

Rapamycin nanoparticles mitigate anti-AAV antibody formation in a mouse model of ornithine transcarbamylase deficiency

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Adeno-associated virus (AAV) vector-based liver gene therapy for inherited diseases has demonstrated efficacy in clinical trials in adults. However, its application to pediatric patients is limited by loss of AAV genomes during hepatocyte proliferation, compromising long-term benefits. Additionally, the anti-AAV immune responses induced after the initial AAV-administration preclude vector re-dosing. One key driver of this immune response is mTOR-dependent activation of dendritic cells. Inhibition of this pathway with rapamycin can promote immune tolerance. We assessed the safety and efficacy of rapamycin-loaded synthetic nanoparticles (ImmTOR) in juvenile OTC^{Spf-Ash} mice, a model of ornithine transcarbamylase (OTC) deficiency (OTCD). Treatment of postnatal day 30 mice with ImmTOR alone transiently activated autophagy and reduced urinary orotic acid levels. Treatment with a liver-specific AAV8 vector encoding codon-optimized human OTC cDNA normalized urinary orotic acid levels and significantly increased ureagenesis, OTC protein expression, and enzyme activity compared with untreated controls. AAV-vector co-administration with ImmTOR prevented IgM and IgG formation and induced a dose-dependent reduction of anti-AAV neutralizing antibodies, consistent with modulation of the humoral immune response. These findings suggest that ImmTOR can mitigate humoral immune responses to AAV vectors in OTC^{Spf-Ash} mice and may enable AAV vector re-administration, although further optimization is required for clinical translation.

INTRODUCTION

Ornithine transcarbamylase (OTC) deficiency (OTCD) is the most common urea cycle disorder¹ with an estimated prevalence at birth of 1 in 35,000 in the United States.² Pathogenic variants in the X-linked OTC gene reduce enzyme activity, affecting the conversion of neurotoxic ammonia into non-toxic urea. Clinical severity of

OTCD largely depends on acute life-threatening hyperammonemia crisis, which can cause neurodevelopmental impairments of variable severity. Residual OTC enzymatic activity, genetic modifiers, and environmental factors contribute to phenotypic variability of the disorder. Hemizygous affected males lacking enzyme activity typically present with a life-threatening hyperammonemia within a few days after birth (early onset OTCD). Survivors experience recurrent crisis and cumulative neurological damage, reducing neurocognitive performances and their long-term survival.³ In females, the symptoms depend on both the type of variant and skewed X chromosome inactivation.

Milder OTCD patients can be managed by dietary protein restriction and ammonia scavenger drugs, but severe OTCD often requires orthotopic liver transplantation (OLT), the only effective therapy available.⁴ However, OLT is an invasive procedure, limited by organ shortage and life-long immunosuppression, highlighting the need for new therapies.

To date, adeno-associated virus (AAV) vector-mediated liver gene therapy has shown efficacy and safety in adult patients, even at high systemic doses of the vector,^{5–8} including a clinical trial for late-onset OTCD patients sponsored by Ultragenix.⁹ However, episomal AAV genomes are lost during hepatocyte proliferation, reducing therapeutic efficacy in growing livers.¹⁰ Pediatric patients may, therefore, require re-dosing later in life. Yet, the induction of anti-AAV capsid humoral immune response, with persistent neutralizing antibody (NAb) titers, precludes repeated dosing.^{11–13} Strategies to remove the anti-AAV NAb from plasma, such as

Received 18 November 2025; accepted 17 June 2026;
<https://doi.org/10.1016/j.omta.2026.201793>.

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plasmapheresis, immunoabsorption, or IgG-cleaving proteases, are effective only at low antibody titers.^{14–18} Preventing anti-AAV NAb formation using immunomodulators or immune tolerizing agents is therefore an attractive alternative.^{19–24} Among these, co-administration of the AAV vector with rapamycin-loaded synthetic nanoparticles (ImmTOR) induces immune tolerance enabling successful vector re-dosing.^{20–23,25–29}

In this study, we evaluated ImmTOR co-administration with an AAV8 vector expressing a codon optimized version of the human OTC cDNA in juvenile OTC^{Spf–Ash} mice, a model of OTCD previously corrected in adults.³⁰ We have shown that this approach resulted in urinary orotic acid within the normal range and in substantial reduction but not total elimination of anti-AAV IgGs and NAb titers. These findings indicate effective immunomodulation, although further optimization may be required to enable vector re-administration in case of loss of therapeutic efficacy.

RESULTS

Dose-escalation studies in juvenile OTC^{Spf–Ash} mice

We previously demonstrated restoration of OTC activity in adult (3-months old) OTC^{Spf–Ash} mice using an AAV8 vector carrying a codon-optimized hOTC cDNA under the transcriptional control of a liver-specific promoter.³⁰ We hypothesized that juvenile OTC^{Spf–Ash} mice may require re-dosing due to the loss of AAV vector genomes associated to hepatocyte duplication during liver growth.^{10,31,32} To address this, we tested the same AAV8-CO21-hOTC vector treatment in combination with ImmTOR nanoparticles to suppress the anti-AAV humoral immune response, enabling the possibility of vector re-dosing.

In an initial experiment, juvenile P30 OTC^{Spf–Ash} mice received 5.0E11 vg/kg of AAV8-CO21-hOTC vector, a dose that resulted in correction of the phenotype in adult animals.³⁰ Mice were euthanized at P90 (Figure S1A). This dose resulted only in partial correction of the phenotype: treated mice remained underweight compared to wild-type (WT) controls, liver OTC protein levels increased but below WT levels (Figures S1B and S1C), and urinary orotic acid concentrations remained elevated (Figure S1D) suggesting that higher AAV doses or AAV re-administration may be needed to achieve full correction in juvenile mice. However, anti-AAV NAb reached titers known to prevent further AAV-mediated gene transfer (Figure S1E).¹¹

To define the minimal effective dose, we performed a dose-response study in P30 OTC^{Spf–Ash} mice using urinary orotic acid excretion, OTC enzyme expression, and catalytic activity as endpoints. (Figure 1A). Age-matched untreated hemizygous OTC^{Spf–Ash} mice were used as controls. We observed a clear dose-dependent reduction of urinary orotic acid (Figures 1B and S2A–S2D), with all treated groups reaching normal levels that were maintained until the end of the experiment. At sacrifice, the livers were harvested and protein extracts were prepared and

were used to measure OTC protein production and enzymatic activity. Liver OTC protein expression and enzyme activity were comparable to WT at the low- and mid-doses (1.0E12 and 2.0E12 vg/kg, respectively), whereas the high-dose (5.0E12 vg/kg) resulted in supraphysiological expression (Figures 1C–1E). Vector genome copies (VGCs) in the liver (Figure S2E) correlated with OTC protein levels. No differences in body weight were observed among the experimental groups (Figure S2F).

