRNA, or 20 μg of AAVR-specific DsiRNA for 72 hours and then analyzed by Western blotting for AAVR expression using antibody against KIAA0319L (ProteinTech, Rosemont, IL, USA) or transduced at MOI of 2000 of Anc80-Luc, with one set of wells receiving Anc80 at MOI of 2000 admixed with 100 μg of ImmTOR. Twenty-four hours after transduction, cells were lysed and assayed for luciferase activity, which was normalized to the average of no DsiRNA wells (n = 6). AAVR expression was quantified by densitometry using the Image Lab software (Bio-Rad, Hercules, CA).

**Tissue preparation and fluorescent microscopy**

The whole slide scans were taken on a Vectra Polaris (made by Akoyna Biosciences). The microscope images were taken with Nikon E-600 with the y-fluorescent attachment and captured using the AmScope software.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 8.0.2. To compare the mouse experimental groups, pairwise multiple t test (for several time points) or Mann-Whitney two-tailed test (for a single time point) was used. One-way analysis of variance (ANOVA) was used to compare data from in vitro assays. Significance is shown for each figure legend (*P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001; not significant, P > 0.05). All data for individual experimental groups are presented as means ± SD (error bars).

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at advances.sciencemag.org/cgi/content/full/7/9/eabd0321/DC1

View request a protocol for this paper from Bio-protocol.

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Enhancement of liver-directed transgene expression at initial and repeat doses of AAV vectors admixed with ImmTOR nanoparticles

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