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Repeated dosing of AAV-mediated liver gene therapy in juvenile rat and mouse models of Crigler-Najjar syndrome type I

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Crigler-Najjar syndrome is an ultra-rare monogenic recessive liver disease caused by *UGT1A1* gene mutations. Complete UGT1A1 deficiency results in severe unconjugated hyperbilirubinemia in newborns that, if not treated, may lead to brain damage and death. Treatment is based on intensive phototherapy, but its efficacy decreases with age, rendering liver transplantation the only curative option. Adeno-associated virus (AAV)-mediated gene therapy has shown long-term correction in adult patients, but loss of viral DNA and therapeutic efficacy are expected in younger patients associated with liver growth. Effective vector re-administration is hindered by anti-AAV neutralizing antibodies generated during the first administration. Here, we investigated AAV vector re-administration by modulating the immune response with rapamycin-loaded nanoparticles (ImmTOR) in Gunn rats (*Ugt1a*$^{-/-}$) and *Ugt1a*$^{-/-}$ mice. We administered a liver-specific AAV8 vector expressing a codon-optimized h*UGT1A*1 cDNA (1.0E11 vg/kg) in P25-P28 mutant animals and, upon loss of efficacy after 3 to 5 weeks, a higher second dose (1.0E12 or 5.0E12 vg/kg) was given. ImmTOR co-administration reduced anti-AAV neutralizing antibodies and immunoglobulin Gs generation in male animals of both models allowing effective re-dosing, underscored by a significant and long-term decrease in plasma bilirubin, although efficacy was affected by low-titer residual anti-AAV antibodies suggesting that re-administration in patients may require combination with other methods.

INTRODUCTION

Crigler-Najjar syndrome (CNS) is an ultra-rare inherited autosomal recessive disorder that affects bilirubin metabolism. It occurs with an incidence of about 1 in 1,000,000 newborns. CNS is caused by mutations in the *UGT1A1* gene, coding for the only enzyme responsible for bilirubin conjugation in the liver, UDP glucuronosyltransferase family 1 member A1 (UGT1A1).[1] The most severe form, CNS type I (CNSI; OMIM number 218800), is characterized by the complete or near complete absence of residual UGT1A1 activity, with progressive accumulation of unconjugated bilirubin (UCB) in the blood and lipid-rich tissues, resulting in permanent neurological damage and the risk of death, if not promptly treated.[2] From birth, patients must receive daily cycles of 12–16 h of exposure to blue light but the effect of this cumbersome treatment is temporary as the efficacy of phototherapy (PT) diminishes with age. So far, the only curative treatment is a liver transplantation, a major surgery having important limitations and risks warranting the development of novel treatments.[3–5]

Efficacy of adeno-associated virus (AAV)-mediated liver gene therapy in preclinical models of Crigler-Najjar provided the basis for a clinical trial in adult patients.[6–9] In fact, this approach resulted in an important decrease in plasma bilirubin levels, discontinuation of phototherapy, with a follow-up of 18 months.[10] While this strategy was shown to be effective in adult patients, with a fully developed liver, the perspective changes considerably when treating neonatal and pediatric patients. Preclinical studies, showing loss of efficacy in neonate and juvenile animals, have raised concerns on sustained efficacy of AAV gene therapy in neonates and children.[8,11–13] The loss of episomal AAV genomes in proliferating hepatocytes during growth leading to diminished correction may require

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re-administration of the AAV vector later in life to re-establish therapeutic efficacy. However, the induction of anti-AAV neutralizing antibodies (NAbs) upon the first AAV treatment renders re-administration of the same vector ineffective.[14–16] Different strategies have been proposed to overcome this obstacle to allow effective vector re-administration, such as transient immunosuppression by the administration of immunomodulators,[13,17–22] albumin masking of the viral capsid,[23] plasmapheresis to lower neutralizing antibody titer,[24,25] serotype switching,[26] saturating the neutralizing antibodies by decoy empty capsids,[27] or the pre-treatment with immunoglobulin (Ig)G-cleaving endopeptidases[28] to remove anti-AAV NAbs from the bloodstream of seropositive subjects.

In the present work, we investigated the potential modulation of the immune response against the AAV vector by the co-administration of rapamycin-loaded nanoparticles (ImmTOR). These nanoparticles were shown to blunt immune responses toward the bacterial enzyme pegadricase enabling monthly dosing and sustained reduction of serum uric acid levels in a Phase 2 trial on gout patients.[29,30] ImmTOR primarily targets the antigen-presenting cell-T cell axis by inducing a tolerogenic phenotype in antigen-presenting cells (APCs), which results in the induction of antigen-specific regulatory T cells when co-administered with the target antigen.[31–33] In preclinical models these particles proved effective in mitigating the antibody response to AAV vectors.[22,31,34,35] We have investigated the potential use of ImmTOR tolerogenic nanoparticles in two relevant animal models of CNS, Gunn rats (*Ugt1a*$^{-/-}$) and *Ugt1a*$^{-/-}$ mice. Both models present severe hyperbilirubinemia due to a mutation in the *Ugt1a* gene, resulting in the complete absence of bilirubin-glucuronidation activity.[36,37] Juvenile mice and rats were repeatedly treated with a good manufacturing practice (GMP)-like batch of the AAV8-AAT-hUGT1A1 vector, admixed with ImmTOR tolerogenic nanoparticles.

RESULTS

Dose- and age-finding studies in juvenile *Ugt1a*$^{-/-}$ mice

To demonstrate effective AAV re-administration by ImmTOR treatment, a dose-finding study in mice of different ages was performed to optimize dosing of the AAV vector in order to induce a humoral immune response against the infused AAV8 vector while achieving transient levels of expression of the *UGT1A*1 transgene. The complexity of these studies derives from the extremely low doses of AAV vectors needed to correct the phenotype in *Ugt1a*$^{-/-}$ mice and the fact that juvenile mice have an immature immune system that leads to variable antibody formation, in particular at low doses. In fact, high vector doses result in the long-term normalization of plasma bilirubin,[6–8] leading to the inability to induce a further decrease of the serological marker (total bilirubin) after vector re-administration. Based on prior experience,[6,7] *Ugt1a*$^{-/-}$ mice were injected either at postnatal day 11 (P11) with the same AAV8-AAT-hUGT1A1 vector at 2.5E11 or 5.0E11 vg/kg, or at P25 or P40 with 1.0E11 or 2.5E11 vg/kg (Figure 1A). We monitored plasma total bilirubin (TB) (Figures 1B–1D), determined the titer of anti-AAV NAbs in treated animals over time (Figures 1E–1G), and euthanized them at P120. To prevent neonatal mortality, all mice were treated with phototherapy up to P30.[36,38] *Ugt1a*$^{-/-}$ mice dosed at P11 showed a transient correction with both AAV doses tested, with a variable anti-AAV8 NAbs response that peaked at P30 (Figures 1B and 1E). In *Ugt1a*$^{-/-}$ mice dosed at P25, the higher dose resulted in sustained correction while the lower dose provided a transient correction and a clear induction of NAbs (Figures 1C and 1F). These vector doses provided a sustained correction in the *Ugt1a*$^{-/-}$ mice dosed at P40 with a clear induction of NAbs (Figures 1D and 1G). The high anti-AAV8 NAb titer at P40 and P60 detected in the mice injected at P25 indicates that the treatment at this age may allow for evaluating the efficacy of ImmTOR in vector re-administration (Figure 1F). Thus, based on these dose-finding studies, treatment of mice at P25 with a dose of 1.0E11 vg/kg was selected for testing the efficacy of ImmTOR (Figure 1C). As expected,[8] the loss of correction was accompanied by a loss of viral genome copies (VGs) from the liver, and by the decrease in the intensity of the UGT1A bands detected by western blot analysis of liver protein extracts (Figures S1A and S1B).