Tolerability of ImmTOR and autophagy activation

To evaluate the safety of ImmTOR particles in OTC^{Spf–Ash} mice, we systemically injected juvenile P30 mice with ImmTOR nanoparticles at different doses or with empty nanoparticles, and monitored body weight, transaminases levels, and OTCD markers for 2 weeks (Figure S3A).

Interestingly, we observed an unexpected reduction in urinary orotic acid with ImmTOR even in the absence of AAV gene therapy. Although the differences did not reach statistical significance, the groups injected with the 8 and 12 mg/kg ImmTOR doses showed a trend in greater reduction in urinary orotic acid at early time points compared to those treated with empty nanoparticles (Figure S3B). At the latest evaluated time point (14 days post-injection), this reduction was lost and all groups showed similar excretion. Body weight increased in all groups and was comparable among them (Figure S3C). Serum transaminase levels remained within the reference range after ImmTOR nanoparticles treatment at all tested doses (Figures S3D and S3E). Altogether, these data indicate that ImmTOR can be safely administered to juvenile OTC^{Spf–Ash} mice.

To further investigate and confirm the beneficial effect of ImmTOR particles, a larger cohort of P30 OTC^{Spf–Ash} mice was systemically injected with ImmTOR nanoparticles or empty-nanoparticles and euthanized at 2 days (Figure 2A) or 14 days post-injection (Figure S4A). ImmTOR-treated animals showed a significant reduction in urinary orotic acid (Figures 2B, S4C, and S4D), confirming the previous results (Figure S3B). Recent work has shown that autophagy enhancers increase hepatic ureagenesis and protect against hyperammonemia.^{33,34} Thus, to investigate whether ImmTOR particles reduce urinary orotic acid and ammonia levels through autophagy enhancement driven by rapamycin-mediated inhibition of the mammalian target of rapamycin complex 1 (mTORC1), we assessed mTORC1 activation and autophagy flux in livers of ImmTOR-treated mice, as previously reported.³³ Livers of ImmTOR-treated mice showed reduced mTORC1 activation, evidenced by decreased phosphorylation of ribosomal protein S6, a major downstream target of mTORC1 (Figures 2C and 2D). Consistently, autophagic flux was enhanced, as indicated by reduced protein levels of the autophagosome marker LC3-II (Figures 2C, 2D, S4E, and S4F). In summary, these data suggest that ImmTOR administration alone activates hepatic autophagy *in vivo*, leading to an improved biochemical phenotype in OTCD without AAV-mediated OTC gene replacement.

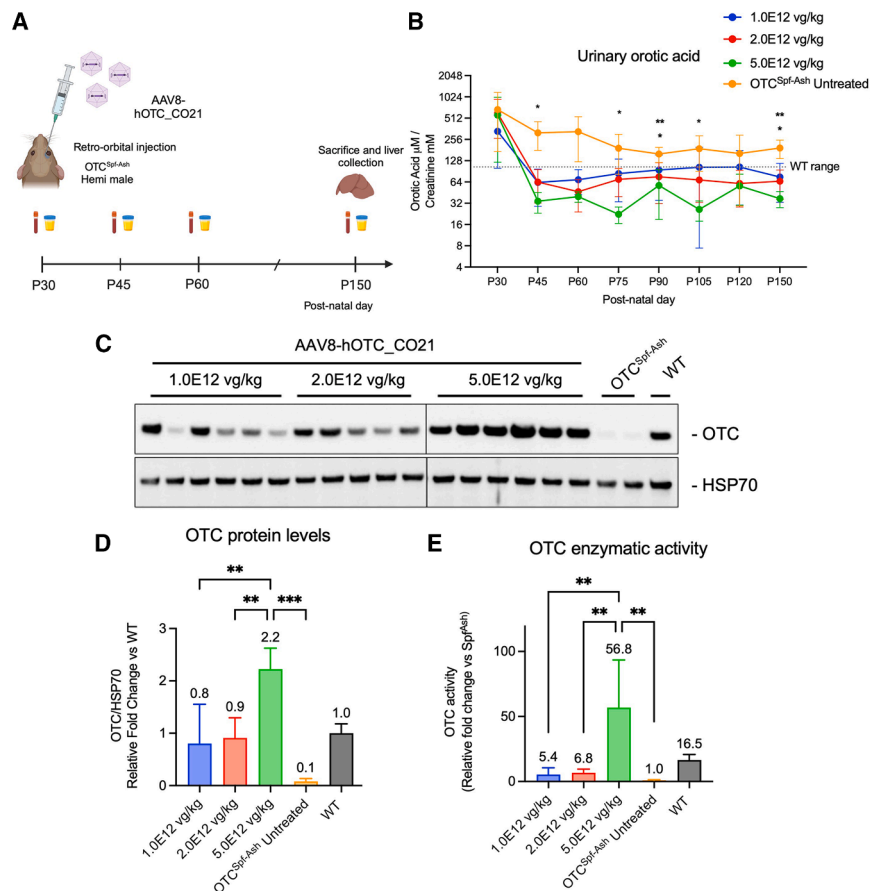


Figure 1. Dose-finding treatment with the AAV8-CO21-hOTC vector in juvenile OTC^{Spf-Ash} mice