Safety of ImmTOR administration in infantile WT and *Ugt1a*$^{-/-}$ mice

To test if a single administration of ImmTOR could result in adverse effects in suckling animals, different doses of ImmTOR (6, 8, and 12 mg/kg) were intravenously administered to P11 male and female wild-type (WT) and *Ugt1a*$^{-/-}$ mice (Figure S2A). Since the clearance of ImmTOR nanoparticles is relatively fast,[33] to be able to observe potential ImmTOR side effects, mice were euthanized at P60. The ImmTOR treatment did not result in major differences in the growth curve, with all mice showing an increase in body weight similar to that of untreated animals (Figures S2B–S2E). *Ugt1a*$^{-/-}$ male mice treated with 12 mg/kg ImmTOR showed a trend toward increased body weight compared with untreated controls, reaching statistical significance for males at P60 (Figures S2E and S3D), excluding growth retardation in mice treated with ImmTOR.

To monitor potential liver damage due to ImmTOR administration, the liver transaminases alanine aminotransferase (ALT), aspartate aminotransferase (AST), and AST/ALT ratio in WT and *Ugt1a*$^{-/-}$ mice were determined. Similarly to what was observed for body weight, we observed no major differences although some of the groups presented statistically significant differences that were not the same in WT and *Ugt1a*$^{-/-}$ groups, suggesting they were not associated with the treatment. For example, we observed a reduction in both ALT and AST in WT mice treated with 8 mg/kg of ImmTOR and an increase in AST levels and the AST/ALT ratio in *Ugt1a*$^{-/-}$ mice treated with 8 mg/kg ImmTOR at 2 months post-injection (Figures S2F, S2I, and S2K). Importantly, AST and ALT levels in animals treated with 12 mg/kg ImmTOR were not significantly different from those of the respective untreated controls for either WT or *Ugt1a*$^{-/-}$ mice and the ALT/AST ratio remained well below the value of 2, suggesting no liver damage (Figures S2J and S2K).

At P60 all animals were euthanized, and the spleen and the liver were dissected and weighed (Figure S3). This revealed small but statistically