(A) Experimental scheme. P30 hemizygous male OTC^{Spf-Ash} mice ($n = 5/6$ per group) were treated with AAV8-hOTC_{Co21} at the dose of 1E12, 2E12, 5E12 vg/kg and compared to untreated age-matched littermates. Mice were sacrificed after 4 months (postnatal day P150). Blood and urine samples were collected during the experiment, and liver were harvested at sacrifice. (B) Urine samples were analyzed by HPLC-MS and values obtained were normalized to creatinine concentration. The graph shows the dose-dependent trend of urinary orotic acid levels in the treated and untreated animals. Data are expressed in log2 scale as mean (SD), two-way ANOVA mixed model ($*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$), the dotted line represents the maximum value urinary orotic acid expected in WT animals. (C) Proteins extracted from liver homogenates were resolved on SDS-PAGE gel and blotted on nitrocellulose membrane and used to determine OTC protein levels. The blot was incubated with anti-OTC and anti-HSP70 specific antibodies. (D) The bar graph represents the quantified and normalized values of the bands obtained from all the animals treated at different doses, expressed as fold change over C57BL/6 WT controls. (E) The bar graph is representative of the OTC catalytic activity in all the groups; values are expressed as fold change ratio over untreated OTC^{Spf-Ash}. Mean (SD), ordinary one-way ANOVA ($*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$).

Co-administration of ImmTOR tolerizing nanoparticles with the AAV8-hOTC-CO21 vector restored OTC expression and urea cycle in juvenile OTC^{Spf-Ash} mice

To evaluate the efficacy of ImmTOR in mitigating the formation of anti-AAV8 NAb, juvenile hemizygous OTC^{Spf-Ash} (P30) received AAV8-CO21-hOTC (2.0E12 vg/kg) admixed with ImmTOR (2 or 6 mg/kg), with additional ImmTOR doses administered on days 28 and 56 (Figure 3A). Mice were monitored for 200 days, and urine and blood samples were collected at intermediate time points.

Assessment of urinary biomarkers showed that ImmTOR co-administration did not affect the overall efficacy of the treatment, confirming previous results. Urinary orotic acid concentrations were within the normal range by 14 days post-AAV injections and remained stable throughout the study (Figures 3B and S5A–S5E). In contrast, mice treated with ImmTOR alone showed urinary orotic acid levels similar to those of untreated OTC^{Spf-Ash} untreated animals.

To directly evaluate restoration of a functional urea cycle, we performed a ureagenesis assay measuring ¹⁵N-Urea enrichment 15

and 30 min after the systemic administration of the isotope-labelled precursor (¹⁵NH₄Cl, 4 mmol/kg; Figure 3C). Mice receiving gene therapy exhibited a significantly higher ureagenesis rate compared to ImmTOR only and untreated groups ($p < 0.001$), consistent with reduced urinary orotic acid. Importantly, ureagenesis in WT mice was comparable to that in the groups treated with AAV8-CO21-hOTC alone or combined with ImmTOR 6 mg/kg, while the AAV8-CO21-hOTC plus ImmTOR 2 mg/kg group showed slightly lower ureagenesis, still markedly higher than untreated control groups (Figure 3C). These results confirm that the beneficial effect of the gene therapy treatment reflects increased OTC enzymatic activity.

At sacrifice (D200), OTC protein levels and enzyme activity in treated livers were higher than untreated controls but lower than WT livers (Figures S6A and S6B). OTC enzyme activity in AAV + ImmTOR groups was similar to WT animals, except for AAV-only mice, which showed slightly reduced activity but remained much higher than AAV-untreated control groups (Figure S6C). VGC numbers correlated with protein levels and enzyme activity, ranging between 0.6 and 2.0 VGC (Figure S6D). These values were lower than those in Figure 1, likely due to viral DNA loss associated with

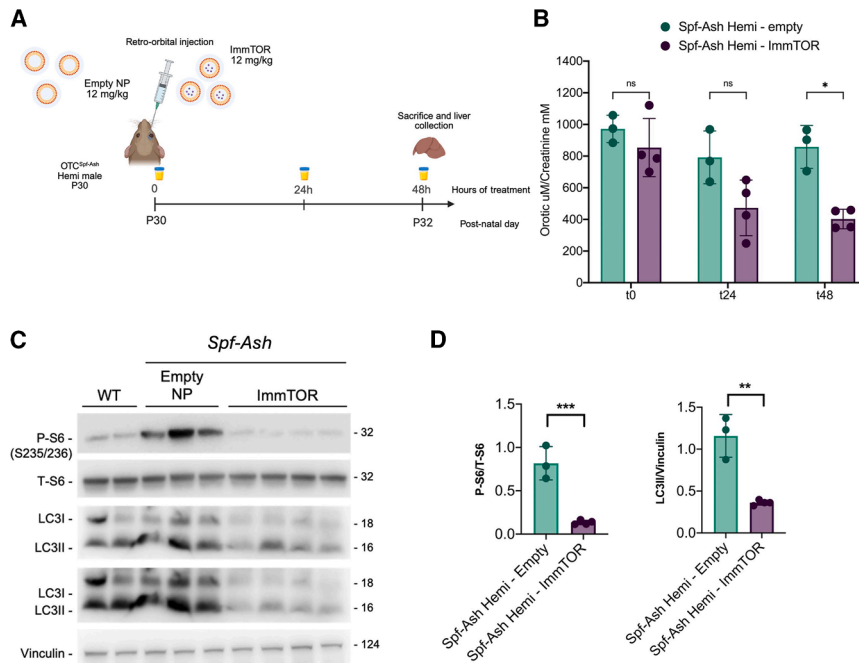


Figure 2. ImmTOR treatment in juvenile OTC^{Spf-Ash} mice

(A) Experimental scheme. Mice were treated at P30 with ImmTOR (12 mg/kg) or empty nanoparticles (NP; 12 mg/kg). Urine samples were taken at 24 h and at sacrifice after 48 h. The liver was collected at sacrifice. (B) The bar graph shows the urinary orotic acid at 24 and 48 h. Each dot represents a single animal. Mixed-model, time, $**p \leq 0.01$; treatment, $*p \leq 0.05$; interaction, NS; Sidak's multiple comparison test, t 48, $p = 0.0184$. (C and D) Western blot analyses and densitometric quantifications of phospho (P)-S6 vs. total (T)-S6 ratio, and LC3 in livers from empty NP- and ImmTOR-treated mice ($n \geq 3$ /group). Vinculin was used as loading control. Two control samples of livers from wild-type mice are also shown. Unpaired Student's *t* test; $**p \leq 0.01$; $***p \leq 0.001$.