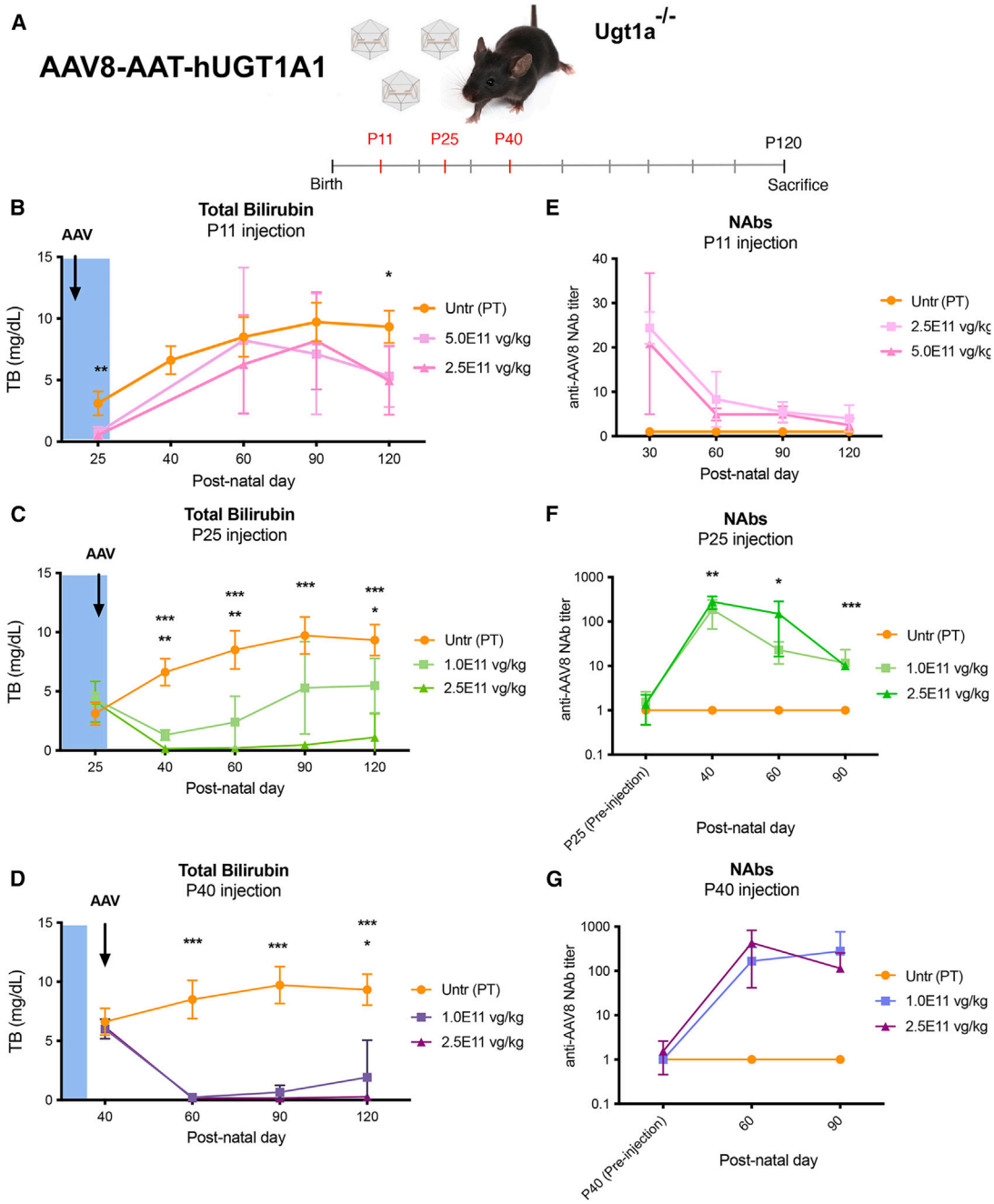

Figure 1. Dose- and age-finding studies in juvenile *Ugt1a*$^{-/-}$ mice

(A) Scheme of the experimental design. *Ugt1a*$^{-/-}$ mice were injected either at postnatal day 11 (P11), P25, or P40 with different doses of the AAV8-AAT-hUGT1A1 vector and euthanized at P120. To prevent neonatal mortality, all mice were treated temporarily with phototherapy (PT-light blue rectangle) up to P30. (B and E) *Ugt1a*$^{-/-}$ mice were injected at P11 with 2.5E11 (n = 4) or 5.0E11 vg/kg (n = 7) of AAV8-AAT-hUGT1A1 vector. Plasma bilirubin (B) and total anti-AAV8 NAbs titer (highest serum dilution giving 50% inhibition of transduction) (E) were determined. Untr (PT), *Ugt1a*$^{-/-}$ mice control animals treated temporarily with PT n = 8; 2.5E11 vg/kg, AAV8-treated *Ugt1a*$^{-/-}$ mice n = 4; 5.0E11 vg/kg, AAV8-treated *Ugt1a*$^{-/-}$ mice n = 7. (C and F) *Ugt1a*$^{-/-}$ mice were injected at P25 with 1.0E11 (n = 5) or 2.5E11 vg/kg (n = 6) of AAV8-AAT-hUGT1A1 vector. Plasma bilirubin (C) and total anti-AAV8 NAbs titer (F) were determined. AAV8-treated *Ugt1a*$^{-/-}$ mice n = 5; 2.5E11 vg/kg, AAV8-treated *Ugt1a*$^{-/-}$ mice n = 6. (D and G) *Ugt1a*$^{-/-}$ mice were injected at P40 with 1.0E11 (n = 4) or 2.5E11 vg/kg (n = 4) of AAV8-AAT-hUGT1A1 vector. Plasma bilirubin (D) and total anti-AAV8 NAbs titer (G) were determined. Linear mixed-effect model followed by post hoc tests. *p < 0.05; **p < 0.01; ***p < 0.001. For all the graphs results are presented as mean (SD).

significant differences in the liver/body weight ratio only in the WT males treated with 8 mg/kg ImmTOR compared with untreated control mice, while it was similar to WT for all female animals and for male mice treated with 6 mg/kg and 12 mg/kg of ImmTOR (Figure S3B), and for both female and male *Ugt1a*$^{-/-}$ mice treated with 8 mg/kg and 12 mg/kg of ImmTOR (Figure S3E). No differences were observed for the spleen/body weight ratio (Figures S3C and S3F). No major differences were observed in body weight, liver to body weight, and spleen to body weight ratios in the *Ugt1a*$^{-/-}$ mice, except for a minor but statistically significant increase in the weight in male mice treated with ImmTOR 12 mg/kg, as already mentioned above (Figures S3D–S3F).

Histological evaluation of sections from liver, spleen, lung, kidney, and reproductive organs of WT and *Ugt1a*$^{-/-}$ mice (males and females) treated with 12 mg/kg of ImmTOR showed no changes indicating that ImmTOR treatment at this young age appeared safe (Figure S4).

Repeated administration of AAV8-AAT-hUGT1A1 in juvenile *Ugt1a*$^{-/-}$ mice

Upon showing safety of ImmTOR in young mice, we evaluated whether the treatment with these investigational nanoparticles would allow effective AAV vector re-administration. Based on the optimal dosing regimen established earlier (Figures 1C–1F), P25 *Ugt1a*$^{-/-}$ mice were treated with 1.0E11 vg/kg of the AAV8-AAT-hUGT1A1 vector, while phototherapy was discontinued just before P30. The AAV vector was co-administered with ImmTOR (12 mg/kg) or with empty nanoparticles (empty NPs) (Figure 2A). Upon this treatment, plasma TB levels decreased in all groups. Similarly to what observed in Figure 1C, at P47 plasma TB started to increase in all female mice and in the males treated with empty NPs. At this moment a second dose (1.0E12 vg/kg) of the AAV vector plus ImmTOR or empty NP admix was administered (Figure 2B). After the second AAV administration, and in contrast with mice treated with empty NPs, the group of male mice treated with ImmTOR maintained low levels of plasma TB through the end of the experiment at week 11 (Figure 2B). In the female mice, at 1 and 2 weeks after the second injection, TB increased at a lower rate in animals receiving ImmTOR compared with those receiving empty NPs, but the difference did not achieve statistical significance (Figure 2B). At the end of the experiment, plasma bilirubin levels of female mice treated with ImmTOR was comparable to that of female animals treated with empty NPs, reaching the levels of untreated animals.

The analysis of anti-AAV8 IgM and IgG antibodies showed a peak of IgM at 1 week after the first dose in the groups treated with AAV plus empty NPs, that later decreased as mice seroconverted to IgG (Figures 2C and 2D), while in the groups treated with AAV plus ImmTOR the IgM peak was delayed and the levels did not decrease (Figures 2C and 2D). Interestingly, the levels of anti-AAV8 IgGs were very high in the groups treated with empty NPs, while in the groups treated with ImmTOR, IgGs were not detected at 4 weeks after the first AAV administration and were maintained very low only in males after the second AAV administration (Figure 2D). The animals treated with ImmTOR presented a significant reduction in anti-AAV neutralizing antibodies, which was more evident in male mice after the second AAV vector and ImmTOR administration (Figure 2E).

Determination of viral genome copy number (VGCN) in the liver showed a minor, but not statistically significant, increase in the group of males treated with ImmTOR, compared with the group treated with empty NPs (Figure 2F). In line with these previous results, an Ugt1a-specific band was detected only in the group of males treated with ImmTOR, as determined by western blot analysis (Figure 2G). The lack of therapeutic efficacy in female mice treated with AAV+-ImmTOR could be related to the combined effect of reduced AAV transduction in female mice[39] and the presence of residual anti-AAV NAbs (Figure 2E).

Dose-finding study of single AAV8-AAT-hUGT1A1 administration admixed with ImmTOR in juvenile Gunn rats

Next, we tested the strategy in a commonly used model of CNS: the Gunn rat.[9,40–43] We determined the optimal single dose of AAV8-AAT-hUGT1A1 able to transiently correct hyperbilirubinemia when co-administered with ImmTOR or empty NPs. Rats were treated at P28 with 5.0E11 vg/kg or 2.0E12 vg/kg of AAV8-AAT-hUGT1A1 combined with ImmTOR or empty NPs at 3 mg/kg, blood samples were periodically taken, and animals were euthanized 20 weeks after treatment (Figure 3A). Serum TB decreased to WT levels 1 week after vector administration in all AAV-treated animals (Figures 3B and 3D). In the rats treated with the highest AAV vector dose, serum TB levels remained low for the duration of the experiment, regardless of the immunomodulation treatment (Figure 3D). On the contrary, in the animals treated with the low dose, the efficacy waned over time, especially in the animals treated with empty NPs, while those treated with ImmTOR lost efficacy at a lower rate (Figure 3B). As previously observed in *Ugt1a*$^{-/-}$ mice, the loss of correction was less pronounced in the animals treated with ImmTOR, probably due to the increased AAV transduction efficacy. No major differences in body weight, AST-, or ALT-liver transaminases, were observed between animals treated with ImmTOR and empty NPs (Figure S5). Importantly, the titer of anti-AAV NAbs as well as IgM and IgG were very high in the rats treated with empty NPs, and it was significantly reduced in the animals treated with ImmTOR (Figures 3C, 3E, and S6). The inhibition of induction of these antibodies was more effective in the rats treated with the low AAV dose, lasting for more than 4 weeks after vector administration (Figures 3C and 3E).

The presence of VGCN at higher levels in the liver compared with the spleen confirmed the liver tropism of AAV8, and mRNA quantification demonstrated the tissue-specific expression of UGT1A1 provided by the alpha1-antitrypsin promoter (Figures S7A–S7D and S7E–S7H, respectively).

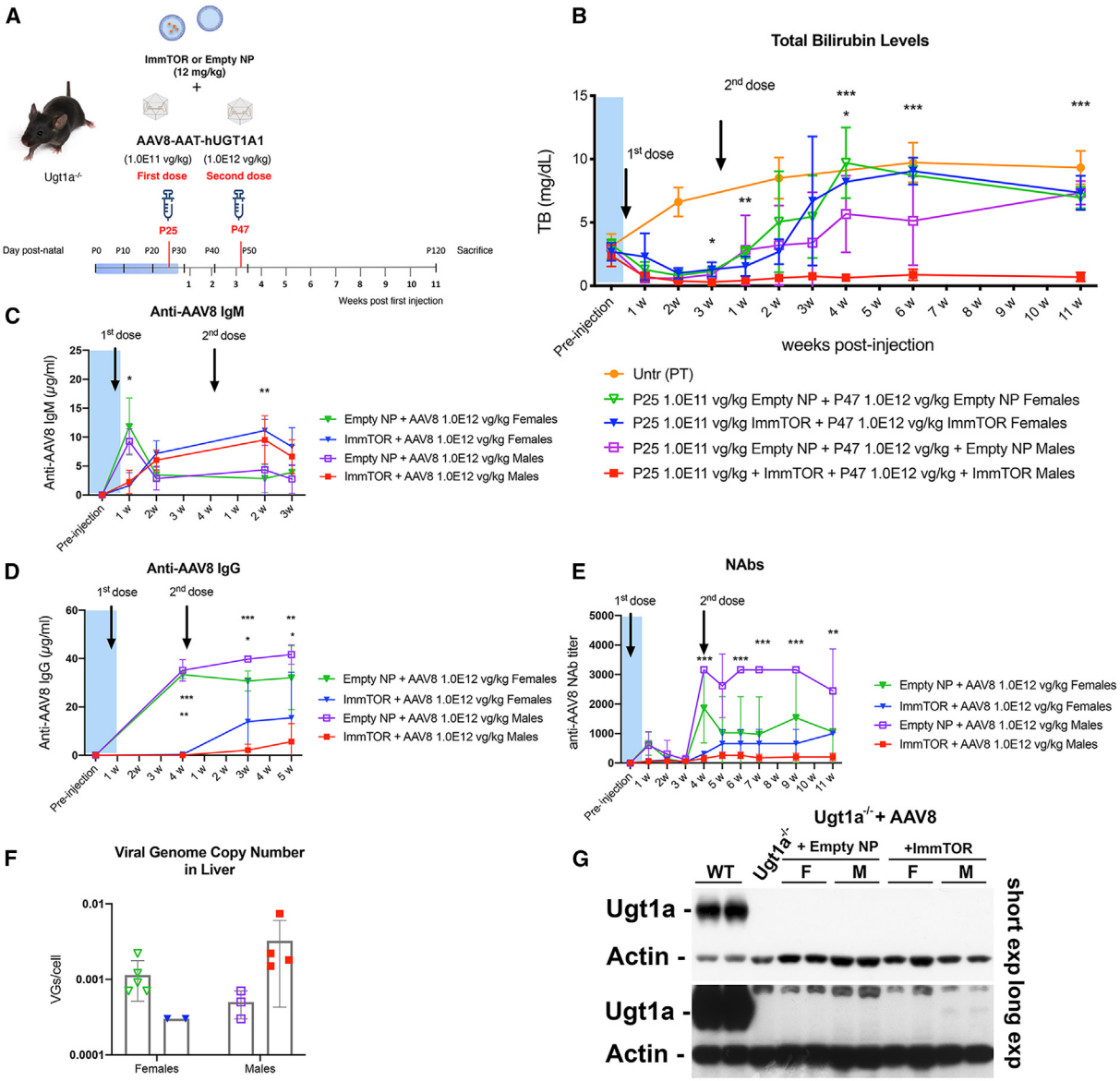

Figure 2. Repeated administration of AAV8-AAT-hUGT1A1 in juvenile *Ugt1a*$^{-/-}$ mice

(A) Experimental design. P25 *Ugt1a*$^{-/-}$ mice were treated with a dose of 1.0E11 vg/kg of the AAV8-AAT-hUGT1A1 vector, while phototherapy (blue box) was continued until P30. The AAV vector was co-administered with ImmTOR (12 mg/kg) or with empty nanoparticles (Empty NPs). Animals received a second dose of AAV8-AAT-hUGT1A1 vector (1.0E12 vg/kg) at P47. Blood samples were periodically taken. Mice were euthanized at P120 (11 weeks after the first administration of AAV8). (B) Time course of total bilirubin levels (mg/dL). Untr (PT), *Ugt1a*$^{-/-}$ mice control animals treated temporarily with PT $n = 8$; Male Empty NP $n = 4$, Male ImmTOR $n = 4$; Female Empty NP $n = 4$; Female ImmTOR $n = 2$ (3 female mice were dosed but one died during sampling for reasons unrelated to the treatment). (C–E) Time course of anti-AAV8 IgM (C), anti-AAV8 IgG (D), and anti-AAV8 NAbs titer (E) in *Ugt1a*$^{-/-}$ mice treated in (B); (F) Viral Genome copy number (VGs/cell) in liver of treated animals. (G) Western blot analysis of liver protein extracts. Actin was used as internal control. A short (upper panels) and a long (lower panels) exposition are shown. Linear mixed-effect model followed by post hoc tests. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. For all the graphs, results are presented as mean (SD).

ImmTOR particles allowed efficient AAV8 re-administration in Gunn rats

Since none of the tested doses resulted in the loss of therapeutic efficacy both in male and female rats, to test whether ImmTOR co-administration could allow for effective re-administration in the rat model of CNS, the initial AAV dose was further reduced to 1.0E11 vg/kg (as done in mice), resulting in a suboptimal therapeutic effect and significant loss of correction at 5 weeks after the vector administration (Figure 4A). At that time, a second AAV dose (5.0E12 vg/kg) was given with or without ImmTOR and the effect on serum TB,