homeostatic hepatocyte turnover during the extended follow-up (120 days vs. 200 days), consistent with the estimated hepatocyte lifespan of ~ 200 days.³⁵

No differences were observed in the body weight growth curves of the AAV-treated mice compared to WT littermates, whereas both control groups (OTC^{Spf-Ash} untreated and ImmTOR-only treated) showed weight decline at later time points (from D150 to D200; Figure S6E), likely reflecting accumulative damage from the reduced OTC activity.

Evaluation of hematological safety

Since ImmTOR has inherent immunosuppressant properties, we evaluated hematological parameters to confirm the safety of the procedure. Blood samples were collected after a 4 h fasting on the day before sacrifice. Values were compared with reference ranges for healthy C57Bl/6 mice (instrument standards and peer-reviewed data)³⁶ (Figure S7). Most red blood cell parameters were within normal limits, although red cell distribution (RDW) differed significantly among groups (Figure S7B). Hemoglobin content (HGB), hematocrit (HCT), and mean corpuscular hemoglobin (MCH) values were comparable to those of untreated OTC^{Spf-Ash} control group (Figures S7C, S7E, and S7F). In contrast, MCH concentration (MCHC) and mean corpuscular volume (MCV) were significantly different in mice treated with ImmTOR alone (6 mg/kg) or AAV + ImmTOR (6 mg/kg) but not in those receiving AAV alone or AAV + ImmTOR (2 mg/kg) (Figures S7D and S7G). Similarly to what was observed for red cells, white blood cell counts also fell within the normal range of healthy C57Bl/6 mice, with occasional intergroup differences (Figures S7H–S7K).

Platelet counts (PLTs) were unaffected (Figure S7L), while mean platelet volume (MPV) and platelet distribution width (PDW) values presented significant differences among groups but remained within the expected normal values and standard detection range of the instrument (Figures S7M and S7N). Blood glucose levels were normal across all groups (Figure S7O). Overall, these findings indicate that ImmTOR treatment, even at high doses, does not induce clinically relevant hematological changes.

ImmTOR co-treatment inhibited anti-AAV IgG, IgM, and NAb

AAV administration triggers a strong humoral immune response against capsid antigens, limiting potential vector re-administrations later in time if therapeutic efficacy wanes. We therefore assessed whether ImmTOR nanoparticles could mitigate the formation of anti-AAV IgG, IgM, and NAb in the same animals treated previously in Figure 3. In the control OTC^{Spf-Ash} group (AAV only, no ImmTOR), IgM and IgG titers increased as expected, peaking at D28 and declined thereafter (Figures 4A and 4B). In contrast, mice receiving AAV + ImmTOR displayed titers comparable to untreated OTC^{Spf-Ash} animals at D14 and D28 (Figures 4A–4D). The higher ImmTOR dose (6 mg/kg, three doses) almost completely suppressed IgM and IgG responses, maintaining titers near baseline throughout the study (Figures 4A–4D). The lower ImmTOR dose (2 mg/kg, three doses) partially reduced antibody levels, which remained below those of controls (AAV only) but above baseline (Figures 4A and 4B).

Importantly, anti-AAV NAb titers showed a clear dose-dependent reduction. Notably at D200, NAb titers in the high ImmTOR group were 1/10 or lower with the exception of one animal, and were generally 10-fold or 100-fold lower than those of mice treated with vector alone (Figure 4E).

We next performed a re-administration experiment by dosing juvenile hemizygous OTC^{Spf-Ash} (P30) with AAV8-CO21-hOTC (5.0E11

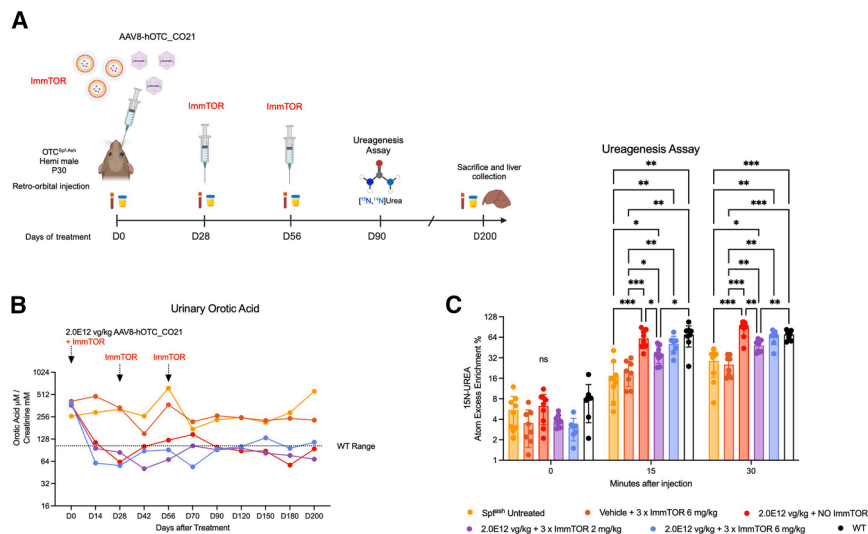


Figure 3. AAV dosing and repeated ImmTOR treatment in juvenile OTC^{Spf-Ash} mice