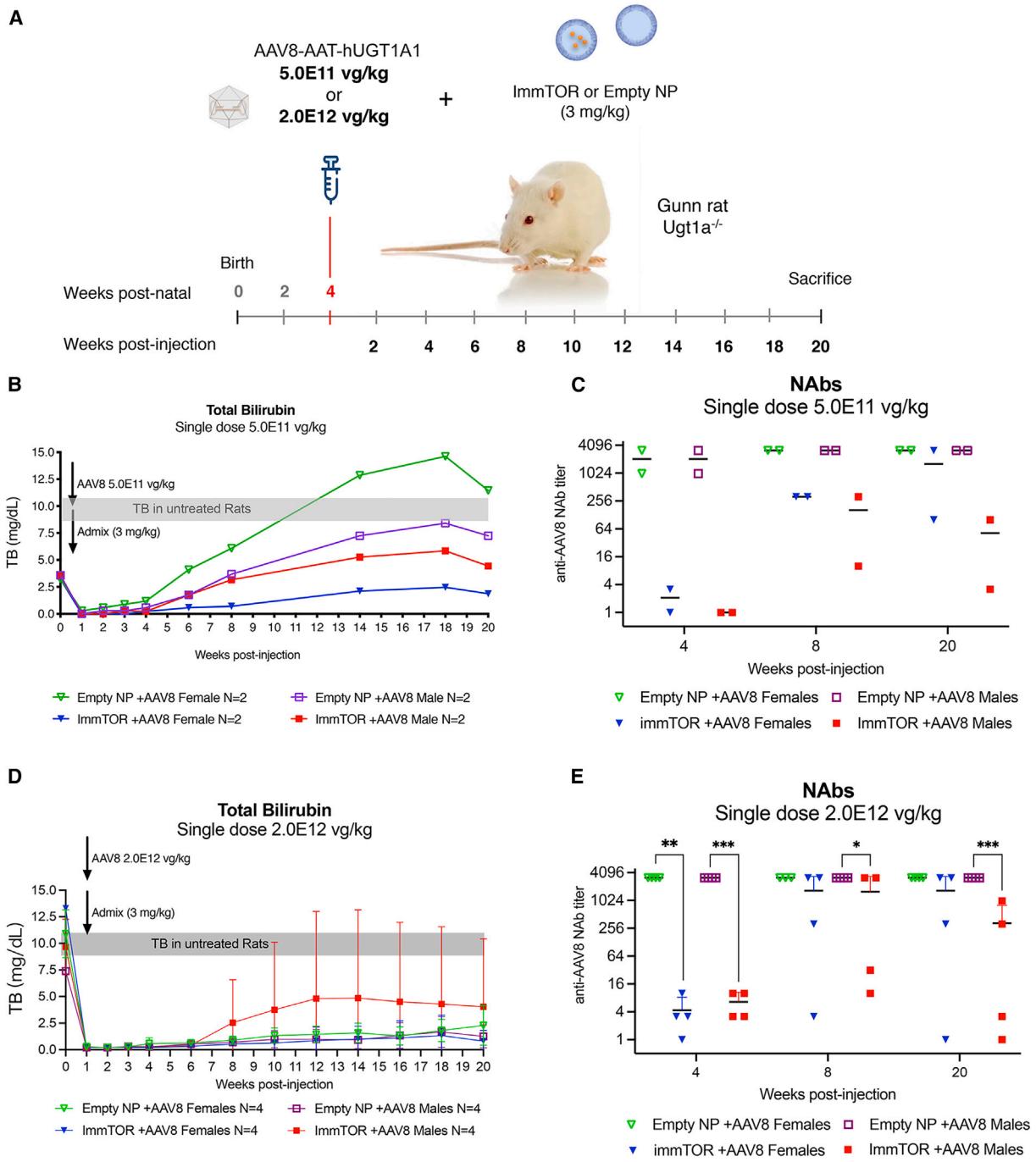

Figure 3. Dose-finding study of single AAV8-AAT-hUGT1A1 administration admixed with ImmTOR in juvenile Gunn rats

(A) Experimental design. Gunn rats were treated at P28 with 5.0E11 vg/kg or 2.0E12 vg/kg of AAV8-AAT-hUGT1A1 combined with ImmTOR or empty nanoparticles (Empty NP) at 3 mg/kg, blood samples were periodically taken, and animals were euthanized 20 weeks after treatment. (B and C) Time course of total bilirubin levels (mg/dL; B) and anti-AAV8 NAbs titer (C) in animals treated with 5.0E11 vg/kg AAV8-AAT-hUGT1A1 combined with ImmTOR or empty NP. (D and E) Time course of total bilirubin levels (mg/dL; D) and anti-AAV8 NAbs titer (E) in animals treated with 2.0E12 vg/kg AAV8-AAT-hUGT1A1 combined with ImmTOR or empty NP. Linear mixed-effect model followed by post hoc tests. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. For all the graphs results are presented as mean (SD).

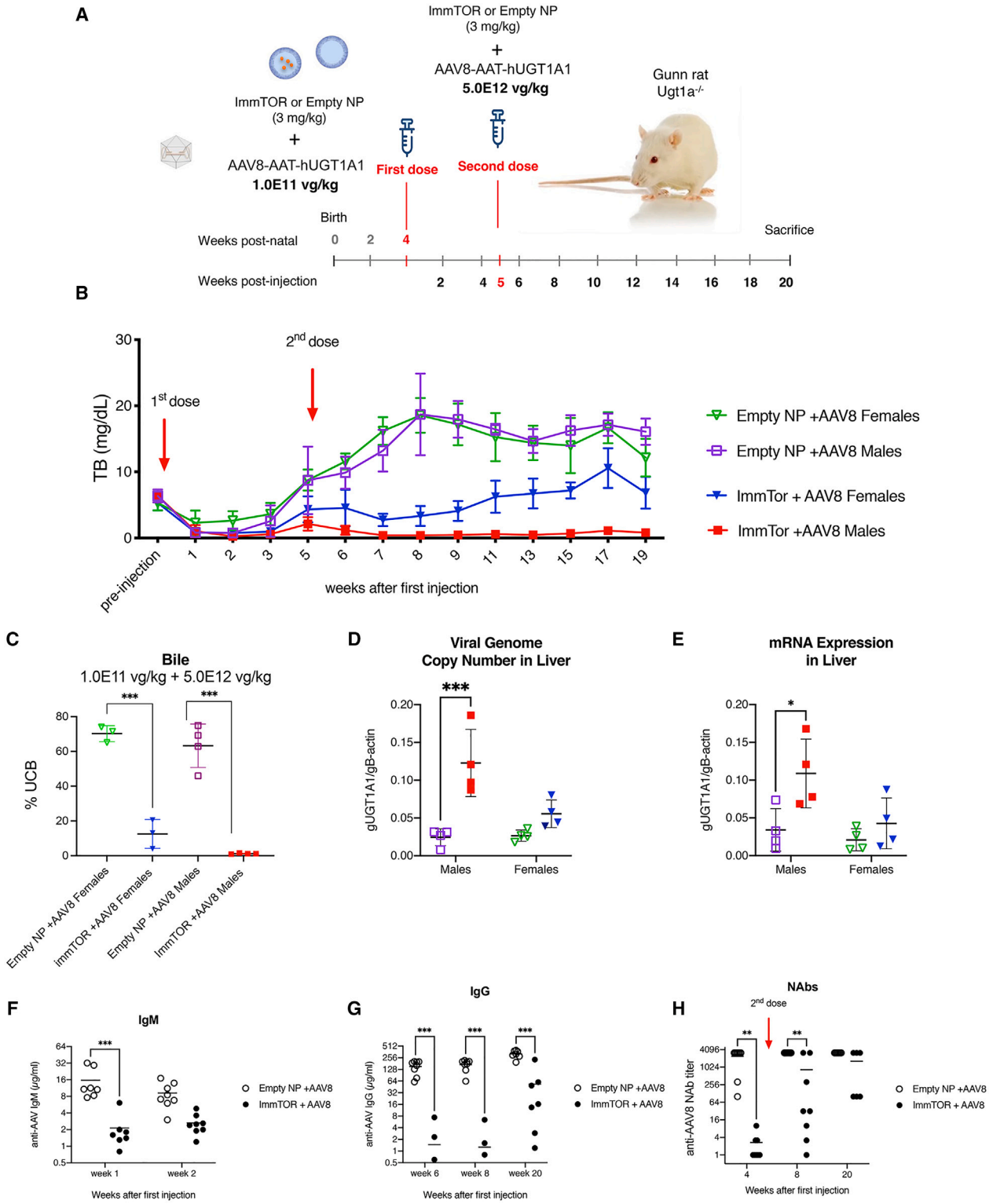

Figure 4. Repeated administration of AAV8-AAT-hUGT1A1 in juvenile *Ugt1a*$^{-/-}$ rats

(A) Experimental design. At 4 weeks of age *Ugt1a*$^{-/-}$ rats were treated with a dose of 1.0E11 vg/kg the AAV8-AAT-hUGT1A1 vector. The AAV vector was co-administered with ImmTOR (3 mg/kg) or with empty NP. Animals received a second dose of AAV8-AAT-hUGT1A1 vector (5.0E12 vg/kg) 5 weeks after the first administration. Blood

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anti-AAV IgM and IgG antibodies, and anti-AAV8 NAb levels were monitored by regular blood sampling until experimental week 20 when the animals were euthanized for further analysis. In accordance with the data obtained in the safety study in WT and *Ugt1a*$^{-/-}$ mice, the ImmTOR regimen did not affect growth of the animals, or cause changes in AST and ALT liver damage markers (Figures S8A–S8C), supporting the safety of this treatment. In both control groups, receiving empty NPs, the TB correction was lost over time and re-treatment did not show any efficacy (Figure 4B). In contrast, in the animals treated with ImmTOR, re-administration of the AAV vector resulted in a further decrease in serum bilirubin in male rats demonstrating effective re-treatment. In females, the effect was less evident, but re-administration did prevent the gradual TB increase overtime seen in the animals not treated with ImmTOR. The significantly lower level of TB in rats treated with ImmTOR compared with the female rats not treated with ImmTOR does demonstrate this compound increases the effectivity of vector re-administration (Figure 4B). As already observed, the correction of hyperbilirubinemia was more effective in male than in female rats, probably due to gender bias in the liver gene transfer efficiency in rodents.[44] Additionally, the bile of the empty NP-treated animals contained significant levels of UCB, illustrating the loss of the gene therapy efficacy in these groups, while the bile of the rats treated with ImmTOR contained only conjugated bilirubin (Figure 4C), demonstrating the efficient restoration of the UGT1A1 enzyme following the second AAV administration. These results were confirmed by the determination of the VGCN in the liver, which showed a statistically significant increase in both male and female rats treated with ImmTOR, compared with those treated with empty NPs (Figure 4D). No differences in VGCN were observed in the spleen, with no detectable presence of the *UGT1A1* gene expression (Figures S8D and S8E). The highest level of transgene expression was measured in male rats treated with ImmTOR, as shown by *UGT1A1* mRNA levels in the liver (Figure 4E). Determination of anti-AAV8 NAbs, IgM, and IgG showed a very strong humoral response in the rats treated with empty NPs compared to the ImmTOR-treated animals at 4 weeks after the first AAV administration (Figures 4F–4H). Upon re-treatment, the difference in NAb titer levels was less clear and was lost at 20 weeks after injection (Figure 4H). The presence of NAbs after the second injection indicated that the initial treatment with ImmTOR did not completely prevent an immunological response and that a second injection was likely to boost the response.

DISCUSSION

A recent report estimated that 3.5%–5.9% of the entire world population is affected by a rare disease, accounting for 262–446 million persons, of whom about 72% are of genetic origin with a vast majority of them with exclusive pediatric onset (69.9%).[45] Many of these inherited diseases require early intervention to prevent subsequent tissue and organ damage. Many liver diseases are orphan in terms of curative treatments and, in some of them, liver transplantation stands as the only curative option. Gene therapy is becoming an attractive option to restore the missing function through delivery of the corrected transgene, especially for the treatment of inborn errors of metabolism with hepatic involvement.[15,46] Since the beginning of the gene therapy era, CNS has been a paradigmatic disease model, being an ultra-rare genetic disease of the liver with pediatric onset and no cure except for liver transplantation, having an easy-to-measure biological marker of efficacy such as blood (plasma or serum) bilirubin. In fact, different integrative and non-integrative gene therapy strategies have been tested using adenoviral vectors, lentivirus, and AAVs.[36,40,42,47–51] AAV-mediated liver gene therapy treatment of adult mouse and rat models of the disease has shown efficacy and safety,[6–9] which led to the successful treatment of adult CNS patients.[10] However, translation of this approach to neonate and young pediatric patients may not be straightforward since hepatocyte replication during liver growth may lead to the loss of viral DNA genomes, with the consequent loss of therapeutic efficacy.[8,11–13] Life-long efficacy in young patients may thus require re-administration of the therapeutic vector. In fact, previous experiments showed that administration of AAV8 or AAV9 vectors to neonatal *Ugt1a*$^{-/-}$ mice and rats results in efficient correction of blood TB levels in the short term, but a gradual loss of therapeutic efficacy over time is observed.[8,13,48,52] Treating the patients early in life would avoid the risks of having peaks of bilirubin resulting in permanent damage,[2,53] early organ transplantation, and would substantially improve life quality.[54] The main barrier to re-administer the therapeutic vector in humans is the presence of high titer anti-AAV NAbs raised after a single AAV vector dosing and that persist for years.[27,55] Different strategies are being investigated to overcome this important limitation, although none of these potential alternatives has yet been evaluated in the clinic.[14,22,23,28,56] The use of ImmTOR tolerogenic nanoparticles to mitigate the formation of anti-drug antibodies is a very promising approach as it allowed re-administration of AAV vectors in different settings and preclinical models.[22,31,34,35,57] This approach was tested in clinical trials in adult individuals.[29,58,59] Thus, co-administration of ImmTOR nanoparticles with the AAV8-AAT-hUGT1A1 vector might mitigate the formation of anti-AAV NAbs allowing effective re-administration of the same therapeutic vector and enabling the treatment of pediatric Crigler-Najjar patients. As a hypothesis, a potential strategy may involve young Crigler-Najjar patients requiring intensive phototherapy co-administered with ImmTOR nanoparticles and the AAV8-AAT-hUGT1A1 vector. In the case of loss of therapeutic efficacy leading to the re-introduction of phototherapy, they could be re-administered with the same AAV vector. To investigate the feasibility of this approach we dosed juvenile animals of

samples were periodically taken. Rats were euthanized 20 weeks after the first administration of AAV8. (B) Time course of total bilirubin levels (mg/dL). (C) Bile analysis in rats injected as single dose or repeated administration in combination with ImmTOR nanoparticles. The percentage (%) of UCB in bile is represented. Each dot represents a single animal. (D) Viral Genome copy number (VGs/cell) in liver. (E) *hUGT1A1* mRNA expression in liver. (F–H) Anti-AAV8 IgM (F), anti-AAV8 IgG (G), and anti-AAV8 NAbs (H) were determined at different timepoints (indicated). Linear mixed-effect model followed by post hoc tests. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. For all the graphs, results are presented as mean (SD).