(A) Juvenile P30 hemizygous OTC^{Spf-Ash} mice ($n = 8/10$) were treated with 2.0E12 vg/kg of AAV8-CO21-hOTC (D0), with and without 3 subsequent administrations of ImmTOR at two different doses of 2 and 6 mg/kg at day 0, 28 and 56. Animals were injected retro-orbitally and sacrificed at P230 (D200). Urine and blood samples were collected at P30 and intermediate time points. At D90 the ureagenesis test was performed to evaluate OTC activity *in vivo*. At sacrifice (D200) liver was harvested for analysis. Age-matched untreated and ImmTOR-noAAV treated hemizygous OTC^{Spf-Ash} mice were used as controls. (B) Graph representative of urinary orotic acid dose-dependent trend in the groups treated and untreated ($n = 8/10$). (C) Ureagenesis assay. ¹⁵N-urea enrichment was evaluated after a starvation period of 3 h upon the intraperitoneal administration of the ¹⁵N-isotope marked precursor (¹⁵NH₄Cl – 4 mmol/kg). Blood samples were collected before, 15 and 30 min after the injection for the determination of ¹⁵N-marked urea enrichment. Average

enrichment values with statistical analysis and sample dispersion at each time point evaluated. Values are expressed as ¹⁵N-urea atom excess enrichment percentage. Mean (SD), Mixed-model two-way ANOVA ($p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$). Each dot represents a single animal.

vg/kg) admixed with ImmTOR or empty nanoparticles. Upon re-administration of the AAV vector (1.0E12 vg/kg) ± ImmTOR at week 8, we clearly observed that ImmTOR treatment effectively prevented the generation of anti-AAV8 NABs following the second AAV dose. In contrast, animals that were re-administered with the AAV vector and received empty nanoparticles (without ImmTOR) showed increased anti-AAV8 NAB levels (Figure S8). These results strongly support the potential of ImmTOR to enable AAV vector re-dosing. However, further optimization of experimental design and dosing regimen will be required to maximize its efficacy and translational potential.

DISCUSSION

Most hepatic inborn errors of metabolism lack definitive cure. Liver transplantation can correct the underlying metabolic defect but is an invasive procedure limited by organ shortage and lifelong immunosuppression.³⁷ In contrast, AAV-mediated liver gene therapy offers a precise, non-invasive alternative to liver transplantation for inborn metabolic liver diseases. It targets only the defective enzyme and is designed as a one-time treatment expected to provide durable correction. Clinical trials in adults have demonstrated safety and efficacy, leading to regulatory approvals of multiple therapeutic AAV vectors by the FDA and the European Medicines Agency (EMA).³⁸

Clinical experience with AAV-based non-integrative therapy for OTCD remains limited to adults. A phase 1/2 trial in late-onset OTCD (Ultragenyx; NCT02991144) confirmed safety and showed promising results, with most patients (7 out of 11) discontinuing ammonia scavengers and protein-restricted diets.⁹ However, translating these outcomes to early onset cases—where irreversible neurological damage can occur within days of birth and intervention must occur in infancy—poses unique challenges.

The episomal nature of AAV vectors is a major concern for pediatric applications. Hepatocyte proliferation during liver growth accelerates vector genome loss, reducing transgene expression and therapeutic efficacy.^{10,31,32,39} Even in adults, homeostatic hepatocyte renewal contributes to gradual episome loss; a recent report established that human hepatocytes divide at an estimated rate of 17%–19% per year,⁴⁰ suggesting that AAV re-dosing may eventually be required to maintain therapeutic efficacy. This challenge is particularly critical for early onset OTCD, where progressive loss of AAV genomes would compromise long-term correction. These limitations are key concerns not only for treatment of OTCD, but also for many inherited childhood diseases.^{41–44}

Thus, mitigation of AAV immunogenicity is essential to enable vector re-administration by preventing anti-AAV NABs formation, thereby sustaining transgene expression and therapeutic efficacy.

In this study, we confirmed that juvenile OTC^{Spf-Ash} mice require higher AAV doses for long-term correction compared to adults (2.0E12 vs. 5.0E11 vg/kg),³⁰ likely due to vector loss during liver growth, as previously reported in OTC^{Spf-Ash} mice and other models.^{31,39,45} Notably, the ability to re-administer AAV vectors could allow lower initial doses, thereby mitigating safety concerns associated with high-dose AAV, including genotoxicity and other adverse events.⁴⁶ These considerations emphasize the need for strategies enabling safe and effective re-dosing.

Several strategies have been explored to overcome anti-AAV immunity, including immunosuppressive regimens and NABs removal by plasmapheresis and IgG-cleaving endopeptidases.^{15–17,22,23,47–49} The strategy we tested here is based in the co-administration of the AAV vector with the ImmTOR (rapamycin-loaded nanoparticles). These

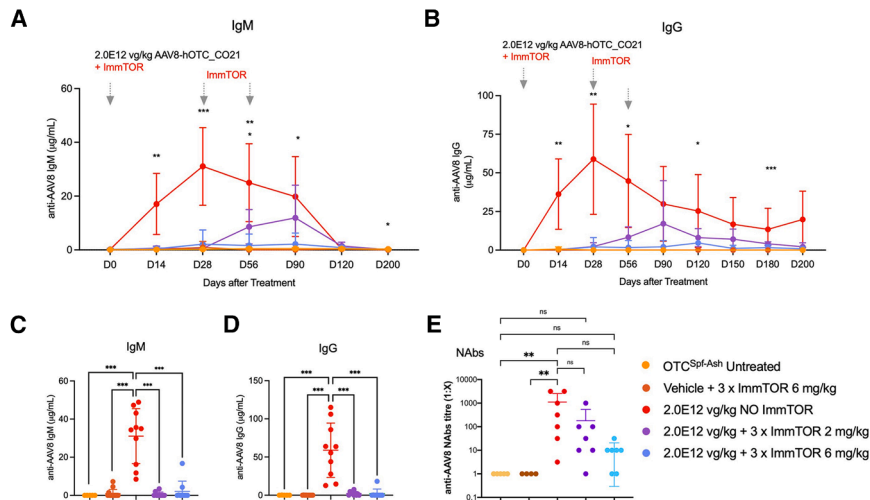


Figure 4. Anti-AAV antibody levels after AAV dosing and repeated ImmTOR treatment in juvenile OTC^{Spf-Ash} mice