two models of CNS. Our results showed that co-administration of the AAV vector with ImmTOR nanoparticles resulted in the modulation of the immune response, with an important reduction of anti-AAV NAbs titer after the first vector administration. It is worth mentioning that the fine-tuning of the re-administration approach in both models required dose-finding studies with the AAV vector. Indeed, to verify its efficacy, it is pivotal to demonstrate a significant decrease in blood TB levels after the AAV vector re-administration. This requires a transient therapeutic effect of the first AAV administration that can be achieved only at very low AAV vector doses especially in the *Ugt1a*$^{-/-}$ mouse, which is likely to induce a low antibody response. Finding the experimental setting that does raise a substantial immune response in animals treated with the AAV vector without immune suppression and, at the same time, provides a suboptimal transient correction, proved challenging and necessitated testing different dosing regimens at different ages. This type of balance between therapeutic efficacy and immune response was not necessary in previous reports because of the higher doses of vector used (2.5–4.0E12 vg/kg)[22,31] and will certainly differ from the potential clinical setting for the treatment of patients. In the clinical setting, the situation is different, as the dose given in the first vector administration should be high enough to have long-term efficacy. In case of efficacy loss, multiple administrations may be needed, raising safety concerns related to potential activation of complement by immunocomplexes by residual anti-AAV antibodies, which need to be addressed. In our models, and especially in the *Ugt1a*$^{-/-}$ mice, the very low dose used to obtain a transient therapeutic effect resulted in a low and variable anti-AAV8 humoral immune response, with a rapid decline in NAbs titer, even in empty NP-treated animals. While doses inducing a reliable humoral response were sub-therapeutic in rats, in *Ugt1a*$^{-/-}$ mice we were not able to demonstrate a phenotypic improvement since plasma TB levels were still very low at the time point of the second AAV dosing, although the steady and long-term low-plasma bilirubin strongly supports the efficacy of vector re-administration. In fact, both in rats and male mice, re-treatment resulted in the long-term reduction of plasma bilirubin, an effect not observed in the absence of ImmTOR co-administration. As already observed in mice, the correction of hyperbilirubinemia was more effective in male than in female rats. Although this gender effect in AAV liver transduction has been reported for murine liver, in rats this is less clear. In a previous study using a suboptimal dose, the correction appeared more persistent in male rats, although the differences were small.[13] In another study we showed that macrophages could play a role in the reduced therapeutic efficacy of AAV vectors in female rats.[60] In that study, the blockade of AAV vector uptake by macrophages, by inhibiting Scavenger Receptor-A, had a more pronounced effect in adult females compared with adult males, resulting in higher hepatocyte transduction and therapeutic effect. Saturating this system will prevent loss of vector by macrophage uptake. Due to their higher macrophage uptake capacity, a higher vector dose will be needed in adult females to overcome this effect. A similar mechanism may have caused the reduced efficacy in the 9-week-old females that were injected at week 4 with this suboptimal dose and re-injected 5 weeks thereafter. Importantly, in both animal models we observed an increase in VGCN in the liver after vector re-administration with ImmTOR, supporting the possibility of re-administering the same AAV vector. Nonetheless, it is important to note that the VGCN present in the re-administered animals were lower than those present in control animals injected only as adults at P40 with a much lower dose (Figures 1D and S1A), suggesting that immunomodulation was incomplete and that very low titers of anti-AAV8 NAbs partially blunted transduction of the re-administered AAV vectors. Even if ImmTOR did not entirely block NAb titer in these Crigler-Najjar murine models, a decrease may be very important as it could enable re-dosing in combination with other strategies, such as IgG depletion through plasmapheresis,[24] immunoadsorption,[61] IgG-specific proteases,[28,62] or B-cell targeting agents.[63] Importantly, the safety studies performed here indicate that the treatment of P11 mice with ImmTOR, an age that in humans may correspond to 3- to 5-year-old children[64] was safe, supporting ImmTOR potential clinical application. These results also suggest that a better therapeutic effect can be obtained by increasing the dose of the second administration, allowing a minor fraction of AAV to bind to the residual NAbs while a therapeutically efficacious concentration of the AAV vector remains available to efficiently transduce hepatocytes, as shown in other models. In fact, a similar strategy was employed by Weber and colleagues, who recently showed efficacy of ImmTOR in blocking the generation of anti-AAV NAbs in a mouse model of progressive familial intrahepatic cholestasis type 3 (PFIC3) and the successful re-administration of the same vector. However, important differences are present between the PFIC3 model and ours, since (1) a much higher dose (8.0E13 vg/kg) was necessary to obtain a sub-therapeutic effect in juvenile 2-week-old PFIC3 mice, (2) the ImmTOR-mediated blockade of anti-AAV NAbs was more efficient in the PFIC3 model, and (3) the dose of second AAV administration was performed only 2 weeks after the first one, with the same dose used in the first administration,[35] which was much higher than the ones used here. In addition, ImmTOR was only co-administered with the first dose of vector, while the second (higher) dose was administered without ImmTOR. Unfortunately, the authors did not report naive rodents injected at 4 weeks (time point of the second AAV injection) to compare the VGCN and therapeutic efficacy with double AAV-ImmTOR injected mice, as we did in our study.

Summarizing, we observed an important reduction in anti-AAV NAbs and IgGs generation in mice and rats. However, ImmTOR only partially inhibited the IgM antibody response, and the neutralizing activity of the residual anti-capsid IgM response seems to affect the efficiency of vector re-administration, resulting in a partial therapeutic effect. Thus, the combination of ImmTOR with other methods mitigating the response or depleting NAbs from plasma may be needed to reduce vector neutralization and enable AAV vector re-dosing in the pediatric setting.

MATERIALS AND METHODS

Animals

Ugta1$^{-/-}$ mice have been described previously.[36] WT littermates were used as a control. Mice were housed and handled according to

institutional guidelines, and experimental procedures approved by International Centre for Genetic Engineering and Biotechnology (ICGEB) board and by the Italian Ministry of Health (authorization N. 971/2017-PR). Animals used in this study were at least 99.8% C57Bl/6 genetic background, obtained after more than nine backcrosses with C57Bl/6 mice. Mice were kept in a temperature-controlled environment with 12/12-h light-dark cycle. They received a standard chow diet and water *ad libitum*.

Ugt1a-deficient Gunn rats (*Ugt1a*$^{-/-}$) from our own breeding colony were housed in a temperature-controlled environment with a 12/12-h light-dark cycle and permitted *ad libitum* consumption of C1000 control diet (Altromin, Triple A Trading, Germany) and water. All animal experiments were performed in accordance with the European Directive 2010/63/EU and with approval of the Institutional Animal Care and Use Committee of the Amsterdam UMC.

AAV vector production and purification

GMP-like AAV8-AAT-hUGT1A1 vector used in this study was produced in 200L-scale bioreactors by adenovirus-free transient transfection method. Suspension HEK293 cells were transfected with PEI (PEIpro, Polyplus) with three plasmids containing the adenovirus helper proteins, the AAV Rep and Cap genes, and the inverted terminal repeats (ITR)-flanked transgene expression cassette. At 24 h after transfection, cells were treated with Benzonase, and, 2 days later, they were lysed with Triton (Sigma, St. Louis, MO) and clarified by filtration. Vectors were purified by a single chromatography column based on AVB Sepharose immuno-affinity (GE Healthcare) before concentration by tangential flow filtration. Purified particles were formulated in Ringer-Lactate solution containing 0.001% Pluronic (F68), vialed, and stored at −80°C. Titers of AAV vector stocks were determined using real-time qPCR. Specific probe and primers were as follows: forward, 5′-GGCGGGCGACTCAGATC-3′; reverse, 5′-GGGAGGCTG CTGGTGAATATT-3′; and probe, 5′-AGCCCCTGTTTGCTCCTC CGATAACTG-3′.

Phototherapy treatment in *Ugt1a*$^{-/-}$ mice

PT was applied as previously described.[38] Briefly, newborn pups were exposed from birth for 12 h/d to blue fluorescent light (20 μW/cm^2/nm, Philips TL 20W/52 lamps) for 27 days after birth. PT treatment was synchronized with the light/dark cycle of the animal housing facility and intensity of the phototherapy units was monitored by Olympic Mark II Bili-Meter (Olympic Medical).

AAV gene transfer procedure

AAV8 delivery to *Ugta1*$^{-/-}$ *mice* was performed at different postnatal days: P11, P25, P40, or P47 by retro-orbital injection. In rats the delivery was by tail vein injection. ImmTOR tolerogenic nanoparticles or empty nanoparticles (NPs) were co-administered with AAV8 in admix as previously described.[34]

ImmTOR tolerogenic nanoparticles

ImmTOR PLGA-based polymer nanoparticles encapsulating rapamycin were manufactured as described previously[22,31,65] and used in mice in different doses as indicated in the experimental design of each experiment (6, 8, 12 mg/kg) in *Ugt1a*$^{-/-}$ mice; in *Ugt1a*$^{-/-}$ Gunn rats they were used at the dose of 3 mg/kg. Empty NPs 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.

Organ histology

Organ biopsies from animals treated with ImmTOR or empty NP were extracted and fixed with 4% paraformaldehyde in PBS overnight at 4°C. Paraffin-embedded sections (5 μm) were stained with hematoxylin-eosin (H&E) and digital scanning was performed at Sponsor's facilities and histopathological evaluation was performed at Sciempath Bio (Lillois, Belgium).

Biochemical analyses of blood samples

In mice, blood samples were collected at indicated time points in *Ugt1a*$^{-/-}$ and WT littermates by facial vein exsanguination or cardiac puncture. Bilirubin determination in plasma was performed as previously described.[8] Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) determination was performed using Abnova assay kit (KA4189 and KA4187) according to manufacturer instructions.

In *Ugt1a*$^{-/-}$ Gunn rats, blood samples were collected by tail vein puncture under isoflurane anesthesia in heparin tubes. Bilirubin, ALT, and AST in serum were quantified on a Roche Cobas c502/702 analyzer (Roche Diagnostics, USA) by the hospital Routine Clinical Chemistry Department.

Bile determination

Bilirubin metabolites in bile were determined by high-performance liquid chromatography (HPLC) as described,[66] with the modification that a pursuit column (Agilent Technologies, the Netherlands) was used.

Viral genomes determination

Total DNA from mouse tissues was extracted using the Wizard SV Genomic DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The AAV8-AAT-hUGT1A1 vector genome copy number was quantified by real-time PCR using specific primers for alpha-1 antitrypsin (AAT) promoter as previously described.[6]

In Gunn rats, the genomic DNA was isolated from the tissues as previously described.[67] The AAV8-AAT-hUGT1A1 vector copy number was quantified by qPCR in a Bio-Rad CFX96 system (Roche Diagnostics, USA) using the TaqMan assay, iQ Supermix (Bio-Rad, Germany) as previously described.[13]

Preparation of total RNA, RT-PCR, and real-time PCR analysis

In Gunn rats, total liver or spleen RNA was isolated as previously described using Trizol reagent.[67,68] Briefly, 2 μg of RNA was used to synthesize cDNA using the RevertAid cDNA Synthesis Kit (Thermo Scientific) with oligo dT and random hexamer primers. qPCR was performed in a Bio-Rad CFX96 system (Roche Diagnostics, USA) using Sensi-FAST SYBR No-ROX kit (Bioline). Results were normalized to β-actin.

Preparation of total protein extracts and western blot analysis

Liver biopsies were homogenized in RIPA Buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl pH8, 2x protease inhibitors) and analyzed by western blot analysis as described previously.[36] Primary antibodies used were as follows: anti-UGT1A rabbit polyclonal 1:1,000 (A01865-1, BOSTER Biological Technology Co, CA, USA), anti-Actin rabbit polyclonal 1:1,000 (A2066, Sigma-Aldrich) was used as loading control.

Detection of anti-AAV8 antibodies in rodent plasma or serum

MaxiSorp 96-well plates (Thermo Fisher Scientific, Waltham, MA) were coated with AAV8 capsids in carbonate buffer at 4°C overnight. Standard curve of rat or mouse IgG or IgM (Sigma-Aldrich, St. Louis, MO) prepared as seven 2-fold dilution steps starting at 1 mg/mL was coated onto the wells. After blocking, plasma or serum samples were added to plates and incubated 1 h at 37°C. Secondary antibody was added into the well (anti-mouse IgG-HRP or IgM-HRP, or anti-rat IgG-HRP or IgM-HRP), plates were developed with 3,3′,5,5′-tetramethylbenzidine substrate, and the optical density was assessed by spectrophotometry at 450 and 570 nm (for background subtraction) on Enspire plate reader (PerkinElmer, Waltham, MA) after blocking the reaction with 5% H_2SO_4.

In vitro AAV8 neutralization assay

The neutralizing antibodies (NAbs) assay was performed as we previously described.[69] Briefly, on day 1, 96-well plates were seeded with 2×10^4 HEK 293 cells per well. An AAV8-Luc vector was then diluted in serum-free DMEM (Thermo Fisher Scientific) and incubated with semi-log fold serial dilutions of the serum samples for 1 h at 37°C. Subsequently, the serum-vector mixtures were added to the cells at a multiplicity of infection of 200. After 24 h, cells were lysed and luciferase activity was measured on a luminometer (EnSpire). Luciferase expression was measured as relative light units (RLUs) per second. The neutralizing titer was reported as the highest serum dilution that inhibited AAV transduction by $\geq$50% compared with the 100% transduction control.

Statistics

The Prism package (GraphPad Software, La Jolla, CA) was used to analyze the data. The normal distribution of the data was confirmed with the Shapiro-Wilk test of normality. Depending on the experimental design, Student's t test, or one-way ANOVA or linear mixed-effect model, with Tukey's post hoc comparison tests, were used, as indicated in the legends to the figures and text. Results are expressed as mean ± SD. Values of $p < 0.05$ were considered statistically significant.

DATA AND CODE AVAILABILITY

The materials presented here are fully available for distribution after the execution of a material transfer agreement (MTA) with the respective institutions.

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AUTHOR CONTRIBUTIONS

X.S., G.B., F.C., G.R., P.B., and A.F.M. contributed to the experimental design, supervised experiments and results interpretation, and wrote the manuscript. X.S., G.B., and F.C. contributed to experimental design, performed experiments, and analyzed the data, and contributed to results interpretation and manuscript preparation. P.-R.L.B., L.t.B., N.G., D.R.d.W., P.S., S.D., and P.V. contributed to the *in vivo* experiments and experimental activities. T.K.K. and F.M. contributed to the experimental design and results interpretation. All authors contributed to the revision and correction of the manuscript.

DECLARATION OF INTERESTS

T.K.K. is an employee and shareholder of Selecta Bioscience; F.M., G.R., F.C., G.B., and A.F.M. are inventors in patents describing the AAV technology and gene therapy-based treatments for Crigler-Najjar syndrome.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2024.101363.

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