(A and B) Time course of anti-AAV IgM (A) and anti-AAV IgG (B) generation after dosing. (C–E) Scatter dot-plot indicating sample size, average and dispersion of anti-AAV IgM at day 28 post-treatment (C) and anti-AAV IgG (D) at day 28 post-treatment (one way ANOVA, $p < 0.0001$, multiple comparisons, $***p \leq 0.001$), and anti-AAV neutralizing antibodies (E) titer at day 200 post-treatment (D200). One-way ANOVA (Kruskal-Wallis test), $p = 0.0006$; multiple comparisons, ns, not significant; $**p \leq 0.01$. Mean (SD).

nanoparticles, when co-administered with the antigen, have been shown to induce antigen-specific tolerance by promoting tolerogenic antigen-presenting cells and antigen-specific regulatory T cells, thereby inhibiting T cell-dependent B cell responses.^{25,50} ImmTOR has shown efficacy in other models,^{20–23,29} but this is its first application in a urea cycle disease.

Several key observations emerged from the present study. First, ImmTOR + AAV co-administration did not induce toxicity; hematological parameters remained within normal ranges, supporting the safety of this approach. Second, ImmTOR strongly modulated humoral immunity: high-dose ImmTOR reduced anti-AAV IgM and IgG to baseline for the at least 6 months and lowered NAb titers by approximately 100-fold compared with AAV alone, reaching levels that have the potential to be compatible with successful re-dosing. This preliminary proof-of-concept experiment, however, needs to be coupled with re-dosing experiments to evaluate both humoral and cellular immune responses against AAV antigens, and confirm that these reduced NAb levels permit successful vector re-administration. In contrast, low-dose ImmTOR maintained tolerance only for 28 days, consistent with previous reports that sustained immunomodulation requires higher doses.^{20,21,23} Residual NAb could potentially be further reduced by combining ImmTOR with B cell-targeting agents, such as anti-B-cell activating factor (BAFF) antibody or with engineered Treg-selective IL-2 muteins, which have been shown to enable repeated administrations even at high AAV doses,^{22,51} or with IgG-cleaving endopeptidases (imilef-dase, IdeS)^{52–54} or other immunomodulatory approaches, such as B cell transient immunosuppression.⁵⁵ Third, beyond immunomodulation, ImmTOR activated autophagy, enhancing ureagenesis and reducing ammonia and urinary orotic acid—findings aligned with prior studies on autophagy enhancers in hyperammonemia.^{33,34,56,57} Fourth, long-term correction in juvenile P30 mice requires a higher dose than the one necessary for adult mice. Dosing even younger animals would probably require higher AAV doses, or earlier vector re-administration.

However, this study also has several limitations. Although ImmTOR significantly reduced anti-AAV IgG and NAb titers, residual antibodies were still detectable, and the ability to achieve complete immune tolerance remains to be confirmed. The study did not include a re-dosing experiment, which is essential to validate whether ImmTOR-mediated immunomodulation effectively enables repeated AAV administrations. Moreover, the long-term safety of ImmTOR, particularly in combination with high AAV doses, requires further evaluation. Future work should focus on optimizing ImmTOR dosing regimens and exploring combination strategies with B cell-targeting agents or IgG-depleting strategies to further suppress humoral immunity. Additional studies in neonatal models and large animals will be necessary to assess efficacy in rapidly growing livers and to confirm translational potential.

It should be noted that an alternative approach that could avoid the need for re-dosing involves permanent genome modification.^{45,58,59,60,61,62} Recent clinical trials for OTCD (OTC HOPE, sponsored by iECURE; <https://otc-hope.com/>) are evaluating *in vivo* gene editing strategies (ECUR-506) in infants up to 7 months of age and have reported encouraging clinical responses. This pioneering effort underscores both the promise of early intervention and the potential need for re-dosing strategies if initial treatment is suboptimal or if therapeutic effect declines over time, both circumstances where ImmTOR-mediated immunomodulation could prove a valuable strategy to enable effective re-administration. In fact, one of the limitations of this approach is the relatively low recombination efficiency, which decreases with patient growth and becomes minimal when the liver enters a quiescent state following proliferation.

Collectively, our results support ImmTOR as a promising strategy to enable AAV vector re-administration, reduce initial dosing and improve safety. This approach could be extended to other urea cycle disorders and inherited metabolic liver diseases, addressing a critical unmet need in pediatric gene therapy.

MATERIALS AND METHODS

Animals

All animal care and experimental procedures were evaluated and approved by the International Center for Genetic Engineering and

Biotechnology (ICGEB) board and the Italian Ministry of Health (Ministero Italiano della Salute, authorization no. 926/2017-PR and no. 996/2017-PR), in full compliance with the EU Directive 2010/63/EU. Breeding pairs of OTC Spf-Ash mice (B6EiC3Sn a/A-OTC^{Spf-Ash/J}) were purchased from Jackson Laboratories (stock no. 001811) and the colony was expanded and maintained at the ICGEB BioExperimentation Facility. B6EiC3Sn C57BL/6 and OTC mice (OTC^{Spf-Ash}) were maintained in a temperature-controlled environment with 12/12 h of light/dark cycles and received a standard chow diet and water *ad libitum*. WT males and heterozygous females were used to maintain and generate hemizygous males used for experimentation.

AAV vectors production and purification

The codon-optimized cDNA was cloned into the pSMD2 vector containing the alpha-1-antitrypsin (hAAT), liver-specific, promoter, and the apolipoprotein E (ApoE) enhancer. Human OTC_{codon optimized coding sequence number 21} (hOTC_CO21) was inserted using SalI and NheI restriction sites into the pSMD2 episomal AAV ITRs containing vectors as described previously.³⁰ AAV8 stocks were provided by Selecta Biosciences, aliquoted and stored at -80°C . Each aliquot was thawed once and used; leftover was discarded.

AAV gene transfer procedure

ImmTOR and AAV treatments were administered intravenously via the retro-orbital route of injection or tail vein injection as indicated in the experimental design. Dosing was performed according to the weight of the animals (vg/kg). Prior to the injections, juvenile (P30) mice were anesthetized with isoflurane. Untreated WT and mutant animals were used as controls.

ImmTOR tolerogenic nanoparticles

ImmTOR PLGA-based polymer nanoparticles encapsulating rapamycin were manufactured as described previously^{21,23,47} and used in mice in different doses as indicated in the experimental design of each experiment (2, 6 mg/kg). Empty NP were manufactured in an identical manner, but without rapamycin. The day of AAV administration the nanoparticles (rapamycin-loaded or empty) were thawed at room temperature and mixed with AAV at desired concentration. After 5–10 min of incubation, the preparation was ready for injection in the animal. Administration occurred within 30–45 min from preparation.

Biochemical analyses of blood samples

Mice were fasted for 4 h before collecting whole blood samples. For glucose analysis, a drop of blood was loaded onto AccuCheck Guide reactive strips (Roche, Monza, Italy) and quantified with the appropriate instrument. For whole blood analyses, 100 μL blood was collected into a Sarstedt Micro sample tube with EDTA K3E (Sarstedt) and readily analyzed at an automated veterinary instrument Mindray BC-2800 Vet (Mindray, Trezzano sul Naviglio, MI, Italy) as previously described.⁶³ Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) determination was performed

using Abnova assay kit (KA4189 and KA4187) according to manufacturer's instructions.

Hematological analysis

For whole blood analyses, 100 μL blood was collected into a Sarstedt Micro sample tube with EDTA K3E (cat. 41.3395.005) and readily analyzed at an automated veterinary instrument Mindray BC-2800 Vet.

Ammonia determination

Ammonia was analyzed spectrophotometrically with the Ammonia Assay Kit from Sigma-Aldrich (AA0100) following the indication provided as described previously.⁴⁵

Ureagenesis assay

Mice were weighed and injected intraperitoneal (i.p.) with a dose of 4 mmol/kg of isotopically marked ammonium chloride ($^{15}\text{NH}_4\text{Cl}$, cat. no 39466-62-1, Merck), in our case 6.25 $\mu\text{L/g}$ of a 0.64 M working solution. At time point 0 and before each treatment, mice were fasted for 3 h to normalize endogenous urea in-between groups. Therefore, blood was collected the day before the treatment (T0), at 15 (T1), and 30 (T2) min after the treatment by retro-orbital bleeding using sterile capillary glass tubes. After collection blood was left to clot for 45 min and centrifuged at 8,000 g, then serum was moved to clean tubes and stored at -80°C until analysis. Serum samples were analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS), with the use of an internal urea standard marked on C and N2 (15N213C-urea; 64_46). In brief, 1 volume of serum was mixed with 3 volumes of pure acetonitrile, vortexed, and centrifuged at max speed for 10 min (repetition time, RT). Supernatant was loaded into HPLC-MS tubes and used for the quantification of all marked (62_44/45, 63_45) and unmarked (61_44) urea isoforms (enrichment %). This method was adapted based on the work from Allegri et al.⁶⁴

Urinary orotic acid quantification by HPLC-MS

Urine samples were collected at intermediate time point and stored at -80°C until use. Orotic acid was quantified with HPLC-MS strictly as described previously.³⁰ Urinary orotic acid biomarker accumulation was analyzed by HPLC-tandem MS as described previously.³⁰ Quantitative experiments were performed using as internal standard 200 μM isotopically labeled 1,3-15N2 orotic acid both in the calibration curve and sample buffers. Quantification was done using a calibration curve with linear range ($R^2 > 0.99$) from 15 to 300 μM . A Bruker (Bremen, Germany) amaZonSL bench-top ion trap mass spectrometer, equipped with an electrospray source, was used. The source was operated in negative-ion mode with a needle potential of 4,500 V and a gas flow of 12 L/min of nitrogen with heating at 200°C . The chromatographic separations for quantitative experiments were performed using a series 1260 Agilent Technologies (Waldbronn, Germany). HPLC with autosampler controlled from the Bruker Hystar data system. A Phenomenex (Torrance, USA) HPLC column Gemini C18 5 μm , 110Å, 2×150 mm was employed. Column flow rate was 0.4 mL/min, and elution was performed using 5 min wash time after

10 μ L injection and a 3 min gradient from water with 0.1% formic acid to 90% acetonitrile with 0.1% formic acid. The tandem MS/MS transitions used for the quantitative experiments (multiple reaction monitoring, MRM) were m/z 155.1 to 111.1 (orotic acid) and 157.1 to 113.1 ($1,3\text{-}^{15}\text{N}_2$ orotic acid). The acquired data were processed using the Bruker Compass Data Analysis proprietary software. Raw orotic acid concentration was normalized by urinary creatinine using the enzymatic mouse creatinine kit (Crystal Chem, cat. 80350) following a protocol optimized from the manufacturer instructions as previously described.³⁰ In brief, urine samples were diluted 1:10 in PBS in a 96-well PCR plate. Samples and standards were equilibrated to RT before starting the assay. For each sample and standard 5 μ L (ranging from 0 to 5 mg/mL) were transferred into a 96-well transparent plate. Buffer 1 was added, incubated at 37°C for 5 min, and absorbance was read on a plate reader to measure the background (T0). Following, buffer 2 was added, incubated at 37°C for 5 min and absorbance was read to determine enzymatic reaction completion (T1). Creatinine concentration was calculated by interpolation to standard curve. Standard and sample concentration in mg/dL was calculated as $(\Delta T - k)/N$ and multiplied by dilution factor (10). The values obtained were expressed in mM.

Viral genomes determination

Genomic DNA was extracted from pulverized liver using the Wizard SV genomic DNA purification system (Promega) following the manufacturer's guidelines. Vector genomes in liver were quantified by real-time PCR using the iQ SYBR green supermix (Bio-Rad), using primers targeting inside the promoter region as previously described.³¹

Preparation of total protein extracts and western blot analysis

Total liver protein extracts were prepared as previously described.³⁰ In brief, livers were collected and reduced in powder using a mortar and liquid nitrogen. Total liver protein extracts were extracted with a homogenizer in lysis buffer (Mitochondrial lysis buffer). Proteins in total liver lysates were separated on a 10% SDS gel or Precast 4%–12% SDS gel (Invitrogen). Proteins were transferred onto either a nitrocellulose membrane or polyvinylidene difluoride (PVDF) membranes, blocked with 1X PBS-Tween 20 0.1%, skim milk 5%, and probed with rabbit anti-human OTC antibody (Abcam, Ab203859; dilution 1/3,000), anti-LC3B (Novus Biologicals; Cat# NB-100-2220; dilution:1/1000), rabbit anti-P-S6 (Cell Signaling Technology; Cat# 2211, dilution 1/1,000), rabbit anti-S6 (Cell Signaling Technology; Cat# 2217, dilution 1/1,000), mouse anti- β -actin (Novus Biologicals; Cat# NB600-501, dilution 1/1,000), and anti-HSP70 antibody (Enzo Life Sciences; dilution 1/8,000). The primary antibody was detected with a goat anti-rabbit IgG horse-radish peroxidase (IgG-HRP) or anti-rat, respectively. Quantification was performed as previously described.^{30,34}

OTC enzyme activity

OTC enzyme activity was determined in liver lysates as described previously.³⁰ Briefly, 1 mg of total liver protein extract (in mitochondrial lysis buffer, HEPES pH 7.4 10 mM, Triton X-100 0.5%, DTT

2 mM)³⁰ was added to 350 μ L of reaction mixture (5 mM ornithine, 15 mM carbamoyl phosphate, and 270 mM triethanolamine [pH 7.7]) and incubated at 37°C for 30 min. The reaction was then stopped by adding 125 μ L of 3:1 phosphoric/sulfuric acid solution followed by 25 mL of 3% 2,3-butanedione monoxime and incubated at 95°C for 15 min in the dark. Citrulline production was determined by measuring the absorbance at 490 nm. For each sample the assay was performed in duplicate.

Detection of anti-AAV8 antibodies

IgG and IgM were measured by ELISA following a protocol designed at Selecta Biosciences. In brief, 96-well plates were coated with 1×10^9 vg/well of AAV-empty capsid diluted in carbonate buffer (1 μ g/mL) by overnight incubation at +4°C. Wells were then washed with wash buffer (1X PBS 0.05% Tween 20) and 200 μ L of blocking buffer (PBS/milk 6%) were added and incubated 2 h at RT. Wells were then washed and serum samples (diluted 1:5 in blocking buffer) were added to the wells and incubated 1 h at 37°C. Wells were rinsed with wash buffer and anti-mouse IgG-IgM/HRP was added and incubated 1 h at 37°C. Wells were again washed and freshly prepared *o*-phenylenediamine dihydrochloride (OPD) substrate was added and incubated 15 min at RT. Stop solution was added and 492 nm absorbance was measured with a plate reader. Each sample was tested in duplicate and measured along with a fresh calibration curve.

In vitro AAV8 neutralization assay

NABs titer was measured following a modified version of the cell-based *in vitro* reporter assay described by Meliani et al.⁶⁵ Briefly, on day one Hek293 E (Epstein-Barr virus infected cells) were seeded in a 96-well plate at a density of 2.0×10^6 cells/well, in a medium supplemented with heat-inactivated fetal calf serum (FCS) and Ponasterone A. The medium was kept antibiotic free. On day two, after assessing no visible contamination of cultured cells, serum samples were thawed on ice and serial semi-log dilutions of samples and titer standard (1:1; 3.16; 10; 31.6; 100; 316; 1,000; 3,160) were prepared together with a maximum (no inhibition) and minimum (no AAV reporter) controls. Then AAV-luciferase reporter at the titer of 2×10^{11} vg/mL was added in 1:1 ratio to the test samples and incubated at 37°C for 1 h. After incubation, the sample/AAV mix was used to infect cells at MOI = 200 and incubated 21–22 h at 37°C in a cell-culture incubator. On day three, the luciferase assay was performed using the Luciferase Assay System from Promega (cat. E4550). Briefly, cells were lysed directly in the plate where luciferase substrate was added to read luminescence intensity with a plate reader. The neutralizing titer of the sample was determined as the first dilution at which 50% or greater inhibition of the luciferase expression was reported.

Figure Layout: Graphical illustrations were designed and assembled using BioRender (<https://www.biorender.com>), an online tool for creating scientific visuals.

Statistics

Statistical analyses were performed for all the assays using GraphPad Prism package. Data in the graphs were plotted as mean with the

corresponding standard deviation (SD). Statistical analysis of data containing different time points was done using a mixed-effect model (REML). The comparison of groups was done using one-way or two-way ANOVA. A p value below 0.05 was considered significant (ns, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$).

DATA AND CODE AVAILABILITY

The authors confirm that data supporting the findings and conclusion of this study are presented within the article and supplemental information. Additional data are available from the corresponding author upon reasonable request.

ACKNOWLEDGMENTS

The authors thank the ICGEB BioExperimentation Facility for help with animal care. This work was supported by ICGEB intramural funding to AFM and by Selecta Biosciences.

AUTHOR CONTRIBUTIONS

P.I., N.B.-P., G.R., T.K.K., and A.F.M. contributed to experimental design and results interpretation; T.K.K. and A.F.M. were responsible for funding acquisition; A.V., F.B., G.R., C.G., F.C., P.V., L.R.S., G.d.S., M.L., H.S., N.T., L.C., A.I., L.M., and G.B. contributed to experimental design, performed experiments, and analyzed the data; N.S., G.R., T.K.K., and A.F.M. supervised the project; A.V. and G.B. prepared the figures; A.V. and A.F.M. wrote the original draft. All authors contributed to the revision and correction of the manuscript.

DECLARATION OF INTERESTS

P.I. and T.K.K. were employees of Selecta Biosciences. T.K.K., G.d.S., and A.F.M. are inventors in patents describing the methods and compositions of OTC constructs and vectors. G.R. is an associate editor of the journal.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omta.2026.201793>.

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